MONASH University

The physiological effects of amniotic insufflation

Benjamin James Amberg

Bachelor of Medical Science (Honours)

Bachelor of Medicine/Bachelor of Surgery

Supervisors:

Professor Stuart Hooper

Associate Professor Ryan Hodges

Dr Philip DeKoninck

Dr Kelly Crossley

A thesis submitted for the degree of Doctor of Philosophy at

Monash University in 2021

Department of Obstetrics and Gynaecology

The Ritchie Centre, Hudson Institute of Medical Research

HUDSON INSTITUTE OF MEDICAL RESEARCH

The Ritchie Centre

This page is left intentionally blank.

I certify that I have made all reasonable efforts to secure copyright permissions for third-party content included in this thesis and have not knowingly added copyright content to my work without the owner's permission.

This thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

Signature:

Print Name: Benjamin James Amberg

Date: 08/07/2019

Table of Contents:

This page is left intentionally blank.

Acknowledgements

"This research was supported by an Australian Government Research Training Program (RTP) Scholarship and a Monash Graduate Excellence Scholarship (MGES)."

This will be the most frequently visited section of my thesis so I make no apologies for its length. Over the last 4 years I've realised that 90% of a PhD is finding a way to manage the people you work with. More specifically, it's like dealing with your huge, extended work family. Everyone needs different things from you and wants to see things presented in a different ways. You don't always see eye to eye and you get in trouble sometimes but, at the end of the day, you all love each other you wouldn't replace anyone for the world. So, to my huge, wonderful, sometimes dysfunctional, work family, thank you for everything and please never change.

Stuart (the family head). When our first lamb insufflated with heated, humidified CO_2 survived the full three hours, you told me that, "just because cold, dry CO_2 was used in humans didn't mean we actually knew it was the best gas to be using." I thought you were a bit mental to be honest until the second and third lambs also survived. From then on, I've been hooked on that incredible feeling of being totally and utterly wrong. Thanks for all your wisdom, experience and helping me develop an ability to question things that "seem" like they're known. I used to

get palpitations responding to angry reviewers or grumpy people in the audience but you've helped me realise that the angrier they seem, the better you've probably presented your argument against what they believe.

Ryan (the father). Safe to say I won't be taking any more photos in the animal house after the backlash we got from this one. The hardest part about going through med and PhD has been dealing with the endless barrage of, "What's next?" or "What kind of doctor do you want to be?" To be honest, I don't really know. What I do know, is that I want to do research and I want to supervise students the way you supervised me. Thanks for being so open with your life and giving me an insight into all the little things that run in parallel to life as a clinician, scientist and leader. I'm also very grateful of, and impressed by, your ability to help me develop my own opinions. Over the course of my thesis I've swung from, "insufflation is killing babies" to "the sheep is the most useless model of anything in the world." Wherever I was on this spectrum you always just checked in to clarify why I thought the way I did and never imposed your own thoughts. I now feel I have a much more rounded and (hopefully) more mature outlook on translational research as a whole.

Kel (the mother). While Ryan, Philip and Stu mainly dealt with my overly ambitious or contentious clinical and scientific endeavours, you mainly dealt with my day-to-day peculiarities. Thanks for pulling me into line when I decided I'd run a human fetal membrane histology project with no ethics, making sure I always had a food break and being totally supportive of (or great at faking interest in) my colour coding in the animal house. I hope you've had as much fun with me as I've had bagging the Tigers, struggling through lab meetings and getting called "Aidan" with you. I could not have asked for a better "cashed up, bogan, work mum" (your words not mine).

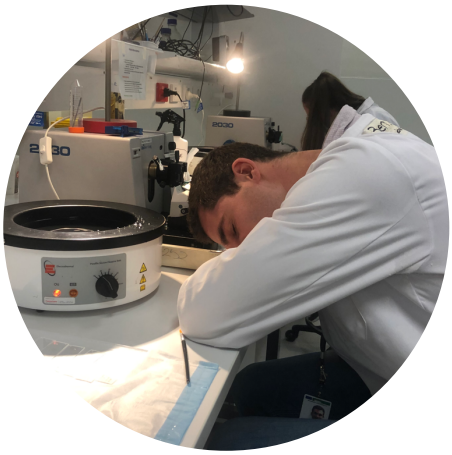
Philip (the god father). Indeed, I think it's taken me 3 years and 8 months to learn how to spell both your first and last name correctly. Here's all the ways I've spelt your name in our emails and paper drafts so far:

- Philip, Phillip, Philipe but luckily not Filip
- DeKoninck, D'Konick, DeKonnick and DeKonick
- I've also accidently put your gmail in the corresponding author section on a paper. . . . oops

We made cardboard Philip because you weren't physically with us in Melbourne for one Hodges lab group photo. But that's where your absence ended. Thank you for the most incredible amount of support with my writing, presenting, clinical thinking and scientific interpretation. Also, as long as IFMSS keeps running I'll try and make sure I have something to present so we can catch up at least once a year.

Kaz (the cool aunty). Thanks for just being so incredibly awesome at what you do. I'd never have acquired the humidifier, learnt to ALWAYS check the ear tag, remembered to fast my sheep, got to cut up a blue flow probe cord, sneak my way into CDH surgeries or had anywhere near as much fun watching 3 hour insufflations without you.

Aidan and Sasha (the older siblings). I'm bloody lucky to have had role models and mentors like you guys around. It's been very reassuring to know I can always do something similar to what Aidan and Sasha did and that will be well and truly enough. Aidan, I still get a little kick out of being mistaken as "Aidan Kashyap" every now and again. It's been an absolute pleasure mate, and I can't wait to see what you get up to next. Sasha, thanks for staying around after BMedSci. I feel like you've been my cheat sheet into all the little things that make running our insufflation studies work.



Val, Ali, Beth, Rob, Jade, Amy and Yen (the lab based neighbours who supervised me when my parents weren't around). "I just have one histology chapter to go which I should be able to knock off by about May next year and hopefully, submit a bit early" – Ben Amberg, November 2019. Safe to say that didn't happen. . . . . As frustrating, unpredictable and resembling witchcraft as the lab is, I think my third chapter was nearly one of my favourites. I don't think that actually had anything to do with the science though, it's probably more a reflection I loved working with you guys. Thanks for answering all of my dumb questions (sometime multiple times) and getting excited with me when my slides went brown. You guys are the best but I don't think I'll miss cutting histology slides.

Mikee (the mate). I'm going to leave this one short deliberately. You are, without a doubt, my best mate and fingers crossed it stays that way.

Annie, Ben, Maddie, Maddy, Paris, Tessa, Manon and Fraser (the crew). When I signed up for PhD I thought it would be 4 years of surgery, publishing papers in the Lancet and winning awards like Aidan at conferences. Turns out the majority was actually prepping the animal house, labelling stuff and wondering why my data didn't really look right. The other thing I didn't predict was that I'd meet a group of the most talented, eccentric and caring people you could ask for. I know everyone will head off in their own direction in the future but I am so grateful for all your support over the last 4 years. Without you guys, it would have felt like real work.

Brodie (the partner). My "one extra BMedSci year" didn't really go as planned hey! I count myself very lucky to have someone so totally supportive of the decisions I make, even if they don't really make sense or seem a bit insane. Thank you for all your love, patience and understanding. You are absolutely my best person.

Mum (the best). It's been a bloody long way from that first year 9 biology essay, "The benefits of unleaded E10" on the way back from Yorkes to, "The physiological effects of amniotic insufflation." Thanks for every piece of Neverland-prolonging advice, phone call, care package, vent, proof read, birthday reminder, safety text, Christmas box and recipe. I could not be more grateful of your endless love and support no matter what crazy ambition I set my mind to. I love you.

I also need to say a big thank you to **Monash School of Clinical Sciences**, the **Hudson Institute**, the **Ritchie Centre** and the **International Fetal Medicine and Surgery Society**. Over the course of my thesis I've been lucky enough to receive funding to that has allowed me to travel within Australia and internationally to present my work. These opportunities have undoubtably been a highlight of my candidature and continue to fuel my enthusiasm for research and science communication moving forward.

This page is left intentionally blank.

Thesis aims:

Incomplete closure of the fetal spine is detected with prenatal ultrasound in approximately 125 Australian pregnancies every year. This congenital abnormality known as a myelomeningocele, causes the fetal spinal cord to become damaged during pregnancy which can lead to permanent leg paralysis, faecal and urinary incontinence and sensory loss. The opening in the fetal spine can also disrupt normal brain development and increase pressure within the skull which is potentially fatal. However, modern medicine has revolutionised the management of these pregnancies. Fetal surgeons can now cover the myelomeningocele in early pregnancy to protect the baby's spinal cord and dramatically improve spinal cord and brain function after birth. These procedures are performed via keyhole (fetoscopic) surgery using a camera inserted into the mothers uterus. To see clearly during fetoscopy and create enough space for surgery, surgeons drain the liquid surrounding the baby and distended the uterus with pressurised carbon dioxide (CO_2) gas. This technique is known as amniotic insufflation.

Despite progressive improvements in fetoscopic myelomeningocele repair since the early 1990's, there are concerns that amniotic insufflation may have detrimental effects on the fetus. Animal data suggest that insufflation causes CO_2 to accumulate in the fetus (hypercapnia) during surgery which lowers blood pH (acidosis). Worryingly, chronic fetal hypercapnia and acidosis is associated with impaired brain development and neurodevelopmental delay. Preliminary human data also suggest that amniotic insufflation increases the risk of preterm fetal membranes rupture after surgery. Preterm membrane rupture increases the risk of preterm birth which also carries significant implications for the developing brain.

Given its ongoing use in humans, it is essential we understand how amniotic insufflation effects the fetus and fetal membranes and develop strategies to mitigate any adverse effects. Heating and humidifying the insufflated CO_2 has been proposed as a potential solution to both fetal acid base disturbances and preterm membrane rupture however, preclinical evidence is still required to support these hypotheses. Therefore, the global aim of this thesis is: **To investigate the effects of amniotic insufflation with unconditioned (cold, dry) or heated, humidified CO_2 on the fetus and fetal membranes in sheep.** Experimental chapters 1 and 2 investigate the physiological effects of amniotic insufflation on fetal and placental physiology while chapter 3 investigates the histological effects of amniotic insufflation on the fetal membranes.

This page is left intentionally blank.

Research output

Publications arising from this thesis:

2018:

1. The effects of partial amniotic carbon dioxide insufflation in an ovine model.
   S. Skinner, K. Crossley, B. Amberg, A. Kashyap, S. Hooper, J. A. Deprest, R. Hodges and P. DeKoninck. T Prenatal diagnosis 2018; 38: 994-1003.

2. Physiological effects of cold-dry versus heated-humidified partial amniotic carbon dioxide insufflation in sheep.
   Amberg B, Hodges R, Kashyap A, Skinner S, Rodgers K, Mcgillick E, Deprest J, Hooper S, Crossley K, Dekoninck P. Ultrasound Obstet Gynecol. 2018 Nov 21. doi: 10.1002/uog.20180.

3. Placental gas exchange during amniotic carbon dioxide insufflation in sheep
   B. J. Amberg, P. L. J. DeKoninck, A. J. Kashyap, S. M. Skinner, K. A. Rodgers, E. V. McGillick, J. A. Deprest, S. B. Hooper, K. J. Crossley and R. J. Hodges. Ultrasound Obstet Gynecol 2019. DOI 10.1002/uog.21933.

2020:

4. Why do the fetal membranes rupture early after fetoscopy? A Review.
   Amberg BJ, Hodges RJ, Rodgers KA, Crossley KJ, Hooper SB and Dekoninck PLJ – Submitted to *Fetal Diagnosis and Therapy*

5. The effects of cold, dry and heated, humidified amniotic insufflation on sheep fetal membranes.
   Benjamin Amberg, Philip DeKoninck, Aidan Kashyap, Karyn Rodgers, Sasha Skinner, Valarie Zhara, Stuart Hooper, Kelly Crossley, Ryan Hodges – Submitted to *Placenta*

Presentations to learned societies:

2017:

1. Monash Health Translation Precinct Research Week, Melbourne, Australia
   "The impact of cold, dry and heated, humidified amniotic insufflation on the sheep fetus"
   Awarded best poster in Obstetrics and Gynaecology

2018:

2. Australian Medical Student Journal, Victorian Research Symposium, Melbourne, Australia
   "The impact of cold, dry and heated, humidified amniotic insufflation on the sheep fetus"

3. International Fetal Medicine and Surgery Society (IFMSS) Annual Congress, Bali, Indonesia
   "Heated humidified partial amniotic carbon dioxide insufflation in sheep"
   Young investigator travel award
   Awarded the Umberto Nicolini Award for best talk from a young investigator

4. Australian Society of Medical Research, Melbourne, Australia
   "The impact of cold, dry and heated, humidified amniotic insufflation on the sheep fetus"

5. Monash Health Translation Precinct Research Week, Melbourne, Australia
   "The effect of heated, humidified amniotic insufflation on placental gas exchange in sheep"

2019:

6. Perinatal Society of Australia and New Zealand (PSANZ), Gold Coast, Australia
   "The effect of heated, humidified amniotic insufflation on placental gas exchange in sheep"
   Awarded the best talk from a young investigator in Obstetrics and Gynaecology

7. International Fetal Medicine and Surgery Society (IFMSS) Annual Congress, Sils Switzerland
   "The effect of heated, humidified amniotic insufflation on placental gas exchange in sheep"
   Young investigator travel award
   Awarded the Umberto Nicolini Award for best talk from a young investigator

2020:

8. Australian Society of Medical Research (ASMR), Melbourne, Australia

"Heated humidified amniotic insufflation mitigates histological evidence of fetal membrane injury in sheep"

Awarded the best short talk

9. Trans-Tasman Fetal and Neonatal Conference, Melbourne, Australia
   "Heated humidified amniotic insufflation mitigates histological evidence of fetal membrane injury in sheep"

10. Trans-Tasman Fetal and Neonatal Conference, Melbourne, Australia
    "The effect of heated humidified amniotic insufflation on placental gas exchange in sheep"

Invited presentations:

2017:

1. Monash University Monash University Paediatric Promotions, Interest and Training Society (MUPPITS) Revision lecture Series
   "Paediatric Gastroenterology"

2018:

2. Monash University Monash University Paediatric Promotions, Interest and Training Society (MUPPITS) Revision lecture Series
   "Paediatric Surgery"

3. Australian Medical Student Journal - Victorian Research Symposium, Melbourne, Australia
   "Pathways to Medical Research – MD/PhD"

2019:

4. Monash University Monash University Paediatric Promotions, Interest and Training Society (MUPPITS) Revision lecture Series
   "Paediatric Surgery"

2020:

5. Monash University Monash University Paediatric Promotions, Interest and Training Society (MUPPITS) Revision lecture Series
   "Paediatric Surgery"

6. Monash University BMedSci (hons) annual congress
   "How to give a great talk"

Other publications during my PhD candidature:

2019:

1. Partial Amniotic carbon dioxide insufflation for fetal surgery.
   Skinner S, Dekoninck P, Crossley K, Amberg B, Deprest J, Hooper S, Hodges R. Prenat Diagn. 2018 Sep 20. doi: 10.1002/pd.5362. Review.

2. Physiologically based cord clamping improves cardiopulmonary haemodynamics in lambs with a diaphragmatic hernia.
   Aidan J. Kashyap, Ryan J. Hodges, Marta Thio, Karyn A. Rodgers, Benjamin J. Amberg, Erin V. McGillick, Stuart B. Hooper, Kelly J. Crossley, Philip L. J. DeKoninck. Arch Dis Child Fetal Neonatal Ed. 2019 May 23. pii: fetalneonatal-2019-316906. doi: 10.1136/archdischild-2019-316906.

3. Antenatal sildenafil treatment improves neonatal pulmonary hemodynamics and gas exchange in lambs with diaphragmatic hernia.
   Kashyap AJ, DeKoninck PLJ, Rodgers KA, Thio M, McGillick EV, Amberg BJ, Skinner SM, Moxham AM, Russo FM, Deprest JA, Hooper SB, Crossley KJ, Hodges RJ. Ultrasound Obstet Gynecol. 2019 Jul 31. doi: 10.1002/uog.20415.

This page is left intentionally blank.

Declarations

Thesis including published works declaration

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes 3 original papers published in peer reviewed journals and 2 submitted publications. The core theme of the thesis is understanding how distending the uterus with pressurized CO_2 during keyhole fetal surgery effects the fetus and fetal membranes. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the student, working within the Department of Obstetrics and Gynecology, School of Clinical Sciences, Monash university under the supervision of Professor Stuart Hooper, Associate Professor Ryan Hodges, Dr Kelly Crossley and Dr Philip DeKoninck.

In the case of experimental chapters 1-3 my contribution to the work is described in the following:

Thesis Chapter	Publication Title	Status	Nature and % of student contribution	Co-author name(s) Nature and % of Co-author's contribution*	Co-author(s), Monash student Y/N*
Experimental Chapter 1.1	The effects of partial amniotic carbon dioxide insufflation in an ovine model	Published	50%. - data collection, data analysis, manuscript preparation and manuscript editing	1. Sasha M. Skinner - data collection, data analysis, manuscript preparation and manuscript editing (30%) 2. Kelly J. Crossley - concept design, data collection and manuscript editing (3%) 3. Aidan J. Kashyap - data collection and manuscript editing (3%) 4. Stuart B. Hooper - concept design, data collection, data analysis and manuscript editing (3%) 5. Jan A. Deprest - concept design and manuscript editing (3%) 6. Ryan J. Hodges - concept design, data collection and manuscript editing (4%) 7. Philip L.J. DeKoninck - concept design, data collection, data analysis, manuscript preparation and manuscript editing (4%)	Yes No Yes No No No No

Thesis Chapter	Publication Title	Status	Nature and % of student contribution	Co-author name(s) Nature and % of Co-author's contribution*	Co-author(s), Monash student Y/N*
Experimental Chapter 1.2	Physiological effects of partial amniotic carbon dioxide insufflation with cold, dry vs heated, humidified gas in a sheep model	Published	70%. - concept design, data collection, data analysis, manuscript preparation and manuscript editing	1. Ryan J. Hodges - concept design, data collection, and manuscript editing (3%) 2. Aidan J. Kashyap - data collection and manuscript editing (3%) 3. Sasha M. Skinner - manuscript editing (3%) 4. K.A. Rodgers - data collection, data analysis and manuscript editing (3%) 5. E.V. Mcgillick - data collection, and manuscript editing (3%) 6. Jan A. Deprest - concept design, and manuscript editing (3%) 7. Stuart B. Hooper - concept design, data analysis and manuscript editing (4%) 8. Kelly J. Crossley - concept design, data collection and manuscript editing (4%) 9. Philip L.J. DeKoninck- concept design, data collection, data analysis, manuscript preparation and manuscript editing (4%)	No Yes No No No No No No No
Experimental Chapter 2	Placental gas exchange during amniotic carbon dioxide insufflation in sheep.	Published	70%. - concept design, data collection, data analysis, manuscript preparation and manuscript editing	1. Philip L.J. DeKoninck - concept design, data collection, data analysis, manuscript preparation and manuscript editing (4%) 2. Aidan J. Kashyap - data collection and manuscript editing (3%) 3. Sasha M. Skinner - manuscript editing (3%) 4. K.A. Rodgers - data collection, data analysis and manuscript editing (3%) 5. E.V. Mcgillick - manuscript editing (3%) 6. Jan A. Deprest - concept design and manuscript editing (3%) 7. Stuart B. Hooper - concept design, data collection, data (4%)analysis and manuscript editing 8. Kelly J. Crossley - concept design, data collection, data (3%)analysis and manuscript editing 9. Ryan J. Hodges - concept design, data collection and manuscript editing (4%)	No Yes No No No No No No No
Experimental Chapter 3	The effects of cold, dry and heated, humidified amniotic insufflation on sheep fetal membranes	Submitted	70%. - concept design, data collection, data analysis, manuscript preparation and manuscript editing	1. Philip L.J. DeKoninck - concept design, data collection, data analysis, manuscript preparation and manuscript editing (5%) 2. Aidan J. Kashyap - data collection and manuscript editing (4%) 3. Sasha M. Skinner - manuscript editing (4%) 4. K.A. Rodgers - data collection, manuscript editing (4%) 5. Stuart B. Hooper - concept design, data analysis andmanuscript editing (4%) 6. Kelly J. Crossley - concept design, data collection and manuscript editing (4%) 7. Ryan J. Hodges - concept design and manuscript editing (5%)	No No No No No No No

I have not renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Student name: Benjamin Amberg

Student signature: Date: 19.01.2021

I hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions to this work. In instances where I am not the responsible author I have consulted with the responsible author to agree on the respective contributions of the authors.

Main Supervisor name: Professor Stuart Hooper

Main Supervisor signature: Date: 19.01.2021

This page is left intentionally blank.

Figures and tables

Figures

Tables

Abbreviations

CD45	Cluster determination 45
CLDN	Claudin
CO_2	Carbon dioxide
COL	Collagen subunit
CSF	Cerebrospinal fluid
CTGF	Connective tissue growth factor
CYR61	Cysteine-rich angiogenic inducer 61
DAMP	Damage associated molecular pattern
EGR1	Early growth response 1
H^+	Hydrogen ions
HCO_3^-	Bicarbonate ions
IL	Interleukin
mmHg	Millimetres of mercury
MMP	Matrix metalloproteases
MOMS	The Management of Myelomeningocele Study
mRNA	Messenger ribonucleic acid
O_2	Oxygen
OCLN	Occludin
PACI	Partial amniotic carbon dioxide insufflation
$PaCO_2$	Arterial partial pressure of carbon dioxide
PAMP	Pathogen associated molecular pattern
PaO_2	Arterial partial pressure of oxygen
PPROM	Preterm prelabour rupture of membranes
TIMP	Tissue inhibitors of matrix metalloproteases
TJP	Tight junction protein
HMGB1	High mobility group box 1
RAGE	Receptor for advanced glycation end products
RPS18	Ribosomal protein S18
TIMP	Tissue inhibitor of matrix metalloprotease
TNF	Tumour necrosis factor
VCAM	Vascular cell adhesion molecule

This page is left intentionally blank.

Literature review

Introduction

Incomplete closure of the fetal spine is detected with prenatal ultrasound in approximately 125 Australian pregnancies every year.[1-3] This congenital abnormality known as a myelomeningocele, causes the fetal spinal cord to become damaged during pregnancy which can lead to permanent leg paralysis, faecal and urinary incontinence and sensory loss.[4, 5] The opening in the fetal spine can also disrupt normal brain development and increase pressure within the skull (hydrocephalus) which is potentially fatal.[4, 5] Open fetal surgery is currently considered the gold standard of care for these pregnancies.[6] Between 18 and 24 weeks gestation, fetal surgeons make an incision in the mothers abdomen (laparotomy) and uterus (hysterotomy) to expose the fetus and surgically repair the fetal spine.[7] This procedure protects the spinal cord and brain from damage during the remainder of pregnancy and significantly improves functional and neurodevelopmental outcomes after birth.[6]

While open fetal surgery protects the fetal spinal cord and brain, the need to incise the uterus causes significant complications for the mother including the risk of uterine rupture and hysterectomy. These complications have driven the development of a "keyhole" alternative to open fetal surgery, known as fetoscopy, which avoids hysterotomy.[6, 8] During fetoscopy, a camera and surgical instruments are inserted into the uterus through ports in the uterus and the fetal spine is then repaired under video-guidance.[8] However, fetoscopy faces its own challenges. The intrauterine space is small and the cloudy amniotic fluid limits visibility using the camera.[8] To overcome these challenges, surgeons partially drain the amniotic fluid and distend the uterus with pressurised carbon dioxide (CO_2) gas, a procedure known as amniotic insufflation.[8]

Despite progressive improvements in fetoscopic myelomeningocele repair since the early 1990's, there are concerns that amniotic insufflation may have detrimental effects on the fetus. Preliminary sheep studies suggest that amniotic insufflation increases fetal blood CO_2 (hypercapnia) and lowers arterial pH (acidosis).[9-12] Growth restricted fetuses exposed to chronic hypercapnia and acidosis show impaired brain development and neurodevelopmental delay after birth.[13] Preliminary human case series also suggests that amniotic insufflation may increase the risk of post-operative preterm prelabour rupture of the fetal membranes (iatrogenic PPROM).[8, 14] Iatrogenic PPROM increases the risk of lung hypoplasia, chorioamnionitis and preterm birth, all of which have significant implications for the infant and potentially offset the benefits of surgery.[15]

Concerns surrounding fetal hypercapnia and acidosis during insufflation and high rates of iatrogenic PPROM must be addressed before fetoscopic myelomeningocele repair can replace the current gold

standard of open fetal surgery. Recently, two small human case series have suggested that heating and humidifying the insufflated CO_2 could mitigate both fetal acid base disturbances and high iatrogenic PPROM rates however, these hypotheses lack physiological and histological support.[16, 17] The following reviews the current management strategies for myelomeningocele, the concerns that underpin the use of amniotic insufflation and the potential benefits of heating and humidifying the insufflated CO_2.

Myelomeningocele

Neurulation and neural tube defects

During embryonic development, the brain, spinal cord and vertebral column arise from a flat mass of cells known as the neural plate.[18, 19] This plate folds down its length to form the neural tube which allows the future skull and vertebral column to wrap and protect the brain and spinal cord (shown in Figure 1). Folding of the neural tube begins on day 22 post conception at the level of the future brainstem.[18, 19] The neural tube then zips together toward each end of the developing embryo in a process known as primary neurulation. Closure of the neural tube in a cranial direction towards the forebrain is completed by day 24 post-conception. Neural tube closure also proceeds in a caudal direction down the vertebral column and reaches the lower sacrum by day 26 post conception.[18, 19] The lower sacral and coccygeal vertebrae are formed through a separate process known as secondary neurulation, that does not involve folding of the neural tube.[20, 21]

A group of congenital malformations known as neural tube defects, occur when there is incomplete closure of the neural tube. [20, 22, 23] When the cranial end of the neural tube fails to close the skull does not completely enclose the back of the brain (shown in Figure 2a). The most common to these cranial defects is known as anencephaly.[20, 22, 23] Spina bifida occurs when the caudal end of the neural tube fails to close and results in incomplete union of the vertebral arches (shown in Figure 2b-c).[19]

Spina Bifida defects can be broadly grouped as open or closed based on the tissues that cover the meninges and spinal cord.[19, 20, 24] In closed defects such as spina bifida occulta or meningocele, the neural tissues are covered by skin and paraspinal muscles (shown in Figure 2b). Open spina bifida occurs when the skin and muscles are absent, which leaves the meninges and spinal cord exposed. Myelomeningocele is the most severe form of open spina bifida accounting for ≈2/3 cases. The spinal cord herniates through the opening in the vertebral arches, stretching the cord and leaving a sack of cerebrospinal fluid (meningeal sack) within the spinal canal (shown in Figure 2c).[19] In Myeloschisis (≈1/3 of open spina bifida), the cord remains within the spinal canal.[19]

Figure 1: The embryonic neural tube

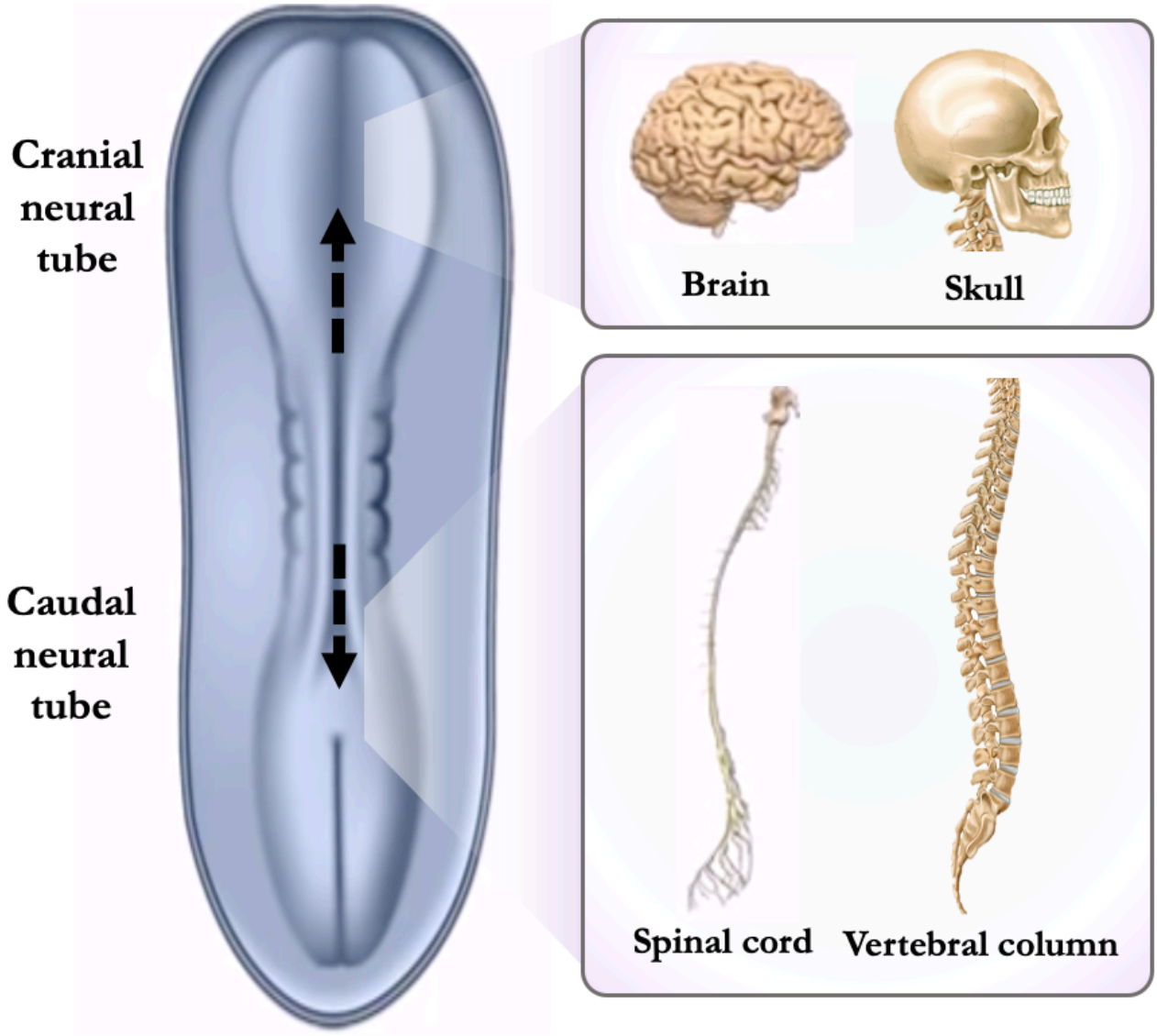


Figure 1: Closure of the neural tube begins at its center and progresses in a cranial and caudal direction. This gives rise to the brain and spinal cord of the central nervous system and the overlying skull and vertebral column – Image adapted from Anatomy TV, Detailed Animation on Neurulation.[25]

Figure 2: Classification of neural tube defects

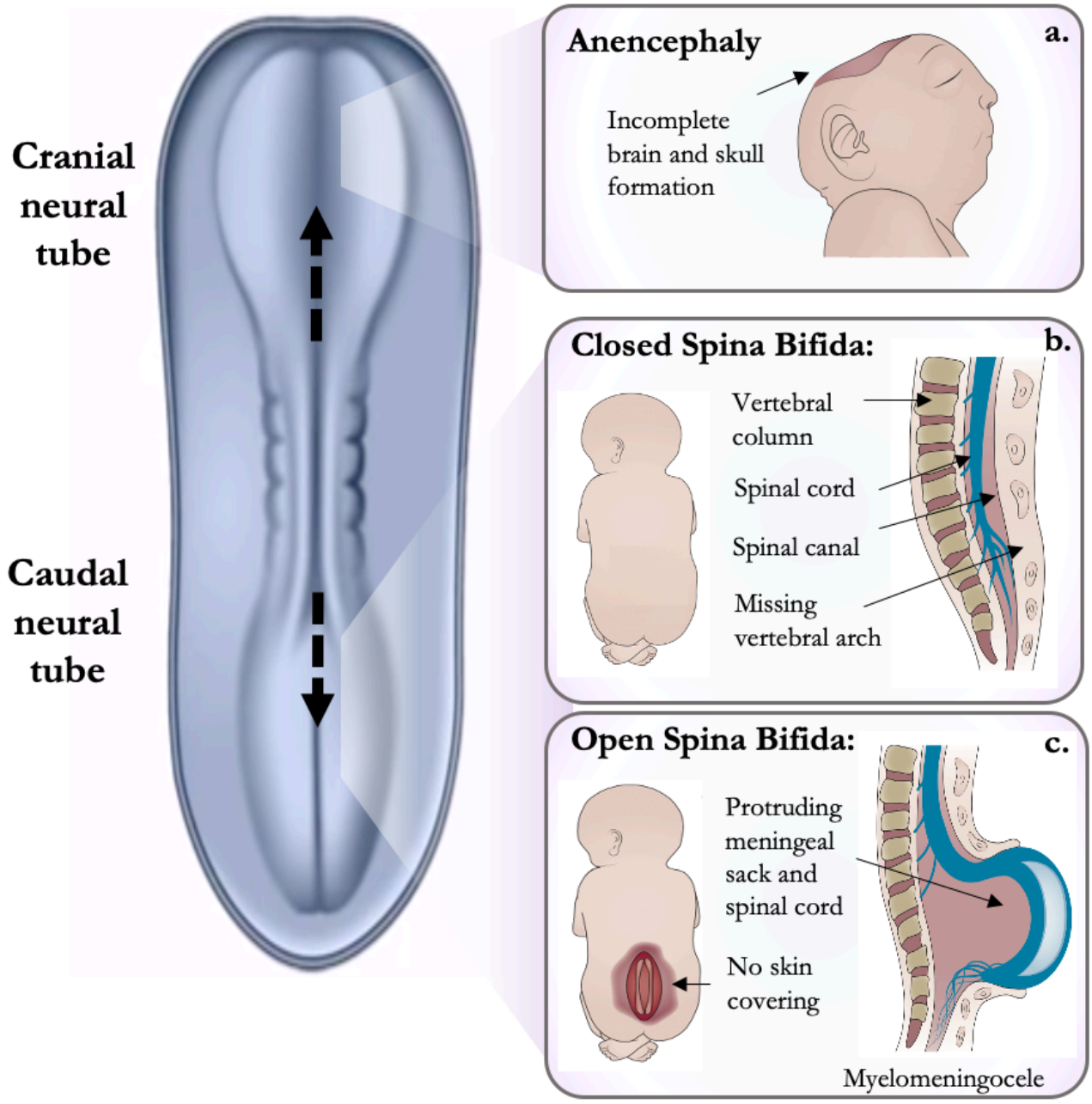


Figure 2: (a.) Incomplete closure of the cranial neural tube results in incomplete brain and skull formation (Anencephaly). (b-c.) Spina bifida occurs when there is incomplete caudal formation of the neural tube. In closed defects such as spina bifida occulta or meningocele, the neural tissues are covered by skin and paraspinal muscles (b). In open spina bifida, the skin and muscles are absent leaving the meninges and spinal cord exposed (c). – Image adapted from Copp et al. and Anatomy TV, Detailed Animation on Neurulation.[19, 25]

Aetiology and risk factors for myelomeningocele

Mutations of genes that control neural tube folding are estimated to account for 60-70% of neural tube defects however, the number of known causative genes is currently limited.[19] Another 10% occur in the context of chromosomal abnormalities such as Patau (trisomy 13) and Edwards (trisomy 18) syndrome.[19] A much smaller proportion occur in conjunction with other congenital abnormalities however, the cause of incomplete neural tube closure in these cases remains unclear.[26]

Maternal folate (vitamin B9) deficiency during the time of neural tube folding is the most widely understood non-genetic risk factor for neural tube defects including myelomeningocele.[19] Folate acts as co-factor for enzymes involved in DNA synthesis and DNA methylation, which alters the activity of genes that regulate neural tube closure.[27] Two large randomised control trials in the early 1990's established that maternal supplementation with a synthetic form of folate (folic acid) around the time of conception could both prevent and reduce the recurrence of neural tube defects.[28, 29] This protective role of folic acid supplementation lead to the mandatory fortification of flour in Australia with folic acid in 2005. However, since 2005 there has been only a modest reduction in rates of neural tube defects (4.8 to 4.4 in 10,000 births).[2, 3]

In addition to low folate, maternal deficiencies of vitamin B12, vitamin C, Choline and Zinc may also play a role in causing neural tube defects. Maternal exposure to pesticides, organic solvents and some medications such as the anticonvulsant valproic acid, have also been shown to increase the risk of neural tube defects.[19]

Epidemiology

Spina bifida affects ≈125 Australian pregnancies (4.4 in 10,000) each year.[1-3] This number has slowly fallen since the introduction of mandatory supplementation of flour with folic acid in Australia in 2005 (4.8 to 4.4 in 10,000 births).[2, 3] Outside of Australia, rates of spina bifida vary significantly between regions. Rates are highest in Asia (24.3 in 10,000 births) and lowest in North America (3.8 in 10,000 births) however, these numbers are likely affected by differences in the reporting of terminated cases.[30]

The morbidity of myelomeningocele

Exposure of the spinal cord during pregnancy leads to significant morbidity and mortality for babies born with myelomeningocele.[31-33] The amniotic fluid and fetal movements against the uterus damage neurons moving down the spinal cord to innervate the fetal lower limbs, bladder and bowel. This damage causes permanent lower limb weakness or paralysis and sensory loss as well as faecal and urinary incontinence.[31-33] Additionally, the meninges surrounding the spinal cord can rupture and leak cerebrospinal fluid (CSF). This draws CSF from within the brain down into the spinal canal and compresses the back of the brain (the hindbrain) into the base of the skull. This compression, known as the Arnold Chiari II malformation or hindbrain herniation (shown in Figure 3), damages cranial nerves and impairs normal brain development.[4, 5]

Hindbrain herniation also obstructs the movement of CSF from within the skull into the vertebral column. This obstruction increases intracerebral pressure and dilates the cerebral ventricles, a complication known as hydrocephalus.[4, 5] Hydrocephalus also impairs normal brain development and, if left untreated, is potentially fatal.[4, 5] To relieve hydrocephalus, a drainage system is surgically inserted between the cerebral ventricles and the abdominal cavity. This complex procedure known as ventriculoperitoneal shunting, improves infant survival however, shunt infection and blockage remain major causes of long-term morbidity and mortality for patients with myelomeningocele.[24, 34-36] Other neurosurgical interventions to relieve CSF pressure within the brain known as third ventriculostomy have also been attempted however, these procedures also carry significant risks.[19, 36]

Even with advances in modern medicine, 2-4% of patients with myelomeningocele die before the age of five and up to 26% die before the age of 40.[37, 38] Deaths most commonly result from complications with ventriculoperitoneal shunting or severe hydrocephalus.[24, 39] Patients that survive require significant social and financial support as less than 50% are able to live independently as adults. The lifetime cost of supported education and living, ongoing medical and surgical care and the loss of productivity is estimated at $750,000 Australian dollars per patient.[40-42]

Figure 3: The morbidity of myelomeningocele

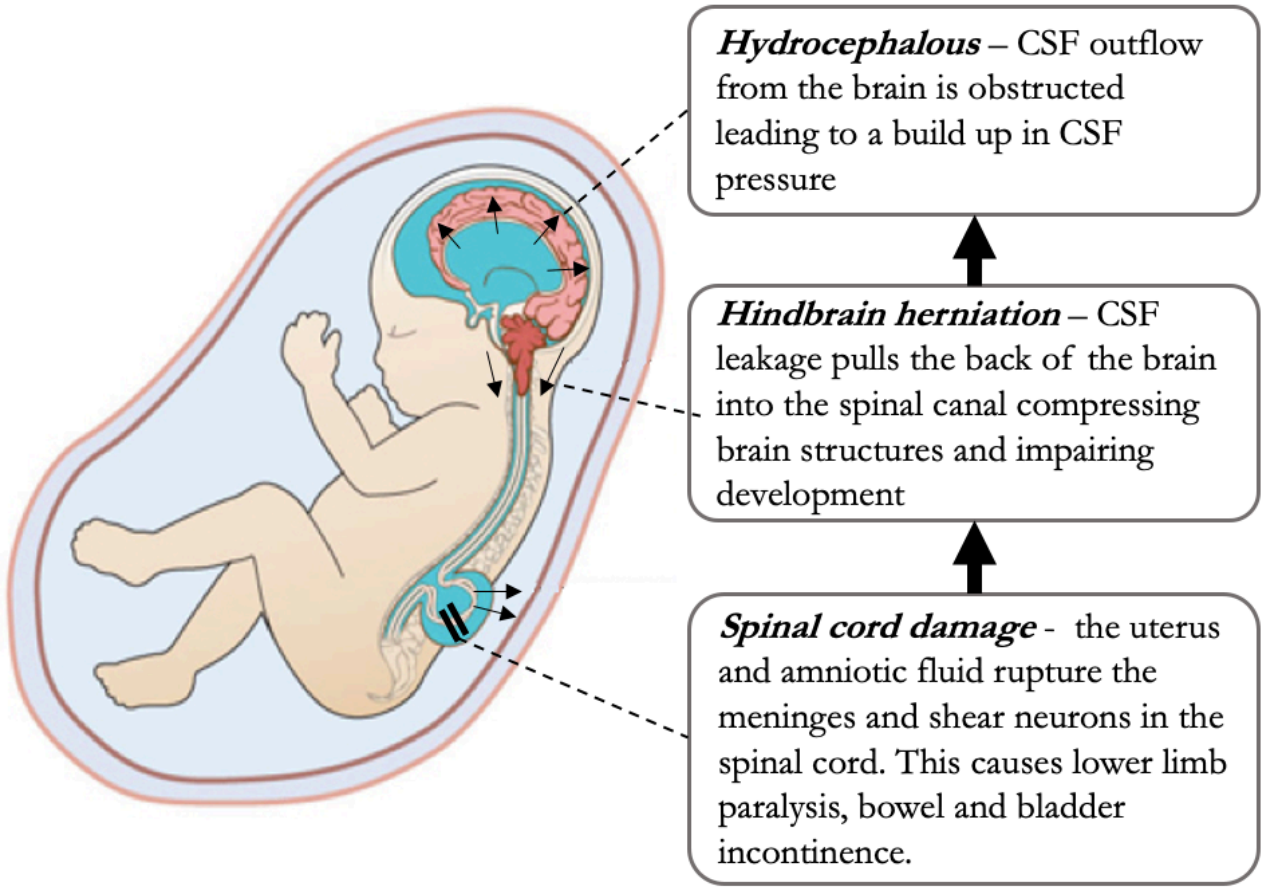


Figure 3: Incomplete caudal formation of the caudal neural tube leaves the spinal cord and meninges exposed. The amniotic fluid and uterine wall damages nerves moving down the spinal cord to innervate the lower limbs and pelvic organs during pregnancy. This results in lower limb paralysis and fecal and urinary incontinence. Cerebrospinal fluid (CSF) leakage from the damaged meninges also pulls the hindbrain into the base of the skull (hindbrain herniation) and causes CSF to accumulate within the brain (Hydrocephalus) – Image adapted from UCSF Fetal Treatment Center.[43]

Diagnosis of myelomeningocele

Imaging

Nearly all myelomeningoceles (90-98%) are detected on routine second trimester fetal ultrasound. The meninges and/or spinal cord can be seen protruding through the hole in the vertebral column as shown in Figure 4.[19, 44] Defects higher in the vertebral column (thoracic or lumbar) typically feature more severe intracranial complications, require ventriculoperitoneal shunting more often and have poorer lower limb function and overall survival than lower (sacral) lesions.[24, 44, 45]

When myelomeningoceles is complicated by hindbrain herniation, ultrasound may also detect changes in the fetal brain.[46] The frontal bones of the fetal skull bulge inward and lose their normal convex appearance (shown in Figure 4e).[46] This appearance, known as the lemon sign, is seen in nearly all cases of myelomeningocele between 16 and 24 weeks.[19, 46-48] Additionally, the cerebellum appears compressed and rounded as it is pulled into the base of the skull (the banana sign) and the cerebral ventricles appear enlarged and distended (ventriculomegaly) (shown in Figure 4e-f).[46]

Fetal ultrasound may also identify the absence of fetal leg movements or abnormal posturing of the foot (talipes).[19] These changes are thought to result from damage to neurons innervating the lower limbs and correlate with worse motor function at 12 months of life.[49, 50] At large fetal therapy centres, ultrasounds suggestive of fetal myelomeningocele are often followed by fetal magnetic resonance imaging (MRI) to more precisely evaluate the spinal defect, skull and brain.[20, 24]

Biochemical Markers

The use of ultrasound and fetal MRI has largely replaced the use of amniotic fluid markers for the diagnosis of fetal myelomeningocele. However, in cases where the diagnosis is unclear, the presence of acetylcholinesterase (a component of fetal CSF) in the amniotic fluid and high levels of maternal serum alfa-fetoprotein (AFP) at 16-18 weeks' gestation can be used to support a diagnosis.[19]

Figure 4: Ultrasound findings in fetuses with myelomeningocele

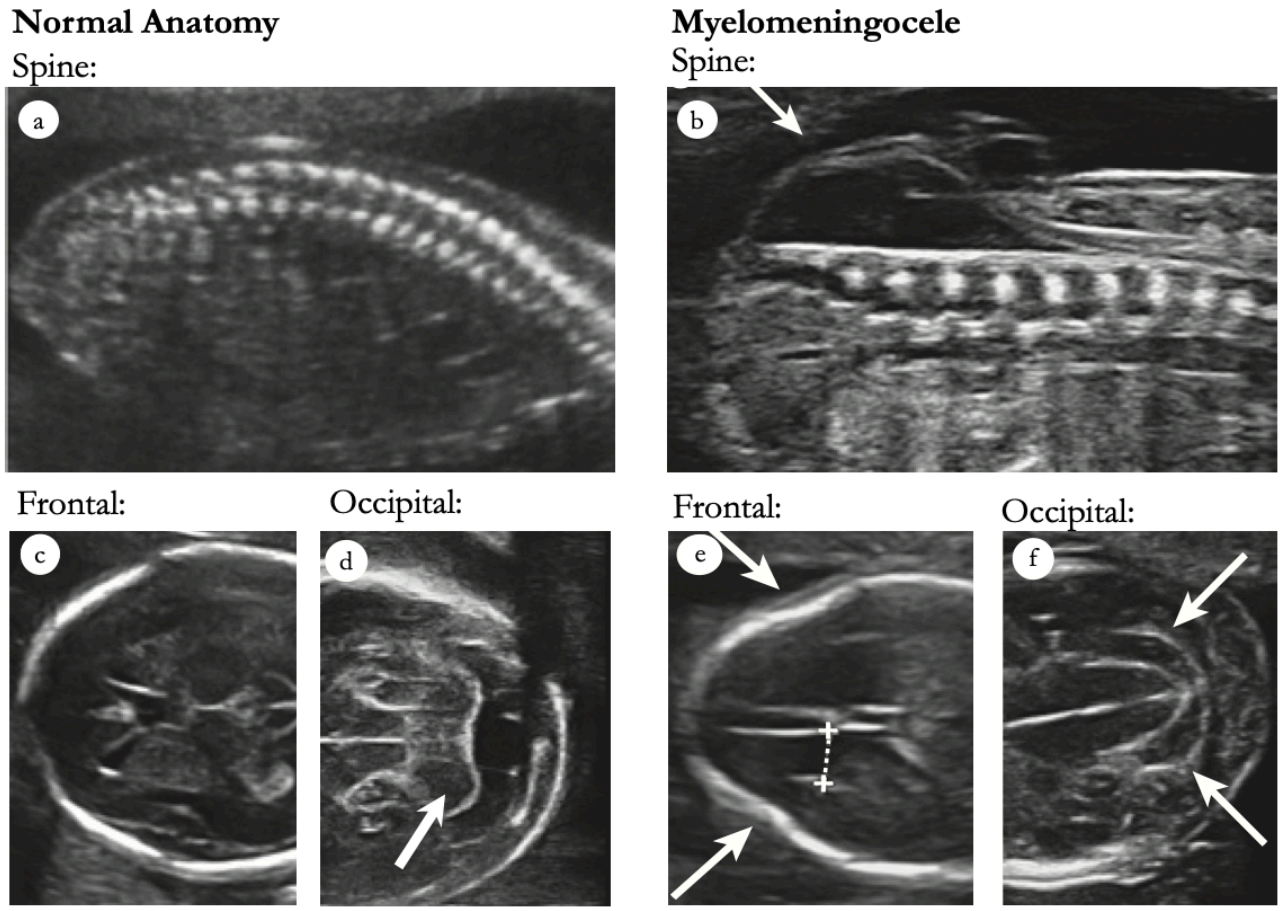


Figure 4: In the normal spine (a), the skin covered, parallel vertebrae are clearly visible, whereas in myelomeningocele the meninges and spinal cord protrude from the vertebral column (b – white arrow). In the normally developed fetus, the frontal bones have a smooth rounded appearance (c) compared to the inward scalloping (e - white arrows) of these bones in myelomeningocele (the lemon sign). The normal cerebellum has a dumbbell shape (d – white arrow) compared to the curved cerebellum (f – white arrows) seen in myelomeningocele (banana sign). – Image adapted from Copp et al.[19]

The management of myelomeningocele

Most parents confronted with a diagnosis of fetal myelomeningocele (44.6 - 75.4%) choose to terminate the pregnancy due to the significant morbidity associated with fetal spinal cord and brain injury.[2, 3, 24] Until the mid 2000s, the remaining cases were carefully monitored during pregnancy and babies underwent surgery to repair the spine within 48 hours of birth (postnatal surgery).[51] However, the irreversible damage to the spinal cord and brain sustained during pregnancy meant that infants undergoing postnatal surgery still had poor spinal cord function and neurodevelopmental outcomes.[7] This progressive injury and ongoing morbidity led to efforts to cover the spinal cord during pregnancy. The following summarises the evolution of fetal myelomeningocele surgery and the key questions yet to be answered in its ongoing refinement.

Postnatal surgery for myelomeningocele

Until 2010, standard treatment for antenatally diagnosed myelomeningocele was surgery within 48 hours of birth (postnatal surgery). A paediatric neurosurgeon would free the neural tissues adhered to the skin (the neural placode) and return them to the spinal canal.[51, 52] They would then close the inner (pia mater) and outer (dura mater) layers of the meninges in separate layers over the spinal cord to prevent CSF leakage (shown in Figure 5).[51, 52] Following this meningeal repair, the tendinous extensions of the paraspinal muscles (the thoracolumbar fascia) would be sutured together over the repaired meninges.[51, 53] For larger defects where the thoracolumbar fascia could not easily be brought together, muscle (paraspinal, latissimus dorsi or gluteal) or periosteal (lumbar vertebrae) flaps could be used.[53] Finally the skin on each side of the defect would be brought together over the thoracolumbar fascia and sutured. Occasionally, parallel skin incisions on either side of the repair (relaxing incisions) were used to relieve tension on the skin sutures.[51] This three-layered neurosurgical repair of the meninges, thoracolumbar fascia and skin remains the gold standard to protect the spinal cord and prevent CSF leakage.[7]

Figure 5: Surgical technique – postnatal closure of myelomeningocele

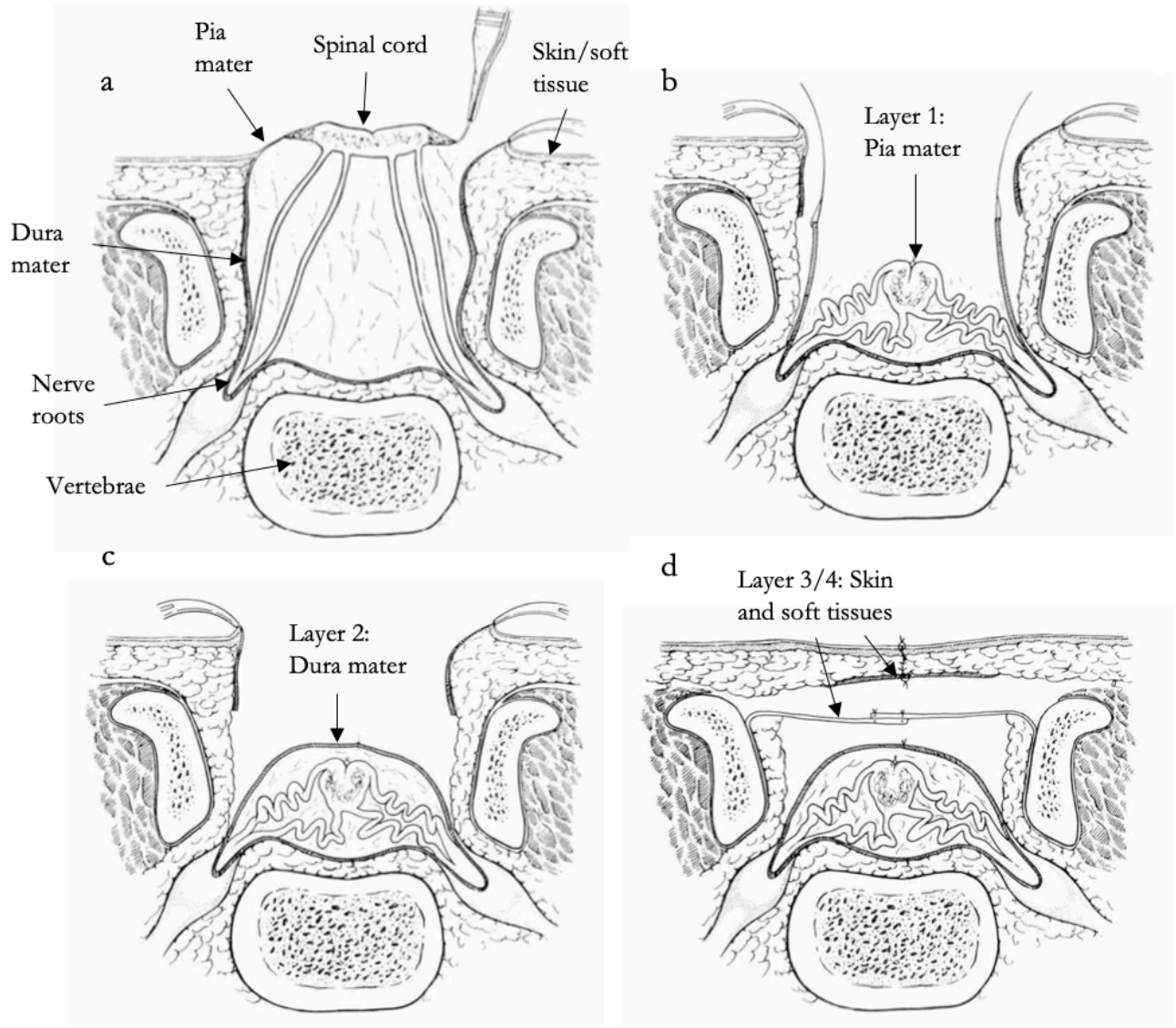


Figure 5: The edges of the exposed tissues (the neural placode) adhered to the skin are incised (a) to allow it to move freely back into the spinal canal (b). The inner pia mater (b) and outer dura mater (c) of the meninges are then closed separately over the spinal cord. Finally, muscle, bone or fascial flaps and the skin are closed over the repaired spinal cord (d). – Image adapted from McCullough et al.[52]

Fetal surgery for myelomeningocele

Preliminary animal studies investigating fetal spinal cord repair: (1984 – 1997)

Fetal repair of a myelomeningocele-like defect was first demonstrated in 1984 in eight primate fetuses with surgically created spinal defects (shown in Figure 6).[54] Five fetuses had their spinal defects immediately covered with an allogenic bone paste and three were left as un-treated controls. At term, the three controls had severe leg weakness, sensory loss and incontinence that resembled infants with myelomeningocele while the fetuses with repaired defects had normal lower limb function and spinal cord histology.[54] Similar findings were subsequently demonstrated in fetal rodents and pigs.[5, 33, 55, 56]

Unlike these initial animal repairs where spinal defects were created and then repaired immediately, human cases of myelomeningocele would remain untreated from the point of incomplete neurulation (day 22-28 post conception) until eventual diagnosis and surgery at 18-24 weeks gestation. During this time, irreversible injury to the spinal cord would have occurred meaning that preliminary animal studies likely overestimated the benefits of fetal myelomeningocele repair.[32] To account for this delay, Mueli et al. surgically created spinal defects in fetal lambs at 75 days gestation and delayed repair until 100 days (term 146 days).[32] At term, lambs that received fetal surgery showed some hind limb weakness, but could stand, walk and climb stairs. Histologically, their spinal cords appeared normal.[32] These results demonstrated that fetal spinal cord repair could improve spinal cord and brain outcomes after birth in a large animal model and justified preliminary human attempts in 1997.

These initial animal studies repairing the fetal spine during pregnancy all used large incisions in the mother's abdomen (laparotomy) and uterus (hysterotomy) to access the fetus.[5, 32, 33, 54-56] This technique was later referred to as open fetal surgery.[7] However, early gestation hysterotomy incisions were known to have significant implications for the mother's future pregnancies. Therefore, before performing their first human fetal myelomeningocele repairs, surgeons developed a "keyhole" alternative to open fetal surgery known as fetoscopy that avoided the need for hysterotomy.[57-59] During fetoscopy, surgeons repaired the fetal spine using a camera and instruments inserted through ports in the uterine wall.[57-59] However, fetoscopy faced its own challenges. The small intrauterine space and the cloudy amniotic fluid limited visibility using the camera which made it difficult to manipulate the fetus and repair the spine.[57, 58] To overcome these limitations, surgeons drained the amniotic fluid surrounding the fetus and distended the uterus with pressurised CO_2 gas. This distension became known as amniotic insufflation.[57, 58] Carbon dioxide was the logical choice for insufflation at this time given its extensive use in endoscopic abdominal and thoracic surgery with minimal adverse effects.[60-62]

Concerns about the fetal safety of amniotic insufflation: (1994 – 2000)

While amniotic insufflation improved visibility during fetoscopy, several groups raised concerns that CO_2 used to distend the uterus may enter exposed fetal blood vessels during surgery and cause hypercapnic acidosis.[9-12] This was particularly worrying as chronic fetal hypercapnia and acidosis impair fetal brain development and are associated with neurodevelopmental delay in early childhood.[13] These concerns lead to a series of studies in pregnant sheep that involved periodically sampling fetal arterial blood over 30-60 minutes of insufflation.[9-12] All four studies (summarised in Table 1) showed progressive increases in the arterial partial pressure of CO_2 ($PaCO_2$) and reductions in arterial pH during insufflation.[9-12]

The interpretation of these animal studies varied significantly. Some authors believed that these results confirmed that the fetus was absorbing CO_2 during amniotic insufflation and that fetoscopic myelomeningocele repair should not be performed in humans until safety could be confirmed in animals.[9-12] Others thought that fetal disturbances were due to over-distension of the sheep uterus and that the thicker human uterus would prevent these disturbances.[8, 14, 36, 63] Despite the lack of evidence supporting the fetal safety of amniotic insufflation in animals, fetoscopic myelomeningocele repair was approved for use in a small number of preliminary human cases (shown in Figure 6).[36, 63, 64]

Table 1: Early animal studies investigating the effect of amniotic insufflation on the fetal lamb

Author		Luks 1994[10]	Pelletier 1995[11]	Saiki 1997[12]	Gratacos 2000[9]
Study details	Species	Sheep	Sheep	Sheep	Sheep
	Gestational Age (days)	95-105 (term 147)	120 (term 147)	104 – 121 (term 147)	92-100 (term 147)
	Number (n =)	8	9	7	10
Insufflation details	Gas	Carbon dioxide	Carbon dioxide	Carbon dioxide	Carbon dioxide
	Amniotic Fluid Drained (ml)	0	All	0	0
	Insufflation pressure (mmHg)	3-4	15	10	4-5
	Duration (minutes)	30	30	30	60
Fetal acid base changes	pH	7.6 → 7.11	7.26 → 7.16	7.30 → 7.2	7.24 → 7.06
	$PaCO_2$ (mmHg)	57.6 → 87.0	64.0 → 83.0	51.0 → 72.0	55.0 → 88.0
	PaO_2 (mmHg)	"Stable"	20.0 → 24.0	24.0 → 28.0	19.0 → 22.0

Table 1: Abbreviations: PaCO2 – arterial partial pressure of dissolved CO2, PaO2 – arterial partial pressure of dissolved oxygen

Figure 6: The landmark studies in fetal surgery for myelomeningocele

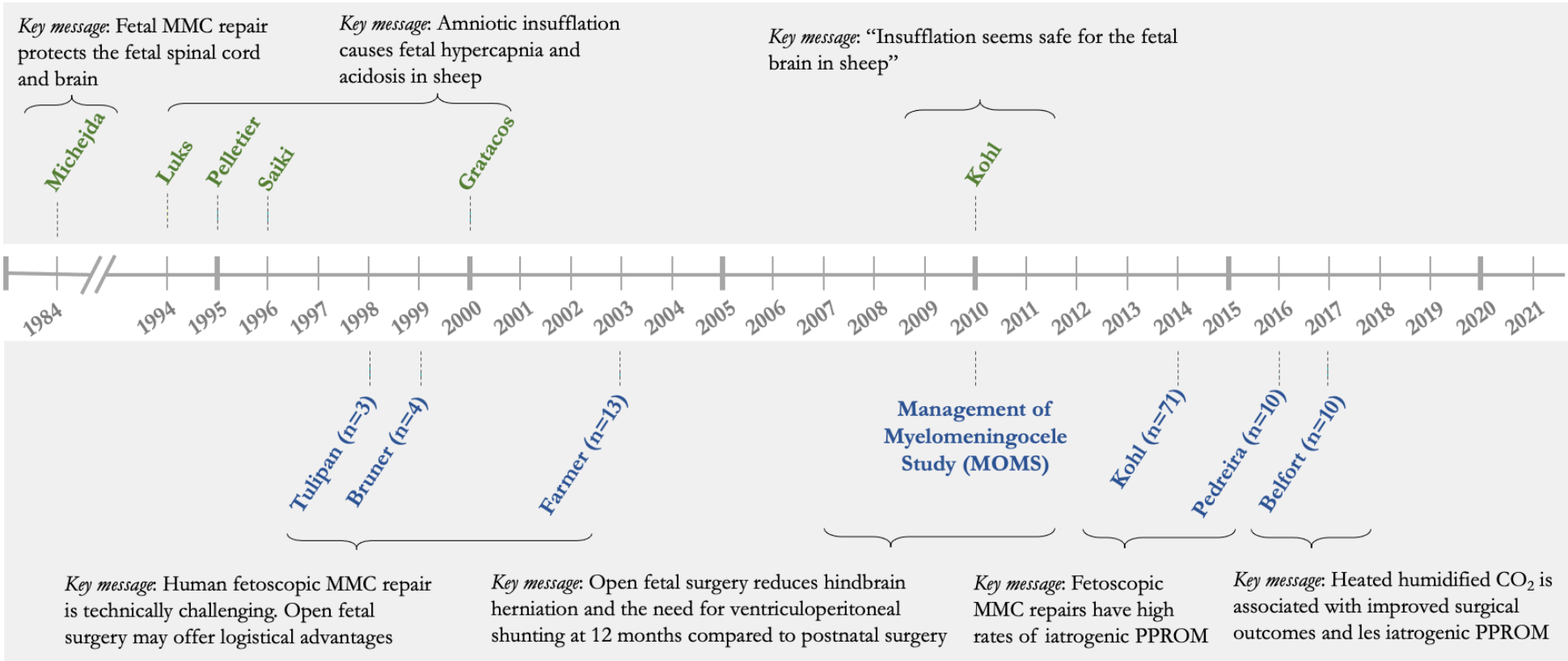


Figure 6: Landmark animal (**green**) and human (**blue**) studies in the fetal management of myelomeningocele (MMC). *1984*: Michejda et al. established that fetal myelomeningocele MMC repair improves spinal cord function at birth using sheep. *1994 – 2000*: Luks et al.., Pelletier et al.., Saiki et al. and Gratacos et al. suggest that amniotic insufflation causes fetal hypercapnia and acidosis in sheep. *1997 – 2003*: Tulipan et al., Bruner et al. and Farmer et al. performed the first human cases of fetal MMC repair using a fetoscopic approach. These cases were largely unsuccessful, and an open approach was then adopted. 2010: The Management of Myelomeningocele Study (MOMS) demonstrated that open fetal surgery improves infant outcomes compared to postnatal surgery. *2010*: Kohl et al. continued to persure fetoscopic repair in sheep and conclude that amniotic insufflation appeared safe for the fetal brain in sheep. *2014 – 2016*: Kohl et al. and Pedreira et al. performed their first human cohorts revisiting fetoscopic MMC repair however, there were concerns surrounding high rates of iatrogenic preterm membrane rupture (PPROM). *2017 – 2018*: Belfort et al. continued to refine the fetoscopic technique in small series suggesting potential fetal benefits to using heated humidified CO_2 for insufflation. – original image

The first human fetal myelomeningocele repairs (proof of principle): (1998-2003)

The first four human fetal myelomeningocele repairs were published between 1998 and 1999 (shown in Figure 6 and summarised in Table 2).[63, 64] Surgeries were initially performed using a variation of the fetoscopic technique where the uterus was partially delivered through a mini-laparotomy and the fetoscopic ports inserted directly into the uterus.[63] Manipulating fetal tissues and repairing the spine using the fetoscopic technique proved very challenging. Instead of a multi-layered spinal closure as performed in open fetal surgery, the neural placode was separated from the skin and covered with a maternal skin graft.[63]

Unfortunately, these preliminary cases were largely unsuccessful.[63] One fetus died intra-operatively and another could not be resuscitated after preterm delivery at 24 weeks gestation due to intraamniotic infection (chorioamnionitis). The two surviving fetuses were born with thin membranous coverings over the myelomeningocele and displayed only mild lower limb deficits at 6 and 30 months of age. However, both were delivered preterm, needed postnatal surgery to re-cover the spinal cord and required ventriculoperitoneal shunting to treat hydrocephalus.[63] Farmer et al. published three additional cases using a similar technique in 2003 however, these too, were largely unsuccessful and included one intrauterine fetal death and one preterm delivery at 31 weeks gestation.[36]

These poor fetal outcomes from the first human fetal myelomeningocele repairs were attributed to difficulties manipulating the fetus and covering the spinal defect with fetoscopic instruments.[63] In response, both Bruner et al. and Farmer et al. adopted the open fetal surgery approach that had demonstrated clear benefits in the preliminary animal studies.[36, 64] This technique allowed surgeons to position the fetus more easily and cover the fetal spinal cord in three layers using a technique that closely resembled postnatal repair.[36, 64, 65] To minimise the risks to the mother, the hysterotomies were kept as small as possible and all cases and future pregnancies were delivered by caesarean section to avoid uterine contractions that may cause the scar to rupture.[36, 64]

Although rates of preterm delivery were still high, open fetal myelomeningocele repair significantly improved neonatal and paediatric outcomes compared to initial human cases.[66-69] Compared to matched postnatal repairs, neonates that underwent open myelomeningocele repair had fewer complications from hindbrain herniation and had better lower leg function at birth and required less ventriculoperitoneal shunting by 12 months of age.[66-69] Ongoing follow up showed that children who had open fetal myelomeningocele repair were also more likely to walk independently at 5 years of age and had better neurodevelopmental outcomes.[70]

While these outcomes suggested that open fetal myelomeningocele repair achieved better neonatal and paediatric outcomes than postnatal surgery, these benefits were based on comparisons to historically matched cases that did not reflect the standard of postnatal care at the time. Additionally, if open fetal surgery was to become the new gold standard, any benefits to the child would need to be considered against the risk of preterm delivery and the maternal implications of a mid-trimester hysterotomy.[36] This equipoise provided the rationale for a multi-centred randomized control trial comparing open fetal myelomeningocele repair and postnatal surgery - The Management of Myelomeningocele Study (MOMS).[7]

Table 2: First attempts at fetal myelomeningocele repair

	Centre	Vanderbilt University Medical Center[63, 64]		University of California San Francisco[36]	
	Cohort	1999, N=4	1998, N=3	2003, N=3	2003, N=10
Uterine Access	Gestational age at surgery (Weeks)	22-24	28-29	19-25	20-23
	Uterine access	Fetoscopic – Exteriorized Uterus	Open fetal surgery	Fetoscopic – Exteriorized Uterus	Open fetal surgery
Defect Closure	Technique	Maternal Split thickness skin graft	Three layer neurosurgical closure	Alloderm or amnion patch	Three layer neurosurgical closure
	Operative time (minutes)	218-383	n.s.	n.s.	n.s.
Fetal Outcomes	Perinatal mortality	2/4	0/3	2/3	2/10
	Gestational age at delivery (weeks$^{+\ days}$)	$24^{+3} – 35^{+1}$	33-36	22-35	22-34
	Preterm delivery <30 weeks	3/4	0/3	1/3	3/10
	Repair dehiscence	2/2	0/3	2/2	3/10
	Requirement for VP shunting at 12 months	2/2	1/3	2/2	3/8
Maternal Outcomes	Preterm prelabour rupture of membranes	1/2	0/3	2/3	7/10
	Uterine scar separation	0/4	1/3	0/3	0/10
	Chorioamnionitis	1/4	0/3	0/3	1/10
	Pulmonary oedema	0/4	0/3	1/3	0/10

Figure 7: Surgical technique – open myelomeningocele repair

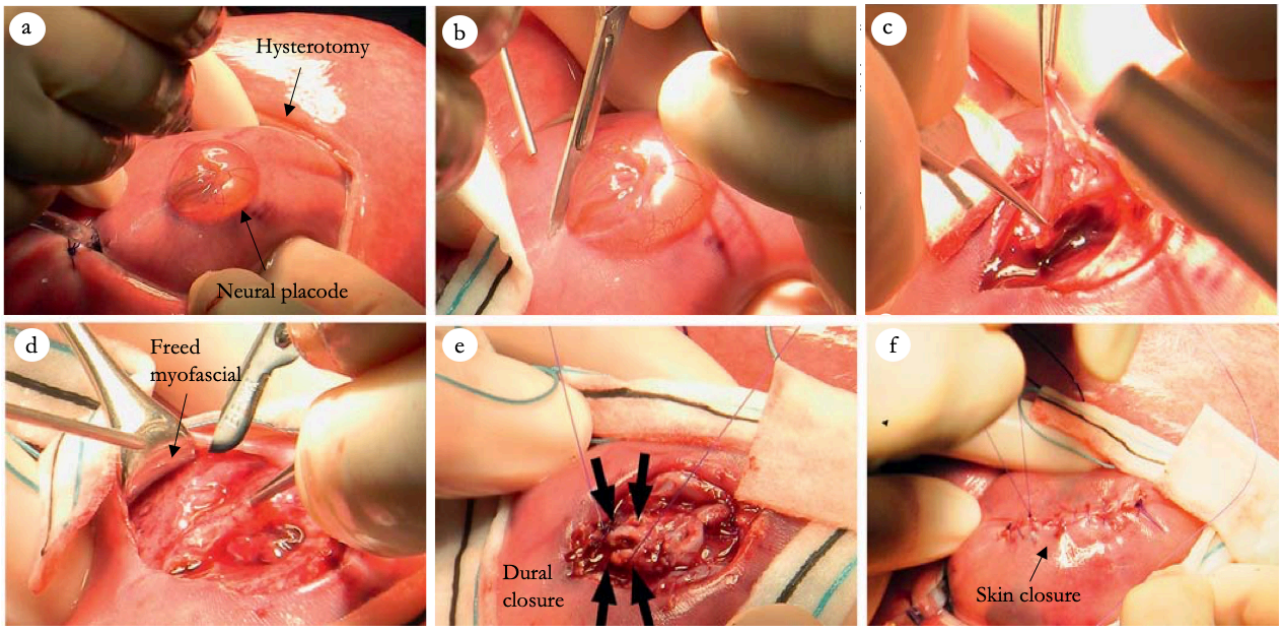


Figure 7: A hysterotomy incision is made to access the fetus (a). The edges of the neural placode are then dissected from the surrounding skin (b). The arachnoid and residual epithelial tissue is dissected from the edges of the freed placode (c). The lateral myofascial is then freed (d) and closed over the repaired spinal cord (e – black arrows). The skin is then closed over the closed myofascial (f) Image adapted from Huer et al. 2015.[65]

The Management of Myelomeningocele Study (MOMS): (2003 – 2010)

The MOMS randomized 183 cases of fetal myelomeningocele to open fetal surgery (n=91) or standard postnatal repair (n=92) between February 2003 and December 2010.[7] Compared to standard postnatal repair, open fetal surgery significantly reduced hindbrain herniation (96 vs. 64%) and the need for ventriculoperitoneal shunting (82 vs. 40%) at 12 months of age. Open fetal surgery also improved gross motor and mental function at 2.5 and 6 years of age as well as improved mobility outcomes by 10 years of age.[7, 71, 72] While both the prenatal and postnatal surgery groups had ongoing issues with urinary incontinence, parental reports suggested that children who underwent open fetal surgery were also more likely to void volitionally than the postnatal group.[73]

Despite significantly reducing paediatric morbidity, the MOMS showed that open fetal surgery still carried significant risks for the fetus and neonate. Compared to standard postnatal repair, open fetal surgery had higher rates of iatrogenic PPROM (36 vs. 6%), oligohydramnios (16 vs. 3%), preterm birth (79 vs. 15%) and respiratory distress syndrome (21 vs 6%). Concerningly, 13% of preterm births in the open fetal surgery group occurred before 30 weeks gestation (extremely preterm) compared to 0% that underwent postnatal repair.[7] This was particularly worrying as extreme preterm birth was known to be associated with poor neurological outcomes and may have offset the benefits of prenatal myelomeningocele repair.[7]

Open fetal surgery also had significant consequences for the mother. Despite attempts to keep the size of the hysterotomies as small as possible at the time of fetal surgery, 25% of scars were described as thin when inspected at caesarean section, 9% had an area of partial separation and 1% had completely separated.[7] Follow up of mothers that underwent open fetal surgery showed that 6-14% suffered uterine rupture and 3% required hysterectomy with subsequent pregnancies.[74] Several of these uterine ruptures were also associated with perinatal death.[74]

The MOMS concluded that, although the maternal implications were significant, open fetal surgery should be offered as the gold standard to improve postnatal outcomes for fetal myelomeningocele.[7, 75] Since the MOMS, the demand from patients and the number of centres offering open fetal surgery has steadily increased, including one Australian centre, the Mater Mothers Hospital in Brisbane.[76] All centres offering the procedure are encouraged to use the same selection criteria, surgical technique and patient follow up protocols to the original MOMS.

Revisiting fetoscopic myelomeningocele repair: (2003 – 2017)

Despite the largely unsuccessful outcomes of preliminary myelomeningocele repairs (summarised in Table 2), two large fetal surgery centres continued to pursue fetoscopic repair in parallel to the MOMS with the aim of reducing maternal hysterotomy related complications.[8, 14, 57, 77-79] However, before fetoscopy could be re-attempted in humans, these centres needed to refine the surgical technique and address concerns about fetal hypercapnia and acidosis during amniotic insufflation.

Kohl et al. performed a series of fetoscopic myelomeningocele repairs on fetal lambs with surgically created spinal defects.[57, 78] However, instead of partially exteriorising the uterus like the initial human studies, the group used a completely percutaneous approach where fetoscopic ports were inserted through the maternal abdomen and uterus.[57, 78] The group delivered the lambs at term and assessed their gross motor and hind leg function to confirm their ability to protect the fetal spinal cord using a fetoscopic approach.[78] Additionally, they collected the brains of the newborn lambs to look for histological evidence of brain injury that may be related to hypercapnia and acidosis during insufflation.[78] Newborn lambs that underwent fetoscopic repair showed normal motor function at delivery and had no histological evidence of brain injury using haematoxylin and eosin staining.[78] The studies concluded that percutaneous fetoscopic repair had improved significantly and that amniotic insufflation seemed safe for the fetal brain in sheep (shown in Figure 6).[57, 78]

However, these sheep preclinical studies had several limitations.[57, 78] They used insufflation pressures (7-15 mmHg) and durations (45-80 min) that were significantly less than those used in the initial human surgeries (>15mmHg for ≈180 minutes) which potentially underestimated the acid base and haemodynamic effects of insufflation on the fetal lamb.[36, 57, 63, 78] Additionally, gross motor testing of newborn lambs may have missed subtle neurological impairment that was clinically significant for the human newborn.[78] Haematoxylin and eosin staining may also have missed inflammatory, neuronal and vascular markers of brain injury that may have been detected on more comprehensive immunohistochemistry.[78, 80]

Despite these limitations, Kohl et al. and Pedreira et al. published their first human myelomeningocele repairs using a percutaneous fetoscopic approach between 2010 and 2016 (shown in Figure 6).[8, 14, 79] Encouragingly, maternal hysterotomy related complications were avoided using fetoscopy and, after negotiating the technical learning curve, rates of hindbrain herniation and ventriculoperitoneal shunting at 12 months appeared similar to open fetal surgery.[7, 8, 14, 79] However, percutaneous fetoscopic myelomeningocele repair still carried inherent risks. Iatrogenic PPROM rates were much higher than the MOMS (83.4-100% vs. 36%) and the gestational age of delivery considerably lower (32^{+2} vs. 34^{+1} weeks).[7, 8, 14] Additionally, the three-layered spinal repair (meninges, thoracolumbar fascia and skin) used

in open fetal surgery and postnatal repair could not be replicated fetoscopically. Instead, a collagen/biocellulose patch was used to cover the spinal cord and sutured into the surrounding skin.[8, 14] 12.5-28% of these patch repairs had separated at delivery and frequently required postnatal surgery to repair the spine. It was suggested that although fetoscopic outcomes had improved significantly using a percutaneous fetoscopic approach, the technique could not replace open fetal surgery until three layered spinal repair could be replicated.[8, 14]

Monitoring the fetus within the insufflated uterus was also challenging during fetoscopy.[8, 14] The large pocket of CO_2 gas within the uterus limited fetal monitoring to observations of umbilical cord pulsations or intermittent ultrasound of umbilical cord loops submerged in residual amniotic fluid.[8, 14] However, both methods were not reliable predictors of fetal distress particularly in the context of fetal general anaesthesia which was thought to blunt fetal heart rate changes.[81-84] Reassuringly, extensive fetal ultrasound immediately following surgery suggested that the fetus was able to tolerate fetoscopic surgery and amniotic insufflation.[8, 14, 85]

Belfort et al. continued to refine fetoscopic myelomeningocele repair and published the outcomes of 22 consecutive cases in 2017 (shown in Figure 6).[17] Although only a small cohort, rates of iatrogenic PPROM and preterm birth were considerably lower than had previously been reported (summarised in Table 3). The group attributed these improvements to refinements in the way the uterus was accessed an insufflated. Like the first fetoscopic myelomeningocele repairs, the group partially exteriorised the uterus through a mini-laparotomy and inserted ports directly into the uterus (shown in Figure 8).[17] They suggested this partially exteriorised approach permitted lower insufflation pressures during surgery which prevented overstretching of the fetal membranes. Additionally, the group plicated the fetal membranes to uterine wall at the point where the ports were inserted to prevent the fetal membranes separating (chorioamniotic separation) and then rupturing early after surgery. To further minimise membrane damage during surgery, the group heated and humidified the CO_2 used for insufflation. Heated, humidified insufflation had previously been introduced in endoscopic abdominal and thoracic surgery as a way of minimising irritation to peritoneal and pleural linings of the distended cavities.[17]

Although partially exteriorising the uterus, plicating the fetal membranes and using heated, humidified CO_2 for insufflation all correlated with lower iatrogenic PPROM in Belfort's study, it remains unclear which, if any, factor was responsible for the improved outcomes. However, these observations have provided several important insights into preventing iatrogenic PPROM after fetoscopy which are discussed in our drafted review paper found in Appendix 1 and in more detail below.

Belfort et al. also made several refinements to the way the fetal spine was closed.[17] Instead of using a patch to cover the spinal cord, the group attempted a layered spinal repair that more closely resembled open fetal surgery and postnatal repair. Although layered repair still proved difficult fetoscopically, they were able to successfully cover the spinal cord with single tissue layer containing the dura, thoracolumbar fascia and skin.[17]

Since 2017, many international fetal therapy centres have started performing fetoscopic myelomeningocele repair. However, there is considerable variability in the techniques used to access and insufflate the uterus, monitor the fetus during surgery, repair the fetal spine, close the fetal membranes and manage the pregnancies post-operatively making it difficult to compare outcomes. A summary of the different approaches to performing fetoscopic myelomeningocele repair is included in our drafted review paper found in Appendix 2.[86] In 2019, an international consortium was established to foster collaboration between these centres and help to identify the optimal fetoscopic technique to compare to open fetal surgery.[86]

Table 3: Fetoscopic myelomeningocele repair revisited in humans

	Centre	Centre for Fetal Surgery & Minimally Invasive Therapy, Germany	Albert Einstein Hospital, Brazil	Texas Children's Hospital, USA
	Author	Kohl et al.	Pedreira et al.	Belfort et al.
	Cohort	2014 N=71	2016 N=10	2017 N=22
Uterine Access	Mean gestational age at surgery (Weeks$^{+\ days}$)	22^{+5}	26^{+4}	26^{+5}
	Uterine access	Fetoscopic	Fetoscopic	Fetoscopic with exteriorized uterus
	Port number and diameter (mm)	3-4 x 5mm	2 x 3.6mm 1 x 5mm	2 x 4mm
	Uterine membrane closure	No Closure	No Closure	Plicated membranes to uterine wall with 2/0 Suture
Defect Closure	Technique	Patch: Collagen/Teflon plus overlying skin	Patch: Biocellulose patch plus overlying skin	Direct Closure: Single layer - Skin with Dura
	Mean Operative time (minutes)	223	242	246
Amniotic Insufflation	Gas	Carbon dioxide	Carbon dioxide	Carbon dioxide
	Heated humidified	No	No	Yes
	Amniotic fluid drained (ml)	0	0	300
	Mean insufflation pressure (mmHg)	15.3	15.6	12.0
Fetal Outcomes	Perinatal mortality	7% (5/71)	20% (2/10)	4.5% (1/22)
	Mean gestational age at delivery (weeks$^{+\ days}$)	32^{+2}	32^{+4}	38^{+1}
	Preterm delivery <30 weeks	13.2% (9/71)	20% (2/10)	0% (0/22)
	Repair dehiscence	28% (20/71)	12.5% (1/8)	32% (7/22)
	Requirement for VP shunting at 12 months	45% (32/71)	43% (3/7)	41% (9/22)
Maternal Outcomes	Preterm Premature Rupture of Membranes	84.3% (43/51)	100% (10/10)	23% (5/22)
	Chorioamniotic separation	5.8% (3/51)	40% (4/10)	32% (7/22)
	Oligohydramnios	13.7% (7/51)	40% (4/10)	14% (3/22)
	Chorioamnionitis	5.8% (3/51)	0% (0/10)	0% (0/22)
	Uterine dehiscence or rupture	0% (0/51)	0% (0/10)	0% (0/22)
	Pulmonary Oedema	1.9% (1/51)	0% (0/10)	9% (2/22)

Figure 8: Uterine access for fetoscopic surgery

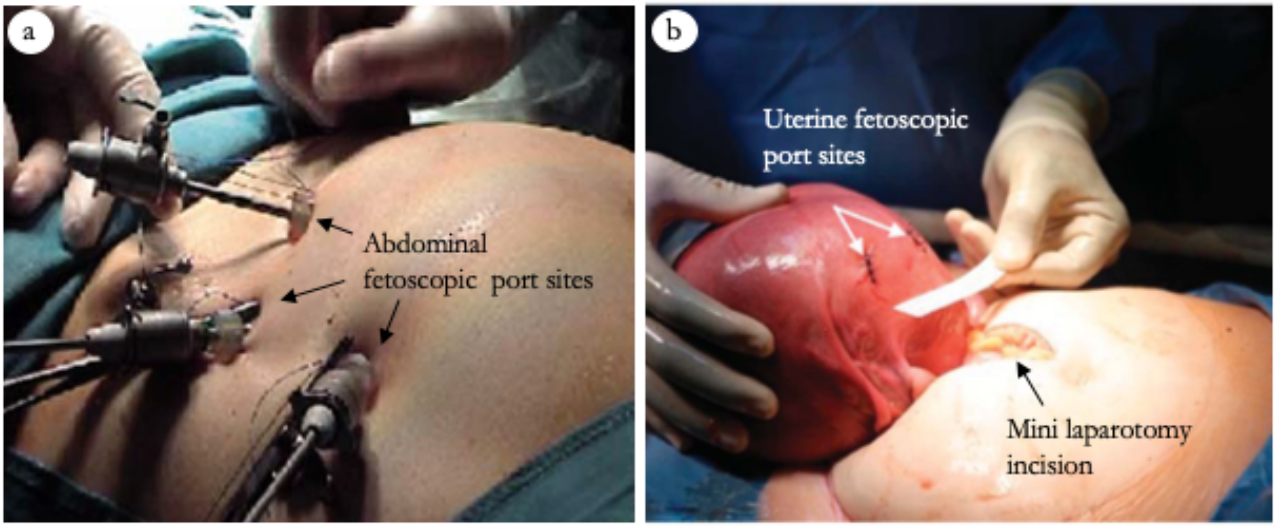


Figure 8: Fetoscopic ports can be inserted through the abdominal and uterine wall into the amniotic space (a.) or directly into the externalized uterus (b.) – Adapted from Kohl et al. and Belfort et al.[8, 17]

The potential effects of amniotic insufflation on the fetus and fetal membranes

The body that oversees the development and implementation of new fetal therapies, the International Fetal Medicine and Surgery Society (IFMSS), strongly encourages that the safety of any new fetal intervention should be demonstrated in a large animal model before it is used in humans.[87] Clearly, at the time of starting this thesis, the fetal safety of amniotic insufflation has not been demonstrated in animals. No animal study has investigated the effect of insufflation on the fetus or fetal membranes at clinically relevant insufflation pressures or durations. Additionally, the negative effects of amniotic insufflation observed in a series of lamb experiments were dismissed by arguing that overdistension of the sheep uterus was likely different to humans. While using heated, humidified CO_2 for amniotic insufflation is associated with improved fetal outcomes and lower rates of iatrogenic PPROM there is a distinct lack of preclinical evidence to support these links. The following reviews the physiology that underpins how amniotic insufflation may affect the fetus and fetal membranes as well as the potential benefits of heating and humidifying the gas.

The potential effects of amniotic insufflation on the fetus

Regulation of blood CO_2 levels

After birth, CO_2 is a normal product of cellular oxidative metabolism and must be transported from the tissues to the lungs for elimination. Transportation occurs in 3 ways (shown in Figure 9). Approximately 10% dissolves in blood plasma while 20% binds to haemoglobin proteins in red blood cells forming carbaminohaemoglobin. An enzyme within red blood cells known as carbonic anhydrase, converts the remining 70% of blood CO_2 into bicarbonate and hydrogen ions (H^+). These ions exist in equilibrium within the plasma which is shown below.[88, 89]

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$$

The production of H^+ makes blood CO_2 levels an important determinant of serum pH. Since maintaining blood pH between 7.35 and 7.45 is essential for normal cellular and enzyme function, blood CO_2 and H^+ levels are tightly regulated.[88, 89] Blood proteins and bicarbonate ions bind excess H^+ and prevent sudden reductions in blood pH (acidosis). However, these buffers can become saturated when CO_2 or H^+ levels rise quickly. As CO_2 and H^+ levels increase, chemoreceptors in the respiratory centres of the brainstem recognise the increase and trigger an increase in respiratory drive that usually involves an increase in respiratory rate and tidal volume. This increases respiratory CO_2 elimination and

lowers blood H^+.[89] Prolonged elevations in H^+ trigger further compensation by the kidney. Cells in the distal convoluted tubule of the nephron remove H^+ from the plasma and resynthesize bicarbonate ions. These additional bicarbonate ions bind free H^+ to help restore normal pH.[88, 89]

During fetal life, blood bicarbonate levels are low, the lungs play no role in CO_2 elimination and the kidneys are not able to resynthesize bicarbonate ions. Instead, blood CO_2 and pH regulation occurs in the placenta (shown in Figure 10). Fetal arterial blood with a high partial pressure of dissolved CO_2 ($PaCO_2$) moves through the umbilical artery into the placenta. Within the placenta, fetal and maternal blood is separated by a single layer of cells that permit rapid diffusion of dissolved gasses between the two placental compartments. This allows CO_2 to diffuse passively down its partial pressure gradient, from high concentrations in the umbilical artery to relatively lower concentrations in maternal blood. Continuous high blood flows through the maternal and fetal placental compartments, particularly on the maternal side, prevents CO_2 from reaching an equilibrium between the compartments, which maximises the efficiency of CO_2 elimination from the fetus.[90-92] Fetal umbilical venous blood leaves the placenta with relatively low $PaCO_2$ and high PaO_2 levels and returns to the fetus.

Several physiological factors exaggerate the $PaCO_2$ difference within the placenta to assist fetal CO_2 elimination. High levels of maternal circulating progesterone during pregnancy increases maternal resting respiratory rate and tidal volume. These changes lower maternal baseline $PaCO_2$ levels to below non-pregnant levels (35-45mmHg to 30-32mmHg), which increases the transplacental $PaCO_2$ and promotes fetal CO_2 elimination from the placenta.[93] Fetal CO_2 elimination is also enhanced by reoxygenation of fetal haemoglobin within the placenta. As oxygen (O_2) diffuses into the fetal compartment within the placenta it binds to fetal haemoglobin and displaces bound CO_2 into the plasma. This displacement by O_2, known as the Haldane effect, increases the local $PaCO_2$ in the fetal placental compartment and increases the partial pressure gradient that drives fetal CO_2 elimination.

Unlike pulmonary ventilation after birth, the placenta has very limited ability to increase the rate of fetal CO_2 elimination to accommodate sudden or prolonged hypercapnia. Given the immaturity of the fetal kidney and blood buffer systems, this limitation of the placenta may leave the fetus vulnrable to hypercapnia and acidosis during amniotic insufflation.

Figure 9: Transportation of carbon dioxide in the blood

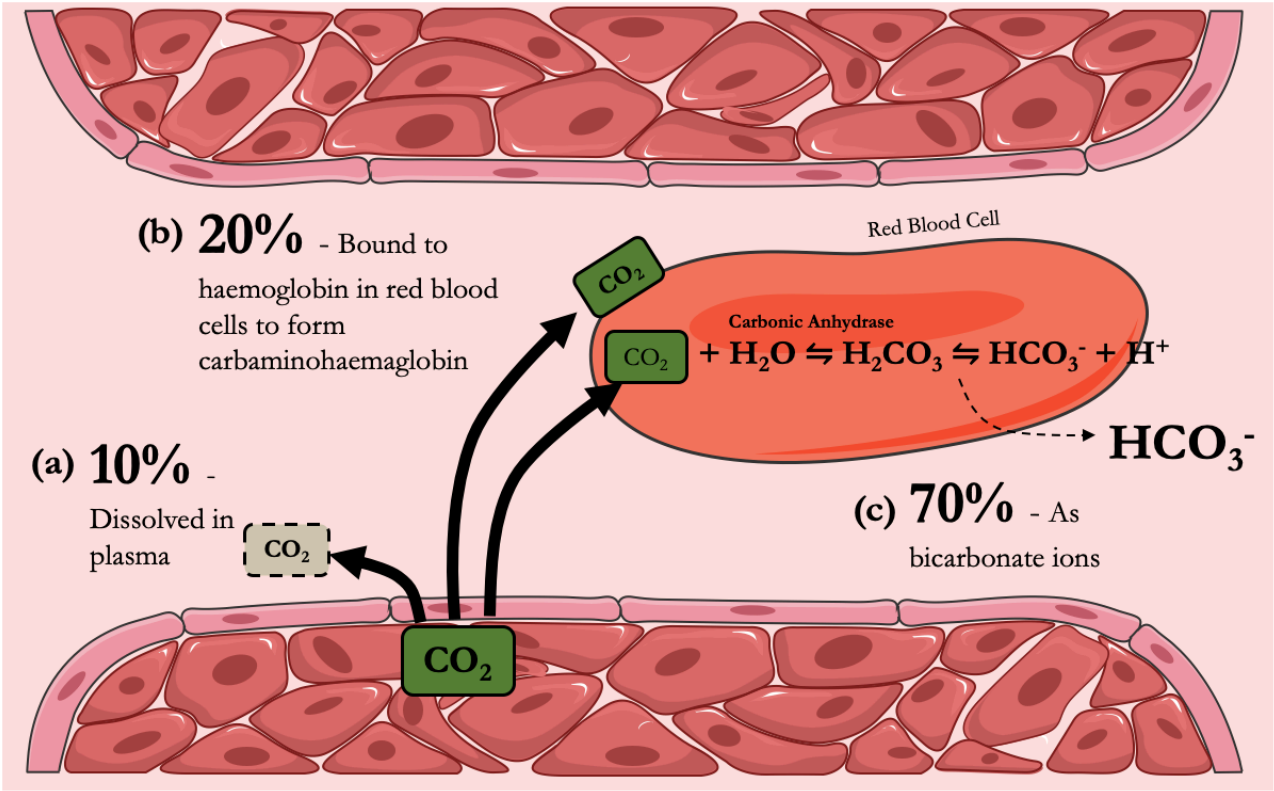


Figure 9: Carbon Dioxide is transported in the blood in 3 ways. 10% dissolves in the serum (a), 20% binds directly to the haemoglobin molecule of red blood cells to form carbaminohaemoglobin (b) and 70% is converted to bicarbonate ions by carbonic anhydrase (c) – original image published in our review – Skinner et al.[94]

Figure 10: Placental exchange of carbon dioxide and oxygen

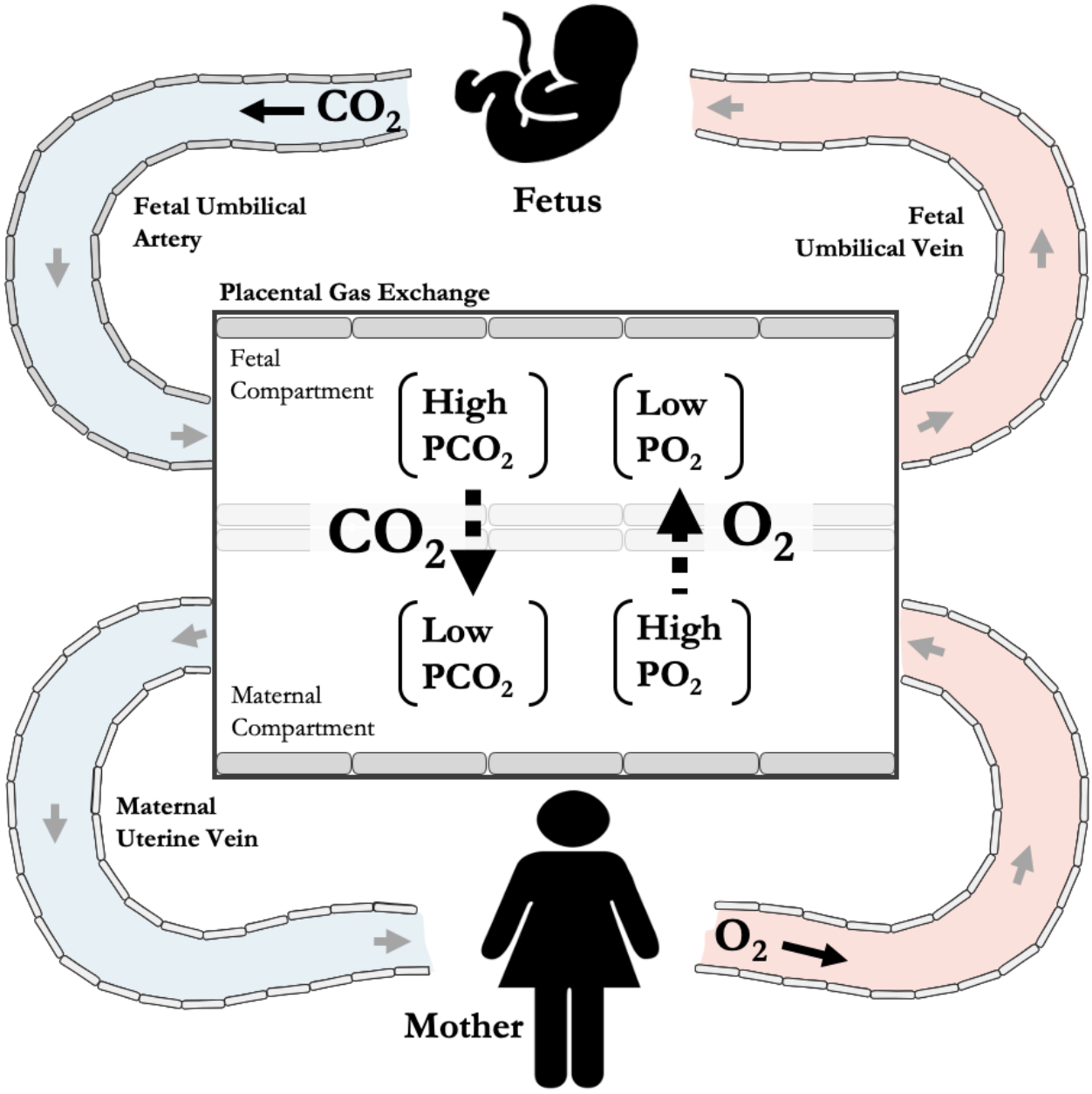


Figure 10: Fetal blood is cleared of carbon dioxide and re-oxygenated in the placenta. Both carbon dioxide and oxygen diffuse from regions of high to low partial pressure across the placental membrane that separates the fetal and maternal compartments. PCO_2 – partial pressure of CO_2, PO_2 – partial pressure of oxygen – original image.

The physiological effects of fetal hypercapnia and acidosis

→ *Cardiovascular effects*

Both hypercapnia and acidosis directly impair the ability of cardiac myocytes to contract.[95-100] To protect the fetus from sudden reductions in cardiac output, the fetal sympathetic nervous system acts directly on cardiac myocytes to increase fetal heart rate and the force of ventricular contraction. These compensatory mechanisms become insufficient to maintain cardiac output when hypercapnia and acidosis is severe (pH <7.2), resulting in a sudden decrease in heart rate (bradycardia) and blood pressure (hypotension).[96]

It is currently unknown if, or how severely, fetal hypercapnia and acidosis effects the fetal cardiovascular system during fetoscopic myelomeningocele repair. Episodes of increased (tachycardia) or decreased (bradycardia) fetal heart rate have only rarely been observed during human fetoscopy.[8, 14] As discussed above, these observations were made on ultrasound of umbilical cord loops that remained submerged in the amniotic fluid during insufflation.[8, 14] However, umbilical artery and vein ultrasound changes poorly correlate with fetal hypercapnia and acidosis. Additionally, both fetal and maternal general anaesthetics may blunt fetal heart rate changes.[81-84] Reassuringly, fetal cardiac function several days after fetal myelomeningocele repair appears normal suggesting that if hypercapnia and acidosis is present intraoperatively it may only be short lived.[85]

→ *Neurological effects*

Growth restricted human fetuses exposed to chronic hypercapnia and acidosis during pregnancy show impaired brain development and an increased incidence of neurodevelopmental delay.[13] Rodent growth restriction studies suggest that these outcomes may be due to impaired neuron oxygen metabolism during hypercapnia and acidosis which causes apoptosis.[101-104] Although fetuses undergoing fetoscopic myelomeningocele repair likely experience much shorter periods of hypercapnia and acidosis than growth restricted infants, they already have congenital brain injury which may leave neurons more susceptible to further damage.

Fetal hypercapnia and acidosis during insufflation may also damage the vasculature supplying the fetal brain. During hypercapnia, the fetal sympathetic nervous system dilates cerebral blood vessels to maintain oxygen delivery to the developing brain, but this also exposes these fragile vessels to high pressures and flows which may cause them to rupture.[99]

Investigating the effects of amniotic insufflation on the human fetus

In 2018, a small case series (n=3) aimed to provide the first human data investigating the effects of amniotic insufflation on the human fetus.[16] Fetal blood gasses were taken via cordocentesis (umbilical vein) after fetal anaesthetic was administered at the start of surgery (prior to insufflation) and then again after desufflation of the uterus. Fetoscopic myelomeningocele repairs lasted an average of 160 minutes and the uterus was insufflated up to a maximum of 8-10 mmHg.[16]

In the first case, fetal umbilical vein $PaCO_2$ rose and pH decreased, thankfully to a lesser degree than seen in earlier animal studies (summarised in Table 4). While these results were reassuring that human fetal acid base changes are less severe than those observed in animals, "post-surgery" blood gasses were taken some time after insufflation and may have underestimated blood gas changes intra-operatively. Additionally, the use of umbilical vein PCO_2 in these human cases may have recorded lower CO_2 values than animal studies sampling blood from the umbilical artery.[16]

Interestingly, in the third case that used heated, humidified CO_2 for insufflation, no rise in umbilical vein CO_2 or decrease in pH was observed.[16] Clearly this small study cannot conclude that heated, humidified insufflation has acid base benefits for the fetus however, it provides an important hypothesis to test in a large animal model.

Table 4: Fetal umbilical venous blood gas values during fetoscopic myelomeningocele repair

	Case 1		Case 2		Case 3	
Details						
Insufflation Gas	Carbon Dioxide		Heated Humidified Carbon Dioxide		Heated Humidified Carbon Dioxide	
Duration (Minutes)	182		159		149	
Blood Gasses	Pre	Post	Pre	Post	Pre	Post
PCO_2 (mmHg)	39	47	32	46	39	37
pH	7.36	7.28	7.46	7.35	7.37	7.36
PO_2 (mmHg)	74	67	59	25	59	61
HCO_3 (mmol/L)	22	21	22	25	22	21
Base Excess (meq/L)	-3	-5	-1	-1	-2	-4

Table 4: Abbreviations: PCO_2 – partial pressure of carbon dioxide, PO_2 – partial pressure of oxygen, HCO_3 – bicarbonate. Adapted from Baschat et al. 2018[16]

Reducing fetal hypercapnia and acidosis during amniotic insufflation

Given the potential implications of hypercapnia and acidosis on the fetus during insufflation, several groups have attempted to develop strategies to mitigate these effects in sheep. Efforts have mainly focused on increasing the mothers respiratory rate and tidal volume (hyperventilation) during insufflation[12, 105] and changing the insufflated gas.[9, 11, 106] Unfortunately, both strategies have shown limited success however, these studies provide important insights into how fetal acid base disturbances may be mitigated in the future.

→ *Maternal hyperventilation*

As described above, fetal CO_2 diffuses down its partial pressure gradient into the maternal placental compartment (Figure 10). Two sheep studies investigated whether hyperventilating the mother during insufflation and lowering maternal $PaCO_2$ could increase fetal CO_2 elimination and mitigate fetal hypercapnia.[9, 12] In both studies, 30-minutes of maternal hyperventilation during insufflation partially mitigated fetal hypercapnia and acidosis.[9, 12] While this appeared promising, an earlier study on fetal lambs had demonstrated that prolonged periods of hyperventilation (>30 minutes) reduced umbilical blood flow, caused fetal hypoxia and metabolic acidosis.[105] These findings suggested that short periods of maternal hyperventilation could be used during human fetoscopy however, would need to be paired with careful monitoring of fetal and placental blood flows.

→ *Changing the insufflation medium*

Groups have also attempted to change the gas used for insufflation to prevent CO_2 entering the fetal blood. While this seems logical, selecting an alternative gas has proven difficult as insufflation gasses must have very specific chemical properties.[61] During amniotic insufflation, small volumes of pressurised gas are forced into fetal (umbilical) and maternal (uterine) blood vessels. The insufflated gas must therefore have a high solubility to avoid forming bubbles in the blood that may move downstream and occlude vessels perfusing vital organs (gas embolism). Gas emboli have limited treatment options and are potentially fatal.[61] Gasses used for insufflation must also be non-combustable, relatively cheap, safe to store and have no other adverse effects on the mother or fetus.[61]

A series of sheep studies demonstrated that amniotic insufflation with helium or nitrous oxide gas causes less fetal hypercapnia and acidosis than using CO_2.[9, 11, 106] Unfortunately, helium has a much lower solubility and is more costly to store than CO_2 while nitrous oxide is potentially teratogenic (summarised in Table 5).[61, 107, 108] Air, nitrogen and argon gasses have also been tested for insufflation in other fields of endoscopic surgery however, all have potential negative implications for the fetus which limit their use within the uterus.[61]

Carbon dioxide gas meets most of the important criteria for amniotic insufflation (Table 5) suggesting that modifying the gas to mitigate its effect on the fetus may be better than changing the gas altogether. The small human series by Belfort et al. and Baschat et al. (summarised in Table 3 and Table 4) suggest that amniotic insufflation with heated humidified CO_2 may improve the fetus' ability to tolerate insufflation.[16] Heating CO_2 from 22°c to 40°c reduces its solubility by ≈42% while humidification lowers the partial pressure of CO_2 within the insufflated gas by ≈7% (Dalton's Law).[109, 110] Together, heating and humidifying the insufflated CO_2 may reduce fetal CO_2 absorption during insufflation and allow the fetus to clear excess CO_2. Clearly, preclinical studies in a large animal model are required to confirm this hypothesis.

Table 5: The chemical properties of gasses used to distend body cavities

	Carbon Dioxide (CO_2)	Heated Humidified CO_2	Helium (He_2)	Nitrous Oxide (N_2O)	Nitrogen (N_2)	Air	Argon (Ar_2)
Solubility (ml/100ml H_2O)	171 (Very High)	≈60* (High)	0.97 (Low)	130 (High)	2.3 (Low)	2.92 (Low)	5.6 (Low)
Supports Combustion	No	No	No	Yes	No	Yes	No
Cost	Low	Low	High	Low	Low	Low	High
Other fetal Safety Concerns	Fetal hypercapnia and acidosis	Evidence still required	High thermal conductivity – burn risk	Suppresses placental DNA synthesis. Teratogenicity	N/A	N/A	Reduces cardiac function.

Table 5: * - the solubility of CO_2 at 40°c is approximately 60ml/100ml water

The potential effects of amniotic insufflation on the fetal membranes

Up to 85-100% of fetoscopic myelomeningocele repairs are complicated by iatrogenic PPROM compared to only ≈36% following open fetal surgery.[7, 8, 14] Although the mechanisms of PPROM are poorly understood, this difference suggests that factors specific to fetoscopy are damaging the fetal membranes during surgery making them more likely to rupture early post-operatively. The following summarises the normal structure and rupture physiology of the fetal membranes and suggests how amniotic insufflation may be contributing to high rates of iatrogenic PPROM. A more comprehensive review of how fetoscopy may be increasing the risk of iatrogenic PPROM can be found in Appendix 1.

Human fetal membrane structure

The fetal membranes surround the fetus during pregnancy and play a critical role in maintaining the pregnancy to term (shown in Figure 11). The innermost membrane, the amnion, provides the majority of structural integrity.[111] This membrane is formed 10-14 days post fertilization, when mesoderm cells migrate from between the layers of the bilaminar embryo to fuse with the ectoderm lining of the amniotic cavity.[112] The ectoderm gives rise to the cuboidal amniotic epithelium which is closest to the fetus and held in place by a basement membrane composed of collagens (Type I and III) and glycoproteins (laminin, nidogen, and fibronectin).[112-114] The amniotic mesoderm forms a layer of compact Type I and III collagen beneath the basement membrane that provides the amnion with its tensile strength.[111, 113, 114] This compact layer is produced and maintained by fibroblasts in an adjacent layer of loose collagen also derived from the mesoderm, known as the fibroblast layer.[15, 113] The human amnion is almost completely avascular, sourcing nutrients and exchanging wastes with the amniotic fluid.[112, 115]

The amnion is separated from the outer chorionic membrane by a space known as the intermediate or spongy layer (shown in Figure 11).[15, 112] This space is liquid filled until 12-15 weeks' gestation when the expanding amnion loosely adheres to the chorion. The collagen adhesions and hydrated proteoglycans within the intermediate layer allow the amnion to slide independently over the chorion which increases the membranes tensile strength.[111, 113, 114]

The chorion surrounds the amnion. This membrane is formed by fusion of the mesoderm with the outer cells of the embryo known as the trophoblasts.[112] The chorionic mesoderm differentiates to become a layer of loose reticular collagen and chorionic fibroblasts that faces the amnion.[112] This collagen is supplied by a network of fetal blood vessels arising from the allantois and its outer surface is lined by the trophoblasts. The outer surface of the chorion is covered by a layer of maternal cells known as the decidua, that were once the endometrium overlying the implanted embryo. The decidua is rich with maternal blood vessels and immune cells and fuses with the chorion during early gestation forming the choriodecidua.[112]

Figure 11: Anatomy and histology of the fetal membranes

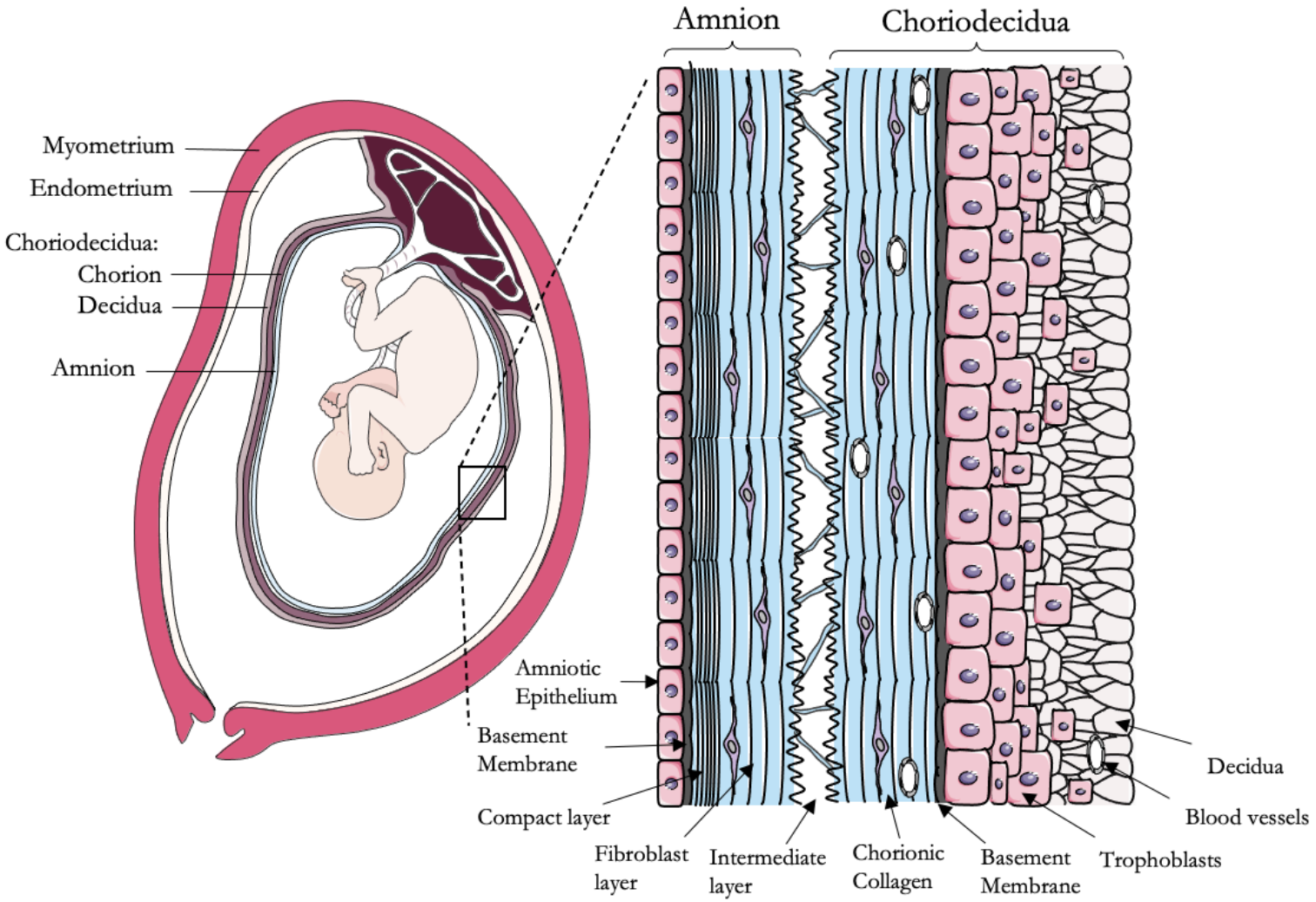


Figure 11: The fetal membranes are made of 3 layers. The innermost amnion, the chorion and decidua. The amnion is composed of a single layer of amniotic epithelium and underlying stroma. The chorionic stroma faces inward and sits on a layer of trophoblasts derived from the placenta. The decidua is in two parts; the decidual epithelium and underlying decidual glands. – original image

Mechanisms of fetal membrane rupture

→ *Term fetal membrane rupture*

During pregnancy there is little change in the composition of the fetal membranes. However, from 37-38 weeks gestation the fetal membranes overlying the cervix progressively thin and weaken.[116-119] This allows the descending fetus and contracting uterus to stretch and eventually rupture the membranes which plays an important role in promoting the onset of labour. The final failing of the membranes is characterised by membrane separation which then promotes rupture of the choriodecidua and amnion in turn.[111]

The progressive membrane weakening that precedes term rupture is thought to be mediated by a family of collagen degrading enzymes known as matrix metalloproteases (MMPs) and their endogenous tissue inhibitors (TIMPs).[120-122] During late gestation, amniotic epithelial cells and chorionic trophoblasts increase the proportion of activated MMPs, particularly MMP-9, within the membrane while reducing TIMPs.[122] This causes degradation of structural membrane collagens and basement membrane proteins which reduces the membrane's tensile strength.[116, 119, 123-131] These focal changes overlying the cervix have been well described as the "zone of high morphological change" and can be clearly seen histologically in both human explants and animal models.[124]

There have been significant efforts to understand the factors that normally induce MMP weakening of the fetal membranes during late gestation. Although a common pathway is yet to be identified, a series of genetic[126, 132-140], epigenetic[141, 142], physical[116, 143-145], inflammatory[116, 146, 147] and hormonal[148, 149] factors have been proposed and are summarised in Figure 12.[114]

→ *Preterm fetal membrane rupture*

Spontaneous fetal membrane rupture before 37 weeks gestation (spontaneous PPROM) occurs in 1-4% of all human pregnancies.[148, 150] The clinical factors associated with spontaneous PPROM can be broadly grouped into those that cause preterm weakening of membrane collagen or abnormal collagen synthesis (summarised in Figure 12).

Membrane weakening

Damage or inflammation within the fetal membranes can prematurely upregulate fetal membrane weakening and predispose preterm rupture. Unlike the focal weakening that occurs over the cervix at term, preterm weakening is often generalized and the site of rupture occurs away from cervix.[151, 152] Bacterial infection of the membranes (chorioamnionitis) is associated with ≈30% of spontaneous PPROM cases.[147, 153, 154] Proteins specific to bacterial cells known as pathogen associated molecular

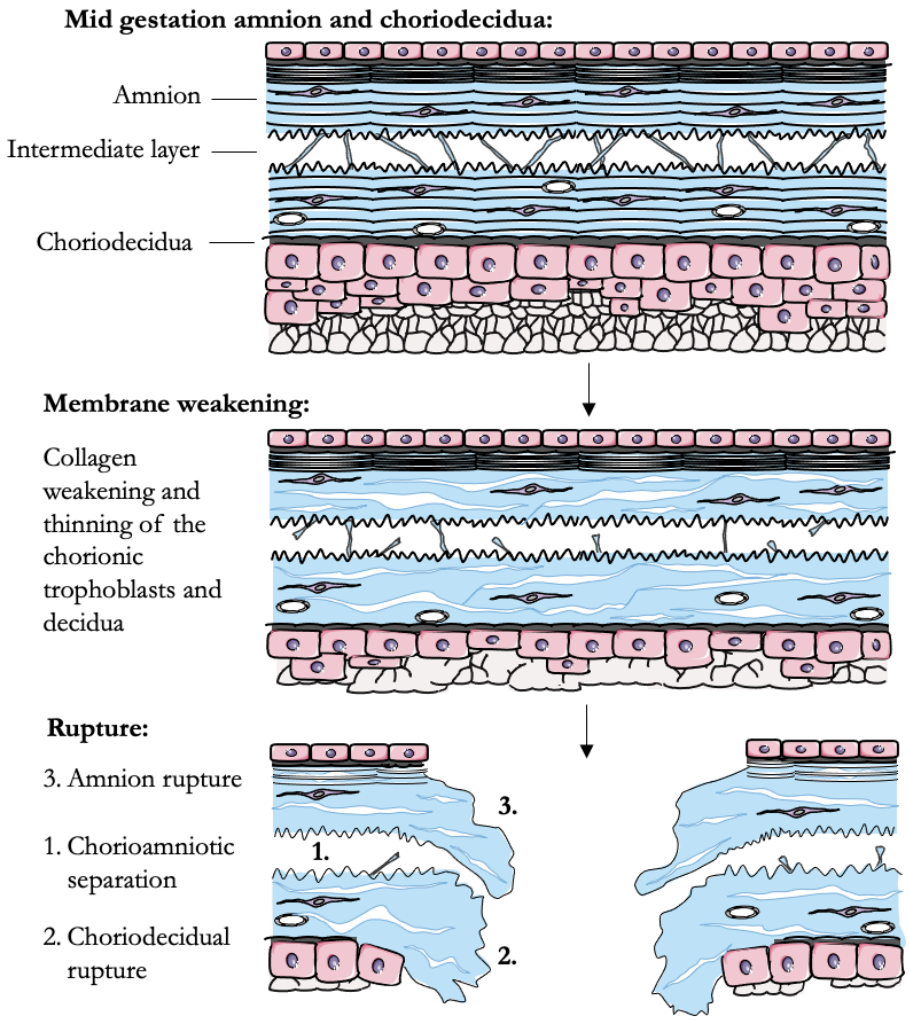
patterns (PAMPs) are recognised by toll-like receptors on membrane fibroblasts and resident macrophages. These cells then release inflammatory cytokines including TNF-alpha that recruit immune cells to the membranes and directly increase the synthesis and activity of MMPs.[116, 155-162] Increased MMP activity causes disorganisation and weakening of membrane collagen and increases the risk of membrane rupture.[159, 161] Some bacteria normally present in vaginal flora including group B Streptococci, Staphylococcus Aureus, Trichomonas Vaginalis and the organisms responsible for bacterial vaginosis also secrete their own MMPs which may also contribute to membrane weakening.[15, 163]

While the fetal membranes tolerate the progressive stretch caused by the growing fetus, excessive stretch, such as in multi-gestation pregnancies or polyhydramnios, also increases the risk of spontaneous PPROM.[15] In addition to physically over distending membrane collagen, excessive stretch is thought to damage the cells of the fetal membranes triggering the release of damage associated molecular patterns (DAMPs).[118, 143, 164, 165] Like PAMPs in the context of infection, this family of proteins causes the release of inflammatory cytokines from the cells of the membrane and upregulates MMP weakening.[166] Other causes of membrane injury such as trauma, antenatal haemorrhage and exposure to bacterial toxins also trigger the release of DAMPs and thus increase the risk of spontaneous PPROM.[116, 148, 166]

Abnormal collagen synthesis

Maternal Vitamin C and Copper deficiencies during early pregnancy are strongly associated spontaneous PPROM.[167-169] Vitamin C is essential for normal collagen synthesis while Copper is an important cofactor for lysyl-oxidase enzymes that give compact membrane collagen its tensile strength. Tobacco smoking during pregnancy is also an independent risk factor for spontaneous PPROM likely due to its ability to lower maternal Vitamin C and Copper levels.[15] Fetuses affected by connective tissue disorders such as Ehlers-Danlos syndrome also have weakened membrane collagen that is predisposed to rupture early.[170]

Figure 12: The physiological mechanisms of term membrane rupture and spontaneous PPROM



Term rupture	Genetic and epigenetic: • Apoptosis of amniotic epithelium and chorionic trophoblasts • Up regulation of MMP promotor genes Physical: • Increasing membrane stretch • Uterine contractions Inflammatory: • Local release of inflammatory cytokines and prostaglandins • Accumulation of oxidative stress Hormonal: • Increase relaxin levels
Spontaneous PPROM	Clinical risk factors: • Previous preterm birth of PPROM Preterm membrane weakening: • Excessive membrane stretch – e.g. multi gestation pregnancies or polyhydramnios • Antenatal hemorrhage • Infection Abnormal collagen synthesis • Connective tissue disorders • Nutritional deficiencies – copper and vitamin C • Smoking

Figure 12: Fetal membrane weakening and rupture: The final failing of the membranes occurs in three phases – 1. The amnion and chorion separate which decreases the tensile strength of the membrane. 2. The decidua (not shown) and chorion rupture causing the amnion to stretch. 3. The amnion ruptures. – original image

Potential effects of amniotic insufflation fetal membrane rupture

Based on the factors known to increase the risk of term and preterm membrane rupture, membrane stretch and tissue damage from exposure to CO_2 may both contribute to higher rates of iatrogenic PPROM after amniotic insufflation.

→ *Fetal membrane distension*

As described above, excessive membrane distension activates MMPs, weakens membrane collagen and is linked with both term and preterm membrane rupture.[15] Membrane distension during amniotic insufflation may trigger similar weakening mechanisms and increase the risk of iatrogenic PPROM. The small series by Belfort et al. (summarised in Table 3) suggested that using lower mean amniotic insufflation pressures (12 vs 15 mmHg) may reduce the risk of membrane rupture.[8, 14, 17, 171] While these trends may represent a protective effect of reducing membrane distension, differences in surgical protocol between these cohorts could also explain these differences.

→ *Exposure of the amniotic epithelium to gas*

The preliminary fetoscopic myelomeningocele repairs summarised in Table 3 also suggest that distending the fetal membranes with unconditioned (cold, dry) CO_2 increases the risk of iatrogenic PPROM while the used of heated, humidified CO_2 mitigates these changes.[8, 14, 17] Other fields of endoscopy have demonstrated peritoneal and pleural damage following exposure to cold, dry CO_2 insufflation.[172-176] While the peritoneum, pleura and amniotic epithelium are anatomically different, they are all liquid lined epithelium and potentially susceptible to damage when exposed to CO_2 for prolonged periods. Membrane damage is known to upregulate MMPs, weaken membrane collagen and increase the risk of membrane rupture.[116, 166] While the results from small human studies suggest that similar changes may occur during cold, dry amniotic insufflation and can be avoided using heated humidified CO_2, pre-clinical studies are clearly required to confirm these hypotheses.

Conclusion and rationale for this thesis

This review has established that surgically covering fetal myelomeningocele at mid gestation improves both spinal cord and brain function for the infant at birth. While open fetal surgery is currently the gold standard, these procedures carry significant risks for the mother. Fetoscopic myelomeningocele repair has recently been proposed as a minimally invasive alternative however, there are concerns that amniotic insufflation used during fetoscopy causes fetal hypercapnic acidosis and iatrogenic PPROM. Heating and humidifying the insufflated CO_2 has been suggested as a potential solution to both fetal and membrane disturbances however, these hypotheses lack physiological and histological data from preclinical studies. Therefore, the global aim of this thesis is;

To investigate the effects of amniotic insufflation with unconditioned (cold, dry) or heated, humidified CO_2 on the fetus and fetal membranes in sheep.

Experimental chapters 1 and 2 investigate the physiological effects of amniotic insufflation on fetal and placental physiology while chapter 3 investigates the histological effects of amniotic insufflation on the fetal membranes.

Experimental chapter 1:
The effect of amniotic insufflation on the fetus

Between 1994 and 2000, four sheep studies demonstrated that fetal lambs exposed to 30-60 minutes of amniotic insufflation at 3-15 mmHg developed progressive hypercapnia and acidosis.[9-12] In my preceding literature review, I provided evidence to suggest that these changes could impair fetal cardiac function during surgery and have detrimental effects on the developing brain. It is also important to note that these sheep studies used lower insufflation pressures and shorter insufflation durations than those being used in humans (≈15mmHg for 180 minutes). As a result, it is possible these sheep studies underestimated the effects of amniotic insufflation on the human fetus. Additionally, these sheep studies used unconditioned (cold, dry) CO_2 for insufflation which does not reflect current clinical practice. In this experimental chapter, we extended the findings of the earlier sheep insufflation studies and investigated how insufflation parameters used in humans effect the physiology of fetal lambs. Using sheep to replicate insufflation allowed us to measure fetal cardiovascular and blood gas changes in real time during insufflation which is currently not feasible in humans.

Experimental chapter 1.1:

Isolating the fetal effects of increasing amniotic pressures

In the first study of this experimental chapter, we investigated the effects of increasing amniotic pressures during insufflation on fetal and maternal physiology. We insufflated the uterus of pregnant ewes with cold, dry CO_2 for 20 minutes at 0, 5, 15 and 25mmHg. These pressures were chosen to cover the range of pressures reported in human case series.[8, 14, 17] During insufflation we periodically sampled fetal blood gasses and continuously recorded uterine blood flows and fetal cardiovascular parameters. We compared these insufflated lambs to a control group of lambs that underwent the same instrumentation and monitoring without amniotic insufflation.

Fetal lambs exposed to cold, dry amniotic insufflation developed progressive hypercapnia and acidosis over the 100 minute insufflation period. Additionally, higher pressures (i.e. 15-25mmHg) significantly reduced uterine blood flow. We suggest these results provide clinicians with a physiological rationale to minimise insufflation pressures during fetoscopic myelomeningocele repair. This manuscript, entitled, **"The effects of partial amniotic carbon dioxide insufflation in an ovine model"** was published in Prenatal Diagnosis and Therapy in 2018. I performed this study in conjunction with Dr Sasha Skinner

who was included as the first author on this manuscript for the purposes of publication. I worked closely with Dr Skinner on the animal experiments and tissue processing, performed all of the histological analysis and helped prepare the final manuscript for publication. A formatted version of the published manuscript is included below.

The effects of partial amniotic carbon dioxide insufflation in an ovine model

Sasha Skinner[1,2,3], Kelly Crossley[1,2], Benjamin Amberg[1,2], Aidan Kashyap[1,2], Stuart Hooper[1,2], Jan A. Deprest[4,5,6], Ryan Hodges[1,2,3] and Philip DeKoninck[1,2,7]

1. The Ritchie Centre, Hudson Institute of Medical Research, Clayton, Victoria, Australia
2. The Department of Obstetrics and Gynaecology, Monash University, Clayton, Victoria, Australia
3. Perinatal Services Monash Health, Monash Medical Centre, Clayton, Victoria, Australia
4. Division of Woman and Child, Department of Obstetrics and Gynaecology, University Hospitals Leuven, Leuven, Belgium
5. Department of Development and Regeneration, Cluster Women and Child, Faculty of Medicine, KU Leuven, Leuven, Belgium
6. Institute of Women's Health, University College London, London, UK
7. Department of Obstetrics and Gynaecology, Erasmus MC, University Medical Center Rotterdam, The Netherlands

Correspondence: Philip DeKoninck, The Ritchie Centre, Hudson Institute of Medical Research, 27-31 Wright St, Clayton, Victoria 3168, Australia. Email: philip.dekoninck@monash.edu

Conflicts of interest: None declared.

Funding information: Monash Health 2018 Emerging Researcher Fellowship awarded to Dr Sasha Skinner.; Great Ormond Street Hospital Charity; Engineering and Physical Sciences Research Council (EPSRC), Grant/Award Number: NS/ A000027/1; Welcome Trust, Grant/Award Number: WT101957

Abstract:

Objective: We aim to assess the effect of partial amniotic carbon dioxide insufflation (PACI) at increasing pressures on fetal acid-base, fetal-placental perfusion, and fetal membrane morphology in an ovine model.

Method: Pregnant ewes and fetuses were instrumented under isoflurane anesthesia at 105 days gestation (term 145 days) to monitor utero-placental blood flow, fetal and maternal blood pressure, heart rate, and blood gas status. One group (n = 6) was exposed to PACI (unheated dry CO2), involving 10 mm Hg stepwise increases in insufflation pressure (5 to 25 mm Hg), for 80 minutes followed by 20 minutes of desufflation. Un-insufflated controls (n = 5) were monitored for 100 minutes. At post-mortem, fetal membranes were collected for histological analysis.

Results PACI at 25 mm Hg caused severe fetal hypercapnia (PaCO2 = 143 ± 5 vs 54 ± 5 mm Hg, P < 0.001), acidosis (pH = 6.85 ± 0.02 vs 7.25 ± 0.02, P < 0.001), hypoxia (SaO2 = 31 ± 4% vs 57 ± 4%, P = 0.01), and reduced uterine artery flow (50 ± 15 vs 196 ± 13 mL/min/kg, P = 0.005) compared with controls. These effects were greater at higher PACI pressures. PACI resulted in leukocyte infiltration in the amnion ($1.77 \times 10{-}5 \pm 0.61 \times 10{-}5$ vs $0.38 \times 10{-}5 \pm 0.19 \times 10{-}5$ cells/μm2, P = 0.04) and chorionic membranes ($2.94 \times 10{-}5 \pm 0.67 \times 10{-}5$ vs $0.84 \times 10{-}5 \pm 0.42 \times 10{-}5$ cells/μm2, P = 0.01).

Conclusion: Higher PACI pressures results in larger disturbances in fetal acid-base, uterine blood flow, and fetal membrane inflammation in sheep. Differences between human and sheep utero-placental structure should be considered.

Introduction

Minimally invasive or fetoscopic alternatives to open fetal surgery are now possible for several fetal anomalies, aiming to reduce associated morbidity and mortality.[177] One of the challenges of fetoscopic surgery is the amniotic fluid environment limiting visibility, ease of hemostasis, fetal immobilization, and instrument maneuverability. This surgical environment is challenging for complex procedures involving dissection, control of hemostasis, and suturing. Partial amniotic carbon dioxide insufflation (PACI) involves draining part of the amniotic fluid and insufflating the uterus with carbon dioxide.[79] Gaseous distension improves visualization, increases working space, and allows easier control of bleeding.[58, 77] PACI is currently used mainly to enable fetoscopic myelomeningocele (MMC) repair. Fetoscopic access aims to reduce the uterine incision dimensions and hence lower uterine dehiscence rates to avoid significant maternal morbidity associated with open fetal surgery.[8, 14, 17, 85, 178-180]

The safety of PACI has been debated for many years as early animal experiments demonstrate progressive fetal hypercapnia and acidosis during PACI.[9-12] However, the relevance of these studies remains unclear in the context of current clinical practice, particularly as recent clinical observations show no obvious evidence of adverse short-term fetal outcomes attributed to PACI. [8, 14, 17, 85, 178-180] Additionally, while initially preterm premature rupture of membranes (PPROM) rates following PACI for fetoscopic MMC patch repair were very high[8, 14, 179] (84-100%), more recent experience using heated-humidified PACI in an exteriorized uterus with surgical closure of the access site suggests this to be less of a problem.[17]

No animal studies have investigated the effect of altering PACI pressures, duration, humidity, or the effect of PACI on fetal membrane ultrastructure. Indeed, these are all parameters of relevance to fetal safety and PPROM rates. Further investigation into the fetal and uterine effects of PACI is therefore appropriate to guide its ongoing clinical application. Our aim was to assess the real-time effects of PACI on fetal and maternal acid-base status, hemodynamics, and utero-placental perfusion at increasing PACI pressures in an ovine model. We also evaluated the effect of PACI on fetal membrane histology.

Methods

All animal experiments were approved by the Monash Medical Centre animal ethics committee and were conducted in accordance with the National Health and Medical Research Council (NHMRC) Australian code of practice for the care and use of animals for scientific purposes.[181]

Fetal and maternal instrumentation

Eleven pregnant date-mated Merino-Border Leicester ewes at 103 to 105 days (term 145 days) gestation of either singleton or twin fetuses were anesthetized using intravenous sodium thiopentone (Pentothal, Boehringer Ingelheim, Australia) bolus induction with 2% to 2.5% inhaled isofluorane (Isoflow, Abbot Pty Ltd, Australia) in room air/oxygen maintenance. We performed midline laparotomy and hysterotomy. The fetus was instrumented (one fetus only if twin gestation) with umbilical and carotid artery flow probes (3 mm ultrasonic flow probe, Transonic Systems, USA), jugular vein and carotid artery catheters (polyvinyl catheters, ID 0.86 mm, OD 1.52 mm, Dural Plastics, Australia) and an esophageal temperature probe. Two amniotic catheter, one for pressure monitoring and another for draining, and CO_2 insufflation tubing were introduced into the uterine cavity. The hysterotomy site was sutured closed in three layers, ensuring an airtight seal around exteriorized catheters and flow probes. A flow probe was placed on the uterine artery prior to laparotomy incision closure. Maternal carotid and jugular catheters (ID 2.6 mm, OD 4.2 mm, Dural Plastics Australia) were inserted. A pneumotac (Respiratory Flow Head 300 L, ADInstruments, Bella Vista, NSW, Australia) was connected in between the maternal endotracheal tube and the ventilation circuit to monitor maternal tidal volume. LabChart (LabChart v8, ADInstruments, Australia) was used to electronically record fetal carotid and umbilical artery flow, fetal and maternal carotid artery pressure and fetal temperature, intrauterine pressure and uterine artery flow. Fetal heart rate was derived from the fetal carotid artery flow waveform, and fetal systolic and diastolic blood pressures were derived from the fetal carotid artery pressure waveform. Maternal heart rate was derived from the uterine artery flow waveform, and maternal systolic and diastolic pressures were derived from maternal carotid pressure waveform. The maternal, fetal, and uterine instrumentation setup is summarized in Figure 13.

Maternal ventilation settings were adjusted to obtain a target maternal $PaCO_2$ of 35 to 40 mm Hg on carotid blood gas, end tidal CO_2 was noted and maintained $\pm$5 mm Hg throughout the experiment. After approximately 10 minutes to allow physiological recordings to stabilize, baseline recordings were taken for 5 minutes, and baseline fetal and maternal blood samples were collected.

Carbon dioxide uterine insufflation

Amniotic fluid was drained and baseline intrauterine pressure was recorded. Ewes were then randomly allocated to be exposed to PACI (experimental group, n = 6) or not (controls, n = 5). In the experimental group, the uterus was insufflated with unheated (21-22°C) dry CO_2 as it expands out of its storage in the liquid phase in medical gas bottles. Insufflation lasted for 80 minutes, and pressures were progressively increased every 20 minutes from 0 to 5, 15, 25 and then reduced to 15 mm Hg. These insufflation pressures were chosen to reflect PACI pressures used clinically in fetoscopic MMC repair ranging from 9 to 30 mm Hg.[8] A duration of 20 minutes at each insufflation pressure was used in an attempt to minimize any accumulating adverse physiological effects of prolonged general anesthesia.[182] After desufflation, normal saline at body temperature was introduced to the uterine cavity to replace drained amniotic fluid. Physiological recording as well as blood gas sampling continued for a further 20 minutes after desufflation. In controls, amniotic fluid was drained, and fetuses were monitored for 100 minutes with warmed normal saline replacing the drained amniotic fluid for the final 20 minutes. In both groups, maternal and fetal carotid artery blood gas samples were taken every 10 minutes. The experimental timeline is summarized in Figure 13.

After the experiment, ewes and fetuses were euthanized with pentobarbitone 100 mg/kg IV, and the fetus (both fetuses in cases of twin gestation) underwent post mortem examination to assess for gross evidence of fetal injury and tissue hemorrhage and record fetal total body and individual major organ weights (heart, lung, liver, spleen, kidney, and brain). In each ewe, 3 × 3 cm segments of the fetal membranes were collected from sites remote from the hysterotomy site, secured to foam to ensure later orientation, fixed in formalin, and embedded in paraffin.

Histological analysis

Inflammation in the amnion and chorion was quantified using CD45 immunostaining. Three tissue sections (5 μm) per animal were mounted on glass slides and deparaffinized using Xylele and rehydrated. Antigen retrieval was performed by incubation with 0.01 M sodium citrate buffer (pH 6) (DAKO #S1699). Endogenous peroxidases and nonspecific antigen binding were blocked using Dako blocking solutions (#S2023, #X0909). The primary antibody was mouse anti-CD45 antibody (BioRad, #MCA2220GA) diluted 1:500 in Dako antibody diluent (#S0809). Secondary antibody was goat anti-mouse antibody (Dako #K4001). Immunostaining was visualized by incubation with 3,3'-diaminobenzidine (DAB) (#K3468) and counterstained with Hematoxylin. CD45 positive cells in the amnion and chorion membranes were counted in five randomly selected, nonoverlapping fields of view using a light microscope (40X; BX-4, Olympus, Oberkochen, Germany), digital camera (Olympus America Inc), and ImageJ software (National Institutes of Health, USA).

Data analysis

LabChart data were analyzed offline by averaging 20-second data epochs taken immediately after changes in PACI pressure and every 5 minutes thereafter. Blood flow recordings were adjusted for fetal weight. Data were tested for normality using a Shapiro Wilk test. Baseline data were compared using a student t test for normally distributed data and a Mann–Whitney test for data not normally distributed (Prism v6, GraphPad Software Inc/SigmaPlot v13.0, Systat Software Inc). Physiological data were compared using a two-way repeated measure analysis of variance with Holm-Sidak post hoc analysis to account for multiple comparisons. All data are presented as mean and standard error of the mean (SEM). P values < 0.05 were considered statistically significant.

Figure 13: Experimental timeline

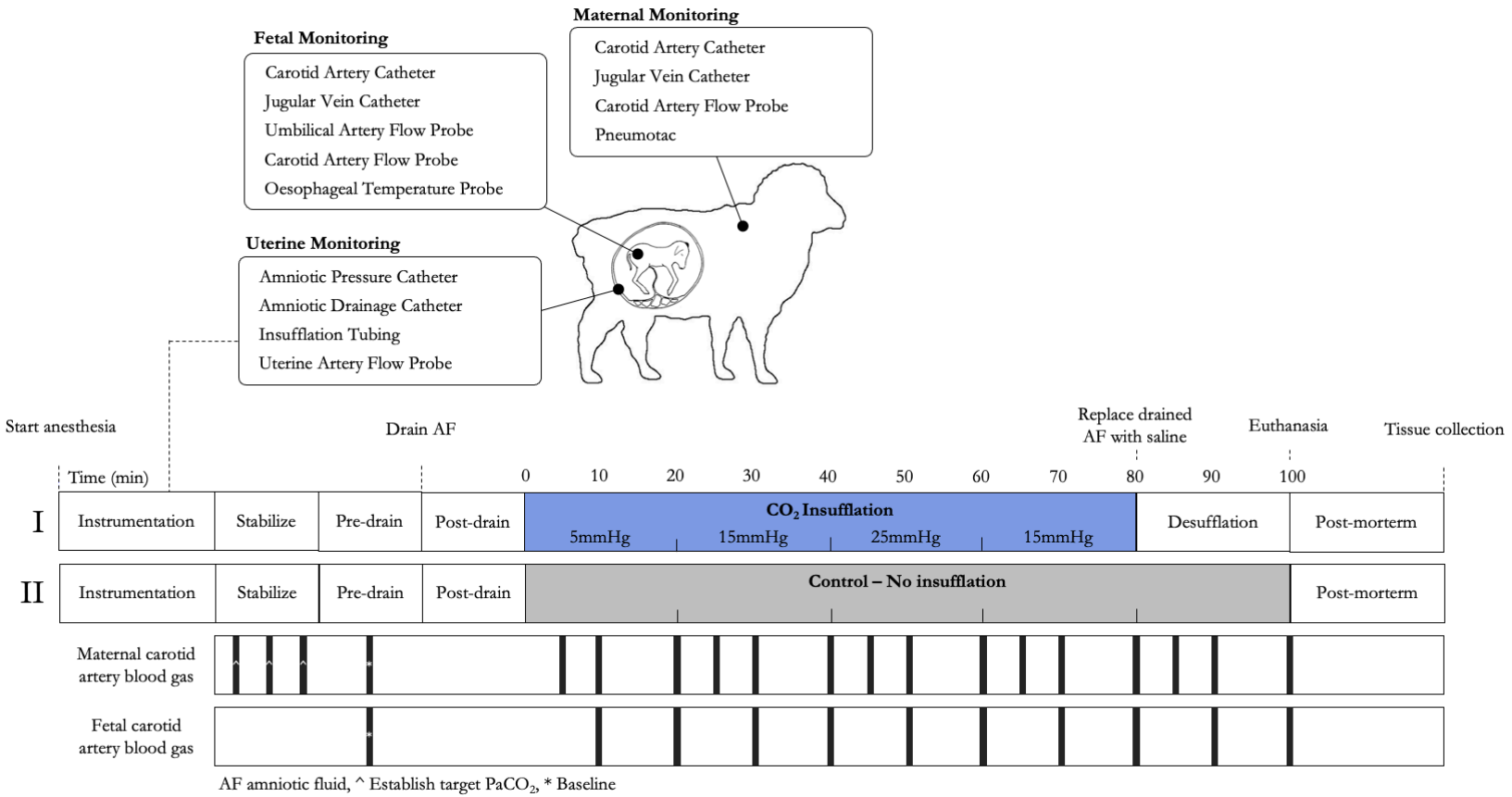


Figure 13: Experimental timeline, maternal, fetal, and uterine instrumentation. Summary of the experimental timeline for group (I): CO_2 insufflation and group (II): controls. Timing of maternal and fetal carotid artery blood gas sampling is shown with (*) denoting timing of baseline samples. Maternal, fetal, and uterine instrumentation is pictorially depicted. Fetal temperature was recorded with an esophageal temperature probe. Fetal carotid and umbilical artery flow probes allowed continuous monitoring of blood flow. Maternal and fetal carotid artery and jugular vein catheters allowed regular blood gas analysis and continuous blood pressure recordings. Maternal tidal volume was measured using a pneumotac. PACI was administered and monitored using an amniotic drainage catheter, insufflation tubing, and intra-amniotic pressure catheter. Uterine artery flow was monitored with a flow probe

Results

Eleven ewes and their lambs (one fetus only in cases of twin pregnancy) were analyzed, six fetuses exposed to PACI and five controls. Fetal weight, gestational age, baseline physiological recordings, and blood gas samples were similar between the two groups (Table 6).

Uterine effects

Uterine artery flow decreased during PACI (n = 6) and increased in controls (n = 5). There were larger reductions in uterine artery flow as PACI pressure was increased, down to 33% of baseline flow at 25 mm Hg (mean minimum −116.2 ± 14.8 vs +26.8 ± 13.4 mL/min/kg, $P < 0.001$). Uterine artery flow increased after PACI pressure was reduced and following desufflation, back to 83% of baseline flow (Figure 14).

Fetal acid-base effects

Fetal $PaCO_2$ increased (mean maximum 142.7 ± 5.3 mm Hg vs 54.1 ± 5.1 mm Hg, $P < 0.001$, at 25 mm Hg) and pH decreased (mean minimum 6.85 ± 0.02 vs 7.25 ± 0.02, $P < 0.001$) over time as PACI pressures increased (Figure 14). Fetal PaO_2 was not significantly different from controls during PACI. However, SaO_2 decreased (mean minimum 30.8 ± 3.8% vs 56.5 ± 3.6%, $P = 0.01$) as PACI pressures increased, with a corresponding decrease in base excess and increase in lactate (Figure 14). Fetal pH, $PaCO_2$, SaO_2, and base excess gradually improved after PACI pressures were reduced and after desufflation (Figure 14). However, fetal lactate continued to rise 20 minutes after desufflation (mean maximum of 8.43 ± 0.6 mmol/L vs 3.98 ± 0.6 mmol/L, $P = 0.001$) (Figure 14). Fetal bicarbonate and temperature were not statistically different from controls during PACI.

Fetal hemodynamic effects

Fetal heart rate increased compared with controls as PACI pressures increased (mean maximum 216 ± 4 vs 164 ± 4 bpm, $P < 0.001$, at 25 mm Hg) and remained higher than controls following desufflation (Figure 14). Fetal carotid artery and umbilical artery blood flow did not change significantly during PACI compared with controls overall. However, on closer analysis, fetuses exposed to PACI could be separated into two distinct subgroups based on their response. In three fetuses, carotid artery flow was maintained, and umbilical artery flow significantly increased compared with controls (Figure 14). In the other three fetal lambs exposed to PACI, carotid artery flow significantly decreased compared with controls and umbilical artery flow tended to decrease. No other differences were found between the two subgroups in any other measured parameters.

Maternal acid-base effects

Maternal end tidal CO_2 remained stable and not different from controls during PACI. However, maternal $PaCO_2$ increased (mean maximum 51.8 ± 1.0 mm Hg vs 36.7 ± 0.9 mm Hg, $P < 0.001$, at 25 mm Hg) and pH decreased (mean minimum 7.35 ± 0.007 vs 7.47 ± 0.007, $P < 0.001$) as PACI pressures increased (Figure 16). Both maternal $PaCO_2$ and pH improved when PACI pressure was reduced and after desufflation, yet remained different from controls (Figure 16). Maternal PaO_2 was not significantly different to controls. However, maternal SaO_2 decreased significantly at PACI pressures of 25 mm Hg (94.7 ± 0.6% vs 98.2 ± 0.6%, $P = 0.005$) but was not different from controls after desufflation (Figure 16). There was no significant difference in maternal lactate (Figure 16), base excess, or bicarbonate compared with controls.

Histology

Compared with controls, CD45 positive cell counts in the amnion ($0.38 \times 10{-}5 \pm 0.19 \times 10{-}5$ vs $1.77 \times 10{-}5 \pm 0.61 \times 10{-}5$ cells/μm2, $P = 0.04$) and chorion ($0.84 \times 10{-}5 \pm 0.42 \times 10{-}5$ vs $2.94 \times 10{-}5 \pm 0.67 \times 10{-}5$ cells/μm2, $P = 0.01$) significantly increased following PACI (Figure 17).

Table 6: Baseline fetal and maternal characteristics

	PACI	Controls	P Value
Fetal Characteristics			
Number	6	5	
% Male	33%	50%	
Gestational age (days)	104.5±0.3	104.5±0.3	0.91
Weight (grams)	1395±63.3	1523±19.9	0.17
Fetal Temperature (°C)	39.2±0.2	39.2±0.2	0.31
Fetal HR (bpm)	173.7±3.5	161.8±3.8	0.06
Fetal Carotid Artery Pressure (mmHg)	40.7±1.4	39.6±2.8	0.73
Fetal Carotid Artery Flow (ml/min/kg)	31.2±3.3	36.5±1.5	0.49
Umbilical Artery Flow (ml/min/kg)	46.8±17.0	49.1.0±13.0	0.66
Fetal pH	7.25±0.02	7.25±0.02	0.96
Fetal $PaCO_2$ (mmHg)	52.3±2.6	59.8±2.7	0.18
Fetal PaO_2 (mmHg)	21.4±1.9	22.6±0.7	0.58
Fetal SaO_2 (%)	49.6±6.3	56.8±4.3	0.39
Fetal Hemoglobin (g/dL)	10.8±0.3	11.4±0.4	0.30
Fetal Lactate (mmol/L)	3.3±0.3	2.6±0.3	0.14
Maternal Characteristics			
Maternal HR (bpm)	89.7±5.5	83.8±8.5	0.59
Maternal Carotid Artery Pressure (mmHg)	64.2±4.5	61.6±4.2	0.51
Uterine Artery Flow (ml/min/kg)	173.0±23.1	133.3±34.1	0.93
Maternal pH	7.45±0.01	7.45±0.01	0.90
Maternal $PaCO_2$ (mmHg)	38.9±1.3	38.3±0.7	0.79
Maternal PaO_2 (mmHg)	182.2±38.3	162.0±16.9	0.79
Maternal SaO_2 (%)	96.9±0.6	97.3±0.6	0.57
Maternal Hemoglobin(g/dL)	8.7±0.6	9.2±0.5	0.57
Maternal Lactate (mmol/L)	1.0±0.1	0.8±0.1	0.57
Maternal end tidal CO_2 (mmHg)	35.5±1.2	32.0±2.2	0.22

Figure 14: Real-time physiological changes in fetal acid-base during PACI.

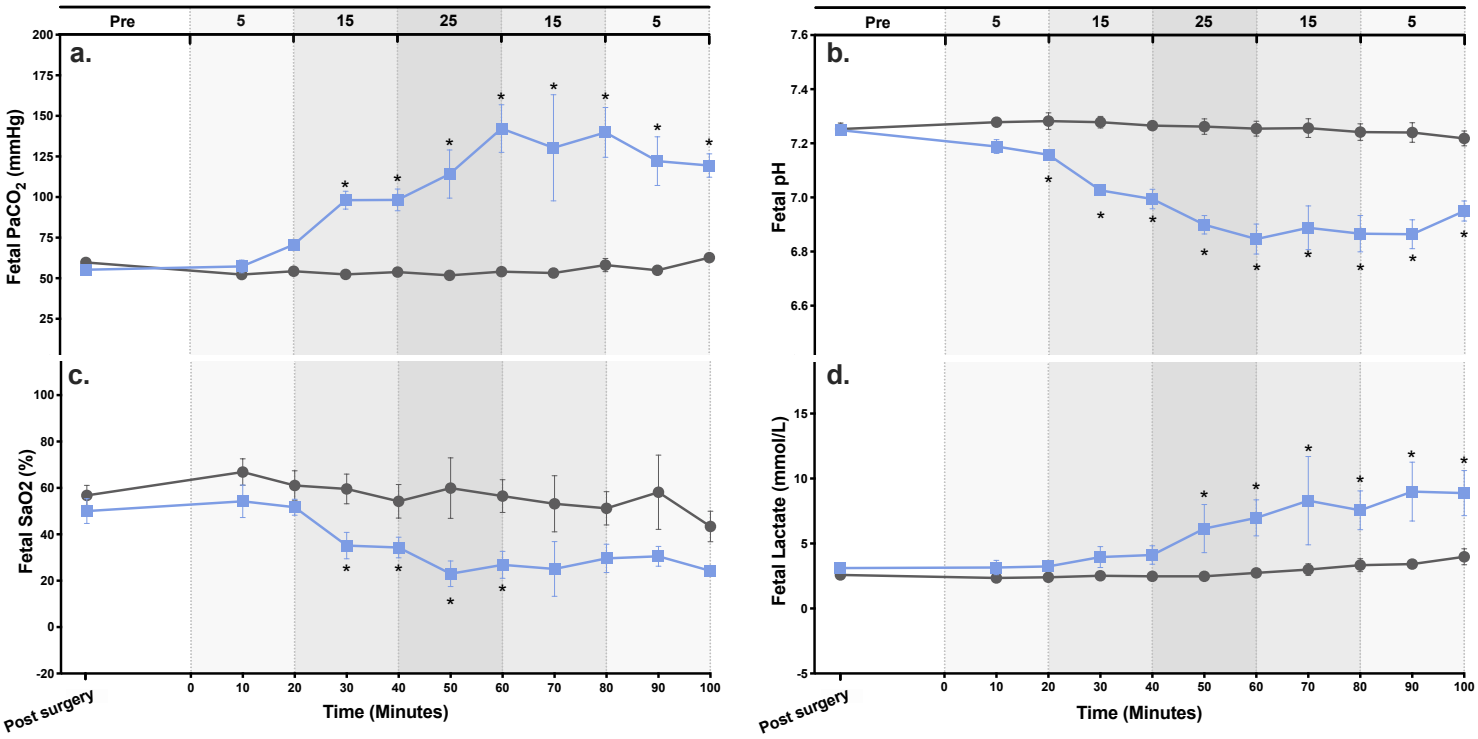


Figure 14: PACI leads to significant fetal hypercapnia A, and acidosis B, greatest at higher PACI pressures and persisting 20 min after desufflation. At high PACI pressures, fetal oxygen saturation decreased but was not significantly different to controls following desufflation C. Fetal lactate continued to increase throughout the experiment D. Pre and post drain denote timing of amniotic fluid drainage. Data are presented as mean ± SEM

Figure 15: Real-time physiological changes in fetal heart rate, uterine, umbilical, and fetal carotid artery flow during PACI.

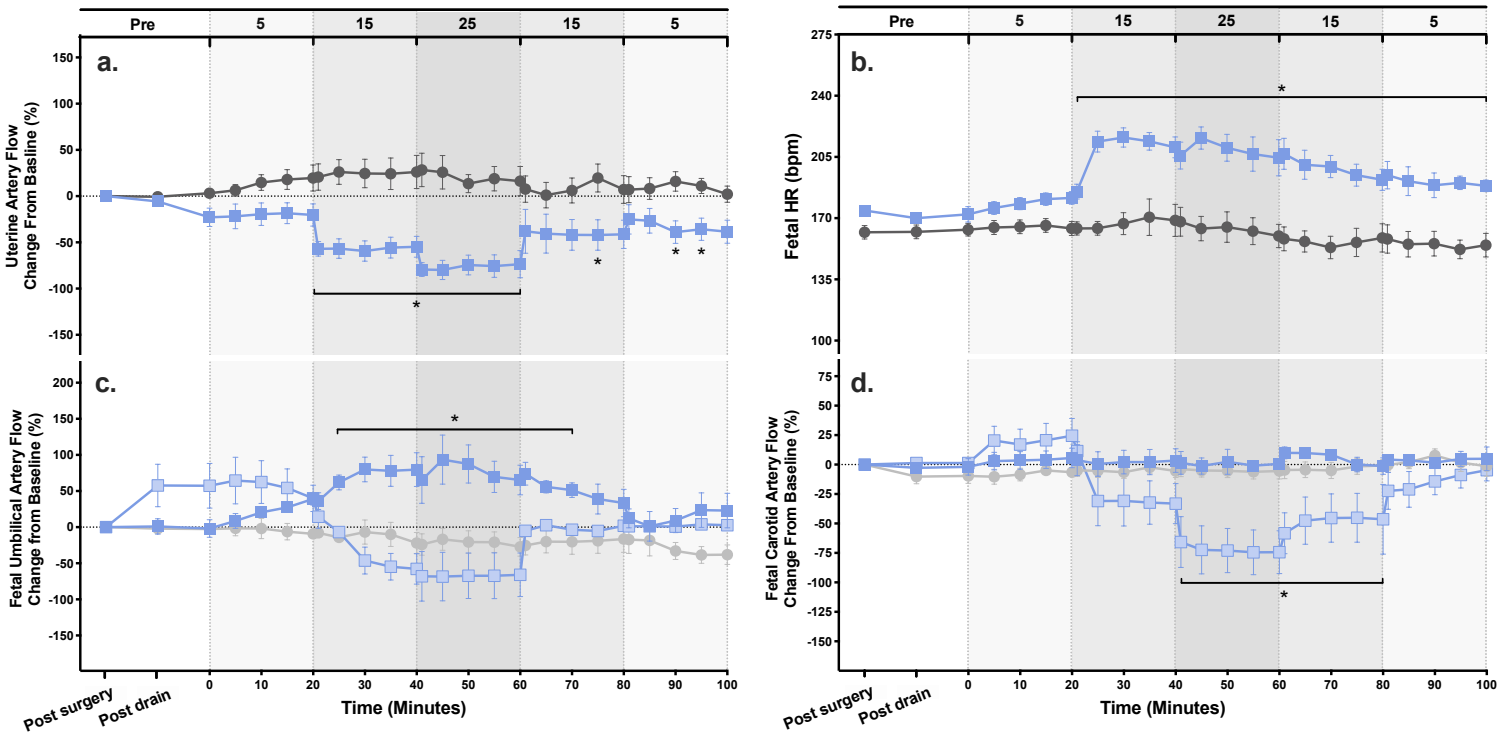


Figure 15: Uterine artery flow decreased as PACI pressures increased A. PACI leads to significant fetal tachycardia B, that persisted 20 min after desufflation. In some fetuses (subgroup A, n = 3), fetal umbilical artery increased during PACI C, and carotid artery flow was maintained D. Conversely in the remaining fetuses (subgroup B, n = 3), fetal umbilical artery flow did not increase and tended to decrease C, while carotid artery flow significantly decreased at high PACI pressures D. Pre and post drain denote timing of amniotic fluid drainage. Data are presented as mean ± SEM. P values < 0.05 (*) are in comparison with controls

Figure 16: Real-time physiological changes in maternal acid-base during PACI.

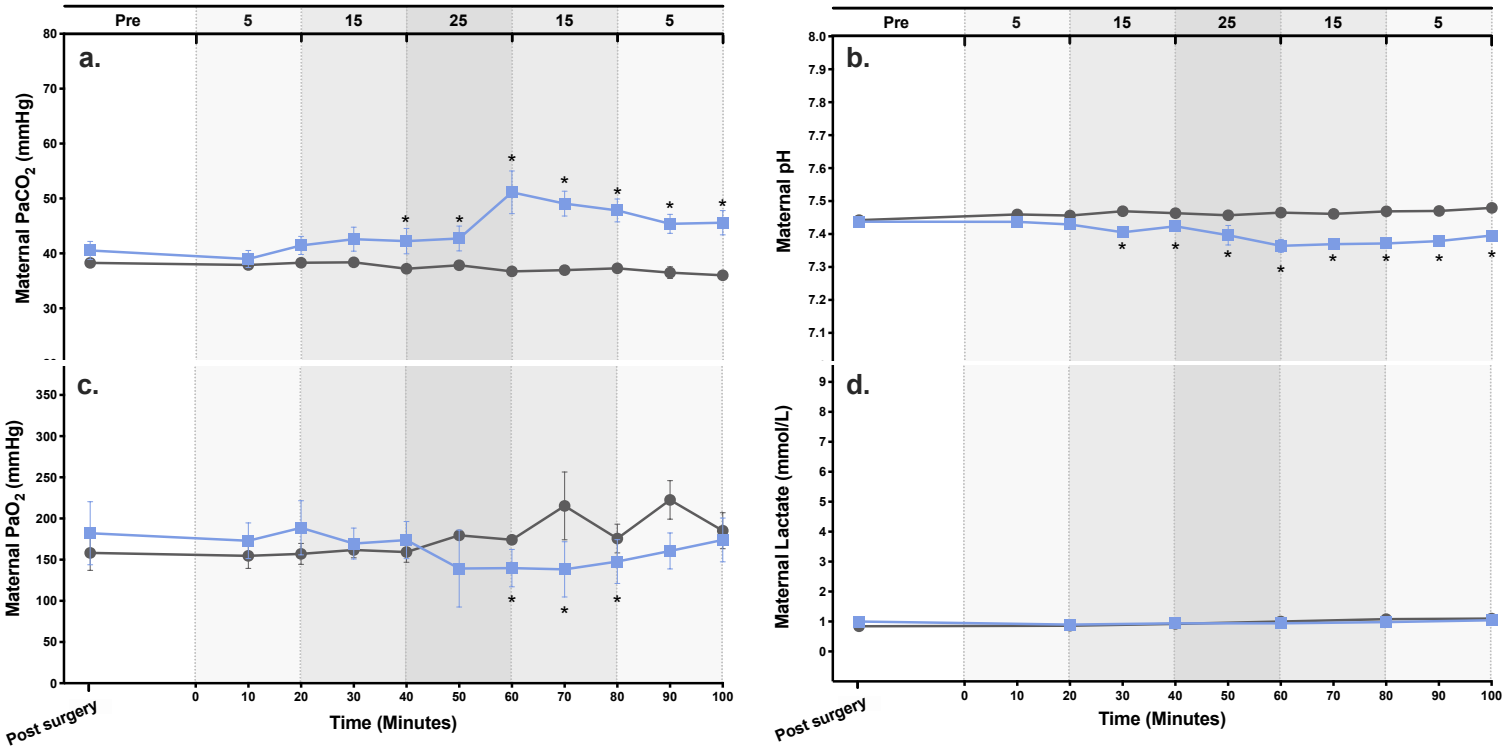


Figure 16: PACI leads to significant maternal hypercapnia A, and acidosis B, greatest at higher PACI pressures and persisting 20 min after desufflation. At high PACI pressures, maternal oxygen saturation decreased but was not significantly different to controls following desufflation C. Maternal lactate was not different to controls throughout the experiment D. Pre and post drain denote timing of amniotic fluid drainage. Data are presented as mean ± SEM

Figure 17: CD45 immunostaining of the fetal membranes.

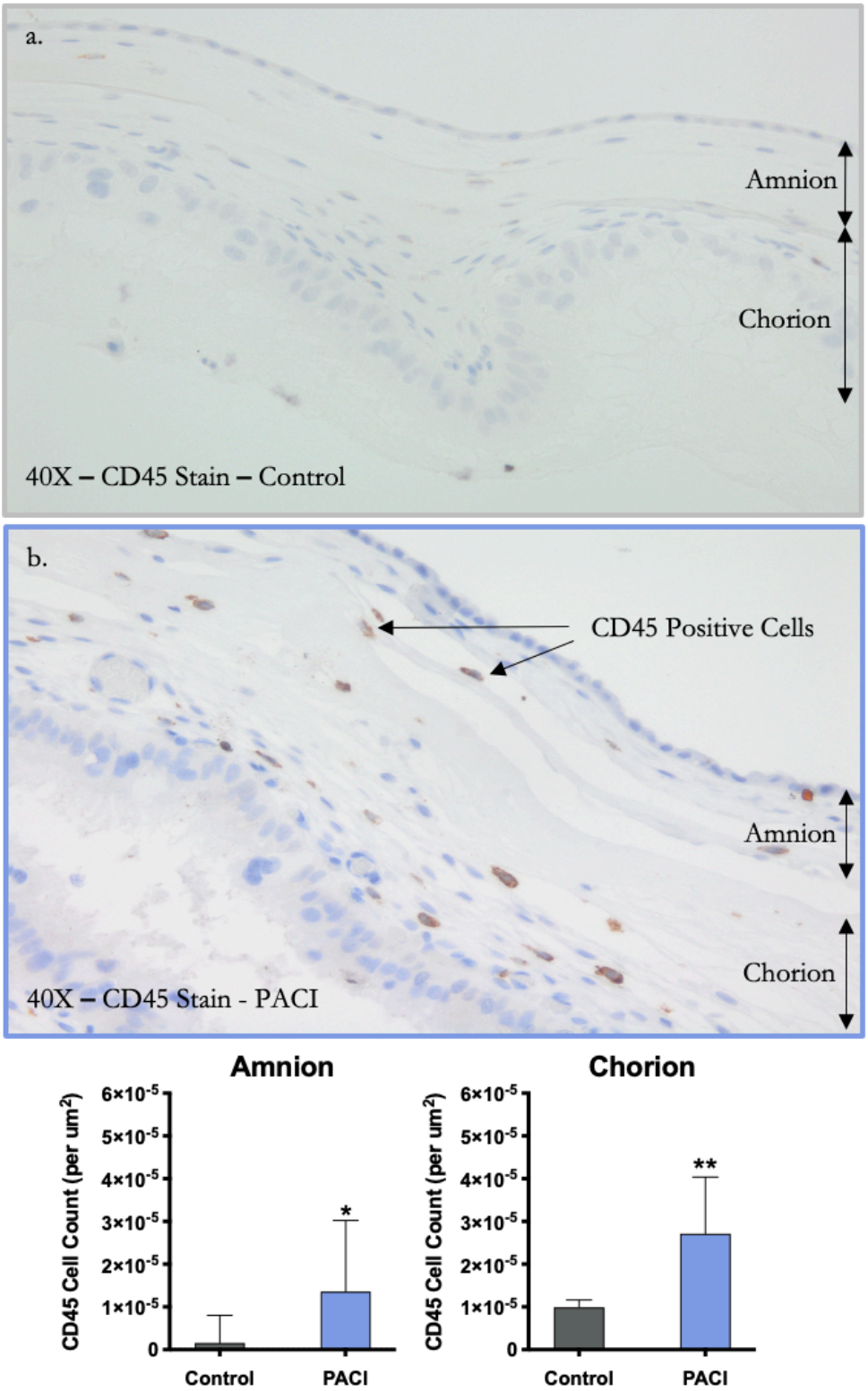


Figure 17: CD45 immunostaining of the fetal membranes demonstrated significant immune cell infiltration in the amnion A, and chorion B, of insufflated membranes C, compared with controls D. Values are presented as mean ± SEM

Discussion

Our study demonstrates that, in pregnant sheep, and using unconditioned CO_2, increasing PACI pressures are associated with significant reductions in uterine artery flow, severe fetal hypercapnia, acidosis, reduced oxygen saturation, and tachycardia. Changes in maternal acid-base status during PACI are minor. Furthermore, PACI was associated with histological signs of inflammation in the fetal membranes.

Stepwise reductions in uterine artery flow with increasing PACI pressures likely results from mechanical uterine distension and associated increased intra-abdominal pressure. In animal studies, increasing intra-abdominal pressure increases peripheral vascular resistance, reducing cardiac venous return and cardiac output, thereby reducing uterine artery flow.[60, 62, 183, 184] Additionally, uterine distension may directly compress the uterine artery. Therefore, it is likely that insufflation using alternatives for CO_2 (such as Helium) would have a similar mechanical effect.[11, 14]

In our study, PACI pressures of 25mmHg lead to significant fetal compromise. Although it is important to assess the effects and safety of extremes of PACI pressures reported clinically (9-30 mm Hg), pressures this high are generally avoided in clinical cases.[8] Likely more relevant are the significant fetal changes already occurring at PACI pressures of 15mmHg, which is more reflective of average PACI pressures reported in the clinical literature. [8, 17, 85]

It is unknown whether the effects of uterine distension will be similar in humans given the sheep uterus appears more compliant and maybe more susceptible to overdistension. As such, for any given pressure, the results we observed in sheep may overestimate the effects that can be expected in humans.[185] Indeed, studies assessing the feasibility of fetoscopic MMC patch repair in sheep models reported lower PACI pressures of 7 to 12mmHg.[57, 185]

In our study, the maternal abdominal wall was closed during insufflation, reflecting more likely the percutaneous fetoscopic approach. However, more recent case series demonstrated better pregnancy outcomes after laparotomy and uterine exteriorization prior to insufflation.[17] Uterine exteriorization likely reduces the resistance created by a closed abdominal wall, potentially reducing the insufflation pressure required to achieve adequate uterine distension, thereby potentially also decreasing the maternal and fetal effects of raised intra-abdominal pressure.

Our study emphasizes the importance of minimizing PACI pressures where possible. Kohl et al. suggested using the "opening pressure" to optimize PACI pressures,[79] which is defined as the pressure

at which gas begins to enter the amniotic cavity. We did not identify an opening pressure but found that the relationship between uterine insufflation, with the associated adverse fetal responses, and CO_2 flow was variable and unpredictable. As such we question whether this is the correct approach for assessing insufflation pressures. However, in view of our findings, it is possible that maternal uterine artery Doppler ultrasound may be considered for intraoperative monitoring of utero-placental function during PACI.[46, 186] Further studies should compare the effects of uterine distension with inert gasses to better delineate the effects of uterine distension on our findings.

We also observed that higher PACI pressures led to more severe fetal hypercapnia and acidosis compared with previous animal studies using lower insufflation pressures.[9-12] We speculate that this may not only be attributed to distention related diminished uterine perfusion but also to an increased pressure gradient for CO_2 diffusion into fetal tissues. Indeed, increased tissue CO_2 absorption is a known consequence of higher pneumoperitoneum insufflation pressures in adult laparoscopic surgery.[187]Additionally, Pelletier et al. observed that ovine fetuses developed hypercapnia and acidosis following uterine insufflation with CO_2 but not with Helium or water. This suggests that CO_2 is an important source of acid-base disturbances rather than purely uterine distension.

Limited fetal capacity to buffer excess CO_2 likely also contributed to the severity of fetal hypercapnia. During PACI, fetal bicarbonate did not change to buffer the large increase in fetal $PaCO_2$, supporting previous studies showing bicarbonate is not excreted by the mid-gestation fetal kidneys and poorly transported across the placenta.[188, 189]Carbonic anhydrases catalyze the reaction of CO_2 and water to produce bicarbonate and hydrogen ions (H^+) and are expressed in fetal tissues with varying degrees of functional activity.[190-194] It has been suggested that the very low levels of carbonic anhydrase enzyme in fetal blood may limit fetal buffering capacity in states of severe hypercapnia. However, hypercapnia and acidosis seen in fetuses exposed to PACI indicate high levels of substrate on either side of the buffering equation (ie, CO_2 and H^+), suggesting against an enzyme insufficiency. Rather, we speculate that fetal susceptibility to hypercapnia and acidosis is increased by a reduced capacity for fetal H^+ plasma buffering and excretion via the immature fetal kidneys.[188, 189]

Additionally, fetal elimination of CO_2 predominantly relies on diffusion into the maternal compartment and thus is limited by decreases in placental perfusion resulting from reduced uterine artery flow. However, the human and sheep placentas have major differences. The ovine placenta is syn-epitheliochorial, with additional tissue layers separating the maternal and fetal circulations compared the human hemochorial placenta.[185, 195] The sheep placenta also consists of multiple distinct fetal cotyledons distributed throughout the uterine wall that are joined by inter-cotelydonary fetal vessels; in contrast,

the human placenta has a singular discoid structure. While gas diffusion and substrate delivery are similar between the two placental structures,[196] it is possible that "stretching" of inter-cotelydonary fetal vessels makes the ovine placenta more susceptible to uterine distension. As such placental gaseous exchange may be impaired more in sheep than in humans. This is consistent with a recent preliminary case series of three fetuses undergoing fetoscopic MMC repair using PACI suggesting that fetal hypercapnia in humans may not be as severe as described in animal models.[16] However, in that study, umbilical vein blood samples were collected at an undefined interval after desufflation and so any intraoperative acid-base disturbances may have been missed as CO_2 is highly soluble.

Fetal blood oxygen levels were reduced at high PACI pressures with significant reductions in fetal SaO_2, despite stable fetal PaO_2 levels, which likely caused some tissue hypoxia as indicated by reduced base excess and increased fetal lactate.[197] This finding may be explained by a pH-induced right shift in the oxygen-hemoglobin dissociation curve causing decreased affinity of fetal hemoglobin for oxygen.[198, 199] While Saiki et al. reported no change in fetal lactate during PACI, lower insufflation pressures in their study (10 mmHg vs up to 25 mmHg) with less severe fetal hypercapnia would expectedly result in smaller reductions in fetal hemoglobin oxygen affinity.[12]

Significant fetal tachycardia was observed at PACI pressures greater than 15 mm Hg, correlating with abrupt changes in fetal acid-base status and uterine blood flow. Clinically, in the absence of invasive fetal monitoring, noninvasive fetal heart rate monitoring and the identification of fetal tachycardia may be a useful indicator of severe physiological disturbance during PACI. However, our study cannot rule out fetal risk during PACI even before a tachycardic response is triggered.

In contrast to gross physiological disturbances in the fetus, PACI caused smaller increases in maternal $PaCO_2$ and reductions in pH. This discrepancy likely reflects a much lower capacity for fetal CO_2 clearance across the placenta compared with maternal CO_2 clearance via the lungs. Importantly, this highlights an inability to predict fetal acid-base status and well-being using maternal monitoring.

Clinically, high PPROM rates remain a major clinical concern for fetoscopic procedures using PACI. [8, 14, 179, 180, 200] Membrane weakening that precedes spontaneous membrane rupture is induced by immune cell infiltration of the amnion and chorion.[116, 201] Acute leukocyte infiltration was clearly demonstrated in our study following PACI exposure. Indeed, this might be a mechanism that contributes to high iatrogenic PPROM rates observed following PACI exposure clinically. Animal studies of CO_2 pneumoperitoneum demonstrate localized acidosis of peritoneal blood vessels and increased inflammatory cytokines.[202, 203] Additionally, increasing pneumoperitoneum insufflation pressures

stretches the peritoneum, limiting peritoneal capillary blood flow, leading to localized hypoxemia and tissue inflammation.[202] We speculate that as fetal membranes in the lamb are also supplied by small capillaries that are distended during PACI, similar biochemical and mechanical effects may mediate observed fetal membrane inflammation.[202, 203]

Interestingly, rates of PPROM were lower in a small clinical series of fetoscopic MMC repairs that used PACI with heated-humidified CO_2.[17] Studies in animals and humans suggest that heated-humidified CO_2 pneumoperitoneum mitigates tissue damage and inflammation compared with unheated dry CO_2.[204-206] Other factors that may contribute to lower PPROM rates in that study include uterine exteriorization through a laparotomy incision and surgical closure of the uterine access site.[17] Nevertheless, further preclinical research is necessary to identify the potential benefits of using heated-humidified PACI to mitigate fetal membrane inflammation hence lower PPROM risk and to examine whether there are any fetal benefits. The greater uterine quiescence in sheep compared with humans limits the relevance of the ovine model to directly assess PPROM risk following PACI.[196, 207] Primates more accurately reflect the human uterine and placental structure; however, this species is financially limiting, and their use is under a moratorium in many places.[208]

While our study focused on assessing the real-time effects of PACI, it is unclear whether our acute observations would translate to poorer outcomes in humans at birth. In fact, in most clinical studies, fetuses seem to tolerate PACI, as no adverse events that are directly attributable to CO_2 insufflation have been reported. Our study is limited by the inherent limitations of the ovine model already discussed above and was not designed to thoroughly assess the risk of neurological deficits at birth. A previous study in sheep however found no obvious neurological impairment or evidence of gross brain injury at low PACI pressures for short durations.[78]

Conclusion

In conclusion, fetal sheep exposed to high PACI pressures experienced more hypercapnia, acidosis, and hypoxia than previously thought, likely exacerbated by significant reductions in uterine artery flow. In this study, we sought to explore the effects of increasing uterine pressures to understand the ontogeny of adverse fetal effects during PACI. While in this context, the sheep model has limitations in its translation to humans, understanding the physiological consequences of PACI is an important step in defining target ranges where PACI can be safely used. Meanwhile, in the absence of safe PACI pressure targets, or effective physiological indicators of uterine insufflation levels, efforts should continue to minimize PACI pressures were possible.

Experimental chapter 1.2:
Investigating the fetal effects of clinically relevant insufflation durations and CO_2 condition

Experimental chapter 1.1 demonstrated that increasing the amniotic pressure from 0 to 25mmHg over 100 minutes reduced uterine blood flow which correlated with worsening fetal hypercapnia and acidosis. However, unlike this first study, human fetuses undergoing fetoscopic myelomeningocele repair would be exposed to longer insufflation periods (180-250 minutes) at relatively stable pressures (mean 15mmHg). Additionally, preliminary human case series used heated and humidified CO_2 for amniotic insufflation.[16, 17] Therefore, in chapter 1.2 we investigated the fetal effects of amniotic insufflation using clinically relevant insufflation pressures (15mmHg), durations (180 minutes) and CO_2 conditions (40ºC, 95-100% humidity).

Like experimental chapter 1.1, we replicated amniotic insufflation in pregnant ewes. One group of instrumented fetal lambs were exposed to amniotic insufflation with cold, dry CO_2 at 15mmHg for 180 minutes. We compared fetal cardiovascular and blood gas changes in this group to a second group that underwent insufflation with heated, humidified CO_2 using the same pressures and durations.

As per chapter 1.1, fetal lambs exposed to cold, dry insufflation developed progressive hypercapnia, acidosis and cardiovascular impairment however, these changes became more severe over the longer insufflation duration. Fetal lambs that underwent amniotic insufflation with heated, humidified CO_2 developed significantly less hypercapnia and acidosis than lambs exposed to cold, dry CO_2 insufflation. These findings suggested that, despite disruptions to uterine blood flow that result from increased amniotic pressure, heated, humidified amniotic insufflation improved the fetus' ability to tolerate the procedure. This study entitled, "**Physiological effects of partial amniotic carbon dioxide insufflation with cold, dry vs heated, humidified gas in a sheep model**," was published in Ultrasound in Obstetrics and Gynaecology in 2018. A formatted version of the manuscript is included below.

Physiological effects of partial amniotic carbon dioxide insufflation with cold, dry vs heated, humidified gas in a sheep model

B.J. Amberg[1,2], R.J. Hodges[1,2], A.J. Kashyap[1,2], S.M. Skinner[1], K.A. Rodgers[1], E.V. Mcgillick[1,2], J.A. Deprest[3,4], S.B. Hooper[1,2], K.J. Crossley[1,2#] and P.L.J. DeKoninck[1,2,5#]

#K. C. and P. D. are joint senior authors.

1. The Ritchie Centre, Hudson Institute of Medical Research, Melbourne, Victoria, Australia;
2. The Department of Obstetrics and Gynaecology, Monash University, Melbourne, Victoria, Australia;
3. Institute of Woman's Health, University College London, London, UK;
4. Department of Development and Regeneration, Cluster Organ Systems, Faculty of Medicine, KU Leuven, Leuven, Belgium;
5. Department of Obstetrics and Gynaecology, Erasmus MC-Sophia Children's Hospital, Rotterdam, The Netherlands

Running Head: Heated-humidified CO_2 insufflation

Keywords: carbon dioxide; fetal membrane inflammation; fetoscopic surgery; myelomeningocele; PACI; partial amniotic CO2 insufflation

Correspondence: Dr P. DeKoninck, The Ritchie Centre, Hudson Institute of Medical Research, 27–31 Wright St, Clayton, Victoria, 3168 (e-mail: Philip.dekoninick@monash.edu)

Abstract:

Objective: Partial amniotic carbon dioxide (CO_2) insufflation (PACI) is used to improve visualization and facilitate complex fetoscopic surgery. However, there are concerns about fetal hypercapnic acidosis and postoperative fetal membrane inflammation. We assessed whether using heated and humidified, rather than cold and dry, CO_2 might reduce the impact of PACI on the fetus and fetal membranes in sheep.

Methods: Twelve fetal lambs of 105days' gestational age (term = 145 days) were exteriorized partially, via a midline laparotomy and hysterotomy, and arterial catheters and flow probes were inserted surgically. The 10 surviving fetuses were returned to the uterus, which was then closed and insufflated with cold, dry (22◦C at 0 – 5% humidity, n = 5) or heated, humidified (40◦C at 100% humidity, n = 5) CO_2 at 15 mmHg for 180min. Fetal membranes were collected immediately after insufflation for histological analysis. Physiological data and membrane leukocyte counts, suggestive of membrane inflammation, were compared between the two groups.

Results: After 180 min of insufflation, fetal survival was 0% in the group which underwent PACI with cold, dry CO_2, and 60% (n = 3) in the group which received heated, humidified gas. While all insufflated fetuses became progressively hypercapnic ($PaCO_2$ > 68 mmHg), this was considerably less pronounced in those in which heated, humidified gas was used: after 120min of insufflation, compared with those receiving cold, dry gas (n=3), fetuses undergoing heated, humidified PACI (n = 5) had lower arterial partial pressure of CO_2 (mean±standard error of the mean, 82.7±9.1mmHg for heated, humidified CO_2 vs 170.5 ± 28.5 for cold, dry CO_2 during PACI, P < 0.01), lower lactate levels (1.4 ± 0.4 vs 8.5 ± 0.9 mmol/L, P < 0.01) and higher pH (pH, 7.10±0.04 vs 6.75±0.04, P<0.01). There was also a non-significant trend for fetal carotid artery pressure to be higher following PACI with heated, humidified compared with cold, dry CO_2 (30.5 ± 1.3 vs 8.7 ± 5.5 mmHg, P = 0.22). Additionally, the median (interquartile range) number of leukocytes in the chorion was significantly lower in the group undergoing PACI with heated, humidified CO_2 compared with the group receiving cold, dry CO_2 (0.7 × 10–5 (0.5 × 10–5) vs 3.2 × 10–5 (1.8 × 10–5) cells per square micron, P = 0.02).

Conclusions: PACI with cold, dry CO_2 causes hypercapnia, acidosis, hypotension and fetal membrane inflammation in fetal sheep, raising potential concerns for its use in humans. It seems that using heated, humidified CO_2 for insufflation partially mitigates these effects and this may be a suitable alternative for reducing the risk of fetal acid – base disturbances during, and fetal membrane inflammation following, complex fetoscopic surgery.

Introduction:

Open fetal surgery is considered the gold standard for antenatal repair of myelomeningocele, following the results of the Management of Myelomeningocele Study.[7] However, significant maternal morbidity associated with open fetal surgery and implications for future pregnancies has driven the development of less invasive, fetoscopic approaches.[7, 8, 74] Unfortunately, the feasibility of complex fetoscopic surgery is hampered by limited space, poor visibility within the cloudy amniotic fluid and surgical instruments that do not work in a fluid environment.[8] Partial drainage of the amniotic fluid and gaseous distention of the uterus with carbon dioxide (CO_2) overcomes these challenges.[8] CO_2 gas seems a logical choice, given its low risk of causing gas embolism, extensive use for endoscopic surgery in adults and infants and the fact that is has virtually no known adverse effects.[183]

However, potential adverse effects of partial amniotic CO_2 insufflation (PACI) on fetal development have been suggested, and its use remains controversial. These safety concerns are based on observations in animal experiments demonstrating fetal hypercapnic acidosis.[9-12] Yet, human clinical case series have reported no adverse effects of PACI during surgery and immediately after delivery.[8, 14, 17, 85, 171] Invasive fetal monitoring during human fetal surgery is not easy and human data are scarce. In a recent case series[16] (n = 3), umbilical venous blood gasses were sampled before and after fetoscopic myelomeningocele repair using PACI. Reassuringly, this study observed only very mild changes in fetal acid–base balance in two of the cases. Interestingly, these changes were absent in the third case, which used heated, humidified CO_2 for insufflation. There are no animal studies investigating the physiological effects on the fetus of PACI with heated, humidified gas.

Additionally, lower rates of postoperative preterm rupture of membranes (PROM) have been observed at fetoscopic centers which use heated, humidified CO_2 for PACI, compared with others that use cold, dry CO_2 (23% vs 75 – 85%)[8, 14, 171] , although procedural differences may explain this in part. We hypothesized that the use of cold, dry CO_2 for PACI triggers an inflammatory process in the choriodecidua and precipitates PROM by dehydrating the membranes.[116, 146] Our aim in this study, therefore, was to investigate in sheep the effects of using heated and humidified, rather than dry and cold, CO_2 for PACI, with clinically relevant insufflation pressures and durations, on the fetal acid–base balance and cardiovascular function, and on fetal membrane inflammation.

Methods:

Surgery

The experimental method was approved by the Monash Medical Centre Animal Ethics Committee and followed a series of experiments in our laboratory to optimize the surgical protocol. Twelve pregnant Merino-Border Leicester ewes between 105- and 108-days' gestation were anesthetized with an intravenous bolus of sodium thiopentone (Pentothal, Boehringer Ingelheim, Warriewood, NSW, Australia). Ewes were ventilated via an endotracheal tube and general anesthesia was maintained with 2 – 2.5% inhaled isoflurane (Isoflow, Abbot Pty Ltd, North Chicago, IL, USA) in air/oxygen. End-tidal CO_2 was measured using a SurgiVet vital signs machine (SurgiVet® Advisor®, Smiths Medial, Dublin, OH, USA). A temperature probe was placed in the ewe's esophagus and a polyvinyl catheter (internal diameter, 2.6mm; Dural Plastics, Sydney, NSW, Australia) was inserted into the carotid artery. Arterial blood gasses were sampled at 10-min intervals to adjust the ewe's ventilation rate and tidal volume to maintain the maternal partial pressure of CO_2 ($PaCO_2$) between 35 and 45 mmHg.

The fetus was partially exteriorized via a midline laparotomy and hysterotomy, and we surgically inserted a carotid artery and jugular vein catheter (internal diameter, 0.86 mm), umbilical vein flow probe (Transonic Systems, Ithaca, NY, USA) and esophageal temperature probe. Insufflation tubing and an amniotic drainage catheter were inserted through the uterine wall into the amniotic sac. All catheters and flow probes were exteriorized and the hysterotomy incision closed in three layers to maintain an airtight seal of the uterus for insufflation. A flow probe was also placed around a large branch of the uterine artery supplying the pregnant horn. The uterus was then returned to the maternal abdomen and the laparotomy incision closed to allow insufflation of the uterus within the abdominal cavity as performed clinically in percutaneous approaches.

Maternal (temperature, carotid artery pressure, heart rate and uterine artery flow) and fetal (temperature, carotid artery pressure, umbilical vein flow and heart rate) parameters were recorded continuously using the LabChart and PowerLab data acquisition system (ADInstruments, Bella Vista, NSW, Australia). Two fetuses did not survive the initial surgery and were excluded, leaving 10 fetuses for insufflation.

Partial amniotic CO_2 insufflation

After surgery, the ewe and fetus were allowed to stabilize for 10 min before baseline physiological recordings were taken and blood gasses sampled (time = 0). The amniotic fluid was drained and ewes were allocated arbitrarily to PACI with cold, dry (22 ◦C at 0–5% humidity, n = 5) or heated, humidified (40 ◦C at 100% humidity, n = 5) CO_2. The intra-amniotic pressure was increased to 15mmHg with an

insufflator (40L High-Performance Insufflator, Stryker South Pacific, St Leonards, NSW, Australia) over a 5-min period, and this pressure was maintained for 180 minutes. The insufflation pressure and duration were selected based on averages obtained from human case series involving insufflation of the uterus within the abdomen.[8, 14, 171] In ewes receiving heated, humidified CO_2, the gas was passed through a laparoscopic humidification system (MR860, Fisher and Paykel Healthcare, Auckland, New Zealand) before it entered the uterus.

Fetal blood gas analysis was performed every 10 minutes for the first 30 minutes and every 30 minutes thereafter. The uterus was desufflated after 180 minutes and monitoring continued for a further 20 minutes. Both ewe and fetus were then euthanized using intravenous pentobarbitone (Lethobarb, Virbac Pty Ltd, Peakhurst, Australia) and post mortem examination was conducted to collect sections of fetal membrane.

Fetal membrane collection and histology

From each ewe, two arbitrarily selected regions of insufflated fetal membrane some distance from the hysterotomy incision were dissected away, immersion-fixed in 10% formalin and embedded in paraffin for histological analysis. Two 5-μm tissue sections were cut from each region, immunostained for CD45-positive cells (leukocytes) and counterstained with Mayer's hematoxylin, as described previously.[209] The number of CD45-positive cells in the amnion and in the chorion were counted by a single observer (B.J.A.), who was blinded to the insufflation group. Counts were performed using ImageJ software (National Institutes of Health, Bethesda, Maryland, USA), in five arbitrarily selected, non-overlapping fields of view at 40× magnification (BX-41 Laboratory Microscope, Olympus America Inc., Center Valley, PA, USA). Each cell count was adjusted for membrane thickness and the average in the amnion and chorion of each ewe calculated.

Data and statistical analysis

Fetal carotid arterial pressure was adjusted for amniotic pressure and umbilical vein blood flow was adjusted for fetal weight. Maternal uterine artery blood flow is expressed as percentage change from baseline. Blood gas and physiological data were normally distributed. These are presented as mean ± standard error of mean (SEM) and compared at matched timepoints using mixed analysis of variance (ANOVA) with Holm-Sidak post-hoc analysis. Cell counts were not normally distributed. These are presented as median (interquartile range (IQR)) and compared using the Mann – Whitney U-test. Statistics were processed and graphed using GraphPad Prism (Prism V7, GraphPad Software Inc., La Jolla, CA, USA). $P < 0.05$ was considered statistically significant.

Results:

Five ewes were insufflated with cold, dry CO_2 and five with heated, humidified CO_2. Blood gas and physiological recordings at baseline and after 120 min of insufflation are given for the fetuses in Table 7 and for the ewes in Table 8. 120min was chosen for comparison because of poor survival in the cold, dry CO_2 group beyond this point.

Fetal effects

All five fetuses in the group receiving cold, dry CO_2 developed severe acid-base disturbances and died before the end of the 180-min insufflation period, two before and three after 120 minutes (range, 63 – 139). Only two of five died in the group receiving heated, humidified CO_2 (120 and 180 minutes). One of the heated, humidified CO_2 group died suddenly following a maternal anesthetic complication, while the other became progressively hypercapnic and acidotic in the same way as the cold, dry CO_2 animals. This second fetus had a much greater reduction from baseline in uterine artery blood flow compared with the other fetuses in the group, which seemingly limited its ability to tolerate acid – base disturbances (data not shown).

While all insufflated fetuses became progressively hypercapnic ($PaCO_2$ > 68 mmHg), after 120 min, the $PaCO_2$ was significantly higher in fetuses insufflated with cold, dry CO_2 compared with those receiving heated, humidified CO_2 (mean ± SEM, 170.5 ± 28.5 vs 82.7 ± 9.1 mmHg, $P < 0.01$) (Table 7, Figure 18a). Additionally, fetal lactate levels were higher (8.5 ± 0.9 vs 1.4 ± 0.4 mmol/L, $P < 0.01$) (Figure 18b) and arterial pH was lower (pH 6.75 ± 0.04 vs 7.10 ± 0.04, $P < 0.01$) (Figure 18c) in those undergoing PACI with cold, dry CO_2 at this timepoint. Serum bicarbonate was similar and remained stable in both groups (Figure 18d). After 120 min, there was a non-significant trend for fetal carotid artery pressure (Figure 18e) and umbilical vein flow (Figure 18f) to be higher in fetuses insufflated with heated, humidified compared with cold, dry CO_2. Fetal esophageal temperatures remained stable and were similar between groups.

Maternal effects

There was no significant difference in the ventilation rates (Table 8, Figure 19a) required to maintain maternal PaCO2 at 35 – 45 mmHg (Figure 19b). Maternal arterial pH (Figure 19c), PaO2, SaO2, temperature and carotid artery pressure were not significantly different between experimental groups at any timepoint. In both groups, uterine artery flow decreased by 30 – 40% immediately after PACI commenced (Figure 19d).

Fetal membrane effects

More CD45-positive cells (leukocytes) were present in chorionic membranes exposed to cold, dry CO_2

during PACI than in those exposed to heated, humidified CO_2 (median (IQR), 3.2 × 10–5 (1.8 × 10–5) vs 0.7 × 10–5 (0.5 × 10–5) cells per square micron, P = 0.02) (Figure 20a). A similar trend was observed in the amniotic membranes, although the difference did not reach signifi- cance (2.4 × 10–5 (1.9 × 10–5) vs 0.2 × 10–5 (0.58 × 10–5) cells per square micron, P = 0.12) (Figure 20b). Representative images of fetal membranes insufflated with cold, dry CO_2 and with heated, humidified CO_2 during PACI are shown in Figure 20c and Figure 20d, respectively.

Table 7: Fetal blood gas and hemodynamic parameters during partial amniotic carbon dioxide (CO_2) insufflation (PACI) with cold, dry CO_2 or with heated, humidified CO_2.

	Baseline			After 120 min insufflation		
	Cold-dry	Heated humidified	P value	Cold-dry	Heated humidified	P value
n	5	5	-	3	5	-
Gestational age (days)	105.8 ± 0.6	105 ± 0.3	0.28	-	-	-
Carotid artery blood gas						
pH†	7.21 ± 0.04	7.24 ± 0.02	0.99	6.75 ± 0.04	7.10 ± 0.04	< 0.01
$PaCO_2$ (mmHg)†	63.4 ± 4.4	67.4 ± 2.5	0.99	170.5 ± 28.5	82.7 ± 9.1	< 0.01
PaO_2 (mmHg)†	22.4 ± 2.2	26.6 ± 0.6	0.02	12.0 ± 0.05	31.6 ± 1.2	< 0.01
SaO_2 (%)	52.5 ± 2.7	61.0 ± 8.3	0.98	9.5 ± 2.0	59.9 ± 4.0	< 0.01
Bicarbonate (mmol/L)	22.4 ± 1.2	26.6 ± 0.6	0.07	24.6 ± 3.8	22.4 ± 1.0	0.96
Lactate (mmol/L)	3.9 ± 0.8	2.8 ± 0.3	0.90	8.5 ± 0.9	1.4 ± 0.4	< 0.01
Physiology						
Carotid artery pressure (mmHg)	36.1 ±2.5	33.0 ±1.5	> 0.99	8.7 ± 5.5	30.5 ± 1.3	0.22
Heart rate (bpm)	155 ± 11	144 ± 6	> 0.99	65 ± 7.1	161.2 ± 3.7	< 0.01
Umbilical vein flow (mL/kg/min)	138.5 ± 8.7	127.6 ± 26	> 0.99	7.4 ± 0.7	124.6 ± 26	0.25
Temperature (°C)	39.7 ± 0.3	39.8 ± 0.2	> 0.99	39.6 ± 0.2	40.2 ± 0.3	> 0.99

Table 7: Data are presented as mean ± standard error of the mean. Baseline values recorded immediately before insufflation (time = 0 min). *Mixed ANOVA with Holm-Sidak post-hoc analysis; P < 0.05 considered statistically significant. †Presented as body temperature-corrected values. $PaCO_2$, arterial partial pressure of carbon dioxide; PaO2, arterial partial pressure of oxygen; SaO2, arterial hemoglobin oxygen saturation.

Figure 18: Fetal blood gas and hemodynamic parameters during partial amniotic carbon dioxide (CO_2) insufflation (PACI) with cold, dry CO_2 or with heated, humidified CO_2.

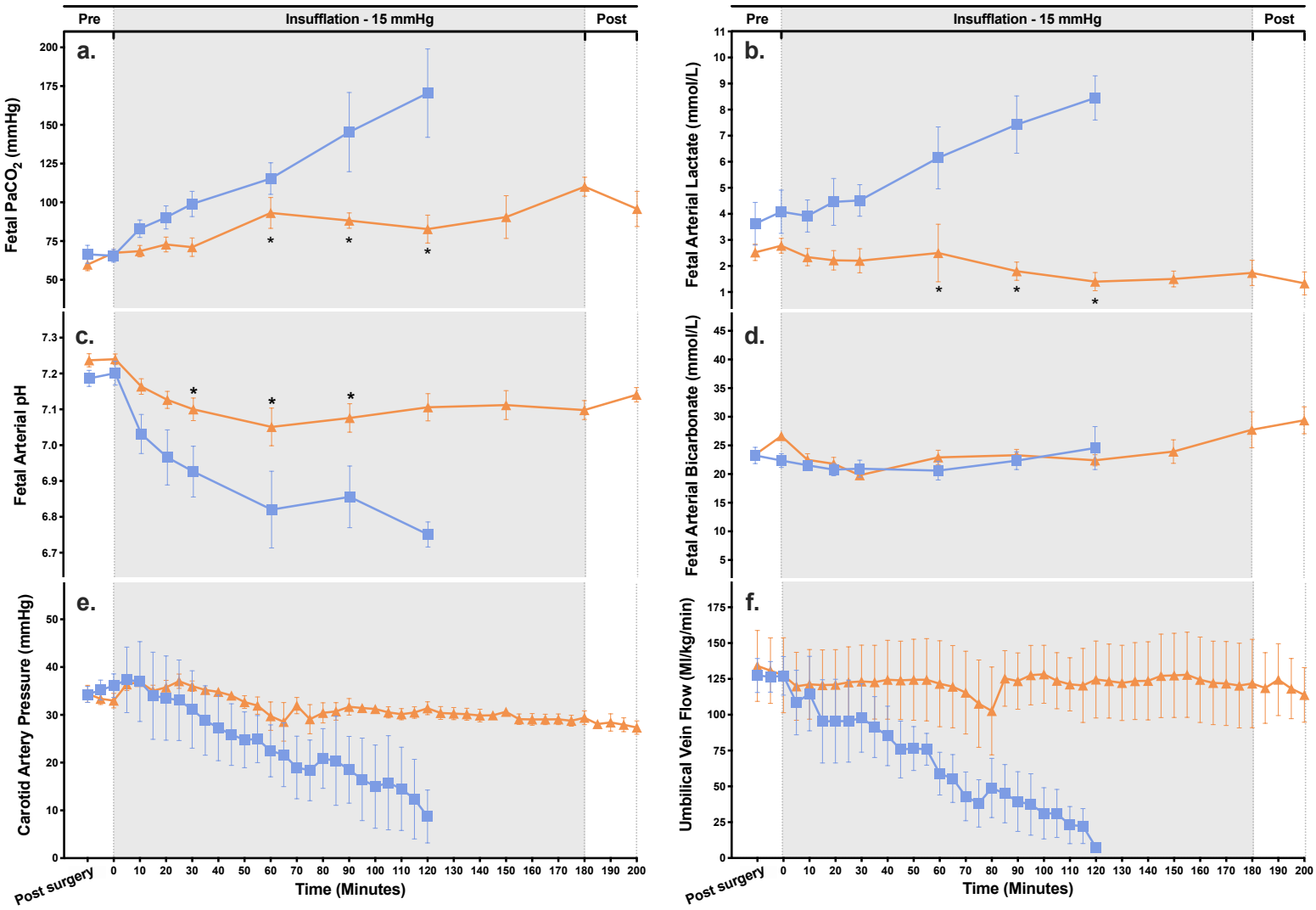


Figure 18: Fetal blood gas and hemodynamic parameters during partial amniotic carbon dioxide (CO_2) insufflation (PACI) with cold, dry CO_2 (■, n = 5) or with heated, humidified CO2 (▲, n = 5). Blood gasses were sampled every 10 min for first 30 min and every 30 min thereafter during 180-min period of insufflation, as well as 10 min before and 20 min after this period. Physiological data were recorded every 5 min. Arterial partial pressure of CO_2 ($PaCO_2$) (a) and lactate (b) were significantly higher and pH (c) significantly lower with cold, dry CO_2 compared with heated, humidified CO_2. Fetal arterial bicarbonate (d) remained stable. Fetal carotid artery pressure (e) and umbilical vein flow (f) decreased progressively during PACI with cold, dry CO_2 and remained stable with heated, humidified CO_2. Data are presented as mean ± standard error of the mean. indicates removal of case from analysis due to fetal death, at 63 and 98 min in cold, dry CO_2 group and at 120 and 130 min in heated, humidified CO_2 group. *P < 0.05 vs PACI with cold, dry CO_2.

Table 8: Maternal blood gas and hemodynamic parameters during partial amniotic carbon dioxide (CO2) insufflation (PACI) with cold, dry CO2 or with heated, humidified CO_2

	Baseline			After 120 min insufflation		
	Cold-dry	Heated-humidified	P value	Cold-dry	Heated-humidified	P value
Carotid artery blood gas						
pH†	7.40 ± 0.02	7.42 ± 0.04	>0.99	7.37 ± 0.03	7.38 ± 0.03	>0.99
$PaCO_2$ (mmHg) †	42.2 ± 1.3	42.2 ±1.9	>0.99	43.6 ± 1.7	41.2 ± 2.1	>0.99
PaO_2 (mmHg) †	199.8 ± 45.7	190.0 ± 15.3	>0.99	114.4 ± 47	157.5 ± 30	>0.99
SaO_2 (%)	95.0 ± 3.5	98.1 ± 0.7	>0.99	90.6 ± 7.1	96.3 ± 1.4	0.97
Bicarbonate (mmol/L)	24.1 ± 1.3	23.6 ± 1.5	>0.99	22.3 ± 1.7	22.6 ± 1.2	>0.99
Lactate (mmol/L)	1.4 ± 0.2	1.1 ± 0.3	>0.99	0.97 ± 0.1	1.1 ± 0.3	>0.99
Physiology						
Uterine artery flow (% change from baseline)	0	0	>0.99	-34.7 ± 24	-22.4 ± 12	>0.99
Carotid artery pressure (mmHg)	66.1 ± 1.1	72.2 ± 6.2	>0.99	48.8 ± 1.0	70.4 ± 9.3	0.62
Heart Rate (beats/min)	93.6 ±9.0	102.1 ±8.6	>0.67	95.5 ±7.8	107.3 ±3.9	0.14
Amniotic pressure (mmHg)	2.6 ± 2.0	3.0 ± 1.9	>0.99	15.6 ± 0.6	15.5 ± 0.4	>0.99
Temperature (°C)	38.7 ± 0.2	38.7 ± 0.2	>0.99	38.2 ± 0.3	38.9 ± 0.3	>0.99
Ventilation rate (breaths/min)	11.5 ± 1.0	11.4 ± 1.2	>0.99	14.0 ± 1.2	13.0 ± 1.1	>0.99

Table 8: Data are presented as mean ± standard error of the mean. Baseline values recorded immediately before insufflation (time = 0 min). *Comparison at each timepoint using mixed ANOVA with Holm-Sidak post-hoc analysis; $P < 0.05$ considered statistically significant. †Presented as body temperature-corrected values. $PaCO_2$, arterial partial pressure of carbon dioxide; PaO_2, arterial partial pressure of oxygen; SaO_2, arterial hemoglobin oxygen saturation.

Figure 19: Maternal blood gas and hemodynamic parameters during partial amniotic carbon dioxide (CO_2) insufflation (PACI) with cold, dry CO_2 or with heated, humidified CO_2

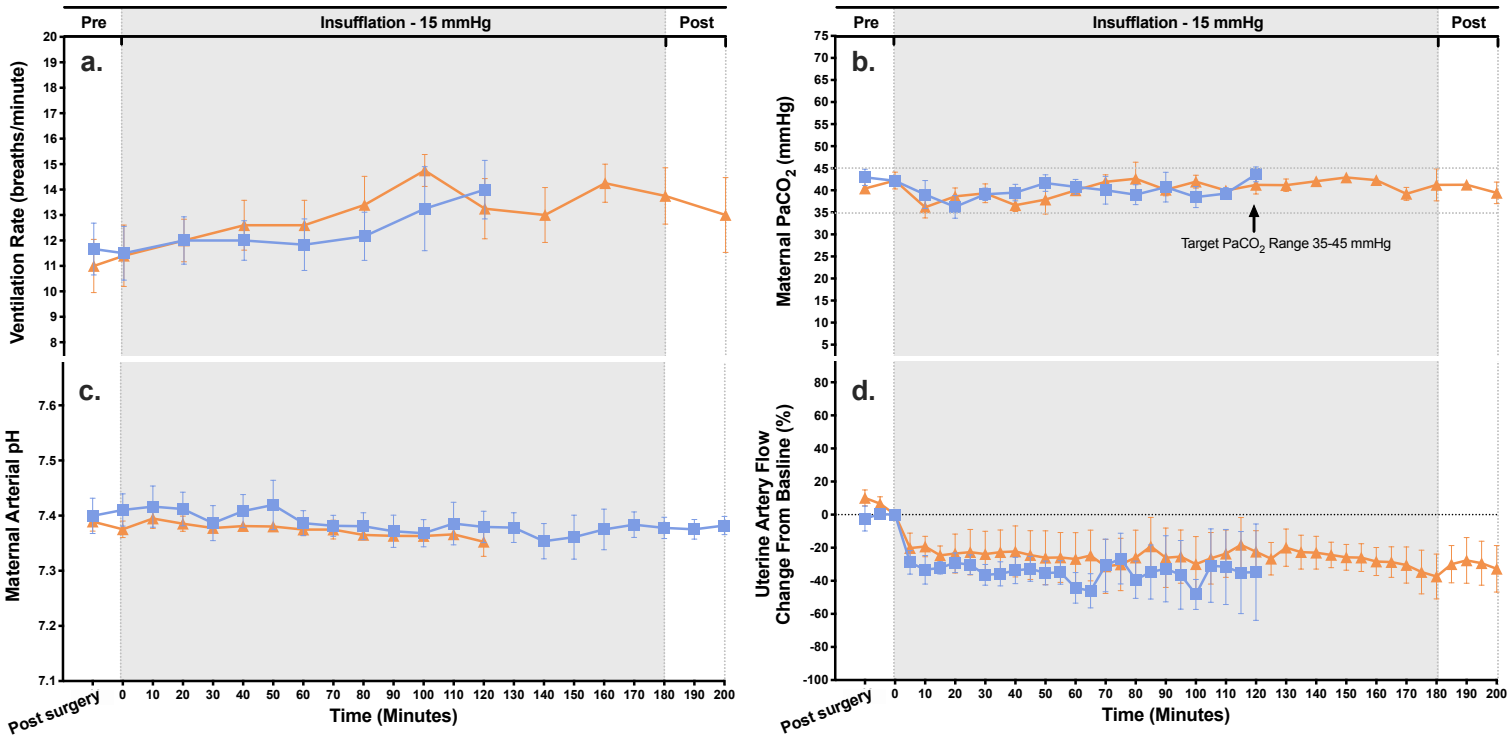


Figure 19: Maternal blood gas and hemodynamic parameters during partial amniotic carbon dioxide (CO_2) insufflation (PACI) with cold, dry CO_2 (■, n = 5) or with heated, humidified CO_2 (▲, n = 5). Maternal ventilation rate was recorded every 20 min, blood gasses sampled every 10 min and physiological data recorded every 5 min. Maternal ventilation rate (a) required to maintain arterial partial pressure of CO_2 ($PaCO_2$) between 35 and 45 mmHg (b) was similar in ewes receiving cold, dry CO_2 and those receiving heated, humidified CO_2 . Maternal arterial pH (c) also remained stable. Maternal uterine artery blood flow (d) reduced to a similar degree in both groups during PACI. Data are presented as mean ± standard error of the mean. indicates removal of case from analysis due to fetal death, at 63 and 98 min in cold, dry CO_2 group and at 120 and 130 min in heated, humidified CO_2 group. There were no significant differences in PACI with cold, dry CO_2 vs heated, humidified CO_2.

Figure 20: CD45-positive cell counts (leukocytes, indicative of inflammation) of insufflated fetal membranes

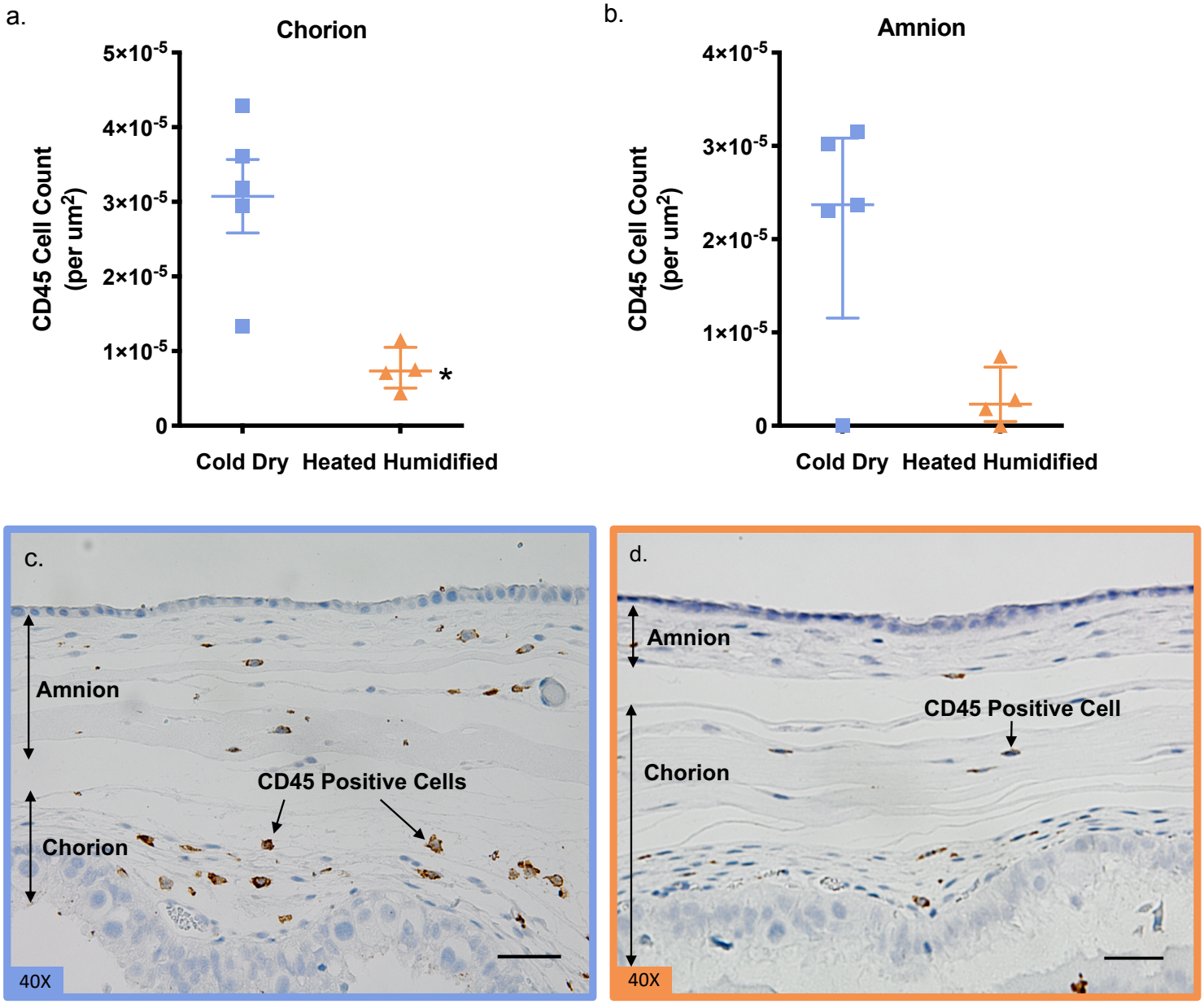


Figure 20: CD45-positive cell counts (leukocytes, indicative of inflammation) of insufflated fetal membranes. Significantly more CD45-positive cells were seen in chorion (a) from fetuses undergoing partial amniotic carbon dioxide (CO_2) insufflation (PACI) with cold, dry CO_2 (■) compared with those insufflated with heated, humidified CO_2 (▲). A similar trend was observed for amnion (b) but this did not reach significance. Data are presented as median (interquartile range). *P < 0.05 vs PACI with cold, dry CO_2. (c,d) Representative images of immunostained tissue sections from fetal membranes that were insufflated during PACI with cold, dry CO_2 (c) or with heated, humidified CO_2 (d). Scale bars represent 50 μm.

Discussion:

In this study, we assessed, using clinically relevant insufflation parameters, the effects on fetal lambs and membranes of PACI with cold, dry and with heated, humidified CO_2. Our results show that the disturbance to fetal physiology and membrane inflammation caused by PACI can be partially mitigated by using heated, humidified rather than cold, dry CO_2. Our findings with cold, dry CO_2 are consistent with those of previous sheep models of PACI, which showed a similar degree of fetal hypercapnia and acidosis over 30 – 60 min of insufflation.[9-12] Additionally, our study shows that prolonged exposure (up to 120min) to cold, dry CO_2 during PACI causes progressive acid – base disturbances that have a significant effect on fetal physiology in sheep.

There are several mechanisms which might help to explain why fetuses insufflated with heated, humidified CO_2 had significantly lower $PaCO_2$ after 120min than had those exposed to cold, dry CO_2. Heating CO_2 gas from 22°C to 40°C reduces its solubility by nearly 40%. Additionally, humidification would have reduced the intra-amniotic partial pressure of CO_2 (from 775 to ≈720 mmHg), due to the contribution of water vapor pressure.[109, 110, 210] Collectively, these may have reduced the absorption of CO_2 in fetuses exposed to heated, humidified gas.

It is also possible that the low temperature of cold, dry gas during PACI may have reduced clearance of CO_2 from the fetal compartment. Reducing the temperature of umbilical vessels from body temperature to 22° C may cause vessel vasoconstriction and reduce umbilical blood flow.[211] Indeed, we observed reductions in umbilical venous flow in fetuses undergoing PACI with cold, dry gas, and the associated reduction in placental perfusion likely reduced CO_2 clearance, adding to the fetal hypercapnia. Heating the gas may have avoided these changes, potentially explaining the improved placental CO_2 clearance and therefore the lower fetal $PaCO_2$ observed in the group in which this was done.

Finally, fetal carbonic anhydrase is most biologically active between 35 and 45° C and has < 50% activity at temperatures below 25° C.[212] Carbonic anhydrase catalyzes the reversible reaction that reforms CO_2 (along with water) from bicarbonate and hydrogen ions in fetal umbilical vessels, allowing it to diffuse into the maternal blood at the placenta.[89] PACI with cold, dry CO_2 may cool the blood moving through the small placental blood vessels and reduce the ability of the fetus to eliminate CO_2 via the mother. PACI with heated, humidified CO_2 may optimize the activity of carbonic anhydrase and improve fetal CO_2 elimination. While fetal and maternal temperatures in our experiment were similar between the two groups, our results were recorded from esophageal temperature probes and not at the placental surface where CO_2 exchange takes place. Future experiments should investigate how heated, humidified CO_2 affects placental clearance of CO_2 and the activity of placental carbonic anhydrase.

Severe hypercapnia and acidosis reduce cardiac contractility directly.[213, 214] We observed progressive reduction in carotid artery pressure in fetuses exposed to cold, dry CO_2 during PACI, all of which ultimately died. Clearly, this physiological disturbance was more severe than that observed in human fetuses, which generally remain stable and survive fetoscopic surgery. Several researchers have suggested that this difference is due to mechanical reductions in uterine blood flow that occur during overdistension of the compliant sheep uterus.[14, 78, 85] Indeed, we observed a 30 – 40% reduction in uterine artery blood flow during both PACI with cold, dry gas and that with heated, humidified gas. Importantly, however, changes in uterine artery blood flow were not different between the group receiving cold, dry gas and that receiving heated, humidified gas, indicating that this reduction in uteroplacental perfusion alone could not explain the metabolic differences observed between the two groups.

The non-significant rise in fetal PaO_2 during PACI with heated, humidified CO_2 is consistent with findings of previous sheep studies of PACI and likely reflects a rightward shift in the fetal oxyhemoglobin dissociation curve during mild hypercapnic acidosis.[11, 12] It is unclear why the fetal PaO_2 was slightly lower at baseline in the group undergoing PACI with cold, dry CO_2; however, the progressive reduction in fetal PaO_2 in this group was likely due to reduced fetal perfusion of the placenta as severe hypercapnic acidosis progressed and cardiovascular function declined.

The etiology of PROM or iatrogenic membrane rupture is not entirely understood, although fetal membrane rupture has been hypothesized to be preceded by a progressive weakening of membrane integrity.[116, 146] This weakening is believed to be caused by inflammation in the choriodecidua and recruitment of leukocytes (CD45-positive cells). We found significantly more immune cells present in chorionic membranes exposed to cold, dry CO_2 during PACI compared with those exposed to heated, humidified CO_2. This suggests that PACI induced an inflammatory response that was partially mitigated by using heated, humidified gas. We speculate that cold, dry CO_2 mediates fetal membrane inflammation by cooling and dehydrating the surface of the amnion. These changes have been seen in the human peritoneum following abdominal insufflation with CO_2 and can be reduced by heating and humidifying the gas.[172, 173] While the peritoneum and fetal membranes are different anatomically, both membranes are exposed to only liquids normally and likely have little capacity to cope with dehydration. As such, it is not surprising that the fetal membranes became inflamed in response to dehydration and that this could be reduced markedly by humidifying the CO_2 to 100%. Although these changes have not been correlated directly with an increased risk of membrane rupture, this may be one of the reasons that relatively low rates of postoperative PROM were observed in recent clinical series using heated, humidified CO_2 for PACI.[17] Future animal studies of PACI should build on our findings

and examine membrane markers of cellular injury, apoptosis and collagen degradation known to correlate with membrane rupture.[215]

Our sheep model of PACI has limitations in predicting the effects of PACI in humans. The cotyledonary arrangement of the sheep placenta increases the surface area for fetal CO_2. Also, the potentially more compliant sheep uterus predisposes to reduction in uterine blood flow during insufflation. These differences may explain the greater fetal acid–base disturbances in sheep compared with humans, as confirmed by three human cases in which only modest acid–base changes were observed in the fetal umbilical vein.[16] It should be noted, however, that our arterial blood gasses were sampled during insufflation, which would be more reflective of the fetal status intraoperatively compared with sampling in human cases after desufflation, when intraoperative changes may have resolved. Future animal studies should compare the effects of PACI with cold, dry and with heated, humidified CO_2 on the developing fetal brain.

In summary, we examined the effects of PACI with cold, dry and with heated, humidified CO_2 on fetal sheep physiology and fetal membranes. Our results support observations in humans suggesting that heating and humidifying the gas used during PACI can mitigate fetal acid–base disturbances caused by cold, dry insufflation and reduce fetal membrane inflammation, which may play a role in reducing postoperative PROM. Further studies are required to identify the mechanisms by which heating and humidifying the gas conveys these benefits.

Experimental chapter 2:
The effect of amniotic insufflation on the placenta

Experimental chapter 1 demonstrated that heating and humidifying the insufflated CO_2 partially mitigated the development of fetal hypercapnia and acidosis over 180 minutes using amniotic pressures of 15mmHg. While these findings aligned with trends in small human case series using heated, humidified insufflation, the mechanisms involved remained unclear which limited any further efforts to refine the insufflation technique. Therefore, we performed a third physiological study in pregnant ewes investigating CO_2 elimination from the fetal placental compartment during insufflation. We hypothesized that fetal hypercapnia and acidosis during cold, dry insufflation were due to vasoconstriction of placental blood vessels that reduced fetal CO_2 elimination. We believed that heating and humidifying the CO_2 was relieving this vasoconstriction and conveyed fetal acid base benefits by maintaining fetal CO_2 elimination.

Using our pregnant ewe model, we sampled paired arterial and venous blood gasses from the umbilical and uterine circulations during insufflation with cold, dry or heated and humidified CO_2. We then used these paired blood gas measurements to calculate blood CO_2 content and, along with measurements of umbilical and uterine blood flow, calculated the rate of CO_2 clearance across the placenta. These data also allowed us to calculate the rate of fetal CO_2 absorption from within the uterus.

Like we observed in experimental chapter 1.2, fetal lambs that underwent amniotic insufflation with heated, humidified CO_2 developed significantly less hypercapnia and acidosis than lambs exposed to cold, dry insufflation. However, amniotic insufflation reduced fetal CO_2 clearance across the placenta independent of insufflation temperature and humidity. We determined that instead of conveying fetal acid base benefits by improving fetal CO_2 clearance as we hypothesised, heated, humidified insufflation resulted in reduced fetal CO_2 absorption from the uterus. We believe these findings provide human fetal surgery centres with a physiological rationale to used heated, humidified insufflation during fetoscopic myelomeningocele repair. This study entitled, **"Placental gas exchange during amniotic carbon dioxide insufflation in sheep"** was published in Ultrasound in Obstetrics and Gynaecology in 2019. A formatted version of this manuscript is included below.

Placental gas exchange during amniotic carbon dioxide insufflation in sheep.

Amberg B.J.[1,2], Dekoninck P.L.J.[1,2,3], Kashyap A.J.[1,2], Skinner S.M[2], Rodgers K.A[1,2], McGillick E.V[1,2], Deprest J.A.[4,5], Hooper S.B.[1,2], Crossley K.J.[1,2] And Hodges R.J.[1,2]

1. The Ritchie Centre, Hudson Institute of Medical Research, Melbourne, Victoria, Australia
2. The Department of Obstetrics and Gynaecology, Monash University, Melbourne, Victoria, Australia
3. Department of Obstetrics and Gynaecology, Erasmus MC-Sophia Children's Hospital, Rotterdam, The Netherlands
4. Department of Obstetrics and Gynaecology, Division Woman and Child, University, Hospitals Leuven, Leuven, Belgium
5. Department of Development and Regeneration, Cluster Organ Systems, Faculty of Medicine, KU Leuven, Belgium

Short Title: Carbon dioxide insufflation and placental gas exchange

Keywords: carbon dioxide, ovine model, fetal therapy, placenta, gas exchange

Disclosure statement: The authors report no conflict of interest

Location of Research: Monash Medical Centre, Melbourne, Victoria, Australia

Corresponding Author: Associate Professor Ryan Hodges – The Ritchie Centre, Hudson Institute of Medical Research, 27-31 Wright St, Clayton, Victoria, 3168. ryan.hodges@monash.edu. (03) 8572 2700

Abstract:

Objectives: Insufflation of the amniotic cavity with carbon dioxide (CO_2) is used clinically to improve visibility during complex fetoscopic surgery. Insufflation with heated, humidified CO_2 has recently been shown to reduce fetal hypercapnia and acidosis in sheep, but the underlying mechanisms are unclear. We have investigated whether differences in placental CO_2 and oxygen (O_2) exchange could explain these findings.

Methods: Fetal lambs at 105 days gestation were instrumented with an umbilical artery and vein catheter and common umbilical vein flow probe. Arterial and venous catheters and flow probes were also inserted into the maternal uterine circulation. Six ewes were insufflated with cold, dry CO_2 (22°C, 0-5% humidity) and seven with heated, humidified CO_2 (40°C, 95-100% humidity) at 15mmHg for 180 minutes. Blood flow recordings and paired arterial and venous blood gasses were sampled from uterine and umbilical vessels. Rates of placental CO_2 and O_2 exchange were calculated. Data are presented as mean±SEM.

Results: At 120 minutes of insufflation, fetuses insufflated with heated, humidified CO_2 had lower arterial CO_2 levels and higher pH compared to those insufflated with cold, dry gas. Insufflation significantly decreased placental gas exchange in both groups as measured by rates of both (i) fetal CO_2 clearance and O_2 uptake and (ii) maternal O_2 delivery and CO_2 uptake from the fetal compartment.

Conclusions: Lower arterial CO_2 and higher pH levels in fetuses insufflated with heated, humidified CO_2 compared to cold, dry CO_2 , could not be explained by differences in placental gas exchange. Amniotic insufflation caused reductions in ovine placental gas exchange independent of CO_2 temperature and humidity.

Introduction:

Detection of congenital anomalies, such as myelomeningocele, during mid-gestation ultrasound provides a window of opportunity for surgical intervention to reduce infant morbidity and improve their long-term outcomes.[7] To reduce the impact of antenatal surgery on the mother, many of these surgeries are performed under video guidance through ports in the uterine wall (fetoscopy).[31, 79, 216] These complex fetoscopic procedures require gaseous distension of the amniotic space with carbon dioxide (partial amniotic carbon dioxide insufflation – PACI) to see clearly and manipulate the fetus during surgery.[79]

Carbon dioxide (CO_2) is a logical gas for insufflation due to its relatively low costs and extensive experience in laparoscopic surgery with minimal adverse effects. Additionally, its high solubility allows gas from the amniotic space to dissolve into fetal and maternal blood rather than forming gas emboli which are potentially fatal.[61] However, this additional dissolved CO_2 must be removed from maternal and fetal blood to avoid lowering blood pH (acidosis). Adults easily eliminate excess CO_2 from the lungs, however the fetal lungs play no role in gas exchange.[217] Instead, excess CO_2 is eliminated from the fetal compartment in the placenta where it enters the maternal circulation and is cleared from the mother's lungs (Figure 1).[217] As fetal placental gas exchange is less efficient than maternal respiratory CO_2 elimination, the fetus may be vulnerable to accumulating CO_2 during insufflation, which is consistent with several animal studies.[9-12, 218, 219]

We have recently shown that using heated humidified CO_2 for insufflation (heated, humidified PACI) greatly reduces fetal CO_2 levels and partially mitigates both the associated acidosis and increased blood lactate levels.[218] While these findings are consistent with trends in small human case series, the mechanisms explaining the fetal benefits remain unclear.[16] Understanding these mechanisms will allow refinement of insufflation parameters to further minimise the fetal effects of PACI during a time of significant physiological stress.

We hypothesised that cold, dry PACI impairs fetal CO_2 elimination by reducing umbilical blood flow and placental gas exchange. On the other hand, we hypothesised that heated, humidified PACI mitigates the decrease in umbilical blood flow and, thereby explains lower fetal CO_2 and higher pH levels. Our aim was therefore, to compare rates of placental gas exchange during cold, dry and heated, humidified PACI in sheep.

Methods:

Surgical Instrumentation:

The experimental protocol was approved by the Monash Medical Centre Animal Ethics Committee and followed a similar protocol as previously described.[218, 219] Thirteen pregnant Merino-Border Leicester ewes (103-106 days gestation) were anaesthetized with Sodium Thiopentone (i.v. Pentothal, Boehringer Ingelheim, Warriewood, NSW, Australia) and intubated with an endotracheal tube. General anaesthesia was maintained with 1.5-2% inhaled isoflurane (Isoflow, Abbot Pty Ltd, North Chicago, IL, USA) in air/oxygen. The ewe's ventilation rate and tidal volume were continuously adjusted to maintain arterial CO_2 levels between 35-45mmHg. Polyvinyl catheters (Internal Diameter (ID) 2.6mm, Dural Plastics, Sydney, NSW, Australia) were inserted into the ewe's carotid artery and jugular vein. Ewes were administered a continuous infusion of saline solution throughout the experiment via the jugular vein.

Fetuses were partially exteriorised via a midline laparotomy and hysterotomy incision made near the tip of the pregnant uterine horn. During instrumentation the exteriorised fetus and uterus were covered with warmed Hartmans solution to prevent heat loss and vessel constriction. A flow probe (Transonic Systems, Ithaca, NY, USA, Size 4) was placed around the fetal common umbilical vein and a temperature probe inserted into the fetal oesophagus. Fetuses were then returned to the amniotic space and a cotyledon close to the placental origin of the umbilical cord was identified. Heparinised saline-filled polyvinyl catheters (internal diameter, 0.86 mm) where introduced into large branches of the cotyledonary artery and vein and the tips advanced into the common umbilical artery and vein, within the cord (Figure 21). Insufflation tubing, a second temperature probe and an amniotic drainage catheter were inserted through the uterine wall into the amniotic sac. All catheters, temperature probes and flow probes were exteriorised and the hysterotomy incision closed in three layers to maintain an airtight seal of the uterus for insufflation as previously described.[218, 219]

A flow probe was placed around a large branch of the uterine artery supplying the pregnant horn and a polyvinyl catheter (internal diameter, 0.86 mm) introduced into the common uterine vein. The uterus was then returned to the maternal abdomen and the laparotomy incision closed to allow uterine insufflation within the abdominal cavity as previously performed in sheep and clinically in humans.[8, 14, 220]

Partial Amniotic CO_2 Insufflation:

Paired maternal (carotid artery and uterine vein) and fetal (umbilical artery and vein) blood gases were sampled immediately after surgery and then after a 10-minute stabilisation period (post-operative and baseline samples). Maternal (ventilation rate, amniotic temperature, heart rate and uterine artery flow) and fetal (temperature, umbilical artery and vein pressure and common umbilical vein flow)

physiological parameters were continuously recorded using LabChart and PowerLab data acquisition system (ADInstruments, Bella Vista, NSW, Australia).

The amniotic fluid was drained and ewes were arbitrarily allocated to cold, dry (22ºC, 0-5% humidity) or heated, humidified (40ºC, 100% humidity) PACI. Intra-amniotic pressures were increased with an insufflator (40L High- Performance Insufflator, Stryker South Pacific, Australia) over five minutes to 15mmHg and maintained for 180 minutes with a flow rate of 0.5L per minute. This pressure and duration were chosen to replicate averages from human case series and our previous ovine study showing a benefit to heated, humidified PACI.[8, 14, 218, 220] In ewes receiving heated, humidified PACI, the gas was passed through a laparoscopic humidification system (MR860, Fisher and Paykel Healthcare, Auckland, New-Zealand) before entering the uterus.

During insufflation, paired maternal blood gases were sampled every ten minutes. Paired fetal gases were sampled every 10 minutes for the first 30 minutes and every 30 minutes thereafter. The uterus was desufflated after 180 minutes in surviving fetuses and monitoring continued for a further 20 minutes. Both the ewe and fetus were then euthanized using intravenous Pentobarbitone (Lethobarb, Virbac Pty Ltd, Peakhurst, Australia) and post mortem examination conducted.

Data analysis and statistics:
Maternal and fetal physiological parameters were assessed every five minutes by determining the mean of each parameter over twenty second epochs. Umbilical vein blood flow was adjusted for fetal bodyweight.

Total blood CO_2 and O_2 content was calculated from each blood gas sample to account for differences in bound and dissolved gas in arterial and venous blood (Table 9, equations 1 and 2).[221, 222] Uteroplacental production of CO_2 and the rate of CO_2 elimination from the umbilical circulation (fetal CO_2 clearance) and uptake by maternal uterine blood (maternal CO_2 uptake) was calculated using equations 3-5 respectively. Similarly, the rate of O_2 loss from the uterine circulation (maternal O_2 delivery) and uptake by fetal umbilical blood (fetal O_2 uptake) was calculated using equations 6 and 7. These equations have been used extensively to calculate rates of placental gas exchange in sheep.[223-226]

Values are presented as mean ± standard error of mean (SEM). Blood gas and physiological data were normally distributed and compared at matched time points using a repeated-measures mixed analysis of variance (ANOVA) with Sidak post hoc analysis. Analysis of variance was also used to compare each time point to baseline values within each group. Linear regression and correlation analysis were used to

assess the relationship between umbilical vein blood flow and fetal placental gas exchange as well as uterine artery blood flow and maternal placental gas exchange.

Table 9: Calculations of blood gas content and placental gas exchange

Name	Equation
1. Total Blood CO_2 Content (mmol/L)[222]	$= PCO_2 \times 10^{(0.91 \times pH - 6.99)}$
2. Total Blood O_2 Content (mmol/L)[221]	$= \frac{Hb \times SaO_2}{100} \times 0.62$
3. Uteroplacental CO_2 production[226]	$= (UtV\ PCO_2 - UtA\ PCO_2) + (UmV\ PCO_2 - UmA\ PCO_2)$
4. Fetal Placental CO_2 Clearance (mmol/L/kg/min)[223-225]	$= \frac{(UmV\ flow \times 0.95)\ (UmA\ TCO_2 - UmV\ TCO_2)}{UmA\ PCO_2 - UtA\ PCO_2}$
5. Maternal Placental CO_2 Uptake (mmol/L/min)[223-225]	$= \frac{(UtA\ flow \times 0.84)\ (UtV\ TCO_2 - UtA\ TCO_2)}{UmA\ PCO_2 - UtA\ PCO_2}$
6. Maternal Placental O_2 Delivery (mmol/L /min)[223-225]	$= \frac{(UtA\ flow \times 0.84)\ (UtA\ TO_2 - UtV\ TO_2)}{UtA\ PO_2 - UmA\ PO_2}$
7. Fetal Placental O_2 Uptake (mmol/L/kg/min)[223-225]	$= \frac{(UmV\ flow \times 0.95)\ (UV\ TO_2 - UmA\ TO_2)}{UtA\ PO_2 - UmA\ PO_2}$

Table 9: PCO_2 – partial pressure of carbon dioxide, PO_2 – partial pressure of oxygen, SaO_2 – arterial haemoglobin oxygen saturation, TCO_2 – total carbon dioxide content, TO_2 – total blood oxygen content, UmA – umbilical artery, UmV – umbilical vein, UtA – uterine artery (maternal carotid artery blood samples have been used provide these values), UtV – uterine vein.

Figure 21: Gas exchange in the placenta and instrumentation of the fetal and maternal placental compartments

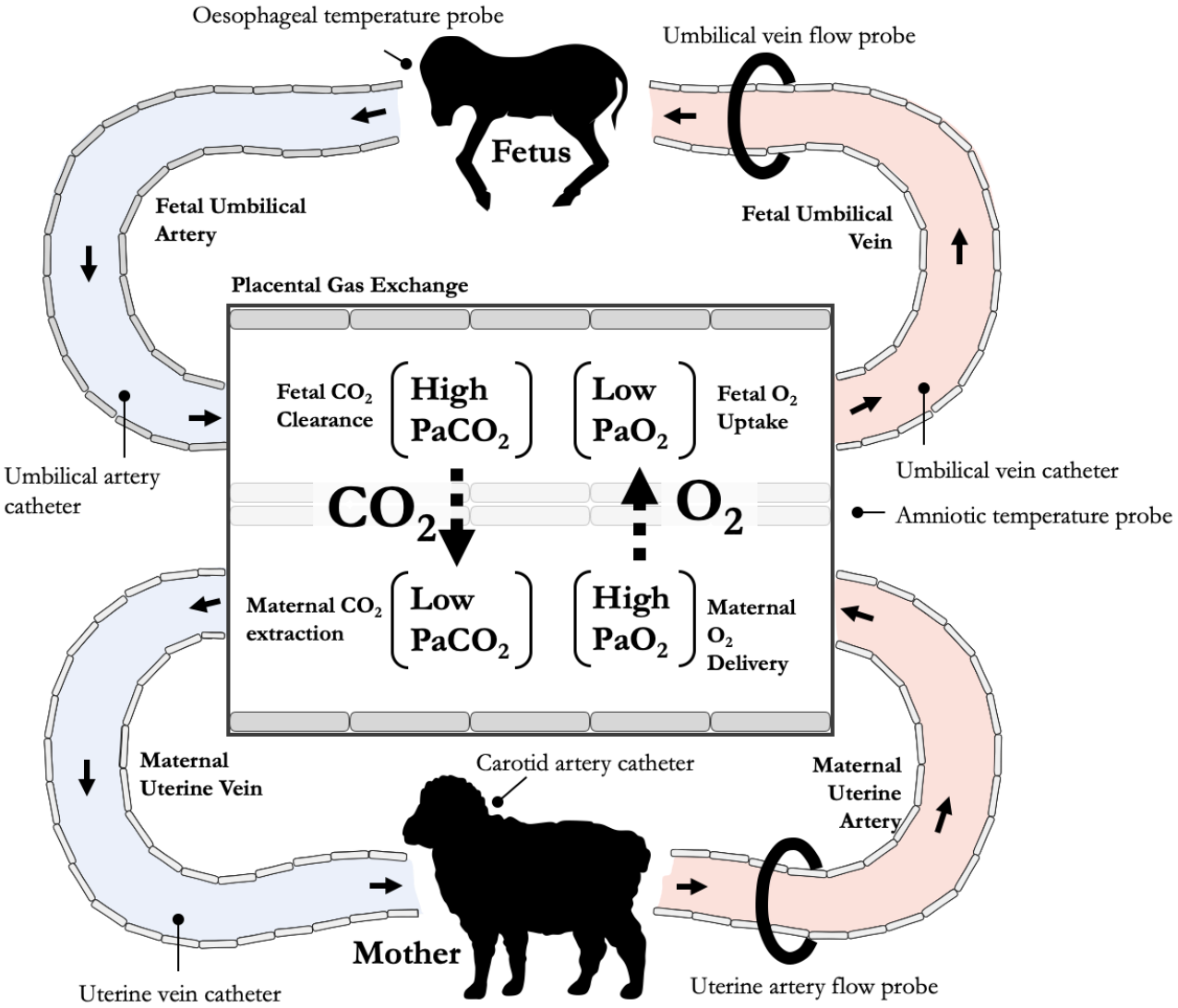


Figure 21: Fetal blood is cleared of carbon dioxide (CO_2) and re-oxygenated by diffusion of dissolved gasses between the fetal and maternal compartments of the placenta. To measure rates of placental gas exchange in sheep the fetus was instrumented with an umbilical artery and vein catheter, umbilical vein flow probe and oesophageal temperature probe. The ewe was instrumented with a carotid artery catheter, uterine vein catheter and uterine artery flow probe. A temperature probe was also inserted into the amniotic space. – original image

Results:

Of the thirteen pregnant ewes undergoing surgery and amniotic insufflation, six were insufflated with cold, dry CO_2 and seven with heated, humidified CO_2. Fetal weight and baseline blood gasses values, physiological parameters and placental gas exchange data were similar between groups. Fetal survival over 180 minutes of insufflation was 33% (2/6) with cold, dry CO_2 and 71% (5/7) with heated, humidified CO_2 (Figure 22a.). 120 minutes was chosen as the timepoint for comparison because of limited survival in the cold, dry group beyond this point.

Fetal Effects

Over 120 minutes of cold, dry or heated, humidified PACI, the partial pressure of CO_2 in the umbilical artery ($PaCO_2$), CO_2 content in the umbilical vein and blood lactate level increased while the pH decreased from baseline (Figure 22b-e.). However, compared to cold, dry PACI at 120 minutes, fetuses insufflated with heated, humidified CO_2 had lower $PaCO_2$ (99.5 ±14.6 vs.167.0 ±5.0 mmHg, $P<0.01$, Figure 22b.) and blood lactate (3.2 ±1.1 vs. 7.2 ±2.1mmol/L, $P<0.01$, Figure 22d.) and higher arterial pH (pH 7.02 ±0.08 vs. 6.78 ±0.10, $P<0.01$, Figure 22e.). Within both insufflation groups, umbilical artery oxygen saturation decreased from baseline while the PaO_2 remained stable (Figure 22f-g). During cold, dry PACI, uteroplacental production of CO_2 increased from baseline and was higher than the heated, humidified group after 120 minutes (Figure 22h).

During both cold, dry and heated, humidified PACI, fetal temperature remained unchanged (Figure 23a.) while umbilical artery and vein pressure increased from baseline immediately after starting insufflation (Figure 23b-c.). Pressures remained elevated in the umbilical vein and gradually returned to baseline in the umbilical artery over 120-minutes. In both groups, the rate of umbilical vein blood flow, placental CO_2 clearance and O_2 uptake decreased from baseline in response to insufflation (Figure 23d-f.). As there were no differences between groups, umbilical blood flow and fetal placental gas exchange data was pooled for linear regression analysis. This showed that rates of fetal CO_2 clearance ($r=0.43$, $P=<0.01$, Figure 23g.) and O_2 uptake ($r=0.64$, $P=<0.01$, Figure 23h.) were positively correlated with umbilical vein blood flow.

Maternal Effects

No changes in amniotic temperature were observed during heated, humidified or cold, dry PACI (Figure 24a.). Maternal ventilation rates required to maintain maternal $PaCO_2$ between 35 and 45mmHg were also similar between groups throughout the experiment (Figure 24b-c.).

Rates of uterine artery blood flow, CO_2 uptake and O_2 loss from the uterine circulation decreased in both groups over 120 minutes of insufflation (Figure 24d-f). As there were no differences between

groups, uterine blood flow and maternal placental gas exchange data were pooled for linear regression analysis. This showed that both maternal CO_2 uptake ($r=0.44$, $P<0.01$, Figure 24g.) and O_2 delivery ($r=0.57$, $P<0.01$, Figure 24h.) were positively correlated with uterine artery blood flow.

Figure 22: Fetal survival and blood gas changes

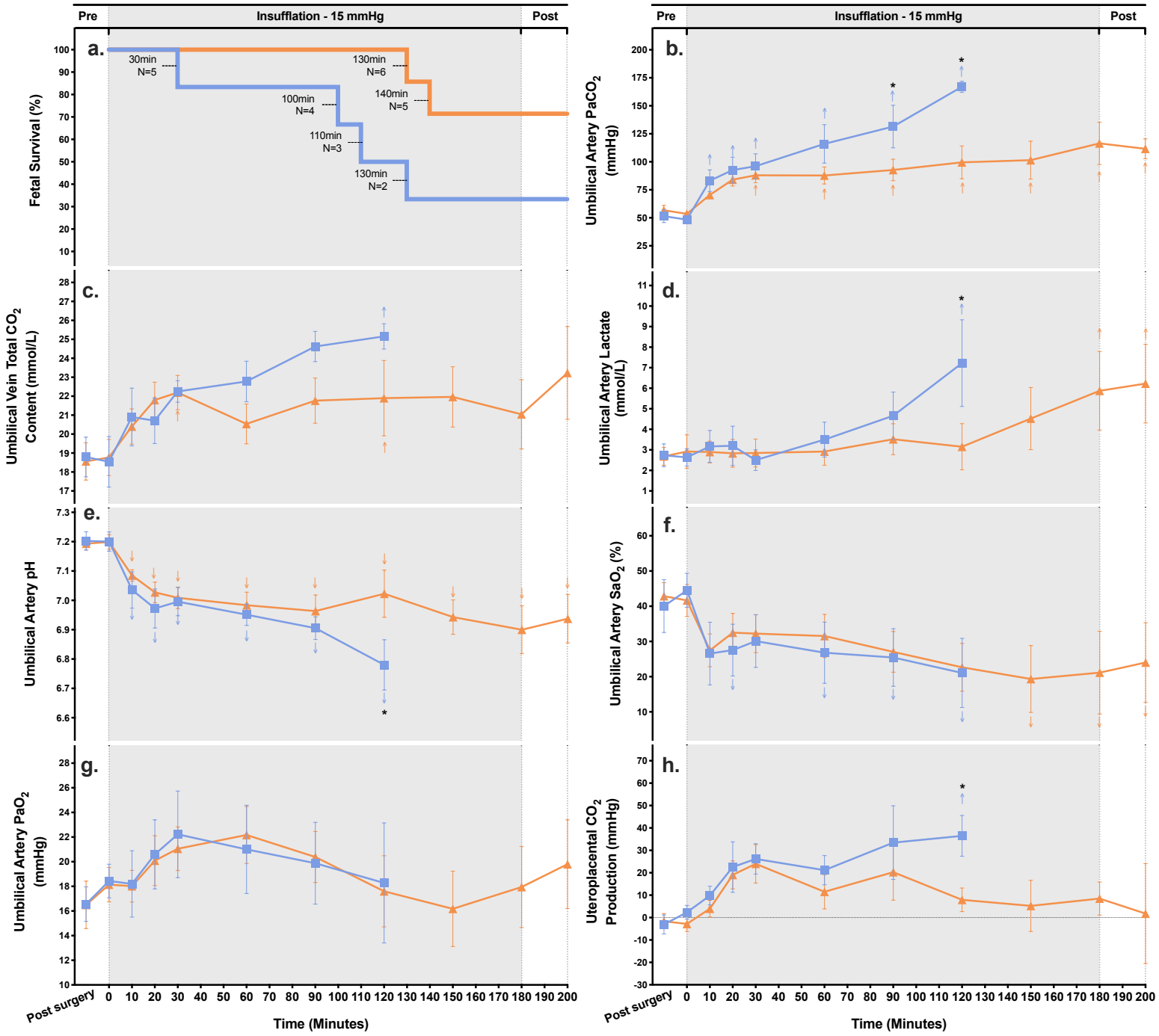


Figure 22: After 120 minutes, heated, humidified (▲) amniotic insufflation resulted in higher fetal survival (a.), lower arterial CO_2 (b.) and lactate (c.) levels and higher arterial pH (d.) compared to cold, dry (■) insufflation. Umbilical vein CO_2 content increased in both groups (e.). Arterial oxygen saturation decreased (f.) and dissolved oxygen remained stable (g.) from baseline in both groups. At 120 minutes uteroplacental production of CO_2 (h.) had increased during cold dry insufflation and was higher than the heated humidified group. Pre insufflation recordings were recorded immediately after surgery (post-surgery) and immediately before drainage of the amniotic fluid and insufflating the uterus (baseline). Data are presented as mean ± SEM. (*) represents $p<0.05$ vs. heated, humidified CO_2. (↑) and (↓) represent a significant ($P<0.05$) increase or decrease compared to baseline values within each group.

Figure 23: Fetal physiology and placental gas exchange

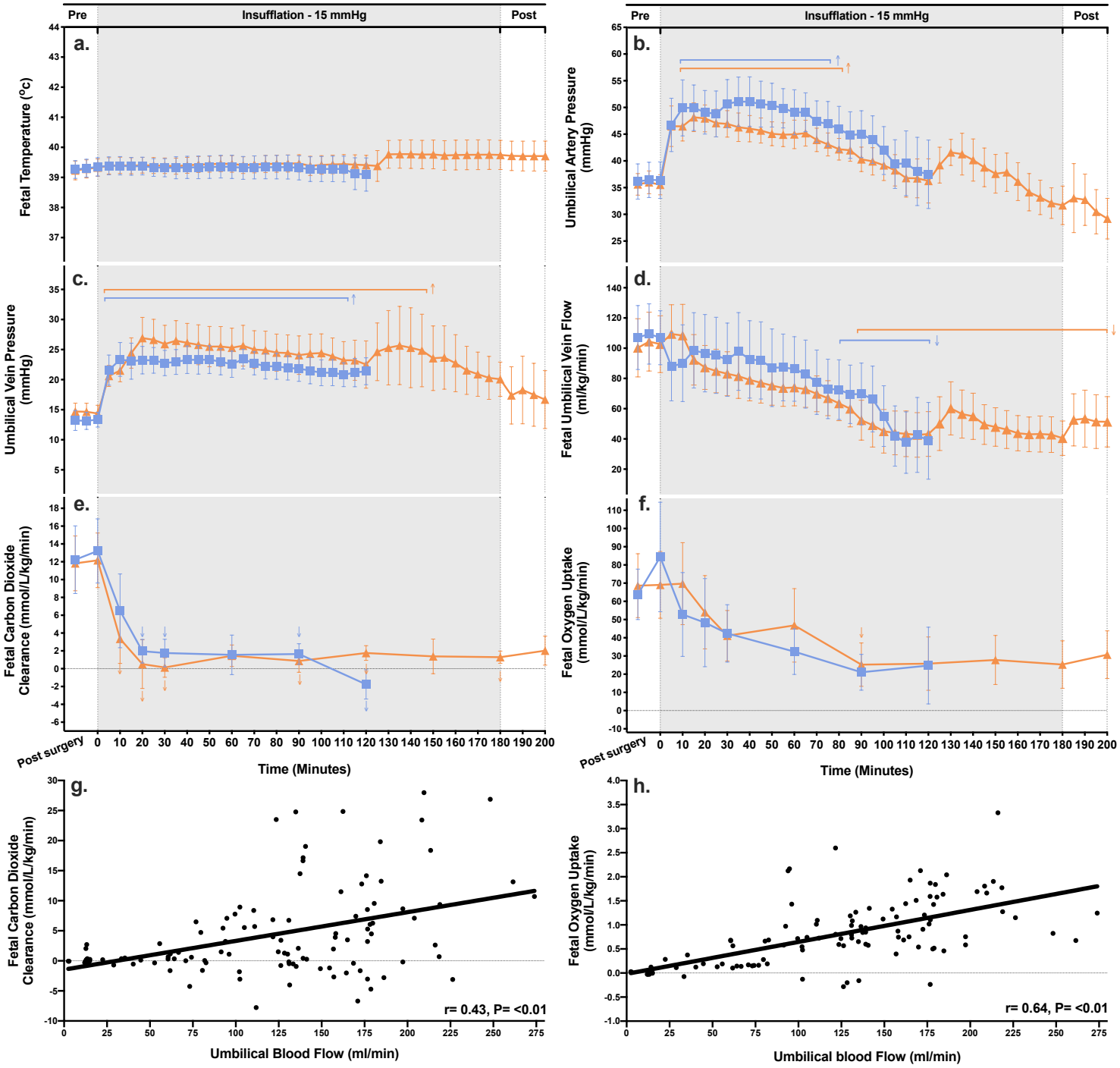


Figure 23: Fetal temperature (a.) remained stable from baseline during cold, dry (■) and heated, humidified (▲) insufflation. Umbilical artery (b.) and vein (c.) pressure increased while umbilical vein blood flow (d.), fetal CO_2 clearance (e.) and fetal O_2 uptake (f.) decreased in both groups independent of CO_2 temperature and humidity. Umbilical vein blood flow negatively correlated with fetal CO_2 clearance (g.) and oxygen uptake (h.). Pre insufflation recordings were recorded immediately after surgery (post-surgery) and immediately before drainage of the amniotic fluid and insufflating the uterus (baseline). Data are presented as mean ± SEM. (*) represents $p<0.05$ vs. heated, humidified CO_2. (↑) and (↓) represent a significant ($P<0.05$) increase or decrease compared to baseline values within each group.

Figure 24: Maternal physiology and placental gas exchange

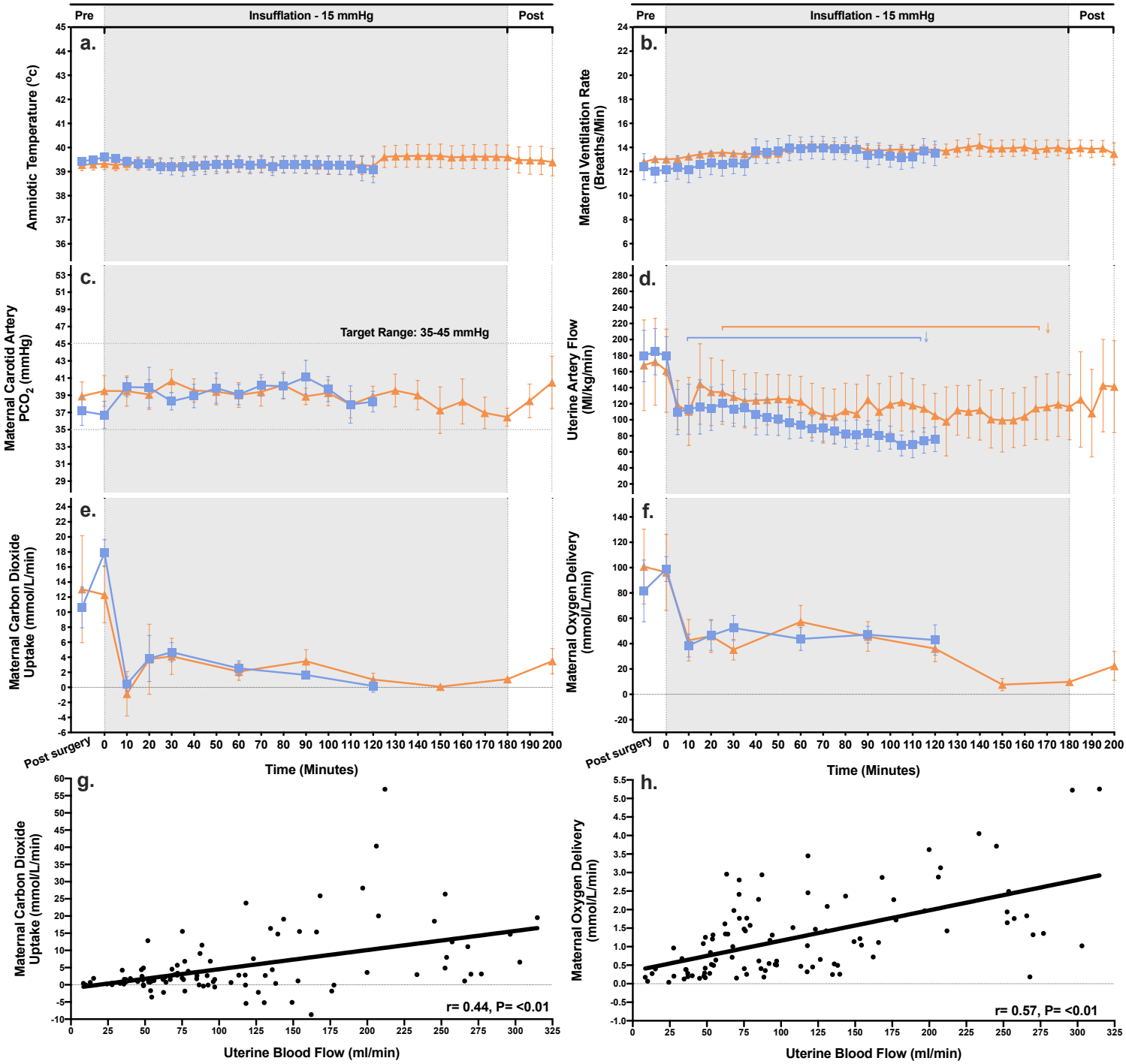


Figure 24: Amniotic temperature (a.), maternal ventilation rate (b.) and arterial CO_2 levels (c.) were the same during cold, dry (■) and heated, humidified (▲) insufflation and remained stable from baseline. Uterine artery blood flow (d.), maternal placental CO_2 uptake (e.) and placental O_2 delivery (f.) decreased in both groups independent of CO_2 temperature and humidity. Uterine artery blood flow negatively correlated with maternal CO_2 clearance (g.) and oxygen uptake (h.) Pre insufflation recordings were recorded immediately after surgery (post-surgery) and immediately before drainage of the amniotic fluid and insufflating the uterus (baseline). Data are presented as mean ± SEM. (*) represents $p<0.05$ vs. heated, humidified CO_2. (↑) and (↓) represent a significant ($P<0.05$) increase or decrease compared to baseline values within each group.

Discussion:

This study investigated the effects of amniotic insufflation with cold, dry or heated, humidified CO_2 on fetal physiology and placental gas exchange. Fetal $PaCO_2$ and blood lactate progressively increased and arterial pH decreased during cold, dry PACI. These acid base changes were partially mitigated when the insufflated CO_2 was heated and humidified. Interestingly, we found that the reductions in fetal (CO_2 clearance and O_2 uptake) and maternal (CO_2 uptake and O_2 delivery) placental gas exchange induced by insufflation were similar in both groups. Fetal and maternal placental gas exchange positively correlated with umbilical and uterine blood flow respectively.

These results confirm previous observations that the fetal hypercapnia and acidosis caused by cold, dry PACI can be mostly mitigated by heating and humidifying the CO_2.[9-12, 218, 219] Like others, we also observed a rapid and sustained reduction in fetal arterial oxygen saturation (within 10mins) which was associated with a non-significant rise in fetal PaO_2.[12, 227] This was likely due to a pH-induced decrease in the affinity of fetal haemoglobin for oxygen.[228] The slightly lower baseline fetal PaO_2 and pH values measured in this study, compared to our previous study, are simply due to the collection of fetal arterial blood from a post-ductal artery (umbilical artery) instead of a pre-ductal (carotid artery).[218]

We suggest that reductions in placental gas exchange during insufflation were caused by the 15mmHg increase in amniotic pressure. As ovine umbilical venous pressure is normally less than 15mmHg, insufflating the amniotic space would have compressed these vessels, potentially causing them to collapse. As a result, the pressure within these vessels must increase during insufflation to re-expand the vessels and restore some blood flow. This would explain why we observed an immediate and sustained increase in umbilical venous pressure during insufflation and an associated reduction in umbilical venous blood flow. Our data and the work of others has shown that umbilical blood flow is essential to maintain diffusion of dissolved gasses between the fetal and maternal compartments.[229] As insufflation pressures were the same in both groups, this explains why reductions in umbilical blood flow and placental gas exchange were independent of CO_2 temperature and humidity. We observed similar pressure related changes in the umbilical artery which would have further reduced placental gas exchange. However, umbilical artery pressure progressively returned to baseline likely as a result of local nitric oxide mediated vasodilation in response to downstream compression.[230, 231]

These results provide a physiological explanation for ultrasound and histological findings in human fetuses undergoing PACI suggestive of increased placental vascular resistance and fetal under-perfusion of the placenta.[232, 233] These results also add to existing experimental evidence suggesting that lower insufflation pressures below human fetal umbilical venous pressure (3-7mmHg) may improve the fetus'

ability to tolerate PACI.[219, 234] As such, ongoing efforts to lower insufflation pressures during human surgery by partially exteriorising the uterus appear justified.[17, 219]

Increasing amniotic pressure was also associated with lower uterine artery blood flow. Similar reductions in uterine blood flow have been observed in human pregnancies with increased amniotic pressure from polyhydramnios.[235] Lower uterine artery blood flow appeared to decrease maternal CO_2 uptake from the fetal compartment which likely contribute to progressive fetal hypercapnia. In both humans and sheep there is normally significant reserve in uteroplacental perfusion to avoid fetal blood gas changes during fluctuations in placental blood flow.[92, 236] However, this reserve may be reduced in the context of PACI where fetal placental gas exchange is also compromised by high amniotic pressure.

Contrary to our hypothesis we found no differences in umbilical venous blood flow or placental gas exchange to explain the fetal benefits of heated, humidified PACI. This suggests that cold, dry CO_2 did not induce umbilical vessel vasospasm like *in vitro* studies using temperatures <28ºC.[211, 237] Although the entry temperature of the insufflated CO_2 was very different (22ºC vs 40ºC) intra-amniotic temperatures were similar between groups. This finding could have been an artefact caused by the temperature probe laying against the warm uterine wall during insufflation in both groups. However, we consider it more likely that the insufflation gas was progressively heated as it entered the mother, particularly as the insufflation gas flow rate was relatively low (0.5L/minute).

Instead of conveying acid base benefits by improving placental gas exchange, heated, humidified CO_2 may slow fetal CO_2 absorption from the amniotic space. This is consistent with less CO_2 entering the fetal blood from the fetal membranes and placenta and the trend for lower umbilical vein CO_2 content during heated, humidified PACI. Heating CO_2 gas from 22 to 40ºc nearly halves its solubility and would reduce fetal absorption, but as indicated above, this effect may only be short lived once inside the uterus. Humidification lowers the partial pressure of CO_2 within the insufflated gas due to the contribution of water vapour pressure (Dalton's Law).[109, 110, 210] At 40ºC, water vapour pressure is ~55mmHg which will reduce the PCO_2 of heated, humidified gas from ~740 to ~685mmHg at normal atmospheric pressures. While this 7% reduction would also reduce fetal CO_2 absorption during heated, humidified PACI, we do not believe it completely explains the fetal acid base benefits. We hypothesize that cold, dry PACI damages the fetal membranes, which increases the rate of fetal CO_2 absorption. Indeed, we have previously shown that PACI with cold, dry CO_2 increases neutrophil recruitment into the fetal membranes.[218]

Although monitoring human fetuses during insufflation is technically challenging, human fetuses clearly tolerate PACI better than sheep fetuses. Rates of human fetal survival are high and, reassuringly, post-

surgical follow up has not been able to identify neurological sequelae attributable to intra-operative acidosis.[8, 14, 16, 17, 220, 238, 239] On the other hand, fetal sheep clearly develop severe hypercapnic acidosis that impacts survival. These differences could be attributed to the cotyledonary arrangement of the sheep placenta which may increase the surface area for fetal CO_2 absorption during PACI or the thinner, more compliant sheep myometrium which may over-stretch and occlude uterine arteries when insufflated. Additionally, the partially keratinized skin of the 103-106-day sheep fetus may more readily absorb CO_2 from the uterus compared the 24-26-week human fetus.[240, 241] While these differences suggest that human fetuses are not experiencing the same severity of fetoplacental disturbances, the principals of fetal CO_2 absorption and reduced placental gas exchange during PACI cannot ignored and should provide the rationale for future human studies aiming to confirm the fetal safety of PACI.

This study showed that PACI reduced placental gas exchange in sheep, independent of CO_2 temperature and humidity. Interestingly, the fetal benefits of heated, humidified PACI could not be explained by differences in placental gas exchange. We speculate that reduced fetal CO_2 absorption from the uterus is therefore the most likely mechanism to explain these benefits.

Acknowledgements:

This work is supported by the Victorian Government's Operational Infrastructure Support Program and also received funding from Monash Health (Emerging researcher fellowship, Dr Sasha Skinner).

Experimental chapter 3:
The effect of amniotic insufflation on the fetal membranes

As discussed in my literature review, human fetal surgery centres using cold, dry amniotic insufflation report markedly higher rates of iatrogenic PPROM (85-100%) compared to centres using heated, humidified CO_2 (10%).[8, 14, 17] While these are small studies, these findings suggest that cold, dry CO_2 insufflation may damage the fetal membranes and precipitate iatrogenic PPROM, whereas heated, humidified CO_2 may partially mitigate this injury and reduce the risk of preterm membrane rupture.

In experimental chapters 1 we collected sections of the fetal membranes exposed to the CO_2 during insufflation. Preliminary histological analysis published in these manuscripts demonstrated that cold, dry insufflation increased inflammatory cell counts, which was partially mitigated by using heated, humidified CO_2. While these studies suggested that heated, humidified insufflation may protect the fetal membranes, the underlying mechanisms of injury remained unclear. We, therefore, decided to undertake a more detailed analysis of molecular and histological markers of damage and inflammation in sheep fetal membranes collected from experimental chapter 2. We collected fetal membrane sections from the anterior region of the uterus directly exposed to either cold, dry or heated, humidified CO_2. Additionally, we collected fetal membranes from non-operated, twin pregnancies to use as non-insufflated controls.

Amniotic insufflation with cold, dry CO_2 increased molecular and histological markers of fetal membrane damage and inflammation relative to non-insufflated controls. These markers were either completely or partially mitigated in fetal membranes exposed to heated, humidified amniotic insufflation. While we were not able to demonstrate that differences in membrane damage and inflammation translated to an increased risk of iatrogenic PPROM, we believe that they provide an important starting point for future preclinical studies aimed at reducing iatrogenic PPROM after fetoscopic myelomeningocele repair. The manuscript entitled**, "The effects of cold, dry and heated, humidified amniotic insufflation on sheep fetal membranes"** has been submitted to Placenta and a formatted copy is included below.

The effects of cold, dry and heated, humidified amniotic insufflation on sheep fetal membranes.

Benjamin Amberg[a,b], Philip DeKoninck[a,b,c], Aidan Kashyap[a,b], Karyn Rodgers[a,b], Valarie Zhara[a,b], Stuart Hooper[a,b], Kelly Crossley[a,b,#], Ryan Hodges[a,b,#]

a. The Ritchie Centre, Hudson Institute of Medical Research, Melbourne, Victoria, Australia
b. The Department of Obstetrics and Gynaecology, Monash University, Melbourne, Victoria, Australia
c. Department of Obstetrics and Gynaecology, Erasmus MC-Sophia Children's Hospital, Rotterdam, The Netherlands

- Kelly Crossley and Ryan Hodges share joint senior authorship

Corresponding Author: Associate Professor Ryan Hodges – The Ritchie Centre, Hudson Institute of Medical Research, 27-31 Wright St, Clayton, Victoria, 3168. ryan.hodges@monash.edu. (03) 8572 2700

Location of Research: The Ritchie Centre, Hudson Institute of Medical Research, 27-31 Wright St, Clayton, Victoria, 3168.

Declarations of interest: none

Keywords**:** Amniotic insufflation, Carbon dioxide, Fetal membranes, Fetoscopic surgery**,** Heated, Humidified**.**

Abstract:

Introduction: Uterine distension with pressurised carbon dioxide (CO_2) (amniotic insufflation) is used clinically to improve visibility during keyhole fetal surgery. However, there are concerns that amniotic insufflation with unconditioned (cold, dry) CO_2 damages the fetal membranes which leads to post-operative preterm prelabour rupture of membranes (iatrogenic PPROM). We assessed whether heating and humidifying the insufflated CO_2 could reduce fetal membrane damage in sheep.

Methods: Thirteen pregnant ewes at 103-106 days gestation underwent amniotic insufflation with cold, dry (22°C, 0-5% humidity, n=6) or heated, humidified (40°C, 95-100% humidity, n=7) CO_2 at 15mmHg for 180 minutes. Twelve non-insufflated amniotic sacs acted as controls. Fetal membrane sections were collected after insufflation and analysed for molecular and histological markers of cell damage (caspase 3 and high mobility group box 1 [*HMGB1*]), inflammation (interleukin 1-alpha [*IL1-alpha*], *IL8* and vascular cell adhesion molecule [*VCAM*]) and collagen weakening (matrix metalloprotease 9 [*MMP9*]).

Results: Exposure to cold, dry CO_2 increased mRNA levels of *caspase 3*, *HMGB1*, *IL1-alpha*, *IL8*, *VCAM* and *MMP9* and increased amniotic epithelial caspase 3 and HMGB1 cell counts relative to controls. Exposure to heated, humidified CO_2 also increased *IL8* levels relative to controls however, *HMGB1*, *IL1-alpha* and *VCAM* mRNA levels and amniotic epithelial HMGB1 cell counts were significantly lower than the cold, dry group.

Discussion: Amniotic insufflation with cold, dry CO_2 damaged the amniotic epithelium and induced fetal membrane inflammation. Heated, humidified insufflation partially mitigated this damage and inflammation in sheep and may prove an important step in reducing the risk of iatrogenic PPROM following keyhole fetal surgery.

Introduction:

Keyhole fetal surgery (fetoscopy) at mid gestation is used clinically to treat fetuses with myelomeningocele.[8, 14, 17] During surgery, the uterus and fetal membranes are distended with pressurised carbon dioxide (CO_2), a technique known as amniotic insufflation, and the fetal spine is repaired under video-guidance.[8] While this approach protects the fetal spinal cord, up to 85-100% of these surgeries are complicated by post-operative preterm prelabour rupture of the membranes (iatrogenic PPROM).[8, 14] Iatrogenic PPROM (iPPROM) increases the risk of lung hypoplasia, chorioamnionitis and preterm birth, all of which have significant implications for the fetus and newborn, and potentially offset the benefits of fetal surgery.[200]

Recently, studies using heated and humidified CO_2 for amniotic insufflation have reported much lower iPPROM rates (10-29%).[17, 242] Although these were small studies with several differences in surgical technique, lower iPPROM rates with heated, humidified CO_2 suggests that insufflation with unconditioned (cold, dry) CO_2 damages the fetal membranes and increases the risk of iPPROM, whereas heated, humidified insufflation may mitigate these effects.[243]

Our recent sheep study demonstrated that fetal membranes exposed to heated, humidified insufflation had lower inflammatory cell counts than membranes exposed to cold, dry CO_2.[244] While these lower cell counts may indicate reduced membrane injury while using heated, humidified CO_2, the cause of these changes remain unclear. We, therefore, assessed molecular and histological markers of damage and inflammation in sheep fetal membranes exposed to amniotic insufflation with cold, dry or heated, humidified CO_2.

Methods:

Animals and tissue collection:

All experimental protocols were approved by the Monash Medical Centre Animal Ethics Committee and carried out in accordance to the National Institute of Health guide for the care and use of laboratory animals.[245] Fetal membranes were collected from a series of insufflation experiments previously reported.[246] Pregnant, Merino-Border Leicester ewes (n=13) were anaesthetized using Sodium Thiopentone (i.v. Pentothal, Boehringer Ingelheim, Warriewood, NSW, Australia) and 2–2.5% inhaled isoflurane (Isoflow, Abbot Pty Ltd, North Chicago, IL, USA) in air/oxygen between 103 and 106 days gestation. Fetuses were partially exteriorised through a laparotomy and hysterotomy made near the tip of the pregnant uterine horn. Fetuses were surgically instrumented for physiological monitoring as previously reported and returned to the amniotic sac.[246] The fetal membranes and hysterotomy were carefully sutured ensuring an air tight seal for insufflation. Amniotic drainage and insufflation catheters were inserted through the uterine wall into the amniotic space adjacent to the hysterotomy. Fetal monitoring and insufflation catheters were then exteriorised from the maternal abdomen and the laparotomy was sutured.

Amniotic fluid was drained and fetuses were randomly allocated to cold, dry (22ºC, 0-5% humidity) or heated, humidified (40ºC, 100% humidity) amniotic insufflation. If ewes were carrying twins, the second fetus and amniotic sac were used as a non-instrumented, non-insufflated control. For both insufflated groups, amniotic sac pressures were increased using an insufflator (40L High- Performance Insufflator, Stryker South Pacific, Australia) over five minutes to 15mmHg and maintained for 180 minutes using a flow rate of 0.5L per minute. These parameters were chosen to replicate averages from human case studies and previous insufflation studies in sheep.[8, 14, 171, 244] For fetuses undergoing heated, humidified insufflation, the CO_2 was passed through a laparoscopic humidification system before entering the uterus (MR860, Fisher and Paykel Healthcare, Auckland, New-Zealand).

In surviving fetuses, amniotic insufflation was ceased and CO_2 released from the uterus after 180 minutes. Amniotic fluid was returned to the uterus for 20 minutes before ewes and fetuses were euthanized with intravenous Pentobarbitone (Lethobarb, Virbac Pty Ltd, Peakhurst, Australia). At post-mortem, two 4x4cm sections of fetal membrane were collected from the anterior (exposed to CO_2) and posterior (still covered with amniotic fluid during experiment) regions of the amniotic sac as shown in figure 1. One anterior and one posterior section were snap frozen in liquid nitrogen and stored at -80ºC for molecular analysis. The other anterior and posterior sections were immersion-fixed in 10% formalin and embedded in paraffin for histology. Only anterior sections of fetal membrane were collected from control amniotic sacs. If the insufflated lamb did not survive the full 200 minutes experimental

protocol, fetal membranes were collected immediately after fetal demise from both the insufflated and control amniotic sacs.

Messenger RNA Quantification:

50mg of frozen fetal membrane from each amniotic sac was homogenized with trizol (Invitrogen, CA, USA) and total RNA isolated using a Purelink isolation kit (Invitrogen, CA, USA). Messenger RNA (mRNA) was reverse transcribed to single stranded complimentary DNA (cDNA) using the SuperScript® III First-Strand Synthesis System (Invitrogen, CA, USA). Quantitative reverse transcription-polymerase chain reaction was performed with the Fluidigm Access Array System (Fluidigm Corporation, CA, USA) by the Monash Health Translation Precinct Medical Genomics Facility. Commercially available, sheep-specific TaqMan primers were for epithelial and basement membrane proteins (occludin 1 [*OCLN1*], claudin 1 [*CLDN1*], tight junction protein 2 [*TJP2*], *TJP3* and basement membrane subunits *COL1A1* and *COL1A3*), damage associated molecular patterns (caspase 3, high mobility group box 1 [*HMGB1*], receptor for advanced glycation end products [*RAGE*], early growth response 1 [*EGR1*], cysteine-rich angiogenic inducer 61 [*CYR61*] and connective tissue growth factor [*CTGF*]), pro-inflammatory cytokines (interleukin 1 alpha [*IL1-alpha*], *IL1-beta*, *IL6*, *IL8*, tumour necrosis factors [*TNF*] and nuclear factor kappa beta [*NFKB*]), vascular adhesion molecules (vascular cell adhesion molecule [*VCAM*] and intracellular adhesion molecule 1 [*ICAM1*]) and collagenases (matrix metalloprotease 9 [MMP9] and tissue inhibitor of MMPs 2 [*TIMP2*]). Targeted genes were chosen to be representative of their respective family of proteins and details of their specific TaqMan primers are presented in supplementary table 1. Housekeeping control primers were for Ribosomal Protein S18 (*RPS18*).

Threshold values (Ct values) for each sample were measured in triplicate and a control sample containing no cDNA was included in each run. Minor differences in the amount of template added to each reaction were adjusted by subtracting the Ct value for RPS18 from the Ct value for the gene of interest (ΔCt). Messenger RNA levels for the genes of interest were normalized using the formula $2^{-\Delta Ct}$ and the results expressed as a fold change from control.

Histology and immunohistochemistry:

Paraffin embedded blocks of fixed chorioamnion were cut into 5μm thick sections and stained with haematoxylin and eosin (H&E) to assess the number of nuclei in the amniotic epithelium and picrosirius red to assess changes in collagen content. For immunohistochemistry, antigen retrieval was performed using citric acid buffer at pH 6.0 and endogenous peroxidase activity was blocked using methyl alcohol/hydrogen peroxide. Non-specific antibody binding was blocked using 2% normal goat serum (NGS) and sections were incubated at 4ºC overnight in the presence of the following primary

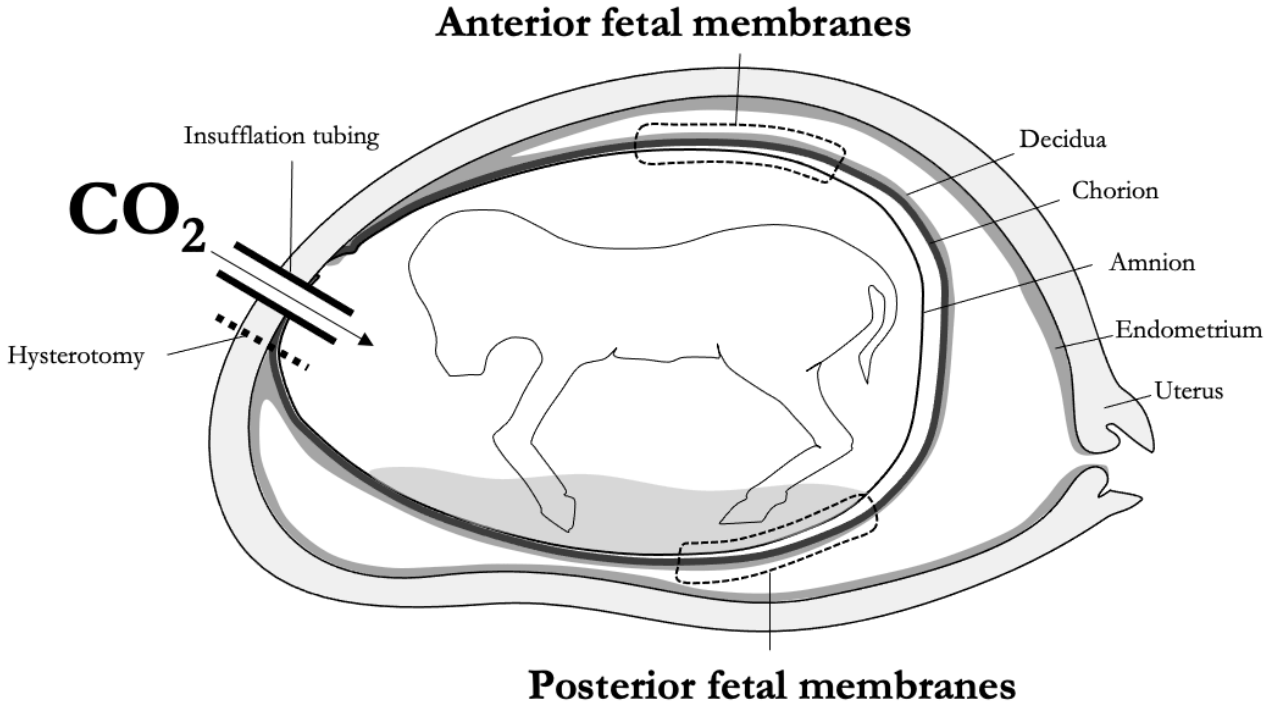
antibodies diluted in 2% NGS: Anti-Caspase 3 (1:50, cat#AF835, R&D Systems, USA), Anti-HMGB1 (1:250, cat#AB18256, Abcam, Cambridge, UK) and Anti-cluster determination 45 (CD45) (1:500, cat#MCA2220GA, BIORAD, CA, USA). Sections incubated in 2% NGS without the primary antibody were used as negative controls (supplementary figure 1). All sections were washed and incubated with the appropriate secondary antibodies diluted in 2% NGS for three hours before antigen/antibody complexes were visualised using an avidin biotin complex reaction (Vector Laboratories, CA, USA) and nickel-diaminobenzidine.

Positively stained cells were counted in the amnion or amniotic epithelium and chorion from three randomly selected, non-overlapping, 40X fields of view (BX-41 Laboratory Microscope, Olympus America Inc., Center Valley, PA, USA). All cell counts were performed by a blinded observer (BJA) using ImageJ software (National Institutes of Health, Bethesda, Maryland, USA). Cell counts were adjusted for membrane thickness or length where appropriate and averages calculated from each animal. Collagen content of the amnion and chorion was calculated as the percentage of membrane area stained with picrosirius red.

Data analysis:

Messenger RNA levels and histology data within each group were tested for normality using a Shapiro-Wilk test in GraphPad Prism 8 software (GraphPad Software, California, USA). Parametric data were compared using a one-way analysis of variance (ANOVA) and Tukey's post-hoc test and presented as mean ± standard error of mean (SEM). Non-parametric data was compared using a Kruskal Wallis analysis of variance and Dunn's post-hoc test and presented as median ± interquartile range (IQR).

Figure 25: Amniotic insufflation and fetal membrane collection.



Animals and **tissue collection:**

All experimental protocols were approved by the Monash Medical Centre Animal Ethics Committee and carried out in accordance to the National Institute of Health guide for the care and use of laboratory animals.[245] Fetal membranes were collected from a series of insufflation experiments previously reported.[246] Pregnant, Merino-Border Leicester ewes (n=13) were anaesthetized using Sodium Thiopentone (i.v. Pentothal, Boehringer Ingelheim, Warriewood, NSW, Australia) and 2–2.5% inhaled isoflurane (Isoflow, Abbot Pty Ltd, North Chicago, IL, USA) in air/oxygen between 103 and 106 days gestation. Fetuses were partially exteriorised through a laparotomy and hysterotomy made near the tip of the pregnant uterine horn. Fetuses were surgically instrumented for physiological monitoring as previously reported and returned to the amniotic sac.[246] The fetal membranes and hysterotomy were carefully sutured ensuring an air tight seal for insufflation. Amniotic drainage and insufflation catheters were inserted through the uterine wall into the amniotic space adjacent to the hysterotomy. Fetal monitoring and insufflation catheters were then exteriorised from the maternal abdomen and the laparotomy was sutured.

Amniotic fluid was drained and fetuses were randomly allocated to cold, dry (22ºC, 0-5% humidity) or heated, humidified (40ºC, 100% humidity) amniotic insufflation. If ewes were carrying twins, the second fetus and amniotic sac were used as a non-instrumented, non-insufflated control. For both insufflated groups, amniotic sac pressures were increased using an insufflator (40L High- Performance

Insufflator, Stryker South Pacific, Australia) over five minutes to 15mmHg and maintained for 180 minutes using a flow rate of 0.5L per minute. These parameters were chosen to replicate averages from human case studies and previous insufflation studies in sheep.[8, 14, 171, 244] For fetuses undergoing heated, humidified insufflation, the CO_2 was passed through a laparoscopic humidification system before entering the uterus (MR860, Fisher and Paykel Healthcare, Auckland, New-Zealand).

In surviving fetuses, amniotic insufflation was ceased and CO_2 released from the uterus after 180 minutes. Amniotic fluid was returned to the uterus for 20 minutes before ewes and fetuses were euthanized with intravenous Pentobarbitone (Lethobarb, Virbac Pty Ltd, Peakhurst, Australia). At post-mortem, two 4x4cm sections of fetal membrane were collected from the anterior (exposed to CO_2) and posterior (still covered with amniotic fluid during experiment) regions of the amniotic sac as shown in figure 1. One anterior and one posterior section were snap frozen in liquid nitrogen and stored at -80°C for molecular analysis. The other anterior and posterior sections were immersion-fixed in 10% formalin and embedded in paraffin for histology. Only anterior sections of fetal membrane were collected from control amniotic sacs. If the insufflated lamb did not survive the full 200 minutes experimental protocol, fetal membranes were collected immediately after fetal demise from both the insufflated and control amniotic sacs.

Messenger RNA Quantification:

50mg of frozen fetal membrane from each amniotic sac was homogenized with trizol (Invitrogen, CA, USA) and total RNA isolated using a Purelink isolation kit (Invitrogen, CA, USA). Messenger RNA (mRNA) was reverse transcribed to single stranded complimentary DNA (cDNA) using the SuperScript® III First-Strand Synthesis System (Invitrogen, CA, USA). Quantitative reverse transcription-polymerase chain reaction was performed with the Fluidigm Access Array System (Fluidigm Corporation, CA, USA) by the Monash Health Translation Precinct Medical Genomics Facility. Commercially available, sheep-specific TaqMan primers were for epithelial and basement membrane proteins (occludin 1 [*OCLN1*], claudin 1 [*CLDN1*], tight junction protein 2 [*TJP2*], *TJP3* and basement membrane subunits *COL1A1* and *COL1A3*), damage associated molecular patterns (caspase 3, high mobility group box 1 [*HMGB1*], receptor for advanced glycation end products [*RAGE*], early growth response 1 [*EGR1*], cysteine-rich angiogenic inducer 61 [*CYR61*] and connective tissue growth factor [*CTGF*]), pro-inflammatory cytokines (interleukin 1 alpha [*IL1-alpha*], *IL1-beta*, *IL6*, *IL8*, tumour necrosis factors [*TNF*] and nuclear factor kappa beta [*NFKB*]), vascular adhesion molecules (vascular cell adhesion molecule [*VCAM*] and intracellular adhesion molecule 1 [*ICAM1*]) and collagenases (matrix metalloprotease 9 [MMP9] and tissue inhibitor of MMPs 2 [*TIMP2*]). Targeted genes were chosen to be representative of their respective family of proteins and details of their specific

TaqMan primers are presented in supplementary table 1. Housekeeping control primers were for Ribosomal Protein S18 (*RPS18*).

Threshold values (Ct values) for each sample were measured in triplicate and a control sample containing no cDNA was included in each run. Minor differences in the amount of template added to each reaction were adjusted by subtracting the Ct value for RPS18 from the Ct value for the gene of interest (ΔCt). Messenger RNA levels for the genes of interest were normalized using the formula $2^{-\Delta Ct}$ and the results expressed as a fold change from control.

Histology and immunohistochemistry:

Paraffin embedded blocks of fixed chorioamnion were cut into 5μm thick sections and stained with haematoxylin and eosin (H&E) to assess the number of nuclei in the amniotic epithelium and picrosirius red to assess changes in collagen content. For immunohistochemistry, antigen retrieval was performed using citric acid buffer at pH 6.0 and endogenous peroxidase activity was blocked using methyl alcohol/hydrogen peroxide. Non-specific antibody binding was blocked using 2% normal goat serum (NGS) and sections were incubated at 4°C overnight in the presence of the following primary antibodies diluted in 2% NGS: Anti-Caspase 3 (1:50, cat#AF835, R&D Systems, USA), Anti-HMGB1 (1:250, cat#AB18256, Abcam, Cambridge, UK) and Anti-cluster determination 45 (CD45) (1:500, cat#MCA2220GA, BIORAD, CA, USA). Sections incubated in 2% NGS without the primary antibody were used as negative controls (supplementary figure 1). All sections were washed and incubated with the appropriate secondary antibodies diluted in 2% NGS for three hours before antigen/antibody complexes were visualised using an avidin biotin complex reaction (Vector Laboratories, CA, USA) and nickel-diaminobenzidine.

Positively stained cells were counted in the amnion or amniotic epithelium and chorion from three randomly selected, non-overlapping, 40X fields of view (BX-41 Laboratory Microscope, Olympus America Inc., Center Valley, PA, USA). All cell counts were performed by a blinded observer (BJA) using ImageJ software (National Institutes of Health, Bethesda, Maryland, USA). Cell counts were adjusted for membrane thickness or length where appropriate and averages calculated from each animal. Collagen content of the amnion and chorion was calculated as the percentage of membrane area stained with picrosirius red.

Data analysis:

Messenger RNA levels and histology data within each group were tested for normality using a Shapiro-Wilk test in GraphPad Prism 8 software (GraphPad Software, California, USA). Parametric data were compared using a one-way analysis of variance (ANOVA) and Tukey's post-hoc test and presented as

mean ± standard error of mean (SEM). Non-parametric data was compared using a Kruskal Wallis analysis of variance and Dunn's post-hoc test and presented as median ± interquartile range (IQR).

Figure 25: Pregnant ewes between 103-106 days gestation underwent amniotic insufflation with cold, dry or heated, humidified CO_2 at 15mmHg for 180 minutes. Two fetal membrane were sections collected from the anterior and posterior part of the amniotic sac. One anterior section and one posterior section were snap frozen in liquid nitrogen and stored at -80ºC for molecular analysis. The other anterior and posterior section was immersion fixed in formalin and embedded in paraffin wax for immunohistochemistry.

Reason for assessment	Gene name	Gene symbol	TaqMan Assay ID
Housekeeping gene	Ribosomal protein S18	RPS18	Oa4906333_g1
Tight junction and basement membrane proteins	Claudin 1	CLDN1	Oa03217991_m1
	Collagen 3A1	COL3A1	Oa04910910_m1
	Collagen 1A1	COL1A1	Oa01463861_gH
	Occludin 1	OCLN1	Oa04728972_m1
	Tight junction protein 2	TJP2	Oa04816038_m1
	Tight junction protein 3	TJP3	Oa04693836_g1
Damage associated molecular patterns	Caspase 3	Caspase 3	Oa04817361_m1
	High mobility group box 1	HMGB1	Ch04812286_s1
	Receptor for advanced glycation end products	RAGE	Bt03231022_m1
	Early growth response 1	EGR1	Oa03237885_m1
	Cysteine-rich angiogenic inducer 61	CYR61	Oa04673852_g1
	Connective tissue growth factor	CTGF	Oa04659069_g1
Markers of inflammation	Interleukin 1 alpha	IL1-alpha	Oa04658682_m1
	Interleukin 1 beta	IL1-beta	Oa04656322_m1
	Interleukin 6	IL6	Oa04656315_m1
	Interleukin 8	IL8	Bt03211906_m1
	Tumour necrosis factor	TNF	Oa04656867_g1
	Nclear factor kappa beta	NFK-beta	Oa04837805_m1
	Vascular cell adhesion molecule	VCAM	Oa04918369_sH
	Intracellular adhesion molecule	ICAM1	Oa04658651_m1
Markers of collagen metabolism	Matrix metalloprotease 9	MMP9	Oa03215996_g1
	Tissue inhibitor of matrix metalloproteases 2	TIMP2	Oa04655716_m1

Supplementary table 1: Details of genes investigated using real time quantitative PCR by Fluidigm. Gene names and symbols have been validated on the National Centre for Biotechnology Information (NCBI) Gene Database. The TaqMan assay ID for each commercially available probe is provided. Target-specific sequences of TaqMan assays are not available due to non-disclosure policies.

Results:

Animals

Of the thirteen pregnant ewes available, six underwent insufflation with cold, dry CO_2 and seven with heated, humidified CO_2. In twelve ewes bearing twins, the non-insufflated amniotic sacs were available as controls. A full description of fetal blood gas and physiological changes in insufflated fetal lambs has been previously described.[246] In summary, fetal survival was 33% (2/6) after 180 minutes of cold, dry insufflation and 71% (5/7) using heated, humidified CO_2.[246] Mean insufflation duration was 122±22 minutes using cold dry CO_2 and 167±8 minutes using heated, humidified CO_2. Mean experimental duration for control lambs was 150±13 minutes.

Markers of membrane damage

Anterior fetal membranes directly exposed to cold, dry CO_2 during insufflation had significantly fewer amniotic epithelial nuclei present on H&E compared to controls (Figure 26a). Additionally, the smooth apical surface of the amniotic epithelium seen in the control and heated, humidified groups (Figure 26b and d) appeared disrupted and desiccated in membranes exposed to cold, dry CO_2 (Figure 26c). Similarly, the nuclear envelope immediately adjacent to the apical surface of amniotic epithelial cells also appeared disrupted. Insufflated fetal membranes collected from the posterior amniotic sac showed no differences in epithelial nuclei count compared to controls (Figure 26a). We, therefore, focussed our analysis of insufflated fetal membranes on anterior sections of the amniotic sac directly exposed to CO_2 during insufflation.

Fetal membranes exposed to cold, dry CO_2 had significantly higher mRNA levels of epithelial proteins *CLDN1* and *COL3A1* (Figure 26e-f) and the DAMPs *Caspase* 3, *HMGB1*, *EGR1*, *CYR61* and *CTGF* compared to controls (Figure 27k-p). Immunohistochemistry identified increased Caspase 3 and HMGB1 cell counts in the amniotic epithelium of membranes exposed to cold, dry CO_2 relative to controls (Figure 27a and Figure 27f). Chorionic HMGB1 cell counts were also significantly increased in the cold, dry group compared to controls (Figure 27g).

Similar to fetal membranes exposed to cold, dry CO_2, fetal membranes exposed to heated, humidified CO_2 had significantly higher mRNA levels of *CLDN1*, *EGR1* and *CTGF* compared to controls (Figure 26e, Figure 27n and Figure 27p). However, the heated, humidified group had significantly lower mRNA levels of the DAMPs *HMGB1* and *CYR61* (Figure 27l and o) as well as amniotic epithelial HMGB1 cell counts (Figure 27f) compared to membranes exposed to cold, dry CO_2. No differences in amniotic epithelial or chorionic Caspase 3 cell counts were identified between the heated, humidified and cold, dry groups (Figure 27a-b and Figure 27f-g).

Markers of membrane inflammation

Fetal membranes exposed to cold, dry CO_2 had significantly higher mRNA levels of the pro-inflammatory cytokines *IL1-alpha, IL1-beta, IL6, IL8, TNF* and *NFKB* as well as the vascular adhesion molecules *VCAM* and *ICAM1* compared to controls (Figure 28f-m). Membranes exposed to cold, dry CO_2 also had significantly higher CD45 cell counts relative to controls within the amnion (Figure 28a). Fetal membranes exposed to heated, humidified CO_2 also had increased mRNA levels of *IL8*, *TNF*, *NFKB* and *ICAM1* relative to controls (Figure 28i-k and Figure 28m) however, had significantly lower *IL1-alpha* and *VCAM* mRNA levels (Figure 28l) as well as amniotic *CD45* cell counts (Figure 28a) compared to the cold, dry group.

Collagenase mRNA levels and membrane collagen content

Fetal membranes exposed to cold, dry CO_2 had significantly higher mRNA levels of *MMP9* relative to controls however, there were no differences in *TIMP2* mRNA levels between these groups (Figure 29f-g). Membranes exposed to heated humidified CO_2 showed no differences collagenase mRNA levels compared to controls (Figure 29f-g). Picrosirius red staining identified no differences in total collagen content between the cold, dry, heated, humidified or control groups (Figure 29a-b).

Figure 26: The effect of cold, dry and heated, humidified amniotic insufflation on amniotic epithelial integrity.

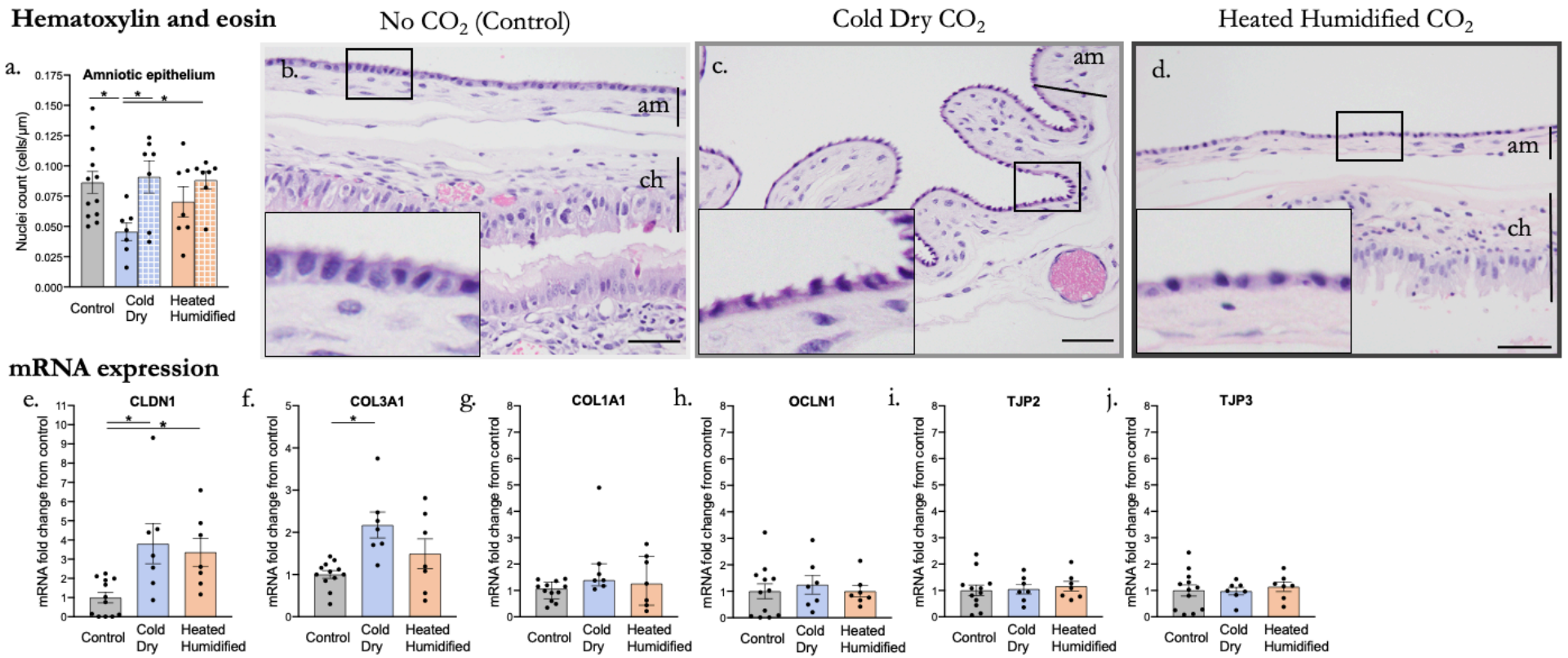


Figure 26: (a) Anterior fetal membranes (solid bars) exposed to cold, dry insufflation had significantly fewer amniotic epithelial nuclei compared to controls. Posterior fetal membranes (checked bar) showed no differences in epithelial nuclei count to controls. (b-d) Representative 40x images of the amnion (am) and chorion (ch) stained with haematoxylin and eosin. Scale bars represent 50um. (e-j) Cold, dry insufflation increased mRNA levels of *CLDN1* and *COL3A1* relative to controls. No differences in *COL1A1*, *OCLN1*, *TJP2* or *TJP3* mRNA levels were identified between the cold, dry and control groups. Only *CLDN1* mRNA levels was increased relative to controls in the heated humidified group. Parametric data were compared using a one-way analysis of variance and presented as mean ± SEM. Non-parametric data were compared using a Kruskal Wallis analysis of variance and presented as median ± IQR. * - $P<0.05$ between indicated groups.

Figure 27: The effect of cold, dry and heated, humidified amniotic insufflation on markers of fetal membrane damage.

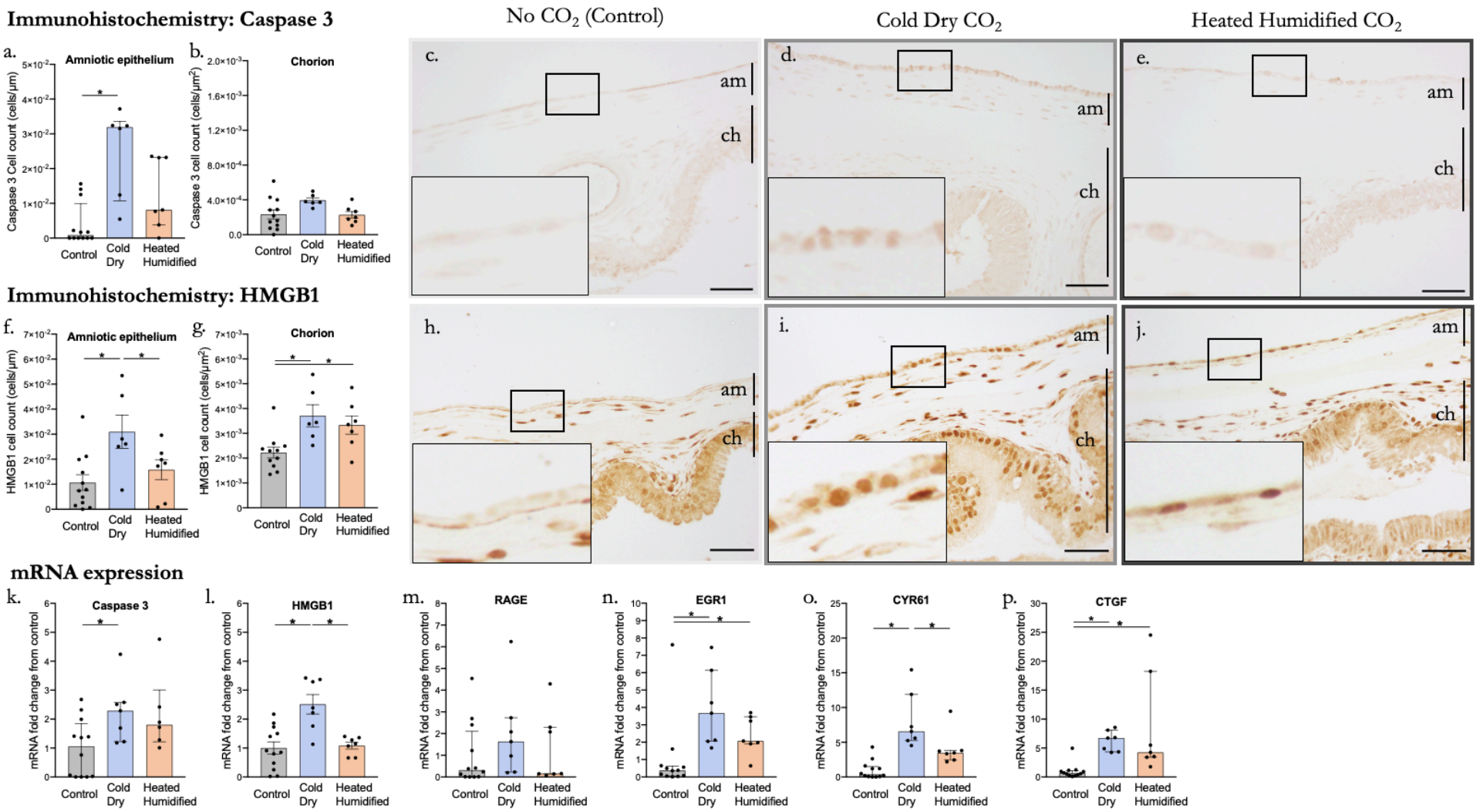


Figure 27: (a-b) Cold, dry insufflation increased caspase 3 cell counts in the amniotic epithelium but not the chorion relative to controls. (c-e) Representative 40x images of the amnion (am) and chorion (ch) immuno-stained for caspase 3. Scale bars represent 50um. (f-g) Cold, dry insufflation increased HMGB1 cell counts in the amniotic epithelium and chorion relative to controls. Membranes insufflated with heated humidified CO_2 also had increased HMGB1 cell counts relative to controls in the chorion however, had lower amniotic epithelial cell counts compared to the cold, dry group. (h-j) Representative 40x images of the amnion and chorion immuno-stained for HMGB1. (k-p) Cold, dry insufflation significantly increased *Caspase 3, HMGB1, EGR1, CYR61* and

CTGF mRNA levels relative to controls. Heated, humidified insufflation also increased *EGR1* and *CTGF* mRNA levels relative to controls however, had lower *HMGB1* and *CYR61* mRNA levels compared to the cold, dry group. Parametric data were compared using a one-way analysis of variance and presented as mean ± SEM. Non-parametric data were compared using a Kruskal Wallis analysis of variance and presented as median ± IQR. * - $P<0.05$ between indicated groups.

Figure 28: The effect of cold, dry and heated, humidified amniotic insufflation on markers of fetal membrane inflammation.

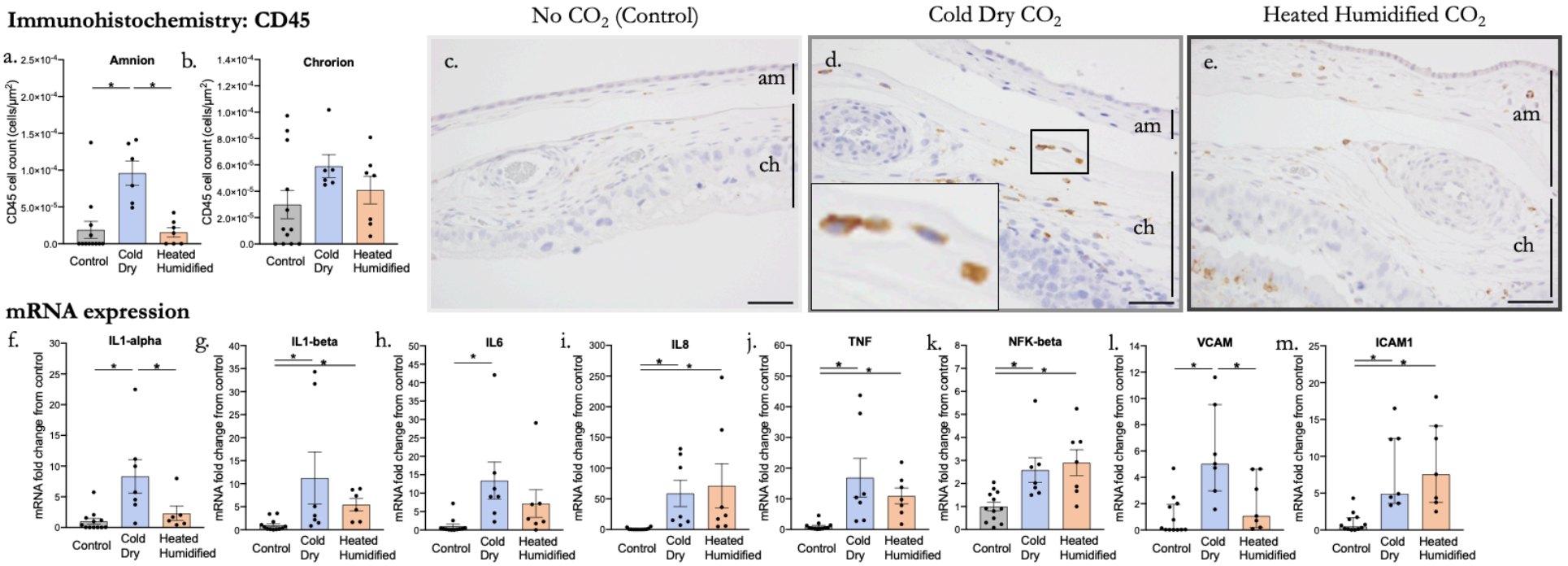


Figure 28: (a-b) Cold, dry insufflation increased CD45 cell counts in the amnion relative to the control and heated humidified groups. No significant differences were seen in the chorion. (c-e) Representative 40x images of the amnion (am) and chorion (ch) immuno-stained for CD45. Scale bars represent 50um. (f-m) Cold, dry insufflation increased mRNA levels of the pro-inflammatory cytokines *IL1-alpha, IL1-beta, IL6, IL8, TNF* and *NFKB* as well as the vascular adhesion molecules *VCAM* and *ICAM-1* relative to controls. Membranes exposed to heated, humidified CO_2 also had increased *IL1-beta, IL8, TNF* and *ICAM1* mRNA levels relative to controls however, had significantly lower *IL1-alpha* and *VCAM* mRNA levels compared to the cold, dry group. Parametric data were compared using a one-way analysis of variance and presented as mean ± SEM. Non-parametric data were compared using a Kruskal Wallis analysis of variance and presented as median ± IQR. * - $P<0.05$ between indicated groups.

Figure 29: The effect of cold, dry and heated, humidified amniotic insufflation on collagen content and collagenase mRNA levels.

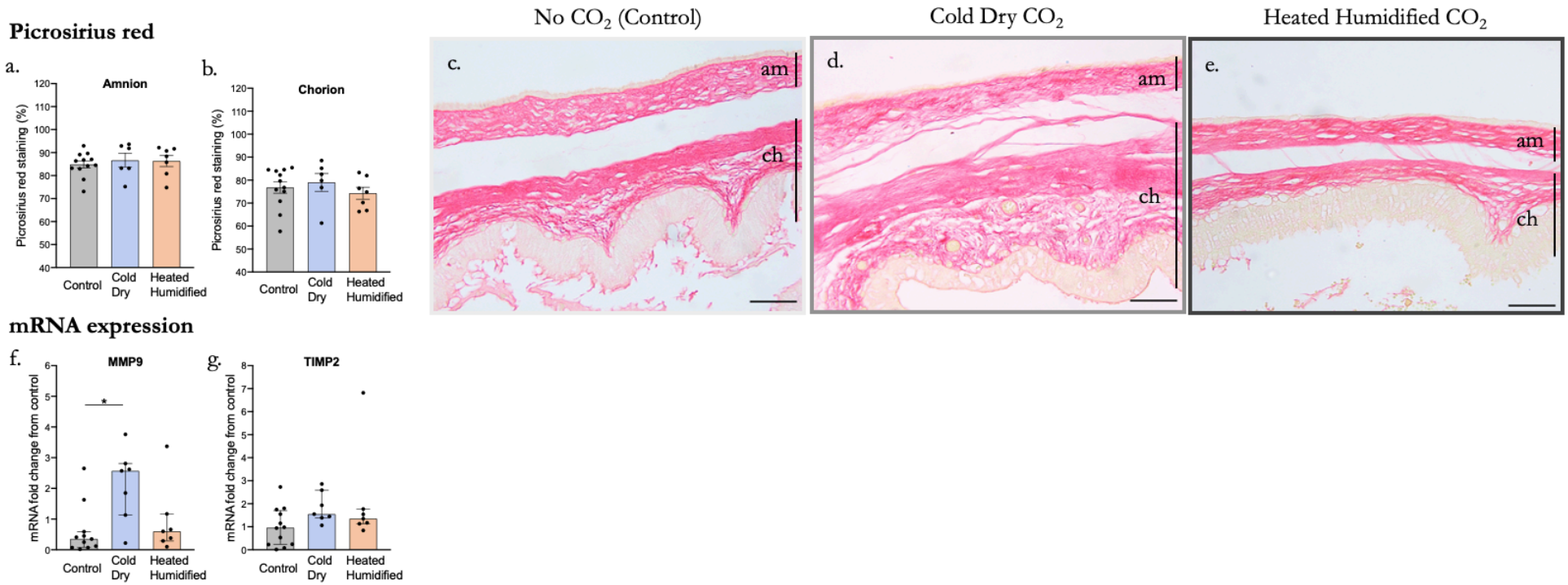
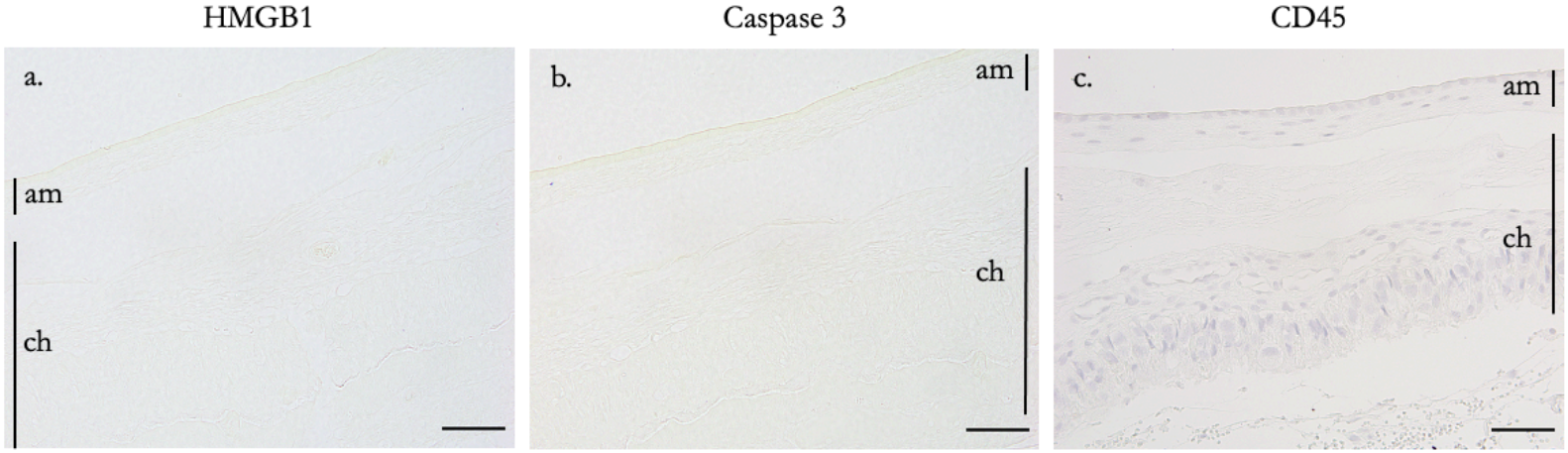


Figure 29: (a-b) No differences in collagen content were identified in the amnion or chorion using picrosirius red staining. (c-e) Representative 40x images of the amnion (am) and chorion (ch) stained with picrosirius red. Scale bars represent 50um. (f-h) Cold, dry insufflation increased mRNA levels of *MMP9* relative to controls however, there no differences in *TIMP2* mRNA levels between these groups. Membranes exposed to heated, humidified CO_2 showed no differences collagenase mRNA levels compared to controls. Parametric data were compared using a one-way analysis of variance and presented as mean ± SEM. Non-parametric data were compared using a Kruskal Wallis analysis of variance and presented as median ± IQR. * - $P<0.05$ between indicated groups.

Supplementary figure 1: Immunohistochemistry negative controls



Supplementary figure 1: High mobility group box 1 (HMGB1) (a), Caspase 3 (b) and Cluster Determination 45 (CD45) (c) immunohistochemistry negative controls. Fetal membrane sections incubated in 2% normal goat serum without the primary antibody were used as negative controls. Representative 40x images of the amnion (am) and chorion (ch) for each negative control section are shown. Scale bars represent 50um.

Discussion:

This is the first pre-clinical study to compare molecular and histological markers of fetal membrane damage and inflammation following cold, dry and heated, humidified amniotic insufflation. Cold, dry insufflation decreased amniotic epithelial nuclei counts compared to controls and increased molecular and histological markers of membrane damage and inflammation. These changes were either completely or partially mitigated in fetal membranes exposed to heated, humidified CO_2. No differences in membrane collagen content were identified between groups.

Our results are consistent with our previous sheep studies showing that cold, dry insufflation increases fetal membrane CD45 cell counts compared to controls and that these changes can be partially mitigated using heated, humidified CO_2 for insufflation.[219, 244] Our data also supports studies on human fetal membranes exposed to heated, humidified insufflation showing similar caspase 3 cell counts to non-insufflated controls at term.[243] Increased interleukin and DAMP mRNA levels as well as increased HMGB1 cell counts in our cold, dry group also resembles previous sheep studies replicating fetal membrane damage and inflammation using lipo-polysaccharide, a model of intra-uterine infection.[247, 248] However, in our study, HMGB1 positive cells cell were mainly seen in the amniotic epithelium rather than surrounding membrane blood vessels.[247]

Loss of amniotic epithelial nuclei and altered epithelial cell morphology, increased DAMP mRNA levels and increased epithelial HMGB1 and Caspase 3 cell counts in our cold, dry group relative to controls suggests that cold, dry CO_2 damaged the amniotic epithelium and initiated apoptosis.[135, 166, 215, 247, 249] We suggest low insufflation temperature and humidity evaporated the fluid lining the amnion and desiccated the apical surface of epithelial cells as seen in figure 2c. This epithelial injury during cold, dry insufflation explains the increased mRNA levels of epithelial and basement membrane proteins, pro-inflammatory cytokines and vascular adhesion molecules as well as increased amnion CD45 cell counts relative to controls.[159, 247, 248]

Cold, dry CO_2 has previously been shown to damage the peritoneal and pleural epithelium following abdominal and thoracic insufflation.[172-176] Additionally, low humidity ventilation gases damage the tracheal epithelium and induce pulmonary inflammation in ventilated lambs.[250-252] While the fetal membranes, peritoneum, pleura and tracheal epithelium are anatomically different, they are all epithelium normally covered in fluid and appear to become damaged when exposed to low humidity gasses for prolonged periods.

In humans, fetal membrane damage and inflammation increases membrane MMP activity which progressively weakens amniotic collagen and eventually predisposes PPROM.[166] The molecular and

histological markers of injury and inflammation we observed in our cold, dry group may therefore, explain high rates of iPPROM at human fetal surgery centres using this insufflation technique.[8, 14] Although we observed no changes in membrane collagen content following cold, dry insufflation, this is not surprising as membranes were collected immediately after our experiment before changes to collagen content had time to develop. However, we did observe significant increases in *MMP9* mRNA levels relative to controls. Furthermore, the structural arrangement of the collagen fibres appeared less ordered in the cold, dry group, which supports the possibility of altered collagen metabolism within the membranes. While we did not have access to polarised light microscopy to compare fibre birefringence like similar studies, these collagen changes suggests that future preclinical studies are necessary to determine whether the signs of acute injury translate to collagen disruption, weakening or loss several days after insufflation.[116, 215]

Lower *HMGB1, CYR61* and *IL1-alpha* mRNA levels as well as amniotic HMGB1 and CD45 cell counts in our heated, humidified, compared to cold, dry group provides further evidence that heated, humidified insufflation partially mitigates fetal membrane damage and inflammation. These findings potentially explain lower iPPROM rates in several small case series using heated, humidified insufflation.[17, 242] While these are promising correlations, larger human case series and preclinical studies are clearly still required to confirm that the use of heated, humidified insufflation reduces fetal membrane weakening and the risk of iPPROM in humans.

Increased chorionic HMGB1 cell counts and mRNA levels of *EGR1* and *CTGF* independent of CO_2 temperature and humidity suggests that the mechanical stretch during amniotic insufflation may be an additional factor causing membrane damage and predisposing iPPROM. This distension related injury is comparable to what is observed in clinical situations associated with excessive fetal membrane stretch, such as in multi-gestation pregnancies or polyhydramnios. These conditions are known to increase markers of membrane damage and are important risk factors for PPROM.[15] Additionally, cohorts reporting mean amniotic insufflation pressures of ≈15mmHg generally report higher iPPROM rates than centres using ≈10-12mmHg.[8, 14, 17, 171, 242] While this difference may represent increased membrane damage and weakening with increased distension, differences in surgical protocols make it difficult to determine the actual impact of insufflation pressures on iPPROM rates.

We acknowledge that the insufflation technique used in this study does not replicate the approach described in recent human studies.[17, 86] Rather than insufflating the uterus within the mothers abdominal cavity, many clinical centres now partially exteriorise the uterus through a mini-laparotomy.[17, 86] Additionally, these centres now report slightly lower mean insufflation pressures (10-12 mmHg) than the 15mmHg used in this study.[17, 242, 253] While we have previously demonstrated that

lowering amniotic pressure have significant effects on uteroplacental blood flow, both insufflation groups were performed using the same insufflation technique.[219, 244, 246] Differences in membrane damage and inflammation between our insufflation groups should therefore, be attributed to heating and humidifying the CO_2.

We also acknowledge that sheep fetal membranes are considerably stronger and more vascular than human fetal membranes which may underestimate how changes in membrane collagen content translates to humans.[117] Given these biomechanical and anatomical differences, we focused our analysis on early markers of membrane damage and inflammation which are consistent between sheep and humans.[254] As such, we emphasize that future preclinical studies using human membrane explants are necessary to confirm our findings.

This study has demonstrated that cold, dry amniotic insufflation damages the amniotic epithelium and induces membrane inflammation in sheep. While we observed no changes in membrane collagen content between groups, this damage is likely an important factor explaining the high rates of iPPROM when using this type of gas for insufflation in a clinical setting.[8, 14] Heating and humidifying the insufflated CO_2 partially mitigated molecular and histological markers of membrane damage and inflammation and may prove an important step toward reducing iPPROM after fetoscopic myelomeningocele repair.

Acknowledgements:

This work is supported by the Victorian Government's Operational Infrastructure Support Program and also received funding from Monash Health (Emerging researcher fellowship, Dr Sasha Skinner).

General Discussion

The key findings of this thesis

In this thesis we have identified that amniotic insufflation with cold, dry CO_2 causes fetal hypercapnic acidosis and fetal membrane damage in sheep. Excitingly, we are the first to provide preclinical evidence that heating and humidifying the insufflated CO_2 partially mitigates both acid base and membrane disturbances.

Fetal hypercapnia and acidosis

When we replicated amniotic insufflation parameters used clinically (cold, dry CO_2 at 15mmHg for 180 minutes) in sheep, fetal lambs developed progressive hypercapnia and acidosis that eventually impaired their cardiovascular function. A summary of these changes is presented in Figure 30. We have identified three components of the insufflation technique that can be altered to partially relieve these disturbances – insufflation pressure, CO_2 temperature and humidity and insufflation duration.

Insufflation pressure

Increasing the amniotic pressure during insufflation compressed umbilical blood vessels, reduced fetal placental CO_2 elimination and caused CO_2 to accumulate in fetal arterial blood. Uterine distension during insufflation also compressed large branches of the uterine artery supplying the maternal placental compartment. This compression reduced maternal CO_2 uptake in the placenta and also contributed to fetal hypercapnia and acidosis. In experimental chapter 1.1 we demonstrated that lower insufflation pressures (0 and 5mmHg) caused significantly less uterine artery compression and this directly correlated with less fetal acid base disturbances and cardiovascular dysfunction.

CO_2 temperature and humidity

We also demonstrated that CO_2 temperature and humidity partially determined the amount fetal CO_2 absorption from the amniotic sac during insufflation. At 20°C and 0-5% humidity, CO_2 readily diffused from the amniotic space into exposed fetal blood vessels in the placenta and fetal tissues. As all insufflated lambs had limited ability to clear CO_2 due to the high amniotic pressure, this absorbed CO_2 progressively accumulated in the fetus and contributed to reduced arterial pH.

Heating (20 → 40°C) and humidifying (0-5 → 95-100% humidity) the insufflated CO_2 lowered the solubility and partial pressure of CO_2 within the uterus and reduced fetal CO_2 absorption during insufflation (shown in Figure 30). This reduced absorption partially mitigated fetal hypercapnia and

acidosis seen during cold, dry insufflation and allowed fetal lambs to survive much longer insufflation periods.

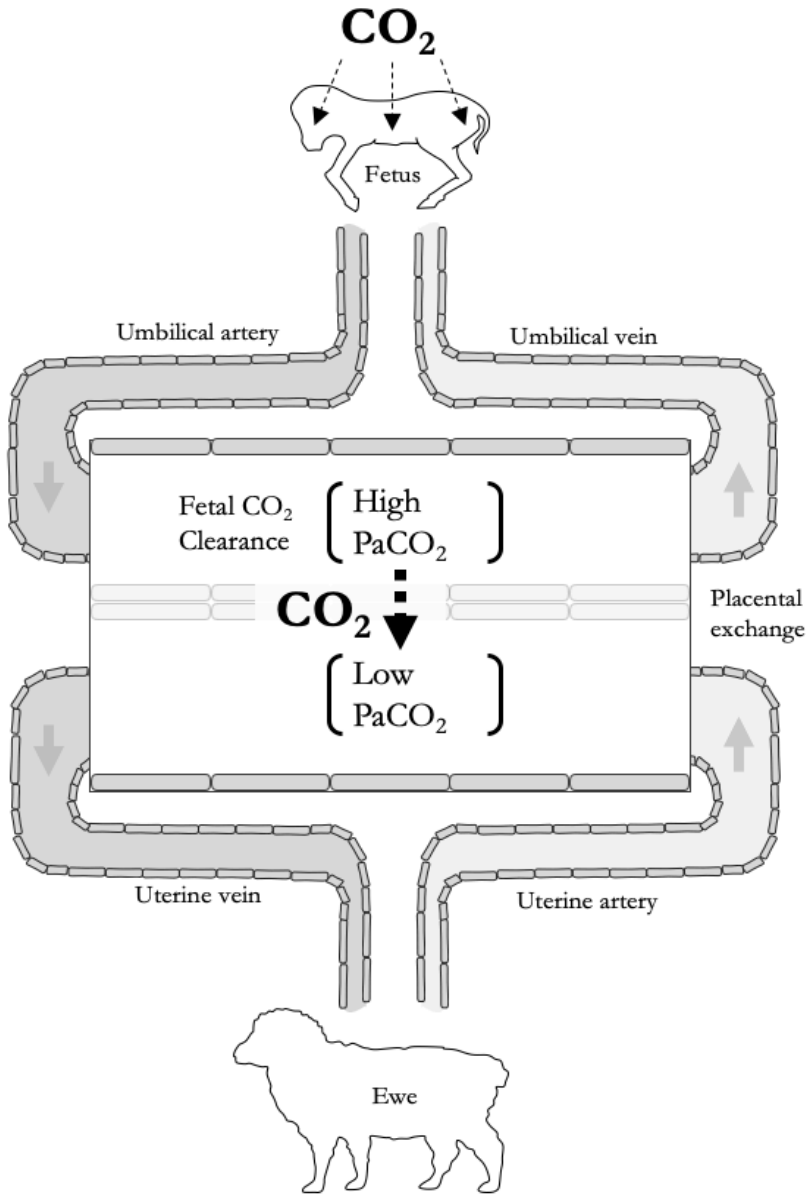
Insufflation duration

Prior to this thesis, studies replicating amniotic insufflation in sheep demonstrated fetal hypercapnia and acidosis over 30-60 minutes of insufflation.[9-12] We demonstrated that fetal acid base disturbances progressively worsen and eventually limit survival over longer insufflation durations that resemble human myelomeningocele repair (100-180 minutes).[8, 14, 17, 86]

Fetal membrane injury

In addition to the effects on the fetus, we showed that cold, dry amniotic insufflation damaged the apical surface of the amniotic epithelium exposed to CO_2 (shown in Figure 31). This damage increased molecular and histological markers of apoptosis and inflammation relative to non-insufflated controls and increased mRNA levels of matrix metalloproteases involved in fetal membrane weakening. Fetal membranes exposed to heated, humidified CO_2 showed some molecular and histological evidence of membrane damage however, this was significantly less than our cold, dry group.

Figure 30: A summary of the fetal, placental and maternal effects of cold, dry and heated, humidified amniotic insufflation in sheep



	Cold dry CO_2 insufflation (15mmHg, 120 min)	Heated humidified CO_2 insufflation (15mmHg, 120min)
Carbon dioxide chemistry		
Temperature	20°c	40°c
Humidity	0-5%	95-100%
Solubility (mol frac x 10^{e-3})	≈7.5	≈4.1
CO_2 Partial Pressure (mmHg)	740	685
Fetal CO_2 absorption (mmHg/L/min)	Ch2: 36.5	Ch2: 8.8
Fetal physiology		
Arterial CO_2 partial pressure (mmHg)	Hypercapnia Ch1.2: 63 ⟶ 170 Ch2: 48 ⟶ 167	Less Ch1.2: 67 ⟶ 83 Ch2: 54 ⟶ 99
Arterial pH	Acidosis Ch1.2: 7.21 ⟶ 6.75 Ch2: 7.21 ⟶ 6.78	Less Ch1.2: 7.24 ⟶ 7.10 Ch2: 7.22 ⟶ 7.02
Carotid artery pressure (mmHg)	Ch1.2: 36.1 ⟶ 8.7	Ch1.2: 33.0 ⟶ 30.5
Umbilical blood flow (% change from baseline)	Ch2: -55%	Ch2: -52%
Placental gas exchange		
Fetal CO_2 clearance (% change from baseline)	Ch2: -111%	Ch2: -77%
Maternal CO_2 uptake (% change from baseline)	Ch2: -94%	Ch2: -95%
Maternal Physiology		
Uterine artery blood flow (% change from baseline)	Ch1.2: -34% Ch2: -56%	Ch1.2: -22% Ch2: -53%

Figure 30: (Left image) – Carbon dioxide (CO_2) from within the uterus is absorbed by the fetus and must then be cleared from the fetal placental compartment. (Right table) – a summary of fetal, placental and maternal data from experimental chapters (Ch) 1.1, 1.2 and 2.

Figure 31: A summary of the fetal membrane effects of cold, dry and heated, humidified amniotic insufflation

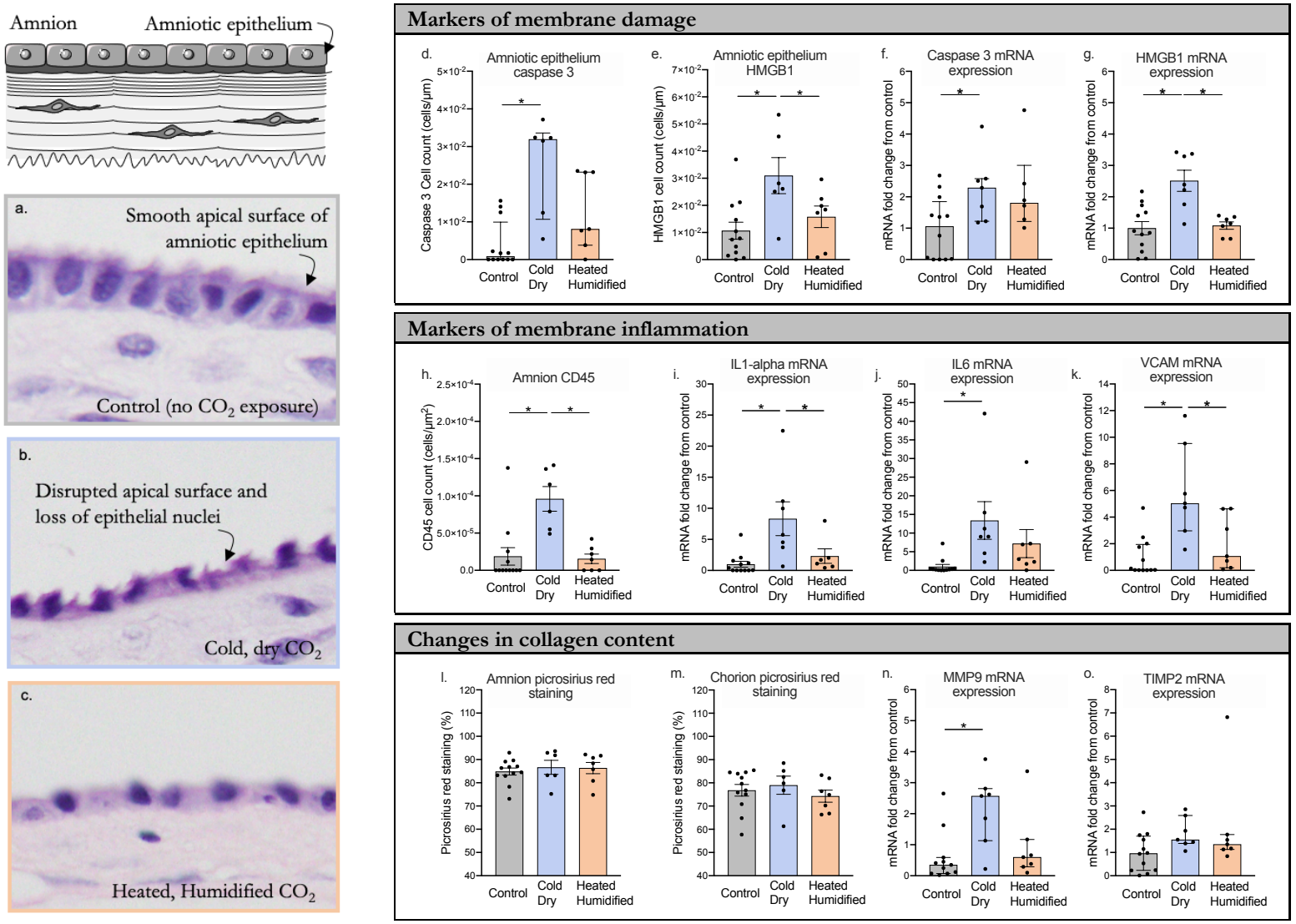


Figure 31: (Left) – Amniotic insufflation with cold, dry carbon dioxide (CO_2) disrupted the smooth apical surface of the amniotic epithelium (c) seen in the heated, humidified (c) and control (a) groups. (Right) – Cold, dry insufflation increased molecular and histological markers of cell damage (d-g) and inflammation (h-k) and increased matrix metalloprotease 9 (*MMP9*) mRNA levels (n). These changes were either completely or partially mitigated in membranes exposed to heated humidified CO_2. No changes in membrane collagen content were identified between groups (l -m).

Thesis limitations

Using sheep to replicate amniotic insufflation

When we replicated human insufflation parameters (cold, dry CO_2 at 15mmHg for 180 minutes) in sheep, fetal lambs developed severe hypercapnic acidosis that caused cardiovascular disturbances and death. However, thankfully, survival rates following human fetoscopic myelomeningocele repair are high and post-surgical follow up studies have not detected neurological sequelae attributable to severe intra-operative acidosis.[8, 14, 16, 17, 220, 238, 239] We suggest these differing outcomes are due to differences in uterine and placental anatomy between sheep and humans.

Sheep have a thinner uterus than humans.

Although the compliance of the sheep and human uterus has not been directly compared, we suggest the thinner sheep uterus is predisposed to over-distend and restrict uterine blood flow during insufflation. In experimental chapter 1.1, stepwise increases in amniotic pressures from 0 to 25mmHg caused stepwise reductions in uterine artery blood flow. Similarly, when we maintained insufflation pressures at 15mmHg we observed a sustained 30-50% reduction in uterine blood flow. Even though there is normally a large reserve in uterine blood flow to protect the sheep fetus from acid base disturbances, we demonstrated that reduced uterine blood flow directly reduced fetal CO_2 clearance and contributed to fetal hypercapnia during insufflation.[92, 236]

Interestingly, a sheep study that insufflated the uterus with air from 5 to 30mmHg, only detected fetal acid base disturbances at pressures above 15mmHg.[255] As these fetal lambs could not be absorbing CO_2 from the uterus, we suggest fetal acid base disturbances were mainly due to reduced fetal CO_2 clearance at high amniotic pressures. We believe these results provide further support for our hypothesis that fetal hypercapnia and acidosis at pressures less 15mmHg are caused by a combination of reduced fetal CO_2 clearance and increased fetal CO_2 absorption from the uterus.

Although the human uterus may not to distend as much as the sheep uterus during insufflation, reductions in uterine blood flow may still be occurring, albeit to a lesser degree.[8, 78] Ultrasound studies have identified reduced uteroplacental blood flow in human pregnancies where the uterus is overdistended due to increased amniotic fluid volume (polyhydramnios).[235] We have shown that similar changes during amniotic insufflation may limit fetal CO_2 clearance and should provide the rationale to monitor uterine artery blood flow during human fetoscopic myelomeningocele repair.

When planning the studies presented in experimental chapters 1.2 and 2 we were aware that using high insufflation pressures (15mmHg) may exaggerate acid base disturbances in our fetal lambs. However,

we considered it likely that, in addition to the physical effects of uterine distension, the fetus was also absorbing CO_2 from the uterus via diffusion. As the partial pressure of CO_2 within the uterus would be an important force driving this diffusion, we thought it essential to replicate insufflation pressures used in humans at the time. Additionally, our physiological studies were aiming to identify if heated, humidified insufflation could mitigate fetal acid base disturbances observed in chapter 1.1 using cold, dry CO_2. We chose insufflation pressures known to be associated with fetal acid base disturbances in sheep so that any differences we identified could be attributed to heating and humidifying the gas. Future animal insufflation studies should investigate the effects of using lower insufflation pressures (8-12mmHg) that more accurately reflect current human case series.[16, 17, 242]

Sheep have a cotyledonary placenta.

The cotyledonary arrangement of the sheep placenta (shown in Figure 32) may also lead to increased fetal CO_2 absorption during insufflation compared to humans. Humans have a single discoid placenta on one side of the uterus that provides a relatively small amount of vasculature to the fetal membranes.[256] On the other hand, the sheep placenta is composed of multiple, smaller placental structures (cotyledons) evenly distributed around the uterus (shown in Figure 32). Cotyledons are interconnected by a large network of fetal blood vessels that pass within the collagen of the amnion and chorion.[257] When the sheep amniotic cavity is distended with CO_2, this membrane vasculature may provide a greater surface area for CO_2 absorption compared to humans, resulting in a greater fetal hypercapnia and acidosis.

Fetal lambs developed a mixed acidosis over prolonged insufflation periods

In experimental chapters 1 and 2, 60-120 minutes of cold, dry amniotic insufflation decreased fetal arterial oxygen saturation and base excess and increased arterial lactate. These blood gas changes suggest that fetal acidosis during prolonged insufflation was partially due to fetal tissue hypoxia. Several groups have suggested these findings are caused by uterine overdistension during insufflation in sheep and that the resulting high blood lactate, rather than fetal CO_2 absorption, is the major contributor to fetal acidosis.[8, 14, 16, 17, 233] While we did identify that amniotic insufflation reduces uterine blood flow in sheep, we suggest that fetal metabolic disturbances occur as a result of progressive CO_2 absorption and hypercapnia.

In experimental chapter 2, we showed that amniotic insufflation caused immediate fetal CO_2 absorption and acidosis while fetal PaO_2 and blood lactate levels remained within normal limits. As fetal hypercapnia and acidosis progressively worsened over 60-120 minutes, we observed a reduction in arterial oxygen saturation and a non-significant rise in PaO_2 (Figure 22). We suggest these changes represent a pH-induced decrease in the affinity of fetal haemoglobin for oxygen (the Bohr Effect)

rather than acute hypoxia induced by uterine overdistension.[228] This progression from fetal hypercapnic to mixed (hypercapnic and metabolic) acidosis also explains the rapid decrease in fetal pH between 60 and 120 minutes of cold, dry insufflation.

As described above, it is unlikely that the majority of human fetuses undergoing amniotic insufflation develop severe hypercapnia that eventually leads to mixed acidosis and cardiovascular dysfunction. However, in addition to isolating the effects of different insufflation parameters, we believe this thesis has established the spectrum of disturbances possible during fetoscopy.

Would a different animal model provide a more accurate representation of human insufflation?
Differences in uterine and placental anatomy between sheep and humans suggest that using a different animal model to replicate amniotic insufflation may better predict the effects of CO_2 insufflation on human fetuses. However, no animal model perfectly models human physiology and other species that are commonly used in scientific research have other limitations which are arguably greater when conducting this type of research.

Rabbits and rodents have a discoid placenta like humans however, their small size prevents invasive physiological monitoring and fetal blood gas sampling (summarised in Table 10).[258] Pregnant pigs have similar uterine thickness to humans however, their placenta occupies the entire internal surface of the uterus (a diffuse arrangement) and would provide an even greater surface area for fetal CO_2 absorption than sheep.[258-260] Non-human primates could provide a more accurate model of the human uterus and placenta however, these are a logistically, financially and ethically limited resource.[261]

Sheep have been used extensively to study fetal and placental physiology as their large size allows invasive fetal and maternal monitoring and, despite differences in placental arrangement, rates of placental gas exchange have been shown to be equivalent to humans.[196] While sheep appear be the best animal models we have to study amniotic insufflation, we must interpret the results generated from these studies carefully. Clearly, differences in uterine and placental anatomy suggest that human fetuses do not experience the same severity of acid base or haemodynamic disturbances as those seen in our fetal lambs. However, the mechanisms of fetal CO_2 absorption and reduced placental gas exchange during insufflation we have identified cannot be ignored and should provide the rationale for future human studies aiming to confirm the safety of amniotic insufflation for the human fetus.

Table 10: Uteroplacental anatomy in domestic species.

	Humans	Non-human Primates	Sheep & Goats	Pig	Rabbits	Rodents
Placental arrangement[258]	Discoid	Discoid	Cotyledonary	Diffuse	Discoid	Discoid
Uterine thickness compared to humans	-	Similar[261]	Thinner[78]	Similar[259, 260]	Thinner[262]	Similar[263]

Figure 32: Arrangement of the sheep and human placenta

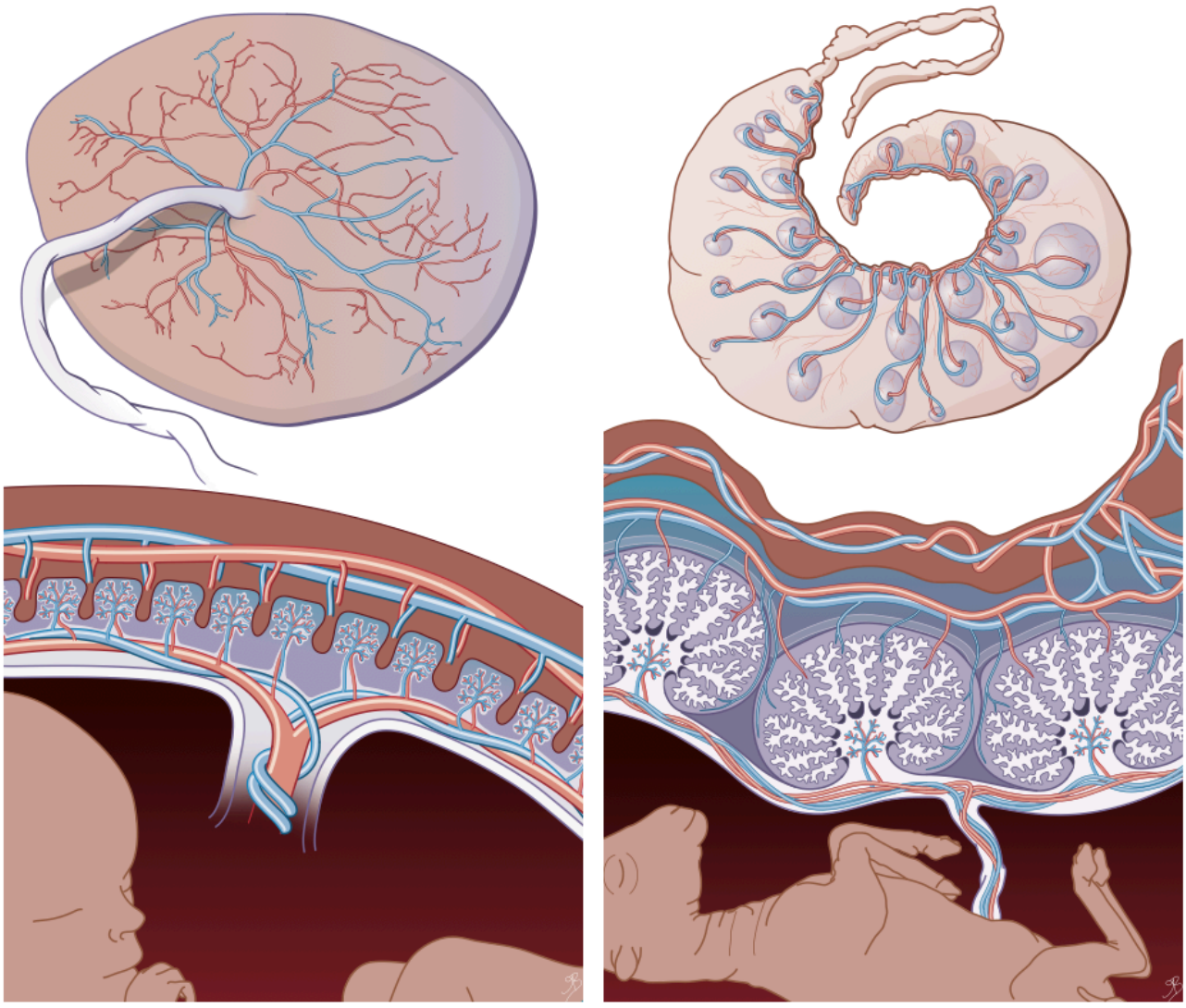


Figure 32: The human has a single discoid placenta located on one side of the uterus. The sheep placenta is composed of multiple cotyledons, interconnected by a series of small blood vessels that run between the fetal membranes (Cotyledonary vessels).

The effects of hypercapnia and acidosis on the fetal brain

The potential hypercapnia and acidosis to cause fetal brain injury during insufflation was one of the major concerns that led our group to investigate the physiological effects of amniotic insufflation. When planning experimental chapters 1 and 2 we aimed to compare histological changes in the brains of fetal lambs exposed to cold, dry and heated, humidified insufflation at clinically relevant insufflation pressures and durations. However, during these physiological studies we observed severe fetal hypercapnia, acidosis, cardiovascular dysfunction and limited survival that was not being observed in parallel human case studies.

Despite these differences in fetal outcomes, we collected the brains of our fetal lambs after immediately after insufflation and performed a preliminary histological analysis using Acid Fuchsin Cresyl Violate staining. As we anticipated, the brains of fetal lambs exposed to cold, dry CO_2 were severely damaged. We observed significant regions of neural apoptosis in the cerebral cortices, loss of vascular integrity in the periventricular white matter and clustering of inflammatory cells around cerebral blood vessels (shown in Figure 33). These histological changes would be expected to correlate with limited survival and severe functional impairment in human infants after birth. Although the brains of fetal lambs exposed to heated, humidified CO_2 showed considerably less injury (data not shown), we believed that the extent of fetal hypercapnia, acidosis and cardiovascular dysfunction significantly limited the clinical relevance of making such comparisons.

We suggest that future studies build on our findings to identify if the acid base benefits of heated, humidified insufflation do translate to improved brain histopathology. These studies should expose un-instrumented fetal lambs to 180 minutes of insufflation using cold, dry or heated, humidified insufflation to isolate the effects of the procedure on the brain. Amniotic pressures should either reflect more recent human case series (8-12mmHg) or cause an equivalent reduction in uterine blood flow to human mothers undergoing amniotic insufflation. Pregnancies should be allowed to continue after insufflation to allow any brain injury to become evident and the brains assessed using a combination of fetal MRI and histological analyses.

Figure 33: Representative Acid Fuchsin Cresyl Violate stained sections of fetal brain from lambs exposed to cold, dry amniotic insufflation.

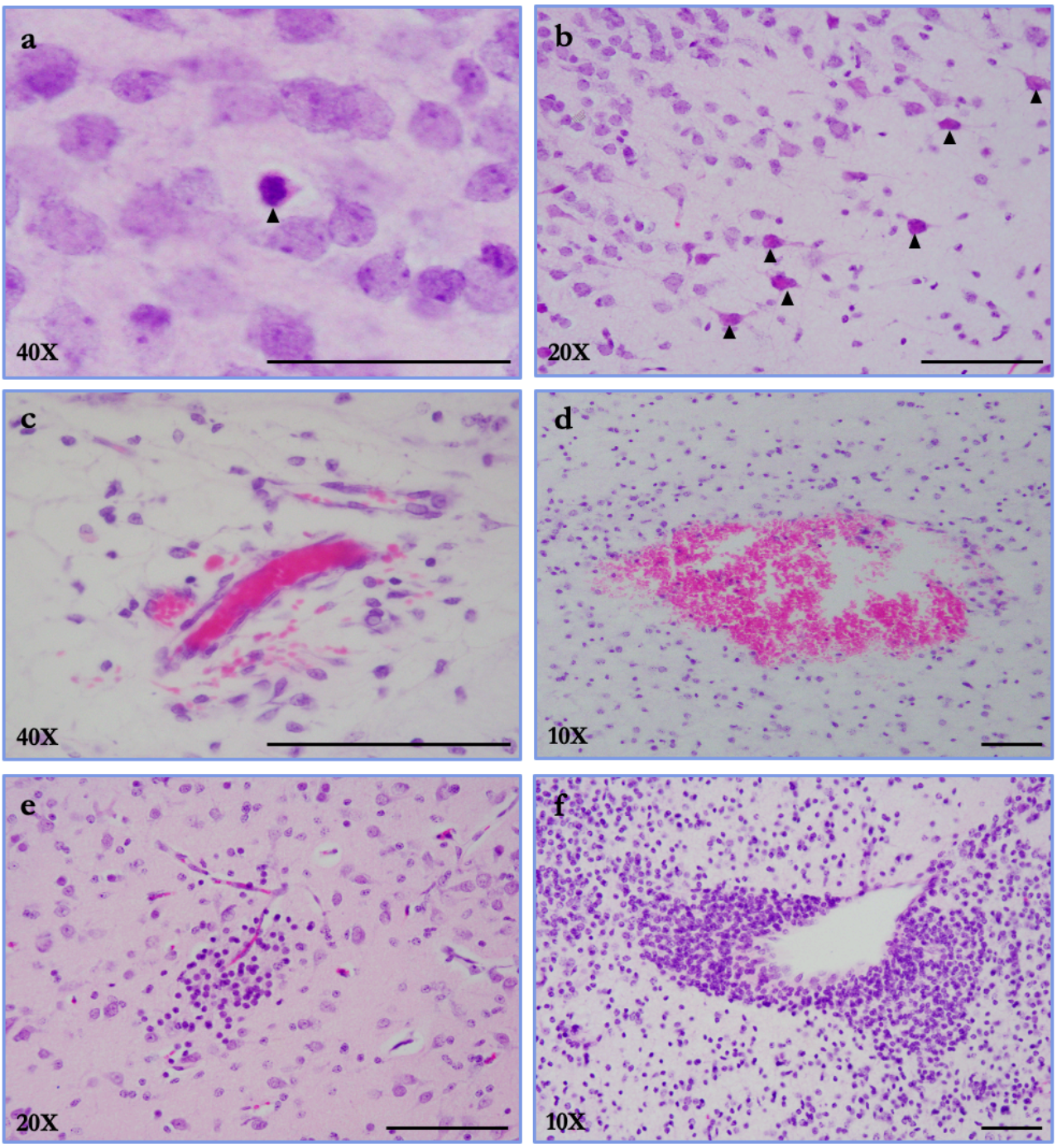


Figure 33: Pyknotic nuclei (▲) could be seen throughout the cerebral cortex of fetal lambs exposed to cold, dry amniotic insufflation (a,b). Extravasation of red blood cells (c) and small bleeds (d) could also be identified in the periventricular white matter while immune cells could be identified surrounding larger cerebral blood vessels (e,f). Scale bars represent 100 μm

Translating the findings of this thesis into clinical recommendations

In this thesis, we have demonstrated that higher amniotic pressures, the use of cold, dry CO_2 and longer insufflation durations all worsened fetal hypercapnia and acidosis during insufflation in sheep. We have also shown that fetal membrane damaged caused by cold, dry CO_2 can be partially mitigated using heated, humidified CO_2. We believe these physiological mechanisms should guide the way amniotic insufflation is performed in humans.

1. **Amniotic insufflation pressures should be minimised.**

Two amniotic insufflation techniques are currently used in humans. Some centres use a completely percutaneous approach where the uterus is insufflated within the mother's abdominal cavity while others use a laparotomy incision and insufflate the uterus exteriorised from the abdomen (shown in Figure 8). Although only small number of cases have been published, centres that exteriorise the uterus report lower mean insufflation pressures than centres using a completely percutaneous approach (8-12 vs 15mmHg).[14, 16, 17, 86, 171, 264] We have found that higher insufflation pressures restrict placental blood flow, limit fetal CO_2 clearance and directly contribute to fetal hypercapnia and acidosis in sheep. Lower insufflation pressures maintained uteroplacental blood flow and directly correlated with less fetal disturbances. While these pressure related effects may be greater in sheep, our data supports exteriorising the uterus during fetoscopy to permit the use of lower insufflation pressures.

2. **The carbon dioxide gas should be heated and humidified.**

We have shown that the CO_2 used to insufflate the uterus should be heated and humidified to reduce fetal CO_2 absorption from the amniotic sac during insufflation. Human fetuses undergoing fetoscopic myelomeningocele repair have a severe malformation of their central nervous system and varying degrees of underlying brain injury.[19] Even small acid base disturbances intra-operatively may harm the developing fetal brain and limit the benefits of fetoscopic myelomeningocele repair. While the acid base benefits of heated, humidified insufflation may be more subtle in humans than sheep, any step toward optimising the surgical conditions and alleviating the adverse effects of these invasive procedures will be critical to improving surgical outcomes.

We also showed that cold, dry amniotic insufflation damages the amniotic epithelium and induces membrane inflammation in sheep. While we observed no changes in membrane collagen content to suggest this injury makes the membranes more likely to rupture early, this injury correlates with high rates of iatrogenic PPROM at human centres using a similar approach.[8, 14] Heating and humidifying the insufflated CO_2 partially mitigated molecular and histological markers of membrane damage and inflammation and may prove an important step toward reducing iPPROM after fetoscopic

myelomeningocele repair. These potential benefits were recently recognized at the International Fetoscopic Myelomeningocele Repair Consortium where there was a general consensus that all centres should be using heated, humified CO_2.[86]

3. **The duration of amniotic insufflation should be minimised.**

In this thesis we have shown that the hypercapnia and acidosis resulting from amniotic CO_2 insufflation was time dependent and likely occurred due to gradual accumulation of CO_2 within the fetal compartment. This suggests that minimizing fetal exposure to amniotic insufflation may be of considerable importance. While there have been significant efforts to shorten operative durations, we suggest amniotic CO_2 insufflation could be restricted to complex parts of myelomeningocele repair that require additional space and visibility for surgery. Less complex parts of the procedure could be performed using fluid distension of the uterus which has shown to be safe in animal models.[255]

Future directions:

Should human amniotic insufflation continue without proven safety in animals?

Yes, but carefully.

In 1982 the International Fetal Medicine and Surgery Society (IFMSS) strongly encouraged that the safety of a fetal intervention should be demonstrated in animals before being performed in humans.[87] However, this did not happen for amniotic CO_2 insufflation. As summarised in my literature review, when fetoscopic myelomeningocele repair was first proposed, four sheep studies clearly demonstrated that amniotic insufflation caused progressive fetal hypercapnia and acidosis.[9-12] These studies suggested that insufflation should not be used in humans until the technique could be shown to be safe in animals.[9-12] However, several authors suggested that these acid base disturbances would not be seen in humans due to limitations of using sheep for insufflation.[8, 14] Therefore, despite the lack of proven safety in animals, amniotic insufflation was adopted into human clinical practice.[8, 14]

Over 200 cases of fetoscopic myelomeningocele repair using amniotic insufflation have been reported since the technique was revisited in 2014 and detailed post-surgical follow up has not been able to identify neurological sequelae attributable to severe intra-operative acidosis.[8, 14, 16, 17, 220, 238, 239] While these outcomes are reassuring and suggest that amniotic insufflation can continue in humans, as we still do not know why humans are less susceptible than sheep, this needs to be done carefully and perhaps be restricted to experienced fetoscopic centres. Indeed, it is possible these experienced centres utilise practises that unknowingly confer additional benefits to human fetuses that may not be performed early in the technical learning curve.

To identify the effects of insufflation on the human fetus, experienced fetoscopic centres should build on the small case series (n=3) by Baschat et al. that collected human umbilical vein blood gasses via cordocentesis before and after surgery (summarised in Table 4).[16] However, new ways to measure fetal CO_2 and pH need to be developed. Cordocentesis carries an ≈1.6% risk of fetal death which seems unacceptable when performed twice during an already invasive procedure like fetoscopic myelomeningocele repair.[265-267] Ideally, fetal arterial CO_2 and pH could be measured continuously during surgery via a patch or implant administered down one of the fetoscopic ports. In addition to determining if amniotic insufflation causes fetal hypercapnia and acidosis in humans, continuous, non-invasive fetal blood gas monitoring could be used as a tool to guide insufflation intra-operatively. Rising fetal CO_2 may prompt surgeons to lower insufflation pressures in an effort to maintain fetal CO_2 clearance in the placenta.

Additional insights into the effects of insufflation on the human fetus may be gained by comparing neurodevelopmental outcomes and cognitive functioning of school aged children that underwent fetoscopic myelomeningocele repair using cold, dry or heated, humidified insufflation. While neurological outcomes may be better in the heated, humidified group due to their surgeries being performed more recently with more refined surgical approach, regression analysis may be able to determine if intraoperative acid base differences observed in our sheep studies translate to functional differences for human infants.

In an ideal translational pathway, our sheep studies would have preceded the wider application of amniotic insufflation in humans and minimised the number of cases performed using cold, dry CO_2 and high insufflation pressures.[268] However, such a linear translational pathway from animals to humans is idealistic and rarely feasible. The fact that this thesis followed preliminary human fetoscopic myelomeningocele repairs serves as an important example of bidirectional translation, where clinical questions are taken back to pre-clinical models to better appreciate the underlying physiology.[268] This "bedside to bench" approach has allowed us to acknowledge the limitations of using sheep for insufflation while still using these studies to provide relevant, physiological based recommendations to refine the insufflation technique in humans.

However, bidirectional translation is a continuous process that requires ongoing collaboration between clinical and pre-clinical colleagues. In 2018, the leading fetal surgery centres performing fetoscopic myelomeningocele repair established an international consortium to foster collaborative research into the technique.[86] In addition to sharing technical advancements and clinical outcomes, this forum would be perfect to also host research centres with the expertise and facilities required to take clinical questions back to pre-clinical models. Excitingly, proceedings from the first meeting of this consortium described a general consensus that all centres should be using heated, humidified CO_2 for insufflation.[86]

Conclusion

Amniotic CO_2 insufflation improves visibility during fetoscopic myelomeningocele repair and provides surgeons with enough space for complex surgery. In a setting where preclinical data has not always guided human clinical practice, this thesis provides human fetal surgery centres with three physiology-based recommendations to performing amnion insufflation using a large animal model.

1- Amniotic insufflation pressures should be minimised to maintain fetal gas exchange in the placenta.
2- The CO_2 gas should be heated and humidified to reduce fetal CO_2 absorption and to prevent fetal membrane damage.
3- The duration of amniotic insufflation should be minimised to reduce fetal exposure to CO_2

We hope these simple recommendations guide the way amniotic insufflation is performed in humans and generate hypotheses for future clinical and preclinical studies aiming to improve the fetus' ability to tolerate fetoscopy and reduce the risk of iatrogenic PPROM post-operatively.

This page is left intentionally blank.

Appendices:

Appendix 1: Why do the fetal membranes rupture early after fetoscopy? A Review

Amberg BJ[1,2], Hodges RJ[1,2], Rodgers KA[1,2], Crossley KJ[1,2], Hooper SB[1,2] and Dekoninck PLJ[1,2,3]

1. The Department of Obstetrics and Gynaecology, School of Clinical Sciences, Monash University, Melbourne, Victoria, Australia
2. The Ritchie Centre, Hudson Institute of Medical Research, Melbourne, Victoria, Australia
3. Department of Obstetrics and Gynaecology, Erasmus MC, University Medical Center, Rotterdam, The Netherlands

Abstract:

Iatrogenic preterm premature rupture of the fetal membranes (iPPROM) remains the Achilles' heel of keyhole fetal surgery (fetoscopy) despite significant efforts in preclinical models to develop new therapies. This limited success is partially due to incomplete understanding why the fetal membranes rupture early after fetoscopy and notable differences in membrane physiology between humans and domestic species. In this review, we summarise aspects of fetoscopy that may contribute to iPPROM, the previous efforts to develop new therapies and limitations of preclinical models commonly used in fetal membrane research.

Introduction

Keyhole fetal surgery (fetoscopy) is used to treat complicated monochorionic twin pregnancies or fetuses with congenital abnormalities.[84, 269] These procedures are generally performed at mid gestation using a camera and instruments inserted into the amniotic space through ports in the uterus and fetal membranes.[270] The ports can be positioned percutaneously or the uterus can be partially exteriorised and the ports inserted directly.[86] More complex fetoscopic procedures require gaseous distension of the amniotic cavity (amniotic insufflation) to improve visibility within the uterus and allow easier manipulation of surgical instruments.[8] While fetoscopy aims to improve postnatal outcomes, post-operative preterm premature rupture of the fetal membranes (iatrogenic PPROM) remains its Achilles' heel. Iatrogenic PPROM (iPPROM) complicates ≈30% of fetoscopic procedures, but rates can be as high as 85-100% following complex procedures using amniotic insufflation.[8, 14, 148, 171, 271, 272] Iatrogenic PPROM increases the risk of lung hypoplasia due to prolonged oligohydramnios, chorioamnionitis and preterm birth, all of which have significant implications for the fetus and potentially offset the benefits of surgery.[200] Although perinatal outcomes are generally improving after fetoscopy, the risk of iPPROM means these procedures are only considered for the most severely affected fetuses, where the potential advantages of surgery outweigh the significant risks.[86, 269, 272]

Techniques to reduce iPPROM after fetoscopy have been investigated extensively in humans and pre-clinical models (summarised in Table 1).[148] Efforts have mainly focused on minimising membrane damage by reducing the number and diameter of fetoscopic ports and using plugs (gelatin or collagen), glues, patches or sutures to seal the holes left in the membranes after surgery.[17, 200, 270, 273-288] More recently, heated and humidified CO_2 gas has been used for amniotic insufflation to prevent the fetal membranes dehydrating and becoming prone to rupture after surgery.[17, 86, 219, 243, 244] Although many of these techniques have been shown to protect the membranes from injury or prevent amniotic fluid leakage (amniorrhexis) in pre-clinical models, there is limited evidence to show they reduce iPPROM in humans.[200, 270, 287, 289] This difficulty developing and translating strategies to reduce iPPROM is largely due to limited understanding why the fetal membranes rupture early after fetoscopy. Herein, we summarise the potential mechanisms and preventions of iPPROM after fetoscopy. In addition, we provide an overview of pre-clinical models commonly used in fetal membrane research and describe their relevance to human fetal membrane physiology.

Main Text

Structure of the human fetal membranes

The fetal membranes surround the fetus during pregnancy and play a critical role in maintaining the pregnancy to term (shown in Fig. 1).[111] The innermost membrane, the amnion, provides the majority of structural integrity.[111] The amnion is formed 10-14 days post fertilization, when mesoderm cells migrate from between the layers of the bilaminar embryo to fuse with the ectoderm lining of the amniotic cavity.[112] The ectoderm gives rise to the cuboidal amniotic epithelium which lines the surface of the amnion facing the fetus and is held in place by a basement membrane composed of collagens (Type I and III) and glycoproteins (laminin, nidogen, and fibronectin).[112-114] The amniotic mesoderm forms a layer of compact Type I and III collagen beneath the basement membrane that provides the amnion with its tensile strength.[111, 113, 114] This compact layer is produced and maintained by fibroblasts in an adjacent layer of loose collagen also derived from the mesoderm, known as the fibroblast layer.[15, 113] The human amnion is almost completely avascular, sourcing nutrients and exchanging wastes with the amniotic fluid.[112, 115]

The amnion is separated from the outer chorionic membrane by a space known as the intermediate or spongy layer (shown in Fig. 1).[15, 112] This space is liquid filled until 12-15 weeks' gestation when the expanding amnion adheres to the chorion. The loose collagen adhesions and hydrated proteoglycans within the intermediate layer allow the amnion to slide independently over the chorion which increases the membranes tensile strength.[111, 113, 114]

The chorion surrounds the amnion and is formed by fusion of the mesoderm with the outer cells of the embryo known as the trophoblasts.[112] The chorionic mesoderm differentiates to become a layer of loose reticular collagen and fibroblasts that faces the amnion (shown in Fig. 1).[112] This collagen is supplied by a network of fetal blood vessels arising from the allantois and its outer surface is lined by the trophoblasts. The outer surface of the chorion is covered by a layer of maternal cells known as the decidua, that were once the endometrium overlying the implanted embryo. The decidua is rich with maternal blood vessels and immune cells and fuses with the chorion during early gestation forming the choriodecidua.[112]

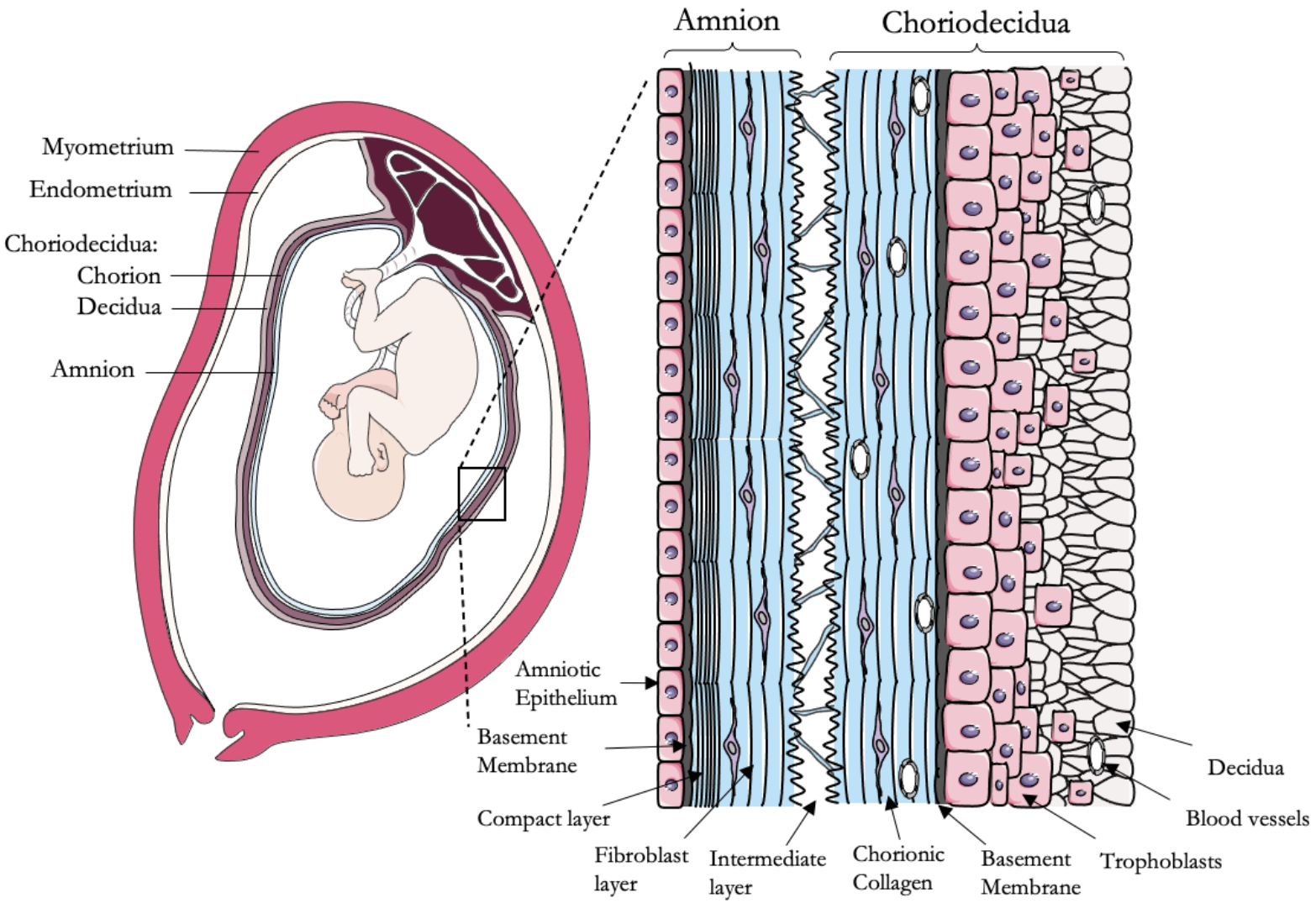


Fig. 1. The arrangement and structure of the human fetal membranes. The human fetal membranes are comprised of the amnion and choriodecidua. The amnion is composed of a single layer of amniotic epithelium, basement membrane and underlying collagen. The chorionic collagen faces the amnion and sits on a layer of trophoblasts derived from the placenta. The decidua fuses with the outer surface of the chorion to form the choriodecidua.

Rupture of the fetal membranes at term

During pregnancy there is little change in the composition of the fetal membranes however, from 37-38 weeks gestation the membranes overlying the cervix thin and weaken.[116-119] This localised weakening makes the amnion and chorion more susceptible to rupture as the descending fetus and contracting uterus progressively stretch the membranes.[15] The final failing of the membranes plays an important role in promoting the onset of labour and is characterised by membrane separation (chorioamniotic separation) which then promotes rupture of the choriodecidua and amnion in turn (shown in Fig. 2).[111]

The progressive membrane weakening that precedes term rupture is thought to be mediated by a family of collagen degrading enzymes known as matrix metalloproteases (MMPs) and their endogenous tissue inhibitors (TIMPs).[120-122] During late gestation, amniotic epithelial cells and chorionic trophoblasts increase the proportion of activated MMPs, particularly MMP-9, within the membrane while reducing TIMPs.[122] Increased MMP activity causes disruption and degradation of structural membrane collagens which reduces the membrane's tensile strength.[116, 119, 123-131] These changes overlying the cervix have been described as the "zone of altered morphology" and can distinguished histologically in human explants as swelling of membrane collagen and thinning of the chorionic trophoblasts and decidua.[114, 124] Similar changes in fetal membrane collagen have been documented in animal models preceding labour.[114, 116, 140, 290, 291]

There have been significant efforts to understand the factors that normally induce MMP-associated weakening of the late gestation fetal membranes. Although a common pathway is yet to be identified, various genetic (apoptosis of the amniotic epithelium and chorionic trophoblasts[126, 132-140]), epigenetic (changes in MMP and TIMP promotor genes[141, 142]), physical (increasing membrane stretch[116, 143-145]), inflammatory (cytokines release by local immune cells and accumulating oxidative stress[116, 146, 147]) and hormonal (increased relaxin levels[148, 149]) factors have been suggested.

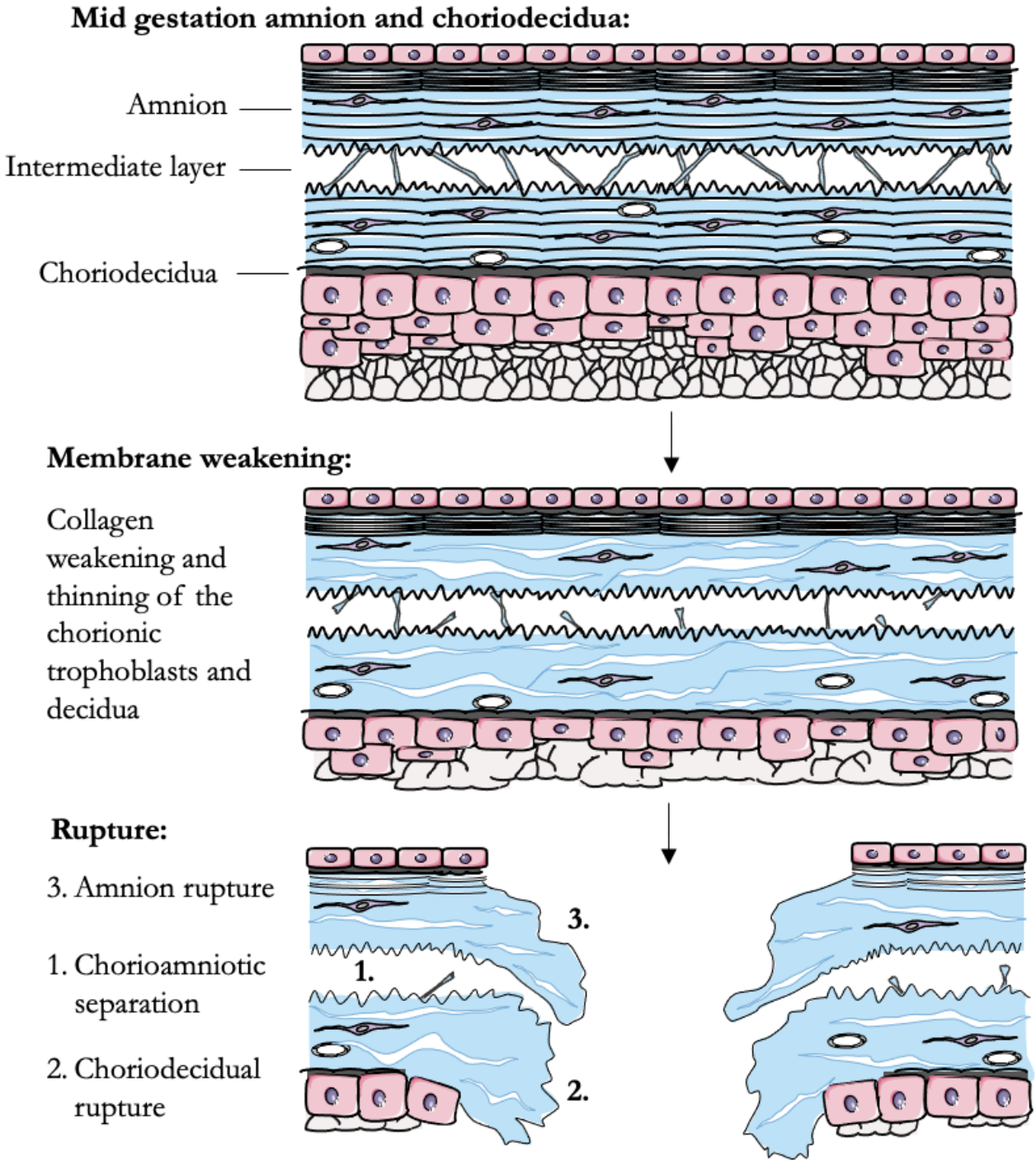


Fig. 2. Fetal membrane weakening and rupture. The amnion and choriodecidua progressively weaken before they rupture both at term and preterm. The final failing of the membranes occurs in three phases – 1. The amnion and chorion separate which decreases the tensile strength of the membrane. 2. The decidua and chorion rupture causing the amnion to stretch. 3. The amnion ruptures.[111]

Possible mechanisms of iatrogenic PPROM

While spontaneous PPROM is a complex, multifactorial pathology, many known risk factors damage the fetal membranes and prematurely upregulate membrane weakening and rupture.[15, 166] Puncturing the membranes with the fetoscopic port, distending the uterus with unconditioned CO_2 and diluting the residual amniotic fluid during fetoscopy may all damage the fetal membranes intra-operatively and increase the risk of iPPROM.

1. Post-operative chorioamniotic separation

Chorioamniotic separation is identified on ultrasound after 20-40% of fetoscopic procedures and significantly increases the risk iPPROM.[14, 17, 86, 292-294] This separation is thought to result from friction between the fetoscopic port and fetal membranes which damages the loose collagen adhesions holding the membranes together.[293, 295] As described above, chorioamniotic separation plays an important biomechanical role in promoting term fetal membrane rupture and similar membrane weakening after fetoscopy may predispose iPPROM.[111]

2. *Membrane apoptosis or damage at the port site*

In addition to causing chorioamniotic separation, the fetoscopic ports may damage the cells surrounding the insertion point. Histological studies have identified apoptosis in the amniotic epithelium and chorionic trophoblasts surrounding the port sites as well as disorganisation of membrane collagen.[295] This injury is thought to be due to shearing forces between the fetoscopic port and fetal membranes as the port is inserted or the instruments are manipulated during surgery.[270] Although, apoptosis is an important trigger of MMPs the effect of this injury on the risk of iPPROM after fetoscopy remains unclear.[126, 132-140, 243]

3. Membrane over-distension

While the fetal membranes tolerate the progressive stretch caused by the growing fetus, excessive stretch, such as in multi-gestation pregnancies or polyhydramnios, triggers membrane weakening increases the risk of spontaneous PPROM.[15] Liquid or gaseous distension of the fetal membranes during fetoscopy may trigger similar weakening mechanisms after surgery and increase the risk of iPPROM. Human case series reporting mean amniotic insufflation pressures of ≈15mmHg report higher iPPROM rates than centres using ≈12mmHg.[8, 14, 17, 171] While this difference may represent increased membrane injury and weakening with increased distension during fetoscopy, differences in surgical protocol between these cohorts such as the use of fetal membrane sutures and heated, humidified CO_2 for insufflation, could also explain the differences in iPPROM rates.[17]

4. *Membrane dehydration and desiccation*

The amniotic fluid plays an important role in hydrating amniotic collagen and maintains the membrane's tensile strength.[296] Prolonged amnion exposure to gas *in vitro* dehydrates amniotic collagen making it brittle and more likely to rupture.[176, 296] While this weakening is reversible *in vitro* when the amnion is re-exposed to liquid, similar dehydration and weakening may occur during amniotic insufflation, increasing the likelihood of iPPROM after fetoscopy.[296]

In addition to dehydrating amniotic collagen, the low temperature (≈22°c) and humidity (0-5%) of insufflated CO_2 may damage the amniotic epithelium, upregulate MMPs and increase the risk of iPPROM.[210] Preliminary sheep studies have shown that amniotic insufflation with unmodified (cold, dry) CO_2 increases membrane inflammatory cell counts which may represent a response to amnion damage.[219, 244] Other fields of endoscopy have demonstrated peritoneal and pleural desiccation following exposure to cold, dry CO_2 insufflation.[172-176] While the peritoneum, pleura and amniotic epithelium are anatomically different, they are all liquid lined epithelium and appear to be susceptible to injury when exposed to CO_2 for prolonged periods.

5. **Diluting the amniotic fluid**

The amniotic fluid is often replaced with warmed Ringer's lactate either during fetoscopy or following amniotic insufflation.[17, 295] As Ringer's Lactate has a lower osmolarity than normal amniotic fluid, surgeons rely on fetal secretions (urine, lung, nasal and salivary) and intramembranous reabsorption to normalize amniotic fluid osmolarity after surgery.[115, 295-298] Complete replacement of the amniotic fluid after fetoscopy is expected to take up to 48 hours.[115] Both *in vitro* and *in vivo* studies have shown that short term membrane exposure to low osmolarity solutions induces apoptosis in the amniotic epithelium and may directly weaken amniotic collagen.[295-297] These changes are likely due to the rapid movement of water from the dilute amniotic fluid into amniotic epithelial cells and between the collagen fibrils. Apoptosis of the amniotic epithelium increases MMP activity which, in combination with weakened collagen, may increase the risk of iPPROM after fetoscopy.[139, 176, 296]

Preventing iatrogenic PPROM

Efforts to prevent iPPROM after fetoscopy have mainly focused on minimising membrane damage around the fetoscopic port and preventing chorioamniotic separation. However, more recently, emphasis has been placed on protecting the amniotic epithelium during fetoscopy by heating and humidifying the insufflated CO_2.

Alternative port insertion techniques

The Seldinger technique was adopted from vascular procedures as a way of minimising local membrane trauma and preventing chorioamniotic separation during fetoscopic port insertion.[270, 282, 299, 300] Instead of directly puncturing the membranes with a large diameter trocar, the port hole is created sequentially using a thin needle, guidewire and conical dilator. While the Seldinger technique has been shown to reduce post-operative amniorrhexis in small human case series, larger cohort studies have not yet identified any reduction in iPPROM rates.[270, 282, 287]

An oblique puncture technique has also been attempted to offset the holes left in the membranes after surgery and prevent chorioamniotic separation. While this technique has shown some success in vitro, it has not been adopted routinely into human fetoscopy.[301] More recently, surgeons have sutured the fetal membranes to the uterine wall during port insertion to prevent chorioamniotic separation.[17] A similar membrane plication technique has shown success in rabbits.[275] While this technique was associated with lower iPPROM rates in a small case series compared to similar procedures, larger cohort studies are still required to confirm this potential benefit.[17, 86]

Reducing the diameter of fetoscopic instruments

One large retrospective study identified that the maximum instrument diameter employed during fetoscopy significantly predicted iPPROM.[200] Although this suggests that reducing port diameters could reduce the risk of iPPROM, similar retrospective studies have not identified this association nor a clinical benefit reducing port diameters (i.e. from 10 to 8 French).[270, 287] Other studies have suggested that factors increasing membrane friction at the port site such as longer surgical durations, more complex procedures, an anterior placenta and inserting instruments without a port may have a greater impact on the risk of iPPROM than the size of the holes left in the membranes.[148, 200, 270]

In addition to reducing port diameter, there have also been considerable efforts to reduce the number of ports employed during fetoscopy.[17] While procedures using 2 or 3 ports generally report higher iPPROM rates than single port procedures, surgical and pathology specific factors make it difficult to isolate the effect of port number on the risk of iPPROM.[200]

Fetal membrane plugs, glues and patches

A variety of fetal membrane plugs[148, 273-280], tissue sealants[275, 278, 288, 302-308], adhesive patches[278, 284-286] and membrane welding techniques[309] have been developed in pre-clinical models to seal punctured or ruptured fetal membranes (summarised in Table 1). Collagen/gelatin plugs are one of the few techniques trialled in humans during fetoscopy however, there is limited evidence to suggest they reduce the iPPROM compared to leaving the membranes unsealed.[281-283] Despite this limited success,

new minimally invasive techniques to apply membrane sealing therapies continue to be developed and show promise in vitro.[310]

Heated and humidified amniotic insufflation

Sheep studies have shown that heating and humidifying the CO_2 used for amniotic insufflation reduces inflammatory cell counts in the membranes compared to cold, dry CO_2.[244] Several fetoscopic centres have recently adopted heated and humidified amniotic insufflation and reported notably lower rates of iPPROM in small case series.[17, 86, 243] Histological studies have also shown similar levels of membrane injury between membranes exposed to heated, humidified CO_2 and non-insufflated controls.[243] However, like many of the factors discussed above, the small number of published cases and variability in surgical technique between centres make it difficult isolate the impact of heated and humidified CO_2 on reducing the risk of iPPROM.

Table 1: Potential mechanisms of iatrogenic PPROM and novel therapies

Potential mechanisms of iatrogenic PPROM	**Novel therapies**	**Pre-clinical studies**	**Clinical studies**
1. Post-operative chorioamniotic separation	Membrane plugs	273-280	281-283
	Membrane patches	278, 284-286	-
	Membrane sutures	275	17, 86
	Tissue sealants and glues	275, 278, 288, 302-308	-
	Oblique port entry	301	-
2. Membrane apoptosis or tissue damage at the port site:	Seldinger technique	-	270, 282, 287
	Reducing port number/diameter	-	17, 200, 270, 287
3. Membrane over-distension:	Reducing insufflation pressures	-	17
4. Membrane dehydration and desiccation:	Heated humidified amniotic insufflation	17, 86, 243	219, 243, 244
5. Diluting the amniotic fluid:	Amniotic fluid substitute	-	-

Pre-clinical models of iPPROM

Given the vulnerability of human fetuses undergoing fetoscopy, it is essential that studies investigating the mechanisms of iPPROM and developing new therapies are performed in pre-clinical models. Human fetal membrane explants, rodents, rabbits, sheep, pigs and non-human primates are the most accessible models and have been used extensively for pregnancy related research (summarised in Table 2).[311] However, there is considerable variability in fetal membrane structure and rupture physiology amongst these models that researchers should be aware of when aiming to translate their findings to human fetoscopy.

Spontaneous membrane rupture

Unlike pregnancy complications such as fetal growth restriction, researchers are yet to develop a pre-clinical model of iPPROM. This is likely because spontaneous PPROM does not appear to occur naturally in other species.[112, 117] Until recently, this has been attributed to the human's evolution of an upright posture that places unique downward forces on the cervical fetal membranes during pregnancy.[117] However, biomechanical studies have also shown that the human fetal membranes are considerably weaker, and rupture more readily than those of many domestic species including sheep and pigs.[117] These biomechanical differences mean that only human fetal membrane explants may accurately combine cellular markers of weakening or damage with biomechanical changes that predispose rupture. However, human explants can only be collected after delivery and the composition of these tissues may not be representative of mid gestation fetal membranes undergoing fetoscopy.[112, 312] This highlights the need to understand the limitations of available animal models in fetal membrane research.

Despite biomechanical differences, the inflammatory responses to injury and membrane weakening cascades involving MMPs are consistent amongst humans and most domestic species.[114, 116, 140, 279, 290, 291] This suggests that pre-clinical animal studies investigating iPPROM and potential therapies should focus on the biology of membrane injury and weakening rather than changes in mechanical rupture properties.[166, 247]

Fetal membrane structure and integrity

There are also anatomical differences between human fetal membranes and those of domestic species. Many mammals have an additional allantoic membrane present at term (summarised in Table 2).[112, 312] Embryologically, the allantois is an out-pouching of the primordial urinary bladder that expands beside the amnion and fuses with the chorion forming the chorioallantois. The allantois is temporary in humans and absent by the time fetal surgery is performed at mid gestation.[112, 312] However, in sheep and

pigs, it persists throughout pregnancy as a large sac filled with fetal urine. A relatively smaller allantois is also seen throughout pregnancy in rabbits.[112, 312] Researchers should be aware of this difference so that interventions, therapies and tissue collection in pre-clinical animal studies of iPPROM are performed on combined sections of chorioamnion that are representative of human fetal membranes. The allantois is easily distinguished from the amnion by the presence of clear, dark fetal urine within the allantoic sac compared to the lighter, cloudy amniotic fluid that contains a mixture of urine, mucous, lung liquid and skin cells.[115]

The fetal membranes of many domestic species also contain considerably more blood vessels than human fetal membranes. Membrane vasculature is largely dependent on the anatomical arrangement of the placenta.[313, 314] Primates (including humans), rabbits and rodents have a discoid placenta that embeds on one side of the uterus (summarised in Table 2).[314] The small number of blood vessels within the chorion arise from the allantois and drain into the fetal sinus venosus. The placenta of sheep is comprised of multiple smaller placental structures (cotyledons) randomly distributed within uterus.[314] Cotyledons are interconnected by a large network of fetal blood vessels running within the chorion, which significantly increases membrane vasculature relative to humans.[313] Increased vasculature is also seen in the fetal membranes of pigs and guinea pigs where the placenta occupies the entire internal surface of the uterus (a diffuse arrangement).[314] Where possible, pre-clinical studies investigating mechanisms of iPPROM or aiming to seal membrane defects should use regions away from dense patches of vasculature.

Spontaneous healing

Unlike humans, the fetal membranes of rodents, rabbits and pigs show some ability to heal after being punctured at mid gestation (summarised in Table 2).[148, 273, 277, 279, 286, 315] In rodent and rabbit membranes, the amniotic epithelium and fibroblasts proliferate around the puncture site and are able to close small defects (<1cm) within 48-72 hours.[148, 273, 277, 279, 315] Larger defects either heal over longer periods or incompletely.[148, 315] Spontaneous membrane healing in these animal models may exaggerate the efficacy of new techniques aiming to prevent iPPROM in humans.

The fetal membranes of sheep and rhesus monkeys have shown limited healing capacity like humans and may therefore, be more appropriate models to test techniques aiming to prevent membrane injury.[279] Sheep also have considerably longer gestations than other animal models and their large size permits invasive monitoring of fetal physiology during pregnancy.[311] These advantages are particularly important as membrane closure techniques should demonstrate efficacy sealing the membranes for several weeks or months and be safe for the fetus when in contact with amniotic fluid.

Table 2: Comparative anatomy and physiology of the fetal membranes in humans and domestic species

	Human	Rodent	Rabbit	Sheep	Pigs	Non-human Primates
Placental and fetal membrane anatomy						
Placental arrangement	Discoid	Discoid	Discoid	Cotyledonary	Diffuse	Bidiscoid
Amnion	Present	Present	Present	Present	Present	Present
Chorion	Fused with decidua	Fused with decidua	Fused with decidua	Fused with decidua	Fused with decidua	Fused with decidua
Allantois at mid gestation	Absent	Absent	Small	Large	Large	Absent
Fetal membrane physiology						
Average Gestational age (days)	266	20-22	30	147	115	168
Number of fetuses	1	5-9	5	1-2	10-14	1-2
Condition of gestational sac at delivery	Partially intact or ruptured	Ruptured	Intact or ruptured	Intact or partially intact	Ruptured	Ruptured
Naturally occurring PPROM	Yes	No	No	No	No	No
Spontaneous membrane healing	No	Yes	Yes	No	Yes	No

Conclusions

The etiology of iPPROM after fetoscopy is not entirely understood however, appears multifactorial. Puncturing the membranes with the fetoscopic port, distending the uterus with unconditioned CO_2 and diluting the residual amniotic fluid during fetoscopy potentially weakens the membranes prematurely and increases the risk of iPPROM. Despite multiple attempts to develop novel preventions in pre-clinical models, iPPROM remains a major concern that limits the benefits of fetoscopy. It is important to consider that the structure and integrity of human fetal membranes are unique and thus promising results from pre-clinical studies will not always translate into clinical improvements. However, careful combination of large animal studies and human membrane explants may shed light on new ways to reduce iPPROM in the future.

Statements

Conflict of Interest Statement

"The authors have no conflicts of interest to declare."

Funding Sources

This research was supported by an Australian Government Research Training Program (RTP) Scholarship awarded to Benjamin Amberg at Monash University. Award number 25129015

Author Contributions

BJA, RJH, KAR, KJC, SBH and PLJD contributed to the conception and planning of this review. BJA and PLJK prepared the original manuscript which was then reviewed and approved by RJH, KAR, KJC and SBH prior to submission.

This page is left intentionally blank.

Appendix 2: The feasibility of Australian fetoscopic myelomeningocele repair

Mr Benjamin AMBERG[1,2], Dr Kelly CROSSLEY PhD[1,2], Prof Stuart HOOPER PhD[1,2], Dr Philip DEKONINCK MD PhD[1,2,3] and A/Prof Ryan HODGES MD PhD[1,2].

1. The Ritchie Centre, Hudson Institute of Medical Research, Melbourne, Victoria, Australia
2. The Department of Obstetrics and Gynaecology, Monash University, Melbourne, Victoria, Australia
3. Department of Obstetrics and Gynaecology, Erasmus MC-Sophia Children's Hospital, Rotterdam, The Netherlands

Concise Title: Fetoscopic myelomeningocele repair in Australia

Abstract Word Count: 92 words

Text Work Count:

Table word Count: 591 words

Key Words:

Conflict of Interest: The authors report no conflict of interest

Abstract:

Fetoscopic myelomeningocele (MMC) repair is rapidly developing as a minimally invasive alternative to open fetal surgery. Although fetoscopic MMC repair is not currently available in Australia, recent improvements in fetal and maternal outcomes overseas suggests that this may occur in the near future. Unfortunately, there is significant variability in surgical technique and the optimal approach for new centres to adopt remains unclear. This review describes the feasibility of establishing fetoscopic MMC repair in Australia by summarising the surgical techniques of the published international programs.

Introduction

Open fetal surgery is currently considered the gold standard management for fetal myelomeningocele (MMC) following the results the Management of Myelomeningocele Study (MOMS).[7] While open fetal surgery clearly improves lower limb function and reduces the need for ventriculoperitoneal shunting after birth, the early gestation hysterotomy used to access the fetus has significant implications for the mother including the risk of uterine rupture and the need to deliver all cases and future pregnancies via caesarean section.[7, 74] This maternal morbidity has driven the development of a minimally invasive, fetoscopic alternative. During fetoscopic MMC repair, the hysterotomy incision is avoided by distending the uterus with pressurised CO_2 and repairing the fetal spine endoscopically.[8]

Fetoscopic MMC repair was first attempted in the late 1990's however, was abandoned because of technical difficulties and poor fetal outcomes.[36, 63] However, several large international centres have recently revisited the technique and their published outcomes now closely resemble gold standard open fetal surgery (Figure 1).[8, 16, 17, 86, 264, 316] This improvement has sparked interest from Australian fetal therapy centres who may soon look to establish similar fetoscopic programs. Unfortunately, there is significant variability in how fetoscopic MMC repair is performed and the optimal surgical approach for new centres to adopt remains unclear. This review describes the feasibility of establishing fetoscopic MMC repair in Australia by summarising the surgical techniques of the published international programs.

Accessing the fetus:

Two techniques are used to access the fetus. Some centres use a completely percutaneous approach where three to four 11-15 French (5.0-7.0mm) ports are inserted into the amniotic space through the mothers abdominal and uterine wall.[8, 316] While this seems to be truly minimally invasive and achieves the best cosmetic outcomes for the mother, several authors report difficulties posturing the fetus fetoscopically, particularly when the mother is obese.[8, 14] Additionally, dislodgement of a port from the uterus can compromise in-utero visibility and is a common reason for procedures being abandoned or converted to open repair.[8]

Other centres partially exteriorise the uterus through a small laparotomy and insert the fetoscopic ports directly into the uterus.[17, 264] This allows surgeons to use their hands to position the fetus and reduces the risk of port dislodgement. Additionally, surgeons are able to use fewer (2-3), smaller diameter (3.3-4.0mm, 9-12 French) fetoscopic ports which has been suggested to minimise injury to the fetal membranes and potentially reduce the risk of post-operative preterm premature rupture of membranes (PPROM).[17, 200, 264]

Repairing the fetal spine:

The optimal surgical technique to protect the fetal spinal cord from injury and prevent CSF leakage also remains unclear.[86] During gold-standard open fetal surgery, the neural tissues adhered to the skin (the neural placode) are incised, the placode is returned to the spinal canal and then the dura, myofascia and skin are approximated in three separate layers.[7] Replicating this multi-layered neurosurgical repair fetoscopically remains challenging. Some centres place a dural patch over the placode instead of suturing the dura and then close the overlying skin. Unfortunately, these centres still report CSF leakage and repair dehiscence in some babies after birth.[17] More recently, the feasibility of a closing the myofascial and skin separately over a dural patch has been demonstrated in a small number of cases with promising results.[86, 264]

Other centres use a fetoscopic patch technique instead of a direct neurosurgical repair. Surgeons return the neural placode to the spinal canal and then cover the placode with a collagen/teflon or biocellulose patch which is sutured into the surrounding skin.[8, 316] Similar rates of repair dehiscence at birth and requirement for ventriculoperitoneal shunting at 12 months suggests that neither patch or direct repair is currently superior. While direct repair more closely resembles the technique used in gold-standard open fetal surgery, patches may be advantageous to cover large MMCs (>15-20mm diameter) or myeloschesis fetoscopically.[316] Authors using the direct technique report some difficulties approximating the tissues and often require large relaxing skin incisions in the fetal flanks to relieve tension on sutures.[17, 264] These relaxing incisions often heal poorly and have poor cosmetic outcomes.[86] Conversely, patches can be cut to size and incorporate an overlying skin substitute to avoid relaxing incisions. Patch repairs are also shorter than direct repairs (≈180 vs. ≈250 min) and may decrease fetal expose to general anesthesia and amniotic insufflation, the impact of which is not completely understood.

There is further variability in how surgical centres manage intra-operative complications. Most centres convert the fetoscopic procedure to open repair while some discontinue the procedure (table 1).[8, 14, 17, 264] An Australian program would need to consider whether conversion to open fetal surgery would be offered, and if so, how this may be supported by the surgeons from the Mater Mothers Hospital in Brisbane who have initial experiences with open myelomeningocele repair (data not published).

Irrespective of the surgical technique chosen for a new Australian program, the fetal surgeons involved must practice the technique in a large animal or simulated model before performing their first human cases.[86] It is also widely accepted that preliminary human cases at new fetoscopic centres are performed

in conjunction with experience surgeons from the leading centres performing fetoscopic MMC repair listed in table 1.[86]

Amniotic insufflation:

All centres performing fetoscopic MMC repair distend the uterus with pressurised CO_2 gas to improve endoscopic visibility and provide more space for surgery. Centres that use a completely percutaneous approach report higher insufflation pressures than those that partially exteriorise the uterus through a mini laparotomy (15 mmHg vs. 6-12 mmHg).[8, 16, 17, 264, 316] This difference likely reflects the additional pressure required to distend the mothers uterus against the abdominal wall. Centres using higher insufflation pressures generally report higher rates of post-operative PPROM (table 1) which may be associated with over-stretching the fetal membranes during insufflation. Additionally, sheep studies of amniotic insufflation show that high insufflation pressures (15-25mmHg) reduce uteroplacental blood flow and contribute to fetal acid base disturbances.[94] While these disturbances appear to be exaggerated in sheep studies of insufflation they provide a physiologic rationale for a new Australian centre to adopt an exteriorised uterus approach.

The International Fetoscopic Myelomeningocele Repair Consortium recently established that the insufflated CO_2 should be heated and humidified in all cases.[86] Sheep studies of amniotic insufflation have shown that heated humidified insufflation reduces fetal CO_2 absorption from the uterus and mitigates fetal membrane inflammation after surgery.[317, 318] Although only a small number of human cases have used heated humidified CO_2, preliminary outcomes show trends towards improved fetal outcomes and reduced rates of post-operative PPROM which may reflect these physiological benefits from the sheep studies.[16, 17, 86]

Monitoring the fetus

Monitoring the fetus within the insufflated uterus remains challenging for all centres. The presence of gas within the uterus limits intra-operative fetal ultrasound to small sections of the umbilical cord submerged in residual amniotic fluid or direct observation of umbilical cord pulsations using the fetoscope.[8, 316] Some centres also use fetal echocardiograms to monitor the fetal heart.[17] Unfortunately, both ultrasound and echocardiography may not reliably predict fetal distress particularly as general maternal-fetal anesthesia may blunt fetal heart rate changes.[84] Reassuringly, fetal ultrasound immediately after surgery and longer term MRI follow up suggest that human fetuses tolerate fetoscopic MMC repair well however, more accurate ways to monitor the fetus intra-operatively are still required.[17, 239]

Maternal and fetal anaesthesia:

Maternal-fetal anesthesia during fetoscopic MMC repair aims to maintain uterine relaxation to allow the uterus to be distended and to keep the fetus still during spinal repair. Until recently, both requirements have been achieved using higher doses of inhaled general anesthetics than typically employed in most surgical cases (2-3% minimum alveolar concentration (MAC)). However, concerns about inducing maternal pulmonary oedema and the effect on the immature fetal brain have led to the development of new techniques. One centre uses a combination of perioperative tocolysis (intravenous Atosiban), intravenous mulscle relaxant (Cis-atracurium) and a continuous remifentantil infusion to lower the required doses of inhaled anaesthetic (Desflurane, 0.5-0.7 MAC).[8] Other centres keep the fetus immobile by directly administering fetal anesthesia (an opioid, anti-cholinergic and muscle relaxant) intra-muscularly or via the umbilical vein (table1).[17] One centre also uses an automated system to carefully control maternal fluid and vasopressor support during surgery to reduce the risk of pulmonary oedema.[8]

Maternal anaesthetic protocols should also target pregnancy specific maternal CO_2 levels. Maternal arterial CO_2 is lower during pregnancy (35-45 → 30-32mmHg) which facilitates fetal CO_2 clearance in the placenta.[317] Maintaining maternal CO_2 between 30 and 32mmHg is particularly important during fetoscopic MMC repair as sheep studies have shown that the fetus must clear additional CO_2 absorbed from the uterus to prevent potentially harmful acid base disturbances.[317]

Planned mode of delivery:

Unlike open fetal surgery that necessitates cesarean section, fetoscopic MMC repair allows women to deliver the infant vaginally. However, two centres suggest that vaginal birth may disrupt the repaired neural placode and plan cesarean section at 39 weeks' gestation.[8, 17] No study has compared rates of fetoscopic repair dehiscence after cesarean or vaginal delivery however, an analysis of unoperated myelomeningoceles has shown that vaginal birth did not increase spinal cord trauma.[319] It therefore seems safe to suggest that a new Australian centre should plan to delivery operated cases after 37 weeks gestation via normal vaginal delivery.

The number of available cases

The number of parents opting for fetoscopic MMC repair in Australia is likely to be fewer than centres in Europe or America. Approximately 180 Australian pregnancies are affected by MMC each year (5.68 in 10,000) of which 75% are terminated.[3] In the MOMS, ≈30% of MMC cases met the criteria for fetal surgery and only 15% of these parents consented to undergo the procedure.[7] Although parents have been shown to become more accepting of fetoscopic MMC repair over time, these figures suggest that

≈6 cases would be available for fetoscopic repair in Australia each year.[320] This is less than most centres who perform 10-20 cases each year (table 1) and suggests that an Australian fetoscopic program would need to be run by a small number of surgeons out of a single specialized centre to focus expertise and negotiate the technical learning curve.[320] All cases would need to be followed up in a specialist MMC clinic until 30 months of age and the neurological and neurodevelopmental outcomes recorded in the common registry established by the International Fetoscopic Myelomeningocele Repair Consortium.

An Australian fetoscopic program may also choose to update the selection criteria used overseas. Most centres base patient selection on the MOMS trial (table 2) however secondary analysis of this cohort suggests that fetuses with large ventricles on antenatal ultrasound (>15mm diameter) may not benefit from fetal surgery.[321] Additionally, some groups have started repairing MMC defects at any spinal level, at slightly later gestational ages (24-28 weeks).[316] While it is difficult to determine if these additional cases benefit from fetal surgery, small case series using these criteria have shown similar rates of perinatal mortality and procedural failure to centres using the original MOMS criteria.[8] Furthermore, all centres need to carefully consider the inclusion of large, flat lesions (Myeloschisis - >20mm diameter) which often require conversion to open repair and still require ventriculoperitoneal shunting after birth.[17, 316]

Conclusions:

Technical advancements and improved fetal outcomes following fetoscopic MMC repair suggests that establishing an Australian program is feasible in the near future. This program would need to be run by a small number of surgeons out of a single specialized centre to account for the small number of cases expected each year. Although published protocols vary significantly, there seems to be several logistical and physiological advantages in placing fetoscopic ports directly into a partially exteriorized uterus and distending it with heated humidified CO_2. The technique used to repair the open spine should aim to emulate multi-layer closure used in gold standard open fetal surgery however, patch repairs may be advantageous for larger defects. Additionally, anesthetists should use perioperative tocolysis to minimize doses of inhaled anesthetics and use pregnancy specific maternal CO_2 ranges intra-operatively.

Acknowledgements:

This work is supported by the Victorian Government's Operational Infrastructure Support Program.

Tables:

Table 1: Current centres performing fetoscopic myelomeningocele repair

	Centre	Centre for Fetal Surgery & Minimally Invasive Therapy, Germany	Albert Einstein Hospital, Brazil	Texas Children's Hospital, USA	Johns Hopkins Centre Fetal Therapy, USA	Hospital Universitari Vall d'Hebron, Spain
	Most recent cohort	2014 N=71	2018 N=47	2017 N=22	2018 N=3	2018 N=5
Local Epidemiology	Spina Bifida – births per 10,000 live births	5.6	14.2	3.2	3.2	1.04
	Spina Bifida termination (% cases)	59.1%	Not Supported	4 - 30 %	4 - 30 %	Not Specified
	Estimated Repairs per year	17	19	11	NA	5
Uterine Access	Mean gestational age at surgery (Weeks$^{+\ days}$)	22^{+5}	26^{+4}	26^{+5}	24-25	23^{+5} -27^{+3}
	Technique	Percutaneous	Percutaneous	Percutaneous with exteriorized uterus	Percutaneous with exteriorized uterus	Percutaneous with exteriorized uterus
	Port number and diameter (mm)	3-4 x 5mm	2 x 3.7mm + 1 x 5mm	2 x 4mm	2-3 x 4mm	3 x 3.3mm
	Uterine membrane closure	No Closure	No Closure	Plicated membranes to uterine wall with 2/0 Suture	Plicated membranes to uterine wall with 2/0 Suture	Sutured with 2/0 suture
Defect Closure	Technique	Patch: Collagen/Teflon plus closure overlying skin	Patch: Biocellulose plus closure overlying skin	Direct Closure: Single layer - Skin with Dura	Direct Closure: Single layer - Skin with Dura	Direct Closure: Two layer – Myofascia + Skin
	Mean Operative time (minutes)	178	175	246	160	180
	Conversion to Open Repair	Not offered	8.5% (4/47)	Not offered	0% (0/3)	0% (0/5)
Amniotic Insufflation	Gas	Carbon dioxide	Carbon dioxide	Carbon dioxide	Carbon dioxide	Carbon Dioxide
	Heated humidified	No	No	Yes	Yes	Yes (Heated)
	Amniotic fluid drained (ml)	0	200 – All	300	Yes – volume n.s.	Yes – volume n.s.
	Mean insufflation pressure (mmHg)	15.3	15.6	12.0	8-10	6-9
	Flow Rate (L/min)	7.0	1.8	0.5	0.8	0.5
Peri-operative Management	Maternal general anaesthetic	Inhaled desflurane (0.5-0.7 MAC) + Intravenous Cis-atracurium and Remifentanil	Intravenous Remifentanil, Propofol, Rocuronium	Inhaled sevoflurane (1-3% MAC)	Inhaled sevoflurane (0.4-3% MAC)	Not Specified
	Direct fetal anaesthetic	None	None	Intramuscular fentanyl, atropine and vecuronium	Intramuscular fentanyl, atropine and rocuronium	Intramuscular fentanyl, atropine and vecuronium

	Pre/Intra-operative tocolysis	Atosiban	None	Indomethacin or nifedipine	Nitro-glycerine + Magnesium Sulfate	Yes - Drug n.s.
	Post-operative tocolysis	Indomethacin (24 hrs)	Atosiban (24 hrs)	Magnesium sulfate	Not Specified	Not Specified
	Planned mode of Delivery	Cesarean section at 39 weeks	Cesarean section at 39 weeks	Spontaneous vaginal delivery	Spontaneous vaginal delivery	Not specified
	Fetal monitoring	Observation of umbilical cord pulsations + intermittent Doppler	Observation of umbilical cord pulsations + intermittent Doppler	Near Continuous echocardiogram	Intermittent Doppler Ultrasound	Intermittent Doppler Ultrasound
	Maternal monitoring	$PaCO_2$ maintained 35-40 mmHg	End tidal CO_2 maintained 35-40 mmHg	Not Specified	End tidal CO_2 maintained 30-32 mmHg	Not Specified
Fetal Outcomes	Perinatal mortality	7% (5/71)	4.4% (2/45)	4.5% (1/22)	NA	0% (0/5)
	Mean gestational age at delivery (weeks$^{+\ days}$)	32^{+2}	32^{+6}	38^{+1}	NA	34^{+1}
	Preterm delivery <30 weeks	13.2% (9/71)	15.6% (7/45)	0% (0/22)	NA	40% (2/5)
	Repair dehiscence	28% (20/71)	17% (8/47)	32% (7/22)	NA	0% (0/5)
	Requirement for VP shunting at 12 months	45% (32/71)	46.5% (20/43)	41% (9/22)	NA	20% (1/5)
Maternal Outcomes	Preterm Rupture of Membranes	84.3% (43/51)	75% (34/45)	23% (5/22)	NA	60% (3/5)
	Oligohydramnios	13.7% (7/51)	n.s.	14% (3/22)	NA	0% (0/5)
	Pulmonary Oedema	2% (1/51)	0% (0/10)	9% (2/22)	NA	0% (0/5)

VP shunting – Ventriculoperitoneal shunting, NA. – not applicable, MAC – minimum alveolar concentration, $PaCO_2$ – arterial partial pressure of carbon dioxide

This page is left intentionally blank.

Thesis References:

1. TORTORI-DONATI P, ROSSI A, CAMA A. Spinal dysraphism: a review of neuroradiological features with embryological correlations and proposal for a new classification. Neuroradiology 2000;42:471-91.
2. HILDER L. Neural tube defects before and after the introduction of mandatory folic acid fortification in Australia 2007–2011. Journal of Paediatrics and Child Health 2017;53:42-43.
3. L.D B. International Clearinghouse for birth defects surveilance and research - Annual Report 2014. In: Defects ICCfB, ed. Annual Reports: International Clearinghouse Centre for Birth Defects, 2014 (vol 2014).
4. MCLONE DG, KNEPPER PA. The cause of Chiari II malformation: a unified theory. Pediatric neuroscience 1989;15:1-12.
5. DANZER E, SCHWARZ U, WEHRLI S, RADU A, ADZICK NS, FLAKE AW. Retinoic acid induced myelomeningocele in fetal rats: characterization by histopathological analysis and magnetic resonance imaging. Experimental neurology 2005;194:467-75.
6. ADZICK NS. Fetal surgery for spina bifida: past, present, future. Semin Pediatr Surg 2013;22:10-7.
7. ADZICK NS, THOM EA, SPONG CY, et al. A Randomized Trial of Prenatal versus Postnatal Repair of Myelomeningocele. New England Journal of Medicine 2011;364:993-1004.
8. KOHL T. Percutaneous minimally invasive fetoscopic surgery for spina bifida aperta. Part I: surgical technique and perioperative outcome. Ultrasound Obstet Gynecol 2014;44:515-24.
9. GRATACOS E, WU J, DEVLIEGER R, VAN DE VELDE M, DEPREST JA. Effects of amniodistention with carbon dioxide on fetal acid-base status during fetoscopic surgery in a sheep model. Surg Endosc 2001;15:368-72.
10. LUKS FI, DEPREST J, MARCUS M, et al. Carbon dioxide pneumoamnios causes acidosis in fetal lamb. Fetal Diagn Ther 1994;9:105-9.
11. PELLETIER GJ, SRINATHAN SK, LANGER JC. Effects of intraamniotic helium, carbon dioxide, and water on fetal lambs. J Pediatr Surg 1995;30:1155-8.
12. SAIKI Y, LITWIN DE, BIGRAS JL, et al. Reducing the deleterious effects of intrauterine CO2 during fetoscopic surgery. J Surg Res 1997;69:51-4.
13. SOOTHILL PW, AJAYI RA, CAMPBELL S, ROSS EM, NICOLAIDES KH. Fetal oxygenation at cordocentesis, maternal smoking and childhood neuro-development. European journal of obstetrics, gynecology, and reproductive biology 1995;59:21-4.
14. PEDREIRA DA, ZANON N, NISHIKUNI K, et al. Endoscopic surgery for the antenatal treatment of myelomeningocele: the CECAM trial. Am J Obstet Gynecol 2016;214:111 e1-11 e11.
15. PARRY S, STRAUSS JF. Premature Rupture of the Fetal Membranes. New England Journal of Medicine 1998;338:663-70.
16. BASCHAT AA, AHN ES, MURPHY J, MILLER JL. Fetal blood-gas values during fetoscopic myelomeningocele repair performed under carbon dioxide insufflation. Ultrasound Obstet Gynecol 2018.
17. BELFORT MA, WHITEHEAD WE, SHAMSHIRSAZ AA, et al. Fetoscopic Open Neural Tube Defect Repair: Development and Refinement of a Two-Port, Carbon Dioxide Insufflation Technique. Obstetrics and gynecology 2017;129:734-43.
18. MULLER F, O'RAHILLY R. The development of the human brain, the closure of the caudal neuropore, and the beginning of secondary neurulation at stage 12. Anatomy and embryology 1987;176:413-30.
19. COPP AJ, ADZICK NS, CHITTY LS, FLETCHER JM, HOLMBECK GN, SHAW GM. Spina bifida. Nature reviews Disease primers 2015;1:15007-07.
20. MITCHELL LE, ADZICK NS, MELCHIONNE J, PASQUARIELLO PS, SUTTON LN, WHITEHEAD AS. Spina bifida. The Lancet 2004;364:1885-95.
21. COPP AJ, GREENE ND, MURDOCH JN. The genetic basis of mammalian neurulation. Nature reviews Genetics 2003;4:784-93.

22. PADMANABHAN R. Etiology, pathogenesis and prevention of neural tube defects. Congenit Anom (Kyoto) 2006;46:55-67.
23. KAPLAN KM, SPIVAK JM, BENDO JA. Embryology of the spine and associated congenital abnormalities. Spine J 2005;5:564-76.
24. ADZICK NS. Fetal myelomeningocele: natural history, pathophysiology, and in-utero intervention. Seminars in fetal & neonatal medicine 2010;15:9-14.
25. ANIMATIONS M. Detailed Animation On Neurulation. In: Animations M, ed. *General Embryology* Youtube, 2015 (vol 2018).
26. BANNISTER CM. Neural Tube Defects – From Origin to Treatment. Cerebrospinal Fluid Research 2006;3:6-6.
27. SCOTT JM, WEIR DG, MOLLOY A, MCPARTLIN J, DALY L, KIRKE P. Folic acid metabolism and mechanisms of neural tube defects. Ciba Foundation symposium 1994;181:180-7; discussion 87-91.
28. COUNCIL MR. Prevention of neural tube defects: results of the Medical Research Council Vitamin Study. MRC Vitamin Study Research Group. Lancet (London, England) 1991;338:131-7.
29. CZEIZEL AE, DUDAS I. Prevention of the first occurrence of neural-tube defects by periconceptional vitamin supplementation. The New England journal of medicine 1992;327:1832-5.
30. ATTA CA, FIEST KM, FROLKIS AD, et al. Global Birth Prevalence of Spina Bifida by Folic Acid Fortification Status: A Systematic Review and Meta-Analysis. American journal of public health 2016;106:e24-34.
31. JOYEUX L, ENGELS AC, RUSSO FM, et al. Fetoscopic versus Open Repair for Spina Bifida Aperta: A Systematic Review of Outcomes. Fetal Diagn Ther 2016;39:161-71.
32. MEULI M, MEULI-SIMMEN C, HUTCHINS GM, et al. In utero surgery rescues neurological function at birth in sheep with spina bifida. Nat Med 1995;1:342-7.
33. HEFFEZ DS, ARYANPUR J, HUTCHINS GM, FREEMAN JM. The paralysis associated with myelomeningocele: clinical and experimental data implicating a preventable spinal cord injury. Neurosurgery 1990;26:987-92.
34. HUTCHINS GM, MEULI M, MEULI-SIMMEN C, JORDAN MA, HEFFEZ DS, BLAKEMORE KJ. Acquired spinal cord injury in human fetuses with myelomeningocele. Pediatr Pathol Lab Med 1996;16:701-12.
35. MEULI M, MEULI-SIMMEN C, HUTCHINS GM, SELLER MJ, HARRISON MR, ADZICK NS. The spinal cord lesion in human fetuses with myelomeningocele: implications for fetal surgery. J Pediatr Surg 1997;32:448-52.
36. FARMER DL, VON KOCH CS, PEACOCK WJ, et al. In utero repair of myelomeningocele: experimental pathophysiology, initial clinical experience, and outcomes. Arch Surg 2003;138:872-8.
37. SPOOR JKH, GADJRADJ PS, EGGINK AJ, et al. Contemporary management and outcome of myelomeningocele: the Rotterdam experience. Neurosurg Focus 2019;47:E3.
38. MASINI L, DE LUCA C, NOIA G, et al. Prenatal diagnosis, natural history, postnatal treatment and outcome of 222 cases of spina bifida: experience of a tertiary center. Ultrasound Obstet Gynecol 2019;53:302-08.
39. KANCHERLA V, DRUSCHEL CM, OAKLEY GP, JR. Population-based study to determine mortality in spina bifida: New York State Congenital Malformations Registry, 1983 to 2006. Birth defects research Part A, Clinical and molecular teratology 2014;100:563-75.
40. YI Y, LINDEMANN M, COLLIGS A, SNOWBALL C. Economic burden of neural tube defects and impact of prevention with folic acid: a literature review. Eur J Pediatr 2011;170:1391-400.
41. TREBLE A, JURANEK J, STUEBING KK, DENNIS M, FLETCHER JM. Functional significance of atypical cortical organization in spina bifida myelomeningocele: relations of cortical thickness and gyrification with IQ and fine motor dexterity. Cerebral cortex (New York, NY : 1991) 2013;23:2357-69.

42. HAMPTON LE, FLETCHER JM, CIRINO PT, et al. Hydrocephalus status in spina bifida: an evaluation of variations in neuropsychological outcomes. Journal of neurosurgery Pediatrics 2011;8:289-98.
43. UCSF FETAL TREATMENT CENTER SF. Spina Bifida (Myelomeningocele)*Conditions and Treatments*. UCSF Fetal Treatment Center Website, 2013 (vol 2018).
44. COLEMAN BG, LANGER JE, HORII SC. The Diagnostic Features of Spina Bifida: The Role of Ultrasound. Fetal Diagnosis and Therapy 2015;37:179-96.
45. VAN DER VOSSEN S, PISTORIUS LR, MULDER EJ, et al. Role of prenatal ultrasound in predicting survival and mental and motor functioning in children with spina bifida. Ultrasound Obstet Gynecol 2009;34:253-8.
46. NICOLAIDES KH, CAMPBELL S, GABBE SG, GUIDETTI R. Ultrasound screening for spina bifida: cranial and cerebellar signs. Lancet (London, England) 1986;2:72-4.
47. PENSO C, REDLINE RW, BENACERRAF BR. A sonographic sign which predicts which fetuses with hydrocephalus have an associated neural tube defect. Journal of ultrasound in medicine : official journal of the American Institute of Ultrasound in Medicine 1987;6:307-11.
48. NYBERG DA, MACK LA, HIRSCH J, MAHONY BS. Abnormalities of fetal cranial contour in sonographic detection of spina bifida: evaluation of the "lemon" sign. Radiology 1988;167:387-92.
49. CORROENNE R, YEPEZ M, PYARALI M, et al. Longitudinal evaluation of motor function in patients who underwent a prenatal or postnatal neural tube defect repair. Ultrasound Obstet Gynecol 2020.
50. JOHNSON MP, BENNETT KA, RAND L, et al. The Management of Myelomeningocele Study: obstetrical outcomes and risk factors for obstetrical complications following prenatal surgery. Am J Obstet Gynecol 2016;215:778.e1-78.e9.
51. CALDARELLI M, ROCCO C, MEMET ÖZEK M, CINALLI G, MAIXNER W. Myelomeningocele Primary Repair Surgical Technique. 2008.
52. MCCULLOUGH DC, JOHNSON DL. Myelomeningocele repair: technical considerations and complications. 1988. Pediatr Neurosurg 1994;21:83-9; discussion 90.
53. LIEN SC, MAHER CO, GARTON HJ, KASTEN SJ, MURASZKO KM, BUCHMAN SR. Local and regional flap closure in myelomeningocele repair: a 15-year review. Child's nervous system : ChNS : official journal of the International Society for Pediatric Neurosurgery 2010;26:1091-5.
54. MICHEJDA M. Intrauterine treatment of spina bifida: primate model. Zeitschrift fur Kinderchirurgie : organ der Deutschen, der Schweizerischen und der Osterreichischen Gesellschaft fur Kinderchirurgie = Surgery in infancy and childhood 1984;39:259-61.
55. STIEFEL D, COPP AJ, MEULI M. Fetal spina bifida in a mouse model: loss of neural function in utero. Journal of neurosurgery 2007;106:213-21.
56. HEFFEZ DS, ARYANPUR J, ROTELLINI NA, HUTCHINS GM, FREEMAN JM. Intrauterine repair of experimental surgically created dysraphism. Neurosurgery 1993;32:1005-10.
57. KOHL T, HARTLAGE MG, KIEHITZ D, et al. Percutaneous fetoscopic patch coverage of experimental lumbosacral full-thickness skin lesions in sheep. Surg Endosc 2003;17:1218-23.
58. KOHL T, TCHATCHEVA K, BERG C, GEIPEL A, VAN DE VONDEL P, GEMBRUCH U. Partial amniotic carbon dioxide insufflation (PACI) facilitates fetoscopic interventions in complicated monochorionic twin pregnancies. Surg Endosc 2007;21:1428-33.
59. KOHL T, WITTELER R, STRUMPER D, et al. Operative techniques and strategies for minimally invasive fetoscopic fetal cardiac interventions in sheep. Surg Endosc 2000;14:424-30.
60. FATUM M, ROJANSKY N. Laparoscopic surgery during pregnancy. Obstet Gynecol Surv 2001;56:50-9.
61. MENES T, SPIVAK H. Laparoscopy: searching for the proper insufflation gas. Surg Endosc 2000;14:1050-6.
62. O'ROURKE N, KODALI BS. Laparoscopic surgery during pregnancy. Curr Opin Anaesthesiol 2006;19:254-9.
63. BRUNER JP, RICHARDS WO, TULIPAN NB, ARNEY TL. Endoscopic coverage of fetal myelomeningocele in utero. Am J Obstet Gynecol 1999;180:153-8.

64. TULIPAN N, BRUNER JP. Myelomeningocele repair in utero: a report of three cases. Pediatr Neurosurg 1998;28:177-80.
65. HEUER GG, ADZICK NS, SUTTON LN. Fetal Myelomeningocele Closure: Technical Considerations. Fetal Diagnosis and Therapy 2015;37:166-71.
66. RINTOUL NE, SUTTON LN, HUBBARD AM, et al. A new look at myelomeningoceles: functional level, vertebral level, shunting, and the implications for fetal intervention. Pediatrics 2002;109:409-13.
67. TULIPAN N, SUTTON LN, BRUNER JP, COHEN BM, JOHNSON M, ADZICK NS. The effect of intrauterine myelomeningocele repair on the incidence of shunt-dependent hydrocephalus. Pediatr Neurosurg 2003;38:27-33.
68. DANZER E, FINKEL RS, RINTOUL NE, et al. Reversal of hindbrain herniation after maternal-fetal surgery for myelomeningocele subsequently impacts on brain stem function. Neuropediatrics 2008;39:359-62.
69. DANZER E, GERDES M, BEBBINGTON MW, et al. Lower extremity neuromotor function and short-term ambulatory potential following in utero myelomeningocele surgery. Fetal Diagn Ther 2009;25:47-53.
70. DANZER E, THOMAS NH, THOMAS A, et al. Long-term neurofunctional outcome, executive functioning, and behavioral adaptive skills following fetal myelomeningocele surgery. Am J Obstet Gynecol 2016;214:269 e1-8.
71. FARMER DL, THOM EA, BROCK JW, 3RD, et al. The Management of Myelomeningocele Study: full cohort 30-month pediatric outcomes. Am J Obstet Gynecol 2018;218:256.e1-56.e13.
72. HOUTROW AJ, THOM EA, FLETCHER JM, et al. Prenatal Repair of Myelomeningocele and School-age Functional Outcomes. Pediatrics 2020;145.
73. BROCK JW, 3RD, THOMAS JC, BASKIN LS, et al. Effect of Prenatal Repair of Myelomeningocele on Urological Outcomes at School Age. J Urol 2019;202:812-18.
74. WILSON RD, LEMERAND K, JOHNSON MP, et al. Reproductive outcomes in subsequent pregnancies after a pregnancy complicated by open maternal-fetal surgery (1996-2007). Am J Obstet Gynecol 2010;203:209 e1-6.
75. OBSTETRICIANS ACO, GYNECOLOGISTS. Maternal Fetal Surgery for Myelomeningocele repair. Number 550, January 2013. Committee Opinion No. 550 American College of Obstetricians and Gynecologists. Obstetr Gynecol 2013;121:218-9.
76. SIMPSON JL, GREENE MF. Fetal Surgery for Myelomeningocele? New England Journal of Medicine 2011;364:1076-77.
77. KOHL T, GEMBRUCH U. Current status and prospects of fetoscopic surgery for spina bifida in human fetuses. Response to Fichter et al: Fetal spina bifida repair--current trends and prospects of intrauterine neurosurgery (Fetal Diagn Ther 2008;23:271-286). Fetal Diagn Ther 2008;24:318-20.
78. KOHL T, ZIEMANN M, WEINBACH J, TCHATCHEVA K, GEMBRUCH U, HASSELBLATT M. Partial amniotic carbon dioxide insufflation during minimally invasive fetoscopic interventions seems safe for the fetal brain in sheep. Journal of laparoendoscopic & advanced surgical techniques Part A 2010;20:651-3.
79. KOHL T, TCHATCHEVA K, WEINBACH J, et al. Partial amniotic carbon dioxide insufflation (PACI) during minimally invasive fetoscopic surgery: early clinical experience in humans. Surg Endosc 2010;24:432-44.
80. PATON MCB, MCDONALD CA, ALLISON BJ, FAHEY MC, JENKIN G, MILLER SL. Perinatal Brain Injury As a Consequence of Preterm Birth and Intrauterine Inflammation: Designing Targeted Stem Cell Therapies. Front Neurosci 2017;11:200-00.
81. AKALIN-SEL T, NICOLAIDES KH, PEACOCK J, CAMPBELL S. Doppler dynamics and their complex interrelation with fetal oxygen pressure, carbon dioxide pressure, and pH in growth-retarded fetuses. Obstetrics and gynecology 1994;84:439-44.
82. RIZZO G, CAPPONI A, ARDUINI D, ROMANINI C. The value of fetal arterial, cardiac and venous flows in predicting pH and blood gases measured in umbilical blood at cordocentesis in growth retarded fetuses. British journal of obstetrics and gynaecology 1995;102:963-9.

83. BASCHAT AA, GALAN HL, BHIDE A, et al. Doppler and biophysical assessment in growth restricted fetuses: distribution of test results. Ultrasound Obstet Gynecol 2006;27:41-7.
84. MOISE KJ, JR., TSAO K, PAPANNA RM, BEBBINGTON MW. Fetoscopic Repair of Meningomyelocele. Obstetrics and gynecology 2015;126:674.
85. ZIEMANN M, FIMMERS R, KHALEEVA A, SCHURG R, WEIGAND MA, KOHL T. Partial amniotic carbon dioxide insufflation (PACI) during minimally invasive fetoscopic interventions on fetuses with spina bifida aperta. Surg Endosc 2018.
86. SANZ CORTES M, LAPA DA, ACACIO GL, et al. Proceedings of the First Annual Meeting of the International Fetoscopic Myelomeningocele Repair Consortium. Ultrasound in Obstetrics & Gynecology 2019;53:855-63.
87. HARRISON MR, FILLY RA, GOLBUS MS, et al. Fetal treatment 1982. The New England journal of medicine 1982;307:1651-2.
88. HALL J. Transport of oxygen and carbon dioxide in blood and tissue fluids *Guyton and Hall Textbook of Medical Physiology* Philadelphia: Saunders 2013 (vol 12).
89. GEERS C, GROS G. Carbon dioxide transport and carbonic anhydrase in blood and muscle. Physiological reviews 2000;80:681-715.
90. OMO-AGHOJA L. Maternal and Fetal Acid-Base Chemistry: A Major Determinant of Perinatal Outcome. Annals of Medical and Health Sciences Research 2014;4:8-17.
91. WILKENING RB, ANDERSON S, MARTENSSON L, MESCHIA G. Placental transfer as a function of uterine blood flow. The American journal of physiology 1982;242:H429-36.
92. WILKENING RB, MESCHIA G. Fetal oxygen uptake, oxygenation, and acid-base balance as a function of uterine blood flow. The American journal of physiology 1983;244:H749-55.
93. OMO-AGHOJA L. Maternal and Fetal Acid-Base Chemistry: A Major Determinant of Perinatal Outcome. Annals of Medical and Health Sciences Research 2014;4:8-17.
94. SKINNER S, DEKONINCK P, CROSSLEY K, et al. Partial amniotic carbon dioxide insufflation for fetal surgery. Prenatal Diagnosis 2018;38:983-93.
95. ORCHARD CH, KENTISH JC. Effects of changes of pH on the contractile function of cardiac muscle. The American journal of physiology 1990;258:C967-81.
96. STENGL M, LEDVINOVA L, CHVOJKA J, et al. Effects of clinically relevant acute hypercapnic and metabolic acidosis on the cardiovascular system: an experimental porcine study. Critical Care 2013;17:R303-R03.
97. TANG WC, WEIL MH, GAZMURI RJ, BISERA J, RACKOW EC. Reversible impairment of myocardial contractility due to hypercarbic acidosis in the isolated perfused rat heart. Critical care medicine 1991;19:218-24.
98. FABIATO A, FABIATO F. Effects of pH on the myofilaments and the sarcoplasmic reticulum of skinned cells from cardiace and skeletal muscles. The Journal of physiology 1978;276:233-55.
99. POURCYROUS M, CHILAKALA S, ELABIAD MT, PARFENOVA H, LEFFLER CW. Does prolonged severe hypercapnia interfere with normal cerebrovascular function in piglets? Pediatric research 2018;84:290-95.
100. SOLARO RJ, EL-SALEH SC, KENTISH JC. Ca2+, pH and the regulation of cardiac myofilament force and ATPase activity. Molecular and cellular biochemistry 1989;89:163-7.
101. FRITZ KI, ZUBROW A, MISHRA OP, DELIVORIA-PAPADOPOULOS M. Hypercapnia-induced modifications of neuronal function in the cerebral cortex of newborn piglets. Pediatric research 2005;57:299-304.
102. DAS S, DU Z, BASSLY S, SINGER L, VICENCIO AG. Effects of chronic hypercapnia in the neonatal mouse lung and brain. Pediatric pulmonology 2009;44:176-82.
103. GOLDMAN SA, PULSINELLI WA, CLARKE WY, KRAIG RP, PLUM F. The effects of extracellular acidosis on neurons and glia in vitro. J Cereb Blood Flow Metab 1989;9:471-7.
104. MACGREGOR DG, CHESLER M, RICE ME. HEPES prevents edema in rat brain slices. Neurosci Lett 2001;303:141-4.
105. MOTOYAMA EK, RIVARD G, ACHESON F, COOK CD. Adverse effect of maternal hyperventilation on the foetus. Lancet (London, England) 1966;1:286-8.

106. TILL H, YEUNG CK, BOWER W, et al. Fetoscopy under gas amniodistension: pressure-dependent influence of helium vs nitrous oxide on fetal goats. J Pediatr Surg 2007;42:1255-8.
107. FUJINAGA M, BADEN JM, SHEPARD TH, MAZZE RI. Nitrous oxide alters body laterality in rats. Teratology 1990;41:131-5.
108. FUJINAGA M, BADEN JM, MAZZE RI. Susceptible period of nitrous oxide teratogenicity in Sprague-Dawley rats. Teratology 1989;40:439-44.
109. CARROLL JJ, SLUPSKY JD, MATHER AE. The Solubility of Carbon Dioxide in Water at Low Pressure. Journal of Physical and Chemical Reference Data 1991;20:1201-09.
110. DUTTON FB. Dalton's law of partial pressures. Journal of Chemical Education 1961;38:A545.
111. ARIKAT S, NOVINCE RW, MERCER BM, et al. Separation of amnion from choriodecidua is an integral event to the rupture of normal term fetal membranes and constitutes a significant component of the work required. Am J Obstet Gynecol 2006;194:211-7.
112. CARTER AM. IFPA Senior Award Lecture: Mammalian fetal membranes. Placenta 2016;48:S21-S30.
113. MALAK TM, OCKLEFORD CD, BELL SC, DALGLEISH R, BRIGHT N, MACVICAR J. Confocal immunofluorescence localization of collagen types I, III, IV, V and VI and their ultrastructural organization in term human fetal membranes. Placenta 1993;14:385-406.
114. STRAUSS JF, 3RD. Extracellular matrix dynamics and fetal membrane rupture. Reprod Sci 2013;20:140-53.
115. BRACE RA, CHEUNG CY. Regulation of amniotic fluid volume: evolving concepts. Adv Exp Med Biol 2014;814:49-68.
116. KUMAR D, MOORE RM, MERCER BM, MANSOUR JM, REDLINE RW, MOORE JJ. The physiology of fetal membrane weakening and rupture: Insights gained from the determination of physical properties revisited. Placenta 2016;42:59-73.
117. BORAZJANI A, WEED BC, PATNAIK SS, et al. A comparative biomechanical analysis of term fetal membranes in human and domestic species. Am J Obstet Gynecol 2011;204:365.e25-36.
118. JOYCE EM, MOORE JJ, SACKS MS. Biomechanics of the fetal membrane prior to mechanical failure: review and implications. European journal of obstetrics, gynecology, and reproductive biology 2009;144 Suppl 1:S121-S27.
119. RANGASWAMY N, ABDELRAHIM A, MOORE RM, et al. Biomechanical characteristics of human fetal membranes. Preterm fetal membranes are stronger than term fetal membranes. Gynecol Obstet Fertil 2011;39:373-7.
120. VETTRAINO IM, ROBY J, TOLLEY T, PARKS WC. Collagenase-I, stromelysin-I, and matrilysin are expressed within the placenta during multiple stages of human pregnancy. Placenta 1996;17:557-63.
121. VADILLO-ORTEGA F, GONZÁLEZ-AVILA G, FURTH EE, et al. 92-kd type IV collagenase (matrix metalloproteinase-9) activity in human amniochorion increases with labor. Am J Pathol 1995;146:148-56.
122. FORTUNATO SJ, MENON R, LOMBARDI SJ. Collagenolytic enzymes (gelatinases) and their inhibitors in human amniochorionic membrane. Am J Obstet Gynecol 1997;177:731-41.
123. MOORE RM, MANSOUR JM, REDLINE RW, MERCER BM, MOORE JJ. The Physiology of Fetal Membrane Rupture: Insight Gained from the Determination of Physical Properties. Placenta 2006;27:1037-51.
124. MCLAREN J, MALAK TM, BELL SC. Structural characteristics of term human fetal membranes prior to labour: identification of an area of altered morphology overlying the cervix. Hum Reprod 1999;14:237-41.
125. MCPARLAND PC, TAYLOR DJ, BELL SC. Mapping of zones of altered morphology and chorionic connective tissue cellular phenotype in human fetal membranes (amniochorion and decidua) overlying the lower uterine pole and cervix before labor at term. Am J Obstet Gynecol 2003;189:1481-8.
126. MCLAREN J, TAYLOR DJ, BELL SC. Increased incidence of apoptosis in non-labour-affected cytotrophoblast cells in term fetal membranes overlying the cervix. Hum Reprod 1999;14:2895-900.

127. MCLAREN J, TAYLOR DJ, BELL SC. Increased concentration of pro–matrix metalloproteinase 9 in term fetal membranes overlying the cervix before labor: Implications for membrane remodeling and rupture. American Journal of Obstetrics and Gynecology 2000;182:409-16.
128. FORTNER KB, GROTEGUT CA, RANSOM CE, et al. Bacteria Localization and Chorion Thinning among Preterm Premature Rupture of Membranes. PLOS ONE 2014;9:e83338.
129. EL KHWAD M, STETZER B, MOORE RM, et al. Term human fetal membranes have a weak zone overlying the lower uterine pole and cervix before onset of labor. Biol Reprod 2005;72:720-6.
130. EL KHWAD M, PANDEY V, STETZER B, et al. Fetal membranes from term vaginal deliveries have a zone of weakness exhibiting characteristics of apoptosis and remodeling. J Soc Gynecol Investig 2006;13:191-95.
131. RANGASWAMY N, KUMAR D, MOORE R, et al. Weakening and Rupture of Human Fetal Membranes – Biochemistry and Biomechanics. 2012.
132. HUNG T-H, HSIEH TS-TA, CHEN S-F, LI M-J, YEH Y-L. Autophagy in the Human Placenta throughout Gestation. PLOS ONE 2013;8:e83475.
133. ATHAYDE N, EDWIN SS, ROMERO R, et al. A role for matrix metalloproteinase-9 in spontaneous rupture of the fetal membranes. Am J Obstet Gynecol 1998;179:1248-53.
134. RETI NG, LAPPAS M, RILEY C, et al. Why do membranes rupture at term? Evidence of increased cellular apoptosis in the supracervical fetal membranes. American Journal of Obstetrics and Gynecology 2007;196:484.e1-84.e10.
135. KUMAGAI K, OTSUKI Y, ITO Y, SHIBATA MA, ABE H, UEKI M. Apoptosis in the normal human amnion at term, independent of Bcl-2 regulation and onset of labour. Molecular Human Reproduction 2001;7:681-89.
136. SAĞOL S, SAĞOL O, OZKAL S, ASENA U. Role of apoptosis, bcl-2 and bax protein expression in premature rupture of fetal membranes. J Reprod Med 2002;47:809-15.
137. KATAOKA S, FURUTA I, YAMADA H, et al. Increased Apoptosis of Human Fetal Membranes in Rupture of the Membranes and Chorioamnionitis. Placenta 2002;23:224-31.
138. ARECHAVALETA-VELASCO F, MAYON-GONZALEZ J, GONZALEZ-JIMENEZ M, HERNANDEZ-GUERRERO C, VADILLO-ORTEGA F. Association of Type II Apoptosis and 92-kDa Type IV Collagenase Expression in Human Amniochorion in Prematurely Ruptured Membranes With Tumor Necrosis Factor Receptor-1 Expression. The Journal of the Society for Gynecologic Investigation: JSGI 2002;9:60-67.
139. FORTUNATO SJ, MENON R, BRYANT C, LOMBARDI SJ. Programmed cell death (apoptosis) as a possible pathway to metalloproteinase activation and fetal membrane degradation in premature rupture of membranes. Am J Obstet Gynecol 2000;182:1468-76.
140. LEI H, FURTH EE, KALLURI R, et al. A program of cell death and extracellular matrix degradation is activated in the amnion before the onset of labor. The Journal of Clinical Investigation 1996;98:1971-78.
141. MONTENEGRO D, ROMERO R, PINELES BL, et al. Differential expression of microRNAs with progression of gestation and inflammation in the human chorioamniotic membranes. American journal of obstetrics and gynecology 2007;197:289.e1-89.e2896.
142. WANG H, OGAWA M, WOOD JR, et al. Genetic and epigenetic mechanisms combine to control MMP1 expression and its association with preterm premature rupture of membranes. Hum Mol Genet 2008;17:1087-96.
143. MARADNY EE, KANAYAMA N, HALIM A, MAEHARA K, TERAO T. Stretching of fetal membranes increases the concentration of interleukin-8 and collagenase activity. Am J Obstet Gynecol 1996;174:843-9.
144. MAEHARA K, KANAYAMA N, MARADNY EE, UEZATO T, FUJITA M, TERAO T. Mechanical stretching induces interleukin-8 gene expression in fetal membranes: a possible role for the initiation of human parturition. European journal of obstetrics, gynecology, and reproductive biology 1996;70:191-96.
145. NEMETH E, MILLAR LK, BRYANT-GREENWOOD G. Fetal membrane distention: II. Differentially expressed genes regulated by acute distention in vitro. Am J Obstet Gynecol 2000;182:60-7.

146. KUMAR D, MOORE RM, NASH A, et al. Decidual GM-CSF is a critical common intermediate necessary for thrombin and TNF induced in-vitro fetal membrane weakening. Placenta 2014;35:1049-56.
147. MENON R, RICHARDSON LS. Preterm prelabor rupture of the membranes: A disease of the fetal membranes. Semin Perinatol 2017;41:409-19.
148. DEVLIEGER R, MILLAR LK, BRYANT-GREENWOOD G, LEWI L, DEPREST JA. Fetal membrane healing after spontaneous and iatrogenic membrane rupture: a review of current evidence. American journal of obstetrics and gynecology 2006;195:1512-20.
149. MILLAR LK, BOESCHE MH, YAMAMOTO SY, et al. A relaxin-mediated pathway to preterm premature rupture of the fetal membranes that is independent of infection. Am J Obstet Gynecol 1998;179:126-34.
150. MERCER BM. Preterm premature rupture of the membranes. Obstetrics and gynecology 2003;101:178-93.
151. TCHIRIKOV M, SCHLABRITZ-LOUTSEVITCH N, MAHER J, et al. Mid-trimester preterm premature rupture of membranes (PPROM): etiology, diagnosis, classification, international recommendations of treatment options and outcome. J Perinat Med 2018;46:465-88.
152. QUINTERO RA, MORALES WJ, KALTER CS, et al. Transabdominal intra-amniotic endoscopic assessment of previable premature rupture of membranes. Am J Obstet Gynecol 1998;179:71-6.
153. ROMERO R, MAYMON E, PACORA P, et al. Further observations on the fetal inflammatory response syndrome: a potential homeostatic role for the soluble receptors of tumor necrosis factor alpha. Am J Obstet Gynecol 2000;183:1070-7.
154. GOLDENBERG RL, CULHANE JF, IAMS JD, ROMERO R. Epidemiology and causes of preterm birth. The Lancet 2008;371:75-84.
155. COX SM, CASEY ML, MACDONALD PC. Accumulation of interleukin-1beta and interleukin-6 in amniotic fluid: a sequela of labour at term and preterm. Hum Reprod Update 1997;3:517-27.
156. MAYMON E, GHEZZI F, EDWIN SS, et al. The tumor necrosis factor alpha and its soluble receptor profile in term and preterm parturition. Am J Obstet Gynecol 1999;181:1142-8.
157. MENON R, SWAN KF, LYDEN TW, ROTE NS, FORTUNATO SJ. Expression of inflammatory cytokines (interleukin-1 beta and interleukin-6) in amniochorionic membranes. American journal of obstetrics and gynecology 1995;172:493-500.
158. VEGA-SANCHEZ R, GOMEZ-LOPEZ N, FLORES-PLIEGO A, et al. Placental blood leukocytes are functional and phenotypically different than peripheral leukocytes during human labor. J Reprod Immunol 2010;84:100-10.
159. KUMAR D, FUNG W, MOORE RM, et al. Proinflammatory cytokines found in amniotic fluid induce collagen remodeling, apoptosis, and biophysical weakening of cultured human fetal membranes. Biology of reproduction 2006;74:29-34.
160. MOORE RM, NOVAK JB, KUMAR D, MANSOUR JM, MERCER BM, MOORE JJ. Alpha-lipoic acid inhibits tumor necrosis factor-induced remodeling and weakening of human fetal membranes. Biology of reproduction 2009;80:781-87.
161. KUMAR D, SCHATZ F, MOORE RM, et al. The effects of thrombin and cytokines upon the biomechanics and remodeling of isolated amnion membrane, in vitro. Placenta 2011;32:206-13.
162. MOORE RM, SCHATZ F, KUMAR D, et al. Alpha-lipoic acid inhibits thrombin-induced fetal membrane weakening in vitro. Placenta 2010;31:886-92.
163. MCGREGOR JA, SCHOONMAKER JN, LUNT BD, LAWELLIN DW. Antibiotic inhibition of bacterially induced fetal membrane weakening. Obstetrics and gynecology 1990;76:124-28.
164. MILLAR LK, STOLLBERG J, DEBUQUE L, BRYANT-GREENWOOD G. Fetal membrane distention: determination of the intrauterine surface area and distention of the fetal membranes preterm and at term. Am J Obstet Gynecol 2000;182:128-34.
165. JOYCE EM, DIAZ P, TAMARKIN S, et al. In-vivo stretch of term human fetal membranes. Placenta 2016;38:57-66.
166. PADRON JG, SAITO REIS CA, KENDAL-WRIGHT CE. The Role of Danger Associated Molecular Patterns in Human Fetal Membrane Weakening. Frontiers in Physiology 2020;11.

167. ARTAL R, BURGESON R, FERNANDEZ FJ, HOBEL CJ. Fetal and maternal copper levels in patients at term with and without premature rupture of membranes. Obstetrics and gynecology 1979;53:608-10.
168. CASEY ML, MACDONALD PC. Lysyl Oxidase (ras Recision Gene) Expression in Human Amnion: Ontogeny and Cellular Localization*. The Journal of Clinical Endocrinology & Metabolism 1997;82:167-72.
169. WIDEMAN GL, BAIRD GH, BOLDING OT. Ascorbic acid deficiency and premature rupture of fetal membranes. Am J Obstet Gynecol 1964;88:592-5.
170. BARABAS AP. Ehlers-Danlos syndrome: associated with prematurity and premature rupture of foetal membranes; possible increase in incidence. Br Med J 1966;2:682-84.
171. LAPA PEDREIRA DA, ACACIO GL, GONCALVES RT, et al. Percutaneous fetoscopic closure of large open spina bifida using a bilaminar skin substitute. Ultrasound Obstet Gynecol 2018.
172. BINDA MM. Humidification during laparoscopic surgery: overview of the clinical benefits of using humidified gas during laparoscopic surgery. Archives of gynecology and obstetrics 2015;292:955-71.
173. OTT DE. Desertification of the peritoneum by thin-film evaporation during laparoscopy. JSLS 2003;7:189-95.
174. MOUTON WG, BESSELL JR, PFITZNER J, DYMOCK RB, BREALEY J, MADDERN GJ. A randomized controlled trial to determine the effects of humidified carbon dioxide insufflation during thoracoscopy. Surgical Endoscopy 1999;13:382-85.
175. MOUTON WG, NAEF M, BESSELL JR, OTTEN KT, WAGNER HE, MADDERN GJ. A randomized controlled trial to determine the effect of humidified carbon dioxide (CO2) insufflation on postoperative pain following thoracoscopic procedures. Surgical Endoscopy 2001;15:579-81.
176. BIRCHER K, EHRET AE, SPIESS D, et al. On the defect tolerance of fetal membranes. Interface Focus 2019;9:20190010.
177. VAN MIEGHEM T, BAUD D, DEVLIEGER R, et al. Minimally invasive fetal therapy. Best Pract Res Clin Obstet Gynaecol 2012;26:711-25.
178. VERBEEK RJ, HEEP A, MAURITS NM, et al. Fetal endoscopic myelomeningocele closure preserves segmental neurological function. Dev Med Child Neurol 2012;54:15-22.
179. DEGENHARDT J, SCHURG R, WINARNO A, et al. Percutaneous minimal-access fetoscopic surgery for spina bifida aperta. Part II: maternal management and outcome. Ultrasound Obstet Gynecol 2014;44:525-31.
180. GRAF K, KOHL T, NEUBAUER BA, et al. Percutaneous minimally invasive fetoscopic surgery for spina bifida aperta. Part III: neurosurgical intervention in the first postnatal year. Ultrasound Obstet Gynecol 2016;47:158-61.
181. COUNCIL N. Australian code for the care and use of animals for scientific purposes: National Health and Medical Research Council, Canberra, 2013.
182. VAN DE VELDE M, DE BUCK F. Fetal and maternal analgesia/anesthesia for fetal procedures. Fetal Diagn Ther 2012;31:201-9.
183. IVANKOVICH AD, MILETICH DJ, ALBRECHT RF, HEYMAN HJ, BONNET RF. Cardiovascular effects of intraperitoneal insufflation with carbon dioxide and nitrous oxide in the dog. Anesthesiology 1975;42:281-7.
184. HO HS, GUNTHER RA, WOLFE BM. Intraperitoneal carbon dioxide insufflation and cardiopulmonary functions. Laparoscopic cholecystectomy in pigs. Arch Surg 1992;127:928-32; discussion 32-3.
185. OSOL G, MANDALA M. Maternal uterine vascular remodeling during pregnancy. Physiology (Bethesda) 2009;24:58-71.
186. MOREL O, PACHY F, CHAVATTE-PALMER P, et al. Correlation between uteroplacental three-dimensional power Doppler indices and true uterine blood flow: evaluation in a pregnant sheep model. Ultrasound Obstet Gynecol 2010;36:635-40.
187. LISTER DR, RUDSTON-BROWN B, WARRINER CB, MCEWEN J, CHAN M, WALLEY KR. Carbon dioxide absorption is not linearly related to intraperitoneal carbon dioxide insufflation pressure in pigs. Anesthesiology 1994;80:129-36.

188. KESBY GJ, LUMBERS ER. The effects of metabolic acidosis on renal function of fetal sheep. The Journal of physiology 1988;396:65-74.
189. AZHIBEKOV T, SERI I. Chapter 6 - Acid-Base Homeostasis in the Fetus and Newborn. In: Oh W, Baum M, eds. *Nephrology and Fluid/electrolyte Physiology (Third Edition)*. Philadelphia: Elsevier, 2019.
190. HOLLAND R, COMIS A. Carbonic anhydrase activity in the blood of adult sheep, fetal sheep and lambs. Comparative biochemistry and physiology A, Comparative physiology 1987;86:673.
191. FISHER DA. Carbonic anhydrase activity in fetal and young rhesus monkeys. Proc Soc Exp Biol Med 1961;107:359-63.
192. LÖNNERHOLM G, WISTRAND P. Carbonic anhydrase in the human fetal lung. Pediatric research 1982;16:407-11.
193. LÖNNERHOLM G, WISTRAND PJ. Carbonic anhydrase in the human fetal kidney. Pediatric research 1983;17:390-7.
194. RIDDERSTRÅLE Y, PERSSON E, DANTZER V, LEISER R. Carbonic anhydrase activity in different placenta types: a comparative study of pig, horse, cow, mink, rat, and human. Microsc Res Tech 1997;38:115-24.
195. SAMMIN D, MARKEY B, BASSETT H, BUXTON D. The ovine placenta and placentitis-A review. Vet Microbiol 2009;135:90-7.
196. BARRY JS, ANTHONY RV. The Pregnant Sheep as a Model for Human Pregnancy. Theriogenology 2008;69:55-67.
197. PALMER J. Acid-base homeostasis and derangements. Equine Fluid Therapy 2015:101-25.
198. BOHR C, HASSELBALCH K, KROGH A. Concerning a biologically important relationship–the influence of the carbon dioxide content of blood on its oxygen binding. Skand Arch Physiol 1904;16:401-12.
199. BURSAUX E, POYART C, GUESNON P, TEISSEIRE B. Comparative effects of CO2 on the affinity for O2 of fetal and adult erythrocytes. Pflugers Arch 1979;378:197-203.
200. BECK V, LEWI P, GUCCIARDO L, DEVLIEGER R. Preterm prelabor rupture of membranes and fetal survival after minimally invasive fetal surgery: a systematic review of the literature. Fetal Diagn Ther 2012;31:1-9.
201. KUMAR D, MOORE RM, NASH A, et al. Decidual GM-CSF is a critical common intermediate necessary for thrombin and TNF induced. Placenta 2014;35:1049-56.
202. MOLINAS CR, KONINCKX PR. Hypoxaemia induced by CO(2) or helium pneumoperitoneum is a co-factor in adhesion formation in rabbits. Hum Reprod 2000;15:1758-63.
203. HANLY EJ, AURORA AR, FUENTES JM, et al. Abdominal insufflation with CO2 causes peritoneal acidosis independent of systemic pH. J Gastrointest Surg 2005;9:1245-51; discussion 51-2.
204. PENG Y, ZHENG M, YE Q, CHEN X, YU B, LIU B. Heated and humidified CO2 prevents hypothermia, peritoneal injury, and intra-abdominal adhesions during prolonged laparoscopic insufflations. J Surg Res 2009;151:40-7.
205. ERIKOGLU M, YOL S, AVUNDUK MC, ERDEMLI E, CAN A. Electron-microscopic alterations of the peritoneum after both cold and heated carbon dioxide pneumoperitoneum. J Surg Res 2005;125:73-7.
206. LARSEN AC, HOLLÄNDER C, DUVAL L, et al. A nationwide retrospective study of perioperative chemotherapy for gastroesophageal adenocarcinoma: tolerability, outcome, and prognostic factors. Ann Surg Oncol 2015;22:1540-7.
207. DREYFUS M, BECMEUR F, SCHWAAB C, BALDAUF JJ, PHILIPPE L, RITTER J. The pregnant ewe: an animal model for fetoscopic surgery. European journal of obstetrics, gynecology, and reproductive biology 1997;71:91-4.
208. DEPREST J, JANI J, LEWI L, et al. Fetoscopic surgery: encouraged by clinical experience and boosted by instrument innovation. Semin Fetal Neonatal Med 2006;11:398-412.
209. WESTOVER A, MELVILLE JM, MCDONALD C, et al. Effect of Human Amnion Epithelial Cells on the Acute Inflammatory Response in Fetal Sheep. Front Physiol 2017;8:871.

210. SAMMOUR T, KAHOKEHR A, HILL AG. Independent testing of the Fisher & Paykel Healthcare MR860 Laparoscopic Humidification System. Minim Invasive Ther Allied Technol 2010;19:219-23.
211. BOURA AL, BOYLE L, SINNATHURAY TA, WALTERS WA. Release of prostaglandins during contraction of the human umbilical vein on reduction of temperature. British journal of pharmacology 1979;65:360-2.
212. DEMIR Y, NADAROGLU H, DEMIR N. Purification and characterization of carbonic anhydrase from bovine stomach and effects of some known inhibitors on enzyme activity. J Enzyme Inhib Med Chem 2005;20:75-80.
213. FISHER DJ, HEYMANN MA, RUDOLPH AM. Myocardial oxygen and carbohydrate consumption in fetal lambs in utero and in adult sheep. The American journal of physiology 1980;238:H399-405.
214. WERNER JC, SICARD RE. Lactate metabolism of isolated, perfused fetal, and newborn pig hearts. Pediatric research 1987;22:552-6.
215. PAPANNA R, MANN LK, MOISE KJ, JR., et al. Histologic changes of the fetal membranes after fetoscopic laser surgery for twin-twin transfusion syndrome. Pediatric research 2015;78:247-55.
216. KABAGAMBE SK, JENSEN GW, CHEN YJ, VANOVER MA, FARMER DL. Fetal Surgery for Myelomeningocele: A Systematic Review and Meta-Analysis of Outcomes in Fetoscopic versus Open Repair. Fetal Diagn Ther 2018;43:161-74.
217. CARTER AM. Placental Gas Exchange and the Oxygen Supply to the Fetus. Comprehensive Physiology 2015;5:1381-403.
218. AMBERG B, HODGES R, KASHYAP A, et al. Physiological effects of cold-dry versus heated-humidified partial amniotic carbon dioxide insufflation in sheep. Ultrasound Obstet Gynecol 2018.
219. SKINNER S, CROSSLEY K, AMBERG B, et al. The effects of partial amniotic carbon dioxide insufflation in an ovine model. Prenat Diagn 2018;38:994-1003.
220. LAPA PEDREIRA DA, ACACIO GL, GONÇALVES RT, et al. Percutaneous fetoscopic closure of large open spina bifida using a bilaminar skin substitute. Ultrasound in Obstetrics & Gynecology 2018;0.
221. GARDNER DS, GIUSSANI DA, FOWDEN AL. Hindlimb glucose and lactate metabolism during umbilical cord compression and acute hypoxemia in the late-gestation ovine fetus. American journal of physiology Regulatory, integrative and comparative physiology 2003;284:R954-64.
222. GATTINONI L, SAMAJA M. Acid-base equilibrium in the blood of sheep. Experientia 1979;35:1347-48.
223. MESCHIA G, COTTER JR, MAKOWSKI EL, BARRON DH. SIMULTANEOUS MEASUREMENT OF UTERINE AND UMBILICAL BLOOD FLOWS AND OXYGEN UPTAKES. Quarterly Journal of Experimental Physiology and Cognate Medical Sciences 1967;52:1-18.
224. KASTENDIECK E, KUNZEL W, KURZ CS. Placental clearance of lactate and bicarbonate in sheep. Gynecologic and obstetric investigation 1979;10:9-22.
225. BATTAGLIA FC. Placental clearance and fetal oxygenation. Pediatrics 1970;45:563.
226. WARD JW, WOODING FB, FOWDEN AL. Ovine feto-placental metabolism. The Journal of physiology 2004;554:529-41.
227. PICKERING BG, PALAHNIUK RJ, COTE J, WADE JG, PASH MG. Cerebral vascular responses to ketamine and thiopentone during foetal acidosis. Can Anaesth Soc J 1982;29:463-7.
228. ANTONINI E, WYMAN J, BRUNORI M, et al. The oxygen Bohr effect of human fetal hemoglobin. Archives of Biochemistry and Biophysics 1964;108:569-72.
229. ADAMSON SL. Arterial pressure, vascular input impedance, and resistance as determinants of pulsatile blood flow in the umbilical artery. European journal of obstetrics, gynecology, and reproductive biology 1999;84:119-25.
230. GARDNER DS, FOWDEN AL, GIUSSANI DA. Adverse intrauterine conditions diminish the fetal defense against acute hypoxia by increasing nitric oxide activity. Circulation 2002;106:2278-83.

231. GARDNER DS, GIUSSANI DA. Enhanced umbilical blood flow during acute hypoxemia after chronic umbilical cord compression: a role for nitric oxide. Circulation 2003;108:331-5.
232. KASSIR E, BELFORT MA, SHAMSHIRSAZ AA, SANZ CORTES M, WHITEHEAD WE, ESPINOZA J. Doppler changes in umbilical artery and ductus venosus during fetoscopic prenatal surgical repair of myelomeningocele. Ultrasound Obstet Gynecol 2018.
233. SANZ CORTES M, CASTRO E, SHARHAN D, et al. Amniotic membrane and placental histopathological findings after open and fetoscopic prenatal neural tube defect repair. Prenat Diagn 2019.
234. WEINER CP, HEILSKOV J, PELZER G, GRANT S, WENSTROM K, WILLIAMSON RA. Normal values for human umbilical venous and amniotic fluid pressures and their alteration by fetal disease. American Journal of Obstetrics & Gynecology 1989;161:714-17.
235. BOWER SJ, FLACK NJ, SEPULVEDA W, TALBERT DG, FISK NM. Uterine artery blood flow response to correction of amniotic fluid volume. Am J Obstet Gynecol 1995;173:502-7.
236. HOOPER SB, WALKER DW, HARDING R. Oxygen, glucose, and lactate uptake by fetus and placenta during prolonged hypoxemia. The American journal of physiology 1995;268:R303-9.
237. ATALIK KE, KILIC M, NURULLAHOGLU ZU, DOGAN N. Effects of cooling and warming on 5-hydroxytryptamine- and acetylcholine-induced contractions of human umbilical vessels: role of nitric oxide. Fundamental & clinical pharmacology 2008;22:37-44.
238. GINE C, AREVALO S, MAIZ N, et al. Fetoscopic two-layer closure of open neural tube defects. Ultrasound Obstet Gynecol 2018;52:452-57.
239. CORTES MS, TORRES P, YEPEZ M, et al. Comparison of brain microstructure after prenatal spina bifida repair by either laparotomy assisted fetoscopic or open approach. Ultrasound Obstet Gynecol 2019.
240. COOLEN NA, SCHOUTEN KCWM, MIDDELKOOP E, ULRICH MMW. Comparison between human fetal and adult skin. Arch Dermatol Res 2010;302:47-55.
241. LYNE AG, HOLLIS DE. The structure and development of the epidermis in sheep fetuses. Journal of Ultrastructure Research 1972;38:444-58.
242. BELFORT MA, WHITEHEAD WE, SHAMSHIRSAZ AA, et al. Comparison of two fetoscopic open neural tube defect repair techniques: single- vs three-layer closure. Ultrasound Obstet Gynecol 2020;56:532-40.
243. SANZ CORTES M, CASTRO E, SHARHAN D, et al. Amniotic membrane and placental histopathological findings after open and fetoscopic prenatal neural tube defect repair. Prenat Diagn 2019;39:269-79.
244. AMBERG BJ, HODGES RJ, KASHYAP AJ, et al. Physiological effects of partial amniotic carbon dioxide insufflation with cold, dry vs heated, humidified gas in a sheep model. Ultrasound Obstet Gynecol 2019;53:340-47.
245. NATIONAL RESEARCH COUNCIL COMMITTEE FOR THE UPDATE OF THE GUIDE FOR THE C, USE OF LABORATORY A. The National Academies Collection: Reports funded by National Institutes of Health. *Guide for the Care and Use of Laboratory Animals*. Washington (DC): National Academies Press (US)

246. AMBERG BJ, DEKONINCK PLJ, KASHYAP AJ, et al. Placental gas exchange during amniotic carbon dioxide insufflation in sheep. Ultrasound Obstet Gynecol 2019.
247. REGAN JK, KANNAN PS, KEMP MW, et al. Damage-Associated Molecular Pattern and Fetal Membrane Vascular Injury and Collagen Disorganization in Lipopolysaccharide-Induced Intra-amniotic Inflammation in Fetal Sheep. Reprod Sci 2016;23:69-80.
248. WESTOVER AJ, HOOPER SB, WALLACE MJ, MOSS TJM. Prostaglandins mediate the fetal pulmonary response to intrauterine inflammation. American Journal of Physiology-Lung Cellular and Molecular Physiology 2012;302:L664-L78.
249. WALLACE MJ, PROBYN ME, ZAHRA VA, et al. Early biomarkers and potential mediators of ventilation-induced lung injury in very preterm lambs. Respiratory Research 2009;10:19.

250. PILLOW JJ, HILLMAN NH, POLGLASE GR, et al. Oxygen, temperature and humidity of inspired gases and their influences on airway and lung tissue in near-term lambs. Intensive Care Med 2009;35:2157-63.
251. TODD DA, BOYD J, LLOYD J, JOHN E. Inspired gas humidity during mechanical ventilation: effects of humidification chamber, airway temperature probe position and environmental conditions. J Paediatr Child Health 2001;37:489-94.
252. TODD DA, JOHN E, OSBORN RA. Tracheal damage following conventional and high-frequency ventilation at low and high humidity. Critical care medicine 1991;19:1310-6.
253. BASCHAT AA, AHN ES, MURPHY J, MILLER JL. Fetal blood gas values during fetoscopic myelomeningocele repair performed under carbon dioxide insufflation. Ultrasound Obstet Gynecol 2018.
254. DEVLIEGER R, GRATACOS E, ARDON H, VANSTRAELEN S, DEPREST J. Factors influencing the flow rate through a surgical defect in human fetal membranes. Prenatal Diagnosis 2002;22:201-05.
255. FAUZA DO, FISHMAN SJ. Fetal carotid blood flow during videofetoscopy. J Pediatr Surg 1998;33:1737-40.
256. CRAWFORD JM. Vascular anatomy of the human placenta. American Journal of Obstetrics & Gynecology;84:1543-67.
257. BRACE RA, GILBERT WM, THORNBURG KL. Vascularization of the ovine amnion and chorion: a morphometric characterization of the surface area of the intramembranous pathway. Am J Obstet Gynecol 1992;167:1747-55.
258. FURUKAWA S, KURODA Y, SUGIYAMA A. A comparison of the histological structure of the placenta in experimental animals. Journal of toxicologic pathology 2014;27:11-8.
259. MANOOGIAN SJ, BISPLINGHOFF JA, KEMPER AR, DUMA SM. Dynamic material properties of the pregnant human uterus. Journal of biomechanics 2012;45:1724-7.
260. MANOOGIAN SJ, MCNALLY C, STITZEL JD, DUMA SM. Dynamic biaxial tissue properties of pregnant porcine uterine tissue. Stapp car crash journal 2008;52:167-85.
261. NYACHIEO A, CHAI DC, DEPREST J, MWENDA JM, D'HOOGHE TM. The baboon as a research model for the study of endometrial biology, uterine receptivity and embryo implantation. Gynecologic and obstetric investigation 2007;64:149-55.
262. LANGEVIN PB, KATOVICH MJ, WOOD CE, JAMES CF, LANGEVIN SO. The effect of nitroglycerin on the gravid uterus in sheep and rabbits. Anesthesia and analgesia 2000;90:337-43.
263. IZUMI H, YALLAMPALLI C, GARFIELD RE. Gestational changes in L-arginine-induced relaxation of pregnant rat and human myometrial smooth muscle. Am J Obstet Gynecol 1993;169:1327-37.
264. GINE C, AREVALO S, MAIZ N, et al. Fetoscopic Two-Layers Closure of Open Neural Tube Defects. Ultrasound Obstet Gynecol 2018.
265. TONGSONG T, WANAPIRAK C, PIYAMONGKOL W, et al. Second-trimester cordocentesis and the risk of small for gestational age and preterm birth. Obstetrics and gynecology 2014;124:919-25.
266. TONGSONG T, WANAPIRAK C, KUNAVIKATIKUL C, SIRIRCHOTIYAKUL S, PIYAMONGKOL W, CHANPRAPAPH P. Fetal loss rate associated with cordocentesis at midgestation. Am J Obstet Gynecol 2001;184:719-23.
267. GHIDINI A, SEPULVEDA W, LOCKWOOD CJ, ROMERO R. Complications of fetal blood sampling. Am J Obstet Gynecol 1993;168:1339-44.
268. SHAKHNOVICH V. It's Time to Reverse our Thinking: The Reverse Translation Research Paradigm. Clin Transl Sci 2018;11:98-99.
269. GRAVES CE, HARRISON MR, PADILLA BE. Minimally Invasive Fetal Surgery. Clin Perinatol 2017;44:729-51.
270. PETERSEN SG, GIBBONS KS, LUKS FI, et al. The Impact of Entry Technique and Access Diameter on Prelabour Rupture of Membranes Following Primary Fetoscopic Laser Treatment for Twin-Twin Transfusion Syndrome. Fetal Diagn Ther 2016;40:100-9.
271. HARRISON MR. Surgically correctable fetal disease. Am J Surg 2000;180:335-42.

272. STIRNEMANN J, DJAAFRI F, KIM A, et al. Preterm premature rupture of membranes is a collateral effect of improvement in perinatal outcomes following fetoscopic coagulation of chorionic vessels for twin–twin transfusion syndrome: a retrospective observational study of 1092 cases. BJOG: An International Journal of Obstetrics & Gynaecology 2018;125:1154-62.
273. DEPREST JA, PAPADOPULOS NA, DECALUW H, YAMAMOTO H, LERUT TE, GRATACÓS E. Closure techniques for fetoscopic access sites in the rabbit at mid-gestation. Human reproduction (Oxford, England) 1999;14:1730-34.
274. LIEKENS D, LEWI L, JANI J, et al. Enrichment of collagen plugs with platelets and amniotic fluid cells increases cell proliferation in sealed iatrogenic membrane defects in the foetal rabbit model. Prenatal Diagnosis 2008;28:503-07.
275. PAPADOPULOS NA, VAN BALLAER PP, ORDOÑEZ JL, et al. Fetal membrane closure techniques after hysteroamniotomy in the midgestational rabbit model. American Journal of Obstetrics and Gynecology 1998;178:938-42.
276. MALLIK AS, FICHTER MA, RIEDER S, et al. Fetoscopic closure of punctured fetal membranes with acellular human amnion plugs in a rabbit model. Obstetrics and gynecology 2007;110:1121-29.
277. GRATACÓS E, WU J, YESILDAGLAR N, DEVLIEGER R, PIJNENBORG R, DEPREST JA. Successful sealing of fetoscopic access sites with collagen plugs in the rabbit model. American Journal of Obstetrics & Gynecology 2000;182:142-46.
278. DEVLIEGER R, ARDON H, VERBIST L, GRATACÓS E, PIJNENBORG R, DEPREST JA. Increased polymorphonuclear infiltration and iatrogenic amniotic band after closure of fetoscopic access sites with a bioactive membrane in the rabbit at midgestation. American Journal of Obstetrics & Gynecology 2003;188:844-48.
279. DEVLIEGER R, RILEY SC, VERBIST L, LEASK R, PIJNENBORG R, DEPREST JA. Matrix Metalloproteinases-2 and -9 and Their Endogenous Tissue Inhibitors in Tissue Remodeling After Sealing of the Fetal Membranes in a Sheep Model of Fetoscopic Surgery. The Journal of the Society for Gynecologic Investigation: JSGI 2002;9:137-45.
280. LUKS FI, DEPREST JA, PEERS KHE, STEEGERS EAP, VAN DER WILDT B. Gelatin sponge plug to seal fetoscopy port sites: Technique in ovine and primate models. American Journal of Obstetrics and Gynecology 1999;181:995-96.
281. PAPANNA R, MOLINA S, MOISE KY, MOISE KJ, JR., JOHNSON A. Chorioamnion plugging and the risk of preterm premature rupture of membranes after laser surgery in twin-twin transfusion syndrome. Ultrasound Obstet Gynecol 2010;35:337-43.
282. CHANG J, TRACY TF, JR., CARR SR, SORRELLS DL, JR., LUKS FI. Port insertion and removal techniques to minimize premature rupture of the membranes in endoscopic fetal surgery. J Pediatr Surg 2006;41:905-9.
283. ENGELS AC, VAN CALSTER B, RICHTER J, et al. Collagen plug sealing of iatrogenic fetal membrane defects after fetoscopic surgery for congenital diaphragmatic hernia. Ultrasound Obstet Gynecol 2014;43:54-9.
284. MANN LK, PAPANNA R, MOISE KJ, et al. Fetal membrane patch and biomimetic adhesive coacervates as a sealant for fetoscopic defects. Acta Biomaterialia 2012;8:2160-65.
285. MICHELETTI T, EIXARCH E, BERDUN S, et al. Ex-vivo mechanical sealing properties and toxicity of a bioadhesive patch as sealing system for fetal membrane iatrogenic defects. Scientific Reports 2020;10:18608.
286. PAPANNA R, MANN LK, TSENG SCG, et al. Cryopreserved human amniotic membrane and a bioinspired underwater adhesive to seal and promote healing of iatrogenic fetal membrane defect sites. Placenta 2015;36:888-94.
287. PETERSEN S, DONE E, GARDENER G, et al. OP09.08: Rate of amniorrhexis is not affected by fetoscopic access cannula diameter. Ultrasound in Obstetrics & Gynecology 2009;34:90-91.
288. KIVELIO A, DEKONINCK P, PERRINI M, et al. Mussel mimetic tissue adhesive for fetal membrane repair: initial in vivo investigation in rabbits. European Journal of Obstetrics & Gynecology and Reproductive Biology 2013;171:240-45.

289. PAPANNA R, MANN LK, MOISE KY, JOHNSON A, MOISE KJ, JR. Absorbable gelatin plug does not prevent iatrogenic preterm premature rupture of membranes after fetoscopic laser surgery for twin-twin transfusion syndrome. Ultrasound Obstet Gynecol 2013;42:456-60.
290. LEI H, KALLURI R, FURTH EE, BAKER AH, STRAUSS JF, III. Rat Amnion Type IV Collagen Composition and Metabolism: Implications for Membrane Breakdown1. Biology of Reproduction 1999;60:176-82.
291. LEI H, VADILLO-ORTEGA F, PAAVOLA LG, STRAUSS JF, 3RD. 92-kDa gelatinase (matrix metalloproteinase-9) is induced in rat amnion immediately prior to parturition. Biol Reprod 1995;53:339-44.
292. ORTIZ JU, EIXARCH E, PEGUERO A, et al. Chorioamniotic membrane separation after fetoscopy in monochorionic twin pregnancy: incidence and impact on perinatal outcome. Ultrasound Obstet Gynecol 2016;47:345-9.
293. PAPANNA R, MANN LK, JOHNSON A, SANGI-HAGHPEYKAR H, MOISE KJ. Chorioamnion separation as a risk for preterm premature rupture of membranes after laser therapy for twin-twin transfusion syndrome. Obstetrics and gynecology 2010;115:771-76.
294. EGAWA M, HAYASHI S, YANG L, SAKAMOTO N, SAGO H. Chorioamniotic membrane separation after fetoscopic laser surgery for twin-twin transfusion syndrome. Prenatal diagnosis 2013;33:89-94.
295. PAPANNA R, MANN LK, MOISE KJ, et al. Histologic changes of the fetal membranes after fetoscopic laser surgery for twin-twin transfusion syndrome. Pediatric Research 2015;78:247-55.
296. BIRCHER K, MERLUZZI R, WAHLSTEN A, et al. Influence of osmolarity and hydration on the tear resistance of the human amniotic membrane. Journal of biomechanics 2020;98:109419.
297. LOCATELLI A, ANDREANI M, GHIDINI A, et al. Amnioinfusion in preterm PROM: effects on amnion and cord histology. J Perinatol 2008;28:97-101.
298. ROBERTSON P, FABER JJ, BRACE RA, et al. Responses of amniotic fluid volume and its four major flows to lung liquid diversion and amniotic infusion in the ovine fetus. Reprod Sci 2009;16:88-93.
299. MERIKI N, SMOLENIEC J, CHALLIS D, WELSH AW. Immediate outcome of twin–twin transfusion syndrome following selective laser photocoagulation of communicating vessels at the NSW Fetal Therapy Centre. Australian and New Zealand Journal of Obstetrics and Gynaecology 2010;50:112-19.
300. DEPREST JA, EVRARD VA, VAN SCHOUBROECK D, VANDENBERGHE K. Endoscopic cord ligation in selective feticide. Lancet (London, England) 1996;348:890-91.
301. GRATACOS E, DEVLIEGER R, DECALUWE H, WU J, NICOLINI U, DEPREST JA. Is the angle of needle insertion influencing the created defect in human fetal membranes? Evaluation of the agreement between specialists' opinions and ex vivo observations. Am J Obstet Gynecol 2000;182:646-9.
302. HARMANLI OH, WAPNER RJ, LONTZ JF. Efficacy of fibrin glue for in vitro sealing of human chorioamniotic membranes. The Journal of reproductive medicine 1998;43:986-90.
303. LOUIS-SYLVESTRE C, RAND JH, GORDON RE, SALAFIA CM, BERKOWITZ RL. In vitro studies of the interactions between platelets and amniotic membranes: a potential treatment for preterm premature rupture of the membranes. American journal of obstetrics and gynecology 1998;178:287-93.
304. REDDY UM, SHAH SS, NEMIROFF RL, et al. In vitro sealing of punctured fetal membranes: potential treatment for midtrimester premature rupture of membranes. Am J Obstet Gynecol 2001;185:1090-3.
305. HALLER CM, BUERZLE W, BRUBAKER CE, et al. Mussel-mimetic tissue adhesive for fetal membrane repair: a standardized ex vivo evaluation using elastomeric membranes. Prenat Diagn 2011;31:654-60.
306. BILIC G, BRUBAKER C, MESSERSMITH PB, et al. Injectable candidate sealants for fetal membrane repair: bonding and toxicity in vitro. American journal of obstetrics and gynecology 2010;202:85.e1-85.e859.

307. OCHSENBEIN-KÖLBLE N, JANI J, LEWI L, et al. Enhancing sealing of fetal membrane defects using tissue engineered native amniotic scaffolds in the rabbit model. American journal of obstetrics and gynecology 2007;196:263.e1-7.
308. MOGAMI H, KISHORE AH, WORD RA. Collagen Type 1 Accelerates Healing of Ruptured Fetal Membranes. Scientific Reports 2018;8:696.
309. MENDOZA GA, ACUÑA E, ALLEN M, ARROYO J, QUINTERO RA. In vitro laser welding of amniotic membranes. Lasers in Surgery and Medicine 1999;24:315-18.
310. DEVAUD YR, ZÜGER S, ZIMMERMANN R, EHRBAR M, OCHSENBEIN-KÖLBLE N. Minimally Invasive Surgical Device for Precise Application of Bioadhesives to Prevent iPPROM. Fetal Diagnosis and Therapy 2019;45:102-10.
311. GRIGSBY PL. Animal Models to Study Placental Development and Function throughout Normal and Dysfunctional Human Pregnancy. Semin Reprod Med 2016;34:11-16.
312. STEMPFLE G, MCGOWEN MR, CARAVAS JA, WILDMAN DE. From PPROM to caul: The evolution of membrane rupture in mammals. Appl Transl Genom 2013;2:70-77.
313. BRACE RA, GILBERT WM, THORNBURG KL. Vascularization of the ovine amnion and chorion: A morphometric characterization of the surface area of the intramembranous pathway. Am J Obstet Gynecol 1992;167:1747-55.
314. HAFEZ S. Chapter One - Comparative Placental Anatomy: Divergent Structures Serving a Common Purpose. In: Huckle WR, ed. *Progress in Molecular Biology and Translational Science*: Academic Press, 2017 (vol 145).
315. SOPHER D. The response of rat fetal membranes to injury. Ann R Coll Surg Engl 1972;51:240-49.
316. PEDREIRA DA, ACACIO GREGÓRIO L, GONÇALVES RODRIGO T, et al. Percutaneous fetoscopic closure of large open spina bifida using a bilaminar skin substitute. Ultrasound in Obstetrics & Gynecology 2018;0.
317. AMBERG BJ, DEKONINCK PLJ, KASHYAP AJ, et al. Placental gas exchange during amniotic carbon dioxide insufflation in sheep. Ultrasound in obstetrics & gynecology : the official journal of the International Society of Ultrasound in Obstetrics and Gynecology 2019:10.1002/uog.21933.
318. AMBERG BJ, HODGES RJ, KASHYAP AJ, et al. Physiological effects of partial amniotic carbon dioxide insufflation with cold, dry vs heated, humidified gas in a sheep model. Ultrasound in obstetrics & gynecology : the official journal of the International Society of Ultrasound in Obstetrics and Gynecology 2019;53:340-47.
319. TOLCHER MC, SHAZLY SA, SHAMSHIRSAZ AA, et al. Neurological outcomes by mode of delivery for fetuses with open neural tube defects: A systematic review and meta-analysis. BJOG : an international journal of obstetrics and gynaecology 2018.
320. ALREFAI A, DRAKE J, KULKARNI AV, et al. Fetal myelomeningocele surgery: Only treating the tip of the iceberg. Prenat Diagn 2019;39:10-15.
321. TULIPAN N, WELLONS JC, 3RD, THOM EA, et al. Prenatal surgery for myelomeningocele and the need for cerebrospinal fluid shunt placement. Journal of neurosurgery Pediatrics 2015;16:613-20.