Cryopreservation of Interferon Tau-Primed Peripheral Blood Mononuclear Bovine Cells

Oklahoma State University Honors Thesis Presentation

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May 5, 2016

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- Biography
  - Eufaula, OK
  - Graduating with a Bachelor or Science in Animal Science
  - Center for Veterinary Health Sciences Class of 2020

- Motivation for completing Thesis
  - Interest in Reproduction
  - Research Experience
  - Culmination of honors and undergraduate studies

Thesis Overview

- Studies have shown significant increases in conception rates by "priming" the female uterus with peripheral blood mononuclear cells or PBMC (lymphocytes and monocytes).
- With the results that have been found, priming the female tract through addition of PBMCs could become a useful reproductive technology
- All of these studies, however, used fresh samples of PBMC. This is not practical for use as a viable reproductive technology considering one is working with live animals. If this were to become a practical reproductive technology, PBMCs would have to be more readily available than fresh PBMCs are.
- This thesis project therefore reviewed the following hypothesis:

Peripheral blood mononuclear cells can be frozen and thawed without loss of cell viability, thus allowing for this procedure to become a practical reproductive technology for increasing pregnancy rates.

Presentation Overview

- Review Female Bovine Reproductive Anatomy and Physiology
- Early Embryo Loss
- Cryopreservation of Immune Cells

Reproductive Anatomy and Physiology of the Female Bovine

With Special Focus on Hormone Signaling for Establishment of Pregnancy

Reproductive Anatomy and Physiology of the Female Bovine

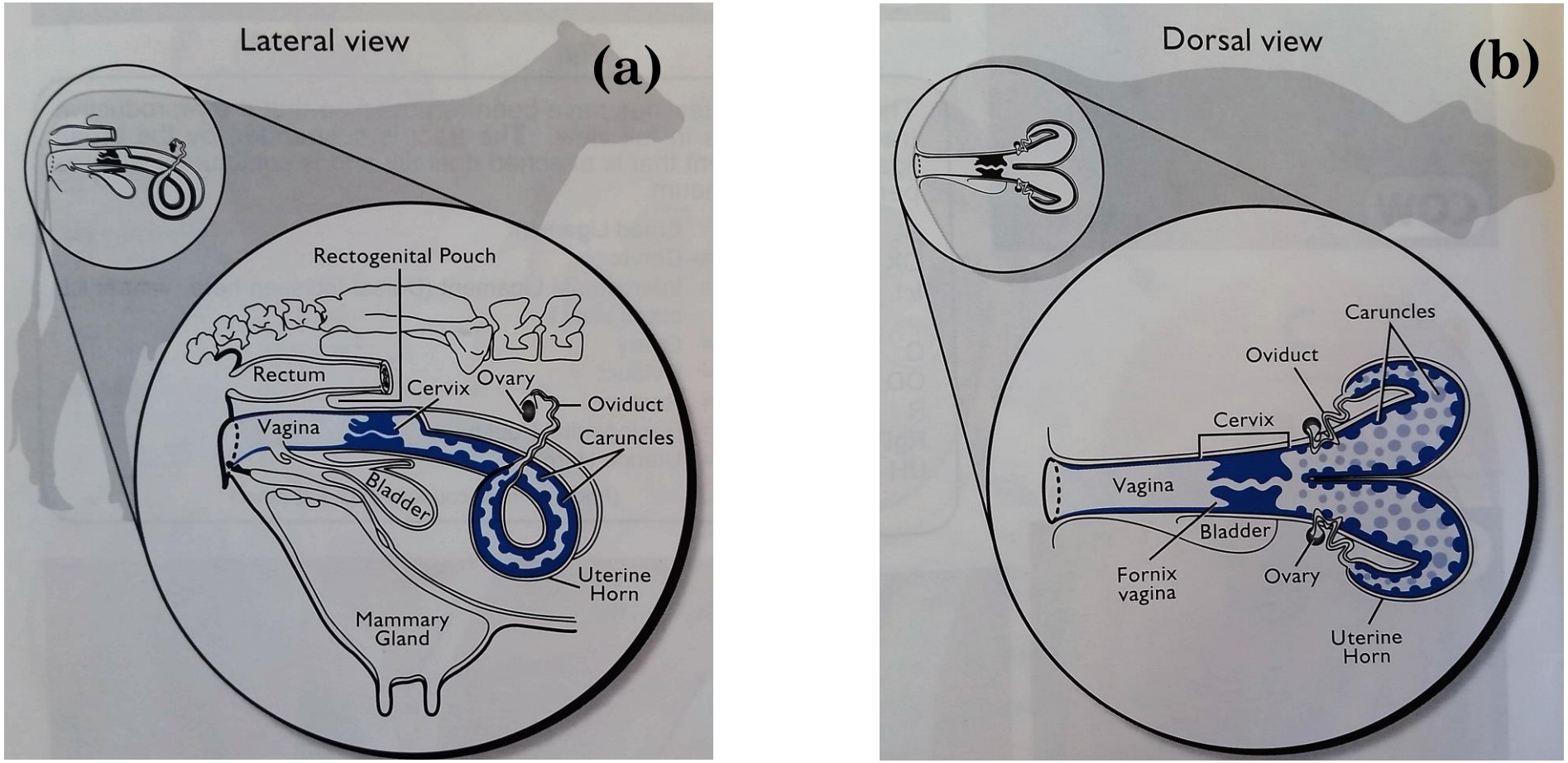


Figure 1: Illustration of the female bovine reproductive tract from the lateral (a) and dorsal (b) views (1).

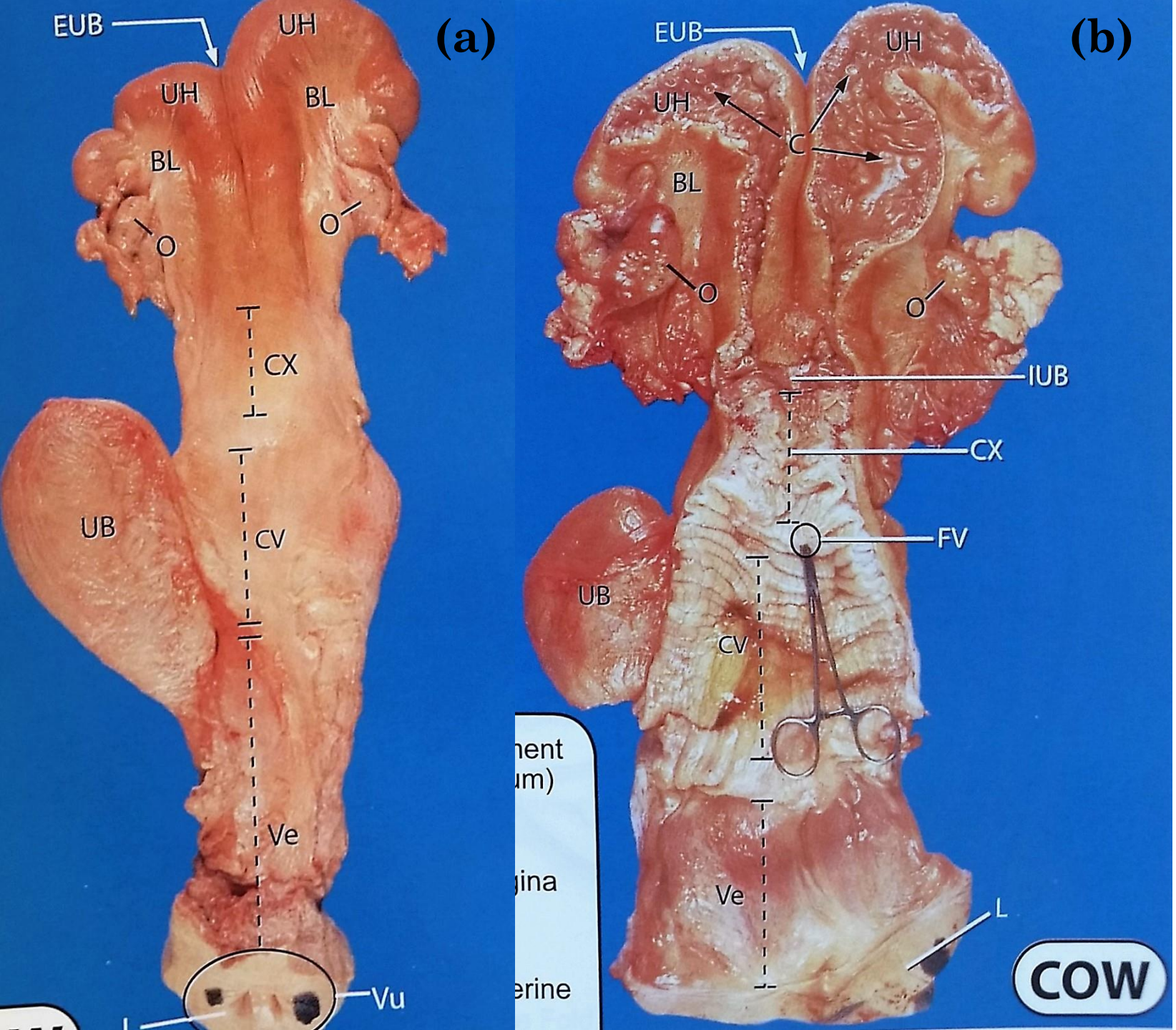


Figure 2: (a) and (b) are dorsal views of an excised female bovine reproductive tract. (b) shows the internal anatomy of the tract while (a) shows the external. (c) is the key for (a) and (b) (1).

BL = Broad Ligament (mesometrium)

C = Caruncle

CV = Cranial Vagina

CX = Cervix

EUB = External Uterine Bifurcation

FV = Fornix Vagina

IUB = Internal Uterine Bifurcation

L = Labia

O = Ovary

OD = Oviduct

UB = Urinary Bladder

UH = Uterine Horn

Ve = Vestibule

Vu = Vulva

(c)

Reproductive Anatomy and Physiology of the Female Bovine

Important Physiological Time-Frame for this Thesis: Days 2-22 after ovulation

- Embryo enters the uterus (approximately day 7)
- At the same time, Corpus luteum is producing the hormone oxytocin
- Maternal recognition of pregnancy must occur or else:
  - Oxytocin will bind in endometrial cells of the uterus
  - Endometrial cells will then be stimulated to produce Prostaglandin $F_{2\alpha}$
  - Prostaglandin $F_{2\alpha}$ will causes luteolysis (corpus luteum death)
  - Luteolysis will cause drop of progesterone, the hormone responsible for maintenance of pregnancy, and embryo will be aborted
- Interferon tau is the signal released by the embryo (day13-21) which blocks the oxytocin receptors and prevents luteolysis (day 15-16)
- Attachment of embryo occurs if signal is received (day 18-22) (1)

Interferon Tau

- Originally known as bovine trophoblast protein 1
- Belongs to the interferon class
- Interferons are cytokines, or immune cells hormones
- Produced by the trophoblastic cells of the blastocyst
- IS NOT luteotrophic: does not enhance progesterone production by the corpus luteum
- Blocks oxytocin receptor synthesis in endometrial cells
- Also binds to cause release of proteins from the uterine glands. These proteins are believed to be critical to preimplantation embryonic survival (1)
- **New research is now suggesting that interferon tau also affects the maternal peripheral blood mononuclear cells which in turn are believed to play a role in establishment and maintenance of pregnancy (2)**

Early Embryo Loss

Early Embryo Loss

- For cattle, 70-80% of early embryo loss tends to occur 8 to 16 days after insemination, with insemination usually occurring within 24 hours of ovulation (3)
- These times correspond with the time frame in which the embryo has entered the uterus and must signal its presence or else it will be aborted

Numerous reasons for why an embryo might be lost. Below are just a few:

- Level of production (low, moderate or high)
  - Evidence of different patterns of embryo loss between the high-producing cow, a heifer and a low producing dairy cow (3)
    - Example: British Friesian versus the Holstein Friesian. These breeds had similar losses for insemination failure and late embryo loss but Holstein had 1.5 times greater early embryo death
- Genetics
  - Chromosomal defects from either diploid formation within the embryo or defects passed down from the parents (3)
    - Example: The 1/29 Robertsonian chromosomal translocation causes reduced fertility individuals that are heterozygous for the mutation

Embryo loss cont.

- Nutrition
  - Imbalances in the nutrient and energy intake can lead to embryo loss
    - Example: Dairy cattle are particularly susceptible to early embryo loss due to negative energy balance as well as of consumption energy and protein rich foods.
      - High levels of protein increase blood urea and ammonia concentrations disturb the microenvironment and could lead to maturation, fertilization and cleavage disruptions.
      - High levels of starch, which can increase ovarian activity, can also affect oocyte and embryo quality after a positive energy balance has been reached (4)
- Environment
  - Outside stressors and toxin sources

Embryo loss cont.

- Progesterone levels prior to and after insemination
  - Lows levels of progesterone prior to insemination may be associated with pre-mature oocyte development
  - Low levels of progesterone 5 to 7 days after insemination is also related to decreased embryo survival (3)
- Signaling failure for implantation
  - If interferon tau is not received by the endometrial cells of the uterus , then the embryo will be aborted

Cryopreservation of Immune Cells

Basis

- *Seminal Fluid and Immune Adaptation for Pregnancy – Comparative Biology in Mammalian Species* by J.E. Schjenken and S.A. Robertson
  - Seminal fluid affects immune status in the female by encouraging a state of immune tolerance that facilitates successful pregnancy. This paper describes recruitment of T lymphocytes which is a result of stimulation by seminal fluid factors. This paper is pertinent to this study because this lead to the idea that immune cells could be deposited directly to facilitate a proper immune status without use of seminal fluid.
- *Intrauterine administration of autologous peripheral blood mononuclear cells increases clinical pregnancy rates in frozen/thawed embryo transfer cycles of patients with repeated implantation failure* by Okitsu, O., M. Kiyokawa, T. Oda, K. Miyake, Y. Sato, and H. Fujiwara.
  - Increased conception rates in individuals who had experienced three or more IVF failures by priming with fresh PBMC

Basis cont.

- *Intrauterine transfer of autologous interferon tau-primed peripheral blood mononuclear cells increases pregnancy rates after embryo transfer in cattle* by J.L. Chase, J.A. Hernandez Gifford, T.L. Ott, D.M. Hallford, and C.A. Gifford
  - Pregnancy rates for PBMC interferon tau-primed cattle were 76% versus 54% for non-primed cattle
  - Study by Ideta et al. had similar results without priming (6)
- *Regulation of interferon-stimulated genes in peripheral blood leukocytes in pregnant and bred, nonpregnant dairy cows* by Gifford, C.A., K. Ralcot, D.S. Clark, K.J. Austin, T.R. Hansen, M.C. Lucy, C.J. Dayles, and T.L. Ott.
  - Interferon tau causes increased expression of interferon regulated genes. These genes, specifically Mx1 gene, were found to be upregulated in peripheral blood leukocytes.

Basis Summary

- T cells (which are mononuclear cells) play a key role in establishing the immune status for establishment of pregnancy. This leads to the idea that the female uterus can be “primed” by depositing mononuclear cells into the uterus and encouraging the proper immune status. This has been seen in humans as well as of species such as bovine.
- Additionally, interferon stimulated phenotype is expressed in natural circulating leukocytes (including mononuclear cells) during pregnancy which suggests that priming the PBMCs with interferon tau before depositing into uterus may be necessary.

These reasons are the basis for conducting this study as well as why the cells were cultured with interferon tau.

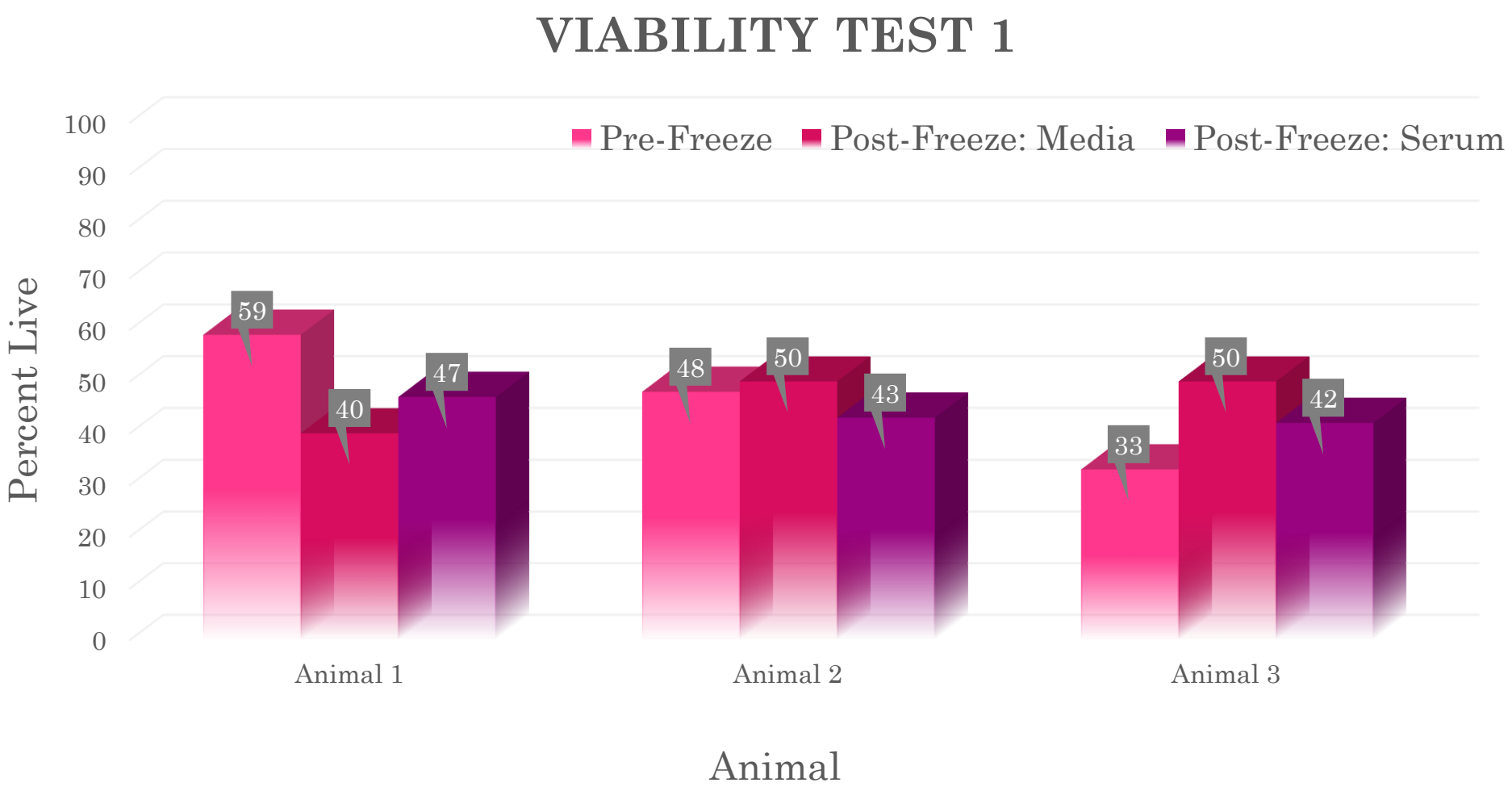
Cryopreservation

- *Development of optimal biopreservation methods and technology for cellular therapy and clinical diagnosis* by Zhiquan Shu.
  - 1.5M Propylene glycol as the preferred freezing agent
    - DMSO, ethylene glycol and glycerol were considered. However, ethylene glycol did not show the proper diffusion rates for successful freezing, glycerol had a higher level of cytotoxicity than desired and DMSO has associated concerns due to its toxicity to the recipient
  - Cooling rate of 1°C per minute
    - Study specifically found that 0.9°C was optimal. However freezing rate of 1°C per minute was more achievable for this study

Cryopreservation of Immune Cells: Procedure

1. Collected whole blood
2. Isolated PBMC
3. Cultured overnight
   - Tests 1 and 2 were primed with interferon tau whereas some samples of Test 3 were not
4. Collected pre-freeze cell viability
5. Added one of two freezing agent mixtures and froze at a rate of 1°C per minute to -80°C
   - 1.5M propylene glycol + Media
   - 1.5M propylene glycol + 90% fetal bovine serum
6. Transferred vials from freezing chamber to liquid nitrogen tank
7. Thawed
8. Collected post-freeze cell viability
9. Ran PCR to determine interferon stimulated gene phonotype between pre- and post-freeze

Test 1 Viability Results



Test 1 Viability Results

Animal 1

- Pre-freeze percent live cells of 59%
- Post-freeze percent live cells with 1.5M propylene glycol + Media of 40%
- Post-freeze percent live cells with 1.5M propylene glycol + 90% fetal bovine serum of 47%

Animal 2

- Pre-freeze percent live cells of 48%
- Post-freeze percent live cells with 1.5M propylene glycol + Media of 50%
- Post-freeze percent live cells with 1.5M propylene glycol + 90% fetal bovine serum of 43%

Test 1 Viability Results

Animal 3

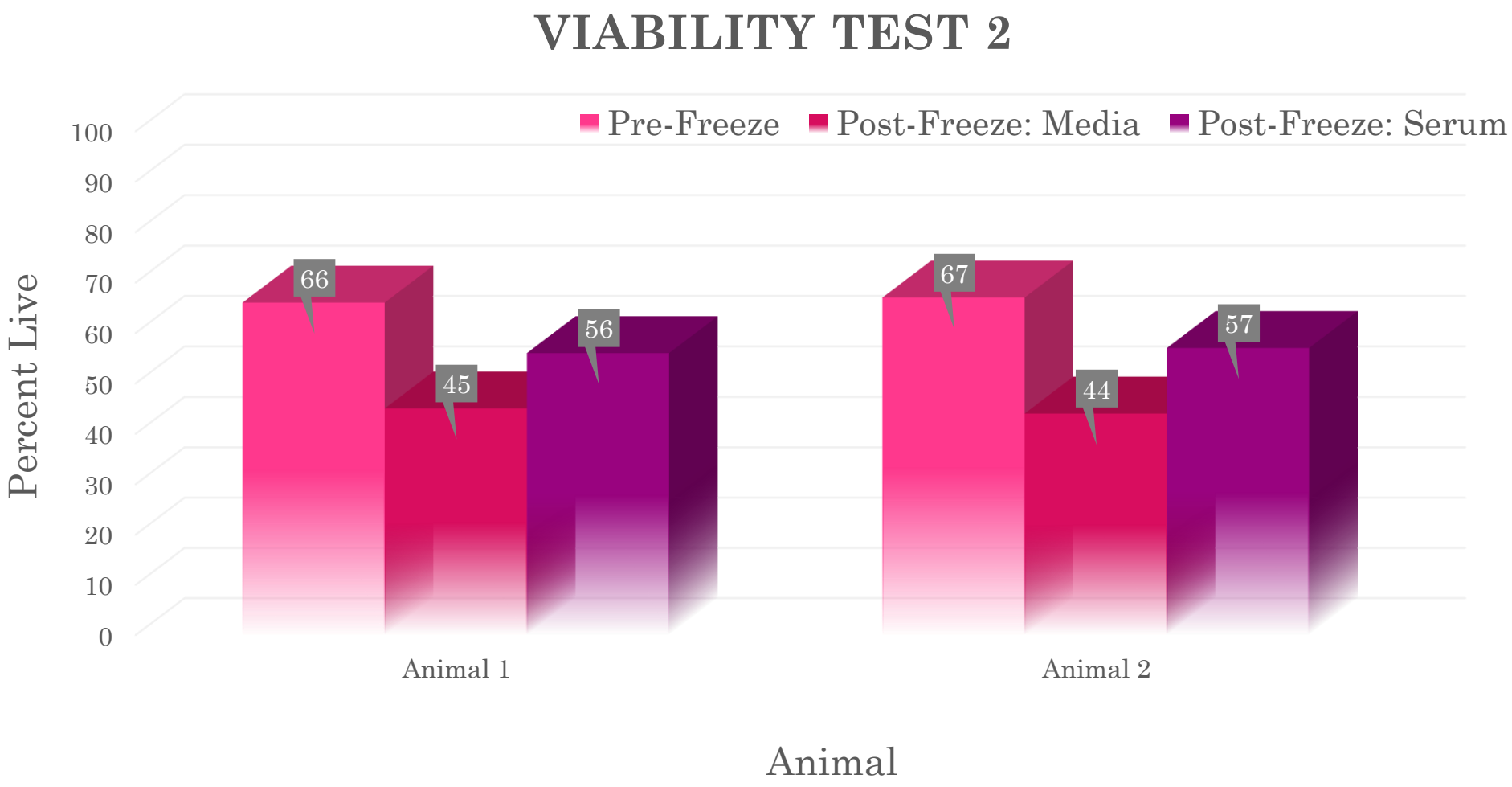
- Pre-freeze percent live cells of 33%
- Post-freeze percent live cells with 1.5M propylene glycol + Media of 50%
- Post-freeze percent live cells with 1.5M propylene glycol + 90% fetal bovine serum of 42%

Test 1 Thoughts

- Overall low percent live in both pre- and post freeze, however difference between pre- and post-freeze in small, suggesting that propylene glycol does seem to work as a freezing agent
- Continue to test whether serum or media is preferred
- Change for Test 2

1. Use a red blood cell lysis buffer to help increase viability
2. Be aware of monocyte “clumping” in preparing samples as this may have also contributed to overall low viability numbers

Test 2 Viability Results



Test 2 Viability Results

Animal 1

- Pre-freeze percent live cells of 66%
- Post-freeze percent live cells with 1.5M propylene glycol + Media of 45%
- Post-freeze percent live cells with 1.5M propylene glycol + 90% fetal bovine serum of 56%

Animal 2

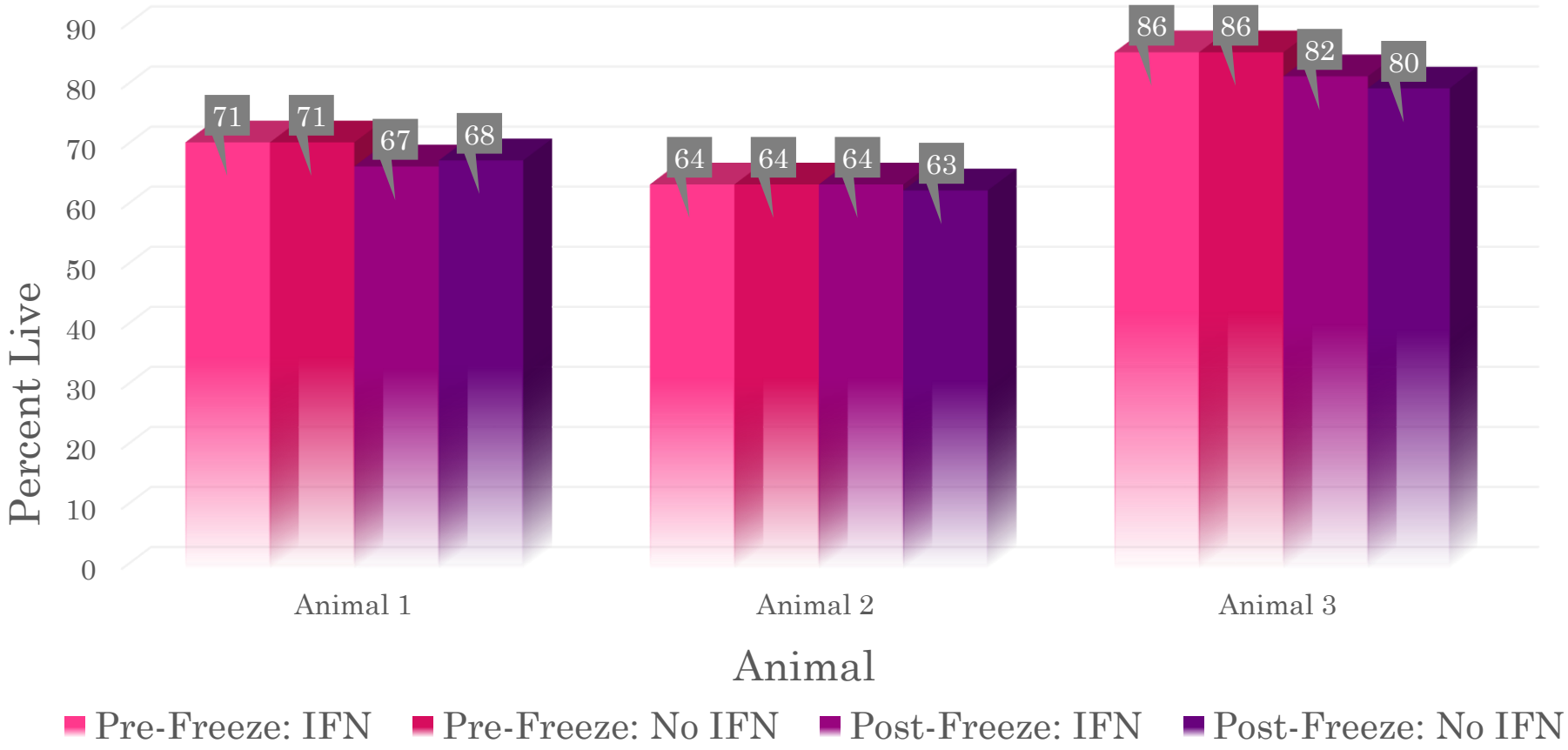
- Pre-freeze percent live cells of 67%
- Post-freeze percent live cells with 1.5M propylene glycol + Media of 44%
- Post-freeze percent live cells with 1.5M propylene glycol + 90% fetal bovine serum of 57%

Test 2 Thoughts

- Use of red blood cell lysis buffer as well as better monocyte clumping management increased percent live but would have preferred an even higher percent live
- This test suggests that 1.5M propylene glycol + 90% fetal bovine serum will allow for higher percent live. This is encouraging because using fetal bovine serum is make this freezing method more practical as it is a more readily available freezing additive
- Change for Test 3:

1. Use different blood collection equipment. Poor needles prevented us from collected more blood samples
2. Use only propylene glycol + 90% fetal bovine serum
3. Freeze non interferon-primed samples
4. Run PCR to determine the effect of freezing on the interferon stimulated phenotype of samples

Test 3 Viability Results



Test 2 Viability Results

Animal 1

- Pre-freeze with Interferon priming percent live cells of 71%
- Pre-freeze without interferon priming percent live cells of 71%
- Post-freeze percent live cells with interferon priming of 67%
- Post-freeze percent live cells without interferon priming of 68%

Animal 2

- Pre-freeze with Interferon priming percent live cells of 64%
- Pre-freeze without interferon priming percent live cells of 64%
- Post-freeze percent live cells with interferon priming of 64%
- Post-freeze percent live cells without interferon priming of 63%

Test 1 Viability Results

Animal 3

- Pre-freeze with Interferon priming percent live cells of 86%
- Pre-freeze without interferon priming percent live cells of 86%
- Post-freeze percent live cells with interferon priming of 82%
- Post-freeze percent live cells without interferon priming of 80%

Thoughts on Test 3 Viability

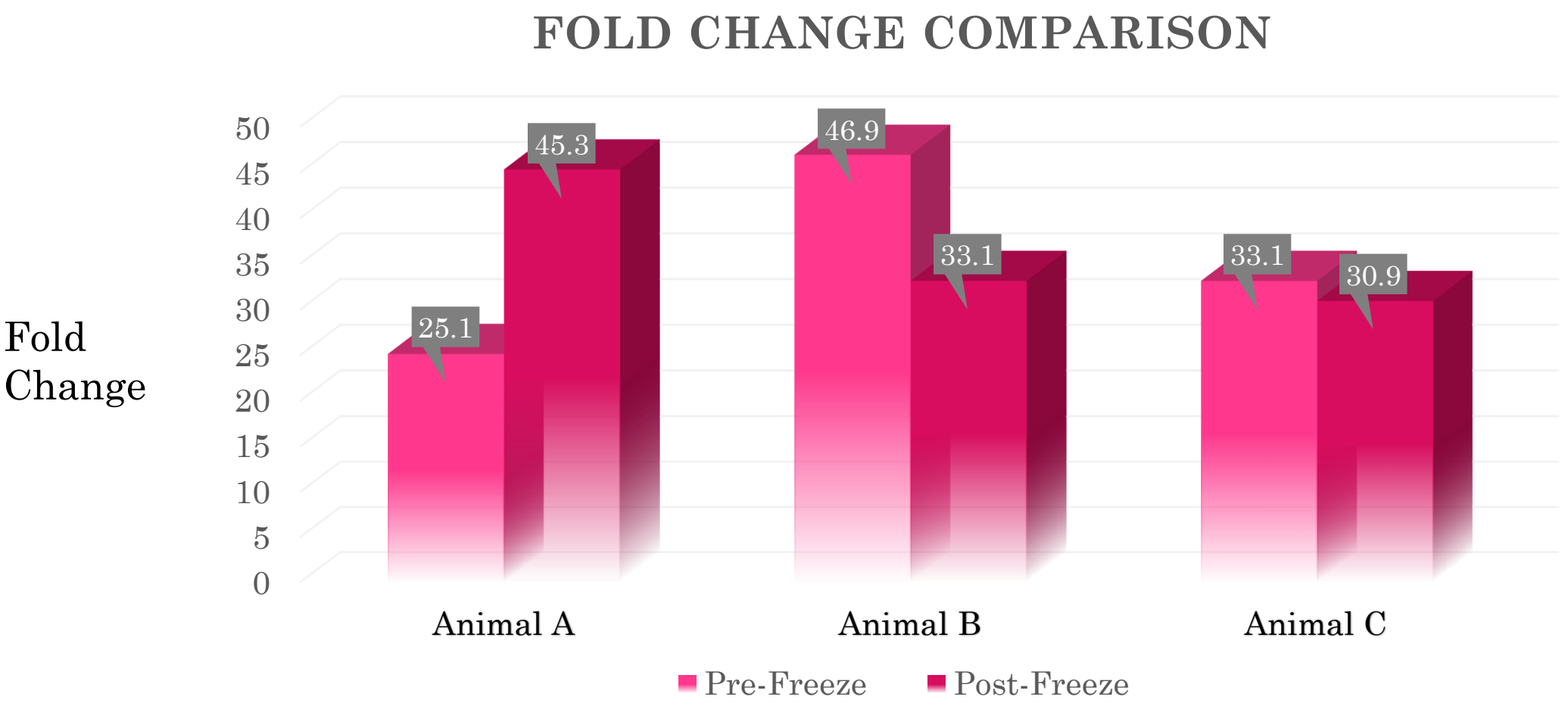
- Finally, percent live was appropriate, with hardly any loses whatsoever between the pre- and post-freeze samples
- Percent live does not seem to be affected by interferon tau priming
- Worth noting that these samples were frozen for the longest duration (about a week) yet experienced the best viability. This is very encouraging because it suggests that these cells can be frozen for tie frames that would make for a more accessible reproductive technology.

PCR results

Fold change is compared for interpretation of this data on the next slide

		B-Actin	B-Actin	AVG	MX1	MX1	AVG	Delta	Delta Delta	Fold Change
Pre-Freeze	A No IFN	18.5	18.4	18.45	29.1	29.3	29.2	10.75	-4.65	25.1
	A IFN	18.2	18.3	18.25	24.1	24.6	24.35	6.1		
	B No IFN	17.9	17.8	17.85	28.3	28.4	28.35	10.5	-5.55	46.9
	B IFN	18	18	18	23	22.9	22.95	4.95		
	C No IFN	18.6	18.7	18.65	30.1	30.3	30.2	11.55	-5.05	33.1
	C IFN	18.4	18.3	18.35	24.8	24.9	24.85	6.5		
Post-Freeze	A No IFN	18.4	18.3	18.35	31	32.9	31.95	13.6	-5.5	45.3
	A IFN	18.2	18	18.1	26	26.4	26.2	8.1		
	B No IFN	18.9	18.8	18.85	29.6	29.1	29.35	10.5	-5.05	33.1
	B IFN	18.3	18.7	18.5	24.1	23.8	23.95	5.45		
	C No IFN	18.6	18.6	18.6	28.9	28.7	28.8	10.2	-4.95	30.9
	C IFN	18.3	18.4	18.35	23.5	23.7	23.6	5.25		
							A	-0.85	-0.85	1.802500925
							B	0.5	-0.5	1.414213562
							C	0.1	-0.1	1.071773463

Comparison of Fold Change for Pre- and Post-Freeze



PCR Thoughts

- PCR shows that interferon stimulated gene phenotype is increased by priming, as indicated by fewer number of cycles to detect Mx1
- PCR and comparison of the fold change value for pre- and post freezing show a minimal difference in interferon phenotype expression, with expression of the interferon stimulated phenotype in sample C being even greater post-freeze versus pre-freeze
- These results therefore suggest that interferon tau priming is causing an interferon phenotype expression and that this expression is not being significantly compromised by the freezing process

Final Thoughts

- Propylene glycol is an appropriate freezing agent, with 90% fetal bovine serum giving higher percent live than simple media
- Cooling rate of 1°C per minute was appropriate
- Decreased percent live in first two tests may have been a result of inexperience on my part
- Duration of freezing (from 24 hours to a week) did not affect percent live cell
- Interferon stimulated phenotype is maintained despite freezing
- Percent live can be maintained without interferon priming

Therefore, these preliminary results show that peripheral blood mononuclear cells, primed with interferon tau or not, can be frozen and thawed without significant loss of cell viability, thus allowing for this procedure to become a practical reproductive technology for increasing pregnancy rates

Looking to the Future

- Repeat this experiment with the following changes:
  - Larger sample set for more accurate results
  - Use a different species such as sheep or goat to determine if freezing affects various ruminant species differently

- Use freeze/thaw PBMC in live cattle to test if conception rates are similar to when fresh PBMC are used

Special Thanks

- Dr. Craig Gifford – Primary Reader
- Dr. David Lalman – Secondary Reader
- Dr. Jennifer Hernandez Gifford
- Ebonie Hill-Williamson

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