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thesis entitled

Decapacitation of Boar Spermatozoa

presented by

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has been accepted towards fulfillment
of the requirements for the

M.S. degree in Large Animal Clinical Sciences

Major Professor's Signature

August 5, 2004

Date

DATE DUE	DATE DUE	DATE DUE

DECAPACITATION OF BOAR SPERMATOZOA

By

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Large Animal Clinical Sciences

2004

ABSTRACT

DECAPACITATION OF BOAR SPERMATOZOA

By

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This thesis describes three studies that were completed to demonstrate the decapacitation ability of boar seminal plasma (SP) when added to incubated, cooled, and frozen-thawed (FT) boar spermatozoa. In the first study, chlortetracycline (CTC) staining analysis was used to investigate the effects of 20% (v/v) SP addition at different time points on the temporal pattern of capacitation in spermatozoa incubated at 39 C or cooled to 5 C and then rewarmed to 39 C. Seminal plasma was able to prevent and reverse capacitation in spermatozoa incubated in a capacitation-supporting environment as well as capacitation induced by cooling the spermatozoa to 5 C. In the second study, the addition of either 10 or 20% (v/v) SP to frozen boar spermatozoa at thawing were investigated using CTC analysis. The addition of either 10 or 20% SP to FT spermatozoa resulted in the reversal of capacitation within 30 min of its addition. In the third study, the decapacitating effects of 10% (v/v) SP addition to FT spermatozoa were examined in different media and at different incubation temperatures. It was demonstrated that the effects of SP were dependent on the temperature of the spermatozoa suspension as well as the media the spermatozoa were suspended in. The results from these three studies demonstrated that capacitation in boar spermatozoa is regulated by some constituent(s) of boar SP.

ACKNOWLEDGMENTS

This project was made possible by the help, support, and encouragement of many people. I am especially grateful to all of those who have provided me with education, training, and guidance. In particular, I thank my advisor Dr. Roy Kirkwood and committee members Dr. David J. Sprecher and Dr. Karen Chou.

I would also like to thank Dr. Gary Althouse for his continued guidance and mentoring through both my undergraduate and graduate education. His influence, support, and encouragement have been extremely influential in where I am and where I am going.

I also acknowledge Dr. John B. Kaneene and his team of graduate students and staff. Their support, wisdom, and optimism have been great strengths to me during my time here at the Michigan State College of Veterinary Medicine.

Finally, I would like to thank both Julie Lanivich and Faith Peterson for their patience and kindness. Without their help I would have been lost! I also thank the staff at the University's Swine Farm.

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LIST OF ABBREVIATIONS

AR ..... Acrosome Reacted

B ..... Capacitated

CTC ..... Chlortetracycline

F ..... Fresh (uncapacitated)

FT ..... Frozen-thawed

GAG ..... Glycosaminoglycan

HA ..... Hyaluronan

HDL ..... High-density Lipoprotein

mM199 ..... Modified Medium 199

MSU ..... Michigan State University

PBS ..... Phosphate Buffered Saline

PS ..... Phosphatidylserine

SP ..... Seminal Plasma

ZP ..... Zona Pellucida

OVERVIEW

Chapters 1, 2, 3, and 4 in this thesis are written in a format suitable for publication independently. Thus, those chapters each have an abstract, introduction, methods, and discussion. A list of literature cited concludes each chapter.

Chapter 1 is a literature review of capacitation across species. It includes a discussion on spermatozoa maturation, fertilizing ability, and decapacitation. Chapter 2 describes our initial study investigating the effects of SP addition on capacitation in vitro. Chapter 3 is a study examining the effects of SP on cryocapacitation caused by the freezing and subsequent thawing of spermatozoa. Chapter 4 describes a study to determine if the effects of SP are dependent on the incubation temperature and/or presence of egg yolk in the spermatozoa suspension. The thesis concludes with Chapter 5, which is an overall summary and synthesis of the findings of this research.

CHAPTER 1

Review: Capacitation of spermatozoa

Abstract

Spermatozoa are required to undergo the processes of capacitation as well as acrosome reaction before they obtain fertilizing ability. The molecular changes of capacitation are still not fully understood. However, it is accepted that capacitation is a sequential process involving numerous physiological changes including membrane destabilization, alteration of membrane potential, and protein phosphorylation. There are no known morphological changes to the spermatozoa during capacitation. The purpose of this review is to summarize the data available on the biochemical aspects of capacitation both in vivo and in vitro.

Introduction

Early work with in vitro fertilization led to the postulate that spermatozoa must reside within the female reproductive tract for a period of time before they acquire the ability to fertilize an oocyte. It was later demonstrated that spermatozoa introduced into rabbit oviducts shortly after ovulation were unable to fertilize the oocytes, despite rabbit ova remaining fertilizable for up to 8 h after ovulation; however, when spermatozoa were inseminated into the oviduct several hours prior to ovulation a high percentage of fertilization occurred [1, 2]. Similarly, fertilization occurred 4 h after rat spermatozoa were deposited into the postovulatory ovarian capsule [3]. This amount of time required before the spermatozoa were capable of fertilizing an oocyte was termed capacitation and was discovered independently by Austin [3] and Chang [4] over fifty years ago.

The evidence for the need for capacitation in various species is based on this temporal pattern between mating or insemination and penetration of the zona pellucida (ZP). Copulation after the time of ovulation in rats demonstrated that spermatozoa were present in the oviduct after 1 hour, but it wasn't until 2-3 hours after copulation that spermatozoa penetrated the oocyte [5]. Also, ejaculated spermatozoa incubated in a golden hamster uterus for several hours fertilized a higher proportion of oocytes 4-5 hours after surgical insemination then did spermatozoa from the epididymis [6].

Capacitation of a freshly ejaculated spermatozoa population appears to take several hours in most mammalian species. For example, in the rabbit, a period of 5-6 hours is required for capacitation [4]. Conversely, within a spermatozoa population, individual cells respond to capacitating conditions at widely different rates [7]. Capacitation within an individual spermatozoon can take place over a short period of time [8]. The length of the time needed for capacitation suggests that capacitation is a sequence of events. Therefore, capacitation is a specific, initiated, and controllable process rather than a natural continuation of maturation [9].

Spermatozoa Maturation

Spermatogenesis is the biological process of maturation of germ cells into spermatozoa within the seminiferous tubules of the testis over a period of time. This process involves cellular proliferation by repeated mitotic divisions, duplication of chromosomes, genetic

recombination through cross-over, reduction-division by meiotic division to produce haploid spermatids, and terminal differentiation of the spermatids into spermatozoa [10].

Maturation of the spermatozoa is not completed in the seminiferous tubules but continues as the spermatozoa are transported through the epididymis. Protein addition and lipid membrane alteration occurs as the spermatozoa transit through the caput, corpus, and cauda epididymides and at ejaculation [11-14]. An example of this is the secretion of the 25 kDa protein, anti-agglutinin, by the epididymal duct of the corpus epididymides and the subsequent binding to the acrosome of spermatozoa [15].

Capacitation (Appendix I)

Capacitation has been described as a biochemical and reversible process which enables the spermatozoa to penetrate the ZP of the ovum [16]. There is much evidence that explain the molecular modifications of the spermatozoa membrane during capacitation. In vivo, capacitation occurs in the isthmus of the oviduct after the spermatozoa contact the oviductal epithelial cells [17-20].

Capacitation in vitro can occur during incubation at ambient temperature in the presence of bicarbonate, calcium, and serum albumin in a balanced salt solution in which the pH is controlled at 7.8 [11, 21, 22]. Incubation of spermatozoa in media devoid of serum albumin, calcium, or bicarbonate components prevents spermatozoa from undergoing capacitation, undergoing a ZP induced acrosome reaction, and fertilizing metaphase II

arrested oocytes in vitro [23]. In each of these cases, tyrosine phosphorylation of the capacitation-associated proteins did not occur.

Several studies have shown that bicarbonate plays a key role in the ability of spermatozoa to undergo capacitation and fertilization both in vivo as well as in vitro [21, 23-25]. Low levels of bicarbonate are maintained in the epididymis, but bicarbonate levels are high in the female reproductive tract where capacitation occurs [21]. This effect of bicarbonate may be one of the initial events in the capacitation process by altering the plasma membrane lipid architecture. This was demonstrated by Harrison et al. [25] using the fluorescent lipophilic molecule merocyanine. Merocyanine is membrane-impermeable and binds to the outer area of the plasma membrane of intact cells in relation to the degree of lipid disorder. Bicarbonate causes an increase in membrane lipid disorder therefore enhancing the ability of spermatozoa to bind merocyanine. The merocyanine-detected change took place within 5 min of spermatozoa exposure to bicarbonate [25].

Bicarbonate activates adenylate cyclase to produce an increase in the intracellular cAMP concentration, which in turn stimulates a protein kinase A to phosphorylate unknown protein components of the spermatozoa membrane [24, 26, 27]. The relationship between these proteins and the plasma membrane lipid architecture is not fully understood; however, they are related to a continual cycle of phosphorylation and dephosphorylation [7]. Recent work by Tardif et al. [28] demonstrated that a 32 kDa tyrosine kinase-like protein is auto-phosphorylated in the capacitating boar spermatozoa. Moreover, the capacitation-associated phosphorylation of this protein appears to be calcium dependent.

The bicarbonate induction of merocyanine binding is temperature dependent and reversible, occurring at 38 C but not at 25 C [25]. The reversibility of the bicarbonate effects may also be related to the continual cycle of membrane protein phosphorylation and dephosphorylation.

Capacitation involves a reversible range of membrane-related events resulting in a decreased membrane stability and increased permeability. These events include the removal of protein 'decapacitation' factors acquired in the epididymis or at ejaculation, a decrease in the cholesterol: phospholipids ratio [29], an increase in membrane lipid fluidity, activation of phospholipases, and a readiness to undergo the acrosome reaction [30, 31]. In boar spermatozoa, capacitation induces changes in lipid membrane structure via a cAMP-dependent protein phosphorylation pathway. These membrane changes include phosphatidylserine (PS) inversion, the relocation of cholesterol to the apical head, and a decrease in lipid packing [26]. The lipid scrambling allows for cholesterol relocation. Lipid scrambling, mainly PS inversion, is a recognized stage of the apoptotic death pathway [32]. However, there is no evidence that PS inversion is related to the apoptotic death pathway [26]. Serum albumin has been considered to act as an acceptor of cholesterol from the cell membrane resulting in the decrease in cholesterol: phospholipids ratio which contributes to the increase in membrane fluidity [29].

The increased membrane fluidity allows for a shift in membrane potential. The membrane potential of the spermatozoa becomes more negative as potassium ions leave the spermatozoa. This change in membrane potential may allow calcium channels to be

opened permitting calcium ions to enter the spermatozoa. Calcium and bicarbonate ions may be critical in the activation of cAMP production leading to protein phosphorylation [23, 33].

Fertilization and Acrosome Reaction

Shortly after binding to the ova's ZP, the spermatozoa undergo a cellular exocytosis referred to as the acrosome reaction. The acrosome is a large, Golgi-derived, lysosome-like organelle that overlies the nucleus in the apical region of the spermatozoa head [34]. It is made up of an inner membrane that overlies the nucleus and an outer membrane that underlies the plasma membrane. Binding of the spermatozoa to the ZP3 protein of the ova initiates the acrosome reaction [35-37].

The acrosome reaction involves multiple fusions between the outer acrosomal membrane and plasma membrane at the anterior region of the spermatozoa head, extensive formation of hybrid membrane vesicles, and exposure of the inner acrosomal membrane and acrosomal contents [30]. Only spermatozoa that have completed the acrosome reaction can penetrate the ZP and fuse with the ova plasma membrane.

Acrosome reacted spermatozoa remain bound to the ZP through their association with ZP2 [38, 39]. Bound spermatozoa must penetrate the ZP and pass through the perivitelline space to reach and fuse with the ova's plasma membrane. Penetration of the ZP is a result of both spermatozoa motility and enzymatic hydrolysis [30, 40]. An

acrosomal serine protease called acrosin catalyzes this enzymatic hydrolysis [41]. When the spermatozoa body is adsorbed into the ova, syngamy ensues.

Decapacitation Factors

Ejaculated spermatozoa are more difficult to capacitate then epididymal spermatozoa. Coating of the spermatozoa surface with seminal plasma proteins that inhibit processes normally associated with capacitation may be responsible for these findings [23]. Seminal plasma proteins have been postulated to act as decapacitation factors that prevent a premature acrosome reaction [42-44]. Their removal may initiate capacitation.

Bovine seminal plasma proteins (BSP-A1/-A2, BSP-A3, BSP-30-kDa) bind to choline phospholipids on the spermatozoa membrane at ejaculation. Subsequently these proteins interact with high-density lipoproteins (HDL) and heprin-like glycosaminoglycans (GAG) in the female reproductive tract resulting in capacitation [45]. In the boar, the heparin-binding protein pB1 has been characterized and identified to bind to lipids containing the phosphorylcholine group on the spermatozoa membrane at ejaculation [28]. Recently, an increase in capacitated spermatozoa were demonstrated after frozen-thawed spermatozoa were incubated with hyaluronan (HA), a GAG [46]. This suggested capacitation-causing actions of HA. Heparin [47, 48] and HDL [45] have also been shown to stimulate capacitation. Glycosaminoglycans have been localized in the pre- and post-ovulatory oviducts of swine reproductive tracts [49] and may play a role in the removal of seminal plasma proteins on the spermatozoa membrane resulting in capacitation both in vitro and in the female reproductive tract.

Conclusion

Spermatozoa's ability to fertilize an oocyte is dependent on their ability to successfully undergo capacitation and the acrosome reaction. The correct environment is required for this to occur whether the spermatozoa are in the oviduct of the female reproductive tract or an in vitro system. Capacitated spermatozoa have a short period of time to meet and fertilize an oocyte. If an oocyte is not present the spermatozoa die shortly thereafter.

A large number of spermatozoa are deposited into the female reproductive tract at artificial insemination. Cryopreservation results in a higher proportion of spermatozoa being effectively capacitated upon thawing [50, 51]. As a result, the spermatozoa have a shorter period of time for oocyte fertilization. In the swine industry, the use of frozen-thawed spermatozoa results in lower farrowing rates and smaller litter sizes [52, 53] in part due to the spermatozoa being effectively capacitated upon thawing.

A complete understanding of the process of capacitation would not only benefit the scientific community but also the food animal production industry. The ability to reverse the capacitation-like effects of cryopreservation would allow for the widespread application and acceptance of frozen-thawed spermatozoa for artificial insemination, specifically within the swine industry. As a result, the biosecurity of the international food animal industry would be greatly enhanced.

References

1. Adams, C., Chang M. The effect of delayed mating on fertilisation in the rabbit. J Exper Zoo, 1962. 151: 155-8.

2. Chang, M. Fertilisability of rabbit ova and the effects of temperature in vitro on their subsequent fertilisation and activation in vivo. J Exper Zoo, 1952. 121: 351-81.

3. Austin, C. Observations on the penetration of the sperm into the mammalian egg. Aust J Sci Res Ser B, 1951. 4: 581-9.

4. Chang, M. Fertilising capacity of spermatozoa deposited into the Fallopian Tubes. Nature, Lond, 1951. 168: 697.

5. Austin, C. The 'capacitation' of the mammalian sperm. Nature, 1952. 170: 326.

6. Hunter, R. Capacitation in the golden hamster, with special reference to the influence of the uterine environment. J Reprod Fertil, 1969. 20: 223-37.

7. Harrison, R.A. Sperm plasma membrane characteristics and boar semen fertility. J Reprod Fertil Suppl, 1997. 52: 195-211.

8. Roldan, E., Vazquez, J. Bicarbonate/CO2 induces rapid activation of phospholipase A2 and renders boar spermatozoa capable of undergoing acrosomal exocytosis in response to progesterone. FEBS Letters, 1996. 396: 227-32.

9. Bedford, J. Report of a workshop, maturation of the fertilizing ability of mammalian Spermatozoa in the male and female genital tract. Biol Reprod, 1974. 11: 346-59.

10. Hess, R. Spermatogenesis, Overview, in Encyclopedia of Reproduction. 1999, Academic Press.

11. Watson, P., Green, C.E. Cooling and Capacitation of Boar Spermatozoa: What do They Have in Common? In: Boar Semen Preservation IV, 2000. 53-60.

12. Bwanga, C.O. Cryopreservation of boar semen I: a literature review. Acta Vet Scand, 1991. 32: 431-53.

13. Rana, A., Majumder, G.C., Misra, S., Ghosh, A. Lipid changes of goat sperm plasma membrane during epididymal maturation. Biochem Biophys Acta, 1991. 1061: 185-96.

14. Nikolopoulou, M., Soucek, D., Vary, J. Changes in the lipid content of boar sperm plasma membranes during epididymal maturation. Biochem Biophys Acta, 1985. 815: 486-98.

15. Harayama, H., Magargee, S.F., Kunze, E., Shidara, O., Iwamoto, E., Arikawa, S., Miyake, M., Kato, S., Hammerstedt, R.H. Changes in epididymal protein anti-agglutinin on ejaculated boar spermatozoa during capacitation in vitro. Reprod Fertil Dev, 1999. 11: 193-9.

16. Chang, M. A detrimental effect of seminal plasma on the fertilizing capacity of sperm. Nature, Lond, 1957. 179: 258-9.

17. Pollard, J., Plante, C., King, W.A., Hansen, P.J., Betteridge, K.J., Suarez, S.S. Fertilizing capacity of bovine sperm may be maintained by binding of oviductal epithelial cells. Biol Reprod, 1991. 44: 102-7.

18. Ericsson, R. Technology, physiology and morphology of spermatozoa capacitation. J Reprod Fertil Suppl, 1967. 2: 65-74.

19. Soupart, P. Studies on the hormonal control of rabbit sperm capacitation. J Reprod Fertil Suppl, 1967. 2: 49-64.

20. Fazeli, A., Duncan, A.E., Watson, P.F., Holt, W.V. Sperm-oviduct interaction: Induction of capacitation and preferential binding of uncapacitated spermatozoa to oviductal epithelial cells in porcine species. Biol Reprod, 1999. 60: 879-86.

21. Harrison, R. Capacitation mechanisms, and the role of capacitation as seen in eutherian mammals. Reprod Fertil Dev, 1996. 8: 581-94.

22. Visconti, P., Kopf, G. Regulation of protein phosphorylation during sperm capacitation. Biol Reprod, 1998. 59: 1-6.

23. Visconti, P.E., Bailey, J.L., Moore, G.D., Pan, D., Olds-Clark, P., Kopf, G.S. Capacitation of mouse spermatozoa. I. Correlation between the capacitation state and protein tyrosine phosphorylation. Development, 1995. 121: 1129-37.

24. Okamura, N., Tajima, Y., Soejima, A., Masuda, H., Sugita, Y. Sodium bicarbonate in seminal plasma stimulates the motility of mammalian spermatozoa through direct activation of adenylate cyclase. J Biol Chem, 1985. 260: 9699-705.

25. Harrison, R.A., Ashworth, P., Miller, N.G.A. Bicarbonate/CO2, an effector of capacitation, induced a rapid and reversible change in the lipid architecture of boar sperm plasma membranes. Molec Reprod Dev, 1996. 45: 378-91.

26. Gadela, B.M. Harrison, R.A. Capacitation Induces Cyclic Adenosine 3',5'-Monophosphate-Dependent, but Apoptosis-Unrelated, Exposure of Animophospholipids at the Apical Head Plasma Membrane of Boar Sperm Cells. Biol Reprod, 2002. 67: 340-50.

27. Visconti, P.E., Galantino-Homer, H., Moore, G.D., Bailey, J.L., Ning, X., Fornes, M., Kopf, G.S. The molecular basis of sperm capacitation. J Androl, 1998. 19: 242-8.

28. Tardif, S., Dude, C., Chevalier, S., Bailey, J.L. Capacitation is associated with tyrosine phosphorylation and tyrosine kinase-like activity of pig sperm proteins. Biol Reprod, 2001. 65: 784-92.

29. Cross, N.L. Role of cholesterol in sperm capacitation. Biol Reprod, 1998. 59: 7-11.

30. Yanagimachi, R. Mammalian Fertilization, in "The Physiology of Reproduction." J.N. E Knobil, Editor. 1994, Raven Press: New York. 189-317.

31. Topfer-Petersen, E., Friess, A.E., Stoffel, M., Schill, W.B. Boar sperm membranes antigens. I. Topography of a mobile glycoprotein of the sperm cell membrane. Histochemistry, 1990. 93: 485-90.

32. Fadok, V.A., Bratton, D.L., Rose, D.M., Pearson, A., Ezekewitz, R.A., Henson, P.M. A receptor for phosphatidylserine-specific clearance of apoptotic cells. Nature, 2000. 405: 85-90.

33. Arnoult, C., Kazam, I.G., Visconti, P.E., Kopf, G.S., Villaz, M., Florman, H.M. Control of the low voltage-activated calcium channel of mouse sperm by egg ZP3 and by membrane hyperpolarization during capacitation. Proceedings of the National Academy of Sciences of the United States of America, 1999. 96: 6757-62.

34. Abou-Haila, A., Tulsiani, D. Mammalian sperm acrosome reaction: formation, contents, and function. Arch Biochem Biophys, 2000. 379: 173-82.

35. Florman, H., Arnoult, C., Kazam, I.G., Li, C., O'Toole, C.M.B. An intimate biochemistry: egg-regulated acrosome reactions of mammalian sperm. Adv Dev Biochem, 1999. 5: 147-86.

36. Bleil, J., Wassarman, W. Sperm-egg interactions in the mouse: sequence of events and induction of the acrosome reaction by a zona pellucida glycoprotein. Dev Biol, 1983. 95: 317-24.

37. O'Toole, C., Arnoult, C., Darszon, A., Steinhardt, R.A., Florman, H.M. Ca2+ entry through store-operated channels in mouse sperm is initiated by egg ZP3 and drives the acrosome reaction. Molec Biol Cell, 2000. 11: 1571-84.

38. Bleil, J., Greve, T., Wassarman, P. Identification of a secondary sperm receptor in the mouse egg zona pellucida: Role in maintenance of binding of acrosome-reacted sperm to eggs. Dev Biol, 1988. 128: 376-85.

39. Mortillo, S., Wassarman, P. Differential binding of gold-labeled zona pellucida glycoprotwins mZP2 and mZP3 to mouse sperm membrane compartments. Development, 1991. 113: 141-51.

40. Bedford, J. Mammalian fertilization misread? Sperm penetration of the eutherian zona pellucida is unlikely to be a lytic event. Biol Reprod, 1998. 59: 1275-87.

41. Eddy, E., O'Brien, D. In: The Physiology of Reproduction, E. Knobil and J. Neill, Editors. 1994, Raven: New York. 29-77.

42. Dukelow, W., Chernoff, H., Williams, W. Properties of decapacitation factor and presence in various species. J Reprod Fertil, 1967. 14: 393-9.

43. Fraser, L., Harrison, R.A., Herod, J. Characterization of a decapacitation factor associated with epididymal mouse spermatozoa. J Reprod Fertil, 1990. 89: 135-48.

44. Oliphant, G., Reynolds, A., Thomas, T. Sperm surface components involved in the control of the acrosome reaction. Am J Anat, 1985. 174: 269-83.

45. Therien, I., Soubeyrand, S., Manjunath, P. Major proteins of bovine seminal plasma modulate sperm capacitation by high-density lipoprotein. Biol Reprod, 1997. 57: 1080-8.

46. Suzuki, K., Asano, A., Eriksson, B., Niwa, K., Nagai, T., Rodriguez-Martinez, H. Capacitation status and in vitro fertility of boar spermatozoa: effects of seminal plasma, cumulus-oocyte-complexes-conditioned medium and hyaluronan. Int J Androl, 2002. 25: 84-93.

47. Therien, I., Bleau, G., Manjunath, P., Soubeyrand, S. Phosphatidylcholine-binding proteins of bovine seminal plasma modulate capacitation of spermatozoa by heparin. Biol Reprod, 1995. 52: 1372-9.

48. Kim, N.H., Day, B.N., Lim, J.G., Lee, H.T., Chung, K.S. Effects of oviductal fluid and heparin on fertility and characteristics of porcine spermatozoa. Zygote, 1997. 5: 61-5.

49. Tienthai, P., Kjellen, L., Pertoft, H., Suzuki, K., Rodriguez-Martinez, H. Localization and quantitation of hyaluronan and sulfated glycosaminoglycans in the tissues and intraluminal fluid of the pig oviduct. Reprod Fertil Dev, 2000. 12: 173-82.

50. Bailey, J., Bilodeau, J., Cormier, N. Semen cryopreservation in domestic animals: a damaging and capacitating phenomenon. J Androl, 2000. 21: 1-7.

51. Maxwell, W., Johnson, L. Chlortetracycline analysis of boar spermatozoa after incubation, flow cytometric sorting, cooling, or cryopreservation. Molec Reprod Dev, 1997. 46: 408-18.

52. Reed, H. Current use of frozen boar semen-future need of frozen boar semen. In: Deep freezing of boar semen., J. LA and L. K, Editors. 1985, Swedish University of Agricultural Science: Uppsala. 225-37.

53. Almlid, T., Hofmo, P. A brief review of frozen semen application under Norwegian AI service conditions. Reprod Dom Anim, 1996. 31: 169-73.

CHAPTER 2

Effect of cooling and the addition of seminal plasma on the capacitation status of fresh boar spermatozoa as determined using chlortetracycline staining

Abstract

Insemination of swine with frozen-thawed spermatozoa results in lower fertility, in part due to spermatozoa having undergone a capacitation-like reaction. The present study employed chlortetracycline staining analysis to investigate the effect of adding 20% (v/v) boar seminal plasma (SP) to boar spermatozoa on the temporal progress of capacitation and the acrosome reaction when spermatozoa were cooled to 5 C or incubated at 39 C. Seminal plasma reversed capacitation induced by incubation at 39 C in a capacitation-supporting medium from 59.7% to 36.6% capacitated spermatozoa ($P<0.001$). Similarly, the addition of SP to boar spermatozoa cooled to 5 C resulted in both the prevention of the capacitation-like reaction and the reversal of an established capacitation-like reaction from 63.3% to 34.2% capacitated ($P<0.001$). These observations indicated that the addition of boar SP can both prevent spermatozoa from undergoing capacitation as well as reverse capacitation in spermatozoa that have already undergone the process.

Introduction

Artificial insemination of swine with frozen-thawed (FT) spermatozoa results in farrowing rates and litter sizes 20 to 30% below those observed following insemination of fresh spermatozoa [1]. This is believed to be a result of spermatozoa acquiring cryoinjury, including a capacitation-like reaction (cryocapacitation), during the freezing

and thawing process. Capacitated spermatozoa do not associate with the oviductal epithelium and so do not enter the functional spermatozoa reservoir [2, 3]. Although capacitated FT spermatozoa are capable of fertilizing oocytes, their life span in the female reproductive tract is considerably shorter than that of freshly ejaculated spermatozoa [4, 5]. Therefore, the use of FT spermatozoa for swine artificial insemination requires that an oocyte be almost immediately available for fertilization.

Capacitation is a sequential process involving numerous physiological changes including membrane destabilization [6], an influx of calcium ions [7], and protein phosphorylation [8]. Cryocapacitation is a similar, capacitation-like, process that displays a different pattern and number of tyrosine-phosphorylated proteins [9]. Membrane destabilization consists of the removal of protein decapacitation factors, acquired during epididymal transport or after mixing with seminal plasma (SP) during ejaculation, as well as cholesterol from the spermatozoa membrane [10]. In the boar, the epididymal protein anti-agglutinin that is bound to spermatozoa acrosomes during maturation is released at an early stage of capacitation; this release was attenuated in spermatozoa incubated in 5 or 10% (v/v) SP [11].

Green and Watson [9] demonstrated that the capacitation-like reaction was induced by the cooling of boar spermatozoa to 5 C and was observed once the spermatozoa were re-warmed to 39 C. Further, the addition of 20% (v/v) boar SP to cooled spermatozoa suspensions resulted in a decreased percent of spermatozoa displaying the capacitation-like reaction [12]. The addition of boar SP also reduced the percent of capacitated

spermatozoa when incubated in a capacitation-supporting media at 39 C [11]. Interestingly, it has been shown that the cold shock-induced membrane damage in ram spermatozoa was reversed following incubation with a solution containing proteins extracted from ram SP [13]. It is evident that SP has a role in the regulation of capacitation. The objective of the present study was to examine the effect of supplemental SP on the cooling-induced capacitation-like changes in boar spermatozoa.

Materials and Methods

All chemicals were of analytical grade. Unless otherwise stated, reagents were purchased from Sigma Chemicals (St. Louis, MO) and solutions prepared with sterile deionized water.

A pool of SP from four boars was accumulated for these studies. The sperm-rich fractions of the ejaculates were centrifuged at 1000 x g for 10 min, and the pellet discarded. The SP was stored in 15 mL conical tubes at –80 C until required. Prior to use, a tube was thawed overnight in a humidified incubator at 39 C and 5% CO_2 in air.

Medium

The capacitation-supporting medium (mM199) consisted of 87% (v/v) Medium 199 Hanks (Gibco Invitrogen Corporation, Grand Island, New York), 12% (v/v) heat-activated fetal calf serum, 1% (v/v) penicillin-streptomycin, 2.3% (wt/vol) bovine serum albumin, 3.05 mM D-glucose, 2.91 mM calcium lactate, and 0.91 mM sodium pyruvate,

with pH adjusted to 7.8. The mM199 was equilibrated overnight in a humidified incubator at 39 C and 5% CO_2 in air prior to use.

Semen collection and assessment

Ejaculates from three Yorkshire boars between 12 and 20 months of age were used for this study. All boars were housed at the Swine Research Facility at Michigan State University where their fertility had been proven. The sperm-rich fraction was collected by a manual method into a thermally protected vessel and transported to the laboratory in a 37 C water bath. Upon arrival to the laboratory, spermatozoa concentration was evaluated using a Bright-Line Hemacytometer (Hausser Scientific, Horsham, PA). The spermatozoa concentration in each treatment suspension was approximately 250 x 10^6 spermatozoa per mL.

Processing for non-cooling treatments (Appendix II)

A 5mL aliquot of each ejaculate was centrifuged at 23 C for 10 min at 600 x g. The spermatozoa pellet was then resuspended in 5mL of mM199 at 39 C and incubated at 39 C in a humidified incubator in 5% CO_2 for a 100 min initial incubation. At the end of the 100 min initial incubation, each suspension was centrifuged for 10 min at 600 x g at 23 C. The pellet was then resuspended in 5mL of mM199 at 39 C and immediately placed in a humidified incubator at 39 C and 5% CO_2 in air for 10 min. This period of 10 min is designated as the warming period. After the warming period, suspensions remained in the incubator for an 8 h incubation period. The 100 min and 10 min periods were chosen to

allow for temporal comparisons with the cooling treatments (see below) and are based on the protocol of Green and Watson [9].

Processing for cooling treatments (Appendix II)

A 5mL aliquot of the ejaculate was centrifuged at 23 C for 10 min at 600 x g, and the spermatozoa pellet resuspended in 5mL of mM199 at 39 C. The spermatozoa suspension was then cooled to 5 C over 100 min in a Digital Temperature Control Electronic Cooler/warmer (TR Enterprises Inc, Aberdeen, NC). At the end of the 100 min cooling period, each suspension was centrifuged for 10 min at 600 x g in a refrigerated centrifuge at 5 C. The pellet was then resuspended in 5mL of mM199 at 5 C. The spermatozoa suspensions were immediately placed in a humidified incubator at 39 C and 5% CO_2 in air for a 10 min warming period; after which, they remained in the incubator for an 8 h incubation period.

Chlortetracycline (CTC) staining analysis (Appendix II)

CTC staining analysis [14] was used to identify the progress of capacitation and acrosome reaction of boar spermatozoa. For CTC staining, 20 µl of 500 µM CTC in a chilled buffer of 20 mM Tris, 130 mM sodium chloride, and 5 mM L-cysteine prepared the day of each treatment was added to a warm (37 C) microscope slide followed by 20 µl of spermatozoa suspension and mixed. After 10 sec, spermatozoa were fixed with 10 µl of 12.5% glutaraldehyde in Tris buffer (pH 7.8) and mixed again. A cover slip was applied, and the slide was compressed between bibulous papers to remove excess fluid and maximize the number of spermatozoa lying flat on the slide prior to observation.

Prepared slides were placed in a black box until read within 2 h of preparation. Spermatozoa were illuminated with a 100 W mercury bulb and examined under a Nikon Optiphot microscope equipped with a 380-425 nm excitation filter, a 520 nm barrier filter, and a 510 nm dichroic mirror (Nikon Co., Tokyo, Japan).

Slides were prepared for CTC staining analysis directly before (designated as 'Pre') and after (designated as 'Post') the 100 min initial incubation or cooling period; directly after the 10 min warming period (Hour 0); and at 1, 2, 4, 6, 7, and 8 h after Hour 0. Two slides were prepared for each sample, and 100 spermatozoa were counted on each slide. The average of both slides was determined. Four fluorescent patterns were used to demonstrate the progress of capacitation and the acrosome reaction. Fresh (F) spermatozoa (uncapacitated) were characterized by a bright anterior region with a bright acrosome cap, a relatively faint posterior region, and a dark half circle at the equatorial region. Those with equally bright anterior and equatorial regions and a less bright posterior region were also scored as F. Capacitated (B) spermatozoa were consistent with a faint anterior region, a bright equatorial region, and a faint posterior region. Acrosome reacted (AR) spermatozoa were characterized by a very faint anterior region, a slightly bright equatorial region, and a faint posterior region.

Bis-Benzimide Hoechst No. 33258 staining analysis

For all treatments, Hoechst 33258 was used to determine effect of treatment on viability of the spermatozoa. A 5 µl sample of chilled stock solution (200 µM Hoechst 33258) was added to 100 µl of spermatozoa suspension and incubated for 15 min at 39 C and 5% CO_2

in the dark. Subsequently, 20 µl of stained spermatozoa suspension was added to a warm (37 C) microscope slide and fixed with 5 µl 12.5% glutaraldehyde. A cover slip was applied and the slide was compressed between bibulous papers. Prepared slides were placed in a black box until read within 2 h of preparation. Spermatozoa were illuminated with a 100 W mercury bulb and examined under a Nikon Optiphot microscope equipped with a 365/10 nm excitation filter, a 400 nm barrier filter, and a 400 nm dichroic mirror (Nikon Co., Tokyo, Japan).

Slides were prepared for staining analysis at Pre; Post; Hour 0; and at 2, 4, 6, and 8 h after Hour 0. Two slides were prepared for each sample, and 100 spermatozoa were counted on each slide. The average of both slides was determined. Spermatozoa exhibiting bright blue fluorescence over the entire head were considered nonviable cells.

Treatments (Appendix III)

Seven treatments were performed with each of the three Yorkshire boars. All treatments were replicated 3 times with boar Y27-10 and performed once each with boars Y43-11 and Y64-11 totaling 5 replicates per treatment. Where indicated, SP was added at 20% v/v. This inclusion level was chosen based on previously demonstrated efficacy [11, 12]. Times for SP addition were chosen to examine its effect on the capacitation-like reaction prior to or following cooling and its efficacy in reversing an established spermatozoa capacitation reaction.

Three non-cooling treatments (Treatments 1, 2, and 3) were completed where the spermatozoa suspensions were incubated at 39 C for an initial 100 min followed by continued incubation for 8 h: Treatment 1 no SP was added; Treatment 2 SP was added immediately prior to the 10 min warming period; Treatment 3 SP was added at Hour 2 of the 8 h incubation.

Four treatments (Treatments 4, 5, 6, and 7) were completed where the spermatozoa suspensions were cooled to 5 C over 100 min followed by warming and incubation: Treatment 4 no SP was added; Treatment 5 SP was added immediately prior to the 10 min warming period; Treatment 6 SP was added immediately before the cooling period, and the pellet was re-suspended in mM199 containing no SP after the cooling period; Treatment 7 SP was added at Hour 2 of the 8 h incubation.

Statistical Analysis

Data were analyzed using Statistical Analysis Systems software (SAS Institute Inc., Cary, NC). Percentage data for each response category (live, fresh, capacitated, and acrosome-reacted) were transformed based on the arcsin of the square roots of the proportions prior to analysis. Repeated measures ANOVA was conducted to determine treatment differences at each time point separately for each of the response categories. Least-squares means for each treatment and time were back-transformed for the purposes of reporting point estimates and preparing figures.

Results

Viability assessment

The percent of viable spermatozoa decreased from 73% at time Pre to 60% at time Post. Thereafter, the percent viable sperm remained relatively constant throughout the experimental period with no significant difference between treatments ($P>0.05$; Figure 2.1).

Non-cooling treatments

In Treatment 1, SP was not included. The percent of F spermatozoa steadily decreased from 59.6% to 10.9% over a total of 10 h of incubation. Concurrently, B spermatozoa rose from 39% to 58.8%, and AR increased from 1.4% to 30.3% (Figure 2.2).

In Treatment 2, SP was added at the end of the initial incubation period. The percent F spermatozoa decreased from 58.1% to 46.6% while the percent B spermatozoa increased from 39.4% to 50.3% by the end of the initial incubation period. The CTC analysis at Hour 0 (57.3% F, 40.8% B) demonstrated that SP reversed capacitation within 10 min of introduction (Figure 2.3). After reversal, the percent of F and B spermatozoa remained relatively constant throughout the 8 h incubation period. The percent of spermatozoa that had undergone an acrosome reaction increased from 2.5% to 11% during the experimental period.

The changes in capacitation status observed in Treatment 3 were similar to those noted in Treatment 1 until SP was added at Hour 2. At Hour 2, the percent F and B spermatozoa

were 32.4% and 62.3%, respectively, but were reversed to 36% and 59.6%, respectively, at Hour 4 (Figure 2.4). The percent F and B remained relatively constant during the remaining incubation period. The percent of spermatozoa that had undergone the acrosome reaction increased throughout the treatment period from 1.4% to 9.75%.

Cooling treatments

In Treatment 4, no SP was included. The percent F and B spermatozoa remained relatively unchanged during the 100 min cooling period. However, a sharp decrease in F spermatozoa from 58.7% to 35.1% and an increase in B spermatozoa from 36.9% to 59.2% occurred upon warming (Figure 2.5). Fresh spermatozoa continued to decline to 11.2% while the percent B spermatozoa increased slowly to 63.7% at Hour 8. Acrosome reacted spermatozoa increased steadily throughout the treatment period from 1.8% at Pre to 25.1% at Hour 8.

In Treatment 5, SP was added after the 100 min cooling period (Figure 2.6). The percent F and B spermatozoa were initially 57.9% and 39%, respectively, and remained relatively constant throughout the treatment period changing only to 47.8% and 39.8%, respectively, by the end of the 8 h incubation. During the treatment period acrosome reacted spermatozoa increased from 3.1% to 12.4%.

In Treatment 6, SP was included in the spermatozoa suspension during cooling but removed for the incubation period. This resulted in the percent F and B spermatozoa remaining relatively constant throughout the experimental period (Figure 2.7). Percent

acrosome reacted spermatozoa increased from 1.4% to 10.9% during the experimental period.

In Treatment 7, changes in percent F and B spermatozoa were similar to those observed in Treatment 4 until the addition of SP at Hour 2. The percent F and B spermatozoa were 29.5% and 64.4%, respectively, at Hour 2 but were reversed to 59.5% and 37.8%, respectively, at Hour 4 (Figure 2.8). Acrosome reacted spermatozoa increased from 0.75% to 6.0% during the experimental period.

Discussion

Our findings confirmed that SP reduced the percent capacitated boar spermatozoa when incubated in a capacitation-supporting media at 39 C [11,15] and extend this observation to demonstrate that capacitation was reversed within 10 min of introduction of SP. The addition of SP also reversed capacitation that had been well established after several hours of incubation.

The present data support previous studies [9, 12, 16] in that rate controlled cooling of boar spermatozoa to 5 C resulted in a capacitation-like reaction. This effect was not evident until the spermatozoa were re-warmed to ambient temperature suggesting a possible involvement of enzymes. The addition of 20% (v/v) SP to the spermatozoa suspension before cooling or after cooling prevented the cooling-induced capacitation-like reaction for a period of at least 8 h. The addition of SP after the warming period or

after 2 h of incubation resulted in the reversal of the cooling-induced capacitation-like reaction.

The data presented are limited to a SP inclusion level of 20% v/v. However, additional treatments that examined the effect of inclusion of 10 or 40% (v/v) SP have indicated almost identical responses (data not shown) and also support previous studies [17, 18]. Therefore, the effect of SP appears to be independent of the time of addition, percent inclusion (10%, 20%, or 40%), or the type of capacitation reaction (time-dependant or cooling-induced).

Seminal plasma, produced by the accessory glands of the male reproductive system, has several reported effects on spermatozoa in vitro: modulating capacitation in epididymal bull spermatozoa [19, 20], reversing capacitation in fresh ejaculated boar spermatozoa [11, 15], and improvement of motility of spermatozoa (boar: [21, 22]; ram, bull, boar: [16]). Further, spermatozoa maintained in their own SP prior to its removal for extension intended for cryopreservation demonstrated increased resistance to cold shock [23, 24]. The composition of SP includes inorganic compounds, amino acids, peptides, as well as low and high molecular-weight proteins [25]. For artificial insemination, SP is highly diluted with an isotonic extension media.

Seminal plasma proteins have been postulated to act as decapacitation factors that prevent a premature acrosome reaction [26], and their removal may initiate capacitation. Bovine SP proteins (BSP-A1/-A2, BSP-A3, BSP-30-kDa) bind to choline phospholipids on the

spermatozoa membrane at ejaculation subsequently interacting with high-density lipoproteins (HDL) and heparin-like glycosaminoglycans (GAG) in the female reproductive tract promoting capacitation [27]. Bovine spermatozoa that have been exposed to SP possess more binding sites for heparin than spermatozoa obtained from the caudal epididymis [20]. In boar, the heparin-binding protein pB1 has been characterized and identified to bind to lipids containing the phosphorylcholine group on the spermatozoa membrane at ejaculation [28]. Recently, when FT spermatozoa were incubated with the GAG hyaluronan (HA) an increase in the percent of B spermatozoa was demonstrated [15]. This suggested a capacitation-promoting action of HA. Heparin [29, 30] and HDL [27] have also been shown to promote capacitation. Glycosaminoglycans have been localized in pre- and post-ovulatory oviducts of swine reproductive tracts [31] and may play a role in the removal of SP proteins on the spermatozoa membrane resulting in capacitation both in vitro and in vivo. As the present study has demonstrated, by adding back a percentage of SP to the spermatozoa, capacitation and the capacitation-like reaction were either prevented or reversed. This prevention and reversal suggests that SP maintained proteins on the spermatozoa membrane or replaced SP proteins previously lost to the membrane. Further investigations are needed to identify the component(s) (i.e. possible proteins) of the SP that result in these outcomes.

An important implication of the ability to reverse capacitation is the possibility to significantly extend the fertile life of cryopreserved spermatozoa. An increased functional spermatozoa reservoir within the oviduct as a result of the addition of SP to extended FT

spermatozoa would improve sow fertility when this technology is employed. The ability to successfully use FT spermatozoa would allow the swine industry to gain the maximum potential in terms of efficiency of spermatozoa use. More importantly, use of FT spermatozoa would allow greater protection of herd health by permitting spermatozoa to be quarantined prior to use in the breeding herd.

References

1. Johnson, L.A., Weitze, K.F., Fiser, P., Maxwell, W.M.C. Erratum to "Storage of boar semen": [Anim. Reprod. Sci. 2000;62:143-72]. Anim Reprod Sci, 2000. 64: 133-4.

2. Fazeli, A., Duncan, A.E., Watson, P.F., Holt, W.V. Sperm-oviduct interaction: Induction of capacitation and preferential binding of uncapacitated spermatozoa to oviductal epithelial cells in porcine species. Biol Reprod, 1999. 60: 879-86.

3. Tienthai, P., Johannisson, A., Rodriguez-Martinez, H. Sperm capacitation in the porcine oviduct. Anim Reprod Sci, 2004. 80: 131-46.

4. Holt, W., Medrano, A. Assessment of boar sperm function in relation to freezing and storage. J Reprod Fertil Suppl, 1997. 52: 213-22.

5. Bailey, J., Bilodeau, J., Cormier, N. Semen cryopreservation in domestic animals: a damaging and capacitating phenomenon. J Androl, 2000. 21: 1-7.

6. Harrison, R. Capacitation mechanisms, and the role of capacitation as seen in eutherian mammals. Reprod Fertil Dev, 1996. 8: 581-94.

7. Yanagimachi, R. Mammalian Fertilization. In: Knobil E, Neill JD (eds): The Physiology of Reproduction. New York: Raven Press, 1994; 189-317.

8. Visconti, P., Kopf, G. Regulation of protein phosphorylation during sperm capacitation. Biol Reprod, 1998. 59: 1-6.

9. Green, C., Watson, P. Comparison of the capacitation-like state of cooled boar spermatozoa with true capacitation. Reproduction, 2001. 122: 889-98.

10. Watson, P., Green, C.E. Cooling and capacitation of boar spermatozoa: What do they have in common? In: Boar Semen Preservation IV, 2000. 53-60.

11. Harayama, H., Magaree, S.F., Kunze, E., Shidara, O., Iwamoto, E., Arikawa, S., Miyake, M., Kato, S., Hammerstedt, R.H. Changes in epididymal protein anti-agglutinin on ejaculated boar spermatozoa during capacitation in vitro. Reprod Fertil Dev, 1999. 11: 193-9.

12. Kaneto, M., Harayama, H., Miyake, M., Kato, S. Capacitation-like alterations in cooled boar spermatozoa: assessment by the chlortetracycline staining assay and immunodetection of tyrosine-phosphorylated sperm proteins. Anim Reprod Sci, 2002. 73: 197-209.

13. Barrios, B., Perez Pe, R., Gallego, M., Tato, A., Osada, J., Muino Blanco, T., Cebrian Perez, J.A. Seminal plasma proteins revert the cold-shock damage on ram sperm membrane. Biol Reprod, 2000. 63: 1531-7.

14. Lin, C. Regulation of sperm capacitation. Doctoral Thesis. Michigan State University, East Lansing, Michigan 1996.

15. Suzuki, K., Asano, A., Eriksson, B., Niwa, K., Nagai, T., Rodriguez-Martinez, H. Capacitation status and in vitro fertility of boar spermatozoa: effects of seminal plasma, cumulus-oocyte-complexes-conditioned medium and hyaluronan. Int J Androl, 2002. 25: 84-93.

16. Maxwell, W., Johnson, L. Chlortetracycline analysis of boar spermatozoa after incubation, flow cytometric sorting, cooling, or cryopreservation. Molec Reprod Dev, 1997. 46: 408-18.

17. Maxwell, W.M.C., Long, C.R., Johnson, L.A., Dobrinski, J.R., Welch, G.R. The relationship between membrane status and fertility of boar spermatozoa after cytometric sorting in the presence or absence of seminal plasma. Reprod Fertil Dev, 1998. 10: 433-40.

18. Maxwell, W.M.C., Johnson, L.A. Physiology of spermatozoa at high dilution rates: The influence of seminal plasma. Theriogenology, 1999. 52: 1353-62.

19. Lee, C., Handrow, R.R., Lenz, R.W., Ax, R.L. Interactions of seminal plasma and glycosaminoglycans on acrosome reactions in bovine spermatozoa in vitro. Gamete Res, 1985. 12: 345-55.

20. Miller, D., Winer, M., Ax, R. Heparin-binding proteins from seminal plasma bind to bovine spermatozoa and modulate capacitation by heparin. Biol Reprod, 1990. 42: 899-915.

21. Okamura, N., Tajima, Y., Soejima, A., Masuda, H., Sugita, Y. Sodium bicarbonate in seminal plasma stimulates the motility of mammalian spermatozoa through direct activation of adenylate cyclase. J Biol Chem, 1985. 260: 9699-705.

22. Rodriguez-Martinez, H., Ekstedt, E., Einarsson, S. Acidification of epididymal fluid in the boar. Int J Androl, 1990. 13: 238-43.

23. Tamuli, M.K., Watson, P.F. Cold resistance of live boar spermatozoa during incubation after ejaculation. Vet Rec, 1994. 135: 160-2.

24. Catt, S.L., O'Brien, J.K., Maxwell, W.M.C., Evans, G. Assessment of ram and boar spermatozoa during cell-sorting by flow cytometry. Reprod Dom Anim, 1997. 32: 251-8.

25. Pursel, V., Johnson, L., Schulman, L. Effect of dilution, seminal plasma and incubation period on cold shock susceptibility of boar spermatozoa. J Anim Sci, 1973. 37: 528-31.

26. Oliphant, G., Reynolds, A., Thomas, T. Sperm surface components involved in the control of the acrosome reaction. Am J Anat, 1985. 174: 269-83.

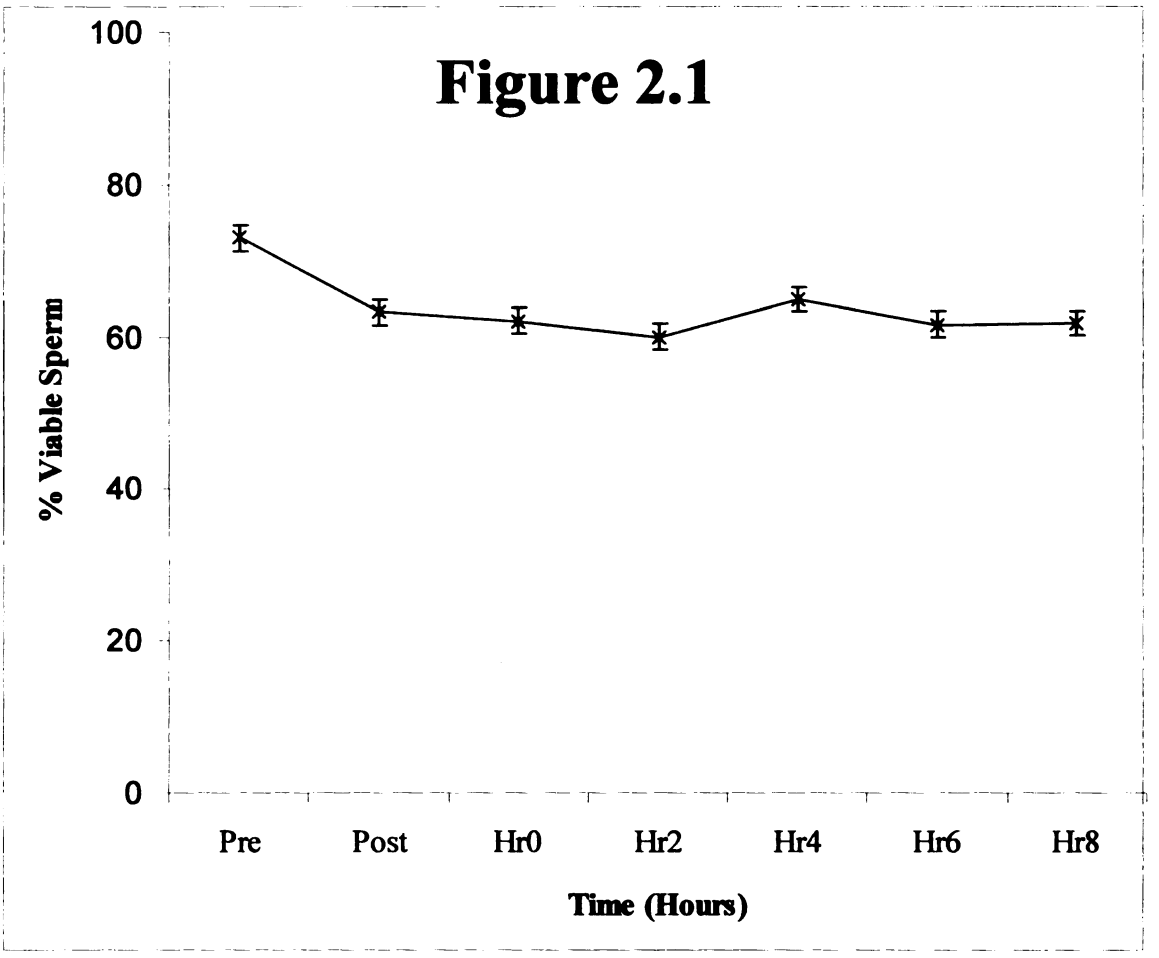
27. Therien, I., Soubeyrand, S., Manjunath, P. Major proteins of bovine seminal plasma modulate sperm capacitation by high-density lipoprotein. Biol Reprod, 1997. 57: 1080-88.

28. Calvete, J., Raida, M., Gentzel, M., Urbanke, C., Sanz, L., Topfer Petersen, E. Isolation and characterization of heparin- and phosphorylcholine-binding proteins of boar and stallion seminal plasma. Primary structure of porcine pB1. FEBS Letters, 1997. 407: 201-6.

29. Therien, I., Bleau, G., Manjunath, P., Soubeyrand, S. Phosphatidylcholine-binding proteins of bovine seminal plasma modulate capacitation of spermatozoa by heparin. Biol Reprod, 1995. 52: 1372-9.

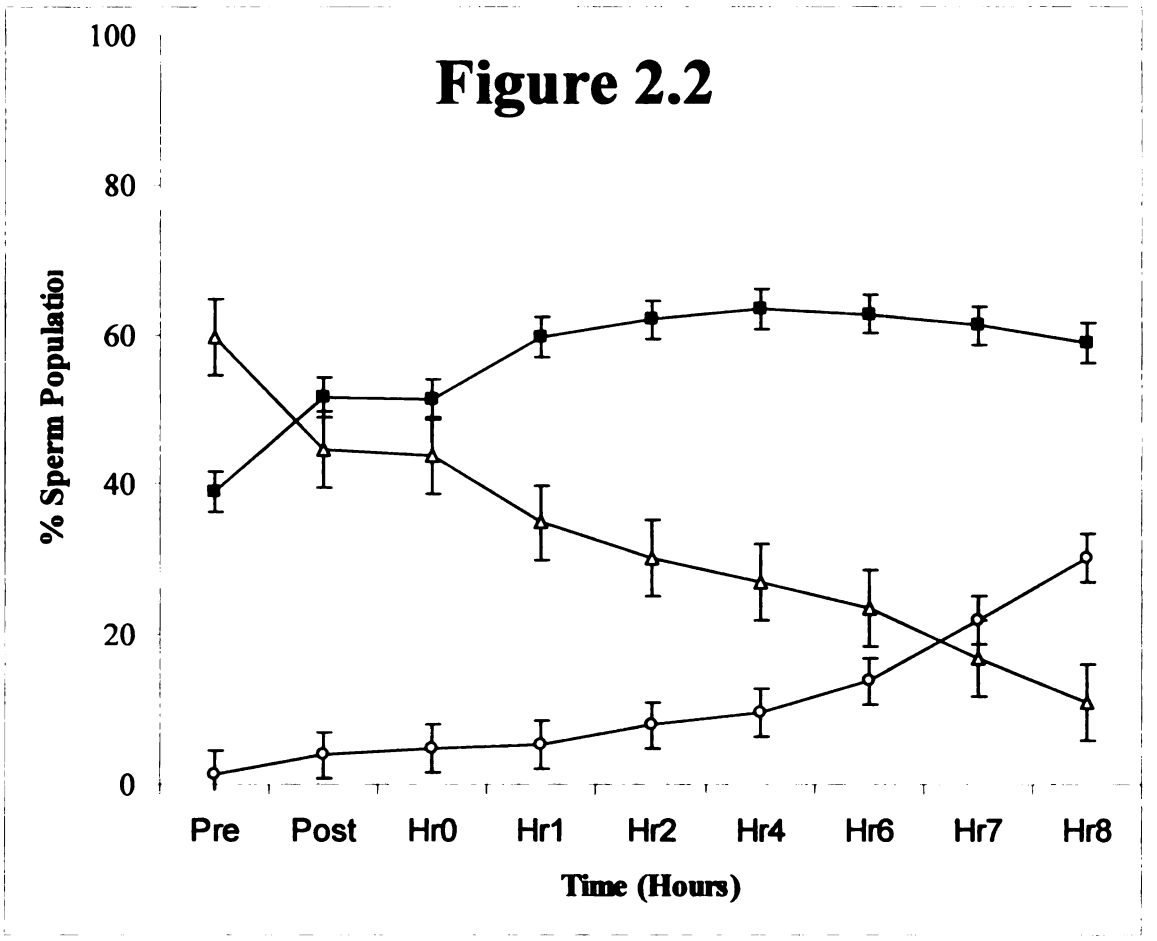
30. Kim, N., Day, B.N., Lim, J.G., Lee, H.T., Chung, K.S. Effects of oviductal fluid and heparin on fertility and characteristics of porcine spermatozoa. Zygote, 1997. 5: 61-5.

31. Tienthai, P., Kjellen, L., Pertoft, H., Suzuki, K., Rodriguez-Martinez, H. Localization and quantitation of hyaluronan and sulfated glycosaminoglycans in the tissues and intraluminal fluid of the pig oviduct. Reprod Fertil Dev, 2000. 12: 173-82.



In Figures 2.1 through 2.8, time points 'Pre' and 'Post' represent readings prior to and after the 100-min pre-incubation or cooling, respectively.

Figure 2.1: Effect of all treatments on viability of spermatozoa. Each point represents the mean ± SE of all determinations.



In Figures 2.2 through 2.8, Δ represent Fresh spermatozoa, ■ represent Capacitated spermatozoa (B), and O represent Acrosome Reacted spermatozoa. Each point represents the average of 5 counts ± SE.

Figure 2.2: Pre-incubation and incubation in mM199 containing 0% SP.

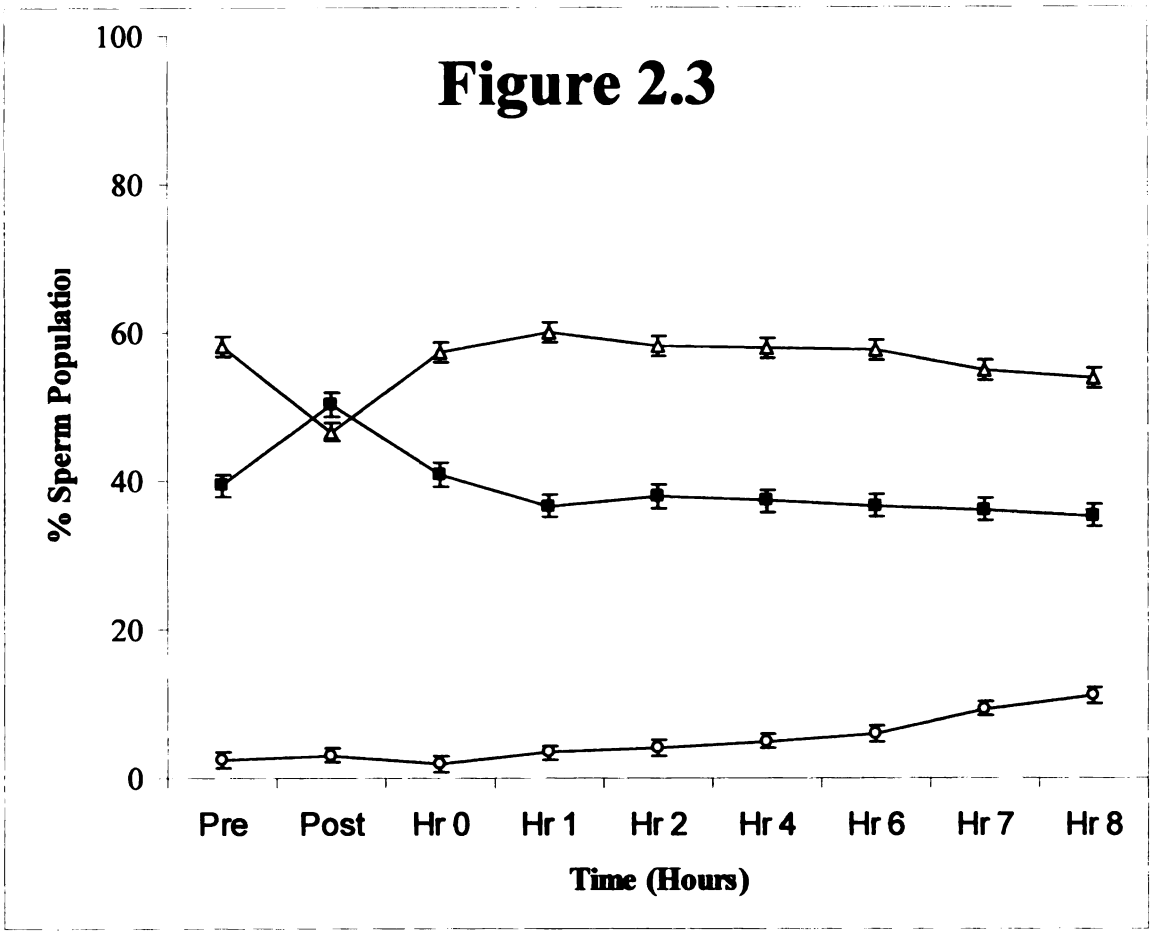


Figure 2.3: Pre-incubation with 0% SP and then incubation in mM199 containing 20% SP.

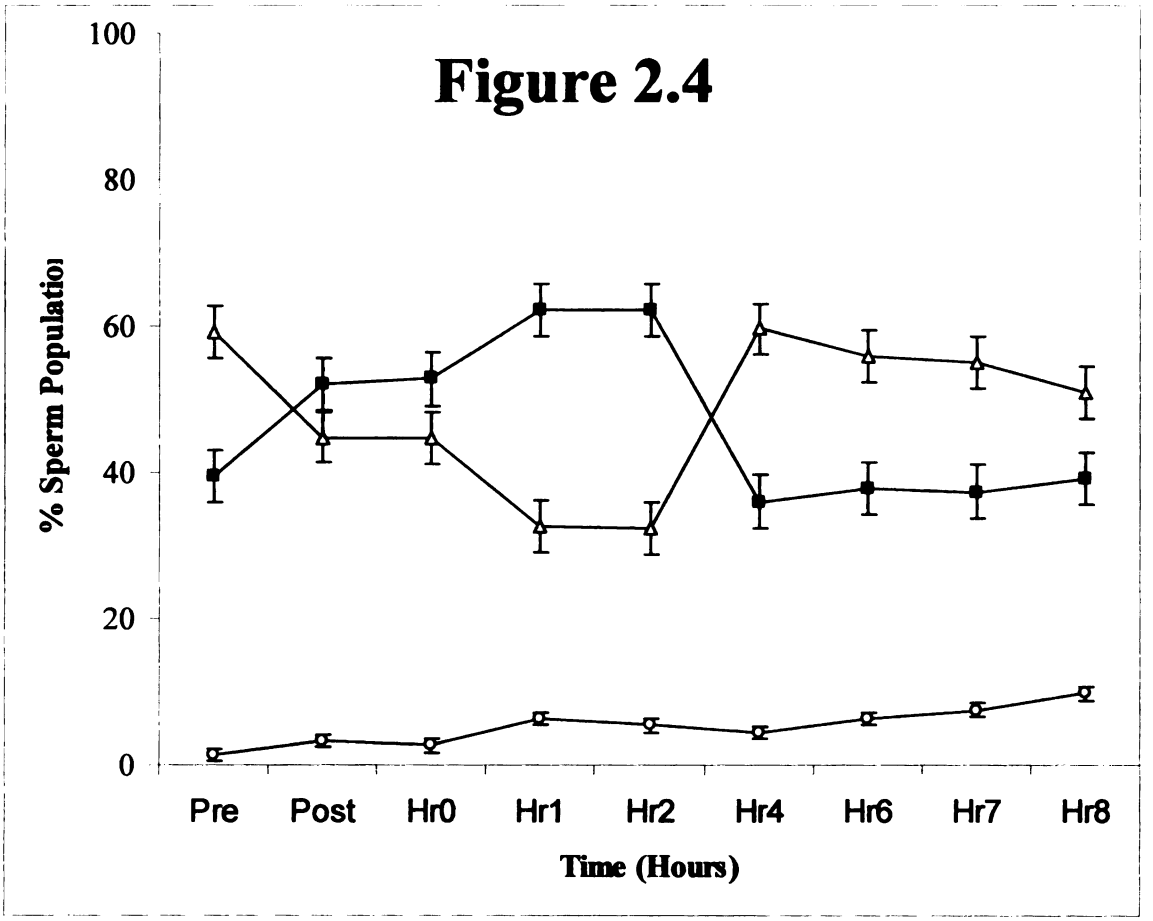


Figure 2.4: Pre-incubation and initial incubation in mM199 containing 0% SP with 20% SP added at Hr 2.

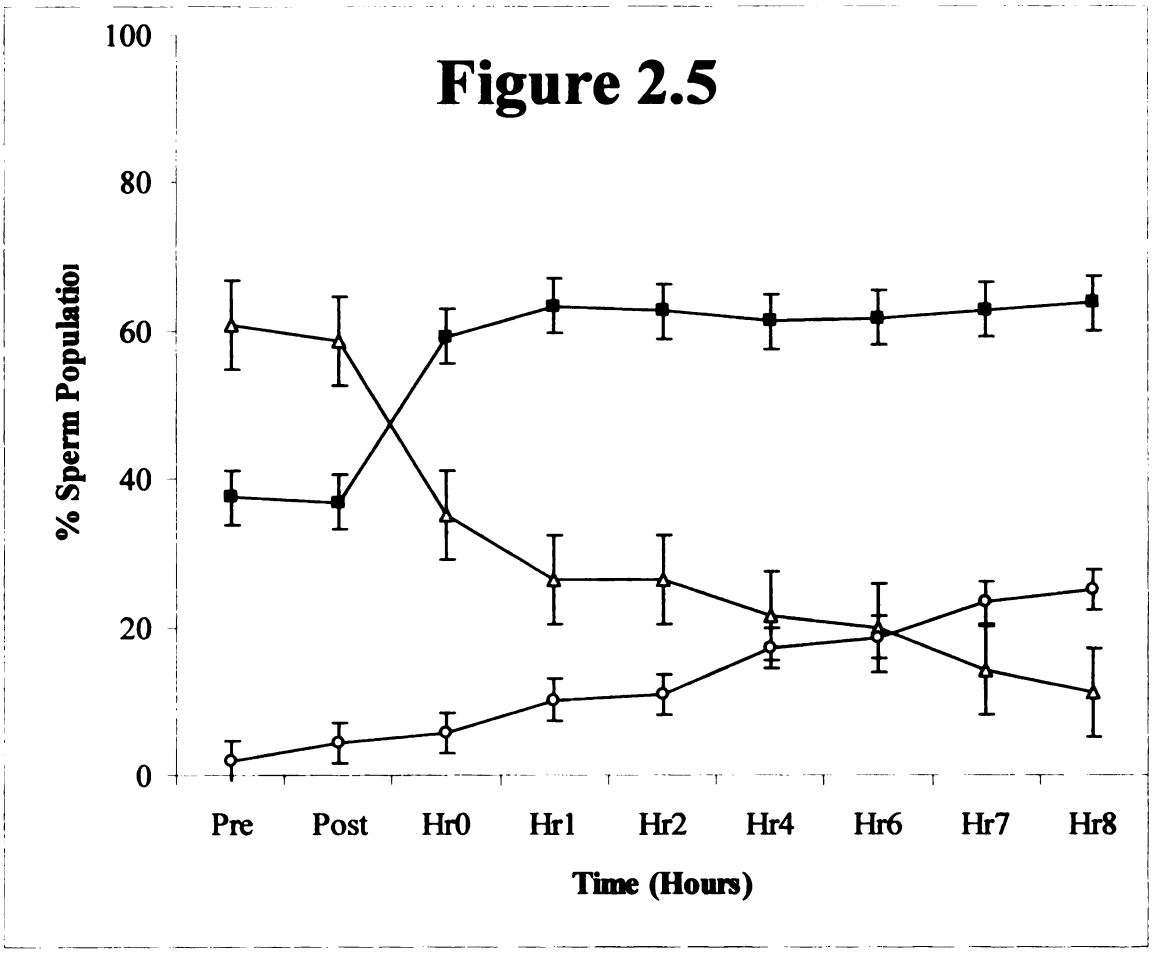


Figure 2.5: Cooling/warming and incubation of spermatozoa in mM199 containing 0% SP.

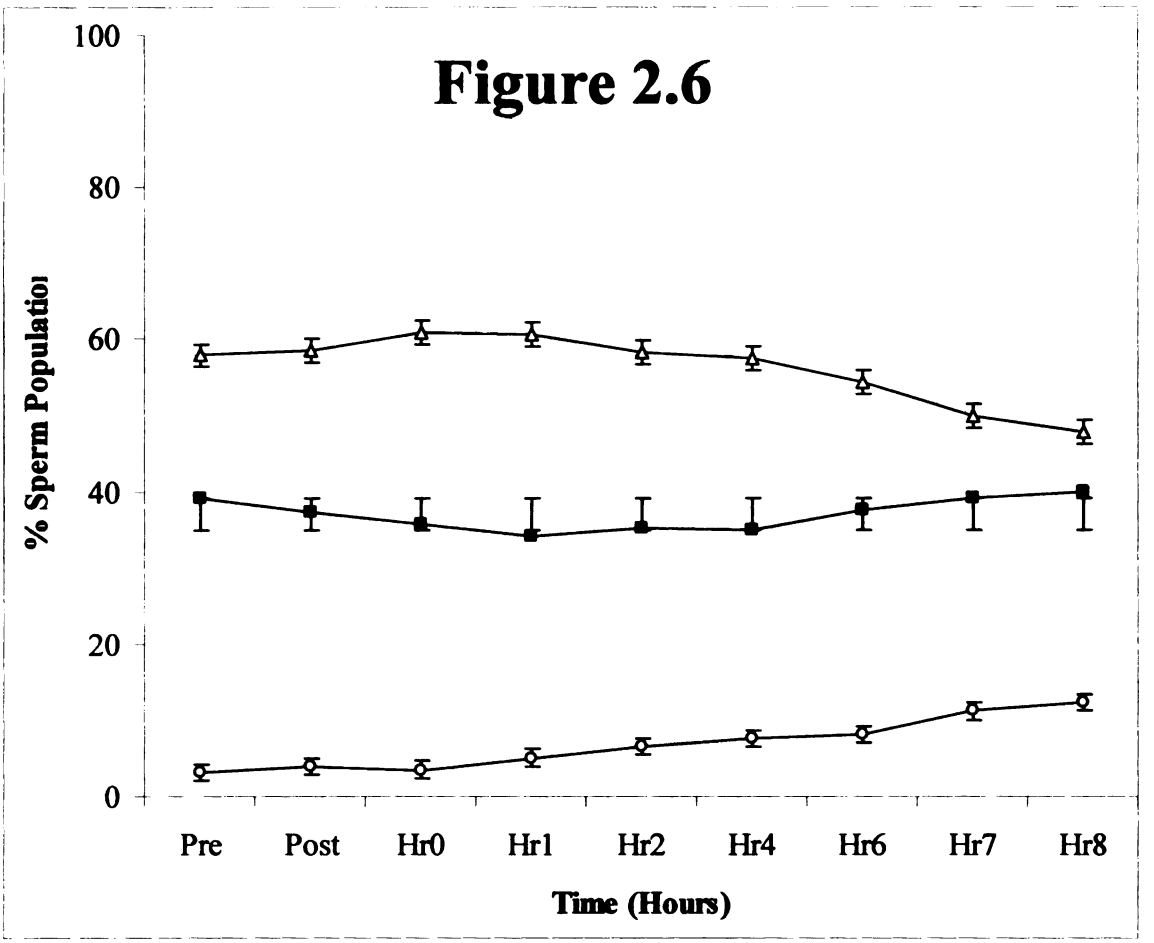


Figure 2.6: Cooling in mM199 containing 0% SP with warming and incubation in mM199 containing 20% SP.

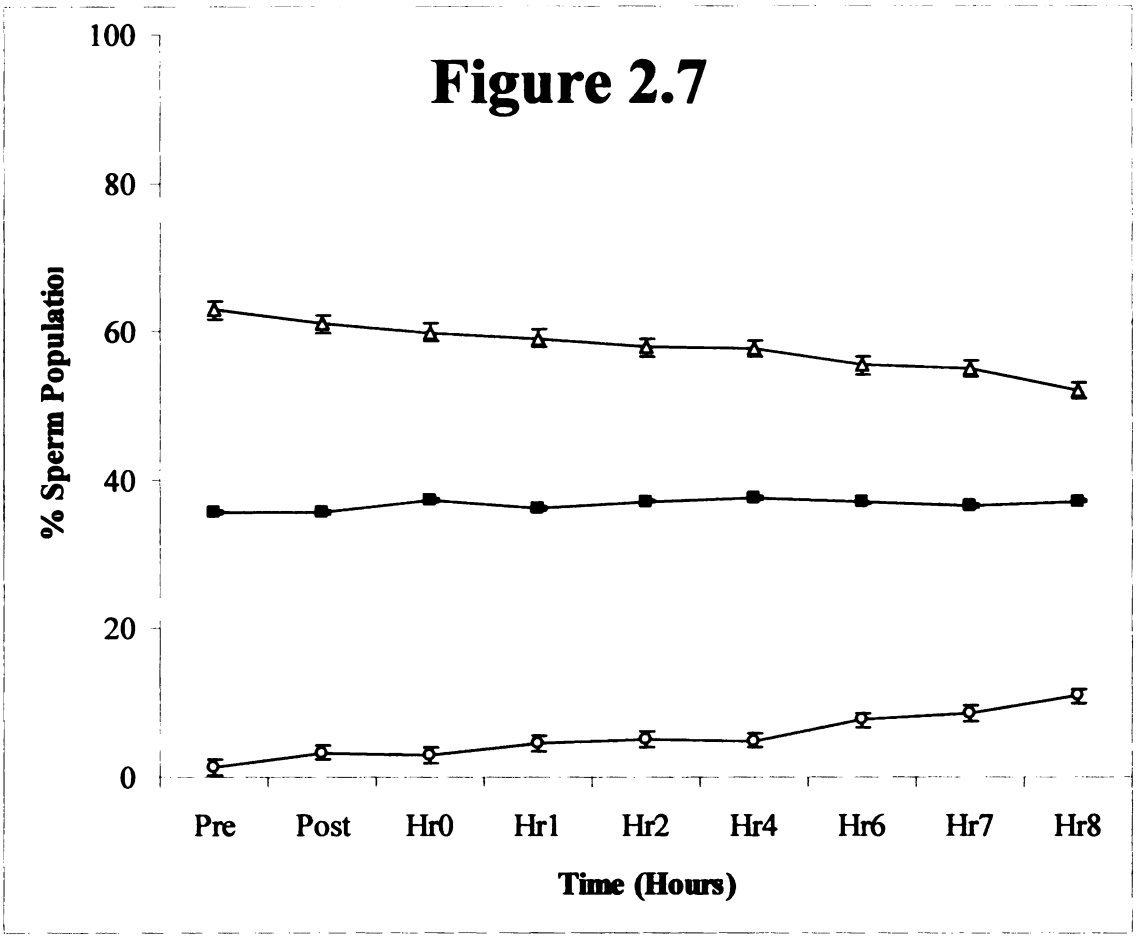


Figure 2.7: Cooling in mM199 containing 20% SP with warming and incubation in mM199 containing 0% SP.

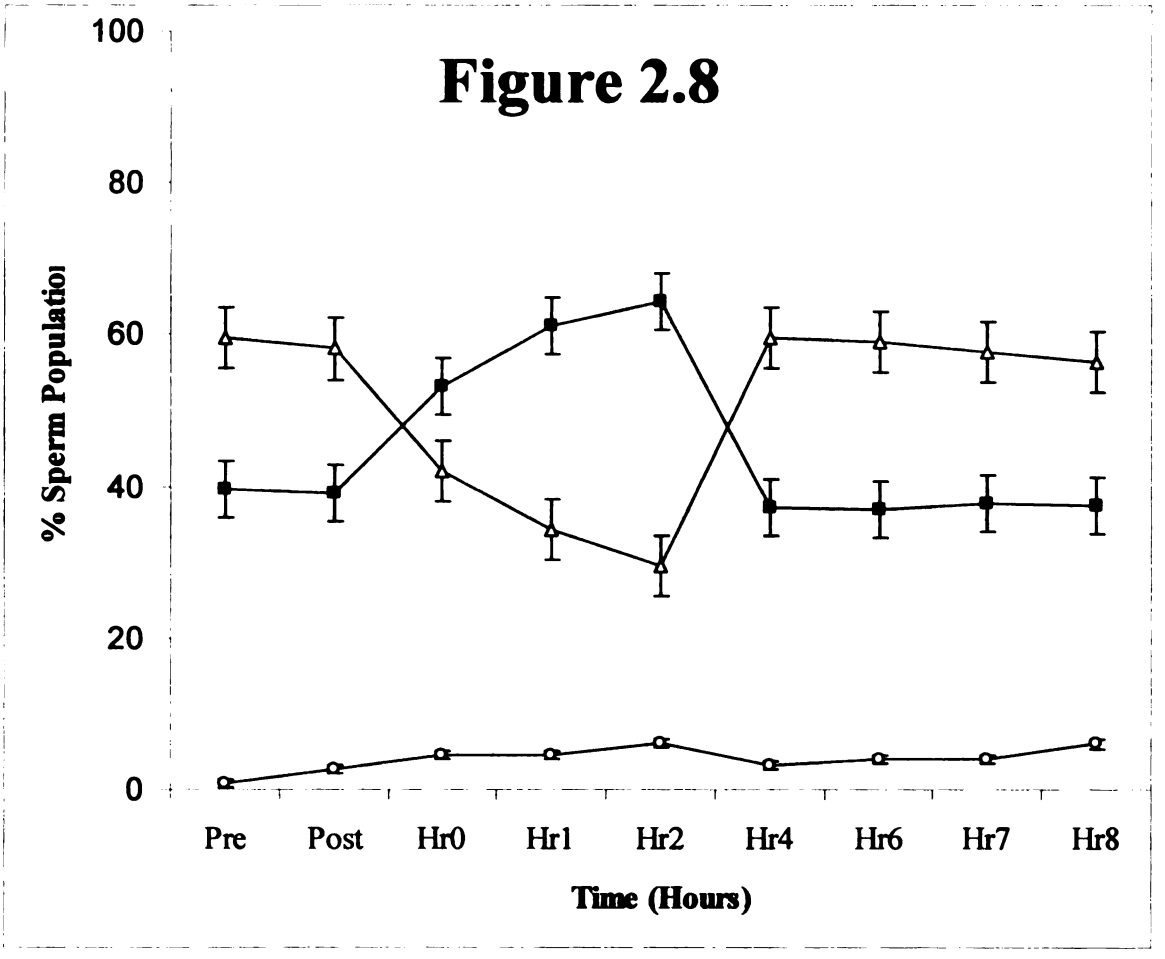


Figure 2.8: Cooling/warming in mM199 containing 0% SP with 20% SP added at Hr 2 of incubation.

CHAPTER 3

The effect of the addition of seminal plasma and the duration of incubation on the post-thaw capacitation status of frozen-thawed boar spermatozoa

Abstract

The present study investigated the effects of adding either 0%, 10%, or 20% (v/v) boar seminal plasma (SP) at thawing to frozen-thawed boar spermatozoa on the status of capacitation. Chlortetracycline staining analysis was used to determine the capacitation and acrosome reaction status of the spermatozoa. One hour after the addition of 10 or 20% (v/v) SP the percent of capacitated spermatozoa decreased from 59.7% to 30.3% and from 59.5% to 26.8%, respectively, ($P<0.001$). The effects of SP on the reversal of capacitation were determined to be complete by 30 min after SP addition to the spermatozoa. These results indicated that SP is able to reverse cryocapacitation of frozen boar spermatozoa within short period of time.

Introduction

The routine use of frozen-thawed (FT) spermatozoa by the swine industry would offer such benefits as enhanced biosecurity with the safe introduction of new genetics into a herd, decreased risk for the dissemination of pathogens capable of transmission via semen, and the preservation of superior genetics. Transmission of viruses such as porcine reproductive and respiratory syndrome virus can occur through boar semen [1]. The use of FT spermatozoa would allow for the identification and regular use of non-infected boars.

Unfortunately, the use of FT spermatozoa results in lower farrowing rates and smaller litter sizes compared to the use of fresh-extended spermatozoa [2, 3]. This effect relates, in part, to cryocapacitation at thawing. The term cryocapacitation describes the capacitation-like process that occurs during cryopreservation and is expressed at the subsequent thawing [4-6]. Capacitated spermatozoa show a decreased ability to bind to the oviductal epithelial cells both in vitro as well as in the female reproductive tract [7, 8] resulting in a decreased number of spermatozoa in the sperm reservoir available to fertilize the ova. Presumably, cryocapacitated spermatozoa demonstrate a similar inability to bind to the oviductal epithelial cells.

Capacitation is a sequential process that may involve the removal of protein decapacitation factors from the spermatozoa membrane that were acquired in the epididymus or at ejaculation [9]. It has been shown that the cold shock-induced membrane damage in ram spermatozoa was reversed following incubation with a solution containing proteins extracted from ram seminal plasma (SP) [10]. In the boar, the epididymal protein anti-agglutinin that is bound to spermatozoa acrosomes during maturation is released at an early stage of capacitation, but this loss was attenuated in spermatozoa incubated in 5 or 10% (v/v) SP [11]. The addition of boar SP also inhibited the capacitation of spermatozoa when incubated in a capacitation-supporting media at 39 C [11, 12].

Our previous work has demonstrated that the addition of 20% SP prevents fresh spermatozoa from undergoing capacitation in a capacitation-supporting environment. Spermatozoa that had already undergone the capacitation-like process experienced a reversal. The objective of the present study was to examine the effect of 10 and 20% (v/v) SP on cryocapacitation of FT boar spermatozoa.

Materials and Methods

All chemicals were of analytical grade. Unless otherwise stated, reagents were purchased from Sigma Chemicals (St. Louis, MO) and solutions prepared with sterile deionized water.

A pool of SP from four boars was accumulated for these studies. The sperm-rich fractions of the ejaculates were centrifuged at 1000xg for 10 min, and the pellet discarded. The SP was stored in 15mL conical tubes at –80 C until required. Prior to use, a tube was thawed overnight in a humidified incubator at 39 C and 5% CO_2 in air.

Medium

To assure the proper environment for capacitation a capacitating-supporting medium (mM199) was used. The medium contained three ingredients that support capacitation in vitro: bicarbonate, bovine serum albumin, and calcium [13, 14]. The mM199 consisted of 87% (v/v) Medium 199 Hanks (Gibco Invitrogen Corporation, Grand Island, New York), 12% (v/v) heat-activated fetal calf serum, 1% (v/v) penicillin-streptomycin, 2.3% (wt/vol) bovine serum albumin, 3.05 mM D-glucose, 2.91 mM calcium lactate, and 0.91

mM sodium pyruvate, with pH adjusted to 7.8. The mM199 was equilibrated overnight in a humidified incubator at 39 C and 5% CO_2 in air prior to use.

Spermatozoa processing for treatments

Frozen semen from three Yorkshire boars was purchased from International Boar Semen (Eldora, IA) in 5mL maxi-straws. Straws were held at room temperature for 7 sec before being submerged horizontally into a 42 C water bath for 43 sec. Immediately after thawing, the straws were emptied into 15mL conical tubes and centrifuged for 10 min at 600xg. The supernatant was discarded, and 5mL of mM199 at 39 C was added. The spermatozoa suspension was then centrifuged for 5 min at 600xg. Again the supernatant was discarded, and the spermatozoa pellet was resuspended in 5mL of mM199 with or without SP at 39 C. A bright-line microscopic assessment (400x) of progressive motility was performed directly after thawing and repeated after the final centrifugation. A percent motile was assigned within 10%. The spermatozoa suspension remained in a humidified incubator in 5% CO_2 in air for the remainder of the treatment period.

Treatments

Four treatments were performed with each of the three boars. In Treatments 1, 2, and 3 the spermatozoa suspensions were incubated at 39 C for 8 h after thawing. In Treatment 4 the spermatozoa suspension was incubated at 39 C for 1 h after thawing. No SP was added in Treatment 1. Treatments 2 and 4 included 10% (v/v) SP added after the final centrifugation. Treatment 3 included 20% (v/v) SP added after the final centrifugation.

Chlortetracycline (CTC) staining analysis (Appendix II)

The CTC staining assay is based on the transfer of neutral, uncomplexed CTC across the spermatozoa membrane resulting in the fluorescent patterns characteristic of the various transitional phases [15] that spermatozoa display (fresh, capacitated, acrosome reaction). Frozen-thawed spermatozoa display an increase in the CTC fluorescence pattern corresponding to the capacitated state [16-18].

For CTC staining [19], 20 µl of 500 µM CTC in a chilled buffer of 20 mM Tris, 130 mM sodium chloride, and 5 mM L-cysteine prepared the day of each treatment was added to a warm (37 C) microscope slide followed by 20 µl of spermatozoa suspension and mixed. After 10 sec, spermatozoa were fixed with 10 µl of 12.5% glutaraldehyde in Tris buffer (pH 7.8) and mixed again. A cover slip was applied, and the slide was compressed between bibulous papers to remove excess fluid and maximize the number of spermatozoa lying flat on the slide prior to observation. Prepared slides were placed in a black box until read within 1 h of preparation. Spermatozoa were illuminated with a 100 W mercury bulb and examined under a Nikon Optiphot microscope equipped with a 380-425 nm excitation filter, a 520 nm barrier filter, and a 510 nm dichroic mirror (Nikon Co., Tokyo, Japan).

For Treatments 1, 2, and 3 slides were prepared for CTC staining analysis directly after the final centrifugation and resuspension (Hour 0); and at 1, 2, 4, 6, 7, and 8 h after Hour 0. For Treatment 4 slides were prepared at Hour 0, 15 min 30 min, 45 min, and at Hour 1. Two slides were prepared for each sample, and 100 spermatozoa were counted on each

slide. The average of both slides was determined. Fluorescent patterns were used to demonstrate the progress of capacitation and the acrosome reaction. Fresh (F) spermatozoa (uncapacitated) were characterized by a bright anterior region with a bright acrosome cap, a relatively faint posterior region, and a dark half circle at the equatorial region. Those with equally bright anterior and equatorial regions and a less bright posterior region were also scored as F. Capacitated (B) spermatozoa were consistent with a faint anterior region, a bright equatorial region, and a faint posterior region. Acrosome reacted (AR) spermatozoa were characterized by a very faint anterior region, a slightly bright equatorial region, and a faint posterior region.

Statistical Analysis

Data were analyzed using Statistical Analysis Systems software (SAS Institute Inc., Cary, NC). Repeated measures ANOVA was conducted to determine treatment differences at each time point separately for each of the response categories (fresh, capacitated, acrosome reacted) and for percent motile. Least-squares means for each treatment and time were back-transformed for the purposes of reporting point estimates and preparing figures.

Results

The average percent of progressive motile spermatozoa was 60% at post-thaw and 55% after the final centrifugation. No significant differences ($P>0.05$) were observed between treatment groups or times.

In Treatment 1, where no SP was included, the percent F spermatozoa remained relatively unchanged throughout the 8 h incubation period only decreasing from 31.2% to 30.5%. The percent B spermatozoa decreased from 59.7% to 48.3% while the percent AR increased from 9.2% to 21.2% (Figure 3.1).

In Treatment 2, where 10% SP was included, there was an increase in the percent of F spermatozoa from 31.7% at Hour 0 to 61.2% at Hour 1. Conversely, there was a decrease in the percent of B pattern spermatozoa from 59.7% at Hour 0 to 30.3% at Hour 1 (Figure 3.2). After reversal, the percent F and B spermatozoa remained relatively constant throughout the remainder of the incubation period. The percent AR spermatozoa increased from 8.7% at Hour 0 to 13% at Hour 8.

In Treatment 3, where 20% SP was included, changes in percent F, B, and AR spermatozoa were similar to those observed in Treatment 2 (Figure 3.3). The percent F spermatozoa were 31.7% and 63.3% at Hours 0 and 1, respectively. The percent B spermatozoa decreased from 59.5% at Hour 0 to 26.8% at Hour 1. The percent AR spermatozoa increased from 8.8% to 12.7% during 8 h of incubation.

In Treatment 4, where 10% SP was included, the percent F spermatozoa increased from 29%, to 55%, to 64% while the percent B spermatozoa decreased from 64.5%, to 39.5%, to 30%, at 0 min, 15 min, and 30 min respectively. Percentages remained relatively unchanged over the next 30 min of incubation (Figure 3.4). The percent spermatozoa that

had undergone the acrosome reaction remained relatively unchanged over the 1 h incubation, increasing from 6.5% to 7.5%.

Discussion

Our findings confirmed that the majority of FT spermatozoa are cryocapacitated upon thawing [5, 20, 21]. The present data also supports our previous studies with cooled, fresh spermatozoa [12] in that 20% (v/v) SP was capable of reversing the capacitation-like process. We now extend those observations to include the observation that 10% (v/v) SP was capable of reversing cryocapacitation and that the effect of SP was evident within 15 min of its addition to the spermatozoa and complete by 30 min. We also determined that centrifugation and resuspension of the FT spermatozoa did not significantly alter their motility.

The entire sequence of events involved in capacitation is not fully understood; however, several major molecular changes have been linked to capacitation. In boar spermatozoa, bicarbonate and exposure to CO_2 cause a reversible increase in plasma membrane lipid disorder resulting in an increased concentration of cholesterol at the apical ridge [22]. This increased apical concentration of cholesterol may be an intermediate capacitation state that allows for albumin-mediated cholesterol extraction [23]. Further membrane destabilization occurs with the loss of proteins and carbohydrates from the plasma membrane. The membrane potential shifts with the increased membrane fluidity causing an influx of calcium ions that drive the cAMP-protein phosphorylation [9].

The observations in the late 1950's that SP caused a reversible inhibition of fertilizing ability in rabbit spermatozoa [24] and ultracentrifugation of rabbit SP sedimented a decapacitation factor [25] have lead to the concept that capacitation involves the removal of coating macromolecules from the spermatozoa plasma membrane. The loss of proteins and carbohydrates from the plasma membrane during capacitation may be the loss of the decapacitation factors resulting in a reduced ability to bind to the oviductal epithelial cells. In bull, cryopreservation resulted in a 70 to 80% decrease in sperm-bound bovine seminal plasma (BSP) proteins [26]. These proteins are responsible for controlling cholesterol efflux and are mediators in spermatozoa-oviduct binding [27].

The results of this study predict that the addition of SP to FT boar spermatozoa would prevent and/or reverse cryocapacitation, thereby, potentially improving fertility after artificial insemination. Greater utilization of FT spermatozoa would allow the swine industry to maximize the efficiency of genetically superior boars. Swine farms could introduce superior genetics, disease-free, without having to quarantine new boars. Through increased biosecurity the industry-wide risks to swine health would decrease.

References

1. Hennings, C., Holler, L., Benfield, D., Nelson, E. Detection and duration of porcine reproductive and respiratory syndrome virus in semen, serum, peripheral blood mononuclear cells, and tissues from Yorkshire, Hampshire, and Landrace boars. J Vet Diagn Invest., 2001. 13: 133-42.

2. Almlid, T., Hofmo, P. A brief review of frozen semen application under Norwegian AI service conditions. Reprod Dom Anim, 1996. 31: 169-73.

3. Reed, H. Current use of frozen boar semen-future need of frozen boar semen., in Deep freezing of boar semen., J. LA and L. K, Editors. 1985, Swedish University of Agricultural Science: Uppsala. 225-37.

4. Curry, M.R., Kleinhans, F.W., Watson, P.F. Measurement of the Water Permeability of the Membranes of Boar, Ram, and Rabbit Spermatozoa Using Concentration-Dependent Self-Quenching of an Entrapped Fluorophore*1. Cryobiology, 2000. 41: 167-73.

5. Bailey, J., Bilodeau, J., Cormier, N. Semen cryopreservation in domestic animals: a damaging and capacitating phenomenon. J Androl, 2000. 21: 1-7.

6. Watson, P.F. The causes of reduced fertility with cryopreserved semen. Anim Reprod Sci, 2000. 60-61: 481-92.

7. Medeiros, C., Forell, F., Oliveira, A.T.D., Rodrigues, J.L. Current status of sperm cryopreservation: why isn't it better? Theriogenology, 2002. 57: 327-44.

8. Tienthai, P., Johannisson, A., Rodriguez-Martinez, H. Sperm capacitation in the porcine oviduct. Anim Reprod Sci, 2004. 80: 131-46.

9. Watson, P., Green, C.E. Cooling and Capacitation of Boar Spermatozoa: What do They Have in Common? In: Boar Semen Preservation IV, 2000. 53-60.

10. Barrios., Perez Pe, R., Gallego, M., Tato, A., Osada, J., Muino Blanco, T., Cebrian Perez, J.A. Seminal plasma proteins revert the cold-shock damage on ram sperm membrane. Biol Reprod, 2000. 63: 1531-7.

11. Harayama, H., Magargee, S.F., Kunze, E., Shidara, O., Iwamoto, E., Arikawa, S., Miyake, M., Kato, S., Hammerstedt, R.H. Changes in epididymal protein anti-agglutinin on ejaculated boar spermatozoa during capacitation in vitro. Reprod Fertil Dev, 1999. 11: 193-9.

12. Vadnais, M.L., Kirkwood, R., Sprecher, D.J., Tempelman, D., Chou, K. Effect of cooling and seminal plasma on the capacitation status of boar sperm as

determined by chlortetracycline staining. Submitted to Anim Reprod Sci, June 2004.

13. Harrison, R. Capacitation mechanisms, and the role of capacitation as seen in eutherian mammals. Reprod Fertil Dev, 1996. 8: 581-94.

14. Visconti, P., Kopf, G. Regulation of protein phosphorylation during sperm capacitation. Biol Reprod, 1998. 59: 1-6.

15. Ward, C., Storey, B. Determination of the time course of capacitation in mouse spermatozoa using a chlortetracycline fluorescence assay. Dev Biol, 1984. 104: 287-96.

16. Cormier, N., Sirard, M.A., Bailey, J.L. Premature capacitation of bovine spermatozoa is initiated by cryopreservation. J Androl, 1997. 18: 461-8.

17. Gillan, L., Evans, G., Maxwell, W. Capacitation status and fertility of fresh and frozen-thawed ram spermatozoa. Reprod Fertil Dev, 1997. 9: 481-7.

18. Perez, L.J., Valcarcel, A., de las Heras, M.A., Moses, D., Baldassarre, H. Evidence that frozen/thawed ram spermatozoa show accelerated capacitation in vitro as assessed by chlortetracycline assay. Theriogenology, 1996. 46: 131-40.

19. Lin, C. Regulation of sperm capacitation. Doctoral Thesis. Michigan State University, East Lansing, Michigan 1996.

20. Maxwell, W., Johnson, L. Chlortetracycline analysis of boar spermatozoa after incubation, flow cytometric sorting, cooling, or cryopreservation. Molec Reprod Dev, 1997. 46: 408-18.

21. Watson, P.F. Cooling of boar spermatozoa and fertilizing capacity. Reprod Dom Anim, 1996. 31: 135-40.

22. Harrison, R.A., Ashworth, P., Miller, N. Bicarbonate/CO2, an effector of capacitation, induced a rapid and reversible change in the lipid architecture of boar sperm plasma membranes. Molec Reprod Dev, 1996. 45: 378-91.

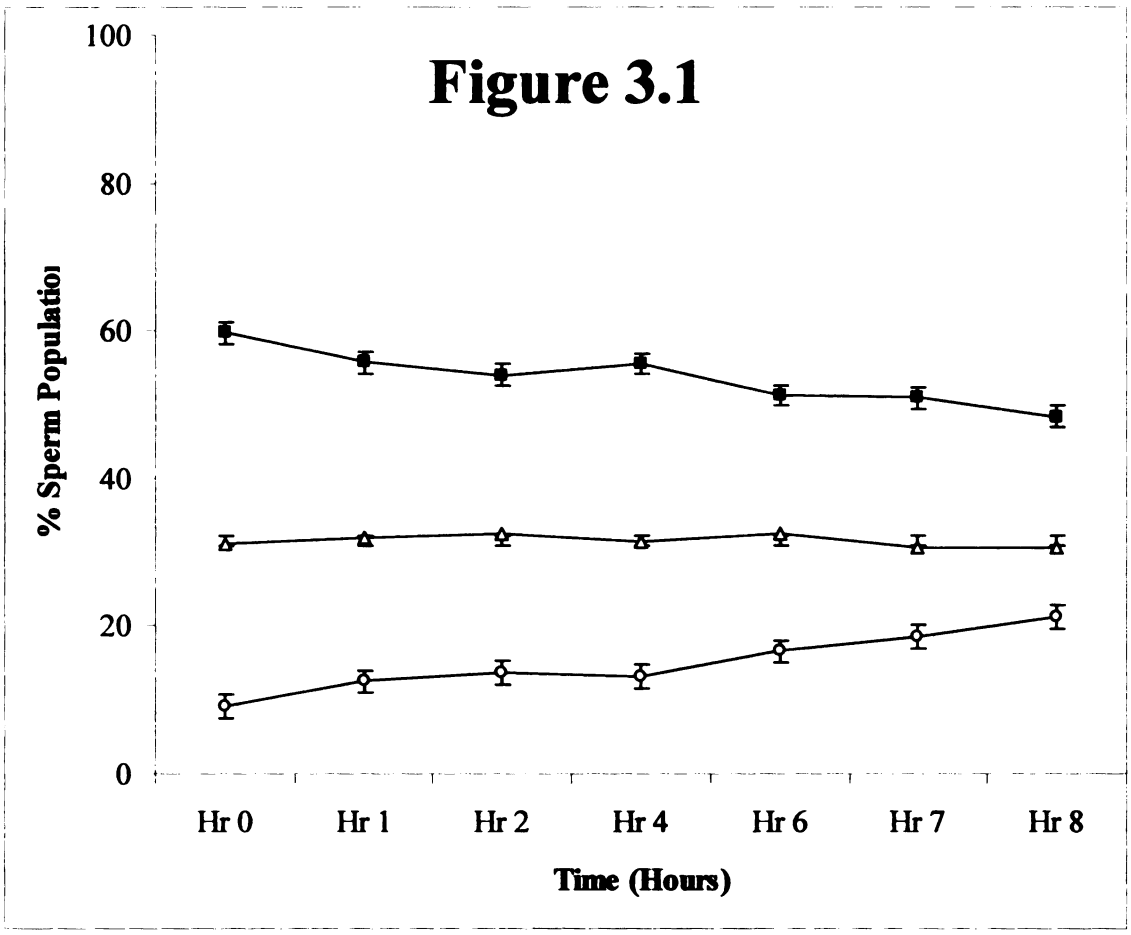
23. Flesch F.M., Brouwers, J., Nievelstein, P., Verkleij, A.J., van Golde, L.M.G., Gadella, B.M., Bicarbonate stimulated phospholipid scrambling induces cholesterol redistribution and enables cholesterol depletion in the sperm plasma membrane. J Cell Biol, 2001. 114: 3543-55.

24. Chang, M. A detrimental effect of seminal plasma on the fertilizing capacity of sperm. Nature, Lond, 1957. 179: 258-9.

25. Bedford, J., Chang, M. Removal of decapacitation factor from seminal plasma by high speed centrifugation. Am J Phys, 1962. 202: 179-81.

26. Nauc, V., Manjunath, P. Radioimmunoassays for bull seminal plasma proteins (BSP-A1/-A2, BSP-30 Kilodaltons), and their quantification in seminal plasma and sperm. Biol Reprod, 2000. 63: 1058-66.

27. Therien, I., Soubeyrand, S., Manjunath, P. Major proteins of bovine seminal plasma modulate sperm capacitation by high-density lipoprotein. Biol Reprod, 1997. 57: 1080-8.



In Figures 3.1 through 3.4, Δ represent Fresh spermatozoa, ■ represent Capacitated (B) spermatozoa, and ○ represent Acrosome Reacted spermatozoa. Each point represents the average of 6 counts ± SE.

Figure 3.1: Frozen spermatozoa thawed in mM199 containing 0% SP.

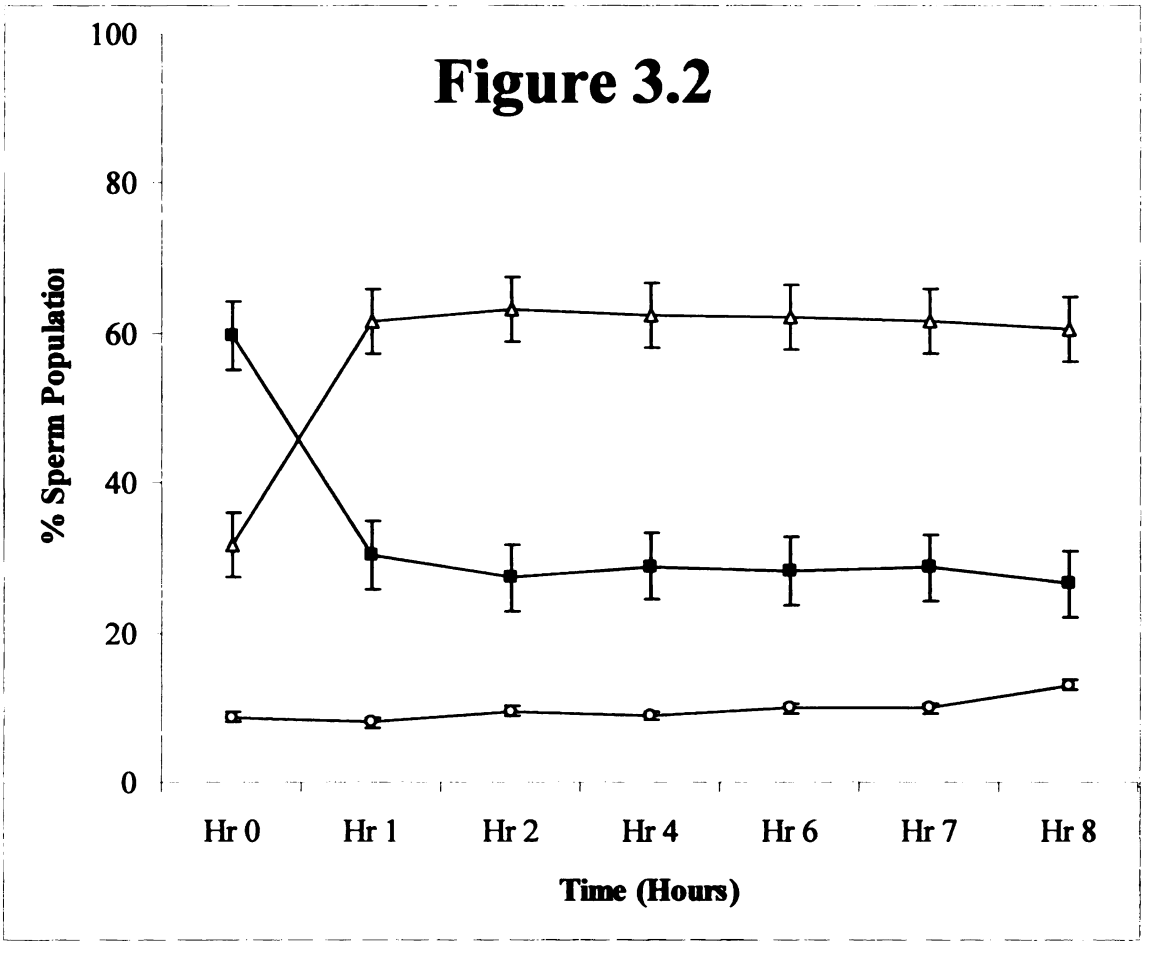


Figure 3.2: Frozen spermatozoa thawed in mM199 containing 10% SP.

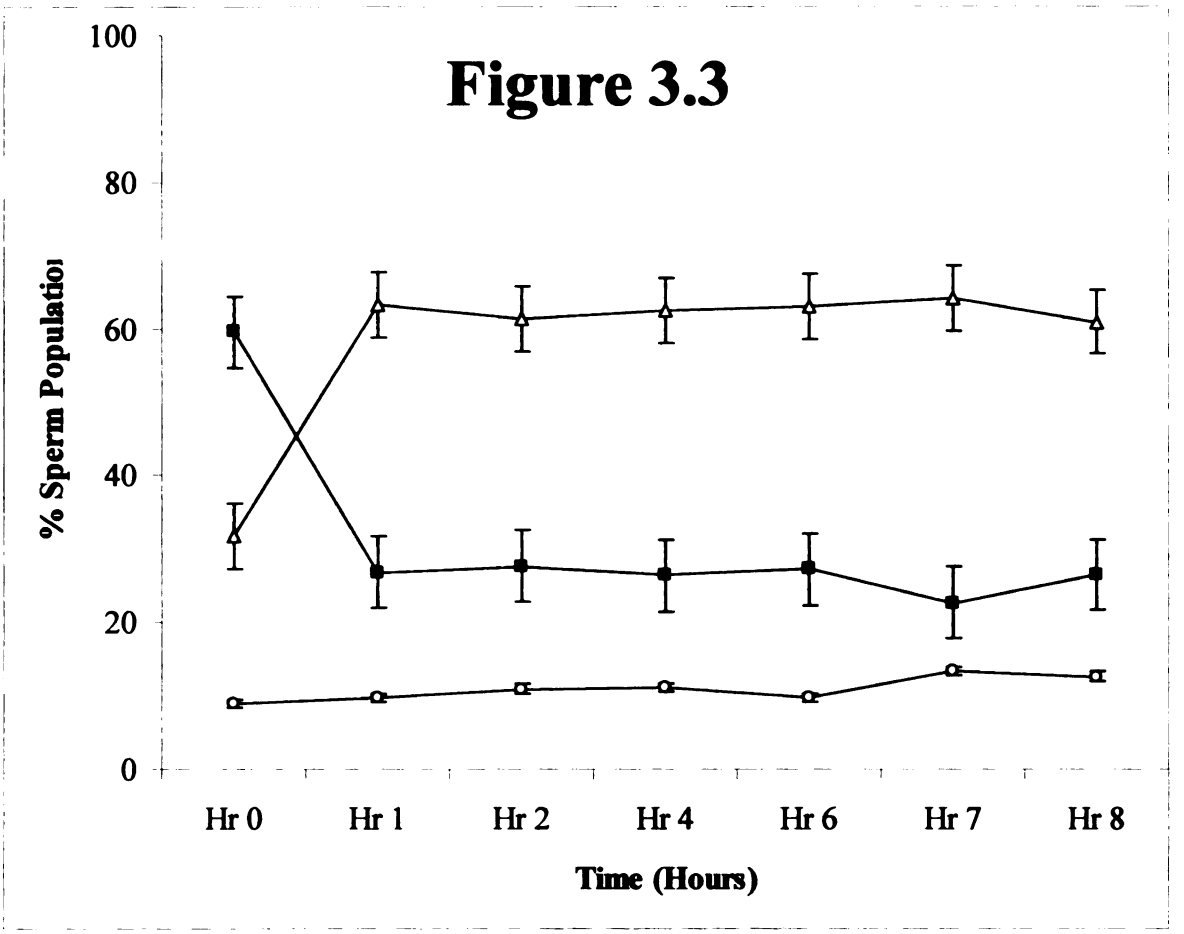


Figure 3.3: Frozen spermatozoa thawed in mM199 containing 20% SP.

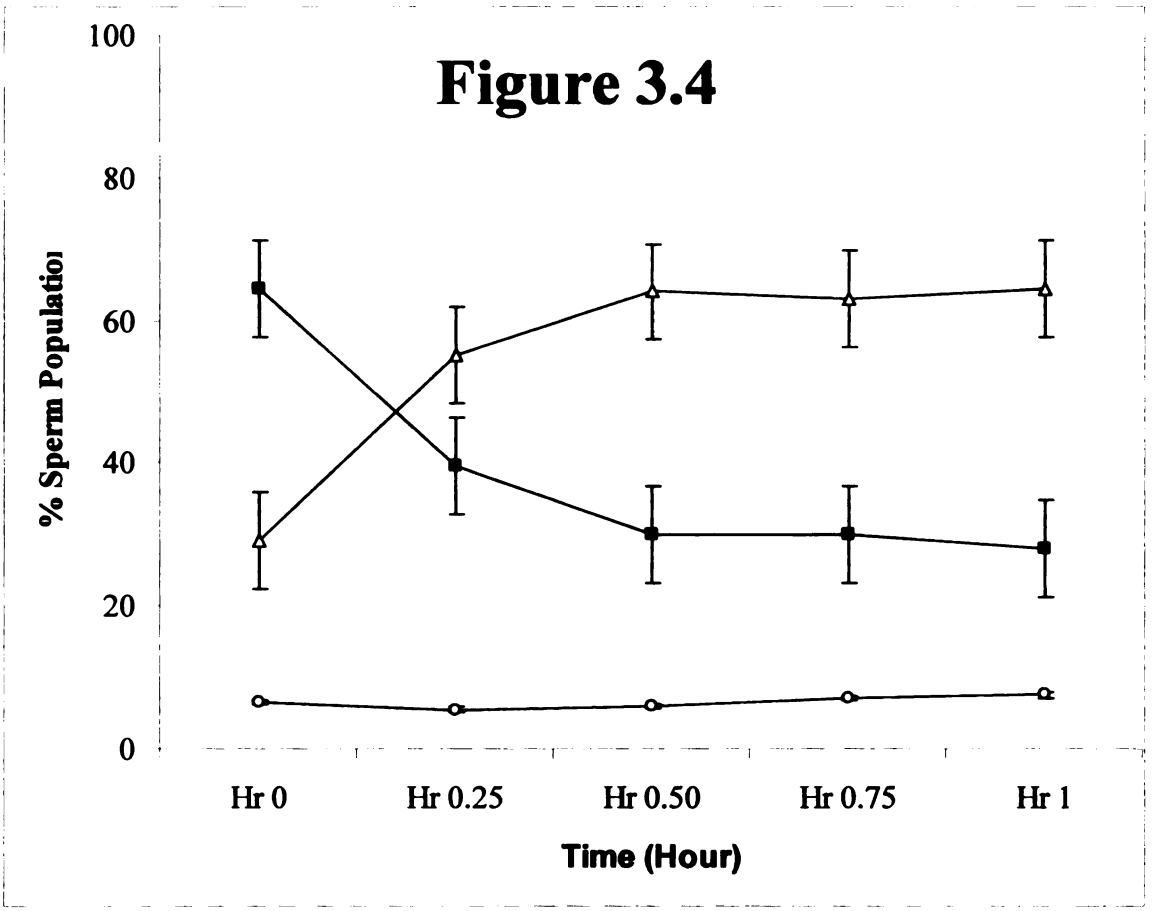


Figure 3.4: Frozen spermatozoa thawed in mM199 containing 10% SP with readings taken at 15 min intervals over 1 h.

CHAPTER 4

The effects of egg yolk freezing extender, incubation temperature, and the addition of seminal plasma on cryocapacitation of frozen-thawed boar spermatozoa as determined by chlortetracycline analysis

Abstract

The addition of seminal plasma (SP) to frozen-thawed (FT) spermatozoa separated from the egg yolk-based freezing extender decreased the percent of capacitated spermatozoa within 30 min of its addition. The present study examined the effect of 10% (v/v) boar SP addition to FT boar spermatozoa extended in either X-cell thawing extender with or without the egg yolk-based freezing extender or in phosphate buffered saline (PBS) at either 17 C or 39 C. No effects of SP resulted from addition to spermatozoa when the egg yolk-based freezing extender was included or when the temperature was maintained at 17 C ($P>0.001$). However, the percent of capacitated spermatozoa were 36.5% and 37.3% ($P<0.001$) when spermatozoa were incubated at 39 C with 10% (v/v) SP for 30 min in either PBS or X-cell thawing extender without the egg yolk-based freezing extender, respectively. The effects of SP addition to the spermatozoa are dependent on both temperature and the type of extender used to support freezing.

Introduction

Regardless of the nearly 30 years of commercial availability of frozen-thawed (FT) boar spermatozoa, it has not received widespread acceptance for commercial breeding in the swine industry. This is in part due to the higher semen costs, lower farrowing rates, and smaller litter sizes associated with the use of FT spermatozoa [1, 2]. The decrease in

fertility with FT spermatozoa is believed to be a result of the spermatozoa being effectively capacitated upon thawing [3-5]. This reaction has been termed cryocapacitation. Capacitated spermatozoa do not enter the spermatozoa reservoir therefore having a shortened period of time to meet and fertilize an oocyte [6, 7]. If there is not an oocyte present, the spermatozoa die shortly after capacitation.

Capacitation is a multi-step process that involves the removal of epididymal and seminal plasma (SP) proteins from the spermatozoa membrane [8]. The boar epididymal protein anti-agglutinin is bound to spermatozoa acrosomes during maturation and is released at an early stage of capacitation, but this loss was reduced in spermatozoa incubated in 5 or 10% (v/v) SP [9]. Similarly, the addition of 10 or 20% (v/v) SP to spermatozoa capacitated in vitro at 39 C decreased the percent of capacitated spermatozoa [9, 10].

In our previous experiments, when 10% (v/v) SP was added to washed FT boar spermatozoa that were incubated at 39 C, cryocapacitation was reversed within 30 min of the SP inclusion. It is evident that SP plays an important role in the capacitation process. It is unclear if the effects of SP addition are dependent on the incubation temperature or the media it is added to. The objective of the present study was to examine the effects of egg yolk freezing extender, incubation temperature, and the addition of SP on the cryocapacitation of FT boar spermatozoa.

Materials and Methods

All chemicals were of analytical grade. Unless otherwise stated, reagents were purchased from Sigma Chemicals (St. Louis, MO) and solutions prepared with sterile deionized water.

A pool of SP from four boars was accumulated for these studies. The sperm-rich fractions of the ejaculates were centrifuged at 1000 x g for 10 min, and the pellet discarded. The SP was stored in 15mL conical tubes at –80 C until required. Prior to use, a tube was thawed overnight in a humidified incubator at 39 C and 5% CO_2 in air.

Media

The Phosphate Buffered Saline (PBS) solution contained 160 mM sodium chloride, 8 mM $NaHPO_4$ (anhydrous), and 2 mM $NaHPO_4 \cdot H_2O$ with pH adjusted to 7.4. The PBS was equilibrated overnight in a humidified incubator at 39 C and 5% CO_2 in air prior to use. The commercial semen extender employed was X-cell (IMV, Maple Grove, MN).

Treatments (Appendix IV)

Frozen semen from three Yorkshire boars was purchased from International Boar Semen (Eldora, IA) in 5mL maxi-straws. For thawing, straws were held at room temperature for 7 sec before being submerged horizontally into a 42 C water bath for 43 sec. Temperature was maintained in either a humidified incubator at 39 C and 5% CO_2 in air or in a Digital Temperature Control Electronic Cooler/warmer (TR Enterprises Inc, Aberdeen, NC) at 17 C for a 30 min incubation period prior to CTC analysis. Where indicated, 10% (v/v)

SP was added to the spermatozoa suspension. A microscopic assessment of progressive motility was performed immediately after thawing, the final centrifugation, and the 30 min incubation period. A percent motile was assigned within 10%.

Experiment 1a: For this experiment, thawed semen was extended directly in X-cell and so included the egg yolk-based freezing extender in the extended semen. Treatments were a 2x2 factorial arrangement with main effects being temperature during the holding period (17 or 39 C) and presence of SP (0 or 10%). After the 30 min holding period a 5mL aliquot was extracted and centrifuged at 600xg for 10min. The pellet was resuspended in 5 mL X-cell at either 17 or 39 C to remove the egg yolk and immediately used for CTC analysis.

Experiment 1b: This experiment was a repeat of experiment 1a, except it was performed in PBS without the egg yolk-based freezing extender included in the final spermatozoa suspension. Immediately after thawing, semen straws were emptied into 15 mL conical tubes and centrifuged for 10 min at 600xg. The supernatant was discarded, and 5mL of PBS at 17 or 39 C was added. The spermatozoa suspension was then centrifuged for 5 min at 600 x g. Again, the supernatant was discarded and the spermatozoa pellet resuspended in 5mL of PBS with or without SP. All procedures were performed at either 17 or 39 C.

Experiment 1c: This experiment was performed using X-cell but without the egg yolk-based freezing extender included in the final spermatozoa suspension. Immediately after

thawing, the straws were emptied into 15 mL conical tubes and centrifuged for 10 min at 600 x g. The supernatant was discarded, and 5mL of IBS thawing extender at 39 C was added. The spermatozoa suspension was then centrifuged for 5 min at 600 x g. Again the supernatant was discarded, and the spermatozoa pellet was resuspended in 5mL of X-cell extender with or without SP, all at 39 C.

Chlortetracycline (CTC) staining analysis (Appendix II)

CTC staining analysis [11] was used to identify the progress of capacitation and acrosome reaction of boar spermatozoa. For CTC staining, 20 µl of 500 µM CTC in a chilled buffer of 20 mM Tris, 130 mM sodium chloride, and 5 mM L-cysteine prepared on the day of each treatment was added to a warm (37 C) microscope slide followed by 20 µl of spermatozoa suspension and mixed. After 10 sec, spermatozoa were fixed with 10 µl of 12.5% glutaraldehyde in Tris buffer (pH 7.8) and mixed again. A cover slip was applied, and the slide was compressed between bibulous papers to remove excess fluid and maximize the number of spermatozoa lying flat on the slide prior to observation. Prepared slides, read within 1 h of preparation, were placed in a black box prior to reading. Spermatozoa were illuminated with a 100 W mercury bulb and examined under a Nikon Optiphot microscope equipped with a 380-425 nm excitation filter, a 520 nm barrier filter, and a 510 nm dichroic mirror (Nikon Co., Tokyo, Japan).

Slides were prepared for CTC staining analysis directly after the 30 min incubation period. Two slides were prepared for each sample, and 100 spermatozoa were counted on each slide. The average of both slides was determined. Four fluorescent patterns were

used to demonstrate the progress of fresh, capacitated, and acrosome reacted spermatozoa. Fresh (F) spermatozoa (uncapacitated) were characterized by either a bright anterior region with a bright acrosome cap, a relatively faint posterior region, and a dark half circle at the equatorial region or by equally bright anterior and equatorial regions and a less bright posterior region. Capacitated (B) spermatozoa were consistent with a faint anterior region, a bright equatorial region, and a faint posterior region. Acrosome reacted (AR) spermatozoa were characterized by a very faint anterior region, a slightly bright equatorial region, and a faint posterior region.

Bis-Benzimide Hoechst No. 33258 staining analysis

Hoechst 33258 was used for all 10 treatments to determine effect of treatment on viability of the spermatozoa. A 5 µl sample of chilled stock solution (200 µM Hoechst 33258) was added to 100 µl of spermatozoa suspension and incubated for 15 min at 39 C and 5% CO_2 in the dark. Subsequently, 20 µl of stained spermatozoa suspension was added to a warm (37 C) microscope slide and fixed with 5 µl 12.5% glutaraldehyde. A cover slip was applied and the slide was compressed between bibulous papers. Prepared slides were placed in a black box until read within 1 h of preparation. Spermatozoa were illuminated with a 100 W mercury bulb and examined under a Nikon Optiphot microscope equipped with a 365/10 nm excitation filter, a 400 nm barrier filter, and a 400 nm dichroic mirror (Nikon Co., Tokyo, Japan).

Slides were prepared at the same time points as for CTC staining analysis. Two slides were prepared for each sample, and 100 spermatozoa were counted on each slide. The

average of both slides was determined. Spermatozoa exhibiting bright blue fluorescence over the entire head were considered nonviable cells.

Statistical Analysis

Data were analyzed using Statistical Analysis Systems software (SAS Institute Inc., Cary, NC). Two-way ANOVA was conducted to infer upon treatment differences separately for each of the response categories (fresh, capacitated, and acrosome-reacted). Incubation temperature and the media used for spermatozoa suspension were used as the variables in the two-way ANOVA.

Results

The effect of seminal plasma and holding temperature on capacitation status of spermatozoa in X-cell commercial semen extender are shown in Table 1. As can be seen, when the egg yolk-based freezing extender was included in the final spermatozoa suspension, there was not effect of seminal plasma on capacitation status at either temperature.

When the egg yolk-based freezing extender was removed prior to extension in PBS (treatments 5 to 8), seminal plasma reversed capacitation ($P<0.001$) only in spermatozoa suspensions held at 39 C (Table 2). Similarly, when extended in X-cell without the freezing extender and held at 39 C (treatments 9 and 10), seminal plasma reversed capacitation ($P<0.001$, Table 2).

Motility and Viability

The average percent of viable spermatozoa was 52% with no significant difference (P >0.05) across treatment groups. Similarly, the average percent motile was 55% with no significance (P>0.05) across treatment groups or time of evaluation.

Discussion

The present data support previous studies [3, 12, 13] in that cryopreservation of boar spermatozoa results in cryocapacitation. These results also agree with our previous work in that addition of 10% SP to FT spermatozoa at 39 C without the egg yolk-based freezing extender results in a decrease in the percent of capacitated spermatozoa. These results indicate that the effects of SP are dependent on the temperature of the spermatozoa suspension as well as the presence or absence of egg yolk in the freezing extender.

When SP was added to spermatozoa at 17 C regardless of the extender used, no decrease in the percent of capacitated spermatozoa was noticed. Similarly, when SP was added to spermatozoa suspended in the egg yolk-based freezing extender regardless of the temperature no decrease in the percent of capacitated spermatozoa was noticed. The addition of 10% (v/v) SP resulted in a reversal of capacitation only when added to spermatozoa suspended in either X-cell thawing extender or PBS at 39 C without the egg yolk-based freezing extender.

Based on our work [10] and work by others [9, 14] a SP inclusion level of 10% (v/v) is recommended to achieve the expected reversal of cryocapacitation. Our previous studies [10] indicated that the effect of SP appears to be independent of the time of addition and percent of inclusion (10, 20, 40%). It was concluded that the de-capacitating effect of SP was complete within 30 min of its addition. This was used to determine that the length of each treatment's incubation period would be 30 min for this study. Incubation temperature and the presence of egg yolk in the freezing extender can interfere with the ability of SP to reverse cryocapacitation in FT boar spermatozoa. Further trials need to be performed to establish these effects in vivo.

Temperature change induces membrane stress and lipid phase change in spermatozoa [15, 16]. The major phase change occurs in the vicinity of 5-15 C [17]. A capacitation-like reaction was induced by cooling boar spermatozoa to 5 C, and this reaction was displayed upon re-warming the spermatozoa to 39 C [18]. Membrane destabilization associated with such cooling increases the fluidity of the plasma membrane [19] but does not affect the fertilizing capacity of the cells. These membranes may be more permeable allowing free calcium ions to enter the cell and stimulate calcium-dependent events associated with capacitation [13].

The extent of cold shock to bull spermatozoa is decreased when the spermatozoa are frozen with a protective compound such as egg yolk [20]. The component(s) in egg yolk and the mechanisms by which they alter the membrane are not fully understood. It is known that egg yolk contains lipids that interacts with the spermatozoa membrane [21-

24]. Presumably, the phospholipids and low density lipoproteins of egg yolk attach to the membrane preventing lipid disorganization. The same level of protection to boar spermatozoa is not achieved with egg yolk alone [20]; however, its protection can be improved by added Orvus Es Paste (OES) to the freezing extender [25]. Orvus Es Paste only offers protection against cold shock when included with egg yolk. This suggests that OES acts to alter the constituents in egg yolk to improve its protection against cold shock in boar spermatozoa. Interestingly, boar spermatozoa incubated in their own SP became resistant to cold shock over 16 h after ejaculation [26]. Similarly, it has been shown that the cold shock-induced membrane damage in ram spermatozoa was reversed following incubation with a solution containing proteins extracted from ram SP [27]. This study demonstrated that the presumed binding of egg yolk constituents to the spermatozoa membrane obstructs the presumed binding of SP constituents to the lipid membrane; therefore, preventing the reversal of cryocapacitation.

The extent of damage that results from temperature change depends on a combination of elements: cholesterol/phospholipids ratio, content of non-bilayer-preferring lipids, degree of hydrocarbon chain saturation, and protein/phospholipids ratio in the membrane [28]. In boar spermatozoa, capacitation induces changes in lipid membrane structure via a cAMP-dependent protein phosphorylation pathway. These membrane changes include phosphatidylserine (PS) inversion, relocation of cholesterol to the apical head, and a decrease in lipid packing [29]. The lipid scrambling allows for cholesterol relocation. Lipid scrambling, mainly PS inversion, is a recognized stage of the apoptotic death

pathway [30]. However, there is no in evidence the PS inversion in temperature induced capacitation is related to the apoptotic death pathway [29].

Seminal plasma is produced by the accessory sex glands and is composed of inorganic compounds, amino acids, peptides, as well as both low and high weight-molecular proteins [31]. The existence of decapacitation factors in SP has been known since Chang's work in the 1950's [32]. Heparin-binding proteins in bovine SP bind to cauda epididymal spermatozoa and regulate capacitation by heparin [33]. In boar, the heparin-binding protein pB1 has been characterized and identified to bind to lipids containing the phosphorylcholine group on the spermatozoa membrane at ejaculation [34]. Recently, when spermatozoa were incubated with the glycosaminoglycan (GAG) hyaluronan (HA) an increase in the percent of capacitated spermatozoa was demonstrated [14]. This indicated an effect of GAG's such as HA and heparin on capacitation mediated through SP proteins bound to the spermatozoa membrane. In addition, when SP was introduced into the uterus before the spermatozoa an increase in fertilization rate and number of accessory spermatozoa in the zona pellucida was obtained [35]. This beneficial affect could be attributed to the decapacitation of spermatozoa therefore increasing the number in the spermatozoa reservoir.

Further investigation is needed to fully identify all constituents of SP and their mechanisms of action on capacitation both in vitro and in vivo. However, it is evident that some constituent(s) of SP acts on the process of capacitation through pathways that are affected by temperature and the cryoprotectants used during freezing. The ability to

allow widespread use of FT spermatozoa in the swine industry would eventually result in the preservation of good genetics, lower production costs, and an increase in herd biosecurity. It would allow safe international trade of good genetics to improve herd production worldwide. This study demonstrates concerns that need to be considered when designing in vivo experiments that test the ability of post-thaw SP addition to the improvement of swine fertility with FT spermatozoa.

References

1. Almlid, T., Hofmo, P. A brief review of frozen semen application under Norwegian AI service conditions. Reprod Dom Anim, 1996. 31: 169-73.

2. Reed, H. Current use of frozen boar semen-future need of frozen boar semen., in Deep freezing of boar semen., J. LA and L. K, Editors. 1985, Swedish University of Agricultural Science: Uppsala. 225-37.

3. Bailey, J., Bilodeau, J., Cormier, N. Semen cryopreservation in domestic animals: a damaging and capacitating phenomenon. J Androl, 2000. 21: 1-7.

4. Curry, M.R., Kleinhans, F.H., Watson, P.F. Measurement of the Water Permeability of the Membranes of Boar, Ram, and Rabbit Spermatozoa Using Concentration-Dependent Self-Quenching of an Entrapped Fluorophore*1. Cryobiology, 2000. 41: 167-73.

5. Watson, P.F. The causes of reduced fertility with cryopreserved semen. Anim Reprod Sci, 2000. 60-61: 481-92.

6. Fazeli, A., Duncan, A., Watson, P.F., Holt, W.V. Sperm-oviduct interaction: Induction of capacitation and preferential binding of uncapacitated spermatozoa to oviductal epithelial cells in porcine species. Biol Reprod, 1999. 60: 879-86.

7. Tienthai, P., Johannisson, A., Rodriguez-Martinez, H. Sperm capacitation in the porcine oviduct. Anim Reprod Sci, 2004. 80: 131-46.

8. Yanagimachi, R. Mammalian Fertilization, in "The Physiology of Reproduction." J.N. E Knobil, Editor. 1994, Raven Press: New York. 189-317.

9. Harayama, H., Magargee, S.F., Kunze, E., Shidara, O., Iwamoto, E., Arikawa, S., Mihake, M., Kato, S., Hammerstedt, R.H. Changes in epididymal protein anti-agglutinin on ejaculated boar spermatozoa during capacitation in vitro. Reprod Fertil Dev, 1999. 11: 193-9.

10. Vadnais, M.L., Kirkwood, R., Sprecher, D.J., Tempelman, D., Chou, K. Effect of cooling and seminal plasma on the capacitation status of boar sperm as determined by chlortetracycline staining. Submitted to Anim Reprod Sci, June 2004.

11. Lin, C. Regulation of sperm capacitation. Doctoral Thesis. Michigan State University, East Lansing, Michigan 1996.

12. Maxwell, W., Johnson, L. Chlortetracycline analysis of boar spermatozoa after incubation, flow cytometric sorting, cooling, or cryopreservation. Molec Reprod Dev, 1997. 46: 408-18.

13. Watson, P.F. Cooling of boar spermatozoa and fertilizing capacity. Reprod Dom Anim, 1996. 31: 135-40.

14. Suzuki, K., Asano, A., Eriksson, B., Niwa, K., Nagai, T., Rodriguez-Martinez, H. Capacitation status and in vitro fertility of boar spermatozoa: effects of seminal plasma, cumulus-oocyte-complexes-conditioned medium and hyaluronan. Int J Androl, 2002. 25: 84-93.

15. De Leeuw, F.E., Chen, H.C., Colenbrander, B., Verkleij, A.J., Cold-induced ultrastructural changes in bull and boar sperm plasma membranes. Cryobiology, 1990. 27: 171-83.

16. Holt, W.V., North, R.D. Partially irreversible cold-induced lipid phase transitions in mammalian sperm plasma membrane domains: freeze-fracture study. J Exper Zoo, 1984. 230: 473-83.

17. Drobnis, E.Z., Crowe, E.Z., Berger, T., Anchordoguy, T.J., Overstreet J.W., Crowe, J.H. Cold shock damage is due to lipid phase transitions in cell membranes: a demonstration using sperm as a model. J Exper Zoo, 1993. 265: 432-7.

18. Green, C., Watson, P. Comparison of the capacitation-like state of cooled boar spermatozoa with true capacitation. Reproduction, 2001. 122: 889-98.

19. Robertson, L., Watson, P. Calcium transport in diluted or cooled ram semen. J Reprod Fertil, 1986. 77: 177-85.

20. Benson, R.W., Pickett, B.W., Komarek, R.J., Lucas, J.J. Effect of incubation and cold shock on motility of boar spermatozoa and their relationship to lipid content. J Anim Sci, 1967. 26: 1078-81.

21. Foulkes, J. The separation of lipoproteins from egg yolk and their effects on the motility and integrity of bovine spermatozoa. J Reprod Fertil, 1977. 49: 277-84.

22. Watson, P.F. The interaction of egg yolk and ram spermatozoa studied with fluorescent probe. J Reprod Fertil, 1975. 42: 105-11.

23. Pursel, V.G., Johnson, LA., Schulman, L.L. Interaction of extender composition and incubation period on cold shock susceptibility of boar spermatozoa. J Anim Sci, 1972. 35: 580-4.

24. Gebauer, M., Pickett, B.W., Romarek, R. Motility of bovine spermatozoa extended in 'defined' diluents. J Dairy Science, 1970. 53: 817-23.

25. Pursel, V.G., Schulman, L.L., Johnson, L.A. Effect of Orvus ES Paste on acrosome morphology, motility and fertilizing capacity of frozen-thawed boar sperm. J Anim Sci, 1978. 47: 198-202.

26. Tamuli, M.K., Watson, P. Cold resistance of live boar spermatozoa during incubation after ejaculation. Vet Rec, 1994. 135: 160-2.

27. Barrios., Perez Pe, R., Gallego, M., Tato, A., Osada, J., Munino Blanco, T., Cebrian Perez, J.A. Seminal plasma proteins revert the cold-shock damage on ram sperm membrane. Biol Reprod, 2000. 63: 1531-7.

28. Medeiros, C., Forell, F., Oliverira, A., Rodrigues, J.L. Current status of sperm cryopreservation: why isn't it better? Theriogenology, 2002. 57: 327-44.

29. Gadela, B.M., Harrison, H.A. Capacitation Induces Cyclic Adenosine 3',5'-Monophosphate-Dependent, but Apoptosis-Unrelated, Exposure of Animophospholipids at the Apical Head Plasma Membrane of Boar Sperm Cells. Biol Reprod, 2002. 67: 340-50.

30. Fadok, V.A., Bratton, D.L., Rose, D.M., Pearson, A., Ezekewitz, R.A., Henson, P.M. A receptor for phosphatidylserine-specific clearance of apoptotic cells. Nature, 2000. 405: 85-90.

31. Pursel, V., Johnson, L., Schulman, L. Effect of dilution, seminal plasma and incubation period on cold shock susceptibility of boar spermatozoa. J Anim Sci, 1973. 37: 528-31.

32. Chang, M. A detrimental effect of seminal plasma on the fertilizing capacity of sperm. Nature, Lond., 1957. 179: 258-9.

33. Miller, D., Winer, M., Ax, R. Heparin-binding proteins from seminal plasma bind to bovine spermatozoa and modulate capacitation by heparin. Biol Reprod, 1990. 42: 899-915.

34. Calvete, J., Raida, M., Gentzel, M., Urbanke, C., Sanz L., Topfer Petersen, E. Isolation and characterization of heparin- and phosphorylcholine-binding proteins of boar and stallion seminal plasma. Primary structure of porcine pB1. FEBS Letters, 1997. 407: 201-6.

35. Willmen, T., Weitze, K.F., Habeck, O., Waberski, D. Effect of seminal plasma and different sperm numbers in the inseminate on fertilization, accessory sperm number, and ovulation time in the gilt. In Proc. 3rd Int. Conf. Pig Reprod. 1989. Nottingham.

Treatment	Egg Yolk Inclusion	Incubation Temperature	SP Inclusion	Fresh	Capacitated	Acrosome Reacted
1	Yes	17 C	No	31.67	58.17	10.17
2	Yes	17 C	Yes	37.83	50	12.17
3	Yes	39 C	No	34	56.6	9.5
4	Yes	39 C	Yes	33	56.3	10.67

Table 1: Effect of seminal plasma on capacitation status of frozen-thawed spermatozoa after a 30 min incubation period in commercial semen extender at 17 or 39 C.

Treatment	Egg Yolk Inclusion	Incubation Temperature	SP Inclusion	Fresh	Capacitated	Acrosome Reacted
5	No	17 C	No	32	57.17	10.83
6	No	17 C	Yes	37	51.17	11.83
7	No	39 C	No	37.5	51.5	11
8*	No	39 C	Yes	55	36.5	8.5
9	No	17 C	Yes	34.83	55.67	9.5
10*	No	39 C	Yes	54.17	37.3	8.5

*P-value<0.001

Table 2: Effect of seminal plasma on capacitation status of frozen-thawed spermatozoa after a 30 min incubation period in phosphate buffered saline (Treatments 5 through 8) or commercial semen extender (Treatments 9 and 10) at 17 or 39 C.

CHAPTER 5

Summary and Conclusions

Summary

The effects of boar SP addition to capacitated boar spermatozoa were examined. Fresh spermatozoa from 3 boars as well as FT spermatozoa from 6 boars were used for these studies. The spermatozoa were capacitated in a capacitation-supporting environment, by cooling the spermatozoa to 5 C over 100 min, or via cryopreservation. The status of capacitation was examined using CTC staining analysis. The viability of the spermatozoa was examined using bis-benzimide stain.

Seminal plasma was added at 0, 10, or 20% (v/v) to the spermatozoa suspension depending on the treatment used. The temporal pattern of capacitation was initially examined. In treatments where fresh spermatozoa were used, capacitation was examined over a 10 hr period of time. In initial treatments with FT spermatozoa the process of capacitation and acrosome reaction were examined oven an 8 hr period. After the decapacitation pattern was repeatedly demonstrated, the effects of SP on FT spermatozoa were examined over an hour period.

Seminal plasma was added at different time points depending on the treatment used. Primary studies with fresh spermatozoa in a capacitation-supporting environment observed the effect of SP addition at the start of the treatment but removed after 100 min, added at 100 min, or added after several hr of incubation. Primary studies with fresh spermatozoa cooled to 5 C over 100 min to initiate capacitation observed the effect of SP

addition at the start of the treatment, added at the start of the treatment but removed after cooling, or added after rewarming to 39 C and several hr of incubation. When FT spermatozoa were used SP addition occurred immediately after thawing.

In experiments 1 and 2 (Chapters 3 and 4, respectively) an egg yolk-based freezing extender was not a factor with the use of fresh spermatozoa and was washed from the FT spermatozoa. Also, the temperatures in both experiments were controlled at 39 C. Temperature as well as the presence and absence of egg yolk in the freezing extender were examined using FT spermatozoa in the third experiment (Chapter 4). The temperature in experiment 3 was at either 17 or 39 C.

Conclusions

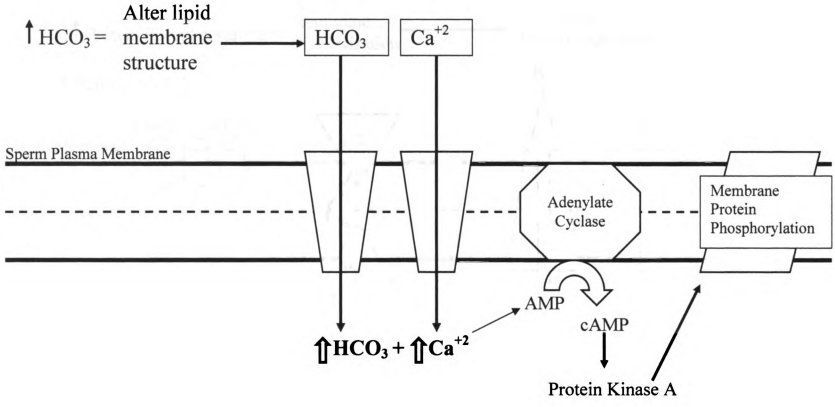
The addition of either 10 or 20% SP was capable of preventing and reversing capacitation in spermatozoa capacitated in a capacitation-supporting environment or in spermatozoa capacitated by slow cooling to 5 C. Similarly, 10 and 20% SP addition were capable of reversing cryocapacitation in FT boar spermatozoa. This reversal is apparent within 15 min of SP addition and is complete within 30 min.

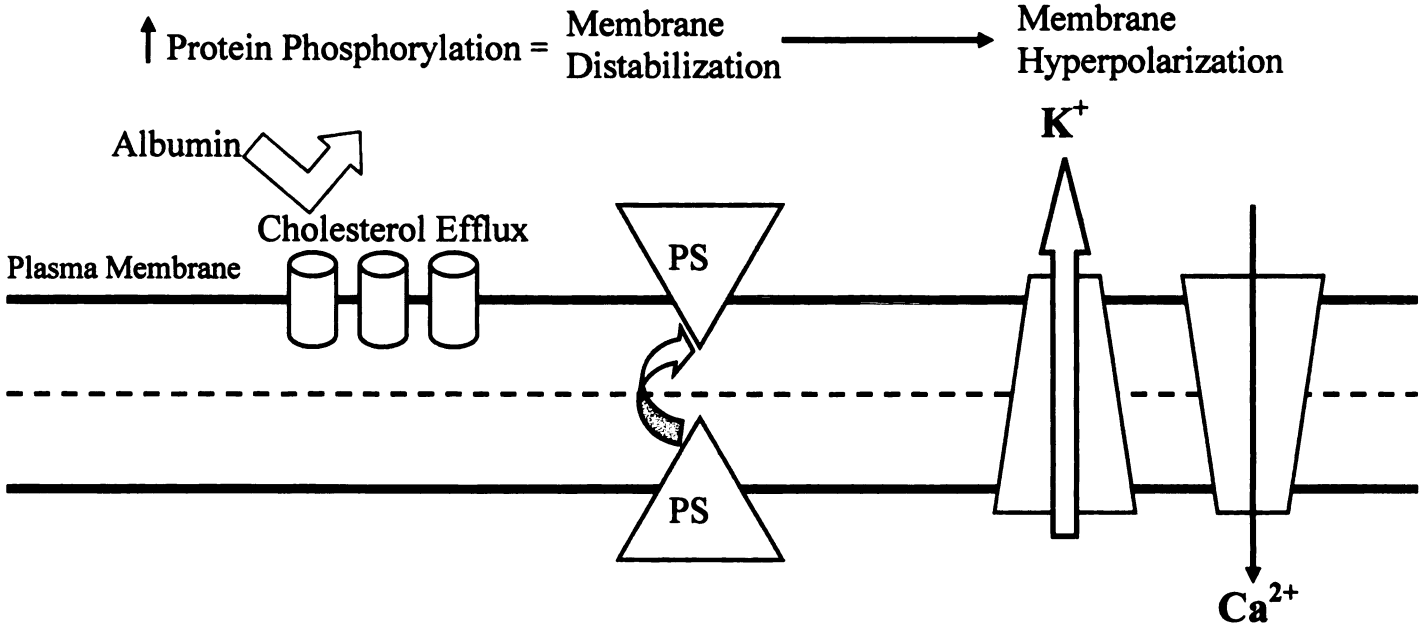
The effects of SP appear to be independent of the time or percent of addition. However, it was demonstrated that the effects of SP are dependent on both the temperature of the spermatozoa suspension at the time of addition as well as the presence of egg yolk-based freezing extender. Seminal plasma is capable of reversing cryocapacitation in

spermatozoa when it is added at ambient temperature (39 C) in the absence of the egg yolk-based freezing extender.

APPENDICES

APPENDIX I

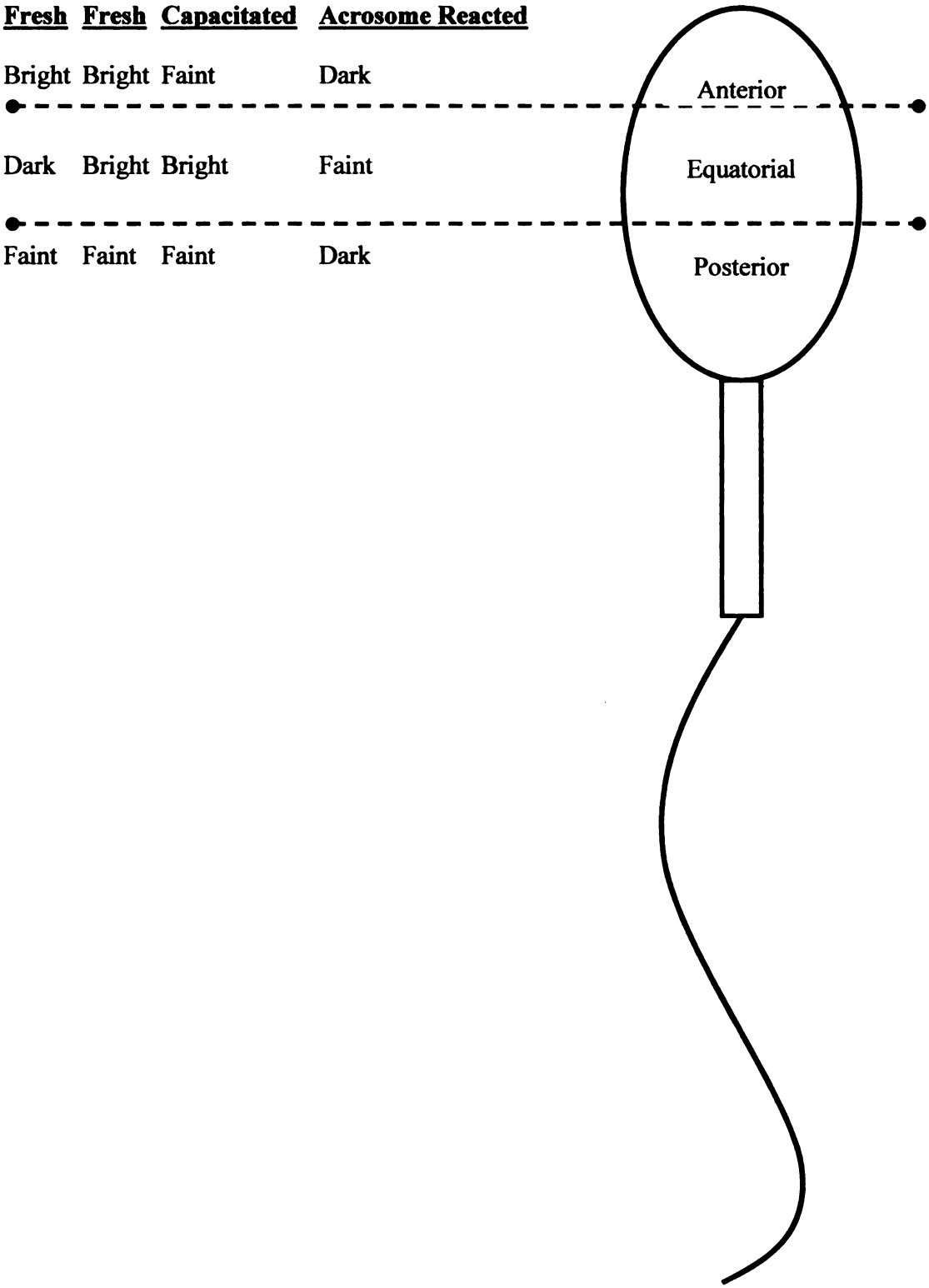

↑ HCO_3 =
Alter lipid membrane structure
HCO_3
Ca^{+2}
Sperm Plasma Membrane
Adenylate Cyclase
Membrane Protein Phosphorylation
AMP
cAMP
⇧HCO_3 + ⇧Ca^{+2}
Protein Kinase A


↑ Protein Phosphorylation =
Membrane Distabilization
Membrane Hyperpolarization
Albumin
Cholesterol Efflux
Plasma Membrane
PS
PS
K+
Ca2+

APPENDIX II

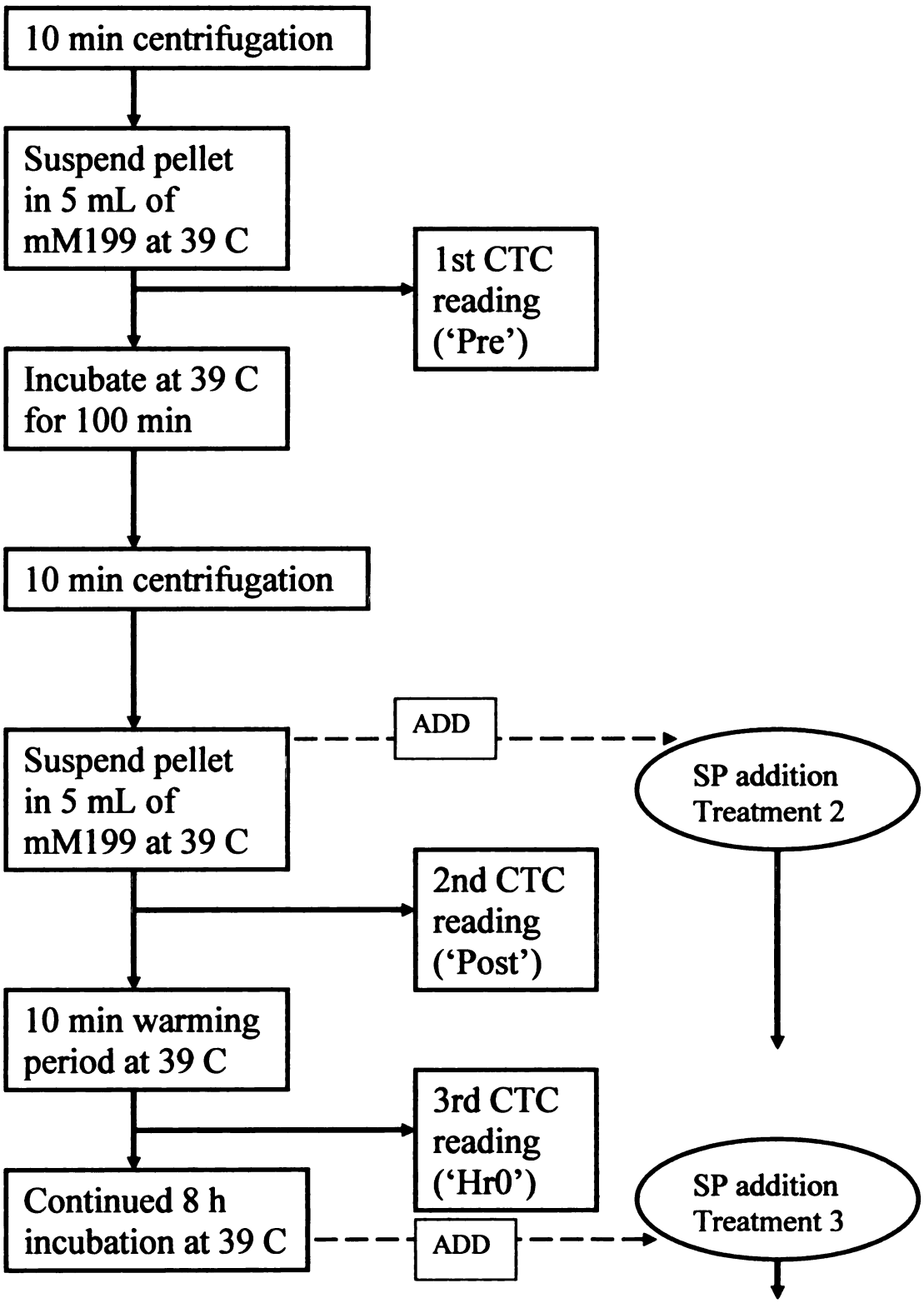
Fluorescent patterns (Fresh, Capaciated, Acrosome Reacted) displayed by spermatozoa after staining with CTC:

Fresh	**Fresh**	**Capacitated**	**Acrosome Reacted**
Bright	Bright	Faint	Dark
Dark	Bright	Bright	Faint
Faint	Faint	Faint	Dark

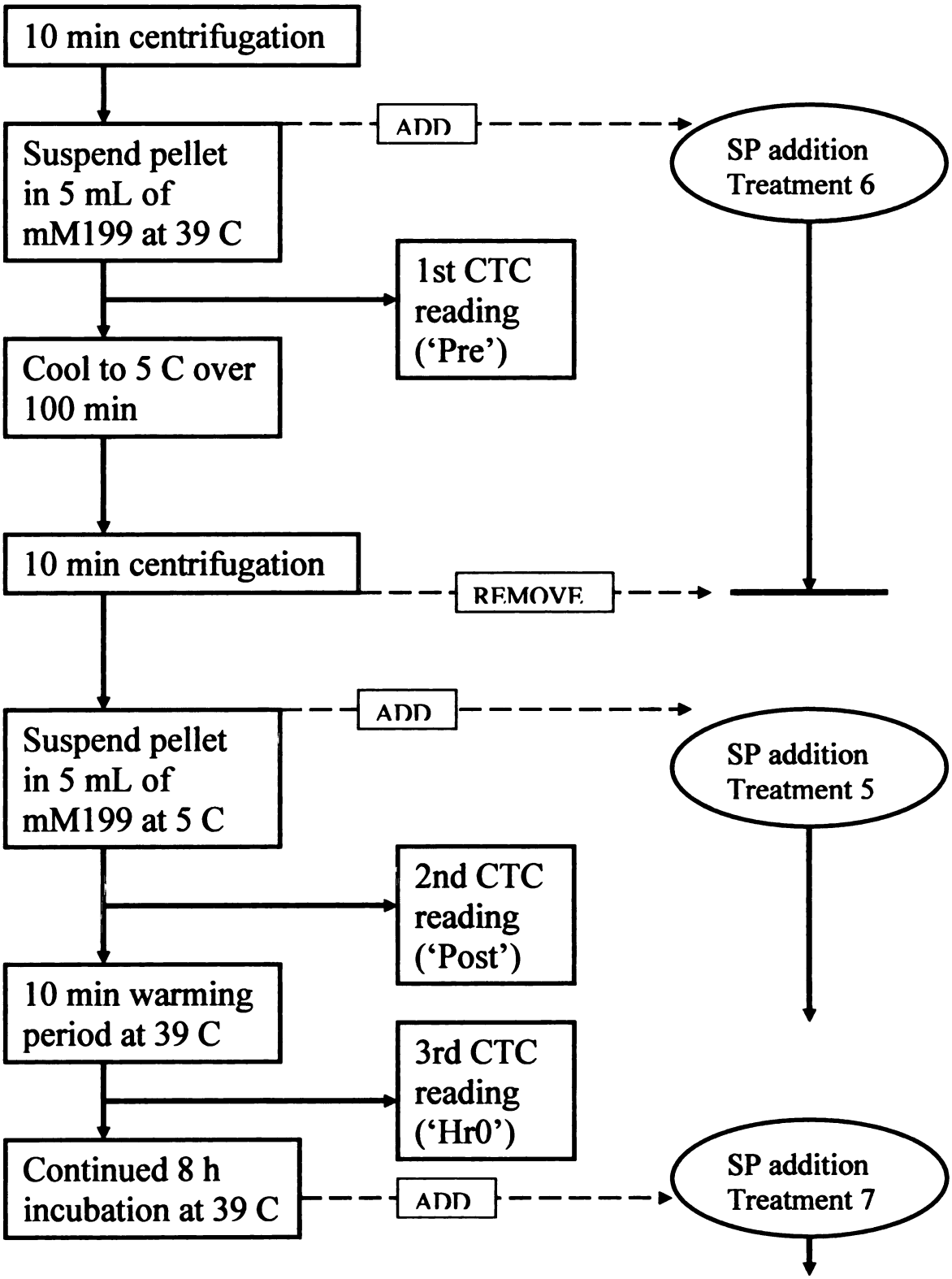


APPENDIX III

Time line depicting spermatozoa preparation and SP addition in non-cooling Treatments 2 and 3 (Treatment 1 0% SP was added):



Time line depicting spermatozoa preparation and SP addition in cooling Treatments 5, 6, 7 (Treatment 4 0% SP was added):



APPENDIX IV

Experimental Design for Experiment 3 (Chapter 4):