EFFECTS OF CAFFEINE, EQUILIBRATION TIME AND THAWING TEMPERATURES ON THE QUALITY OF BOAR INSEMINATION DOSES

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Abstract

The effect of cryopreservation on boar sperm is more detrimental than on sperm of other farm animals. The purpose of our study was to improve the steps of storage and subsequent thawing so that cryopreservation might find wider applications in swine reproduction. We focused on three factors which can influence the quality of insemination doses. The first factor was the length of the equilibration phase where we compared the difference between 7 and 26 hours. The longer time of equilibration appears more convenient for maintaining the motion characteristics of sperm. Another factor was the thawing temperatures and times of thawing of the insemination doses where we compared 38°C for 30 seconds with 70°C for 8 seconds. The rapid method with the higher temperature showed to be preferable because the sperm retained better movement characteristics. The last factor was the effect of caffeine, added to the thawing medium, which resulted in a significant improvement in the quality of sperm movement. However, on the other hand, a considerable impairment of sperm viability occurred.
Key Words: Boar, spermatozoa, cryopreservation, caffeine, thaw medium

The process of sperm cryopreservation consists of many steps that can dramatically affect the quality of insemination doses and, consequently, the success rate of insemination. Boar spermatozoa are more sensitive to these factors than spermatozoa of other farm animals, and therefore, cryopreserved boar semen is not used in artificial insemination as widely as bull semen.

The cold shock is a big problem (Pursel et al. 1972) which is explained by the different cholesterol content and unsaturated/saturated fatty acid ratio in the boar sperm cytoplasmic membrane (Parks and Lynch 1992). To mitigate the effects of the cold shock, boar semen is usually stored in cooling medium at 17°C. The doses maintained under these conditions can be stored for a few days, depending on medium type. This period of preservation, when collected sperm are extended with pre-warmed cooling medium, carefully cooled and then stored at 17°C, is called the equilibration phase. In our experiment, we investigated the effect of the equilibration time of 7 or 26 hours on sperm.

Besides freezing, the thawing process also has a considerable influence on sperm quality. We compared the two most common thawing procedures. In the first one, insemination straws are thawed at 38°C for 30 seconds. This is the body temperature which is natural for sperm, but the process of thawing lasts longer than in the second procedure where sperm is exposed to the temperature of 70°C for 8 seconds. This temperature would kill the sperm if the timing were not strictly observed. However, under the aforementioned conditions, the thawing process is very fast.

Our final goal was to influence the thawed insemination dose by adding a low concentration of caffeine to the thawing medium. Positive effects of caffeine on sperm and the reproductive tract of pigs are explained by preventing the inflammatory response which occurs during insemination (Yamaguchi et al. 2013). Caffeine stimulates sperm metabolism, enhances fructose utilization, improves respiration and causes an increase in cAMP levels, which results in an improvement of sperm motility (Makler et al. 1980, Salem et al. 1992, Zeldan 2002, Milani et al. 2010, Yamaguchi at al. 2013, Spalekova et al. 2014). We investigated the effect of caffeine on boar sperm after cryopreservation.

Material and Methods

Semen collected from 3 boars was frozen according to the method of Westendorf et al. (1975) modified by Thurston et al. (1999). At the Insemination Centre, ejaculated semen was extended with a short-term extender VIP5 (HEMA Malšice s.r.o) immediately after collection. Then, within 7 hours, it was transported to our laboratory at 17°C where part of it was immediately frozen, and part was frozen after 26 h of equilibration in the aforementioned extender. Two methods of semen thawing in a water bath were compared: at 38°C for 30 seconds and at 70°C for 8 seconds. The thawing medium VIP5 was used without or with the addition of 1mM caffeine (Caffeine anhydrous, Sigma-Aldrich®). Sperm analysis included the evaluation of motility, progressivity of movement, viability and acrosome integrity, carried out immediately after thawing and 2 and 4 hours later. Motility and progressive movement were assessed by microscopy, counting motile sperm in fields of view at a magnification of 200x. Viability (percentage of live spermatozoa with intact plasma membrane) was determined by intravital eosin-nigrosine staining (World Health Organization 2010). Acrosome integrity was determined by morphological evaluation of spermatozoa under a phase-contrast microscope at a magnification of 1000x.

Results and Discussion

The effect of the equilibration time during cryopreservation of boar semen

Obtained values are shown in Table 1. In maintaining sperm motility, progressivity of movement and viability, the longer equilibration time before semen freezing appeared preferable. Spermatozoa were likely to have more time to deal with the decreasing temperature and develop a higher resistance to thermal shock. It was found that at this stage, seminal plasma proteins exert a positive effect. These proteins are incorporated into the plasma membrane, helping to maintain its integrity (Casas and Althause 2013). Later cryopreservation had a negative impact on sperm acrosome integrity and the occurrence of morphological defects. These

results are consistent with the Conejo-Nava et al. (2003). Naturally, these defects will increase with the passage of time. Dubé et al. (2004) reported a reduction in viability. The increase of these defects will be stopped by freezing. It appears that 26 hours is a good compromise between the positive and negative effect of equilibration due to sperm aging (Tomás et al. 2014) .

The effect of temperature and the time of exposure during thawing of insemination doses

Results are shown in Table 2. The comparison of semen thawing methods showed that boar spermatozoa retained better features at a higher thawing temperature for a shorter time, both at the initial time and after 2 and 4 hours post-thawing. The high temperature exerted for a short time did not have an adverse effect on sperm and it was shown that a longer thawing period caused more damage to the sperm. Higher sperm motility and acrosome integrity during rapid thawing was also shown by Fisher et al. (1993), Erisson and Rodrigues-Martinez (2000), Cordova et al. (2006), Muino et al. (2008) and Tomás et al. (2014). A rapid thawing rate results in a reduced recrystallization of intracellular ice, a process that can lead to the formation of larger and more stable ice crystals that could damage the mitochondria (Fiser and Hairfull 1990).

The effect of caffeine in the thawing medium.

Results are shown in Table 3.

The addition of caffeine was able to increase motility and progressive movement of sperm, but adversely affected sperm viability, acrosome integrity and the percentage of morphologically normal sperm, with the highest effect on viability. The negative effects of caffeine to acrosome integrity describes Zeldan (2002). Morphological changes manifested themselves in acrosome swelling. It is surprising that the viability of spermatozoa treated with caffeine is lower than their motility. Caffeine made the sensitive membrane even more sensitive and the subsequent effect of eosin-nigrosine was too aggressive. The viability detection method using eosin-nigrosine may be not the best for this purpose because authors using Annexin V, SYBR-14 or YO-PRO-1 staining did not observe this phenomenon. They reported no effect on viability and a significant positive effect on sperm motility (Makler et al. 1980, Maxwell et al. 1995, Yamaguchi et al.2013, Spalekova et al. 2014).

Table 1. The effect of equilibration time on frozen/thawed boar spermatozoa at 0 h - 4 h after thawing

	Equilibration time 7 h			**Equilibration time 26 h**		
	0 h	**2 h**	**4 h**	**0 h**	**2 h**	**4 h**
Sperm motility %	34 ± 8.0 (26 - 42)	25 ±14.0 (10 - 38)	14 ± 10.8 (2 - 23)	35 ± 2.5 (33 - 38)	38 ± 6.9 (34 - 46)	29 ± 3.0 (26 - 32)
Progressive motility of sperm%	32 ± 7.5 (23 - 38)	22 ± 13.0 (8 - 34)	12 ± 10.2 (1 - 21)	32 ± 2.0 (30 - 34)	34 ± 6.5 (29 - 41)	25 ± 2.0 (23 - 27)
Sperm viability %	42 ± 5.2 (38 - 48)	35 ± 7.2 (29 - 43)	31 ± 7.1 (23 - 37)	47 ±12.8 (33 - 58)	41 ± 13.2 (33 - 57)	35 ± 7.1 (28 - 43)
Sperm with intact acro-somes %	43 ± 18.5 (22 - 57)	31 ± 7.6 (22 - 36)	29 ± 9.0 (19 - 36)	37 ± 6.5 (30 - 43)	28 ± 6.2 (21 - 33)	17 ± 5.2 (14 - 23)
Morphologically normal sperm%	35 ± 11.8 (21 - 42)	26 ± 6.7 (19 - 32)	22 ± 6.0 (15 - 26)	32 ± 5.6 (26 - 37)	24 ± 5.6 (18 - 28)	15 ± 4.0 (11 – 19)

Data are presented as mean ± standard deviations and (range)

Table 2. The effect of the thawing temperature on frozen/thawed boar spermatozoa at 0 h – 4 h after thawing

Thawing	**38°C / 30 sec**			**70°C / 8 sec**		
	0 h	**2 h**	**4 h**	**0 h**	**2 h**	**4 h**
Sperm motility %	32 ± 10.1 (20 - 38)	23 ± 9.5 (12 - 30)	19 ± 9.5 (9 - 28)	34 ± 12.2 (20 - 42)	33 ± 12.7 (18 - 40)	28 ± 16.2 (9 - 28)
Progressive motility of sperm%	28 ± 8.4 (18 - 33)	21 ± 6.3 (14 - 26)	16 ± 8.5 (7 - 24)	31 ± 11.0 (18 - 38)	29 ± 12.7 (14 - 36)	23 ± 15.0 (6 - 32)
Sperm viability %	38 ± 8.8 (28 - 44)	32 ± 9.5 (26 - 43)	23 ± 7.3 (16 - 31)	45 ± 11.7 (31 - 52)	35 ± 10.6 (23 - 42)	18 ± 8.1 (9 - 24)
Sperm with intact acro-somes %	67 ± 2.5 (64 - 69)	61 ± 3.4 (57 - 65)	59 ± 0.6 (58 - 59)	63 ± 5.3 (59 - 69)	62 ± 4.8 (56 - 65)	57 ± 7.6 (49 - 64)
Morphologically normal sperm%	58 ± 3.8 (54 - 62)	53 ± 1.8 (51 - 55)	51 ± 2.2 (49 - 53)	55 ± 5.5 (51 - 61)	51 ± 4.5 (46 - 55)	48 ± 8.0 (40 - 55)

Data are presented as mean ± standard deviations and (range)

Table 3. The effect of Caffeine on frozen/thawed boar spermatozoa at 0 h – 4 h after thawing

Thawing medium	Caffeine free			with Caffeine		
	0 h	2 h	4 h	0 h	2 h	4 h
Sperm motility %	35 ± 2.5 (33 - 38)	38 ± 6.9 (34 - 46)	29 ± 3.0 (26 - 32)	46 ± 4.7 (41 - 50)	43 ± 5.5 (37 - 47)	34 ± 4.0 (30 - 38)
Progressive motility of sperm%	32 ± 2.0 (30 - 34)	34 ± 6.5 (29 - 41)	25 ± 2.0 (23 - 27)	42 ± 4.1 (37 - 45)	39 ± 5.2 (33 - 42)	31 ± 4.0 (27 - 35)
Sperm viability %	47 ±12.8 (33 - 58)	41 ± 13.2 (33 - 57)	35 ± 7.1 (28 - 43)	28 ± 6.0 (22 - 34)	27 ± 4.9 (23 - 32)	21 ± 6.7 (16 - 29)
Sperm with intact acro-somes %	37 ± 6.5 (30 - 43)	28 ± 6.2 (21 - 33)	17 ± 5.2 (14 - 23)	35 ± 7.6 (27 - 42)	27 ± 8.6 (18 - 35)	15 ± 5.7 (10 - 21)
Morphologically normal sperm%	32 ± 5.6 (26 - 37)	24 ± 5.6 (18 - 28)	15 ± 4.0 (11 – 19)	29 ± 6.4 (22 - 34)	21 ± 6.7 (14 - 27)	12 ± 3.0 (9 -15)

Data are presented as mean ± standard deviations and (range)

Conclusion

Even though the presented results were so far obtained from a limited number of animals, we can assume that the 26 -hour equilibration period is suitable for cryopreservation of boar semen. Within this period, the collected ejaculate can be extended with a cooling medium in an AI centre and sent to a distant specialized laboratory where, on the following day, cryopreserved insemination doses can be produced.

The advice "the faster the better" applies to thawing of boar semen straws. Therefore, it is preferable to use 70°C for 8 seconds.

Caffeine might be able to improve kinetic features of sperm, but due to its effect, the plasma cell membrane becomes more sensitive to the influences of the environment. The question remains whether, in an *in-vivo* environment, caffeine-activated sperm could reach the fallopian tube of a sow to create a functional reservoir and fertilize an egg.

References

CASAS I., ALTHOUSE GC. (2013) The protective effect of a 17°C holding on boar sperm plasma membrane fluidity after exposure to 5°C. Cryobiology 66:69-75.

CONEJO-NAVA J., FIERRO R., GUTIERREZ C.G., BETANCOURT M. (2003): Membrane status and in vitro capacitation of porcine sperm preserved in long-term extender at 16 degrees C. Arch. Androl.: 49:287-295.

CÓRDOVA-IZQUIERDO A., OLIVA J.H., LLÉO B., GARCÍA-ARTIGA C., CORCUERA, B.D.,PÉREZ-GUTIÉRREZ, J.F. (2006): Effect of different thawing temperatures on the viability, in vitro fertilizing capacity and chromatin condensation of frozen boar semen packaged in 5 ml straws. Anim. Reprod. Sci. 92:145-154.

DUBÉ C., BEAULIEU M., REYES-MORENO C., GUILLEMETTE C., BAILEY J.L. (2004): Boar sperm storage capacity of BTS and Androhep Plus: viability, motility, capacitation, and tyrosine phosphorylation. Theriogenology 62: 874–886.

ERIKSSON B.M., RODRIGUEZ-MARTINEZ H., (2000): Effect of freezing and thawing rates on the post-thaw viability of boar spermatozoa frozen in FlatPacks and Maxi-straws. Anim Reprod. Sci. 63: 205–220.

FISER P.S., FAIRFULL R.W. (1990): Combined effect of glycerol concentra-tion and cooling velocity on motility and acrosome integrity of boarspermatozoa frozen in 0.5 ml straws. Mol. Reprod. Dev. 25:123–129.

FISER P.S., FAIRFULL R.W., HANSEN C., PANICH P.L., SHRESTHA, J.N.B., UNDER-HILL, L. (1993): The effect of warming velocity on motility and acrosome integrity of boar sperm as influenced by the rate of freezing and glycerol level. Mol. Reprod. Dev. 34:190-195.

MAKLER A., MAKLER E., ITZKOVITZ J., BRANDES J.M. (1980): Factors affecting sperm motility. IV. Incubation of human semen with caffeine, kallikrein and other metabolically active compounds. Fertil Steril 33:624-630.

MAXWELL W.M., ROBINSON S.J., ROCA J., MOLINIA F.C., SANCHES-PARTIDA L.G. (1995): Motility, acrosome integrity and fertility of frozen ram spermatozoa treated with caffeine, pentoxifyline, cAMP, 2-deoxyadenosine and kallikrein. Reprod Fertil 7:1082-1087.

MILANI C., FONTBONNE A., SELLEM E., STELLETTA C., GE´RARD O., ROMAGNOLI S. (2010): Effect of post-thaw dilution with caffeine, pentoxifylline, 2'-deoxyadenosine and prostatic fluid on motility of frozen-thawed dog semen. Theriogenology 74:153-164.

MUINO R., RIVERA M.M., RIGAU T., RODRIGUEZ-GIL J.E., PENA A.I. (2008): Effect of different thawing rates on post-thaw sperm viability, kinematic parameters and motile sperm subpopulations structure of bull semen. Anim. Reprod. Sci. 109:50-64.

PARKS, J. E., AND LYNCH, D. V. (1992): Lipid composition and thermotropic phase behavior of boar, bull, stallion, and rooster sperm membranes. Cryobiology 29:255-266.

PURSEL V.G., JOHNSON L.A., SCHUMAN L.L. (1972): Interaction of extender composition and incubation period on cold shock susceptibility of boar spermatozoa. J Anim Sci 35:580-584.

SALEM M.H., MEKKAWY M.Y., AHMED N.A. (1992): Effect of cyclic-AMP on fructose utilization, progresive motility and protein-synthesis by ram spermatozoa. Theriogenology 37:1061-1074.

SPALEKOVA E., MAKAREVICH A.V., KUBOVICOVA E., OSTRO A., CHRENEK P. (2014): Effect of caffeine on functions of cooling-stored ram sperm in vitro. Acta vet Brno 83:019-025.

THURSTON L.M., WATSON P.F., HOLT W.V. (1999): Sources of variation in boar spermatozoa fertility following cryopreservation. Cryobiology 39:355.

TOMÁS C., GOMEZ-FERNANDEZ J., GOMEZ-IZQUIERDO E., MERCADO E. (2014): Effect of the holding time at 15°C prior to cryopreservation, the thawing rate and the post-thaw incubation temperature on the boar sperm quality after cryopreservation. Anim Repro Sci 144:115-121.

WESTENDORF P., RICHTER L., TREU H. (1975): Zur Tiefgefrierung von Ebersperma. Labor und Besamungsergebnisse mit dem Hulsenberger Pailletten-Verfahren. Dtsch Tierarztl Wochenschr 82:261-267.

World Health Organization (2010): WHO laboratory manual for the examination of human semen. 5th ed. WHO Press, Geneva, Switzerland, 271 p.

YAMAGUCHI S., SUZUKI C., NOGUCHI M. (2013): Effects of caffeine on sperm characteristics after thawing and inflammatory response in the uterus after artificial insemination with frozen-thawed boar semen. Theriogenology 79:87-93.

ZELDAN A.E.B. (2002): Semen quality, enzymatic activities and penetrating ability of spermatozoa into she-camel cervical mucus as affected by caffeine addition. J Camel Pract Res 9:153-161.

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