Egg Yolk Protective Effect in Boar Spermatozoa Cooled at 5ºC

Alexandru-Vasile Rusu, Vasile Miclea, Marius Zahan

University of Agricultural Sciences and Veterinary Medicine, 400372, Cluj-Napoca, Calea Manastur str., 3-5, Romania

Abstract
Nowadays, many boar reproduction researches are directed to improve extenders and to increase cold shock protection of semen. Little research is focused on the influence of egg yolk combined with alternative cold shock protective media. Egg yolk could interfere with other compounds present in the extender composition. The influence of egg yolk addition was assessed in boar sperm cells, cooled at 5ºC, to elucidate its effect on motility and membrane integrity. Flow Cytometry and Computer Assisted Semen Analysis (CASA) were used to determine the rate of sperm with intact plasma and acrosomal membrane, respectively the sperm cells motility. Statistical analyses (T-Test) were performed using GraphPad Prism version 5.00. Androhep Plus supplemented with 20% egg yolk (AhPlus+20%EY) indicated a higher cold shock protection in progressive motility (93.9±2.64%) and membrane integrity (79.78±4.14%), rather than the extender without egg yolk ($p<0.01$, respectively $p<0.05$). The results of the this study showed that egg yolk addition to AhPlus do not interfere with its compounds, the data being in a close range with those obtained by using the standard Lactose Egg Yolk extender ($p>0.05$). The combination egg yolk-AhPlus seems to be an alternative to standard extenders, conferring stability in boar sperm cells against cold shock.

Keywords: Androhep Plus, boar sperm, CASA, cold shock, egg yolk, flow cytometry.

1. Introduction

Sperm preservation protocols vary among animal species owing to their inherent particularities that change the extenders composition used for refrigeration and freezing industry. Boar semen differs in several aspects from the semen of other domestic animals. It is produced in large volumes and is extremely sensitive to sudden cooling immediately following collection (the so-called "cold shock") [1]. In many cases the conditions of on-farm storage temperature of semen are inappropriate resulting in a decreased sperm quality. The reason for the increase in the abnormalities percent of semen stored under optimal temperature is unclear [2]. It is suggested that substantial membrane damages occurs already during cooling at temperatures lower than 15°C, being decisive for storage and/ or cryopreservation results. The success of storage and/or cryo-storage boar semen depends on our understanding of how several factors influence the capacity of spermatozoa to survive cold shock conditions while maintaining their ability to fertilize. These factors are either internal, such as the inherent characteristics of spermatozoa and the existing differences among boars and ejaculates, or external, such as the extenders composition, type and concentration of the cold shock protective agent applied, rates of extension and cooling or equilibration, and the method of freezing and thawing of the semen [3] Unfortunately, only these external factors can be modified in order to understand and optimize the cold shock protection of semen [1]. Many studies recommend egg yolk to be included in boar semen cryopreservation protocols, referring to its benefic effect mostly as

* Corresponding author: Alexandru Rusu
Email:rusu_alexandru@hotmail.com

part of LEY extender. Still little research is focused on the influence of egg yolk combined with alternative extenders. Androhep Plus is known as a long term extender that contains a Cell Shield Plus (CSP) substance which provides protection for sperm cells when are exposed to temperature fluctuations. CSP actively plays a role in membrane stabilization and protects against premature sperm cell capacitation. Concomitantly bovine serum albumin (BSA) is a substance with a positive effect on sperm membranes and motility. Egg yolk addition could interfere with such compounds present in the extender composition. Assessment of egg yolk effect on boar sperm cells requires sensitive diagnostic methods, beyond the standard spermatology evaluation. For a precise semen evaluation, the methodological spectrum includes Computer Assisted Sperm Analysis (CASA) and assessment of plasma & acrosomal membrane integrity by Flow Cytometry. Flow cytometry allows counting and examining microscopic particles (cells and chromosomes), stained and suspended in a stream of fluid and then passed through an electronic detection apparatus. Information about cells is acquired by measuring visible and fluorescent light emissions. Computer Assisted Semen Analysis (CASA) is a catch-all phrase for automatic or semi-automatic semen analysis techniques. Most CASA systems are based on image analysis, but alternative methods exist such as tracking cell movement on a digitizing tablet. CASA analysis for sperm motility provides detailed information taking into consideration more than 10 motility parameters. The aim of this study is thereby to evaluate the influence of egg yolk addition in boar sperm cells, cooled at 5ºC and to characterize its effect on motility and membrane integrity.

2. Materials and methods

Sample preparation. Semen of commercial boar hybrids was collected using the glowed-hand method and extended (1:1 [vol/vol]) in Androhep Plus (Minitube). After a previously equilibration at 20°C 1.5h and at 15°C 2.5h semen was centrifugated (2400×g , 3 min). Each pellet was splitted in three equal parts, that were extended ($1.5x10^9$/ml) with LEY, AhPlus respectively AhPlus+20% egg yolk. A third equilibration phase was performed at 5°C 1.5h. The pH and osmolarity of each extender were previously checked. Semen motility was evaluated using a computer assisted sperm analyzer (Sperm Vision^{TM}, Minitube). Plasma and acrosomal membrane integrity were determined on a flow cytometer (Partec DAKO Galaxy). To ensure a high accuracy of semen evaluation, the samples were previously extended in Androhep Plus to a final concentration of $40x10^6$/ml. Statistical analyses (T-Test) were performed using GraphPad Prism version 5.00.

CASA. Semen samples were loaded in a prewarmed Leja standard count four chambers. For each sample, ten optical fields were used to count a minimum number of 800 spermatozoa per sample. Total sperm motility, progressive motility, local motility and immotile spermatozoa parameters were analyzed, for a precise assessment of boar semen motility.

The following characteristics of sperm motility were taking into consideration: percentages of total sperm motility, progressive motility, local motility and immotile spermatozoa.

Flow Cytometry assessment. A combination of four fluorescent stainings was used: propidium iodite (PI) fluorescein isothiocyanate conjugated with Peanut Agglutinin (FITC-PNA), fluorescein isothiocyanate conjugated with Pisum Sativum Agglutinin (FITC-PSA) and Hoechst 33342. 10 µl of each stained sperm sample was added to 890 µl HEPES saline buffer (HBS 300mOsm/kg, pH 7.4) and easily vortexed. A number of 10000 events were evaluated using a flow cytometer, for each sample. Four sperm populations were analyzed through flow cytometry: (Q1) membrane damaged but acrosome intact (red fluorescence), (Q2) membrane and acrosome damaged (red and green fluorescence), (Q3) membrane and acrosome intact (no fluorescence) and (Q4) membrane intact but acrosome damaged (green fluorescence).

3. Results and discussion

Boar spermatozoa have the highest sensitivity to cold shock, among all the domestic animals, a sensitivity that could be caused by the plasma membrane special composition, in phospholipids and cholesterol [4]. As boar spermatozoa can acquire resistance to cold shock, if incubated at low extension rates, for 1-5 h at ambient temperatures [5, 6], a 4h equilibration time of the sperm was applied before

centrifugation, in the present study. The equilibration time may protect the sperm not only by the cold shock but also by the centrifugation stress.

Cold shock (i.e. damage due to rapid cooling above 0°C) could be caused by lipid phase transition effects [7, 8]; phase transitions might be involved in the manifestation of cold shock injury both on cooling and also on rewarming of the sperm cells [9]. Considering the fact that many studies recommend egg yolk to be included in boar cryopreservation protocols, as a cryoprotector agent and the Androhep Plus protection effect on sperm cells in fluctuating temperature conditions, three different extender compositions: Ah Plus, Ah Plus + 20% EY, LEY, were chosen in the present study.

The hypothesis regarding cryopreservation of boar sperm cells is that the cooling phase may be determinative for the success of freezing. This may be also an indicator regarding the level of protection conferred by egg yolk and also about its possibility by using it in combination with Ah Plus on temperature stress conditions. The main advantage of CASA consists in a precise establishment of sperm motility, eliminating also the subjectivity of the usual microscope sperm motility assessment. The results showed in table 1 indicate that egg yolk might confer stability on sperm motility in cold shock conditions.

Table 1. Boar sperm motility cooled at 5°C in different extenders

	Ah Plus	Ah Plus+20%EY	LEY
Total Motility	90.48±0.296[a]	96.36±0.587[b]	96.57±0.750[b]
Progressive motility	85.80±1.068[a]	93.90±1.320[b]	94.13±0.404[b]
Local motility	4.68±0.907[a]	2.46±0.763[a]	2.45±0.349[a]
Immotility	9.52±0.296[a]	3.64±0.587[b]	3.43±0.750[b]

Note: Different superscripts in the same row indicate significant differences. Values in the table represent means±SEM

Even if when using Ah Plus, the spermatozoa motility registered high results, after cooling at 5°C, the addition of 20% egg yolk showed a higher a sperm motility protection under cold shock conditions. Total and progressive motility of boar spermatozoa is significantly higher for Ah Plus +20%EY ($p<0.001$ respectively $p<0.01$). There are no significantly results on all the motility parameters ($p>0.05$) between Ah Plus + 20% EY and the standard LEY extender used in boar semen cryopreservation protocols. It appears that egg yolk does not affect the type of motility, the percentage between total sperm motility and progressive motility being in a normal range of the boar sperm. Egg yolk is able to confer motility protection in bought cases having also no interference with Ah Plus compounds. Concerning the sperm local motility ($p>0.05$) there are no significantly results between all three extenders. When egg yolk is lacking from the extender, there are no significantly results ($p>0.05$) on the percent of sperm characterized by local motility, existing anyway significantly results on the immotile sperm. It seems that cold shock injuries may have a rough impact, producing mostly immotile spermatozoa. Egg yolk is able to confer enough protection to keep a low percent of immotile sperm cells, while having high percentages of sperm cells characterized by progressive motility. As in previously studies egg yolk prevented sperm flagellae from bending into a rigid ‘‘bow-like’’ configuration, [10] during cooling,, abolishing the tendency for these flagellae to undergo sudden, irreversible, midpiece bending through 180° when the temperature declined to about 12–14°C [9]. Even in cold-shock conditions by this experimental design used for cooling the sperm to 5°C were obtained high results on sperm motility for all three extenders.

Four sperm populations (Q1, Q2, Q3 and Q4) were assessed for each sample, through Flow Cytometry (figure 1), based on the high advantages of this technique for semen evaluation. Fluorescently labeled spermatozoa were analyzed by means of flow cytometry, enabling the evaluation of larger numbers of spermatozoa in a short time, assessing the function of the semen sample without the interference of fat globules or other non-stained material and the differences between the sperm populations more consistently and clearly.

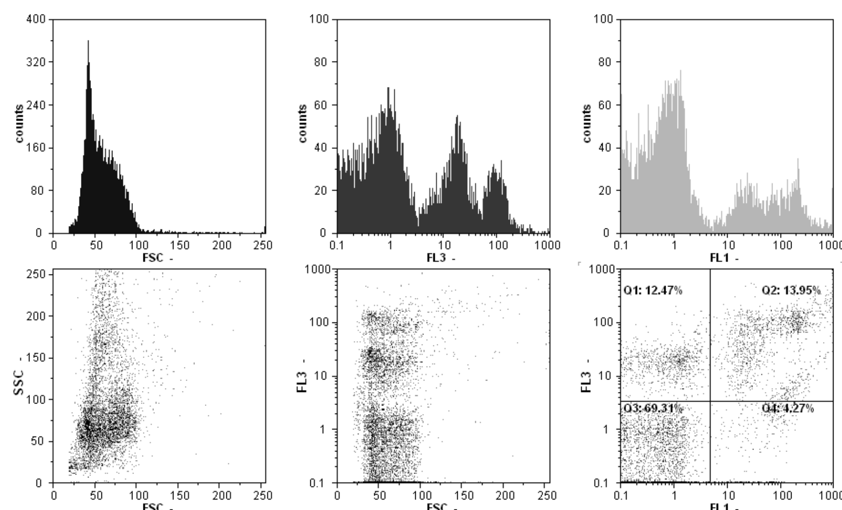

Figure 1. Simultaneous evaluation by Flow Cytometry of plasma and membrane integrity of boar spermatozoa. The diagrams represent the 4 boar semen populations (Q1, Q2, Q3 and Q4).

The sperm plasma membrane integrity, which is essential for the fertilizing capacity, is often synonymous with sperm viability, and most viability assays actually assess whether the sperm plasma membrane is intact or damage. The major problem of the conventional methods (such as eosin/nigrosin and trypan blue in light microscopy) used for the assessment of membrane integrity of spermatozoa is that spermatozoa may show partial staining or even unstained damaged sperm cells, making difficult to interpret the results.

The non-fluorescent stainings for evaluating the acrosomal status (Giemsa, Trypan blue/Bismark brown/Rose Bengal (triple stain) or Spermac®) are low sensitivity techniques, meanwhile fluorescent stainings (fluorescein isothiocyanate conjugated with Peanut Agglutinin (FITC-PNA) and fluorescein isothiocyanate conjugated with Pisum Sativum Agglutinin (FITC-PSA)) are considered high accuracy stainings, enabling to reveal even subtle acrosomal membrane damages [11].

Table 2. Membrane integrity evaluation of boar sperm cooled at 5°C in different extenders

Sperm population	Ah Plus	Ah Plus+20%EY	LEY
Q1	3.94±1.475[a]	4.41±0.620[a]	5.23±1.125[a]
Q2	9.22±1.264[a]	7.80±1.588[a]	7.30±0.677[a]
Q3	71.89±2.171[a]	79.78±2.072[b]	81.56±0.543[b]
Q4	14.95±1.748[a]	8.01±2.774[b]	5.92±1.345[b]

Note: Different superscripts in the same row indicate significant differences. Values in the table represent means±SEM

Beside the sperm motility protection, egg yolk seems to have also a positive influence on boar sperm membrane integrity (table 2). Ah Plus supplemented with 20% egg yolk indicate significantly higher results ($p<0.05$) rather than Ah Plus regarding the sperm cell population having a membrane and acrosome intact.

There are no significantly results on all the motility parameters ($p>0.05$) between AhPlus+20%EY and the standard LEY extender used in boar semen cryopreservation protocols.

It`s interesting to point out that regarding the Q 1 and Q2 sperm populations, no significant differences have been observed between the three extenders used. This concurs with other results obtained in various scientific works.

Freeze-fracture electron microscopy failed to demonstrate that egg yolk influenced the extent of intramembranous particle aggregation induced when ram, bull or boar spermatozoa were cooled and stored at 0–5°C [12, 13]. Taking particle aggregation as evidence of phase transitions having occurred, no prevention of the effect by the presence of egg yolk was evident [9]. The direct modulation of sperm plasma membrane lipid phase transition behavior by interaction with egg yolk is an attractive idea, but one which has little evidence in its favor [9]. However, until the action

of egg yolk in conferring membrane protection is better understood, progress in the search for alternatives or substitutes can be made. The active component of egg yolk is a low-density lipoprotein [4], but direct evidence for its mode of action is still elusive.

The results indicate that the acrosomal membrane is highly affected by the cold shock conditions mainly in case of egg yolk absence. This population of sperm cells may be in an early stage of a complex process called cryocapacitation. There are significantly results ($p<0.05$) between Ah Plus and Ah Plus+20% EY regarding the sperm population with damage acrosomal membrane. Egg yolk addition to Ah Plus seems to not interfere with its compounds, conferring a higher stability to acrosomal membranes in temperature stress conditions. This effect is revealed in normal pH and osmolarity condition (Ah Plus+20%EY 305mOsm/kg, pH 6.89), as also in pH and osmolarity stress conditions (LEY 373mOsm/kg, pH 5.67).

The data registered using AhPlus+20%EY, are in a close range with those obtained by using the standard Lactose Egg Yolk extender ($p>0.05$), both extenders conferring sperm membrane protection.

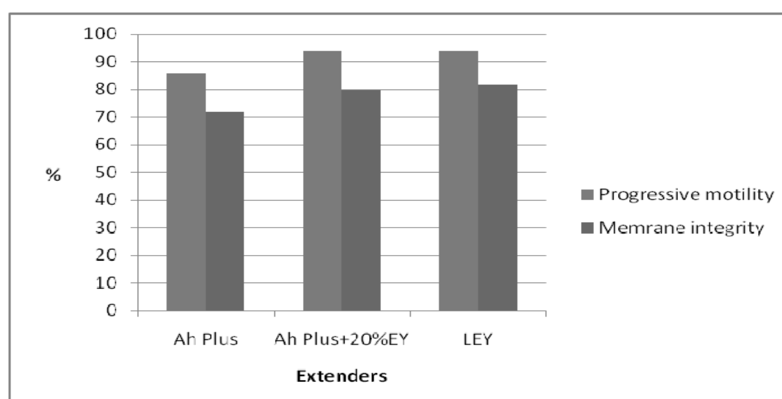

Figure 2. Progressive motility and membrane integrity of boar sperm samples cooled at 5°C in different extenders

Even so, high motility results were registered for all three extenders, sperm membrane integrity was unable to maintain such high values, registering a decrease compared to sperm motility (figure 2). It seems that cold shock may affect more the structure of the membranes (mainly the acrosomal membrane), most of these injuries having no immediately affect on sperm motility.

To evaluate only the motility of boar spermatozoa, may lead to unspecific results, other sperm characteristics having high impact on the fertility, should be considered, as those could be affected rough by temperature stress factors.

In any case, in further trials it would be interesting to evaluate the behavior of sperm cells extended in Ah Plus+20% EY during low temperatures (12-5°C) storage time periods.

4. Conclusions

The results of this study proved the positive effect of egg yolk on sperm motility and its membrane integrity in temperature stress conditions. In inappropriate transport and storage conditions, Ah Plus 20% EY may be a future option, conferring stability in boar sperm cells against cold shock; further studies in this field are still required. The cold shock protective effect of egg yolk may also be seen as an indicator for finding other compounds, which can provide sperm protection on low temperatures. The decrease of the storage temperatures may prove beneficial aspects for prolonging the storage time, while still satisfying conditions of sperm quality are maintained.

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