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Bovine serum albumin and skim-milk improve boar sperm motility by enhancing energy metabolism and protein modifications during liquid storage at 17 °C

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ABSTRACT

Both bovine serum albumin (BSA) and skim-milk have been reported to improve sperm quality, primarily by enhancing sperm motility, but the underlying molecular mechanism remains unknown. In this study, boar semen samples were collected and diluted with Androstar® Plus extender containing different concentrations (0, 2, 4 g/l) of BSA and skim-milk. On days 0, 3, 5 and 7, the sperm motility parameters were determined using computer-assisted sperm analysis (CASA), and the ATP concentrations, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity and mitochondrial membrane potential were evaluated using commercial kits. The levels of protein phosphorylation, acylation and ubiquitination were analyzed by western blot. The results showed that supplementation with BSA and skim-milk provided higher sperm motility parameters, ATP levels, GAPDH activity and mitochondrial membrane potential than the control group ($P < 0.05$). Interestingly, we found that the levels of protein phosphorylation, acetylation and succinylation of the spermatozoa in the treated groups were dramatically higher than those in the control group ($P < 0.05$). Though the protein ubiquitination level had a decreasing trend, the change in ubiquitination modification was not significantly different between the control group and treated groups. Moreover, the changes in protein modifications between the BSA treated group and skim-milk treated group were not distinctly dissimilar. Taken together, these results suggest that BSA and skim-milk had a positive role in the regulation of boar sperm motility by influencing sperm protein modifications changes as well as increasing the GAPDH activity, mitochondrial membrane potential, and intracellular ATP content. This research provides novel insights into the molecular mechanisms underlying BSA and skim-milk protective effects on boar sperm in the male reproductive system and suggests the feasibility of using skim-milk instead of BSA as a boar semen extender supplement.

1. Introduction

Modern pig reproduction, in most breeds, is based on the use of artificial insemination (AI). AI has dramatically increased worldwide due to the use of extended semen, which is liquid stored for several days [1]. Because of a low cholesterol/phospholipid ratio, boar sperm is prone to damage at low temperatures [2]. Moreover, cryopreservation, which results in low fertility of boar semen, also has a serious impact on AI in the swine industry. Therefore, liquid storage at 17 °C is an effective way to preserve boar semen and results in a similar pregnancy rate and litter size compared with natural mating.

The majority of inseminations conducted worldwide are used with diluted semen, which can be preserved up to 5 days [1]. The quality of boar semen distinctly decreased with the extension of preservation time *in vitro*. One of the major causes of these adverse effects is an imbalance in reactive oxygen species (ROS). Indeed, suitable ROS concentrations in sperm can cause the acrosome reaction, hyperactivation, and capacitation [3]. However, excessive ROS can directly damage organelles, membranes, and the DNA of sperm, leading to apoptosis and cell death [3,4]. Numerous studies have demonstrated that the addition of protectants can provide an effective defense against the detrimental effects of oxidative stress [5].

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Bovine serum albumin (BSA) is a common semen protective agent that requires high production costs and complex productive technology. Previous studies have reported that BSA could decrease the lipid peroxidation in the plasma membrane caused by ROS and protect the plasma membrane efficiently [6]. Skim-milk is a non-enzymatic antioxidant that functions by compensating for the loss resulting from seminal plasma removal [5]. Furthermore, the harmful bacteria existing in low-cost skim-milk is in line with the World Health Organization standards. It is well-established that both BSA and skim-milk can protect domestic animal sperm against oxidative stress [6,7]. Thus, many researchers tend to use skim-milk instead of BSA for the sake of reducing production costs.

Most previous studies about semen quality evaluation mainly focus on sperm motility, acrosome integrity, and membrane integrity, which are the common macro-indexes of spermatozoa. Numerous studies have indicated that BSA could effectively maintain boar sperm motility during liquid storage at 17 °C, and the recommended optimum concentration of BSA was 4 g/l. Moreover, very high concentrations of BSA may decrease sperm motility, plasma membrane integrity and acrosome integrity [6]. In addition, Namula et al. reported that the addition of 7.5 g/l skim milk could improve sperm motility and viability after long-term preservation, but the supplementation of skim milk had no significant effects on plasma membrane integrity, acrosome integrity or sperm penetrating ability [8]. Though BSA and skim milk have been proven to be useful supplements for improving the motility of boar sperm, the underlying molecular mechanisms of the protective effects of BSA and skim-milk on boar sperm remain unclear. Importantly, the majority of the physiological processes in sperm, for instance, energy metabolism and protein modifications, are related to sperm motility and fertility. Furthermore, it has been reported that protein phosphorylation and acylation can directly or indirectly influence sperm motility or energy metabolism [9]. However, so far little attention has been paid to the impact of BSA and skim-milk on sperm energy metabolism, protein phosphorylation and acylation during liquid storage at 17 °C. Therefore, with the goal of contributing to this subject, we have comprehensively assessed the changes of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity, mitochondrial membrane potential and ATP concentrations in sperm, and protein phosphorylation, acylation and ubiquitination were determined to explore the effects of BSA and skim-milk supplementation on sperm function. Our study contributes guidelines for using skim-milk instead of BSA as a boar semen extender supplementation.

2. Materials and methods

2.1. Animals

Twelve sexually mature and healthy Large White boars (2-3 year-old) were selected from Shanghai Sunsing livestock Co., Ltd (Shanghai, China). All of them with high sperm quality have been proven fertility (over 80% pregnancy rates).

2.2. Semen collection

The semen samples were collected from twelve boars and transported to the laboratory at 37 °C within 20 min. Only semen with motility >70% was included in this study.

2.3. Diluent preparation

In this study, the basic diluent was Androstar® Plus extender (Minitube, Tiefenbach, Germany), and it was prepared in the laboratory. The treatment groups were Androstar® Plus extender supplemented with various concentrations (2, 4 g/l) of BSA (EMD Millipore Corporation, Billerica, MA USA) and skim-milk (Foodhold USA LLC, Landover MD, USA). Compared to the treated groups, the control group was not supplemented with BSA or skim-milk.

2.4. Semen processing

Semen samples were diluted with Androstar® Plus extender at a ratio of 1:2 and centrifuged at 800 × g for 5 min. After removing one third of the supernatant, the rest of semen was suspended in Androstar® Plus extender at a total volume of 20 ml and a final concentration of 1×10^8 cells/ml. The diluted semen was divided into five equal fractions; one fraction served as the control group, and the other fractions were supplemented with 2 g/l BSA, 4 g/l BSA, 2 g/l skim-milk, or 4 g/l skim-milk for the treatment groups. It is important to note that before supplementation, the skim-milk was sonicated using an ultrasonic cell crusher (Hielscher Ultrasonics Gmbh, UP50H, Germany) on ice for 40 min (Amplitude: 80%, Cycle: 0.5), and then it was centrifuged at 12,500 × g for 10 min to detect whether there were pellets in the plastic centrifuge tubes, which ensured that the skim-milk was sonicated completely. Then, the samples were stored in a 17 °C incubator, which was sterilized with 75% alcohol. During storage, the samples were shaken twice daily to prevent the sperm from clumping together.

2.5. Sperm motility assessment

An aliquot of 500 μl from each semen sample (a control group and four treatment groups) was taken from 17 °C incubator on each experimental day (day 0, 3, 5, 7). After 20 min incubation in 5% CO_2 incubator at 37 °C, 5 μl of semen sample was pipetted into pre-warmed disposable counting chambers (standard count four-chamber 20 μm slides, Leja, Nieuw Vennep, the Netherlands). The computer-assisted semen analysis (CASA) system (Hamilton Thorne Research, Beverly, MA, USA) was used to evaluate sperm movements. The variables measured mainly include total motility (MOT), progressive motility (PRO), curvilinear velocity (VCL), and straight line velocity (VSL). A minimum of eight fields per sample were assessed, counting a minimum of two hundred sperm per sample [10]. For the sake of simplifying the following experiments, we choose the BSA and skim-milk treatment groups which emerge higher motility parameters to examine the metabolic enzymes activity and the level of protein modifications.

2.6. Measurement of ATP content

A bioluminescent ATP assay kit (FLASC, Sigma) was used to measure the ATP concentrations of boar sperm. An aliquot of 1 ml from each specific semen sample (a control group and two higher concentration treatment groups (4 g/l BSA, 4 g/l skim-milk)) was taken from the 17 °C incubator on each experimental day (day 0, 3, 5, and 7) and washed with cold phosphate buffer solution (PBS) (8 g NaCl, 0.2 g KH_2PO_4, 1.15 g Na_2HPO_4, and 0.2 g KCl, diluted with DDH_2O to 1 l). Intracellular ATP was collected by the addition of the manufacturer-supplied lysis buffer. The extracts of ATP and a series of standard ATP dilutions were mixed with luciferase before using the luminometer (Thermofisher Evolution 300) to capture the fluorescent signal. Finally, the observed measurements were compared to dilutions of an ATP standard [10].

2.7. Measurement of GAPDH activity

An aliquot of 1 ml from each specific semen sample (a control group and two higher concentration treatment groups (4 g/l BSA, 4 g/l skim-milk)) was taken from the 17 °C incubator on each