Pre-conditioning with Xanthine oxidase to improve post thawed quality of bull sperm

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ABSTRACT

The purpose of this study was to examine the effects of sub-lethal concentration of Xanthine oxidase (XO) on the post-thawed bull sperm quality. Semen samples were collected from four Holstein bulls, twice a week and during three consecutive weeks (n = 24 total ejaculates). After collection in each replicate, semen samples were pooled and then frozen by semen extender containing different concentrations [0 (XO-0), 0.05 (XO-0.05), 0.5 (XO-0.5), 5 (XO-5), 50 (XO-50) and 500 (XO-500) \(\mu\)M] of XO. After thawing, motion parameters (SCA), plasma membrane functionality (HOST), apoptosis status (Phosphatidylserine translocation assay), mitochondrial activity (Rhodamine 123), and acrosome integrity (PSA), were evaluated. The results showed that total motility, VAP, VSL, VCL, STR, and LIN were lower in XO-50 and XO-500 compared to other groups (P < 0.05). Progressive motility were higher in XO-0.05 and XO-0.5 compared to XO-0, XO-50, and XO-500 (P < 0.05). Mitochondrial activity was highest in XO-0.05 and XO-0.5 groups. Sperm plasma membrane functionality was significantly greater in XO-0, XO-0.05, XO-0.5, and XO-5 than that of XO-50 and XO-500. Xanthine oxidase had not significant effects on acrosome integrity and dead spermatozoa. Higher percentage of live spermatozoa was recorded for XO-0, XO-0.05, XO-0.5, and XO-5; however, the lower amount of apoptotic spermatozoa was detected in the aforementioned groups (P < 0.05). In conclusion, it seems that XO at lower doses may have beneficial effects on post-thawed bull sperm quality.

1. Introduction

Artificial insemination is a technique by which thousands of cattle could be inseminated by the sperm provided from just a few numbers of bulls that helps to spread the superior genetics [32]. Cryopreserved semen, which is widely used in artificial insemination, together with some other reproductive technologies is applied to save endangered species [34]. Improvement in semen technology (successful storage of semen in liquid and frozen condition) is one of the fundamental elements in continuous trend of genetic progress in dairy cattle [33]. However, frozen sperm undergo some structural and biochemical damages during freeze-thawing process [12], which in turn results in impaired function of sperm and fertility reduction [34]. Although reactive oxygen species (ROS) are required for optimal sperm function [30], the impaired function of sperm after cryopreservation is attributed to the excessive production of ROS [3]. Therefore, attempts are made to discover an optimal strategy to overcome the negative effects of ROS during cryopreservation [33]. Accordingly, one of the most used strategies is supplementation of semen extenders with antioxidants to protect sperm against ROS activity [2,14,29,35] which has been shown to be relatively successful. However, some studies believe that antioxidant therapy is not a fully reliable strategy, because sperm cannot use the entire antioxidant capacity and also, the antioxidants may be converted to toxic components which adversely affect sperm performance [1,28]. In recent years a new strategy based on preconditioning of sperm is being developed to protect sperm during cryopreservation process [9,25,28]. It has been suggested that induction of mild sub-lethal stress to the sperm prior to cryopreservation, improves sperm protection against probable damages during semen preservation and cryopreservation [9,13,17,28]. In some studies, it has been shown that induction of mild oxidative stress by nitric oxide improved post thawed bull and rooster sperm quality through curtailing the caspase-3 activity and therefore, constrained the sperm apoptotic pathways [9,28]. Mild sub-lethal stress can result in increment of some especial proteins from...
chaperone family which are involved in cellular mechanisms such as energy and fatty acid metabolism, redox regulation, protein stabilization and dispose of impaired proteins [5]. It is well established that heat shock proteins (molecular chaperones) exist on sperm surface [4,21] which can keep up the sperm homeostasis during stress conditions [26]. Xanthine oxidase is a form of Xanthine Oxidoreductase which produce ROS, it is present in sperm as well as all biological cells, and it can reduce the molecular oxygen to produce superoxide anion and hydrogen peroxide (H$_2$O$_2$) [10]. In a study, researchers have reported that incubation of stallion sperm with XO (as a ROS generator), increased H$_2$O$_2$ levels in stallion sperm and decreased sperm motility and sperm cryosurvival [8]. In this study we used XO to produce the sub-lethal oxidative stress before cryopreservation; therefore, this study was intended to examine the effect of XO-derived mild stress on quality parameters of bull sperm after freeze-thawing process.

2. Materials and methods

2.1. Chemicals

All chemicals applied in this study were provided from Sigma (St. Louis, MO, USA) and Merck (Darmstadt, Germany) company. In case of using chemicals from other companies, the producer company is noted.

2.2. Farm management and semen collection

Approval for the study was provided by the Research Ethics Committees of University of Tehran. By means of artificial vagina, semen samples (n = 24 total ejaculates) were obtained from four Holstein bulls, twice a week and for three consecutive weeks (six replicates). Holstein bulls were kept at ZarGene AI stud, Firoozkooh, Tehran, Iran under standard situation. They were fed a standard diet according to NRC 1998 and they had free access to water. Instantly after collection, semen samples were moved to a 37 °C water bath and primitive evaluations were performed to select the appropriate samples for cryopreservation (semen concentration of ≥ 1.0 × 10^9 spermatozoa/mL, motility ≥ 60%, and ≤15% abnormal morphology). Then, semen samples were put together to eliminate the individual differences. Afterwards, semen samples were split into six equal parts.

2.3. Semen preparation and cryopreservation

Each part of semen sample was extended with Optidyl® (Biovet, France) extender, containing a specific amount of XO enzyme, as an oxidative stressor, to reach the final concentration of 100 × 10^6 spermatozoa/mL. Different XO concentrations used in the present study were as following: 0 (XO-0), 0.05 (XO-0.05), 0.5 (XO-0.5), 5 (XO-5), 50 (XO-50) and 500 (XO-500) μM. Afterwards, the extended semen samples were slowly cooled to reach the final temperature of 4 °C. After 3 h equilibrium time, samples were packed in 0.25 mL French straws (IMV Technologies, L’Aigle Cedex, France) and then frozen in a computer controlled freezing system [(Digit Cools, IMV Technologies), −3 °C/min from 4 °C to −10 °C, −40 °C/min from −10 °C to −100 °C, −20 °C/min from −100 °C to −140 °C]. Thereupon, the samples were maintained in liquid nitrogen until they were used for post thawed assessments [7]. Before each assessment, the frozen semen samples were conveyed to a 37 °C water bath for 30 s to be thawed.

2.4. Post thawed evaluations

2.4.1. Motility and motion parameters

Motion characteristics were assessed by means of sperm Class Analysis software (SCA) [28]. A 5 μL of thawed semen was put on a 37 °C chamber slide (20 μm, Leja 4, Leja Products Luzernestraat B.V., Holland), several characteristics of sperm were recorded. The parameters evaluated included: total motility (%), progressive motility (%), average path velocity (VAP, μm/sec), straight line velocity (VSL, μm/sec), curvilinear velocity (VCL, μm/sec), amplitude of lateral head displacement (ALH, μm), straightness (STR, %), and linearity (LIN, %).

2.4.2. Plasma membrane functionality

Plasma membrane functionality was estimated through Hypo-osmotic swelling (HOS) test [27]. Briefly, 30 μL of semen samples were mixed with 300 μL of HOS solution (9 g fructose and 4.9 g sodium citrate were dissolved in 1 L of distilled water, 100 mOsm/kg, pH = 7) and then incubated at temperature of 37 °C for 30–40 min. Next, a droplet of the blend was loaded on a microscope slide, covered with a coverslip and observed under a phase contrast microscope (× 400 magnifications, CKX41, Olympus, Tokyo, Japan). A minimum of 200 spermatozoa were observed and the percentage of spermatozoa with swollen and non-swollen tails were calculated.

2.4.3. Acrosome integrity

Acrosome integrity was determined by means of Pismum sativum agglutinin (PSA) [20]. Briefly, 5 μL of sperm sample was mixed with 100 μL of 96% purity ethanol. 15 min later, 10 μL of the mixture was blended with 30 μL of PSA on a glass slide. Finally, in each slide, 200 spermatozoa were counted by a fluorescent microscope (BX51, Olympus) equipped with fluorescence illumination and a fluorescein isothiocyanate (FITC) filter (excitation at 455–500 nm and emission at 560–570 nm) at × 400 magnification. The sperm heads taken green fluorescent were counted as sperm with integrated acrosome and the others which had not taken green fluorescence were counted as sperm with damaged acrosome.

2.4.4. Mitochondrial activity

By means of fluorescent dyes, Rhodamine 123 (R123, Invitrogen TM, Eugene, OR, USA) and propidium iodide (PI), mitochondrial activity was assessed. Concisely, 5 μL of Rhodamine 123 solution was added up to 250 μL of semen sample and incubated at 25 °C in the dark for 30 min. Afterwards, 5 μL of PI was added to the mixture and consequently analyzed with a FACS Calibur Flow cytometer (Becton Dickinson, SanJose, CA, USA). Finally, the percentage of live sperm with active mitochondria was determined (positive for R123 and negative for PI) [28].

2.4.5. Phosphatidylserine translocation assay

To observe the movement of phosphatidylserine (PS) in the sperm plasma membrane, a commercial PS Detection Kit (IQP, Groningen, Netherlands) was applied based on the producers’ directions. Concisely, semen samples were washed with a calcium buffer and re-extended to reach the concentration of 1 × 10^6 spermatozoa/mL. Following that, 10 μL of Annexin V-FITC was added to the sperm suspension and incubated at room temperature for 20 min. Then, 10 μL of PI was added to the sperm suspension and incubated at 25 °C in a dark place for 15 min. Finally, the mixture was analyzed with a FACS Calibur Flow cytometer (Becton Dickinson, San Jose, CA, USA). The analysis classified the sperm to three different groups as follows: 1) viable sperm (negative for both annexin-V and PI), 2) early apoptotic sperm (positive for annexin-V and negative for PI), 3) dead sperm (positive/negative for annexin-V and positive for PI).

2.5. Statistical analysis

This trial was carried out in a completely randomized design with six replicates. Data were analyzed through Proc GLM of SAS 9.1 (SAS Institute, version 9.1, 2002, Cary, NC, USA). The results were denoted as mean ± SEM and Tukey’s test was applied to compare the mean of the treatments and differences with values of P < 0.05 were considered to be statistically significant.
3.3. Phosphatidylserine translocation assay between the semen samples received di 

XO-50 and XO-500 (56.5, 54.5 and 50.9, respectively (SEM = 3.5)) spermatozoa with functional membranes was significantly, and acrosome integrity of post thawed sperm. The percentage of a-c Means with different superscript in each row differ significantly.

3. Results

3.1. Motility and motion parameters

Data for motility and velocity parameters are represented in Table 1. Total motility, VAP, VSL, VCL, STR, and LIN were lower in XO-50 and XO-500 compared to other groups (P < 0.05). The highest percentage of progressive motility was recorded for XO-0.05, XO-0.5 and XO-5 groups (P < 0.05), while the lowest value was recorded for XO-500(P < 0.05).

3.2. Plasma membrane functionality, acrosomal integrity, and mitochondrial activity

Table 2 shows plasma membrane functionality, mitochondrial activity, and acrosome integrity of post thawed sperm. The percentage of spermatozoa with functional membranes was significantly lower in XO-50 and XO-500 (53.5 and 48.5, respectively (SEM = 3.1)) compared to other groups (ranging from 72 to 76.1 (SEM = 3.1)), while no differences were identified among other groups.

The highest percentage of post thawed sperm with active mitochondria was detected in XO-0.05 and XO-0.5 (80.4 and 82.1, respectively (SEM = 3.5)) compared to other groups (ranging from 72 to 76.1 (SEM = 3.1)), while no differences were identified among other groups.

For acrosomal integrity, no significant differences were observed between the semen samples received different concentrations of XO.

3.3. Phosphatidylserine translocation assay

The post thawed sperm apoptosis status is illustrated in Table 3. Xanthine oxidase treatment caused a decrease in viable sperm in XO-50 and XO-500 (52.6 and 48.4, respectively (SEM = 4.53)) compared to other groups (ranging from 77 to 81 (SEM = 4.5)), while there were no significant difference among the other groups. Furthermore, the highest percentage of apoptotic sperm was recorded for XO-0.05 and XO-500 (35 and 39.1, respectively (SEM = 3.86)) (P < 0.05).

About dead spermatozoa, no differences were found among different groups (P > 0.05).

4. Discussion

In this study, addition of XO to the freezing extender affected post thawed quality of bull sperm. Progressive motility and mitochondrial activity were significantly higher in XO-0.05 and XO-0.5; however, no significant differences were identified between the mentioned groups and control in terms of other parameters evaluated in this study. Improvement in the quality of sperm in the present study is comparable with the results of Feyzi et al. [9] and Sharafi et al. [28] who reported that post thawed motion parameters of rooster and bull sperm were enhanced following induction of mild sub-lethal oxidative stress to the semen samples before freezing. Sub-lethal stress can stimulate the phosphorylation and production of heat shock proteins which can result in an increase in sperm resistance, repair and stabilize the DNA during stress condition, and finally re-establish sperm homeostasis [5,26]. Hydrogen peroxide is one of the ROS members which is produced by XO [6], and it has been stated that it can increase the level of heat shock proteins [18]. Also, it is well established that heat shock proteins have a protective role against ROS [24] and ROS are known as a factor involving in mitochondrial activity reduction [31]. Mitochondria, as an ATP generator in sperm, have a key role in sperm motility. Therefore, the improved progressive motility in this study might be due to sub-lethal oxidative stress-induced production of heat shock proteins and consequently improved mitochondrial activity. Another reason for improved sperm progressive motility might be due to nitric oxide activity. It is suggested that nitric oxide plays a role in activation of GMP/protein kinase G signaling pathway and consequently can improve sperm motility [22]. Since it is reported that XO has a nitrite reductase activity and it can produce nitric oxide from nitrite [10,16], a probable pathway by which XO could improve sperm progressive motility in the current study might be through production of nitric oxide.

Plasma membrane functionality was the lowest in XO-50 and XO-500 groups. This finding was similar to those described by Sharafi et al. [28] who stated that the higher levels of oxidative stress stimulators (nitric oxide) results in functional membrane reduction. However, our results conflicted with findings of Dodaran et al. [7] who reported that induction of mild stress to bull semen via low doses of ethanol had no significant effect on sperm membrane functionality. Our results seem to indicate that usage of 50 and 500μM of XO are too much to be considered as a mild stressor. Therefore, higher production of XO-induced oxidative stress might be a reason for lower sperm membrane functionality in the mentioned groups. Also, the contradiction between our results and that of Dodaran et al. [7] might be due to different stress stimulators and different dosage of them.

The treatments used in our study had no effect on sperm acrosome integrity. These findings were concurrent with previous reports which showed no significant effect of ethanol or nitric oxide supplementation of semen samples, as oxidative stress stimulators, before freezing on sperm acrosome integrity [7,9,28]. The presence of an intact membrane

<table>
<thead>
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<th>Parameter</th>
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<th>SEM</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total motility (%)</td>
<td>XO-0</td>
<td>74.6 ± 3</td>
<td>42.1 ± 3</td>
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<td>Progression (%)</td>
<td>XO-0.05</td>
<td>81.8 ± 3</td>
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<td>Mitochondrial activity (%)</td>
<td>XO-0.5</td>
<td>82.3 ± 3</td>
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</tr>
<tr>
<td>XO-500</td>
<td>28.7 ± 3</td>
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**Means with different superscript in each row differ significantly.**
and acrosome are very important for a normal fertilization process. Therefore, evaluation of these parameters are essential in assessment of the fertilizing ability of sperm. Although, using sublethal concentrations of XO significantly improved several quality indicators of post-thawed sperm, the membrane functionality and acrosome integrity were not affected under effects of low concentrations of XO. Although there is no reports on the effects of XO on the plasma membrane functionality and acrosome integrity, these results are not confirmed by the other studies in which sublethal concentrations of other oxidant such as nitric oxide and hydrogen peroxide improved the membrane functionality of sperm post thawing. The reason for this observation is not completely understood but it is probably that XO have interaction effects with other components of extender and in such situation, XO couldn’t apply the cryoprotective effects. Also, it has been postulated that sublethal beneficial effects of XO are more intracellular than extracellular.

In the present study, data related to the apoptosis which was considered using phosphatidylserine translocation assay, showed a discrepancy with the reports of Feyzi et al. [9] and Sharafi et al. [28]. The low concentration of oxidative stress induced by XO could not improve the apoptotic status of post thawed sperm; however, the greater doses of XO (XO-50 and XO-500) used in the current study significantly decreased viable sperm and increased apoptotic sperm. It has been stated that preconditioning of different types of live cells with sublethal oxidative stress increase the production of heat shock proteins which in turn increase the cell’s resistance [15]. Moreover, it has been noted that heat shock proteins inhibit the apoptotic pathways in embryos [11]. However, this pathway was not significantly affected by the low doses of XO in our study. Although it is not completely understood that why low doses of XO had no effects on the apoptotic like changes, a possible reason for the discrepancy might be different cell types used in these studies. Also, it is worth noting that with measurement of other apoptotic factors such as caspase enzymes, we may obtain a more reliable results to be discussed for apoptotic pathway under effects of sublethal oxidative stress induced by XO. Still, it should be kept in mind that higher doses of XO (XO-50 and XO-500) negatively affected the apoptotic status of sperm in the present study. It has been reported that ROS can stimulate the activation of caspases and finally induce apoptosis [23]. Thus, it seems that greater doses of XO used in the present study have caused hyper production of ROS which subsequently increased apoptotic and decreased viable spermatozoa.

Comparable to the results of Dodaran et al. [7], our data showed a significant enhancement in sperm mitochondrial activity in semen samples exposed to sub-lethal oxidative stress. As we described previously, this result may be due to the protective effect of heat shock proteins against ROS, a detrimental factor for mitochondrial activity [51], which in turn can result in improved mitochondrial activity. It is worth mentioning that our results showed a rational association between mitochondrial activity and sperm motility. It is well documented that these two parameters are highly related to each other [19].

5. Conclusion

According to the results of the present study preconditioning of bull sperm prior to cryopreservation with an ROS generator, XO, at lower doses (lower than 50 μM) may have beneficial effects on post thawed quality of bull sperm. Supplementation of semen extenders with antioxidants is one of the most used strategies to maintain the sperm performance after freeze-thawing. However, some researchers believe that addition of antioxidants to the semen extender is not a fully reliable strategy, because sperm cannot use the entire antioxidant capacity and also, the antioxidants may be converted to toxic components which adversely affect the sperm performance. With regard to the aforementioned problems implied for antioxidant therapy during semen cryopreservation, developing the new strategies, such as preconditioning of sperm, to upgrade the cryopreservation protocols are of high importance.

Acknowledgement

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cryobiol.2019.07.003.

References


Table 3

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<td></td>
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<td>XO-5</td>
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<tr>
<td></td>
<td>XO-50</td>
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<td>Apoptotic (%)</td>
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<tr>
<td></td>
<td>XO-500</td>
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<tr>
<td>Dead (%)</td>
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* Means different superscript in each row differ significantly.
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