Antioxidant Effects of Seminal Plasma on Cellular Morphological Viability of Swine Semen Post-Cryopreservation

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Abstract

The aims of this study were to measure the antioxidant enzyme activity of superoxide dismutase (SOD) and glutathione peroxidase (GPX1) in seminal plasma (SP), the influence of temperature overtime on such enzymes and, to assess post-thaw viability of semen supplemented with autologous or homologous SP by eosin-nigrosin (EN) and hypoosmotic tests (HOST). A total of 48 sperm-rich fractions from 8 boars were collected and equally divided for SP antioxidant activity determination or cryopreservation experiments. Marked differences in SP antioxidant activity were found amongst individuals. SOD values ranged from 7.88 ± 0.04 U/g to 12.01 ± 0.07 U/g. Restricted maximum likelihood variance components estimates (REML) indicated that 98% of the variation resided between individuals (p<0.05). In addition, GPX1 activity ranged from 0.03 ± 0.001 U/g to 0.05 ± 0.005 U/g with a REML between and within individual of 56% and 42%, respectively (p<0.05). Further, temperature largely influenced SOD and GPX1 activity ( Spearman’s $r > 0.77$; main effect $p<0.01$). Regardless, sperm viability improved significantly in groups supplemented with 20% (v/v) SP compared with control as per EN 27 ± 0.59 % vs. 26 ± 0.23 % or HOST 28 ± 0.27 % vs. 18 ± 0.27 % (p<0.05). Although there was an additive effect of SP on sperm viability, the antioxidant levels were not strongly correlated to sperm morphology. Therefore, other factors in seminal plasma are contributing to sperm viability and overall fitness towards a successful fertilization.

Keywords: Seminal plasma; Antioxidant; Sperm viability; Cryopreservation

Introduction

Successful swine fertility programs can be achieved using extended cooled semen when compared to those using natural service. Although the former is a routine procedure, its use is limited because sperm cells remain viable for about 3 to 5 days [1]. Cryopreservation would offer a solution to the erstwhile problem by long-term storage. However, post-thaw viability of cryopreserved semen in swine species is generally compromised with an inherent reduction in reproductive performance [2]. Reportedly, boar sperm has been found particularly sensitive to cryoinjuries compared with other species. During freezing about 50% of sperm cells lose viability due to several factors such as heat shock, intracellular ice crystal formation, oxidative stress and cryocapacitation [3,4] that negatively affects fertility rates [5,6]; effects include motility impairment, chromatin damage and membrane alterations like decreased mitochondrial membrane potential [6,7]. Consequently, these cellular changes may potentially shorten the life span of spermatozoa by modifying regulatory pathways such as those of the acrosome reaction and thus, leaving a significant portion of the sperm population unable of interacting with the oviduct or ultimately of fertilizing the eggs [8].

A body of evidence indicates that seminal plasma (SP) ameliorates cryoinjuries and likely extends sperm longevity by delaying the undesired early onset of capacitation and acrosome responsiveness [5,6,9-11]. The protective effects of SP may be attributed to critical components such as antioxidant enzymes and other stabilizing proteins capable of modulating sperm function [12-14]. However, SP is removed prior cryopreservation to dilute the semen at a specific concentration in the extender. This dilution effect on SP may result in oxidative damage to unprotected membranes hence, compromising sperm integrity [15]. Additionally, dead spermatozoa contribute towards the cumulative ROS levels, exacerbating the process of damage to viable sperm [16-18]. Furthermore, controversial results may hamper the practical use of SP on cryopreserved semen i.e., while some studies support beneficial effects of SP [19-22], others are confounded by intrinsic experimental designs [23-25] or even detrimental effects have been reported [26,27]. It is plausible to infer that the variation in SP antioxidant activity between boars may carry a significant weight since adding SP from good freezers to frozen-thawed semen with mediocre performance resulted in improved viability and membrane integrity [23].

One of the attributes of SP is the antioxidant activity from critical enzymes such as glutathione peroxidases (particularly GPX1), superoxide dismutases (SOD) and catalases (CAT). Glutathione peroxidases are key players in one of the mayor eukaryotic redox systems i.e., glutathione, by keeping a balance on its reduced (GSH) and oxidized (GSSG) forms [28]. Additionally, SOD catalyzes the transfer of electrons to free radicals resulting in $\text{O}_2 ^*$ or $\text{H}_2\text{O}_2$ and CAT degrades...
hydrogen peroxide excess. Altogether, these enzymes are considered the main barrier as per sperm antioxidant protection [14] and their concentrations may not only have an effect at cryopreservation but on the overall boar fertilizing ability [3,29]. Despite some discouraging results of SP as an additive for cryopreservation, literature supporting its beneficial effects is outweighing e.g., protection against cold shock, reduction of cryocapacitation [5,6,14,15] and, improvement on the percentage of viability, motility and integrity of both plasma and acrosome membrane [23,30,31]. Recently, a seasonal variation of antioxidant activity in fluids from the vesicular glands has been reported and intriguingly, this effect is independent from the antioxidant enzyme activity of epididymal fluids [32]. The aim of this study was 1) to measure the levels of enzymatic activity of SP antioxidants during the hot weather in Tropical Mexico and 2) to evaluate the effect of the addition of either autologous or homologous SP to frozen-thawed semen on morphological viability.

**Material and Methods**

**Reagents and media**

Unless otherwise stated, chemicals used in this study were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA).

**Experimental design**

Procedures involving animals were approved by the Institutional Animal Care and Use Committee at the University of Veracruz, Mexico. Boar selection was based on the following criteria: two to four years old, clinically healthy, without pathologies associated to the male reproductive tract and with a proven history of fertility after four years old, clinically healthy, without pathologies associated to the species. Boars were housed in individual pens, fed twice daily with 1.5 kg of concentrate that contained 15% ADE) appropriate to the species. Boars were thawed and co-incubated with their corresponding seminal plasma i.e. autologous or with counterpart SP i.e. homologous. Boar were the random variable to account for between-and-within SP variation among individuals. The endpoint was to measure the effects of SP co-incubations on semen viability and to compare them against the following groups: freeze-thawed semen only, freeze-thawed-incubated without SP i.e. control, and fresh extended semen.

**Semen collection and sample processing**

A total of 48 ejaculates from eight boars (commercial breed Pietrain x Hampshire) identified as A,B,C,D,E,F,G and H were obtained by the gloved-hand technique. Additionally, only sperm-rich fractions were collected and filtered through sterile gauze. Each sample with ≥ 70% gross motility, ≥ 80% normal morphology and intact acrosome ridges (assessed by phase contrast microscopy) was equally allotted in two aliquots immediately after collection i.e. one for SP collection and the other for semen cryopreservation that was subsequently extended in pre-warmed Vitasem LD* (Magapor, Spain; 1:2 v/v). Both samples were transported to the laboratory within 2 hr at 21±1°C. To collect SP, raw semen was transferred to 50 ml tubes, cooled at 15°C for 120 min and centrifuged at 800×g for 10 min keeping the same temperature at all times. Thereafter, the supernatant containing the SP was transferred to another tube, centrifuged twice at 800×g for 10 min and stored at −20°C until further use to determine antioxidant enzyme activity.

Semen was cryopreserved in a freezing medium containing 80% (v/v) Tris-citric acid-glucose solution (111 mM Trizma base, 31.4 mM monohydrate citric acid, 185 mM glucose) and 20% egg yolk.

**Experiment 1: Antioxidant enzyme activity of seminal plasma:** Frozen samples of SP corresponding to each ejaculate from each boar were allocated to measure the enzyme activity of GPX1 and SOD, expressed in units per gram (U/g) of total protein using a randomized analysis of variance where boar was the random variable to account for between-and-within variation of SP of each individual. The goal of this experiment was to pinpoint any difference in enzyme antioxidant activity among boars. An additional objective was to determine a potential effect that temperature may have exerted on the level of antioxidant activity.

**Experiment 2: Morphological viability post-thaw:** In a randomized block design, cryopreserved semen samples from each boar were thawed and co-incubated with their corresponding seminal plasma i.e. autologous or with counterpart SP i.e. homologous. Boar was the random variable to account for between-and-within SP variation among individuals. The endpoint was to measure the effects of SP co-incubations on semen viability and to compare them against the following groups: freeze-thawed only i.e. thawed, freeze-thawed-incubated without SP i.e. control, and fresh extended semen.

**Results**

**Figure 1:** Diagram illustrating the criteria used in sample selection with subsequent experiments on SP enzyme antioxidant activity and sperm morphology variables.
supplemented with 100 µg/mL kanamycin sulfate (pH 7.2; 295–300 mOsm/kg). Briefly, upon reception semen samples were centrifuged at 800g for 10 min and the supernatant was discarded. Pelleted semen was immediately diluted in the freezing medium to a concentration of 1200 x 10^6 sperm per mL and cooled from 22° to 4°C within 180 min. Freezing medium containing 12% glycerol was gradually added to the cooled semen within 40 min (10 min apart) to obtain a final concentration of 6% glycerol and 600 x 10^6 sperm per mL. Samples were loaded into 0.5 mL plastic straws, sealed with PVA and exposed to liquid nitrogen vapor (4 cm) for 20 min before plunging them into liquid nitrogen. Stored straws were thawed for 30 s in a water bath at 37°C and treatment samples were supplemented with SP 20% (v/v) accordingly.

**Morphology assessment**

Vitality was determined using eosin-nigrosin stain (EN; HYSEL®, Mexico). Plasma membrane integrity was assessed by the hypoosmotic test (HOST) that consisted on incubating 10 µL of semen sample in 1 mL of a citrate-fructose solution (150 mOsm/kg) for one hour at 37°C and cells with any type of helical twist on the tail were considered as intact. In addition, both tests were carried out in at least three replicates by the same researcher on a 40X magnification with a minimum of 200 cell counts per reading.

**SP total protein and enzyme activity assays**

Total protein in the SP was determined by the biuret test where protein bonds were detected in an alkaline solution supplemented with copper ions (Merck®, USA) giving violet-colored coordination complexes that were subsequently read by spectrophotometry in g/dL [33]. The enzyme activity of SOD was measured by colorimetry using a commercial kit (Ransod®, USA) giving violet-colored coordination complexes that were subsequently read by spectrophotometry in g/dL. The enzyme activity of SOD was measured by colorimetry using a commercial kit (Ransod®, USA). Briefly, when provided in solution xanthine oxidase reacts with xanthine and forms superoxide radicals inhibited by the extent of SOD levels. In addition, GPX1 activity was determined by an NADPH-dependent UV kinetic method (Ransel®, USA) in which cumene hydroperoxide-induced oxidation of glutathione (GSSG) is catalyzed by GPX1. Subsequent reduction to GSH is achieved by providing glutathione reductase and exogenous NADPH yielding NADP+ and the decrease in absorbance was measured. All these procedures were performed as per manufacturers' instructions. Results were expressed in units per milliliter (U/mL) of SP and subsequently corrected for total SP protein values, thus, enzymatic activity was expressed in units per gram of protein (U/g).

**Statistical analyses**

Analysis of variance (ANOVA) between groups was performed using JMP PRO version 11 (Statistical Discovery from SAS Institute). Percentage (EN or HOST) or numerical (GPX1 or SOD) data not being normally distributed were transformed to arcsine or natural logarithm values, respectively, with appropriate back-transformation for presentation of the results. Intra and inter assay coefficient of variation of GPX1 were 4.73% and 6.12%, respectively. Likewise, Intra and inter assay coefficient of variation of SOD were 5.87% and 5.91%. We anticipated differences amongst individuals; thus, boar was assumed as the random variable with corresponding restricted maximum likelihood (REML) variance components estimates to account for differences between and within individual. Should the main effect was significant; means were compared using the post hoc test Tukey HSD. A probability of P<0.05 was considered statistically significant and P ≤ 0.1 a trend. Since we hypothesized a possible correlation between temperature and enzyme antioxidant activity, we analyzed the readings corresponding to average temperature at collection, SOD and GPX1 with a multivariate model using Spearman’s rank correlation coefficient (Spearman’s ρ). Should correlation was strong we plotted the data in a linear regression model to appreciate the effects of temperature on SP antioxidant activity levels.

**Results**

**Experiment 1: Antioxidant enzyme activity of seminal plasma**

Analysis regarding total protein in seminal plasma (SP) indicated that the average values corresponding to boars A, B and C were numerically higher than the mean (9.30 ± 0.39, 9.08 ± 0.69, 8.74 ± 0.64 vs. 8.58 ±0.30 g/dL). Additionally, REML indicated that there was less variation in SP total protein between than within individual 26.5% and 73.55%, respectively; however, this effect was not statically significant (p>0.05, Figure 3). In terms of SOD, the mean value for its enzyme activity was 9.71 ± 0.25 units per gram of SP protein (U/g). Significant differences were found between boars G and H expressing the highest values in SOD activity (12.01 ± 0.07 U/g, 11.80 ± 0.12 U/g)
compared with boars A, B and C (8.02 ± 0.12 U/g, 7.88 ± 0.04 U/g, 8.44 ± 0.02 U/g, respectively). Restricted maximum likelihood variance components estimates indicated that 98% of the variation resided between individuals (p<0.05; Figure 4). Furthermore, GPX1 analysis showed that boar E expressed the highest value of antioxidant enzyme activity (0.05 ± 0.005 U/g) and it was significantly different compared to boars A, B and C (0.03 ± 0.002 U/g, 0.03 ± 0.003 U/g and 0.03 ± 0.001 U/g; p<0.05) with a REML between and within individual of 58% and 42%, respectively (Figure 5).

Multivariate correlation analysis did in fact revealed a strong correlation between temperature and SOD and GPX1 enzyme activity levels (main effect p<0.01; Spearman’s ρ ≥ 0.77). In a subsequent linear regression model we found a positive effect of temperature over SOD and GPX1 (p<0.01). The first model indicates that one unit increase in average temperature resulted in a 0.44 ± 0.05 U/g unit increase of SOD activity. Likewise, a one unit increase in average temperature resulted in 0.003 ± 0.0005 U/g unit increase of GPX1 activity (Figure 6).

Experiment 2: Morphological viability post-thaw

Results indicated that the vitality of the sperm assessed by EN stain was significantly affected by treatment condition. As expected, fresh extended semen showed the highest values compared with thawed and control treatments (61 ± 0.36 % vs. 29 ± 0.28 % and 21 ± 0.20 %, respectively). However, when semen was supplemented with autologous SP vitality counts were higher than control and not different from homologous SP treatment (27 ± 0.59 % and 26 ± 0.23 %; p<0.01; Figure 7). Regarding membrane integrity assessed by HOST, results show a similar trend when compared to EN experiment. Fresh semen showed the highest values as opposed to control with the lowest values (38 ± 0.45 % vs. 18 ± 0.27 %). Further, membrane integrity of sperm treated with autologous and homologous SP were not different from each other and, not different from thawed ones (28 ± 0.27 %, 25 ± 0.13 % and 25 ± 0.32 %, respectively; p<0.01; Figure 8).

Discussion

Seminal plasma as an additive to cryopreserved semen has been deemed controversial. In our study, using SP 20% (v/v), we intended to measure the levels of two important antioxidants i.e. SOD and GPX1 and their effects on sperm morphology variables post-thaw. Another objective was to measure the levels of SP antioxidant activity overtime in a tropical environment. Results on antioxidant enzyme activity are in agreement with those reported elsewhere [34-36] except of those reported by Hernández et al. [23] in which ours seem to differ as per SOD activity. In addition, according to the classification of Hernández et al. [23] boars in this study fall under the category of bad freezers since sperm viability is below 50% in thawed semen. However, viability in this study was determined by other methods i.e. EN and HOST whereby readings are lower than those using the fluorescent triple stain approach [37]. Moreover, the same authors in Hernández et al. [23] also acknowledged that the enzyme activity does not have an absolute effect on thawed semen viability.

Seminal plasma protein-corrected SOD and GPX1 values were not only different amongst individuals but also enzyme antioxidant activity
The present study, we found that treatment with either autologous or homologous SP resulted in higher viability rates as compared with control and, no detrimental effects when using the homologous kind. Further, our results are in agreement with those of Kaeoket et al. [14] where addition of autologous or heterologous SP 50% (v/v) to extender yielded better viability as well as motility rates than in controls.

It is not new that seminal plasma components are highly variable among individuals [23,40]. Indeed, our results show a boar-dependent variation in antioxidant activity. Nonetheless, this variation among individual was controlled for in our statistical models thereby allowing us to detect potential differences when using either autologous or homologous SP supplementation; thus, given the similarity of those results we suspect other factors within SP are affecting the overall quality of sperm. Binder of sperm proteins (BSP; previously known as bovine seminal plasma proteins) have an active role in the biology of the sperm membrane after ejaculation. In bovine species, there are 3 BSPs reported in the binding of the sperm to the tubal epithelium i.e. BSP1, BSP3 and BSP5 and the release thereof during ovulation to acquire acrosome responsiveness prior fertilization of the egg [13]. In addition, BSPs (BSP1 in particular) play a role in sperm membrane configuration including removal of cholesterol and phospholipids during capacitation [13]. Therefore, it is plausible to hypothesize that BSPs are also removed during sperm cryopreservation and adding SP improved viability by restoring some of the lost BSPs.

Antioxidant activity was strongly correlated to temperature but weakly correlated to EN and HOST outcomes (data not shown). These results are in agreement with those of Lasota et al. [41] and Villa et al. [42] reporting a poor correlation between GXP1 and SOD on sperm viability. This finding is of particular importance since SOD and GPX1 were correlated to each other based on Spearman’s \( r = 0.6 \) (\( p < 0.05 \)); hence, other factors in SP may also have contributed to improve sperm viability post-thaw. As mentioned above, GXP1 is one of the most important peroxidases within the Glutathione redox system. The second, but not least, important redox system in eukaryote cells is the Thioredoxin system composed by NADPH, Thioredoxin reductases (TRXR1 in particular) and thioredoxin (TRX; [43]). GSH and TRX systems interact with each other albeit they are not mutually exclusive; they are the most important antioxidant systems against oxidative stress. Since both GPXs and TRXRs are selenoenzymes more research is needed to unveil a possible interaction in the seminal plasma, because the former, as the primary redox buffer, is competing with the latter minimized cryopreservation-induced membrane damage [5,9,38] or early onset of acrosome responsiveness post-thaw [16,38] due to stabilizing proteins or antioxidant enzymes in SP that provide protection to the sperm cells [6,13,31]. Results in our study indicate that addition of SP to frozen-thawed semen ameliorated the negative effects of cryopreservation compared with control sperm as reflected in higher EN as well as HOST percent values (Figures 7 and 8). A work from Caballero et al. [39] indicated that the addition of 10% (v/v) autologous SP consistently improved membrane integrity, mitochondrial function and motility compared to those incubated without PS. However, positive effects from homologous SP exposure depended on boar origin since viability ranged from beneficial to detrimental likely due to an altered concentration of certain components such as stabilizing proteins. In the present study, we found that treatment with either autologous or homologous SP resulted in higher viability rates as compared with control and, no detrimental effects when using the homologous kind. Further, our results are in agreement with those of Kaeoket et al. [14] where addition of autologous or heterologous SP 50% (v/v) to extender yielded better viability as well as motility rates than in controls.

**Conclusion**

Herein, adding seminal plasma to frozen-thawed semen has resulted in improved morphological viability assessed by EN and HOST. Additionally, SOD and GPX1 enzymatic activity was influenced by average temperature. Since SP antioxidant enzyme activity did not have a strong effect on viability per se, we believe an unknown factor(s) played a significant role towards improved viability post-thaw. It seems possible that a stabilizing protein i.e. BSP or an additional redox buffer system i.e. TRX may better explain the variation in morphological viability of cryopreserved semen in swine species. Further research is needed to answer those questions.
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