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Characterization of Angiotensin-Converting Enzyme before and after Cryopreservation of Gir Semen

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Abstract

The aim of this study was to characterize the angiotensin-converting enzyme (ACE) in Gir semen before and after cryopreservation. The ejaculate of five sexually mature bulls was used. After collection, one 1-mL aliquot of fresh semen was analyzed immediately, and the rest of the semen was cryopreserved in liquid nitrogen for subsequent analysis. Freshly collected semen and thawed cryopreserved semen were centrifuged twice with Tyrode's albumin lactate pyruvate medium (TALP) to remove plasma and extender, respectively. Samples were then subjected to western blotting, immunocytochemistry, and enzymatic activity techniques. At least one 100 kDa band was observed in every bull analyzed using western blotting with an anti-ACE monoclonal antibody, and band intensity decreased by 70% ($p < 0.05$) after cryopreservation. Immunocytochemistry showed periacrosomal ACE localization, and the area stained by the fluorescent antibody significantly decreased ($p < 0.05$) after cryopreservation. Enzyme activity was evaluated using FAPGG substrate hydrolysis, which was significantly lower ($p < 0.05$) in cryopreserved semen than in fresh semen. Therefore, the process of cryopreservation decreases ACE band intensity and enzyme activity in Gir bull semen, and reduces the stained area in immunocytochemistry.

Keywords

Bovine, Spermatozoid, Seminal Proteomics

1. Introduction

The process of semen cryopreservation becomes fundamental since acquiring a

bull of high genetic value is costly. Artificial insemination with frozen semen achieves satisfactory pregnancy rates, but the process of cryopreservation can still lead to approximately 50% loss in sperm viability [1]. During this process, the plasma membrane undergoes structural alterations, mainly during the cooling stage (from 19˚C to 8˚C), where it passes from a liquid to a gel state [2], and these changes are the main cause of intracellular ice crystal formation, excessive influx of calcium ions, and cell dehydration, resulting in decreased sperm fertilization capacity [3].

Sperm alterations resulting from cryopreservation also include changes in protein composition of both seminal fluid and the sperm membrane [4]. Nauc and Manjunath [5] reported a 70% - 80% decrease in the concentration of BSP-A1-A2, BSP 0A3, and BSP-30 kDa proteins in bovine seminal plasma after cryopreservation when compared to fresh bovine semen, and these proteins were later positively correlated with the freezing capacity of bovine semen [3]. Westfalewicz *et al.* [3] studied protein changes in sperm membrane due to cryopreservation, and reported altered expression of 16 proteins, which mostly decreased after the freezing/thawing process.

Angiotensin-converting enzyme (ACE) is one of the sperm membrane proteins. It is a zinc-dependent metalloprotease that is anchored to the cell plasma membrane, with most of its mass exposed on the cell surface [6]. This enzyme has a somatic and a testicular isoform. The somatic isoform (sACE) has a molecular weight of 150 - 180 kDa, is found in several organs, and plays a fundamental role in blood pressure control, converting angiotensin I (inactive peptide) into angiotensin II (vasoactive peptide) in the renin-angiotensin-aldosterone system, in addition to inhibiting bradykinin (vasodilator peptide), which results in increased blood pressure. This action is inhibited by some substances, such as captopril and enalapril, which are widely used to treat high blood pressure [6] [7].

The testicular isoform (tACE) has lower molecular weight (90 - 110 kDa). tACE and sACE are transcribed by the same gene, but tACE uses a specific testicular promoter and is found only in germinal testicular cells after meiosis [6]. Its expression is 2.5-fold higher on the sperm surface of abnormal human spermatozoa [8], and its enzyme activity increases in the spermatozoa of infertile men [9]. The larger amount in abnormal spermatozoa is due to a failure in ACE release during fertilization [8]. Its presence is fundamental for correct fertilization, since tACE knockout mice exhibit compromised fertility [10].

Therefore, data in the literatures on how the cryopreservation process changes ACE characteristics are scarce. Thus, the aim of this study was to evaluate intensity, immunolocalization, and enzymatic activity of ACE protein before and after cryopreserving the semen of bulls.

2. Materials and Methods

2.1. Animals, Semen Collection and Cryopreservation

Semen samples from five sexually mature Gir bulls (*Bos taurus indicus*) were

used. Collections were performed using the electroejaculation method. After collection, motility, vigor, concentration, and sperm morphology of each ejaculate were evaluated [11].

Each bull's ejaculate was diluted in tris-egg yolk extender (2.42% tris, 1.36% citric acid, 1% fructose, 7% glycerol, 20% egg yolk, and 0.7% gentamicin) at the concentration of 8×10^7 spermatozoa/mL, and stored in 0.5 mL straws. The semen was frozen using a TK 4000 portable programmable cryopreservation system (TK Reproduction Equipment, Uberaba, Brazil). This system uses a −0.25˚C/min cooling rate until reaching 5˚C, holds this temperature for 5 hours, and then a −15˚C/min freezing rate from 5˚C to −80˚C, and then −10˚C/min until reaching −140˚C [12]. Finally, the straws were placed in racks and stored in cryogenic tanks at −196˚C for at least two days before the evaluation.

2.2. Sperm Pellet Preparation

Fresh semen sperm pellets were prepared immediately after collection, using one 1-mL sample that was centrifuged twice at 700 × *g* for 30 minutes with TALP [13], at room temperature, to remove seminal plasma. Supernatant was discarded, and the pellet was used for subsequent analyses.

Frozen/thawed semen sperm pellets were prepared 48 hours after storage in cryogenic tanks. Ten straws were thawed in a 37˚C water bath for 30 seconds. The semen was then placed in the same tube and centrifuged twice at 700 × *g* for 30 minutes, at room temperature, with TALP to remove the extender. Supernatant was discarded, and the pellet was used for subsequent analyses.

2.3. Western Blot

The sperm pellet concentration of fresh and frozen/thawed semen was measured to detect ACE, and 100-µL aliquots containing 1×10^7 spermatozoa were subjected to protein extraction with 25 µL sample buffer (10% DL-Dithiothreitol, 0.004% bromophenol blue, 20% glycerol, 4% sodium dodecyl sulfate), and heated at 100˚C for 5 minutes in a thermoblock heating system. Samples were then centrifuged at 10,000 × *g* for 5 minutes.

The supernatant was electrophoresed (SDS-PAGE) and transferred to nitrocellulose membranes in transfer buffer (25 mM Tris, 0.2 M glycine, and 20% methyl alcohol, pH 8.5) at a constant voltage of 100 V [14]. The resulting nitrocellulose membranes were stained with 0.2% Ponceau S in acetic acid to determine electrotransfer efficacy. Subsequently, nonspecific binding sites were blocked using 3% skim milk in tris-buffered saline + Tween-20 (TTBS) (20 mM Tris, pH 7.8, containing 0.1% Tween-20) for 60 minutes. The membranes were then incubated (overnight at 4˚C) with an anti-ACE primary antibody (anti-ACE, clone E2, Millipore, 1:5000 in TTBS). Then, the membranes were washed three times with TTBS, and incubated with the secondary antibody (goat anti-mouse IgG, HRP conjugate, Millipore, 1:500 in TTBS) for 60 minutes at

room temperature. After washing three times with TTBS, the protein bands were identified by chemiluminescence [15].

To confirm that all samples had the same amount of protein, another gel was prepared with the same samples, which was then examined by western blotting with an anti-β-tubulin antibody (1:10,000) [16].

Pixel intensity of protein bands was compared using the ImageJ 1.40 software. The gray scale ranges from zero to 250, where zero represents a totally black image, and 250 represents a totally white image. Therefore, the closer to zero the band pixel intensity is, the more protein in the sample.

2.4. Immunocytochemistry

To determine ACE localization in spermatozoa, 10 µL of fresh and frozen/thawed semen pellet was diluted in 1 mL of PBS. Spermatozoa were fixed with 4% paraformaldehyde in PBS, and then permeabilized with 0.5% (v/v) Triton X-100 in PBS. Aliquots of 50 µL (1×10^7 spermatozoa) were deposited on slides previously treated with poly-L-lysine and incubated at room temperature for 60 minutes. Next, excess spermatozoa were washed with PBS and nonspecific sites were blocked with 3% BSA in PBS for 60 minutes. Subsequently, the slides were incubated with an anti-ACE primary antibody (anti-ACE, clone E2, Millipore, 1:250) diluted in 0.05% BSA-PBS at 4˚C overnight. After washing three times with PBS, the samples were incubated with an anti-mouse IgG secondary antibody (goat anti-mouse IgG, HRP conjugate, Millipore, 1:400). After washing three more times with PBS to remove excess antibody, the slides were stained with 5 µg/mL Hoechst 33258, and 5 µg/mL *Pisum sativum* fluorescein isocyanate (FITC-PSA), and then mounted with 1.4-Diazabicyclo[2.2.2]octan (DABCO) [15]. The slides were evaluated under fluorescence microscopy (excitation peak 550 nm and emission 570 nm), 200 cells were evaluated.

2.5. Enzyme Activity

ACE activity was determined based on the hydrolysis of furyl-acryloyl-L-phenylalanyl-glycyl-glycine (FAPGG) to furanacryloyl-L-phenylalanine and glycyl-glycine in a continuous spectrophotometric assay, as previously described by Holmquist *et al.* [17] and Ball *et al.* [18]. The fresh semen and frozen/thawed semen pellets were resuspended at a concentration of 1×10^8 spermatozoa/mL, and incubated in TALP with 1% Triton X at 4˚C for 60 minutes. After detergent extraction, samples were centrifuged at 10,000 × *g* for 30 min, and then 100 µL of supernatant was added to 900 µL of FAPGG (45 mM in DMSO) diluted 1:30 in 0.05 M Tris, pH 7.5, containing 0.3 M of NaCl. FAPGG degradation was measured every minute by the decrease of absorbance reading in spectrophotometer for eight minutes, and these values were converted to enzyme activity expressed in mU/mL^{-1}. One unit is equivalent to the hydrolysis of 10^{-6} M of FAPGG. Samples were evaluated in triplicate and reaction specificity was determined by adding the ACE inhibitor captopril (10 µM).

2.6. Statistical Analysis

The mean values of areas stained in immunocytochemistry, mean number of pixels in protein bands, and enzyme activity were subjected to analysis of variance (test F), and the differences between groups were compared using the Student's test, considering a 0.05 significance threshold.

2.7. Ethics Approval

The project underwent ethical review and was given approval by an institutional animal care and use committee (Protocolo 5132013). All international guidelines for the care and use of animals for scientific purposes were followed in this experiment.

3. Results

3.1. Western Blot

The anti-ACE monoclonal antibody recognized at least one protein band of approximately 100 kDa in the sperm suspension before (Figure 1(a)) and after (Figure 1(b)) semen cryopreservation from each of the five sexually mature Gir bulls analyzed. The process of cryopreservation resulted in an increased number of pixels in protein bands, and in the gray scale, the mean intensity was 82.973 pixels before freezing, and 174.64 pixels after freezing (Figure 2), representing a 47.5% reduction in mean protein amount ($p < 0.05$).

To confirm if the same amount of protein was added to all samples, western blotting was performed with an anti-β-tubulin antibody. Band intensities were the same for the five bulls (Figure 3), with no difference in pixel number among samples ($p > 0.05$), showing that the same amount of protein was added to each sample.

3.2. Immunocytochemistry

ACE immunodetection in the spermatozoa of five Gir bulls showed periacrosomal ACE localization, and the cryopreservation process did not change ACE localization (Figure 4(a) and Figure 4(b)). The area stained in fresh semen was 519.6 pixels, while in the cryopreserved semen it was 238.33 pixels, representing a 55% reduction (Figure 5, $p < 0.05$).

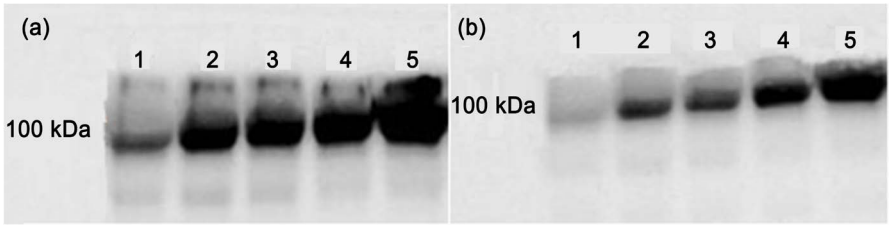

Figure 1. ACE immunodetection before (a) and after (b) cryopreservation of Gir semen. Western blot technique with anti-ACE monoclonal antibody. Each column represents a bull.

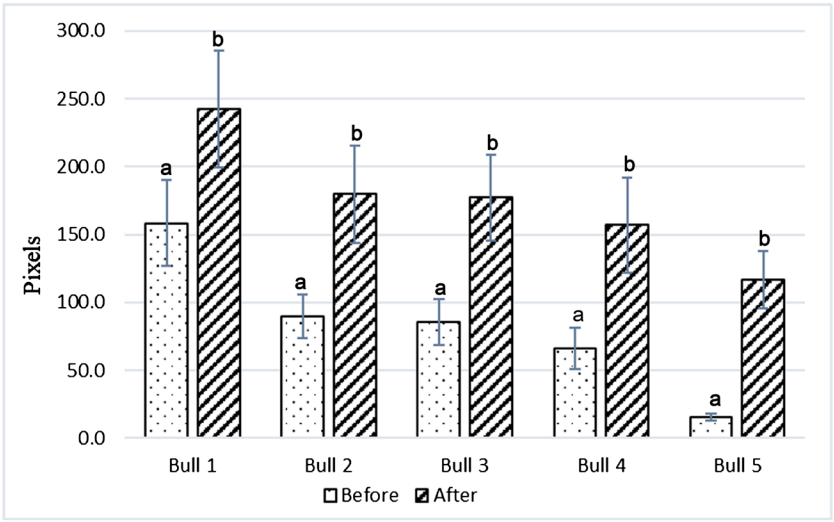

Figure 2. Pixel number in protein bands obtained using western blot and evaluated by ImageJ software. The higher the number of pixels, the clearer the protein band and the less protein detected. Mean band intensity decreased significantly after cryopreservation. Different letters represent a significant difference, determined by the t test ($p < 0.05$).

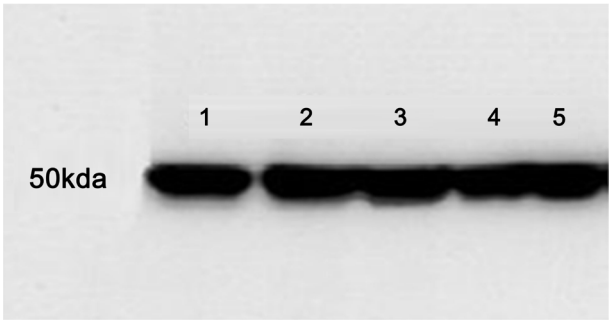

Figure 3. β-tubulin immunodetection in Gir semen. Western blot using an anti-β-tubulin monoclonal antibody. Each column represents a bull.

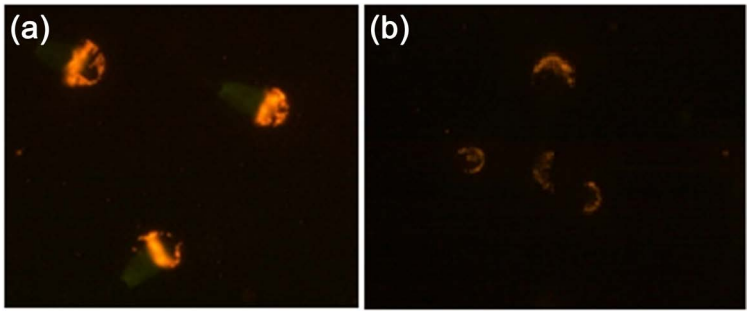

Figure 4. ACE immunolocalization in spermatozoids before (a) and after (b) cryopreservation of Gir semen. ACE was localized in the periacrosomal region.

3.3. Enzyme Activity

Figure 6 shows ACE enzyme activity before and after cryopreservation. The cryopreservation process led to a 47% reduction in ACE enzyme activity ($p < 0.05$).

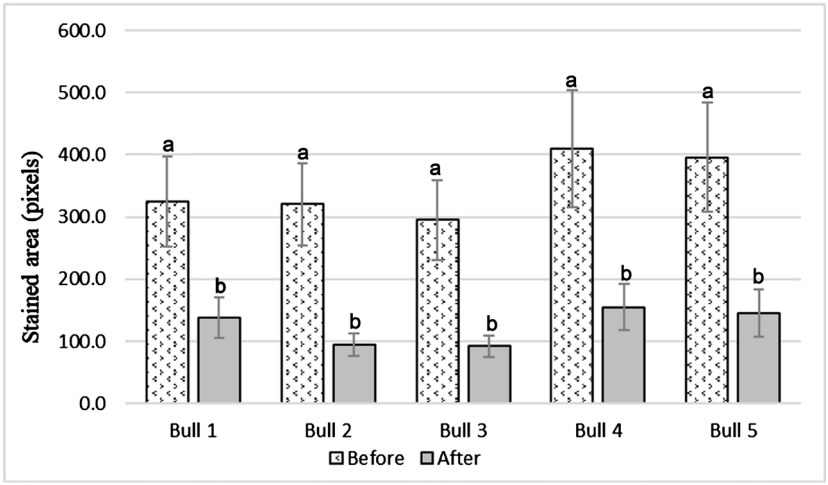

Figure 5. Mean spermatozoid stained area using an anti-ACE antibody conjugated to Cy3 fluorochrome was evaluated using Image J software. Results were obtained in pixels. Different letters represent a significant difference, determined by the t test ($p < 0.05$).

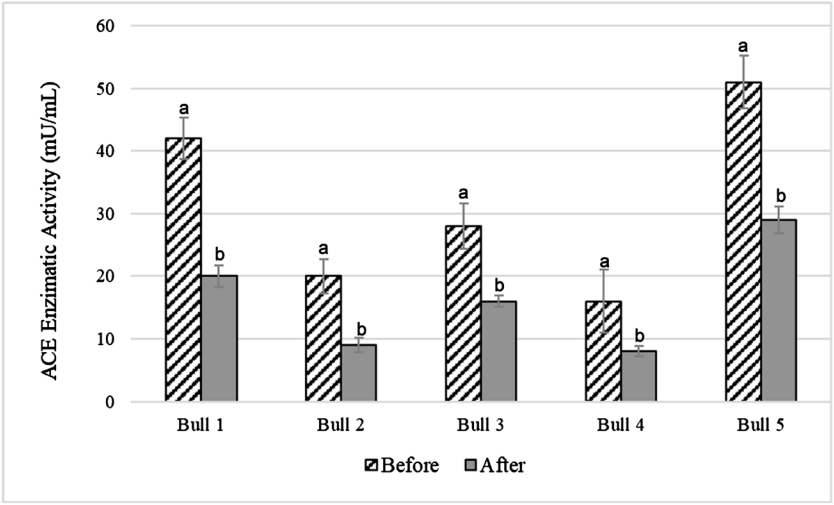

Figure 6. ACE enzyme activity before and after semen cryopreservation of five Gir bulls. ACE activity was determined by FAPGG hydrolysis. Each unit is equivalent to the hydrolysis of 10^{-6} M of FAPGG. Different letters represent a significant difference, determined by the t test ($p < 0.05$).

4. Discussion

The western blot technique with fresh sperm extract from five bulls identified at least one protein band with a molecular weight of 100 kDa (**Figure 1(a)**) using an anti-ACE antibody. This result corroborates what is described for the ACE testicular isoform in other species, such as rabbits [19], pigs [20], humans [21], rats [22], sheep [23] and Holstein cattle [14]. However, Sabeur *et al.* [24], working with dogs, and Dobrinski *et al.* [25], working with horses, found tACE with molecular weights of 65 - 70 and 68 - 70 kDa, respectively. According to these authors, this difference is due to different glycosylation levels among species, since the tACE molecular mass is 20% carbohydrate [19]. However, Nikolaeva *et*

al. [8] suggest that this difference could be due to the use of a polyclonal antibody in these studies, which could have detected other enzymes. This present study used a monoclonal antibody, and the molecular weight found is compatible only with tACE.

After cryopreservation, the tACE bands found in the samples (**Figure 1(b)**) presented the same molecular weight as those found in fresh semen. However, a reduction in band intensity of approximately 47% was observed in samples after cryopreservation (**Figure 2**). Nauc and Manjunath [5] studied other proteins and reported an 84%, 79%, and 74% reduction in the amounts of BSP-A1/-A2, BSP-A3, and BSP-30-kDa, respectively, in cryopreserved bovine sperm, while Yoon *et al.* [26] reported that sperm cryopreservation from bull epididymis changed the amount of nine proteins, seven of which showed increased expression (AKAP, F1-ATPase, TPI, NDPK7, NDUFV2, CAPZB, and SOD2), and two showed decreased expression (ODF2 and an unidentified protein), like the tACE in this study.

A quantity of ACE is released during sperm capacitation, while another part is released during the passage through the female reproductive tract [27]. Singh *et al.* [28] showed this release in cattle, and the reduction in ACE band intensity observed in this study corroborates these results, since according to Medeiros *et al.* [29], membrane alterations due to cryopreservation are similar to alterations observed in sperm capacitation. So, ACE would be released from the sperm membrane, similarly to what occurs in capacitation.

These changes in the protein composition of the sperm membrane and seminal plasma are one of the main causes of decreased sperm fertility after cryopreservation, since these proteins are involved in processes that are fundamental for fertilization, such as motility, acrosome reaction, transportation inside the female reproductive tract, zona pellucida attachment, and oocyte fusion [30]. Kondoh *et al.* [31] reported that tACE could release the extracellular portion of glycosylphosphatidylinositol (GPI) anchored protein; this ACE function would be directly related to oocyte fertilization by the sperm, independently of the previously known ACE proteolytic activity. Testicular germ cells are rich in anchored GPI proteins, and the authors identified a 110 kDa factor during a systematic search for new factors capable of releasing such proteins that, after purification and proteomic analysis, was identified as tACE.

Many studies have correlated sperm parameters and bull fertility with some sperm proteins. For example, osteopontin (OPN), BSP 30 kDa, phospholipase A2, heparin-binding proteins (HBP), and P25b are positively correlated with fertility [32] [33]. On the other hand, spermadhesin z13 and clusterin showed a negative correlation [33] [34]. Similarly, Nikolaeva *et al.* [8] showed that tACE expression is altered in spermatozoa with low motility and/or morphological changes. These authors found a 2.5-fold higher tACE expression on the surface of abnormal human spermatozoa, corroborating the results by Siems *et al.* [35], and Kohn *et al.* [36]. Shibahara *et al.* [9] also found higher tACE activity in in-

fertile human spermatozoa, which had reduced motility, and suggested that the released tACE in normal spermatozoa activates the fertilizing capacity. However, in defective cells, this enzyme cannot detach from the membrane, resulting in a greater ACE amount in these cells. On the other hand, tACE knockout mice exhibit reduced fertility, suggesting that tACE expression in spermatozoa is necessary for correct fertilization [10].

tACE localization seems to vary among species, and some studies reported different localizations within the same species. This protein has already been detected in the intermediate and initial portions of the flagellum in rat and mouse spermatozoa within the testes [37], in spermatids and cytoplasmic droplets of pig epididymal spermatozoa [20], around the spermatid head, spermatozoa and cytoplasmic droplet of rabbit seminal fluid [38], and in the acrosome or in the whole head, postacrosomal region, intermediate portion, flagellum, and equatorial region of human spermatozoa [36]. Nikolaeva *et al.* [8], detected tACE in the head and acrosome of human spermatids and spermatozoa using a monoclonal anti-ACE antibody, and according to these authors, the differences found in tACE localization, as also suggested by western blotting, are due to the low specificity of the polyclonal anti-ACE antibody.

In the present study, an anti-ACE mouse monoclonal antibody was used and detected ACE only in the periacrosomal region of Gir spermatozoa (**Figure 4(a)**). Costa and Thundathil [14] found the same localization using Holstein bulls. This periacrosomal localization was also described in other species, such as horses [25] and dogs [24].

Similar to western blot results, a decreased amount of tACE after cryopreservation was observed using immunocytochemistry, demonstrated by a 55% decrease in the mean stained area (**Figure 5**). However, enzyme localization in spermatozoa was not altered (**Figure 4(a)** and **Figure 4(b)**), suggesting that the process of cryopreservation releases ACE from the plasma membrane, corroborating the results obtained by Mohan *et al.* [39], where cryopreservation decreased the amount of ACE in spermatozoa and increased the amount in seminal plasma.

ACE *in vitro* enzymatic activity can be evaluated using two methods that use two different substrates. The method described by Kasahara and Ashihara [40] uses the synthetic compound hippuryl-histidyl leucine (hip-his-leu) as a substrate, and the method described by Holmquist *et al.* [17] uses FAPGG as substrate. Jaiswal *et al.* [41] found ACE enzyme activity in epididymal and testicular homogenates of albino rats, mice, pigs, guinea pigs, goats, rabbits, and buffalos, all using hip-his-leu as a substrate. ACE enzyme activity using FAPGG as a substrate was already found in human spermatozoa [36], canine testis [24], equine testis [18], and Holstein spermatozoa and seminal plasma [14].

In this study, ACE enzyme activity using FAPGG substrate was found in the semen of the five Gir bulls analyzed, corroborating the results obtained by Costa and Thundathil [14], who found ACE enzyme activity in both spermatozoa and

seminal plasma of Holstein bulls also using FAPGG as substrate. Singh *et al.* [28] and Heder *et al.* [42] also found ACE enzyme activity in bovine semen, however, they used hip-his-leu as substrate. In addition, **Figure 6** shows that ACE enzyme activity decreased in all cryopreserved samples compared to fresh samples, in accordance with western blot and immunocytochemistry results, which also showed decreased sperm ACE after cryopreservation.

ACE appears to behave differently in bird semen compared to mammalian semen. Mohan *et al.* [39] compared ACE enzyme activity in rooster, buffalo, and bull semen using hip-his-leu as a substrate, and concluded that ACE activity in birds is higher in seminal plasma than in spermatozoa, unlike what was observed in buffalos and bulls, which presented enzymatic activity 6.5-fold greater in spermatozoa than in plasma. In addition, after undergoing cold thermal shock (0˚C for 10 minutes), enzyme activity in bird spermatozoa and plasma did not change. In buffalo and bull semen, ACE activity decreased in spermatozoa and increased in plasma.

The process of cryopreservation led to decreased ACE enzyme activity in Gir sperm as determined by decreased protein band intensity, without changing the molecular weight, and in immunofluorescence, without changing enzyme localization. All these results show that sperm lose ACE during the process of cryopreservation.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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