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Cryoprotective effect of vaccenic acid supplemented in bull semen extender

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Abstract: The aim of this study was to investigate whether vaccenic acid (VA) added to TRIS semen extender has an effect on freezing bull semen. The semen was collected at intervals of three days through an artificial vagina then pooled. The pooled ejaculates were divided into five components as the control (C; no supplement) and VA (25, 50, 100, and 200 µg / mL) groups then extended with the TRIS-based medium. The samples were left in a cold air cabinet for cooling process (4 °C), then they were equilibrated for 4 h. Afterward, the samples in the groups were frozen by using a programmable freezer. The greatest progressive and total motility results were obtained in the VA25 group ($p < 0.001$). Besides, the total abnormality and chromatin damage was determined to be on the lowest level in the VA25 group ($p < 0.05$). Except for VA200, the glutathione level was greater in the treatment groups in comparison to the control group ($p < 0.05$), while the lowest malondialdehyde was determined in VA25. In conclusion, VA supplemented with TRIS-based extender at the concentration of 25 µg / mL improves the progressive and total motility properties of spermatozoa, reduces the abnormal spermatozoa rate, and has a positive effect on chromatin integrity.

Keywords: Antioxidant, bull semen, cryopreservation, chromatin integrity, oxidative stress

1. Introduction

Genetic improvement in dairy cattle is an important component of accurate and profitable production. Artificial insemination (AI) using frozen semen for cattle breeding is the oldest and most effective method of this process [1]. This technique has many advantages such as preventing the spread of venereal diseases, preserving genetic material, using male breeding animals with superior genetic characteristics for years, and has a wide range of applications [2,3] The effective use of AI depends on the proper freezing of semen under in vitro conditions and the characteristics of the semen extender used during the semen freezing process [4,5]. Reactive oxygen species (ROS) are chemically reactive compounds that consist of superoxide radicals, and they have detrimental effects on biological materials, such as proteins and lipids, by lipid peroxidation [6]; the plasma membrane of mammalian spermatozoa is rich in unsaturated fatty acids. For this reason, oxidative stress that occurs during the freezing stages of semen has either a lethal effect or a reduction effect on the motility and fertility potential of spermatozoa [7].

Enzymatic and nonenzymatic antioxidant substances in the seminal plasma have an active role in preventing spermatozoa damage during the semen dilution and cooling procedure before freezing [8]. However, due to the limited amounts of these antioxidant substances, it is usually not possible to reduce or eliminate the oxidative stress [9]. As a consequence, studies have reported that the supplementation of novel antioxidant substances to a semen extender will strengthen the antioxidant defense system of spermatozoa in preventing possible negative effects [10,11].

Fatty acids are essential in providing the membrane fluidity and preventing sperm damage triggered by ice crystals during semen freezing [12]. It has been reported

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that fatty acids obtained at different densities and from different sources have positive effects on spermatozoa motility and viability by increasing the compressibility and flexibility properties of the spermatozoa membrane structure [13]. Vaccenic acid (VA) is a trans fatty acid naturally found in the organism [14]. It is found in the fatty tissues of ruminants, milk, yogurt, and butter. It was reported that VA found in mammalian tissues is converted to rumenic acid and then to conjugated linoleic acid [15]. Besides, it is among the reports that VA has anticarcinogenic effects [16].

The aim of this study is to investigate whether VA, a trans fatty acid, added to the hydroxymethyl-aminomethane (TRIS) semen extender, has an effect on the freezing process of bull semen. There has been no study reported investigating the efficacy of VA on semen freezing. This study is the first in this respect.

2. Materials and methods

2.1. Design

The semen used in this study was obtained from four 2 to 4 year old healthy Holstein bulls that were raised at the Sultansuyu Agriculture Business (Sultansuyu, Malatya, Turkey) affiliated to the General Directorate of Agricultural Enterprises. The semen was collected at intervals of three days through an artificial vagina and left in a water bath (37 °C). The total semen volume was read through a graded collection tube, and the concentration of the semen was measured by using a photometer (Minitube GmbH, Tiefenbach, Germany). Then, the mass activity of the native semen, which was left on a hot plate, was scored. Ejaculates with a volume of 5 mL or more, mass activity greater than 3 [on a scale of 1 to 5], and a density of 800×10^6 per mL or higher were used as a semen source. In the next step, the semen was pooled to eliminate differences from the bulls and increase the volume. In order to avoid any standard errors, the procedures were repeated 10 different times (fifty pooled ejaculates) during the same seasonal period (from January to March). The acceptability of the study for animal experiments was confirmed, and its ethical approval was granted by the Animal Care Committee of the Faculty of Veterinary Medicine at Afyon Kocatepe University, with the approval number 49533702 / 29.

2.2. Freezing of semen

The main extender in this study was a TRIS based extender (TRIS 30.7 g, citric acid 16.4 g, fructose 12.6 g, distilled water 1000 mL, egg yolk 20% v / v and 6% glycerol [17]. After the extender was prepared according to the published formulation, its pH value was adjusted to 6.8. Trans - VA (CAS Number: 1173097 – 62 - 5; molecular weight: 283.45) from Sigma–Aldrich Chemical (Novagentek LLC, Ankara, Turkey) was used. A total of 10 mg of the VA was dissolved within 1 mL of ethanol (Merck, 99%), and the VA stock solution was prepared. The pooled semen ejaculate was divided into 5 equal components. One of these components was identified as the control group (additive-free). The others were diluted with the TRIS diluent, which included 25, 50, 100, or 200 µg / mL VA with their osmolarities set to 310 mOsm, and were decided to be administered after preliminary studies. The samples were left in the cold air cabinet for cooling (4 °C) and then equilibrated for 4 h. After this step, the samples were placed in 0.25 mL straws with 16×10^6 sperm cells in each straw and were frozen by using a programmable freezer (SY LAB Gerate GmbH, Neupurkersdorf, Austria) based on the guidelines reported by Avdatek et al. [18]. The frozen semen was left in a liquid nitrogen container (–196 °C). At least three months later, the straws were thawed in a 37 °C hot water bath for 30 s and were assessed for spermatological properties after the freezing and thawing procedures.

2.3. Assessment of spermatozoa motility and movement parameters

Spermatozoa motility values and motility characteristics were determined by using the computer-assisted semen analyzer (CASA) system (Microptic S.L., Spain). CASA was set to assess bull semen before the analysis. A semen sample of 5 µL which was diluted at a rate of 1 : 4 with the Lactated Ringer's solution was placed on a pre-heated 20 - mm chamber slide (Leja 4, Leja Products BV, The Netherlands). The progressive motility, nonprogressive motility, and total motility of the sperm samples were analysed using a 10 × microscope lens at 37 °C. The spermatozoa motility values were set by following to Avdatek et al.'s [18] protocol. In the next step, the following motility motion characteristics were measured: average path velocity (VAP) µm / s, curvilinear velocity (VCL) µm / s, straight linear velocity (VSL) µm / s, amplitude of beat cross frequency (BCF), lateral head displacement (ALH) µm, linearity (LIN, [VSL / VCL] × 100), straightness (STR, [VSL / VAP] × 100), wobble (WOB, [VAP / VCL] × 100) and hyperactivity µm s - 1. For each measurement, an average of 300 sperm cells was microscopically analysed in eight different areas.

2.4. Determination of spermatozoon abnormality

The abnormal spermatozoa ratio in the semen specimens was determined by using the liquid fixation method. In order to implement this method, first, Hancock's stain was prepared to contain distilled water (500 mL), buffer solution (150 mL), and saline solution (150 mL) with formalin (37%, 62.5 mL). To achieve fixation, a 1 / 100 working solution was prepared. After the liquid was mixed thoroughly, 6 µL of the working solution was placed on a slide and covered with a cover slide. By counting 400 spermatozoa in a 100 × phase-contrast microscope through an immersion lens, the percentage of the abnormal spermatozoa was determined. Structures other

than a normal spermatozoon structure were considered abnormal, and the head, mid-piece, and tail anomalies of any spermatozoon were analysed and recorded separately [19].

2.5. Analyssis of chromatin damage

The chromatin integrity of the sperm samples was analysed by using the method of alkaline single-cell gel electrophoresis (COMET Assay). The samples (in slides) were examined under a fluorescent microscope (Olympus CX31), and the resultant images were scored using the Comet Score software (TriTek, V. 1.5). In every specimen, a total of two hundred spermatozoa observed on six different zones were examined [20].

2.6. Determination of oxidative stress

Glutathione (GSH) content was measured spectrophotometrically at 412 nm, and the concentration was calculated in units of mU / mL [21]. A commercial malondialdehyde - 586 (MDA) kit (OxisResearch, Portland, USA) was used to evaluate lipid peroxidation (μmol / mL) [22].

2.7. Statistical analysis

Before the significance test, the gathered data were determined in terms of normality by Kurtosis, one of the parametric test assumptions. Descriptive statistics for each variable were calculated and presented as "mean ± standard error" (Mean ± SE). Statistical analysis of data was performed by the general linear model (GLM) multivariate measures of SPSS 22.0 (SPSS Inc. Headquarters, Chicago, IL, USA). The significance of differences among the means was tested by Duncan post hoc analysis, and the results were considered significant at $p < 0.05$.

3. Results

As presented in Table 1, when the treatment groups were compared to the control group in terms of their progressive and total motility, greater results were obtained in the treatment groups except for VA200 ($p < 0.001$). The greatest progressive and total motility results were obtained in the VA25 group. Although the lowest total motility results were obtained in VA200, these results were not significantly different from those in the control group. According to the results of the analyses on the spermatozoa motility characteristics, the VAP, VSL, WOB, LIN ($p < 0.001$), STR, and hyperactivity ($p < 0.05$) values were determined to be different among the groups, while there was no significant difference detected among the groups based on their VCL, ALH, and BCF values ($p > 0.05$). VAP and VSL were found to be lower in the treatment groups in comparison to the control group ($p < 0.001$). The WOB values were statistically similar in all groups except for VA200 ($p < 0.001$).

As seen in Table 2, the lowest total abnormality value was obtained in the VA25 group ($p < 0.05$). Additionally, the lowest mid-piece abnormality rates were determined in VA25 ($p < 0.001$).

Based on the analysis results, the spermatozoa chromatin damage values were significantly different among the groups, as shown in Table 3 ($p < 0.05$). According to the tail DNA and tail moment parameters, chromatin damage was determined on the lowest level in the VA25 group ($p < 0.05$), whereas the results obtained in the other treatment groups were not significantly different in comparison to the control group (Figure).

Table 1: Mean (±SE) sperm motility values in frozen thawed bull semen.

Analysis	Control	25μg/mL	50μg/mL	100μg/mL	200μg/mL	p
Progressive motility (%)	25.41 ± 0.44^{b}	30.62 ± 0.51^{d}	27.43 ± 0.76^{c}	28.91 ± 0.56^{c}	21.75 ± 0.21^{a}	**
Total motility (%)	45.59 ± 0.28^{a}	58.88 ± 0.78^{d}	53.87 ± 0.80^{c}	51.88 ± 0.87^{b}	45.75 ± 0.32^{a}	**
VAP (μm/s)	73.92 ± 1.23^{b}	64.00 ± 2.34^{a}	69.41 ± 1.62^{b}	71.88 ± 1.78^{b}	70.71 ± 1.57^{b}	*
VSL (μm/s)	59.11 ± 1.63^{b}	47.04 ± 1.88^{a}	55.31 ± 1.80^{b}	55.63 ± 1.51^{b}	56.95 ± 3.44^{b}	*
VCL (μm/s)	102.17 ± 1.26	98.84 ± 1.77	98.93 ± 1.45	99.47 ± 4.30	96.10 ± 1.05	-
ALH (μm/s)	3.85 ± 0.66	3.77 ± 0.05	3.87 ± 0.05	3.77 ± 0.06	3.92 ± 0.09	-
BCF (Hz)	10.55 ± 0.27	10.35 ± 0.19	10.45 ± 0.27	10.64 ± 0.53	10.43 ± 0.39	-
LIN (%)	57.67 ± 1.56^{a}	56.27 ± 1.11^{a}	54.38 ± 1.90^{a}	56.95 ± 2.20^{a}	48.84 ± 1.15^{b}	*
STR (%)	79.76 ± 1.24^{a}	78.57 ± 0.66^{a}	77.64 ± 0.80^{a}	78.87 ± 1.32^{a}	73.48 ± 0.59^{b}	**
WOB μm s^{-1}	72.22 ± 0.68^{b}	71.55 ± 0.89^{b}	69.90 ± 1.34^{b}	72.07 ± 1.67^{b}	66.42 ± 1.27^{a}	*
Hyperactivity μm s^{-1}	25.53 ± 2.14^{b}	28.91 ± 1.33^{c}	26.46 ± 1.19^{b}	27.20 ± 2.38^{bc}	20.12 ± 1.10^{a}	**

$^{a, b, c, d}$ Different superscripts within the same row demonstrate significant differences (*$p < 0.05$), (**$p < 0.001$)

$^{-}$ No significant difference ($p > 0.05$)

Table 2: Mean (±SE) sperm abnormality values in frozen thawed bull semen.

Analysis	Control	25µg/mL	50µg/mL	100µg/mL	200µg/mL	p
Head abnormalities (%)	9.15 ± 0.90[b]	7.93 ± 0.26[a]	9.28 ± 0.41[b]	10.64 ± 0.22[c]	13.03 ± 0.47[d]	**
Mid-piece abnormalities (%)	4.55 ± 0.41[b]	3.31 ± 0.17[a]	3.55 ± 0.37[a]	4.22 ± 0.34[ab]	5.12 ± 0.20[b]	*
Tail abnormalities (%)	2.60 ± 0.26[ab]	2.00 ± 0.16[a]	2.53 ± 0.55[ab]	3.12 ± 0.31[bc]	3.31 ± 0.22[c]	*
Total abnormalities (%)	16.31 ± 0.51[b]	13.25 ± 0.36[a]	15.37 ± 0.74[b]	17.99 ± 0.51[c]	21.46 ± 0.70[d]	**

[a.b. c. d] Different superscripts within the same row demonstrate significant differences (*p < 0.05), (**p < 0.001).
[-] No significant difference (p > 0.05).

Table 3: Mean (±SE) chromatin damage values in frozen thawed bull semen.

Analysis	Control	25µg/mL	50µg/mL	100µg/mL	200µg/mL	p
Tail lenght (µm/s)	5.45 ± 0.23[ab]	4.36 ± 0.62[a]	5.87 ± 0.26[b]	13.24 ± 1.07[c]	21.83 ± 0.28[d]	**
Tail DNA (%)	31.36 ± 0.98[b]	23.78 ± 1.39[a]	31.88 ± 0.56[b]	56.32 ± 1.67[c]	71.80 ± 1.01[d]	**
Tail moment (µm/s)	8.06 ± 0.11[b]	5.65 ± 0.77[a]	7.79 ± 1.36[b]	16.24 ± 1.04[c]	16.60 ± 1.64[c]	**

[a.b. c. d] Different superscripts within the same row demonstrate significant differences (**p < 0.001)
[-] No significant difference(p > 0.05)

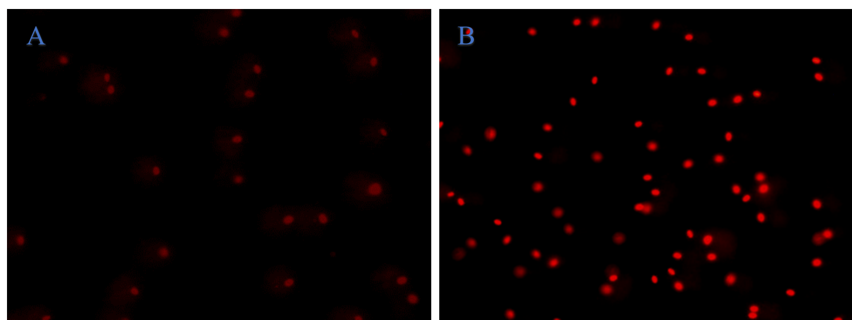

Figure. Control (A) and VA25 (B) comet analysis images.

As seen in Table 4, the obtained GSH and MDA levels were different among the groups (p < 0.05). Except for VA200, the GSH level was greater in the treatment groups in comparison to the control group (p < 0.05). While the lowest result in terms of MDA was determined in the VA25 group, the other treatment groups did not reveal a significant curative effect in comparison to the control group (p < 0.05).

4. Discussion

Motility, which is one of the important features in determining the fertility ability of spermatozoa, is defined as the ratio of spermatozoa moving in a straightforward direction to all spermatozoa in the examined area [23,24]. Studies have shown that motility is also an indicator of spermatozoa viability and structural integrity [25]. As presented in this study, improvement was obtained in terms of progressive motility and total motility in all treatment groups, except for the VA200 group with the highest dose of VA supplementation. The most significant improvement was determined in the VA25 implementation (p < 0.001). However, there was no improvement with respect to the sperm motility characteristics, although there were differences among the groups (p < 0.05; p < 0.001). Unlike our study, in another study conducted in bulls, it was reported that curcumin, crocin, and GSH added to the BIOXcell semen extender did not improve the semen's progressive motility, while especially the 1- and 3-mM doses of crocin had detrimental effects on progressive motility [26]. It was considered that these discrepancies were due to the different antioxidants and extenders used in different studies, as well as the mechanism of action of

Table 4: Mean (±SE) glutathione (GSH) and malondialdehyde (MDA) activities in frozen thawed bull semen.

Analysis	Control	25µg/mL	50µg/mL	100µg/mL	200µg/mL	p
GSH (mU/mL)	5.39 ± 0.03^{a}	5.86 ± 0.06^{c}	5.63 ± 0.08^{b}	5.64 ± 0.09^{b}	5.34 ± 0.05^{a}	**
MDA (µmol/mL)	8.36 ± 0.16^{b}	7.34 ± 0.18^{a}	8.16 ± 0.28^{b}	10.83 ± 0.37^{c}	11.87 ± 0.16^{d}	**

[a, b, c, d] Different superscripts within the same row demonstrate significant differences (**$p < 0.001$)
[-] No significant difference ($p > 0.05$)

the supplemented antioxidants. Inconsistently with the results obtained in our study, it was shown that linoleic acid addition to a TRIS-based extender had no curative effect on progressive and total motility [27]. It is thought that these different findings were due to the different densities of the antioxidants that were used by these different studies.

In line with our study, another study conducted in buffalo bulls reported that 1% green tea extract added to a TRIS citric acid-based semen extender had a healing effect on progressive motility [28]. However, the progressive motility value (56.67 ± 1.67) obtained in their study was found to be greater than the best result we obtained in ours (30.62 ± 0.51). In accordance with our results, it was revealed that the addition of 0.5 mM GSH [29] and 5–10 ng / mL alpha - linolenic acid [30] to the semen extender enhanced the total motility values. It was observed that equilibration of bull semen with poor freezing ability for 30 h with an additive consisting of linoleic acid albumin had an improving effect on motility results [31]. Although the used antioxidants were different, the findings were compatible with the motility results we obtained in this study, and this was interpreted as that the antioxidants that were used effectively provided the membrane fluidity at the freezing stage, in compatibility with the cell membrane's phospholipid structure, and they prevented the fragmentation of the spermatozoa's membrane texture. It was reported that CASA motility characteristics may be used to predict the fertility ability of spermatozoa, as well as progressive motility [1]. Contrary to the conclusions reached in this study, it was reported that the combinations of 1- and 5 - mM glutathione [32] and 10 mM quercetin - dimethylacetamide [33] improved the sperm motility properties in studies using different antioxidant substances in different species. Results in parallel with our study were obtained with regard to the effects of antioxidant substances used in bull semen studies, which were different from our findings in terms of their spermatozoa progressive motility data among spermatozoon motility characteristics. The effects of antioxidant substances on spermatozoon motility characteristics were found to be contradicting our findings in some other studies on bull semen [26,27]. It is among the reports that conjugated linoleic acid added to the bull semen extender does not provide an improvement on motility characteristics [34].

As seen in the findings in this study, chromatin integrity was achieved on the greatest level in the VA25 group ($p < 0.05$). This result was interpreted as that the 25 µg / mL VA implementation increased the cryoresistance potential of the spermatozoa against in vitro stress factors that occur during the cooling and freezing processes. It is a known scientific fact that that is a negative correlation between chromatin damage and oxidative stress [17]. Consistent with this study, it was determined that 25 µg / mL quercetin addition to a TRIS - based extender in the freezing process of bull semen [18] and 100 mM resveratrol addition in the freezing process of buffalo bull semen [35] reduced chromatin damage. In other studies, in disagreement with our results, it was determined that green tea extract did not have a positive effect on chromatin integrity [28]. A semen extender based on nano lecithin supplemented with glutathione peroxidase, an enzymatic antioxidant, did not have a healing effect on chromatin damage in bull spermatozoa after freezing and thawing [36]. These differences between studies were considered to occur more likely due to osmotic stress than oxidative stress.

The abnormal spermatozoa rate is directly associated with poor fertility [25] In our study, the lowest abnormal spermatozoa results were obtained in the VA25 application ($p < 0.05$; $p < 0.001$). When alpha-linolenic acid was used together with bovine serum albumin and methyl - beta cyclodextrin in the freezing process of boar semen, it decreased the rate of abnormal spermatozoa [37], and similar results were obtained in bulls when alpha-linolenic acid was used alone [30] where the results of these studies corresponded with the results in this study. In this study, the results were interpreted as that VA at the concentration of 25 µg / mL strengthens the phospholipid structure of the spermatozoon plasma membrane, and thus, provides an advantage against freezing-related damage, and VA application above the threshold value, depending on the treatment dose used, causes an increase in the rate of abnormal spermatozoa.

MDA is a marker that forms due to an excessive increase in ROS during the freezing process of semen, and it has negative effects on the spermatozoon's membrane

structure, and naturally, its viability [38]. In agreement with these findings, a significant positive effect was determined between Rhodiala sacra extract and MDA levels in frozen boar semen [39]. Contrary to our findings, in two studies on bulls, linoleic acid did not have a positive contribution to reducing MDA levels [30], and it had no enhancing effect on GSH activity [27]. It is thought that the difference between these studies occurred depending on the treatment dose, as well as the antioxidant substances that were used. In our study, the reason for the increase in the MDA level with the increase in the VA dose may be the oxidative reactions caused by the unstable molecules in the VA content.

In conclusion, it was determined that VA supplemented to the TRIS - based extender at the concentration of 25 µg / mL improves the progressive and total motility properties of spermatozoa, reduces the rate of abnormal spermatozoa, and has a positive effect on chromatin integrity.

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