Animal Reproduction Science 204 (2019) 101–110

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

Serum and seminal plasma IGF-1 associations with semen variables and effect of IGF-1 supplementation on semen freezing capacity in buffalo bulls

Pradeep Kumar[a,*,1], Suman[a,1], Shikha Pawaria[a], Jasmer Dalal[a,b], Sonam Bhardwaj[a], S. Patil[a,b], A. Jerome[a,*], R.K. Sharma[a]

[a] Animal Physiology and Reproduction Division, ICAR-Central Institute for Research on Buffaloes, Hisar 125001, Haryana, India
[b] Department of Veterinary Gynaecology and Obstetrics, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar 125001, Haryana, India

ARTICLE INFO

Keywords:
Buffalo
Semen
IGF-1
Cryopreservation
Spermatozoa

ABSTRACT

The objective of the study was to establish correlation of seminal and serum IGF-1 with seminal attributes, estimate antioxidant potential of IGF-1 by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) assays and to study the effect of IGF-1 supplementation on semen cryopreservation. For this study, buffalo bulls were divided into sub-fertile (n = 2) and normal (n = 5) on the basis of sperm mass motility and individual motility. The serum IGF-1 concentration of normal bulls was greater than in sub-fertile bulls, but there was no difference in the seminal IGF-1 concentration among the groups. The values from correlation analyses indicated that serum IGF-1 concentration is positively correlated with semen mass motility and sperm concentration. In the second experiment, IGF-1 did not have antioxidant activities when assessed with DPPH and FRAP assays. In the third experiment, the ejaculates of normal and sub-fertile bulls were cryopreserved using semen extender in which there was IGF-1 supplementation at 0 (control), 50, 100, 150, 200, 250, 350 and 450 ng/mL of extender. Supplementation of IGF-1 at 250 ng/ml resulted in improved sperm motility, longevity and membrane intactness as compared to control after cryopreservation of semen from normal buffalo bulls, but not sub-fertile bulls. In summary, serum IGF-1 concentration was correlated with sperm mass motility and concentration in buffalo bulls and supplementation of IGF-1 protected sperm during the cryopreservation process but effects were not due to direct antioxidant activity.

1. Introduction

During cryopreservation, there is excessive generation of reactive oxygen species (ROS) that cannot be neutralized by the seminal antioxidant defense system. This leads to sperm membrane phospholipid layer damage with decreased sperm viability (Roca et al., 2005). This is more evident in buffalo due to the greater susceptibility to ROS and cryoinjury leading to poor semen quality and conception rate (Kadirvel et al., 2009; Seifi-Jamadi et al., 2016). Insulin-like growth factor-1 (IGF-1), a component of seminal plasma secreted by Leydig and Sertoli cells (Fabbrocini et al., 2000), has an important role in spermatogenesis and steroidogenesis (Griffeth et al., 2014; Padilha et al., 2012; Lee et al., 2016). Seminal plasma IGF-1 is primarily of testicular or epididymal origin and its

* Corresponding authors.
E-mail addresses: drpradeepkrvet@gmail.com (P. Kumar), jerome210982@gmail.com (A. Jerome).
[1] These authors contributed equally and are considered as first authors.

https://doi.org/10.1016/j.anireprosci.2019.03.010
Received 17 September 2018; Received in revised form 5 February 2019; Accepted 18 March 2019
Available online 20 March 2019
0378-4320/

production is controlled by the gonadotropins FSH and LH (Lejeune et al., 1996). There are inconsistent results regarding the IGF-1 concentrations in serum and seminal plasma in relation to male fertility. In horses, greater pregnancy rates were reported when inseminated with stallion semen with greater seminal plasma IGF-1 concentrations (Macpherson et al., 2002). In boars, however, the seminal plasma IGF-1 concentration was not related to values for fresh semen variables, but there was an association with increased duration of sperm motility (Zangeronimo et al., 2013). Seminal IGF-1 concentration was correlated to sperm concentration and morphology in men (Glander et al., 1996). Thus, quantification of free IGF-1 concentration is important for determination of the physiological functions in spermatozoa (Cohen et al., 1991). Likewise, abnormalities in the serum and/or seminal IGF-1 milieu is associated with male infertility (Colombo and Naz, 1999; Macpherson et al., 2002; Cao et al., 2003; Hassan et al., 2008; Lee et al., 2016), but there is no information regarding effects of IGF-1 for buffalo species.

Supplementation of IGF-1 improves the semen quality in men (Miao et al., 1998), dogs (Sang-Min et al., 2014), stallions (Champion et al., 2002), boars (Miah et al., 2008), rams (Padilha et al., 2012), yak (Pan et al., 2015), bulls (Henricks et al., 1998) and buffalo (Selvaraju et al., 2009, 2016) and there have been feeding strategies developed (Kumar et al., 2017). It is presumed that the protective function of IGF-1 was due to its antioxidant activities (Selvaraju et al., 2016), but there are no reports of IGF-1 antioxidant activity when IGF-1 is supplemented in semen extenders. The present study was, therefore, conducted to investigate the relationship between the values for sperm variables, serum IGF-1 and seminal IGF-I in buffalo bulls. Further, there was assessment of the antioxidant potential of IGF-1 using specific assays for 2, *2-Diphenyl-1-picrylhydrazyl* (DPPH) and ferric reducing antioxidant power (FRAP). The optimized IGF-1 dose in semen extender for buffalo semen cryopreservation was investigated.

2. Materials and methods

2.1. Study location

The present study was conducted with seven Murrah buffalo bulls (age 3–5 years) maintained in the bull housing facilities of the Semen Freezing Laboratory, ICAR-Central Institute for Research on Buffaloes, Hisar, Haryana, India. All experimental procedures were conducted per the guidelines of the Institutional Animal Ethics Committee (IAEC).

2.2. Experiment 1: phenotype and semen variables of buffalo bulls and correlations with seminal and serum IGF-1

2.2.1. Animals, semen collection and processing

Based on sperm mass motility (0–5 scale) and individual sperm motility (%), the buffalo bulls were divided as sub-fertile (Group I, $n = 2$, mass motility < 3, individual motility < 70; Supplementary file S1) and normal (Group II, $n = 5$, mass motility ≥ 3, individual motility ≥ 70; Supplementary file S2). Because the ejaculates of the sub-fertile bulls consistently had backward and circular motility with coiled tails, the semen of these bulls did not qualify for freezing. The semen of these bulls was, however, desirable for being included in a sub-fertile group. It, however, would have been desirable to have more bulls in this group, but it was difficult to find such bulls for inclusion in large numbers from the ongoing breeding program. Semen of these bulls was collected twice a week using an artificial vagina. The semen was collected in 15 mL graduated glass tube (0.1 mL accuracy). The sperm concentration was estimated using the Accucell bovine photometer (IMV, L'Aigla, France). Mass sperm motility was assessed immediately following semen collection. For this assessment, 10 µL of undiluted semen was placed on a warmed slide placed on a stage warmer (37 °C) and scored on a scale of 0 to 5 on the basis of the relative presence of waves and eddies with a 10 x objective lens using a phase contrast microscope. The individual sperm motility was determined by placing a very small drop of diluted semen on a warm glass slide then a cover slip was placed over the sample and there was observation using the microscope with a 20 x objective lens magnification.

2.2.2. Blood collection, IGF-1 estimation, determination of age, body weight and scrotal circumference

Blood (10 mL) was collected on the day of semen collection from both groups. Serum and seminal plasma were separated from blood and semen, respectively following centrifugation at 5000 rpm for 15 min and stored at −20 °C until further processing. The IGF-1 (seminal plasma and serum) concentration was quantified using an ELISA kit (USCN Life Science Inc., Lot No. L130904110). The sensitively of the assay was < 27.3 pg/mL. The scrotal circumference of buffalo bulls was recorded at the start of the study. Scrotal measurements were taken with a flexible tape after proper restraining of the bull. For measurement of scrotal circumference testicles were pushed firmly into bottom of the scrotum by placing the thumb and fingers laterally on the side of neck of the scrotum and pushing ventrally. A flexible tape was formed into a loop and slipped over the scrotum, and scrotal circumference was measured in centimeters by pulling the tape the scrotal area of greatest diameter. Body weight was measured using a digital weighing balance at the start of the study.

2.3. Experiment 2: determination of antioxidant activities of IGF-1

2.3.1. DPPH (2, 2-diphenyl-1-picrylhydrazyl) assay

The different concentration of IGF-1 dissolved in semen extender and methanol extract of the extenders were prepared as described by Nimalaratne et al. (2011) with some modifications. Briefly, 1 mL extender was extracted with 10 mL of 80% methanol (80:20, vol/vol) adjusted to pH 1.5 with 1 M HCl. The sample was vortexed for 2 min and centrifuged at 6000 *g* for 10 min at 4 °C. The supernatant was evaporated until 1 mL sample remained and the sample was subjected for further analyses. The effect of DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical-scavenging was evaluated using the method of Chen and Ho (1995) with some modifications. For

conducting this procedure, 20 µL of sample and 280 µL of DPPH working solution were added and incubated for 1 h at room temperature. After 1 h, the absorbance was read at 517 nm using a microplate reader. The percentage DPPH inhibition was calculated as $[(A_0\text{-}A_s)/A_S] \times 100$, where, A_0 was the absorbance of the control (distilled water) because this was the absorbance of IGF-1.

2.3.2. Ferric reducing antioxidant power (FRAP)

Ferric reducing antioxidant power (FRAP) of IGF-1 was determined by using the methods previously describe by Benzie and Strain (1999) with slight modifications. In this assay, Fe^{3+} is reduced to Fe^{2+} ion at a low pH resulting in formation of a deep blue ferrous-probe complex from a colorless ferric-probe complex. Briefly, FRAP reagent is prepared by mixing 300 mM sodium acetate buffer (pH 3.6), 10 mM TPTZ solution in 40 mM hydrochloric acid, and 20 mM ferric chloride (III) solution at the ratio 10:1:1 (v/v/v), respectively. A 10 µL sample (different concentration of IGF-1 dissolved in distilled water) was added to 300 µL of FRAP reagent and incubated at room temperature for 3 min. The absorbance at 593 nm was immediately measured using the microplate reader. An aqueous solution with a known $FeSO_4.7H_2O$ concentration (0, 0.2, 0.4, 0.6, 0.8 and 1.0 mM) was used for development of standard curve. The values obtained were expressed as µM of ferrous equivalent Fe (II) per mL of sample.

2.4. Experiment 3: supplementation of IGF-1 in extended semen and evaluation of sperm membrane integrity, viability and kinetics variables

2.4.1. Animals and semen collection

For the third experiment, the same buffalo bulls ($n = 7$) were used. Semen of these bulls was collected twice a week using an artificial vagina technique and freezing was performed using standard procedures. The effect of supplementation of IGF-1 on semen cryopreservation was studied in the normal and sub-fertile bulls in separate replicates of the study.

2.4.2. Dose selection, supplementation of IGF-1 and cryopreservation

Following semen collection, the ejaculate (five ejaculates from each bull) was divided into nine equal fractions and diluted to final concentration 80 million sperm/mL using Tris egg-yolk glycerol extender (Kumar et al., 2015a) fortified with a different concentration of IGF-1 (MP Biomedicals, LLC Pvt. Limited; Cat No. 08982241) as Control (no supplementation), 50 (IGF-50), 100 (IGF-100), 150 (IGF-150), 200 (IGF-200), 250 (IGF-250), 350 (IGF-350) and 450 (IGF-450) ng/mL of extender with normal ($n = 5$) and sub-fertile ($n = 2$) bulls in separate replicates of the study. The extended semen was subsequently placed in French mini straws (IMV, L'Aigle, France), and slowly cooled to 4 °C and equilibrated for a period of 4 h in a cold cabinet (IMV, L'Aigle, France) and was subsequently frozen using a programmable biological freezer (Mini Digi-cool, IMV Technologies, L'Aigle, France) using procedures that have been previously described (Kumar et al., 2015b). Briefly, each semen sample was initially cooled at the rate of −5 °C/ min from 4 to −10 °C. Between −10 and −100 °C, freezing rate was −40 °C/min and then from −100 to −140 °C, its rate was −20 °C/ min. After reaching −140 °C, semen straws were immediately plunged into liquid nitrogen at −196 °C for storage.

2.4.3. Sperm motility and kinetic variables

Sperm kinetics and motility of frozen-thawed semen were assessed using a computer-assisted sperm analyzer (CASA) system (IVOS12.1, Hamilton-Thorne Biosciences, Beverly, MA, USA) as previously described (Kumar et al., 2015b). Before analysis using CASA, the semen sample was diluted with pre-warmed tris buffer to give a sperm concentration of about 40×10^6 sperm/mL. The prepared semen sample (1 µL) was loaded in a pre-warmed (38 °C) eight chamber Leja slide (depth 20 µm) and sperm kinetic and motility variables were assessed. The following motion characteristics were recorded: total motility (TM, %), progressive motility (PM, %), rapid motility (RM, %), straight linear velocity (VSL, µm/s), average path velocity (VAP, µm/s), curvilinear velocity (VCL, µm/s), lateral head displacement (ALH, µm), beat cross frequency (BCF, Hz), straightness (STR, %) and linearity (LIN, %) of the sperm. The CASA software settings were as follows: temperature = 38 °C, frame rate = 60 Hz, frames acquired = 30, minimum contrast = 35, minimum cell size = five pixels, cell size = nine pixels, cell intensity = 110 pixels, progressive cells (VAP cut-off = 50 µm/s, STR cut-off = 70%), slow cells (VAP cut-off = 30 µ/s and VSL cut-off = 15 µ/s).

2.4.4. Sperm incubation test

Sperm incubation assessments were conducted using the procedures that were previously described by Kumar et al. (2015a). For these assessments, each semen sample was thawed in a water bath at 37 °C for 30 s. The content of straws (0.23 mL) was transferred to 2 mL tubes maintained in a dry bath at 37 °C. Sperm individual motility was assessed subjectively under phase contrast microscope equipped with a warm stage (37 °C) at 200× magnification at 0, 30, 60, 90 and 120 min.

2.4.5. Sperm plasma membrane integrity

Plasma membrane integrity was evaluated using hypo-osmotic swelling test (HOST) as described by Kumar et al. (2015b). The assay was performed by mixing 100 µL of frozen-thawed semen with 1 mL hypo-osmotic solution (0.735 g sodium citrate dihydrate and 1.351 g fructose in 100 mL distilled water). After incubation for 60 min at 37 °C, sperm tail bending/coiling was assessed by placing 15 µL of well-mixed sample on a warm slide (37 °C) using light microscopy at 400× magnification. At least 200 spermatozoa were observed per slide. The sperm with coiled tails after incubation were considered to have intact plasma membranes.

2.5. Statistical analysis

The data were analyzed using SPSS (Version 18) software package. All replications of sperm variables of each bull were examined

Table 1
Values for seminal variables of buffalo bulls.

Variables	Group I (n = 2)	Group II (n = 5)	P value
Age (Days)	1419 ± 217	1424 ± 132	0.98
Body weight (Kg)	965 ± 79	616 ± 31	0.002**
Scrotal circumference (cm)	38.50 ± 0.50	31.00 ± 1.51	0.03*
Semen volume (mL)	2.82 ± 0.42	2.83 ± 0.44	0.98
Sperm concentration (million/mL)	1206 ± 59	794 ± 81	0.03*
Total sperm (millions)/ ejaculate	3375 ± 341	2173 ± 305	0.05*
Mass activity (0-5)	1.850 ± 0.35	2.95 ± 0.20	0.03*
Individual motility (%)	44.64 ± 4.64	73.25 ± 1.26	0.000**
IGF-1 in seminal plasma (ng/mL)	49.34 ± 9.17	50.34 ± 6.12	0.98
IGF-1 in serum (ng/mL)	1202 ± 51	1634 ± 61	0.04*

* Mean value differs ($P \leq 0.05$).
** Mean value differs ($P < 0.01$).

for normal distribution using the Shapiro-Wilk test. In cases where there was not a normal distribution, arcsine transformation of data was performed to normalize the distribution before analysis. Also, homogeneity of variances between the two study groups was confirmed using the Levene test of the SPSS statistical package prior to estimation of difference between the groups using the *T*-test and the correlation with seminal and serum IGF-1 in the normal group was assessed using the Pearson's correlation co-efficient. In Experiment 3, the difference in means between groups was compared using an analysis of variance (ANOVA) followed by use of the Tukey's *post-hoc* analysis. Results were considered significant at $P \leq 0.05$.

3. Results

3.1. Phenotype and semen variables of buffalo bulls and the correlation with IGF-1 concentrations

Values for age, body weight, scrotal circumference, seminal variables (semen volume, concentration, total sperm, mass activity, individual motility) and IGF-1 concentration (serum and seminal plasma) of both groups are shown in Table 1. There were no differences in age of bulls of both the groups, but body weights of Group I bulls were heavier ($P = 0.002$) as compared to Group II. The scrotal circumference, sperm concentration and total sperm of Group I bulls were greater than that of Group II, but mass motility and pre-freeze individual motility of Group I bulls were less (as per Minimum Standard Protocol for bull semen production, Gov. of India). The IGF-1 concentration in serum of Group II bulls was greater ($P = 0.05$) than Group I, but there was no difference in concentration of IGF-1 in seminal plasma. Also, with consideration of correlation analyses there was a positive relationship ($r = 0.94$; $P = 0.01$) between serum IGF-1 and sperm mass activity and a trend between serum IGF-1 and sperm concentration ($r = 0.78$; $P = 0.05$) in normal buffalo bulls (Table 2).

3.2. Antioxidant activity of IGF-1

Antioxidant activities of IGF-1 were assessed using the DPPH and FRAP assays. The IGF-1 did not neutralize the DPPH, a well-known free radical. Further, IGF-1 functions to reduce the ferric to ferrous ion but at the concentrations of IGF-1 included in the extender did not have ferric reducing capacity (Fig. 1).

Table 2
Correlation between seminal plasma and serum IGF-1 concentration with values for seminal variables in buffalo bulls.

Variables	Seminal plasma IGF-1 concentration r (P)	Serum IGF-1 concentration r (P)
Age	0.17 (0.74)	−0.12 (0.82)
Body weight	0.17 (0.74)	−0.12 (0.82)
Scrotal circumference	0.28 (0.59)	0.00 (1.00)
Semen volume	0.68 (0.14)	−0.49 (0.33)
Sperm concentration	−0.22 (0.67)	0.78 (0.05)*
Total sperm/ejaculate	0.65 (0.17)	−0.06 (0.92)
Mass activity	−0.64 (0.17)	0.94 (0.01)*
Individual motility	0.11 (0.84)	0.65 (0.16)
IGF-1 in seminal plasma	–	−0.39 (0.44)
IGF-1 in serum	−0.39 (0.44)	–

* Significant.

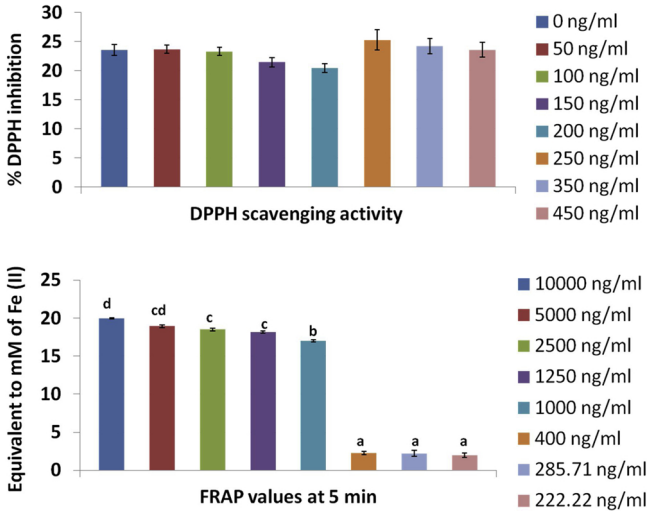

Fig. 1. DPPH and FRAP assays. Values with different letters differ ($P < 0.05$).

3.3. Effect of supplementation of IGF-1 in extender on sperm cryopreservation

3.3.1. Effect of supplementation of IGF-1 on sperm cryopreservation of normal bulls

Values for buffalo sperm kinematic variables were different ($P < 0.01$) between the treated and control groups. Sperm VAP (μm/s) and VSL (μm/s) were greater ($P < 0.01$) in the IGF-50, 150 200 and 250 groups as compared with the control group, while VCL (μm/s) was increased in a dose dependent manner with greatest value ($P < 0.01$) being in the IGF-150 (204.3 ± 2.31) and IGF-200 (204.4 ± 5.86) groups. In contrast, for sperm ALH, BCF, STR and LIN there were no differences among the groups (Fig. 2). Total sperm motility (%) differed ($P < 0.01$) among the groups being greatest in the IGF-50 group (57.56 ± 2.08) with the next ranked motilities being the IGF-150 (54.53 ± 1.64) and IGF-250 (56.28 ± 1.97) groups. Furthermore, progressive sperm motility (%)

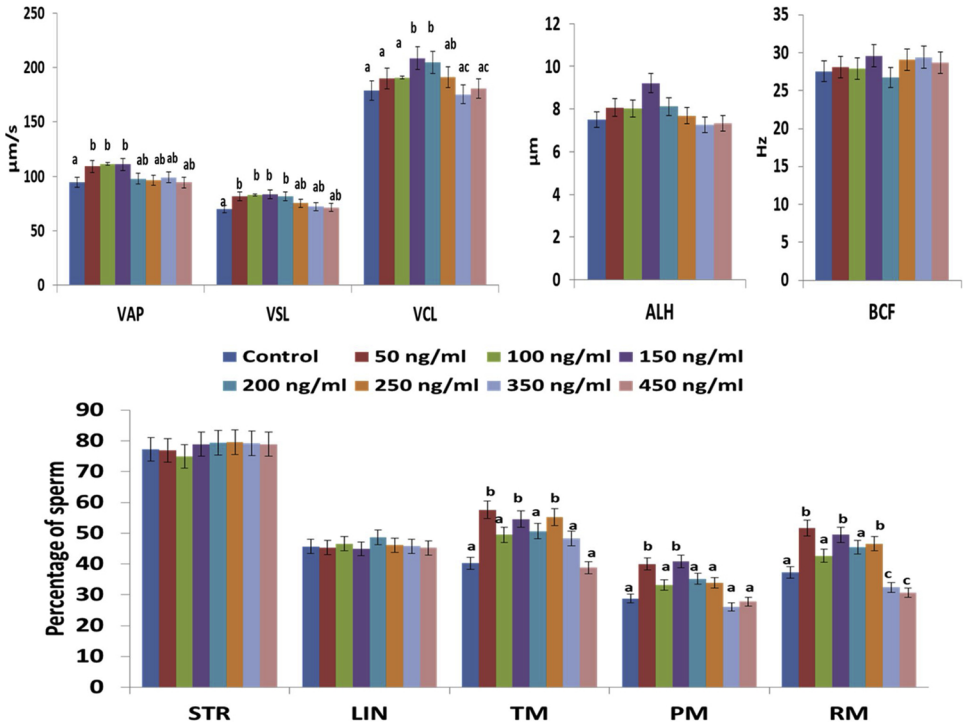

Fig. 2. Values for sperm kinetic and motility variables in normal bulls; VAP: average path velocity, VSL: straight linear velocity, VCL: curvilinear velocity, ALH: lateral head displacement, BCF: beat cross frequency, STR: straightness, LIN: linearity, TM: total sperm motility, PM: progressive sperm motility, RM: rapid sperm motility; Values with different letters differ ($P < 0.05$).

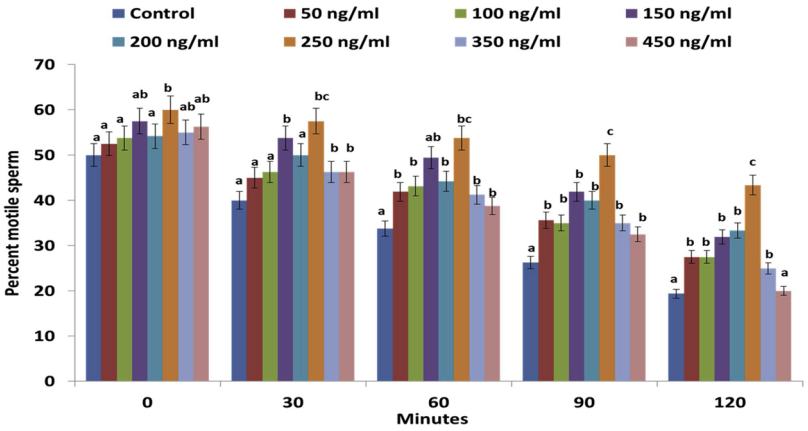

Fig. 3. Sperm post-thaw motility following incubation test in normal bulls; Values with different letters differ ($P < 0.05$).

differed ($P < 0.01$) in IGF-1 treated samples as compared to control with the greatest values being in IGF-50 group (39.97 ± 1.47) and the next greatest values being in the IGF-150 (37.41 ± 1.31), IGF-200 (35.17 ± 1.71) and IGF-250 (34.48 ± 1.22) groups. Likewise, rapid sperm motility (%) was greater ($P < 0.01$) in all IGF-50 (51.65 ± 1.53), 150 (51.45 ± 1.18), 200 (45.4 ± 1.92) and 250 (47.84 ± 2.76) groups in comparison to control group (Fig. 2).

With the incubation test, there was a group and time effect ($P < 0.01$) on sperm longevity. The IGF-250 group had a greater (52.92%; $P < 0.0001$) sperm longevity as compared to the other groups including the control. With respect to time, all IGF-treated groups sperm longevity differed ($P < 0.01$) in comparison to control at different times of sperm incubation (Fig. 3).

With the sperm membrane integrity test, the IGF-200 (48.29%) and 250 (56.38%) groups had greater ($P < 0.05$) plasma membrane integrity as compared with the other groups including the control. The HOST reactive percentage increased in a dose dependent manner with the least percentage being in IGF-450 and control group (Fig. 4).

3.3.2. Effect of supplementation of IGF-1 on sperm cryopreservation of sub-fertile bulls

For all values for sperm kinematic variables (VAP, VSL, VCL, ALH, BCF, STR and LIN), there were no differences among the groups (Fig. 4). Total sperm motility (%), however, differed ($P < 0.01$) among the groups being greatest in the control group but sperm motility was less ($P < 0.01$) than that of normal bulls (Fig. 5). The sperm longevity and plasma membrane integrity of sub-fertile bulls were not enhanced with IGF-1 supplementation to the minimum cut off value to qualify for use of artificial insemination (Figs. 6 and 7).

4. Discussion

For the first time, serum IGF-1 concentration was estimated in buffalo breeding bulls in the present study with ranges in concentration being 927 to 2247 ng/mL (average 1555.22 ng/mL). The mean serum IGF-1 concentration has been reported in red deer with the average being 63.6 ng/mL (57.4–79.9 ng/mL; Ditchkoff et al., 2001), and in rabbits as 200 ng/mL (Bielohuby et al., 2014).There are no reports of serum IGF-1 concentration in bovine bulls of breeding age, but in young bulls the IGF-1 concentration ranged from 300 to 400 ng/mL with a gradual increase with age and body weight (Lee et al., 2005; Brito, 2015; Bourgon et al., 2017). In the present study, the IGF-1 concentration of seminal plasma of buffalo bulls (50.34 ± 6.12 ng/mL) was less than that of cattle (116–144 ng/mL; Henricks et al., 1998), but greater than that of stallions (10.2 to 10.4 ng/mL; Macpherson et al., 2002) and boars

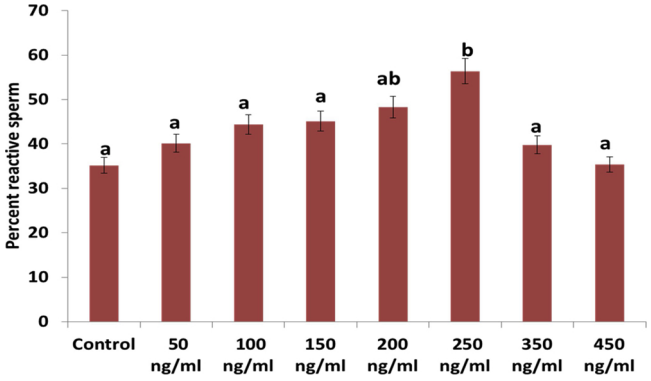

Fig. 4. Sperm plasma membrane integrity in normal bulls; Values with different letters differ ($P < 0.05$).

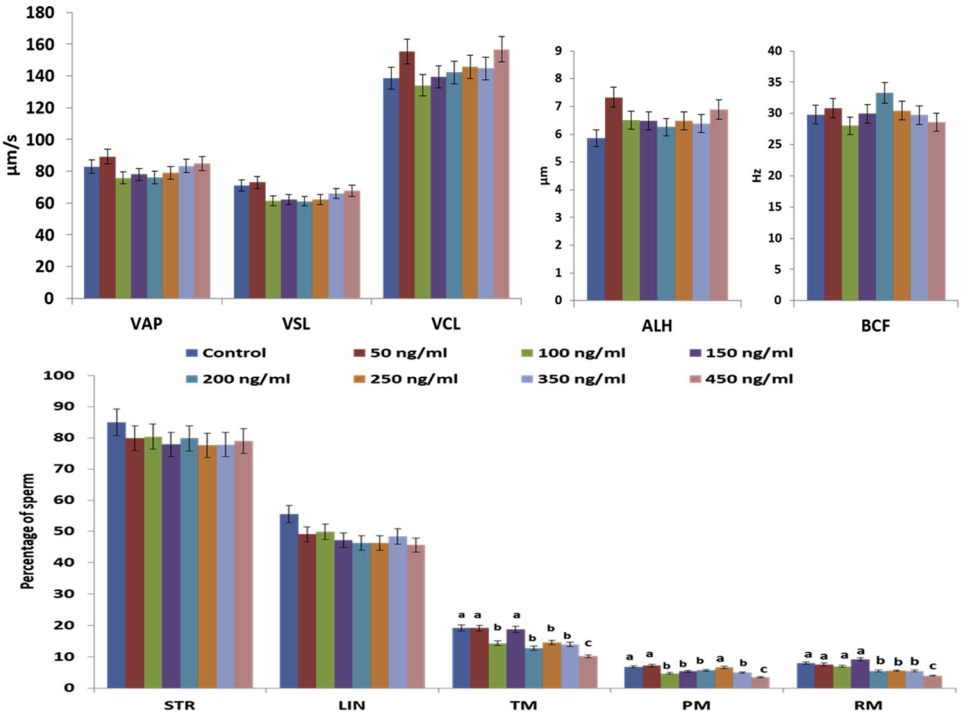

Fig. 5. Values for sperm kinetic and motility variables in sub-fertile bulls; VAP: average path velocity, VSL: straight linear velocity, VCL: curvilinear velocity, ALH: average lateral head displacement, BCF: beat cross frequency, STR: straightness; LIN: linearity, TM: total sperm motility, PM: progressive sperm motility, RM: rapid sperm motility; Values with different letters differ ($P < 0.05$).

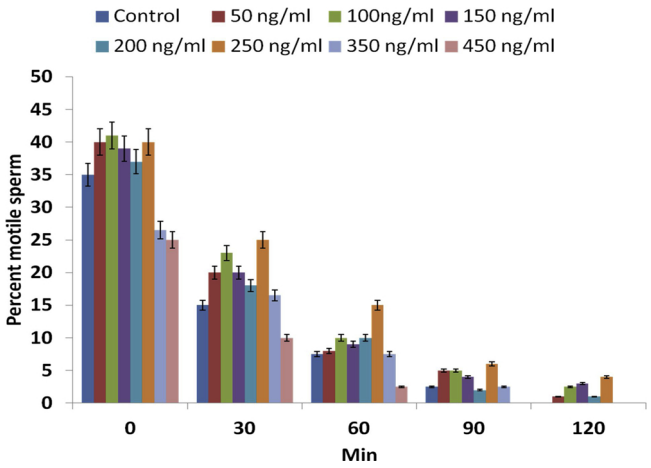

Fig. 6. Values for sperm post-thaw motility following incubation in sub-fertile bulls; Values with different letters differ ($P < 0.05$).

(1.39–2.44 ng /mL; Zangeronimo et al., 2013). Likewise, for the first time, the relationship between the serum IGF-1 concentration and values for semen variables were reported in buffalo bulls. There was a lesser serum IGF-1 concentration in sub-fertile buffalo bulls, but interestingly there was no difference in seminal IGF-1 concentrations among treatment groups. Results of the present study are consistent with those of a previous study in boars, wherein seminal IGF-I was not related to initial values for fresh semen variables (Zangeronimo et al., 2013). Similar to the findings in the present study, there was not a detectable association between values for semen and seminal IGF-1 variables in men (Lee et al., 2016). In this study, however, there was an association between sperm concentration and mass motility with serum IGF-1 concentration. There were also lesser serum IGF-1 concentrations in the sub-fertile bulls in the present study which is consistent with that reported of an earlier study (Lee et al., 2016). In stallions, IGF-I concentrations did not differ between samples from individual animals with differing sexual activity, but IGF-I concentrations varied between stallions (Macpherson et al., 2002). Absence of correlation between the seminal IGF-1 concentrations with values for semen variables

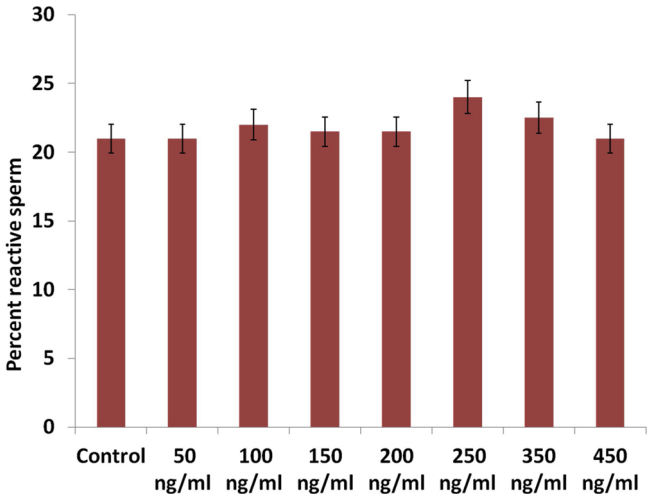

Fig. 7. Values for sperm plasma membrane integrity in sub-fertile bulls; Values with different letters differ ($P < 0.05$).

is consistent with the previous findings of Macpherson et al. (2002) in boars confirming the limited role of IGF-I in regulation of the duration of sperm motility after collection and not with the extent of motility following ejaculation. Several reports indicate that genetics, nutrition, steroids and growth hormone concentration contribute to variations in serum and/ or semen IGF-I concentrations (Henricks et al., 1998; Macpherson et al., 2002; Lackey et al., 1998; Selvaraju et al., 2009). Furthermore, numerous autocrine/ paracrine factors (ATP, calcium, kinases and phosphatases) affect sperm motility (Pereira et al., 2017) and any dysfunctions in synthesis, secretion and actions may contribute to asthenospermia. Results of a study in Japanese Black beef bulls indicated that a lesser insulin-like peptide 3 concentration is associated with a lesser sperm motility as well as a lesser IGF-1 concentration (Weerakoon et al., 2018). Further investigations of these variables are warranted for studying the underlying causes of buffalo bull sub-fertility. Likewise, IGF-1 regulates sperm signaling pathways through other key proteins (Selvaraju et al., 2016) and the autocrine/paracrine actions of IGF-1 in buffalo spermatogenesis needs to be further studied.

The second and third experiment were conducted to validate the hypothesis that supplementation of IGF-1 in semen extender allows for antioxidant functions to the extent that there is an enhancement in values for sperm functional variables during cryopreservation (Padilha et al., 2012; Selvaraju et al., 2009, 2016). Antioxidant activity of any compound is complex usually occurring through several mechanisms of action that cannot be ascertained using one method. It is, therefore, essential to perform more than one type of antioxidant capacity measurement to take into account the various mechanisms of antioxidant action (Schlesier et al., 2002; Wong et al., 2006). Two complementary tests (i.e., DPPH and FRAP assays) were conducted to assess the antioxidant activity of IGF-1 in the present study. The DPPH assay is based on electron and H atom transfer, while the FRAP assay is based on an electron transfer reaction (Huang et al., 2005). With DPPH being a stable, the synthetic free radical remains intact in water, methanol, or ethanol (Aksoy et al., 2013) and the free radical scavenging activities of a solution depend on the capacity of antioxidant compounds to lose hydrogen and the structural conformation of these components (Fukumoto and Mazza, 2000). Thus, the DPPH free radical, can easily receive an electron or hydrogen from antioxidant molecules to become a stable molecule (Soares et al., 1997). In the present study, non-neutralization of DPPH confirmed that IGF-1 does not donate electrons or hydrogen atoms in sufficient amounts to allow for DPPH to be a stable compound. Interestingly, however, when conducting the FRAP assay the ferric reducing capacity was greater when the concentrations of IGF-1 were greater; but the antioxidant property of IGF-1 was not evident at the concentrations used in the present experiment. Thus, the findings with use of these assays validated that there was an improvement in post-thaw semen quality after supplementation of IGF-1 in semen extender but that this improvement was not due to its antioxidant activity as it has been previously reported (Selvaraju et al., 2016).

In normal buffalo bulls, IGF-1 supplementation to buffalo semen resulted in improvements of buffalo sperm motility as previously reported for several species (Henricks et al., 1998; Miao et al., 1998; Champion et al., 2002; Miah et al., 2008; Padilha et al., 2012; Pan et al., 2015; Sang-Min et al., 2014), but in sub-fertile bulls there was no improvement in sperm motility, sperm functional integrity and sperm longevity as a result of IGF-1 supplementation to semen extender. Also, IGF-1 supplementation of buffalo semen resulted in an increased total and progressive sperm motility during the incubation period (Selvaraju et al., 2009, 2016). There are IGF-1 receptors in sperm (Henricks et al., 1998; Naz and Padman, 1999), therefore, it is possible that the chemokinetic effect of IGF-I in maintaining spermatozoal motility might be direct by activation of IGF-I receptors of spermatozoa (Henricks et al., 1998; Zangeronimo et al., 2013). Also, the presence of insulin-like growth factor binding protein in semen (Hwa et al., 1999) could contribute to the modulation of IGF-1 action which needs further investigation. In addition, insulin-like growth factors increase the intracellular calcium concentrations by enhancing the ion transport resulting in greater sperm progressive motility (Miah et al., 2008). The lack of improvement of sperm motility and values for functional variables in semen of sub-fertile buffalo bulls following IGF-1 supplementation might be due to dysfunctions of mechanisms related to sperm motility and there needs to be greater investigation of the intricacies of male infertility in buffalo.

In conclusion, the results of the present study indicate that serum IGF-1 concentration is related sperm mass activity and sperm concentration of buffalo bulls. There was no antioxidant activity of IGF-1 in the semen extender, but IGF-1 supplementation (at 250 ng/mL) improved the post-thaw semen quality of buffalo.

Conflict of interests

The authors declare no conflict of interests with respect to this research

Acknowledgments

The authors thank the Director, ICAR-CIRB for providing the necessary facilities and the funding of this research under AICRP on 'Nutritional and Physiological approaches for enhancing reproductive performance in animals' by Indian Council of Agricultural Research, New Delhi is duly acknowledged.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.anireprosci.2019.03.010.

References

Aksoy, L., Kolay, E., Ağılönü, Y., Aslan, Z., Kargıoğlu, M., 2013. Free radical scavenging activity, total phenolic content, total antioxidant status, and total oxidant status of endemic Thermopsis turcica. Saudi J. Biol. Sci. 20 (3), 235–239.
Benzie, I.F., Strain, J.J., 1999. Ferric reducing/antioxidant power assay: direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. Methods Enzymol. 299, 15–27.
Bielohuby, M., Zarkesh-Esfahani, S.H., Manolopoulou, J., Wirthgen, E., Walpurgis, K., Toghiany Khorasgani, M., Aghili, Z.S., Wilkinson, I.R., Hoeflich, A., Thevis, M., Ross, R.J., Bidlingmaier, M., 2014. Validation of serum IGF-I as a biomarker to monitor the bioactivity of exogenous growth hormone agonists and antagonists in rabbits. Dis. Model. Mech. 7 (11), 1263–1273.
Bourgon, S.L., Diel de Amorim, M., Miller, S.P., Montanholi, Y.R., 2017. Associations of blood parameters with age, feed efficiency and sampling routine in young beef bulls. Livestock Sci. 195, 27–37.
Brito, L.F.C., 2015. Endocrine control of testicular development and initiation of spermatogenesis in bulls. In: Hopper, R.M. (Ed.), Bovine Reproduction. John Wiley & Sons, Inc, Oxford, pp. 30–38.
Cao, E., Chen, Y., Cui, Z., Foster, P.R., 2003. Effect of freezing and thawing rates on denaturation of proteins in aqueous solutions. Biotechnol. Bioeng. 82, 684–690.
Champion, Z.J., Vickers, M.H., Gravance, C.G., Breier, B.H., Casey, P.J., 2002. Growth hormone or insulin-like growth factor-I extends longevity of equine spermatozoa in vitro. Theriogenology 57, 1793–1800.
Chen, C.W., Ho, C.T., 1995. Antioxidant properties of polyphenols extracted from green and black tea. J. Food Lipids 2, 35–46.
Cohen, P., Peehl, D.M., Lamson, G., Rosenfeld, R.G., 1991. Insulin-like growth factors (IGFs), IGF receptors, and IGF-binding proteins in primary cultures of prostate epithelial cells. J. Clin. Endocr. Metab. 73, 401–407.
Colombo, J.B., Naz, R.K., 1999. Modulation of insulin-like growth factor-1 in the seminal plasma of infertile men. J. Androl. 20, 118–125.
Ditchkoff, S.S., Spicer, L.J., Masters, R.E., Lochmiller, R.L., 2001. Concentrations of insulin-like growth factor-I in adult male white-tailed deer (Odocoileus virginianus): associations with serum testosterone, morphometrics and age during and after the breeding season. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 129 (4), 887–895.
Fabbrocini, A., Del Sorbo, C., Fasano, G., Sansone, G., 2000. Effect of differential addition of glycerol and pyruvate to extender on cryopreservation of Mediterranean buffalo (B.bubalis) spermatozoa. Theriogenology 54, 193–207.
Fukumoto, L.R., Mazza, G., 2000. Assessing antioxidant and prooxidant activities of phenolic compounds. J. Agric. Food Chem. 48 (8), 3597–3604.
Glander, H.J., Kratzsch, J., Weisbrich, C.H., Birkenmeier, G., 1996. Insulin-like growth factor I and α-2-macroglobulin in seminal plasma correlate with semen quality. Hum. Reprod. 11, 2454–2460.
Griffeth, R.J., Bianda, V., Nef, S., 2014. The emerging role of insulin-like growth factors in testis development and function. Basic Clin. Androl. 24, 12.
Hassan, A., Abo-Azma, S.M., Fayed Mostafa, T., 2008. Seminal plasma cotinine and insulin-like growth factor-I in idiopathic oligoastheno teratozoospermic smokers. BJU Int. 103, 108–111.
Henricks, D.M., Kouba, A.J., Lackey, B.R., Boone, W.R., Gray, S.L., 1998. Identification of insulin-like growth factor I in bovine seminal plasma and its receptor on spermatozoa: influence on sperm motility. Biol. Reprod. 59, 330–337.
Huang, D., Ou, B., Prior, R.L., 2005. The chemistry behind antioxidant capacity assays. J. Agric. Food Chem. 53 (6), 1841–1856.
Hwa, V., Oh, Y., Rosenfeld, R.G., 1999. The insulin-like growth factor-binding protein (IGFBP) superfamily. Endocr. Rev. 20, 761–787.
Kadirvel, G., Satish, K., Kumaresan, A., 2009. Lipid peroxidation, mitochondrial membrane potential and DNA integrity of spermatozoa in relation to intracellular reactive oxygen species in liquid and frozen-thawed buffalo semen. Anim. Reprod. Sci. 114, 125–134.
Kumar, P., Saini, M., Kumar, D., Balhara, A.K., Yadav, S.P., Singh, P., Yadav, P.S., 2015a. Liposome-based semen extender is suitable alternative to egg yolk-based extender for cryopreservation of buffalo (Bubalus bubalis) semen. Anim. Reprod. Sci. 159, 38–45.
Kumar, P., Kumar, D., Sikka, P., Singh, P., 2015b. Sericin supplementation improves semen freezability of buffalo bulls by minimizing oxidative stress during cryopreservation. Anim. Reprod. Sci. 152, 26–31.
Kumar, A., Singh, P., Bhakat, M., Singh, S., Nitharwal, K., Gupta, A.K., 2017. Effect of feed energy levels on semen quality and freezability of young murrah buffalo bulls. Buffalo Bull. 36 (2), 415–426.
Lackey, B.R., Boone, W.R., Gray, S.L.L., Henricks, D.M., 1998. Computer-assisted sperm motion analysis of bovine sperm treated with insulin-like growth factor I and II: implications as motility regulators and chemokinetic factors. Arch. Androl. 41 (2), 115–125.
Lee, H.G., Hidari, H., Kang, S.K., Hong, Z.S., Xu, C.X., Kim, S.H., Seo, K.S., Yoon, D.H., Choi, Y.J., 2005. The relationships between plasma insulin-like growth factor (IGF)-1 and IGF-binding proteins (IGFBPs) to growth pattern, and characteristics of plasma IGFBPS in steers. Asian-Aust. J. Anim. Sci. 18 (11), 1575–1581.
Lee, H.S., Park, Y.S., Lee, J.S., Seo, J.T., 2016. Serum and seminal plasma insulin-like growth factor-1 in male infertility. Clin. Exp. Reprod. Med. 43 (2), 97–101.
Lejeune, H., Chuzel, F., Thomas, T., Avallet, O., Habert, R., Durand, P., Saez, J., 1996. Paracrine regulation of Leydig cells. Ann, Endocrinol. (Paris) 57, 55–63.
Macpherson, M.L., Simmen, R.C., Simmen, F.A., Hernandez, J., Sheerin, B.R., Varner, D.D., Loomis, P., Cadario, M.E., Miller, C.D., Brinsko, S.P., Rigby, S., Blanchard, T.L., 2002. Insulin-like growth factor-I and insulin-like growth factor binding protein-2 and -5 in equine seminal plasma: association with sperm characteristics and fertility. Biol. Reprod. 67, 648–654.
Miah, A.G., Salma, U., Takagi, Y., Kohsaka, T., Hamano, K.I., Tsujii, H., 2008. Effects of relaxin and IGF-I on capacitation, acrosome reaction, cholesterol efflux and utilization of labeled and unlabeled glucose in porcine spermatozoa. Reprod. Med. Biol. 7, 29–36.

Miao, Z.R., Lin, T.K., Bongso, T.A., Zhou, X., Cohen, P., Lee, K.O., 1998. Effect of insulin-like growth factors (IGFs) and IGF-binding proteins on in vitro sperm motility. Clin. Endocrinol. 49, 235–239.
Naz, R.K., Padman, P., 1999. Identification of insulin-like growth factor (IGF)-1 receptor in human sperm cell. Arch. Androl. 43 (2), 153–159.
Nimalaratne, C., Lopes-Lutz, D., Schieber, A., Wu, J., 2011. Free aromatic amino acids in egg yolk show antioxidant properties. Food Chem. 129, 155–161.
Padilha, R.T., Magalhaes-Padilha, D.M., Cavalcante, M.M., Almeida, A.P., Haag, K.T., Gastal, M.O., Nunes, J.F., Rodrigues, A.P., Figueiredo, J.R., Oliveira, M.A., 2012. Effect of insulin-like growth factor-I on some quality traits and fertility of cryopreserved ovine semen. Theriogenology 78, 907–913.
Pan, Y., Cui, Y., Baloch, A.R., Fan, J., He, J., Li, G., Zheng, H., Zhang, Y., Lv, P., Yu, S., 2015. Insulin like growth factor I improves yak (Bos grunniens) spermatozoa motility and the oocyte cleavage rate by modulating the expression of Bax and Bcl-2. Theriogenology 84, 756–762.
Pereira, R., Sá, R., Barros, A., Sousa, M., 2017. Major regulatory mechanisms involved in sperm motility. Asian J. Androl. 19, 5–14.
Roca, J., Rodríguez, M.J., Gil, M.A., Carvajal, G., Garcia, E.M., Cuello, C., Vazquez, J.M., Martinez, E.A., 2005. Survival and in vitro fertility of boar spermatozoa frozen in the presence of superoxide dismutase and/or catalase. J. Androl. 26 (1), 15–24.
Sang-Min, S., Suhee, K., Jin-Gi, H., Yong-Jun, K., 2014. IGF-I improves mitochondrial membrane potential during hypothermic storage of canine spermatozoa. J. Vet. Med. Sci. 76, 1065.
Schlesier, K., Harwat, M., Böhm, V., Bitsch, R., 2002. Assessment of antioxidant activity by using different in vitro methods. Free Radic. Res. 36 (2), 177–187.
Seifi-Jamadi, A., Kohram, H., Shahneh, A.Z., Ansari, M., Macias-Garcia, B., 2016. Quercetin ameliorate motility in frozen-thawed turkmen stallions sperm. J. Equine Vet. Sci. 45, 73–77.
Selvaraju, S., Reddy, I.J., Nandi, S., Rao, S.B., Ravindra, J.P., 2009. Influence of IGF-I on buffalo (*Bubalus bubalis*) spermatozoa motility, membrane integrity, lipid peroxidation and fructose uptake in vitro. Anim. Reprod. Sci. 113, 60–70.
Selvaraju, S., Krishnan, B.B., Archana, S.S., Ravindra, J.P., 2016. IGF1 stabilizes sperm membrane proteins to reduce cryoinjury and maintain post-thaw sperm motility in buffalo (*Bubalus bubalis*) spermatozoa. Cryobiology 73, 55–62.
Soares, J.R., Dinis, T.C., Cunha, A.P., Almeida, L., 1997. Antioxidant activities of some extracts of Thymus zygis. Free Radic. Res. 26 (5), 469–478.
Weerakoon, W.W.P.N., Sakase, M., Kawate, N., Hannan, M.A., Kohama, N., Tamada, H., 2018. Plasma IGF-I, INSL3, testosterone, inhibin concentrations and scrotal circumferences surrounding puberty in Japanese Black beef bulls with normal and abnormal semen. Theriogenology 114, 54–62.
Wong, S.P., Leong, L.P., Koh, J.H.W., 2006. Antioxidant activities of aqueous extracts of selected plants. Food Chem. 99 (4), 775–783.
Zangeronimo, M.G., Silva, D.M., Murgas, L.D.S.A., Sousa, R.V., Rocha, L.G.P., Pereira, B.A., Faria, B.G., Veras, G.C., 2013. Identification of insulin-like growth factor-i in boar seminal plasma and its influence on sperm quality. Arch. Zootec. 62 (239), 411–418.