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Original article

In vitro evaluation of frozen thawed kangayam bull semen enriched with antioxidants

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Abstract

The aim of this study was to assess the *in vitro* penetration rate of antioxidant enriched frozen thawed Kangayam bull semen. For the current investigation, 5-7-year-old Kangayam bulls were used. The semen was collected twice per week and two ejaculates were collected each time. They were subsequently transported to the laboratory for processing of semen and maintained in a water bath at 34°C. On the day of semen collection, three groups of semen were prepared with Tris-egg-yolk Glycerol Extender (TEYG) (group I), TEGY extenders with hyaluronan (group II), and TEGY extenders with metformin (group III) and stored in a water bath at 34°C. According to the group, the semen sample was first diluted at a ratio of 1:1 with the appropriate extender (TEYG, hyaluronan enriched or metformin enriched) and kept in laminar air flow at 22°C for seven minutes. Each semen sample was then extended using the appropriate semen extender in accordance with the dilution rate. After filling, sealing and printing, the final diluted semen sample was subjected to equilibration. Freezing was done as per the standard protocol. Oocytes were collected from cyclical animals on days 1, 5, 9 after estrus using the ultrasound guided transvaginal ovum pick-up method after ablation of the day 0 pre-ovulatory follicle. After oocyte and sperm maturation, the co-incubation of oocyte and sperm was done and the *in vitro* penetration rate was recorded. The overall *in vitro* penetration rate recorded in Kangayam cows was 46.66 per cent. Maximum *in vitro* penetration rate was observed in group II (52 per cent) followed by group III (46 per cent) and in group I (42 per cent). Based on the above findings, it was concluded that hyaluronan enriched semen may be used as a pragmatic approach for cryopreservation of Kangayam bull semen in order to augment the *in vitro* penetration rate in Kangayam.

Keywords: Hyaluronan, *in vitro* penetration rate, kangayam, metformin, oocyte

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Introduction

India is a major source of unique cattle genetic resources (20th Livestock Census 2019). The population of exotic and crossbred animals increased by 26.90 per cent during the previous census, while the population of native cattle decreased by 6 per cent during the same period. The male population decreased very dramatically, by 29.10 per cent, whereas the female population increased by 10 per cent. Crossbreeding with exotic breeds, being less economically viable, losing their value, having smaller herds and the extensive mechanization of agricultural operations are some of the causes of the depletion of native breeds. Indigenous breeds must be preserved for future genetic security, scientific investigation, as a component of our ecosystem, cultural and ethical requirements, and as a source of energy.

Cryopreservation techniques, according to numerous studies, cause cold shock, ice crystal formation, oxidative stress, osmotic changes, and lipid amino acid changes within the cell membrane, all of which interfere with normal sperm functions and result in loss of motility, viability, and fertilizing ability, in addition to deterioration of acrosomal and membrane integrity and fundamental harm to DNA (Vishwanath and Shannon 2000). Oxidative stress is caused by an imbalance between reactive oxygen species (ROS) generation and scavenging processes (Agarwal and Prabakaran 2005), which causes membrane integrity loss, enzyme inactivation, DNA damage and cell death, subsequently resulting in infertility. It is known that the production of reactive oxygen species (ROS) causes oxidative stress to have a negative impact on spermatozoa (Agarwal and Prabakaran 2005). Spermatozoa physiologically produce ROS. ROS levels in cattle play an important role in the reproductive processes such as sperm-oocyte interactions, implantation and early embryonic development, sperm capacitation, acrosome reaction, fertilizing ability maintenance and stabilization of the mitochondrial capsule in the mid-piece of sperm (Agarwal and Prabakaran 2005 and Desai et al. 2009).

Kangayam being a vulnerable breed due to infertility and unnatural conditions, recent reproductive techniques have provided a solution for the perpetuation of the species. Frozen semen technology is a widely accepted and followed reproductive technique that could help in the perpetuation of these vulnerable animals and efforts are still being made to produce high-quality frozen semen straws (FSS) from pedigree Kangayam bulls. The freezing and thawing procedures being unnatural, the sperm are more prone to cryo-damage, resulting in poor post thaw viability and motility due to the production of ROS. Hence the addition of antioxidants could be a scientifically acceptable option to overcome this issue which has not yet been studied in Kangayam bulls. Based on the above views, a scientific attempt has been made to investigate the effect of antioxidants (hyaluronan and metformin) on the *in vitro* oocyte penetration rate of Kangayam bull semen.

Materials and Methods

Source of experimental animals

Kangayam bull Nos. NK 16 (Ram), NK 17 (Lakshman) and NK 30 (Barathan) aged 5-7 years maintained at the Frozen Semen Bank, Department of Veterinary Gynaecology and Obstetrics, Veterinary College and Research Institute, Namakkal, were used for this study. No ethics agreement was required for this study.

Semen collection

The semen was collected on Tuesday and Friday of every week during the trial period from the above mentioned bulls. During semen collection, additional Kangayam bulls kept in the semen station were used as dummies. The second ejaculate was used for the study. Before semen collection, two false mounts were given each time. The penis was guided into the AV on the third mount and the semen was collected in the collecting tube (Rabadan et al. 2012). The collected ejaculate was immediately sent to a water bath at 34°C in the semen processing laboratory. In total, 24 ejaculates were used in this study (8 ejaculates/bull).

Assessment of total motility and acrosomal integrity

A drop of the neat semen sample diluted with Tris was placed on a clean, grease free glass slide and covered with a clean cover slip. The sample was examined using a phase contrast microscope (20x objective) to calculate the percentage of total motility. The semen ejaculates possessing more than 80 per cent total motility were selected for further processing. The total motility was also monitored at 24 hours after freezing and 30 days after freezing (Elamurugan 2021).

The integrity of the acrosome was assessed using the Giemsa staining procedure. A small drop of neat semen was smeared on a clean, grease-free glass slide and dried in the air. The smear was fixed in methanol by immersing for 10 minutes. The fixed smear was immersed in a working solution of Giemsa and left for 5 hours at room temperature. The slide was then washed

in running water and allowed to air dry. A minimum of 200 spermatozoa were counted in different fields under oil immersion (100x objective). Semen samples possessing more than 80 per cent intact acrosome were utilized for further processing. The integrity of acrosome was also monitored at 24 hours after freezing and 30 days after freezing (Elamurugan 2021).

Extension of semen

Initially the neat semen was diluted at 1:1 ratio with the TRIS (hydroxymethyl aminomethane) egg yolk--glycerol extender [TEYG]. The 1:1 diluted semen samples were kept at 22°C for 9 – 10 minutes in a laminar air flow chamber. During this time, the temperature of the semen samples was reduced from 34°C to 22°C (Elamurugan 2021). After reaching 22°C, the 1:1 diluted semen sample was finally extended with respective semen extender with antioxidants which were maintained at 22°C as per the dilution rate and the group details are given below.

Group I: Semen diluted with extended in TRIS-egg yolk-glycerol extender (control)

Group II: Semen diluted with extended in TRIS-egg yolk-glycerol extender and hyaluronan

Group III: Semen diluted with extended in TRIS-egg yolk-glycerol extender and metformin

In Group I, the semen was extended in TRIS-egg yolk-glycerol extender, so as to contain 80 million spermatozoa per ml of diluted semen. In Group II and III, the semen was extended in TRIS-egg yolk-glycerol extender with hyaluronan (0.25 mg ml^{-1}) and metformin (50 μM) respectively, so as to contain 80 million spermatozoa per ml of diluted semen.

Preparation of hyaluronan working solution:

Hyaluronan is available as 1 gram powder form. To make stock solution, 250 mg of hyaluronan was added to 10 ml of distilled water. For every 100 ml of semen diluent, 1 ml (25 mg) of hyaluronan stock solution was added.

Preparation of metformin working solution:

Metformin is available in 500 mg powder form, which is mixed with 10 mL of distilled water to make stock solution (50 mg/ml). This stock solution was diluted with 1 litre of distilled water to make working solution. To each semen sample, 1.65 ml of working solution was added irrespective of volume which is equivalent to 50 μM metformin.

Each semen sample was then extended using the appropriate semen extender in accordance with the dilution rate (Elamurugan 2021). After filling, sealing and printing, the final diluted semen sample was subjected to equilibration. The freezing and thawing procedures were done as stated by Elamurugan (2021).

Collection of immature oocytes

Cows maintained at the Livestock Farm Complex, Veterinary College and Research Institute, Namakkal were used as a source of immature oocytes. Oocytes were collected from cyclical animals on days 1, 5, 9 after oestrus using the ultrasound guided transvaginal ovum pick-up method after ablation of the day 0 pre-ovulatory follicle. The oocytes were collected in oocyte collection medium containing penicillin (100 IU/ml) and streptomycin (50 mg/ml) maintained at 38°C in a sterile graduated collection tube.

Ovum pick-up (OPU) procedure

The retrieval of oocytes from the live animals was performed using the ultrasound guided transvaginal ovum pick-up technique (Manik et al. 2003). The donor cows were restrained in a squeeze chute and the dung materials were removed through rectal palpation. The perineal region of the cows were cleaned with plain water and disinfected with antiseptic solution. The cows were restrained with epidural injection lignocaine (2 per cent). Transrectal ultrasonography was done to ascertain the presence of follicles in both the ovaries. The ultrasound probe was guided through the vagina after retracting the ovaries towards the vagina through rectal palpation until the ovaries were visualized through the probe. An echogenic needle of 18- gauge was pierced through the vaginal wall and into a follicle and suction (100 mg Hg) was provided using a foot operated suction apparatus to aspirate the follicular fluid with the oocyte. All the follicles measuring from 3 mm to 6 mm diameter were aspirated. The oocytes were aspirated into the oocyte collection medium pre-incubated at a temperature of 37°C. The needle was withdrawn and the same procedure was repeated on the other ovary. The media containing the follicular fluid and oocytes were transported to the laboratory maintained at a temperature of 37°C.

Oocyte grading and selection

Oocytes were assessed based on cumulus cell investment and ooplasm homogeneity using a stereo zoom microscope (Nandi et al. 1998).

In Vitro maturation (IVM) of oocytes

The selected oocytes were washed four times in TCM 199 containing 10 per cent fetal bovine serum

Table 1. Grading of bovine oocytes based on the layers of cumulus cells and homogeneity of ooplasm.

Grade	Description
Grade A:	Oocytes having greater than 3 layers of cumulus cells and homogenous cytoplasm
Grade B:	Oocytes having thin or fewer than 3 layers of cumulus cells and homogenous cytoplasm
Grade C:	Oocytes with few cumulus cells or none at all
Grade D:	Denuded oocytes

Only COCs of grades A, B and C were used for *in vitro* maturation.

Table 2. Assessment of bovine oocyte maturation based on expansion of cumulus cells.

Degree	Description	Expansion
Degree 2	Cumulus cells were evenly distributed and clustered cells were no longer.	Full cumulus cell expansion
Degree 1	Cumulus cells were slightly increased, although clustered cells remained.	Moderate cumulus cell expansion
Degree 0	There was no morphological difference when compared to fresh COCs.	Slight or no expansion

The oocytes with degree 2 and degree 1 cumulus expansion were considered as matured.

(FBS - GIBCO: Invitrogen, USA). The oocytes were washed once in *in vitro* maturation (IVM) medium before culture. The *in vitro* maturation consisted of TCM-199 supplemented with 10 per cent FBS, 1 µg/ml of Folltropin, 0.02 IU/ml of luteinizing hormone and 1 µg/ml of estradiol. The culture dishes were prepared by adding 50 µl droplets of *in vitro* maturation medium in 35 mm petridishes covered with sterile mineral oil and pre-equilibrated for 2 hours at 38.5°C under 5 per cent CO_2 in air to prevent evaporation. Ten COCs were transferred to each droplet and cultured for 24 hrs at 38.5°C in a humidified atmosphere of 5 per cent CO_2 in air.

Morphological assessment of oocyte maturation

The maturation rate was assessed and classified based on the degree of cumulus expansion (Ravindranatha et al. 2002).

Oocyte preparation for *in vitro* penetration

At the end of the maturation period, COCs were washed three times in pre-equilibrated washing medium containing TCM 199, followed by gentle pipetting for partial removal of the cumulus cells, and then washed twice in IVF-TALP. The fertilization medium was prepared with IVF-TALP added with heparin (100 µg/ml) and 50 µl droplets were prepared in 35 mm petridishes covered with sterile mineral oil pre-incubated for 2 hours at 38.5°C under 5 per cent CO_2 in air. Ten to fifteen matured oocytes were added to each IVF pre-incubated droplet (Gopikrishnan 2012).

Sperm preparation for IVF by swim-up

Motile sperms were obtained from the frozen semen sample using the swim-up technique for IVF. The frozen semen sample (2 mL) of 3 groups were taken and diluted with 5 ml of pre-equilibrated SpTALP by centrifugation at 1200 rpm for 5 min at room temperature (RT). After the removal of the supernatant fresh SpTALP was added and the above procedure was repeated twice. One ml of SpTALP medium was then added in three sugar tubes and preincubated at 38.5°C. Finally, 200 µl of the sperm pellet was placed under one ml of SpTALP medium in each tube and incubated at a 45° angle for swim up at 38.5°C in 5 per cent CO_2 in air for 30 minutes. At the end of incubation the superficial layer of 0.4-0.6 ml of the medium containing the motile fraction from each tube were taken and pooled in a 15 ml centrifuge tube. The sperm containing supernatant was washed with 10 ml SpTALP by centrifugation at 1200 rpm for 5 min at RT. After centrifugation, the supernatant was removed and the sperm pellet was collected. The concentration of the final sperm pellet was determined with a haemocytometer and the sample was diluted with IVF TALP to yield a concentration of $1\text{-}2\times10^6$ sperm/ml (Gopikrishnan 2012).

Co-incubation of sperm and oocyte

The motile sperm suspension obtained using the swim up technique were used to inseminate the IVF droplets containing oocytes. The sperms were introduced to the IVF droplet in a 5000:1 sperm-to-oocyte ratio and co-incubated for 24 hours at 38.5°C in a humidified environment of 5 per cent CO_2 in air (Gopikrishnan 2012).

In Vitro culture

The presumptive zygotes were cultured for 18-22 hrs after co-incubation of oocytes with sperms. The embryo culture media used for *in vitro* culture of embryos was synthetic oviductal fluid (SOF). The presumtive zygotes from the fertilization petridishes were placed into micro centrifuge tubes. Cumulus cells and sperms

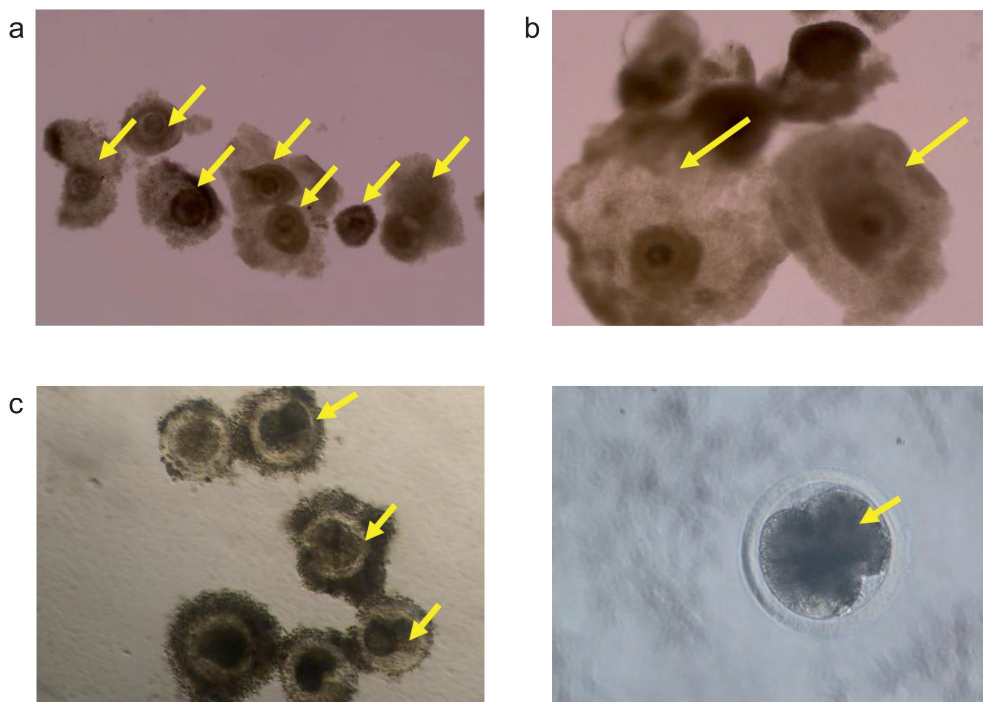

Fig. 1. *In vitro* penetration assay
a. aspirated immature bovine oocytes by transvaginal oocyte aspiration technique viewed under stereo zoom microscope (x4).
b. matured bovine oocytes after 24 hours of incubation viewed under stereo zoom microscope at x8 and x6 magnification.
c. cleaved bovine oocyte after 48 hours of co-incubation under inverted microscope at x10 and x40 magnification.

Table 3. Post thaw total motility and acrosomal integrity of bull semen extended in different anti-oxidants at 24 hours after freezing and 30 days after freezing.

	24 hours after freezing				30 days after freezing			
	Group I	Group II	Group III	F value	Group I	Group II	Group III	F value
Total motility (per cent)	$59.12^{a}\pm1.78$	$64.38^{a}\pm2.22$	$61.29^{a}\pm1.95$	1.753	$54.08^{a}\pm1.56$	$62.04^{b}\pm1.80$	$59.04^{ab}\pm1.93$	5.139**
Acrosome integrity (per cent)	$74.96^{a}\pm2.32$	$76.62^{a}\pm2.10$	$76.08^{a}\pm1.68$	0.172	$71.08^{a}\pm2.17$	$73.88^{a}\pm2.13$	$71.38^{a}\pm2.29$	0.487

Means bearing common superscript within each row between columns (a,b,c) do not differ significantly
Means bearing different superscript within each row between columns differ significantly ($p<0.05$)* and ($p<0.01$)**

from embryos were removed by vortexing or gentle pipetting using hyaluronidase. The *in vitro* culture medium was prepared by inserting 50 µl droplets covered with sterile mineral oil and pre-incubated before the transfer of zygotes. The presumptive zygotes were washed three times in IVC media before being placed into pre-equilibrated 50 µl IVC droplets (10-15/droplets). The presence of cleaved oocyte was assessed 24 and 48 hours after fertilization (Gopikrishnan 2012).

Statistical analysis

The collected data were statistically examined using the One-Way ANOVA test to compare groups for total motility and acrosomal integrity at post thaw 24 hours and 30 days. To assess the *in vitro* penetration rate among the 3 different groups the Chi-Square test was used.

Results

Total Motility

The mean (±SE) total motility of Kangayam bull neat semen was 79.42±0.48 per cent, ranging from 76.00 to 83.00 per cent. The mean (±SE) total motility of frozen thawed semen in groups I, II and III was 59.12±1.78, 64.38±2.22 and 61.29±1.95 at 24 hours after freezing; and 54.08±1.56, 62.04±1.80 and 59.04±1.93 per cent, at 30 days after freezing, respectively (Table 3). Post thaw evaluation of semen diluted in different groups revealed that there was no significant

Table 4. *In vitro* penetration assay using frozen thawed kangayam bull semen.

Groups	Group I (control)	Group II (hyaluronan)	Group III (metformin)	Overall per cent	Chi-square value
No. of oocytes	50	50	50	-	-
No. of embryos produced	21 (42)	26 (52)	23 (46)	46.66	1.02^{NS}
No of oocytes remaining intact	29 (58)	24 (48)	27 (54)	53.33	
In between groups					
Association between group I and group II					1.00^{NS}
Association between group II and group III					0.36^{NS}
Association between group I and group III					0.16^{NS}

^{NS} – Non-significant association at 5% level (p>0.05)
Figures in parenthesis indicates percentage

difference between groups I, II and III at 24 hours. However, a highly significant difference was observed between groups I and II but no difference was observed between groups I and III and groups II and III on the 30th day.

Acrosomal Integrity

The mean (±SE) percentage of sperms with an intact acrosome in the semen sample was 96.46±0.22, ranging from 95 to 98. The mean (±SE) acrosomal integrity of frozen thawed semen in groups I, II and III was 74.96±2.32, 76.62±2.10 and 76.08±1.68 per cent at 24 hours after freezing; and 71.08±2.17, 73.88±2.13 and 71.38±2.29 per cent, at 30 days after freezing, respectively (Table 3). The acrosomal integrity of frozen thawed semen at 24 hours and on the 30th day did not differ significantly among the three groups.

In-Vitro Penetration Rate

The *in-vitro* penetration rate (Table. 4) observed was 42.00, 52.00 and 46.00 per cent in groups I, II and III, respectively. The maximum penetration rate was observed in group II (52 per cent) followed by groups III (46 per cent) and I (42 per cent). Statistical analysis using Chi-square values revealed that there was no significant difference among the 3 groups in *in vitro* penetration rate.

Discussion

Certain stages of cryopreservation (Wang et al. 2004) and extended *in vitro* incubation of semen (Calamera et al. 2010) have been demonstrated to involve the production of detrimental reactive oxygen species which harm sperm motility and viability (Aitken and Clarkson, 1988) and genomic integrity (Foote et al. 2002). Maxwell and Stojanov (1996) reported that the motility and acrosome integrity of ram spermatozoa were enhanced by antioxidants and the survival of spermatozoa increased linearly with increasing antioxidant dose. Following freezing and thawing, the percentage of motile bull spermatozoa increased when glutathione, either with or without superoxide dismutase, was added to a whole milk glycerol extender (Foote et al. 2002).

Regarding the post-freeze total motility, similar results were also reported by Bucak et al. (2007) and Bucak et al. (2008), who showed a significant increase in ram sperm motility related to higher catalase activity of frozen sperm treated with taurine and cysteine. Grandhaye et al. (2020) supplemented metformin in canine semen and reported that metformin could lessen oxidative stress and enhance post thaw motility. In contrast, the addition of 5 mM metformin partially reduced the motility of fresh boar sperm (Hurtado de Llera et al. 2012). In the current experiment, the increased sperm total motility in antioxidant groups (groups II and III) in contrast to the control group (group I) might be due to the alleviation of sperm damages caused by the glycerol in the extender, which is a potent osmolyte regulator which could affect the total motility (Setyawan et al. 2009). Furthermore, none of the three groups had a deleterious effect on sperm motility, evidenced by high fertility in Kangayam cows in each group, with or without antioxidant enrichment.

Greater intact acrosome values were reported by Ansari et al. (2017), Akther (2023) and Kapadiya et al. (2018) in the TEYG extender than in our experiment. A lower percentage of intact acrosomes was observed by Bhakat et al. (2016) in Karan Fries bulls (30.68 per cent) than in the present study. The addition of hyaluronan (Bertoldo et al. 2014 and Grandhaye et al. 2020) and metformin (Bucak et al. 2009) did not alter the amounts of acrosomes in bull semen. Even though there

was no difference among the groups in the present study, there were difference among the studies, which might be due to the concentration of the antioxidants used, semen handling differences, age and breed of the bull and season of collection.

According to Yang et al. (2020), metformin at 40 and 80 µM activated human sperm function through a mechanism related to AMPK, enhancing the tyrosine phosphorylation. The authors also suggested that metformin supplementation improved human sperm function, thereby improving the *in vitro* penetration ability of the spermatozoa (Yang et al. 2020). Similar studies were performed in mice by Tsuda et al. (1994) and Tsuda et al. (1996) and suggested that the metformin addition increased the rate of *in vitro* fertilization and decreased the number of defective zygotes. However, different results were obtained in studies on mouse, stallion, chicken, and boar species, which reported that metformin did not affect sperm viability or the acrosome (Bertoldo et al. 2014 and Nguyen et al. 2014). In our investigation, we noticed that metformin has better *in vitro* penetration rate than the control group. This could be attributed to the fact that metformin has the potential to regulate energy production and motility via an active AMPK pathway.

Similar to the results of the present investigation, Pena et al. (2004) recorded that hyaluronan supplementation increased sperm motility, viability and membrane integrity during freezing and thawing techniques and reduced the incidence of polyspermy. According to Suzuki et al. (2002), exogenous hyaluronic acid increased the rate of oocyte penetration and monospermy in swine. Funahashia and Sano (2005) noticed that adding 5 mM cysteine to a preservation solution containing seminal plasma significantly enhanced the viability and penetrability of spermatozoa *in vitro*. In the present investigation, the higher *in vitro* penetration rate in the hyaluronan group could be attributed to the presence of hyaluronan receptors in all parts of the spermatozoa (head, mid-piece, tail), which facilitated the binding ability of exogenous hyaluronan, as well as an increase in phosphorylation and ATP levels, which improved spermatozoa motility due to increased flagellar activity (Ghosh et al. 2002). In the present investigation, there were no marked differences among the analyzed groups (Group I, II and III) related to the acrosomal integrity and total motility of spermatozoa and this might be the reason behind the insignificant differences in *in vitro* penetration rate among the groups. The *in vitro* penetration rate is highly influenced by the laboratory and environmental conditions which might be the additional for the insignificant results in the *in vitro* penetration rate.

Conclusion

There were no marked differences between the control and the treated groups, and neither hyaluronan nor metformin was better than the control when analyzed by the *in vitro* penetration rate.

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