Original article

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Running title: Encapsulation and cryopreservation of buffalo and bovine semen

Alginate encapsulation preserves the quality and fertilizing ability of Mediterranean Italian water buffalo (*Bubalus bubalis*) and Holstein Friesian (*Bos Taurus*) spermatozoa after cryopreservation

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The use of artificial insemination (AI) in buffalo is limited by the poor ovarian activity during the hot season, the seasonal qualitative patterns in semen, the low resistance of sperm cells in the female tract, the difficulties in detecting and variable length of estrous. Although AI procedures are commonly used in bovine breeding, these techniques are limited in the buffalo (*Bubalus bubalis*). In zootechnical field, different studies were conducted to develop new techniques for the improvement of fertilizing ability of buffalo spermatozoa after AI.

In this study, for the first time, the alginate encapsulation and cryopreservation of buffalo spermatozoa was described to improve AI efficiency, and the same procedure was performed for bovine semen. Overall results obtained from *in vitro* analyses indicate that the encapsulation process does not lead to detrimental effects (respect to the control) in the quality parameters (membrane integrity, progressive motility, path average velocity), also after the cryopreservation in either species. The fertilizing potential of encapsulated-cryopreserved semen was evaluated after AI in 25 buffalo and 113 bovine females: pregnancy rate is not jeopardized in either bovines or buffaloes. This work provide the efficiency proof of concept of frozen semen controlled release devices in the buffalo breeding.

Keywords: artificial insemination, bovine, buffalo, cryopreservation, encapsulation.

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1 **Introduction**

2 Reproductive technologies and biotechnologies are widely used in veterinary
3 and zootechnical fields. In particular, artificial insemination (AI) improves genetic diffusion
4 by partitioning the ejaculate into multiple doses, reducing the number of males and enhancing
5 the scheduling of insemination interventions and health conditions in the farm [1]. During the
6 last 50 years, AI techniques in bovine species have been developed and standardized, as have
7 procedures including semen extension, cooling, freezing and thawing [23]. AI is currently
8 performed through the trans-cervical route, with the placement in the uterine body of
9 approximately 20 million spermatozoa, most of which are phagocytized by leukocytes or
10 removed by the backflow action of the uterus [18]. Compared to natural mating, bovine AI
11 with cooled semen guarantees similar fertility results (over 80%), whereas AI with frozen
12 semen reduces fertility to approximately 60% [23,26]. Additionally, the number of
13 spermatozoa used for AI affects the fertilization rate, and the current trend aims to reduce the
14 cell number while maintaining high yields in terms of pregnancy and parturition rate [3]. AI
15 with low doses of bovine spermatozoa, including those recovered by sex sorting [10], is
16 typically performed in the uterine horns with promising results [24].

17 Although AI procedures are commonly used in bovine breeding, these techniques are limited
18 in the buffalo (*Bubalus bubalis*) [5] because of poor ovarian activity during the hot season, the
19 long period between two consecutive births, the late maturity of females [27], and the
20 difficulties in detecting variable length of estrous [19]; all these factors combine to reduce the
21 female's productivity. In particular, estrus timespan ranges from 5 to 27 hours, and ovulation
22 occurs 6–21 hours after estrus ends [22]. Moreover, they often show 1 or 2 non-ovulatory
23 follicular waves, followed by the ovulatory one and non-ovulatory and ovulatory follicles
24 have approximately identical diameters [4]: the echographic ovulation diagnosis is therefore
25 unreliable; furthermore estrus signs are generally less evident than those in cows.

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Some strategies, such as ovulation synchronization, prostaglandin or progesterone administration to control the luteal phase, or hormone combination to control follicular development and ovulation, can limit the effects of these pitfalls [5]. Other problems relate to the short lifespan of frozen/thawed spermatozoa in the female reproductive tract and the narrow uterine cervix of the females, which makes intrauterine artificial semen delivery difficult [21].

Some of these obstacles could be overcome by adopting the sperm encapsulation technique, which was previously demonstrated in bovine [13,17,18,33] and swine species [7,28,29,31] with promising results. Alginate sperm encapsulation can limit sperm damage and allow prolonged sperm cell release, and the alginate bioadhesiveness properties can prevent semen backflow. To our knowledge, the sperm encapsulation technique has not been applied in buffalo, and the freeze/thaw process for encapsulated spermatozoa has not been optimized. The encapsulation technique combined with cryopreservation could be an optimal solution to optimize the use and yield of buffalo semen.

Aim of this work is to provide the efficiency proof of concept of frozen semen controlled release devices in Mediterranean Italian water buffalo breeding: semen *in vitro* analyses (membrane integrity, progressive motility, path average velocity) were performed, and fertilizing potential of encapsulated-cryopreserved semen was evaluated after artificial insemination.

Materials and Methods

Sample collection

Eight ejaculates from six Mediterranean Italian buffaloes (*Bubalus bubalis*) and seven Holstein Friesian bulls (*Bos taurus*), respectively, of proven fertility was collected (May-July) using an artificial vagina (semen collection interval range 7-20 days). All subjects were

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healthy, as verified by regular clinical objective examinations, and reared under a natural photoperiod in an Italian breeding farm. After collection, each ejaculate was diluted 1:1 in a solution of BULLXcell™ commercial extender (IMV Technologies, France). BULLXcell™ is a concentrated semen medium used to freeze bull and buffalo semen. It is composed of Tris, citric acid, sugars, glycerol as a penetrating cryoprotectant agent, ultra-pure water and antibiotics. Before using the BULLXcell™ extender solution, it was diluted with three volumes of pure water and one volume of 22% egg yolk-TRIS. After dilution, the ejaculate (batch) was characterized based on the concentration, plasma membrane integrity, and motility of the spermatozoa and then divided into two aliquots (each aliquot was diluted to obtain the dose of 20 million spermatozoa in 0.5 ml). The first aliquot was submitted to freezing, and the second aliquot was encapsulated and then frozen. All procedures were performed at room temperature. For each species (bovine and buffalo), four different groups were considered: diluted spermatozoa, used as the control (C_{bov} and C_{buf} for bovine and buffalo, respectively), and diluted encapsulated spermatozoa (E_{bov} and E_{buf} for bovine and buffalo, respectively). Each sample was placed in straws to obtain 20 million spermatozoa/straw and cryopreserved. After the freezing and thawing processes, diluted frozen/thawed spermatozoa (FT-C_{bov} and FT-C_{buf}) and diluted encapsulated frozen/thawed spermatozoa (FT-E_{bov} and FT-E_{buf}) were analyzed. Artificial inseminations were performed in bovine and buffalo females to evaluate the fertility potential of frozen/thawed diluted (FT-C_{bov} and FT-C_{buf}) and frozen/thawed diluted-encapsulated (FT-E_{bov} and FT-E_{buf}) semen (Figure 1).

Spermatozoon encapsulation

For each batch, the spermatozoa suspension (diluted with BULLXcell™) was added to a sodium alginate solution (1% w/v) (Sigma Aldrich, Italy). The alginate sperm suspension was added drop-wise with a needle (32 G) into a continuously stirred BULLXcell™-egg yolk

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solution containing $BaCl_2$ at 50 mM (Carlo Erba, Italy). The barium ions diffused into the droplets, reacted with the alginate chains and formed alginate gel beads. After 10 minutes, the beads were collected by filtration through a nylon mesh cell strainer (Corning, Falcon™, USA) with 100 µm pores, rinsed twice with BULLXcell™-egg yolk extender solution, and re-suspended in the same solution. The 0.5 mL straws were then filled with the bead suspension using a vacuum pump. The final suspension volume was calculated using the following equation:

$$Sv = N*V/20$$

where Sv = the final suspension volume (mL), N = the number of extruded spermatozoa (in million), V = the total straw volume (mL), and 20 = the factor used to obtain 20 million spermatozoa/dose.

A sample of 200 beads was photographed on a digital video camera connected to an image analyzer (CV 9000 Ver. 4.0 Image Analyzer, FKV Srl, Italy), and the whole diameter was measured.

The freezing/thawing processing of spermatozoa

The straws were equilibrated for 3 hours at 4°C and then frozen using an automatic Mini Digitcool freezer (IMV technologies, France). The bovine freezing program included the following steps: 4 to -10°C (rate: -5°C/min), -10 to -100°C (rate: -30°C/min) and -100 to -140°C (rate: -20°C/min). The buffalo freezing program included the following steps: 4 to -10°C (rate: -3°C/min), -10 to -110°C (rate: -40°C/min) and -100 to -140°C (rate: -20°C/min). When the freezer reached -140°C, the frozen straws were immediately plunged and stored in liquid nitrogen.

For the thawing process, two straws from the same frozen batch were thawed for 1 minute in a 37°C water bath (DC5 Haake, EN.CO. S.r.l., Italy), pooled in a tube, and incubated at the same temperature for 14 minutes.

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1 **Semen quality evaluation**

2 After the freezing and thawing processes, the diluted frozen/thawed spermatozoa ($FT\text{-}C_{bov}$
3 and $FT\text{-}C_{buf}$) and diluted encapsulated frozen/thawed spermatozoa ($FT\text{-}E_{bov}$ and $FT\text{-}E_{buf}$)
4 were analyzed. The diluted spermatozoa were characterized after incubation in a 37°C water
5 bath for 14 minutes, whereas the encapsulated spermatozoa were characterized after the
6 dissolution of the beads in an isotonic 3% v/v sodium citrate and 5% v/v EDTA-saline
7 solution (pH 7.4) for approximately 10 minutes at 37°C, as reported by Nebel *et al.* [17].
8 Aliquots from one tube (two straws from the same sample) were used to determine the total
9 cell concentration and plasma membrane integrity (MI) using an integrated fluorescence
10 microscope (NucleoCounter® SP-100™ Chemo-Metec A/S, AllerøDM Denmark). Briefly, the
11 samples were loaded into a SP1-Cassette®. Propidium iodide that was immobilized in the
12 SP1-Cassette® diffused into the cells that had lost membrane integrity and stained their DNA.
13 An integrated computer performed the image analysis and counted the dead sperm cells in the
14 sample. The total number of cells was determined using a lysis buffer (Reagent S100,
15 Chemo-Metec A/S, AllerøDM Denmark) to disrupt the plasma membrane of the sperm cells,
16 thus rendering the nuclei susceptible to staining with propidium iodide. The membrane
17 integrity percentage was calculated using the following equation:

18 MI% = 100 - [(non-viable cells / total cells) * 100]

19 The total motility (TM), progressive motility (PM), and average path velocity (VAP) were
20 determined by a computerized image analyzer (CASA system) using a HTM-IVOS
21 instrument v.12.3 (Hamilton Thorne, Beverly, MA, USA). Ten microliters of semen from
22 each sample (tube) was placed in two Makler® counting chambers (Sefi-Medical Instruments,
23 Israel) at 37°C. The spermatozoon motility was determined for at least 200 cells per sample in
24 a minimum of four microscopic fields. Spermatozoa with a VAP >25 μm/s were defined as

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motile, whereas sperm with a straight-line velocity/path velocity >0.8 were defined as progressively motile [34].

Artificial inseminations

The artificial inseminations were performed from November 2012 to March 2013. This time interval was selected because it is the best buffalo and bovine reproductive season in Italy. For this study, 25 Italian Mediterranean buffalo and 113 Holstein Friesian bovine females were used. Buffalo inseminations were conducted on one farm, whereas bovine inseminations were conducted on two different farms.

Ovulation synchronization and fixed-time inseminations in buffalo

Estrus synchronization was induced using the Ovsynch protocol [6,11]. Briefly, the animals were synchronized using a sequence of GnRH (buserelin acetate, 12 mg; Receptal®; Intervet, Italy) on Day 0 followed by PGF2α (cloprostenol sodium, 524 mg; Estrumate®; Intervet) on Day 7 and an identical GnRH treatment on Day 9. The buffaloes were inseminated once at a fixed time (16 hours after the second GnRH treatment) in the body of the uterus using conventional AI. Inseminations with FT-C_{buf} and FT-E_{buf} semen at 20 million sperm per dose were performed using one straw per insemination. To facilitate the random field use of the doses, the straws were stored two-by-two inside the canister, so that a blind procedure was used. Pregnancy was diagnosed using an ultrasound examination 60 days after insemination, and the pregnancy rate was coded as a binomial event (1 = pregnant; 0 = not pregnant). All field activities were conducted by one veterinarian.

Artificial inseminations in bovine specie

Two dairy farms (A and B) were involved in this study. On farm A, inseminations were performed after estrus synchronization with the Ovsynch protocol. On farm B, artificial insemination was performed without the synchronization protocol. Inseminations with FT-C_{bov} and FT-E_{bov} semen at 20 million sperm per dose were performed using one straw per

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insemination. Pregnancy was diagnosed with an ultrasound examination 60 days after insemination, and the pregnancy rate was coded as a binomial event. All field activities were conducted by one veterinarian at each farm.

Statistical analysis

The MI, TM, PM, and VAP values were analyzed using two-way repeated-measures ANOVA considering species and treatment (encapsulation and/or cryopreservation) as fixed factors and the semen collection time as a repeated-measures variable. One-way ANOVA was performed followed by Tukey's multiple comparison test to assess the differences between the treatment groups for sperm characterization. The coefficient of variation (CV) was calculated to evaluate the homogenous loading of samples in the straws. A chi-square test was performed to evaluate the significance of differences among the pregnancy rates with frozen/thawed diluted (FT-C) and frozen/thawed diluted-encapsulated (FT-E) semen. The statistical analyses were performed using SAS 9.1 software, and the significance level was $p<0.05$.

Results

In vitro results

The bead size, determined by optical microscopy using a sample of 200 beads, was 2.31±0.15 mm, which is suitable for loading in a 2.5-mm-diameter straw. After loading, the mean numbers of non-encapsulated spermatozoa were 18.30±2.32 million/straw (bovine) and 18.42±3.75 million/straw (buffalo), whereas the mean numbers of encapsulated spermatozoa were 23.00±8.00 (bovine) and 20.37±2.49 million/straw (buffalo). In bovine species, the CV, or index of homogeneous straw loading, was lower for $FT\text{-}C_{bov}$ (12.70, n=30) than for $FT\text{-}E_{bov}$ (37.72, n=29). However, for buffalo species, the $FT\text{-}E_{buf}$ presented a CV of 12.22 (n=32), whereas the $FT\text{-}C_{buf}$ showed a CV of 20.37 (n=33).

A significant treatment effect ($p<0.05$) was observed for the MI percentage, whereas the

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species effect was borderline significant ($p=0.064$), most likely because of the high variability of the data (Figure 2A and 3A). For each treatment group, the mean MI values for bovine spermatozoa were higher than those for buffalo sperm cells. The freezing/thawing process reduced the MI% parameter in the semen of both bovines and buffaloes. No significant differences were observed between the control and bead groups within each species, either before or after cryopreservation.

Overall, significant TM differences were recorded among the treatment groups ($p<0.0001$), but there was no obvious species effect ($p=0.087$). A reduction in TM for both species was observed when encapsulation or cryopreservation processes were performed (Figure 2C and 3C). Our results showed that bovine spermatozoa were more sensitive to cryopreservation treatment than buffalo spermatozoa. In fact, after freezing and thawing, the diluted spermatozoa lost approximately 44% of their TM, whereas the diluted encapsulated sperm lost approximately 38% of their TM. The diluted and encapsulated buffalo spermatozoa lost approximately 23 and 16% of their TM, respectively.

A treatment effect ($p<0.0001$), but not a species effect ($p=0.22$), was also noted in terms of PM (Figure 2B and 3B). The freeze/thaw process significantly reduced PM in both species. Surprisingly, the encapsulated bovine spermatozoa presented the highest PM (Figure 2B).

The statistical analysis indicated that both treatment and species influenced VAP ($p<0.001$). In particular, the VAP values for the bovine spermatozoa were higher than those for the buffalo sperm cells ($p<0.001$). In the bovine species, the encapsulation process slightly increased the VAP (Figure 2D). In buffaloes, the VAP of the E_{buf} group was higher than C_{buf} (79.90 ± 28.93 and 73.39 ± 25.94 μm/s for E_{buf} and C_{buf}, respectively), whereas the cryopreservation process significantly ($p<0.05$) reduced the VAP of the spermatozoa (60.77 ± 15.52 and 56.54 ± 25.92 μm/s for the FT-C_{buf} and FT-E_{buf} groups, respectively) (Figure 3D).

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1 ***In vivo* results**

2 The pregnancy rates following artificial insemination in female buffaloes are reported in 3 Table 1. Although the pregnancy rate obtained with encapsulated spermatozoa ($FT\text{-}E_{buf}$ 4 group) was higher than that obtained with conventional semen ($FT\text{-}C_{buf}$), no significant 5 differences were shown between the two treatment groups (p=0.087).

6 The results obtained after artificial insemination in bovine species are reported in Table 2. The 7 chi-square test did not show significant differences in the pregnancy rates between the FT-8 C_{bov} and $FT\text{-}E_{bov}$ groups for either farm A or B. Moreover, the chi-square test performed on 9 the total results, obtained by the addition of pregnant and non-pregnant individuals in each 10 treatment group, did not indicate significant differences between the $FT\text{-}C_{bov}$ and $FT\text{-}E_{bov}$ 11 groups. All buffaloes and bovines maintained good health after parturition and the weight of 12 born animals was not different ($p > 0.05$) between two treatment groups (FT-C and FT-E).

13

14 **Discussion**

15 Alginate encapsulation of buffalo spermatozoa is a promising technique in AI to prolong *in* 16 *utero* cell release and prevent semen backflow. However, the technology has not yet reached 17 in industrial scale, and encapsulated spermatozoa are not commercially available. Moreover, 18 standard method for cryopreservation of encapsulated buffalo semen has not been reported, 19 and the availability of frozen semen is key to the application of such techniques. In the 20 present work, an encapsulation and cryopreservation method is proposed for bovine and 21 buffalo spermatozoa, and the quality of frozen semen was evaluated.

22 The overall results obtained from *in vitro* semen analyses indicate that the encapsulation 23 process does not lead to a decrease in the quality parameters also after the cryopreservation in 24 either species. Moreover, the qualitative pattern observed in buffalo semen was similar to that 25 seen in bovines, although the recorded parameters were somewhat reduced. Only the

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percentage of motile spermatozoa was decreased by the encapsulation process (82.10±7.65 vs. 70.93±15.62% for C_{bov} and E_{bov}, respectively), while Vishwanath *et al.* [32] observed that the bovine spermatozoa did not have reduced TM after encapsulation in poly-L-lysine.

In buffalo specie, the values of MI, TM and PM found here were generally lower than those reported in the literature, which could be related to the seasonal variability in semen quality, as reported by Bahga and Khokar [2] for buffalo; in fact, in our work, the semen was collected in spring and summer (May-July).

Considering MI parameter, Leite *et al.* [15] and Forero-Gonzalez *et al.* [9] cryopreserved bovine spermatozoa and observed lower values with respect to ours, approximately 20%. For buffalo specie, the percentages of MI were reduced after freezing/thawing procedure [14,16]; in the present study, the MI in buffalo spermatozoa was lower than that reported in literature but notably sparse, as denoted by the high standard deviation probably due to high variability of semen quality during hot season. The cryopreservation reduces also the semen quality in term of PM and VAP, as reported by Orgal *et al.* [20] for bovine specie.

Because the *in vitro* semen characterization does not predict the fertilizing potential of spermatozoa with complete reliability, we conducted *in vivo* artificial inseminations in both bovines and buffaloes. In buffaloes, the pregnancy rates were higher for encapsulated spermatozoa (FT-E_{buf}) compared to the control group (FT-C_{buf}); however, the chi-square test did not show differences between the two treatment groups.

Artificial inseminations in bovines produced similar results. The pregnancy rate obtained in synchronized animals was not significantly different between encapsulated and non-encapsulated semen. In 1997, Vishwanath *et al.* [32] conducted an *in vivo* trial to evaluate the fertilizing properties of bovine encapsulated spermatozoa in an alginate poly-L-lysine matrix. For that study, heifers were synchronized with a CIDR-B® device and were artificially inseminated with encapsulated or non-encapsulated spermatozoa. The pregnancy rate they

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reported (45%) was similar to ours (40%). Recently, Standerholen *et al.* [30], used 85 bovine semen doses, immobilized in alginate-based matrix, for an *in vivo* trial. The authors concluded that there are no differences between the outcome of AI using encapsulated or conventionally diluted frozen sperm.

In addition we conducted *in vivo* artificial inseminations without hormonal treatment, but pregnancy rates were lower than in synchronized animals, and also in this case no differences were shown between the encapsulated and control groups. Many researchers have investigated the effect of freezing on encapsulated mammalian cells, but to our knowledge, only Herrler *et al.* [12], for humans and bovine, and Shah *et al.* [25], for dogs, have applied similar techniques to spermatozoa.

The world's critical economic situation requires competent management practices to increase the productivity of buffalo operations. Excellent reproductive efficiency is essential to increase net returns. The use of artificial insemination technologies has become important, particularly to improve the genetic health of buffalo herds. However, the time and effort required to detect estrus have limited the extensive application and success of this technology. This work provide the efficiency proof of concept of frozen semen controlled release devices in Mediterranean Italian water buffalo breeding. In our opinion, the results represent an encouraging starting point to develop further strategies, thus fulfilling the needs of the peculiar buffalo breeding.

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1 **Conflict of Interest**

2 There is no conflict of interest.

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1 Switzerland, 2010.

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1 **Table 1.** Pregnancy rates in female buffaloes after AI using FT-C_{buf} and FT-E_{buf} semen at 20

2 million sperm per dose. Values with the same superscripted letter did not differ significantly

3 ($p>0.05$)

Treatment group	Pregnant (n)	Non pregnant (n)	Total events	Pregnancy rate (%)
FT-C_{buf}	3	11	14	21.4[a]
FT-E_{buf}	6	5	11	54.5[a]

4 Values with the same superscripted letter did not differ significantly ($p>0.05$).

5

6 **Table 2.** Pregnancy rates in female bovines after AI using FT-C_{bov} and FT-E_{bov} semen at 20

7 million sperm per dose considering farms A and B

Farm	Treatment group	Pregnant (n)	Non pregnant (n)	Total events	Pregnancy rate (%)
A	FT-C_{bov}	13	21	34	38.2[a]
	FT-E_{bov}	6	9	15	40.0[a]
B	FT-C_{bov}	8	41	49	16.3[b]
	FT-E_{bov}	2	13	15	13.3[b]
A+B	FT-C_{bov}	21	62	83	25.3[c]
	FT-E_{bov}	8	22	30	26.7[c]

8 Values with different superscripted letters differ significantly ($p<0.05$).

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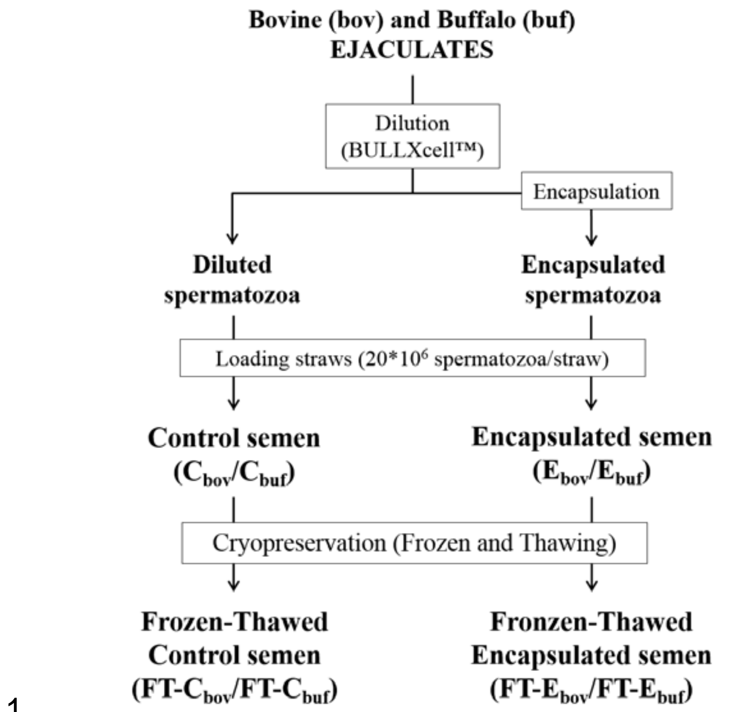
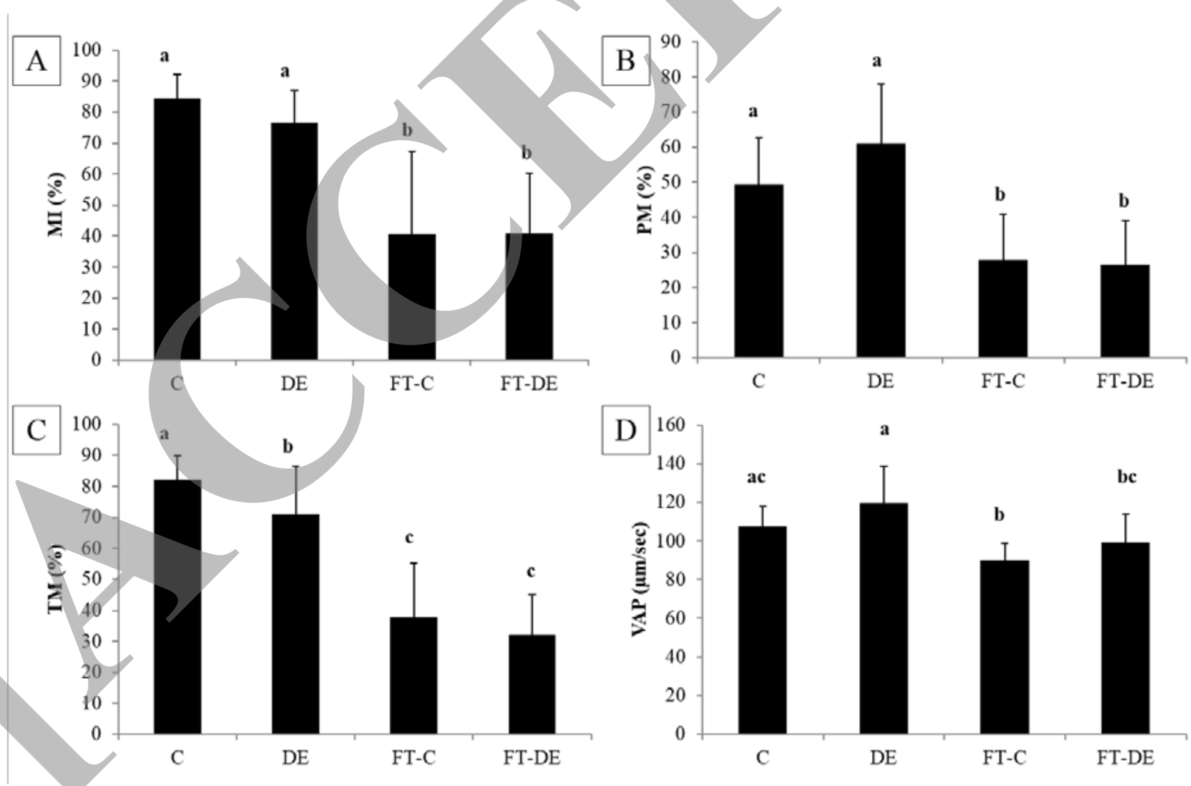


2 **Fig. 1.** Graphical experimental design of the study.

3

4



5 **Fig. 2.** Bargraphs (means ± SD of eight bovine bulls) for the spermatozoon: A) membrane

6 integrity (MI), B) progressive motility (PM), C) total motility (TM), and D) average path

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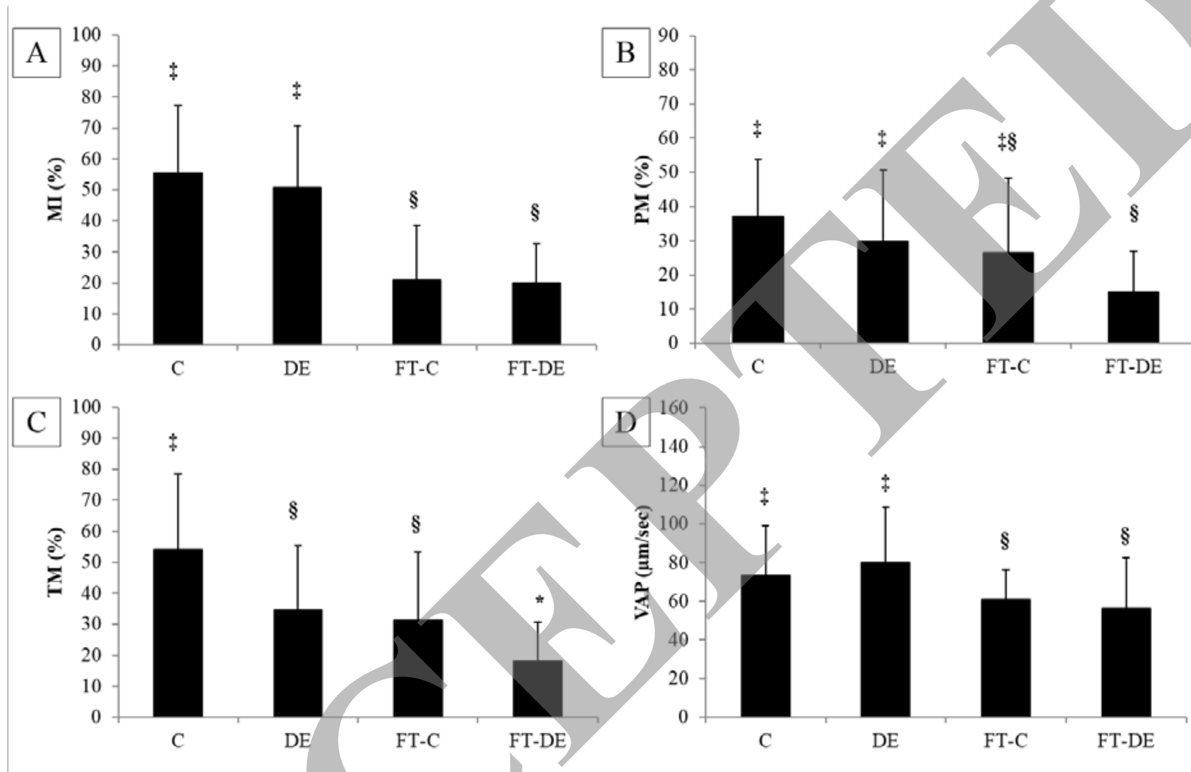
1 velocity (VAP) in the four treatment groups (C: control spermatozoa; E: encapsulated
2 spermatozoa; FT-C: frozen-thawed control spermatozoa; FT-E: frozen-thawed encapsulated
3 spermatozoa) for bovine specie. Values with different superscripted letters differ significantly
4 (p<0.05) among groups.
5



6

7 **Fig. 3.** Bargraphs (means ± SD of eight buffalo bulls) for the spermatozoon: A) membrane
8 integrity (MI), B) progressive motility (PM), C) total motility (TM), and D) average path
9 velocity (VAP) in the four treatment groups (C: control spermatozoa; E: encapsulated
10 spermatozoa; FT-C: frozen-thawed control spermatozoa; FT-E: frozen-thawed encapsulated
11 spermatozoa) for buffalo species. Values with different superscripted symbols differ
12 significantly (p<0.05) among groups.
13

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