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The role of bicarbonate in the modulation of capacitation, spontaneous acrosome reaction and motility of equine fresh and frozen spermatozoa

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ABSTRACT

In this study, we defined the composition of the culture medium that yield a significant percentage of alive and functional equine spermatozoa during enough time before artificial insemination. The effects of sodium bicarbonate were analyzed in fresh and frozen semen in respect to sperm viability, capacitation, spontaneous acrosome reaction and several kinetic parameters such as total motility, progressive motility, VCL, VSL, ALH, BCF, LIN. Moreover, employing Bayk-6844 and Nifedipine, the involvement of L-type voltage-gated calcium channels in the modulation of intracellular calcium concentrations was investigated. Results evidenced an immediate effect of sodium bicarbonate in reducing fresh sperm viability and in increasing capacitation and spontaneous acrosome reaction of fresh and frozen spermatozoa. Furthermore, it affected total and progressive motility of fresh and frozen semen. Because of the sudden effects of the compound, we think that a buffer lacking sodium bicarbonate is more suitable to preserve the viability and the functional state of equine spermatozoa required to undergo at the right time those modifications necessary for fertilization. We also demonstrated that intracellular calcium modifications induced by Bayk-8644 and Nifedipine are not involved in signals related to vitality, capacitation, spontaneous acrosome reaction and motility.

1. Introduction

When a sperm cell travels through the female reproductive tract, it experiences several modifications that will affect the structure of its cellular membrane and its motility. All these changes are called with the unique term of capacitation and include the modification of the ratio between membrane phospholipids and cholesterol, the reorganization of proteins membrane and hyperactivation [1]. They have the purpose of making the spermatozoon go through the necessary steps to achieve the acrosome reaction (AR) when it comes close to the oocyte. The AR is an exocytotic process under a strong physiological regulation that avoids its occurrence at the wrong time in the cascade of events that will lead to fertilization. Under some conditions, spontaneous acrosome reaction (sAR) can occur [2] and this increases the failure of fertilization. In vitro both capacitation and AR can be induced by incubating spermatozoa in the presence of several compounds such as bovine serum albumin (BSA) [3], sodium bicarbonate [4], low concentrated Naloxone [5] for capacitation and calcium ionophore A23187 or progesterone or heparin for AR [6]. It has been demonstrated that AR is a calcium dependent process [7]. In a previous paper we demonstrated that L-type voltage gated calcium channels are expressed on equine spermatozoa and that their incubation in the presence of agonist (Bayk-8644)/antagonist (Nifedipine) of these channels results in modifications of intracellular calcium concentrations ($[Ca^{2+}]_i$) [8] and that these modifications are related to the state of the semen: fresh (f) or frozen (fz). Moreover, we demonstrated that sperm functional modifications induced by cooling or cryopreservation can be easily evidenced using a combination of three fluorescent dyes [9].

The aim of the present work was to understand whether the incubation of equine spermatozoa in culture medium containing BSA and sodium bicarbonate could modify the physiology of the equine spermatozoon by increasing the rate of sAR and/or its

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kinetic. Moreover, we wanted to establish if cryopreservation could increase equine sperm susceptibility to sAR and whether the presence of agonist/antagonist of L-type voltage-gated calcium channels could have a role in sAR and equine sperm kinetics.

2. Materials and methods

2.1. Reagents

All reagents were obtained from Sigma Aldrich (Milan, Italy), unless otherwise specified. Bayk-8644 (Bayk) or Nifedipine (Nif) were dissolved in ethanol to produce 10 mM stock solution and successively 1 mM sub-stock solution in 0.16 M NaCl and used in this work at the final concentration of 1 μM to reduce ethanol concentration lower than 1% avoiding any effect on sperm cell motility as previously reported by Shi and Roldan [10].

2.2. Semen collection

The study was conducted on six stallions (14 ± 2 years old) of proven fertility routinely employed in the artificial insemination (AI) programs at the Veterinary Campus of the University of Bari Aldo Moro, Italy. Semen was collected during the equine official breeding season (February to July at our latitude: 41°2′46"68 N; 16°53′9"24 E). A Colorado artificial vagina (CSU) equipped with the warming bag and an in-line gel filter was employed; volume of the gel free fraction was recorded, then semen was diluted 1:2 in INRA 96 (IMV Technologies, Piacenza, Italy) divided in two samples; the first used as fresh semen (f) the second followed the freezing procedure in INRA Freeze kit (IMV Technologies, Piacenza, Italy) at the concentration of 200 million sperm cells/mL in 0.5 mL straw and named frozen semen (fz). The freezing procedure was achieved by the CL 3300 programmable temperature controller (Cryologic, Blackburn, Australia) and the Cryo-Genesis V4 software. The following fast cooling temperatures were used: 0 °C to −10 °C per 1.5 min; −10 °C to −40 °C per 1 min; and −40 °C to −120 °C per 2 min. Then, for each ejaculate, straws were stored in liquid nitrogen for 24 h until used for all tests described.

For examination, f and fz semen (thawed for 30 s at 37 °C in a water bath) were divided in two aliquots and both centrifuged for the elimination of cryoprotectants, dilutors and seminal plasma as previously reported [5]. Each pellet from f and fz semen was diluted in 1.5 mL non-capacitating buffer (NCB) or in the same volume of capacitating buffer (CB). The composition of the NCB (111 mM NaCl, 3.1 mM KCl, 2 mM $CaCl_2$, 0.4 mM $MgSO_4$, 0.3 mM KH_2PO_4, 50 μg kanamycin/ml, 20 mM Hepes, 5 mM glucose, 21.7 mM sodium lactate, 1 mM sodium pyruvate, and 7 mg/mL BSA) was a modification of the Tyrode's buffer (96 mM NaCl, 3.1 mM KCl, 2 mM $CaCl_2$, 0.4 mM $MgSO_4$, 0.3 mM KH_2PO_4, 50 μg kanamycin/ml, 20 mM Hepes, 5 mM glucose, 21.7 mM sodium lactate, 1 mM sodium pyruvate, 15 mM $NaHCO_3$ and 7 mg/mL BSA) without sodium bicarbonate. This latter buffer was previously employed by Rathi et al. [1] and by our group [8] to evaluate functional modifications of equine spermatozoa in respect to the freezing procedure. In this paper, we refer to it with the name of CB buffer. Moreover, to test the involvement of L-type voltage calcium channels on sperm functional and kinetic parameters, f and fz semen diluted in CB or in NCB buffer were divided in three 0.5 mL subaliquots containing 15×10^6 cells each and added with 1 μM Bayk or 1 μM Nif or with no other substance for the control condition (C). For each test described in this paper, the mean value from two different experiments/horse/month was obtained, as reported in the workflow diagram (Diagram 1), and used for the statistical analysis.

2.3. Evaluation of sperm functional parameters

2.3.1. Viability, capacitation and acrosome reaction

Subaliquots (0.5 mL/each) were incubated in a covered twelve multidish plate (CytoOne, Starlab, Milano, Italy) at 37 °C under 5% CO_2 in air as previously reported [5]. To evaluate at the same time sperm viability and the condition of capacitation/AR, 200 μL aliquots of sample from each experimental condition were examined, at T0 and after 4.5 h (T4) of incubation by employing Hoechst 33258/Chlortetracycline (H258/CTC) staining as previously described [5]. Samples were observed with a Nikon E600 microscope under epifluorescence illumination using 346/460 nm (UV-2A) and 450/630 nm (B-3A) excitation/emission filters for H258 and CTC, respectively. Spermatozoa with bright blue fluorescent nuclei were classified as dead (D) and not assessed for capacitation and AR. On the contrary, spermatozoa showing bright green fluorescence uniformly distributed over the entire sperm head were classified as live non-capacitated (L/NC), those showing green fluorescence over the acrosomal region and a dark post-acrosome as live capacitated (L/C) when they showed green fluorescence only in the post-acrosomal region or no fluorescence on the head were classified as acrosome reacted (L/AR). At least 200 spermatozoa were scored per slide.

2.3.2. Sperm kinetic

Concentration, morphology and the kinetic pattern of equine spermatozoa under different experimental conditions were evaluated by a Computer Assisted Sperm Analyzer (CASA) (Hamilton Thorne Sperm Analyzer (Hamilton Thorne Sperm Analyzer, IVOS II, Software version 12.3 Hamilton Thorne, USA) with the optical set up previously reported [11]. From each experimental condition at T0 and T4, 2 μL aliquot of semen sample were loaded into a Leja slide 4 chambers to record the following kinetic parameters: percentage of motile (MOT) and progressive motile (PMOT) cells, percentage of rapid medium, slow and static cells, curvilinear velocity (VCL), straight-line velocity (VSL), threshold straightness (STR), linearity (LIN), beat cross frequency (BCF), amplitude of lateral head displacement (ALH).

2.4. Statistical analysis

The Statistical Package for Social Science (SPSS, version 19) software was employed to evaluate the statistical significance of the collected data. The analysis was preceded by the verification of the normal distribution of the obtained data by the Kolmogorov-Smirnov test. Despite the logarithmic transformation of the data, their distribution remained not normal so that the analysis was conducted by non-parametric tests. To highlight statistical significances among correlated variables the Friedman test was used, on the contrary for independent variables the Kruskal-Wallis and the Mann-Whitney tests were used. To evaluate correlations among variables the Spearman test was employed. P values < 0.05 were considered significant.

3. Results

3.1. Functional parameters: viability, capacitation and sAR

We found that the state of the semen (f or fz) the buffer composition (CB or NCB) and the incubation time (T0 or T4) affected the number of dead cells, all results are summarized in Fig. 1, panel A. Over time the effects of CB buffer were comparable in f and fz semen, in fact the increase in the percentage of dead cells

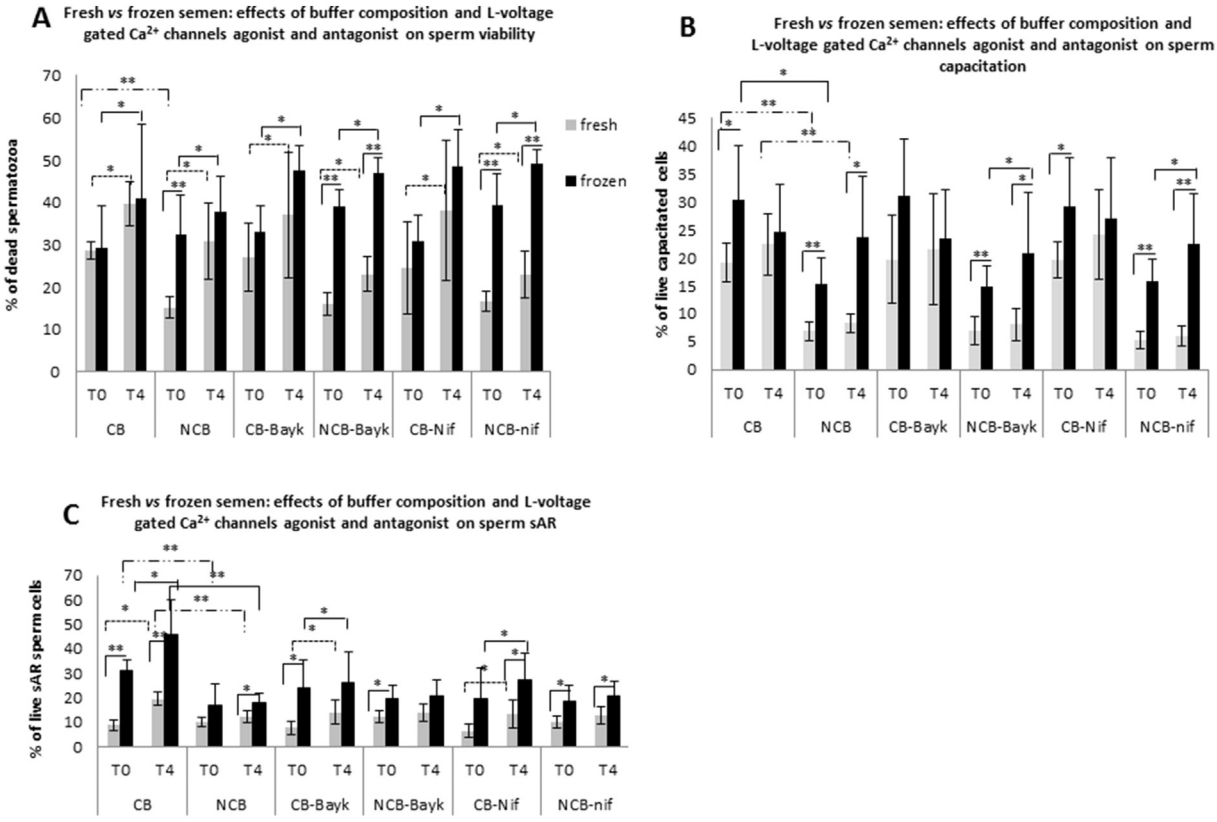

Fig. 1. The histogram shows comparisons between the percentage of dead (panel A), live capacitated (panel B), sAR (panel C) from fresh and frozen semen incubated in the specified buffer. Statistically significant differences are indicated by symbols. * = $P < 0.05$; ** = $P < 0.01$. Dotted lines were used to highlight comparisons between fresh semen at two time points: T0 and T4; on the contrary continuous lines were used for frozen semen in the same conditions.

from T0 to T4 was statistically significant ($P < 0.05$) for both. The addition of Bayk and Nif to CB buffer did not change these results. When NCB buffer was employed, the difference in the percentage of dead spermatozoa between f and fz semen was statistically significant at T0 ($P < 0.01$). The addition of Bayk and Nif induced similar differences between f and fz semen both at T0 and T4 (f *vs* fz $P < 0.01$) even if the two compounds did not behave as a couple of agonist/antagonist. Moreover, when f semen was diluted in CB buffer, the number of dead cells increased immediately (CB *vs* NCB at T0, $P < 0.01$).

In panel B of Fig. 1, the effects of the same variables on capacitation were analyzed. In both buffers at T0, there was a difference between f and fz semen in the number of capacitated cells (CB, f *vs* fz $P < 0.05$ and NCB, f *vs* fz $P < 0.01$), moreover in NCB buffer the difference was also significant at T4 (NCB, f *vs* fz $P < 0.05$).

The percentage of capacitated cells of f semen was higher in CB buffer both at T0 and T4 (CB *vs* NCB $P < 0.01$), in fz semen the difference was only significant at T0 (CB *vs* NCB $P < 0.05$).

Panel C summarizes the results related to sAR. We found that the state of the semen affected the percentage of sAR spermatozoa in both buffers at T4 (CB, f *vs* fz $P < 0.01$ and NCB, f *vs* fz $P < 0.05$), and only at T0 in CB (f *vs* fz $P < 0.01$) because of the high SD recorded. The incubation time affected sAR only in CB buffer both in f and fz semen (T0 *vs* T4, $P < 0.05$). The effect of buffer composition was only significant at T4 in f semen ($P < 0.01$), on fz semen the effect was clear at both time points ($P < 0.01$).

3.2. Sperm kinetic

The percentage of MOT and PMOT spermatozoa (Fig. 2, panels A and B respectively) were both influenced by the state of the semen (f *vs* fz), by buffer composition (CB *vs* NCB) and by the incubation time (T0 *vs* T4).

As detailed in panel A of the figure, the difference in the percentage of motile spermatozoa between f and fz semen was statistically significant in both buffers at T0 and T4 ($P < 0.01$); moreover at T0, MOT values were always significantly higher in CB buffer both in f and fz semen (CB *vs* NCB; $P < 0.01$).

In both buffers, the incubation time had a statistically significant effect on MOT of fz semen only ($P < 0.01$). Although Bayk and Nif had no significant effects on MOT in both buffers, their presence induced more homogeneous MOT values in the population of spermatozoa so that the reduced SD highlighted the effect of the incubation time ($P < 0.05$).

Panel B of Fig. 2 shows in detail the effects of the different variables on PMOT. While PMOT of fz semen decreased significantly over time in both buffers ($P < 0.05$), PMOT of f semen decreased only in CB buffer (T0 *vs* T4, $P < 0.05$). At T0, PMOT of f and fz semen was higher in CB buffer (CB *vs* NCB; $P < 0.01$); while this difference was still present at T4 for fz semen (CB *vs* NCB; $P < 0.01$). Fz semen showed always a lower PMOT in respect to f semen in both buffers at the two fixed time points (f *vs* fz, T0 *vs* T4; $P < 0.01$).

Among kinetic parameters, VAP, VCL, VSL and LIN were influenced by the state of the semen (f *vs* fz) and by the incubation time (T0 *vs* T4) as detailed in Fig. 2 (panel C and D for VAP and VCL), in Fig. 3 (panel A for VSL) and in Fig. 4 (panel A for LIN). BCF of fz semen resulted more affected by buffer composition (CB *vs* NCB at T0 and T4; $P < 0.01$); moreover, at T4 Bayk increased BCF in NCB buffer ($P < 0.01$) and Nif reversed this effect (NCB-Bayk *vs* NCB-Nif; $P < 0.05$). STR was affected by all three variables: buffer composition (CB *vs* NCB), state of the semen (f *vs* fz) and incubation time (T0

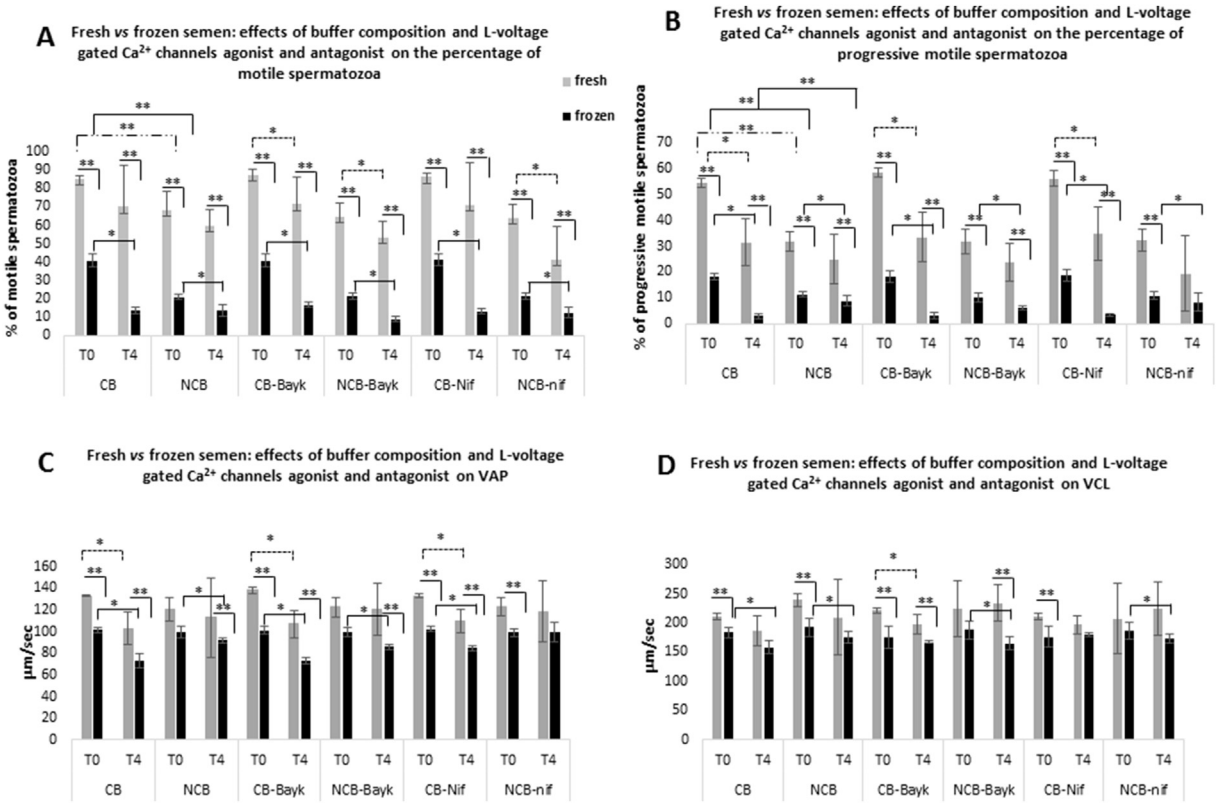

Fig. 2. The histogram shows comparisons between the percentage of motile spermatozoa (panel A), the percentage of progressive motile spermatozoa (panel B), VAP (average path velocity) values (panel C) and VCL (curvilinear velocity) values (panel D) from fresh and frozen semen incubated in the specified buffer. Statistically significant differences are indicated by symbols. * = $P < 0.05$; ** = $P < 0.01$. Dotted lines were used to highlight comparisons between fresh semen at two time points: T0 and T4; on the contrary continuous lines were used for frozen semen in the same conditions.

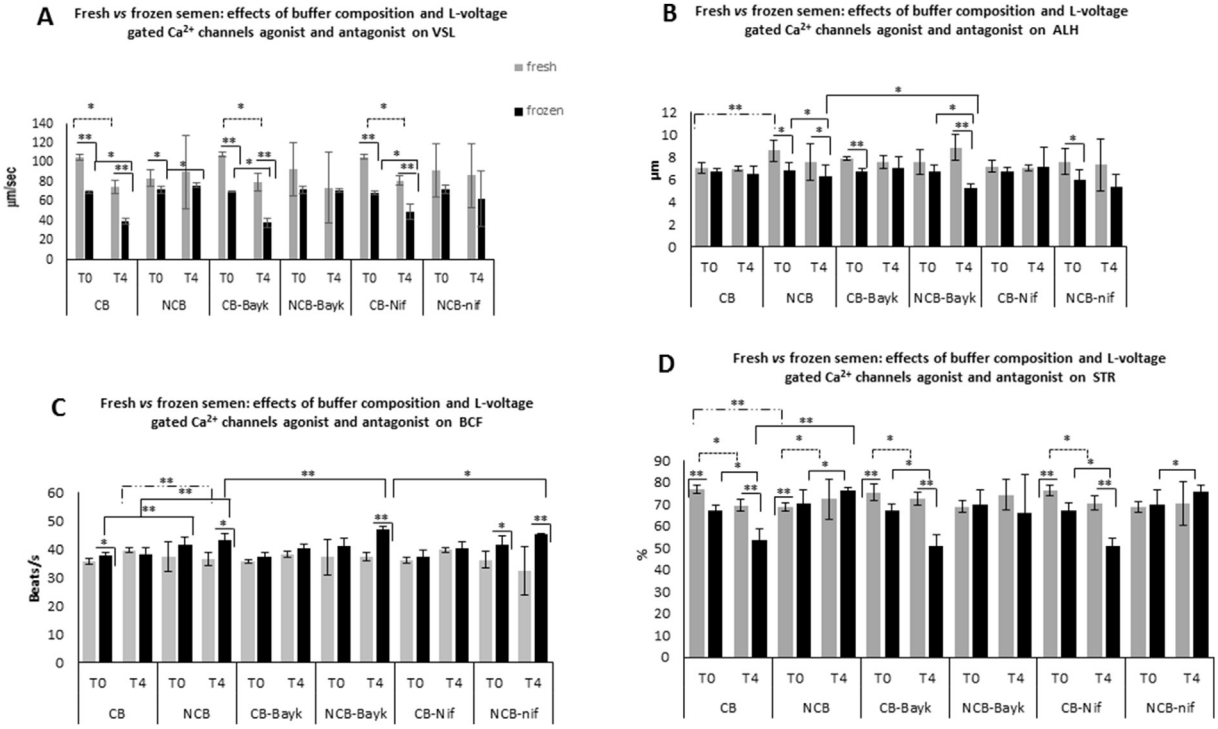

Fig. 3. The histogram shows comparisons between VSL (straight-line velocity) values (panel A), ALH (amplitude of lateral head displacement) values (panel B), BCF (beat cross frequency) values (panel C) and STR (straightness = VSL/VAP) values (panel D) from fresh and frozen semen incubated in the specified buffer. Statistically significant differences are indicated by symbols. * = $P < 0.05$; ** = $P < 0.01$. Dotted lines were used to highlight comparisons between fresh semen at two time points: T0 and T4; on the contrary continuous lines were used for frozen semen in the same conditions.

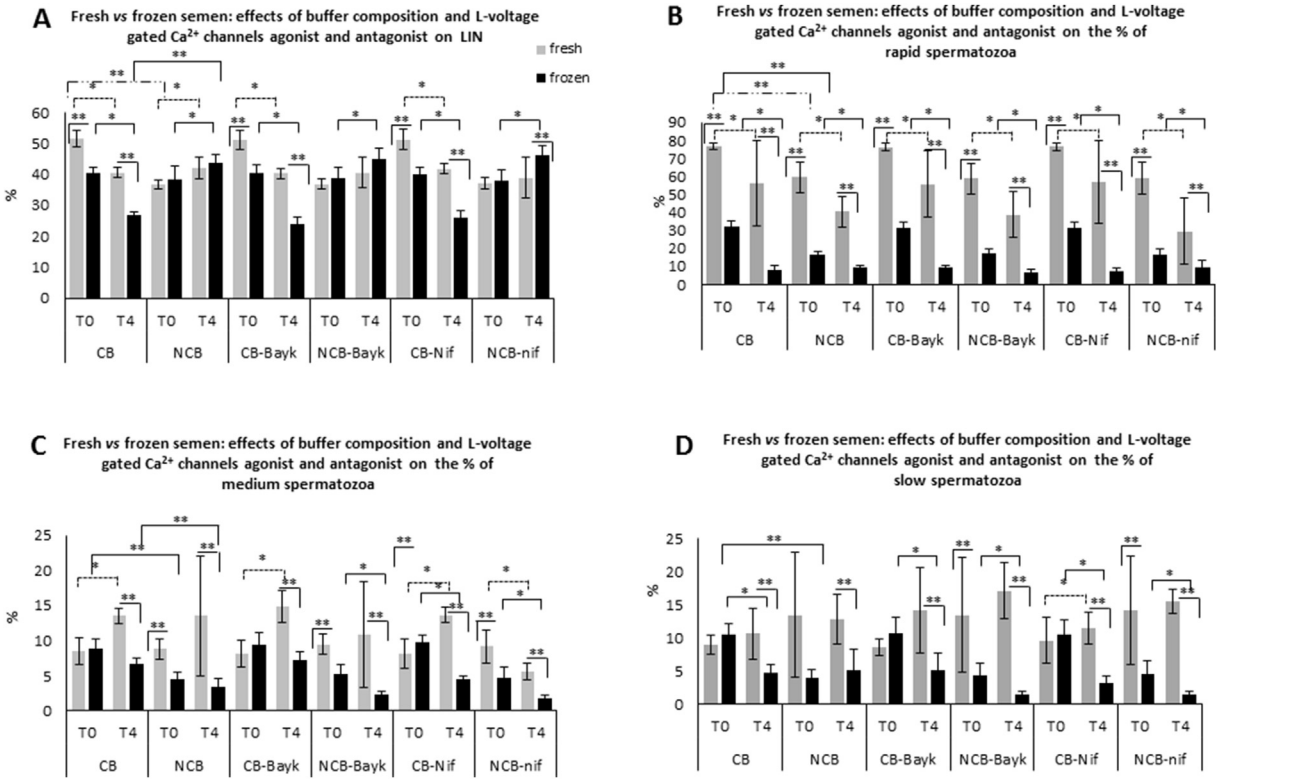

Fig. 4. The histogram shows comparisons between LIN (straight-line velocity) values (panel A), the percentage of rapid spermatozoa (panel B), the percentage of medium spermatozoa (panel C) the percentage of slow spermatozoa (panel D) from fresh and frozen semen incubated in the specified buffer. Statistically significant differences are indicated by symbols. * = P < 0.05; ** = P < 0.01. Dotted lines were used to highlight comparisons between fresh semen at two time points: T0 and T4; on the contrary continuous lines were used for frozen semen in the same conditions.

vs T4, Fig. 3, panel D). ALH of f semen was significantly influenced by buffer composition (P < 0.01) and in NCB buffer ALH values of fz semen were always significantly lower than that of f semen (f *vs* fz; P < 0.05; Fig. 3, panel B).

The percentage of rapid spermatozoa (Fig. 4, panel B) decreased over time both in f and fz semen (T0 *vs* T4; P < 0.05) and the difference of the percentage of rapid spermatozoa between f and fz semen was always statistically significant (f *vs* fz; P < 0.01); moreover, NCB buffer decreased immediately the number of rapid cells both in f and fz semen (CB *vs* NCB at T0; P < 0.01).

The percentage of progressive motile spermatozoa with medium velocity (Fig. 4, panel C) was dependent on the state (f *vs* fz; P < 0.01) except for T0 of CB buffer and the number of medium spermatozoa increased over time in f semen and decreased in fz semen (T0 *vs* T4; P < 0.05). In fz semen the number of medium spermatozoa was higher in CB buffer (CB *vs* NCB; P < 0.01) both at T0 and T4.

The percentage of slow spermatozoa of fz semen decreased over time in CB buffer (T0 *vs* T4; P < 0.05) (Fig. 4, panel D), while NCB buffer decreased immediately the number of slow cells (CB *vs* NCB at T0; P < 0.01); moreover, at T4 the difference between f and fz semen in the percentage of slow spermatozoa was statistically significant despite buffer composition (f *vs* fz; P < 0.01).

While the percentage of static spermatozoa of f semen increased over time only in NCB buffer, (T0 *vs* T4; P < 0.05), that of fz semen increased despite buffer composition (T0 *vs* T4; P < 0.05) (Fig. 5, panel A); the difference between f and fz semen resulted also statistically significant in both buffers and at both time points (f *vs* fz; P < 0.01). NCB buffer increased immediately the number of static spermatozoa both in f and fz semen (CB *vs* NCB at T0; P < 0.01). Bayk and Nif had no statistically significant effect on the percentage of progressive, medium, slow and static spermatozoa in both buffers.

3.3. Correlations among variables

Data evidenced that sAR was inversely correlated to sperm viability (R = -0.3; P = 0.001), MOT (R = -0.5; P = 0.000), PMOT (R = -0.6; P = 0.000), VAP (R = -0.05; P = 0.000), VCL (R = -0.4; P = 0.000), VSL (R = -0.5; P = 0.000), ALH (R = -0.03; P = 0.001), BCF (R = -0.26; P = 0.001), STR (R = -0.3; P = 0.000), LIN (R = -0.3; P = 0.000), % of rapid cells (R = -0.5; P = 0.000), % of medium cells (R = -0.19; P = 0.024), % of slow cells (R = -0.3; P = 0.000), % of static cells (R = -0.51; P = 0.000) and positively correlated to capacitation

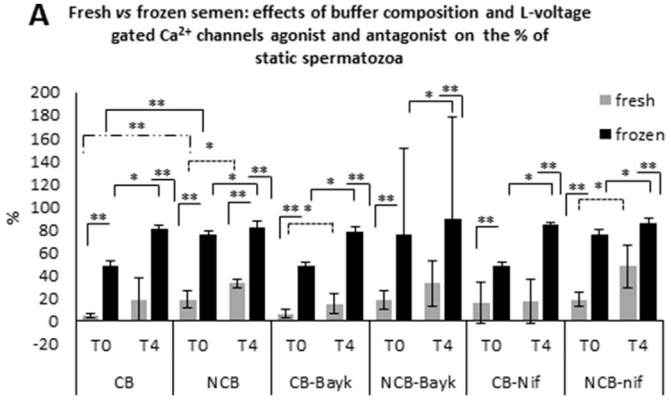

Fig. 5. The histogram shows comparisons between the percentage of static spermatozoa (panel A) from fresh and frozen semen incubated in the specified buffer. Statistically significant differences are indicated by symbols. * = P < 0.05; ** = P < 0.01. Dotted lines were used to highlight comparisons between fresh semen at two time points: T0 and T4; on the contrary continuous lines were used for frozen semen in the same conditions.

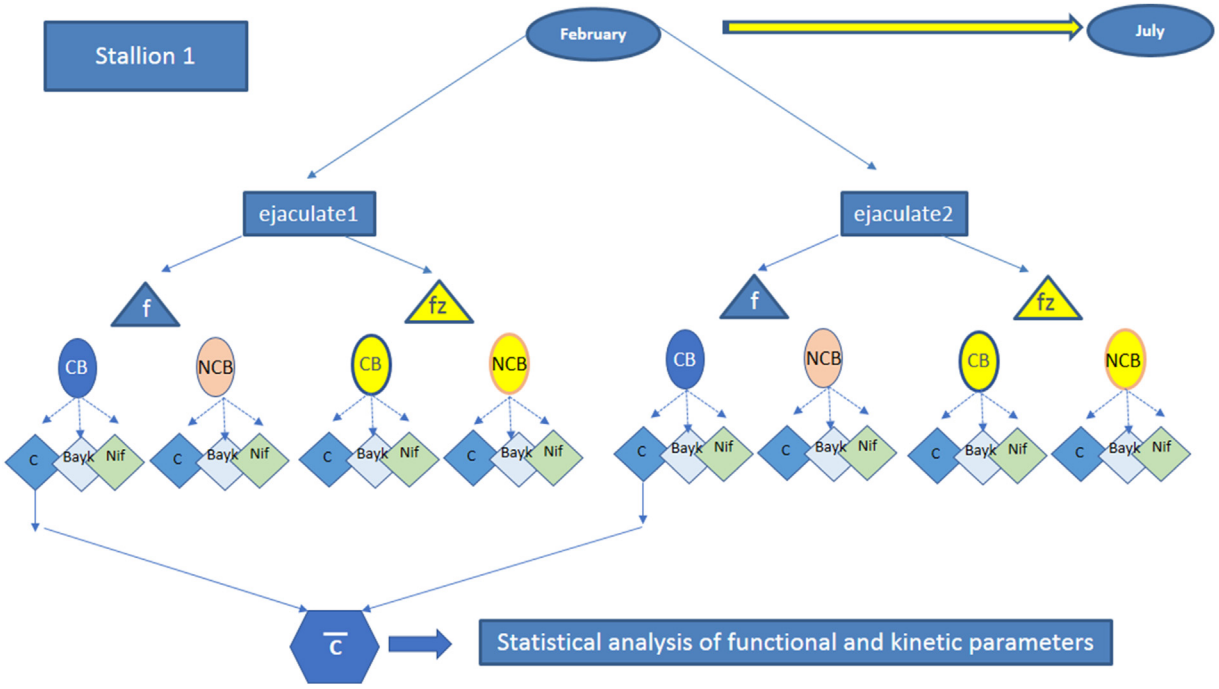

Fig. 6. Workflow diagram of the experimental design. A representative scheme describes the protocol used during the breeding season on each of the six stallions enrolled in the study. Twice a month an ejaculated was recovered and divided in two aliquots (f and fz) according to the protocol described in the materials and methods section. Each sample was subdivided in two aliquots to test the effect of sodium bicarbonate (CB) in respect to the control (NCB) and successively analyzed for the effects of Bay k-8644 (Bayk) and Nifedipine (Nif) in respect to the control (C). For each test (to evaluate morphological and kinetic parameters of the semen) the average of the results coming from experiments on the two ejaculates were calculated.

(R = 0.35; P = 0.000), state of the semen (f or fz) (R = 0.46; P = 0.000) and incubation time (R = 0.31; P = 0.000).

4. Discussion

Artificial insemination is an important management tool in the equine breeding industry so that estimating semen quality is a key requisite. In the management of breeding procedures often a lag period of several hours between semen collection and insemination occurs and nowadays more and more use are made of refrigerated and cryopreserved semen. Discovering the best medium for short period storage or for semen exchanges among breeders preserving sperm viability, acrosomal integrity and motility is essential to maintain the fertilizing ability of in vitro-stored spermatozoa until A.I. has been achieved. For this reason, the specific object of this work was to determine whether sodium bicarbonate, that is commonly added to the incubation medium for equine spermatozoa and it is known inducing sperm capacitation in vitro, was similarly effective for equine semen storage in the case of f and fz semen. This specific need stands on the different physiological condition of f and fz semen. In fact, cryopreservation is based on the intrinsic contradiction between the purpose in keeping spermatozoa alive and the functional modifications deriving from cryoinjury that at the end can impact on sperm physiology and consequently on the reproductive performance. Sodium bicarbonate is used for its ability in buffering the reduction of pH produced by sperm cells and contaminating bacteria during storage that is responsible for reduction of sperm metabolism, motility, and viability [12]. In this study we checked whether fz semen could react differently from f semen to it. We recorded functional modifications of equine f and fz semen concerning cell viability, capacitation, sAR and kinetic occurring during a 4.5 h period of observation in a medium with or without sodium bicarbonate. Results evidenced that when added to f semen the compound immediately increased the percentage of dead cells and even if not statistically significant because of the high SD values, the percentage of dead cells was still higher in CB at T4. The high SD values recorded in this study are caused by analyzing results from evaluations repeated several times on semen collected from each horse in the different months of the reproductive season. On the other hand, because our scope was to have a global idea of the semen conditions during the breeding season, we decided to do not analyze separately results from each month. In a previous paper [8], we showed that equine spermatozoa express L-Type voltage-gated calcium channels on the neck and on the principal piece of the tail and that they are functional active in increasing/decreasing $[Ca2+]_i$ in the presence of their specific agonist/antagonist. In this paper, we aimed to study the possible involvement of these channels in sperm viability, sAR, in the functional modifications occurring during capacitation and in sperm kinetic too. As shown in panel A of Fig. 1, Bayk doesn't induce any modification in the number of viable cells nor in CB or in NCB buffer and spermatozoa are also insensitive to Nif; so that $[Ca2+]_i$ induced by Bayk at the concentration used [8] are not involved in the death signaling. In panel B of the same figure, the sudden effect of sodium bicarbonate in inducing the capacitation of f semen is shown, the capacitating effect lasts until T4. In panel C the percentage of sAR cells is shown; sodium bicarbonate, responsible of the early capacitating effect, seems also involved in the increase of sAR and, in our knowledge, this is the first report highlighting its direct involvement in inducing AR of equine spermatozoa. On the other end it has been demonstrated that only capacitated spermatozoa can experience AR [13] and the sudden increase in the percentage of capacitated cells in CB buffer could be the reason why in this buffer sAR is also significantly higher after 4.5 h of incubation.

A clear role couldn't be assigned to Bayk and Nif in modulating cellular events necessary to the spermatozoon to acquire the fertilizing ability. Most of the reactions are calcium dependent and it is well known that the maintenance of appropriate $[Ca^{2+}]_i$ is under the control of several calcium channels such as the plasma

membrane Ca^{2+}-ATPase, the Na^{+}/Ca^{2+} exchanger and the K^{+}-dependent Na^{+}/Ca^{2+} exchanger [14–16]. This can be the reason why Bayk and Nif, that are responsible of $[Ca^{2+}]_i$ modifications through L-type voltage operated calcium channels both in f and fz equine semen [8], require other participants to regulate the appropriate $[Ca^{2+}]_i$ necessary for the acquisition of the fertility competence.

Concerning sperm kinetic, sodium bicarbonate shows immediately an effect on MOT and PMOT, that increase at T0 in f and fz semen; the main consequence is the significant reduction ($P < 0.01$) of the number of frozen spermatozoa displaying progressive motility at T4. This difference between the effects exerted on f and fz semen could be related to the different rearrangement of the components of the sperm membrane in relation to the state (f *vs* fz) [17]. Moreover, 4.5 h of incubation in sodium bicarbonate containing medium induces kinetic variations characteristic of the hyperactivated semen such as increased ALH and decreased LIN values [18]. The only effect associated to the presence of Bayk in NCB buffer is a reduction of ALH and an increase of BCF (reverted by Nif) of fz semen. This let us to speculate on the possible different functional condition of L-type voltage-gated channels of the membrane of fz spermatozoa in respect to f semen that expose them to the interaction with their specific ligands.

5. Conclusions

In our knowledge, this study is the first to investigate on sAR of equine spermatozoa under in vitro culture conditions. We found that sAR is time dependent being sAR and the incubation time positively correlated. Moreover, we evidenced that sodium bicarbonate induces a significant increase of sAR both in f and fz semen after 4.5 h of incubation.

This work adds new information in the debate concerning the importance of extracellular calcium concentration in the regulation of capacitation [19]. We demonstrated that L-type voltage-gated calcium channels are not active participants in the cascade of events that modulate this process, but they contribute marginally to the modulation of sperm kinetic (acting on ALH and BCF) when the membrane is in an arranged state characteristic of fz semen. Moreover, we showed that the lack of sodium bicarbonate in the incubation medium increases the chances of survival of equine spermatozoa until 4.5 h reducing that observed effect of *"exhausted cells"* due to a fast ATP expenditure. This effect explains why in CB buffer the percentage of dead spermatozoa is higher as well as the percentage of progressive motile spermatozoa. Overall, the absence of sodium bicarbonate protects the cell to experience those modifications associated to capacitation and sAR that make the spermatozoon a cell with a rearranged membrane with reduced chances of surviving to temperature variations.

CRediT authorship contribution statement

Maria Albrizio: Conceptualization, Methodology, Writing – original draft, writing. **Giovanni Michele Lacalandra:** Draft improvement and critical revision. **Mario Cinone:** Formal analysis, Methodology, Writing – original draft, Writing-critical revision. All the authors approved the final version of the manuscript.

Declaration of competing interest

The authors declare no competing interests.

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