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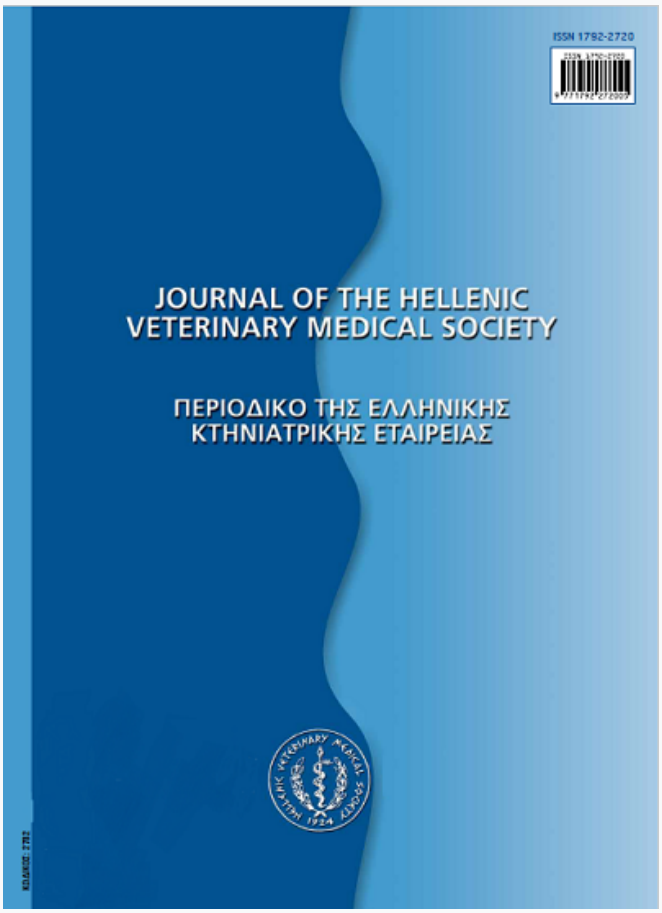

Μελέτη της in vitro επίδρασης χημικών ουσιών στην κινητικότητα και την ακεραιότητα της χρωματίνης των σπερματοζωαρίων του κάπρου

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Evaluation of common *in vitro* used chemicals' effect on motility and chromatin stability of boar spermatozoa

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Μελέτη της *in vitro* επίδρασης χημικών ουσιών στην κινητικότητα και την ακεραιότητα της χρωματίνης των σπερματοζωαρίων του κάπρου

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ABSTRACT. Chemicals such as heparin, calcium ionophore and dimethyl sulfoxide (DMSO) have been used in sperm's function evaluation assays, since they are facilitating the sperm capacitation and the induction of acrosome reaction. Many researchers modified porcine *in vitro* protocols purposing the improvement and validation of *in vitro* assays. The modification of the above mentioned chemicals' concentration could have a detrimental effect on sperm characteristics, relative to normal sperm function and fertilizing ability. The present study aimed to investigate *in vitro* effect of various concentrations of, a) heparin (2, 4, 6, 8 and 10 μg/ml), b) calcium ionophore A23187 (10, 20, 30 μM) and c) DMSO (1, 2, 3 % v/v), that have been used in sperm evaluation techniques, on chromatin instability and total motility of boar spermatozoa. Eight boars were used as semen donors, providing 20 ejaculates. Acridine orange test and subjective evaluation of semen by a microscope equipped with a heated plate were performed in order to evaluate chromatin integrity and motility, respectively. The results of this study showed that the addition of heparin, A23187 and DMSO at concentrations of 8 - 10 μg/ml, 10 μM and 1%, respectively, can be used for in vitro handling of boar spermatozoa without reduction of their motility and chromatin quality.

Keywords: boar sperm, chromatin, heparin, DMSO, calcium ionophore A23187

ΠΕΡΙΛΗΨΗ. Χημικές ουσίες όπως η ηπαρίνη, το ιοντοφόρο ασβέστιο (A23187) και το διμεθυλοσουλφοξείδιο (DMSO) χρησιμοποιούνται ευρέως κατά την εφαρμογή *in vitro* δοκιμών εκτίμησης της λειτουργικότητας των σπερματοζωαρίων, μεταξύ των οποίων η ενεργοποίηση και η πρόκληση της αντίδρασης του ακροσώματος. Το DMSO, πέραν της διαδεδομένης χρήσης

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του ως κρυοπροστατευτικό, χρησιμοποιείται επιπλέον ως διαλύτης πλήθους χημικών ενώσεων σε *vitro* πειραματισμούς. Σύνηθες φαινόμενο είναι η τροποποίηση των πρωτοκόλλων των διαγνωστικών δοκιμών μέσω της αλλαγής της συγκέντρωσης των προαναφερόμενων χημικών ουσιών. Οι μεταβολές αυτές θα μπορούσαν να επηρεάσουν χαρακτηριστικά του σπέρματος που σχετίζονται άμεσα με τη γονιμοποιητική του ικανότητα. Σκοπός της παρούσας εργασίας ήταν η διερεύνηση της *in vitro* επίδρασης διαφόρων συγκεντρώσεων α) ηπαρίνης (2, 4, 6, 8 και 10 μg/ml), β) ιοντοφόρου ασβεστίου A23187 (10, 20, 30 μM) και γ) DMSO (1, 2, 3 % ο/ο), στην κινητικότητα και την ακεραιότητα της χρωματίνης των σπερματοζωαρίων του κάπρου. Επιλέχτηκαν και χρησιμοποιήθηκαν 8 σπερματοδότες κάπροι και εξετάστηκαν 20 εκσπερματίσματα σε χρονικό διάστημα 5 μηνών (Οκτώβριο με Φεβρουάριο). Η εκτίμηση της κινητικότητας του σπέρματος έγινε με τη χρήση μικροσκοπίου με θερμαινόμενη επιφάνεια, ενώ η κατάσταση της χρωματίνης του πυρήνα των σπερματοζωαρίων μελετήθηκε με την εφαρμογή της δοκιμής πορτοκαλόχρουν της ακριδίνης. Τα αποτελέσματα της έρευνας έδειξαν ότι η προσθήκη ηπαρίνης, A23187 και DMSO σε συγκεντρώσεις 8 - 10 μg/ml, 10 μM και 1%, αντίστοιχα, είναι ασφαλής για την *in vitro* μεταχείριση του σπέρματος του κάπρου, δίχως κίνδυνο υποβάθμισης της κινητικότητας και της ποιότητας της χρωματίνης του πυρήνα των σπερματοζωαρίων.

Λέξεις ευρετηρίασης: σπέρμα κάπρου, ακεραιότητα χρωματίνης, ηπαρίνη, διμεθυλοσουλφοξείδιο, ιοντοφόρο ασβέστιο

INTRODUCTION

Capacitation and acrosome reaction of spermatozoa are essential stages of the mammalian fertilization process (Yanagimachi 1994). Heparin is used for *in vitro* capacitation of bovine (Parrish et al. 1988), equine (Varner et al. 1993), boar sperm (Dapino et al. 2006), as well as for assessment of the susceptibility of boar sperm chromatin to decondensation (Fraser and Strzezek 2007). Heparin's common *in vitro* use is attributed to its property to enhance capacitation by rising sperm intracellular pH, Ca^{++} and protein phosphorylation (Chamberland et al. 2001). Moreover, heparin acts like glycosaminoglycans (GAGs), which are components of the extracellular matrix, contributing to cell recognition, cellular adhesion and growth regulation (Dapino et al. 2006). In addition, heparin-binding proteins synthesized by accessory sex glands may play a role in fertilization by attaching to sperm surfaces, enabling heparin-like GAGs in the female reproductive tract to induce capacitation (Nass et al. 1990).

Dimethyl sulfoxide (DMSO) is commonly used as cryoprotectant. However, a lot of researchers used it as a vehicle of some *in vitro* used substances like A23187, as well as chemicals. Usually, in reproductive toxicological *in vitro* experiments, DMSO is added directly to raw diluted semen as chemical solvent aiming to mimic the *in vivo* effect of e.g. a toxin on sperm cells (Tsakmakidis et al. 2007). Previous studies have shown that DMSO affects canine sperm motility (Songsasen et al. 2002), as well as the acrosome reaction of sea urchin sperm (Mikami-Takei et al. 1987). An earlier study in pigs demonstrated that DMSO permeates the boar sperm plasma membrane slowly (Gilmore et al. 1998).

In vitro common protocols for boar sperm capacitation, induction of sperm acrosome reaction, zona binding assays and toxicological studies are using different concentrations of heparin, calcium ionophore and its major vehicle DMSO. As the purpose of these *in vitro* techniques is the investigation of the effect of various factors on spermatozoa, as well as the evaluation of sperm function, it is essential that sperm motility and chromatin stability are not affected. Of all sperm characteristics, motility is the most important one because it is significantly correlated with farrowing rate and total number of piglets born (Gadea et al. 2004, Tardif et al. 1999). In mice, Yildiz et al. (2008) estimated motility in order to correlate raw and frozen thawed sperm quality with sperm chromatin integrity and *in vitro* fertility rate. In addition, earlier toxicological studies evaluated sperm motility in order to investigate potential spermatotoxic effects (Pant and Srivastava 2003, Verma et al. 2006). Furthermore, sperm chromatin integrity is essential for the precise transmission of genetic information and the maintenance of good health in the next generations (Agarwal and Said 2003). Although DNA damaged spermatozoa can fertilize the oocyte and produce early-stage embryo, it fails to produce a successful term pregnancy (Ahmadi and Ng 1999). Especially for the boar, a high incidence of nuclear chromatin instability of semen is associated with reduced breeding efficiency of boars (Evenson et al. 1994).

In order to investigate the effect of additive substances on sperm function or to improve the *in vitro* assays' protocols, many *in vitro* studies modified the chemicals' concentrations of the medium, components

and solvents. However, the individual possible effect of the chemicals to male gametes could decrease semen quality leading in false estimation regarding the effect of the tested substances.

On the basis of the aforementioned considerations, the aim of the present study was to investigate the *in vitro* effects of different concentrations of heparin, calcium ionophore A23187 and DMSO that are used in sperm evaluation techniques, on chromatin instability (CI) and motility (M) of boar spermatozoa. Definition of the potentially negative effects of the tested chemicals could contribute to a technical improvement of *in vitro* assays in reproductive and toxicological studies, by avoiding the use of detrimental concentrations of basic medium components or solvents.

MATERIALS AND METHODS

Chemical reagents and media

All chemicals used in this study were purchased from Sigma-Aldrich, Greece (Heparin H-3149, Calcium ionophore C-7522, DMSO D-5879). Milli-Q purified water (Ultrapure Water Systems, Millipore Corporation, Prograd™ 2, Bedford, USA) was used for preparation of semen extender, media and solutions. A commercial extender was used for semen dilution (Androhep, Minitub®, Germany). A modified Sperm-TALP medium was used for capacitation of boar sperm, which consisted of NaCl (96 mM), KCl (3.1 mM), $NaHCO_3$ (15 mM), NaH2PO4 (0.3 mM), Hepes (20 mM), phenol red (0.01 mg $ml^{-}1$), 60% sodium lactate syrup (21.6 mM), $MgCl_2$ $6H_2O$ (0.4 mM), $CaCl_2$ H_2O (2 mM), Na-pyruvate (1 mM), bovine serum albumin (0.6 mg $ml^{-}1$) and gentamycin (0.05 mg $ml^{-}1$), pH 7.35–7.40, osmolarity 300 mOsm $kg^{-}1$ (Green and Watson 2001). Sperm nuclear chromatin integrity was evaluated by acridine orange dye (A6014) (Sigma-Aldrich, Greece).

Semen collection and initial evaluation

Eight adult crossbreed boars of proven fertility (>85% farrowing rate, >11 litter size) were selected and used in this study. The boars were housed in individual pens of a pig farm, they were fed according to standards of Nutrients Requirements of Swine (1998), they were provided water ad libitum and were routinely used for artificial insemination. The sperm-rich fraction of ejaculates was collected by the 'gloved hand' technique and was assessed in the farm sperm laboratory. Ejaculates of > $0.1x10^9$ sperm/ml and >75% viability were extended to $30x10^6$ sperm/ml with Androhep extender (Minitub®, Germany). After dilution, the semen was transported to the laboratory in an isothermal box, at 16-17°C, within an hour (Johnson et al. 2000).

Semen parameters evaluation

Semen motility (M) was determined as the percentage of spermatozoa with forward motion. M was evaluated in a light microscope equipped with a heated plate regulated at 37°C. Six microlitres of semen sample were applied on a prewarmed slide and covered with a pre-warmed coverslip (22 x 22 mm). Ten different optical areas per sample were evaluated by two individual experienced examiners. Sperm CI was evaluated by acridine orange test (Tejada et al. 1984), which measures the susceptibility of sperm nuclear DNA to acid-induced denaturation *in situ* by quantifying the metachromatic shift of acridine orange fluorescence from green (native DNA) to red (denatured DNA). Acridine orange stained normal double-stranded DNA green and denatured single-stranded DNA red. Slides were examined under a fluorescence microscope (Olympus BX 41, Japan) equipped with a digital camera and image analyzer computer software (U-TV 0.35. C-2, Imaging Software System GmbH, Olympus, Japan). Two hundred spermatozoa per slide were estimated in ten different optical areas for determination of percentage of spermatozoa with denatured DNA.

Experimental design

The experiments of the present study were completed during a 5-month period (October to February), whereas one replication per week was performed using 8 boars (20 replications in total). Each one of the four boars provided three ejaculates (12 ejaculates), while each one of the remaining four boars provided two ejaculates (8 ejaculates).

Four experiments were conducted in total and their protocols are explained in detail in the following paragraphs. Furthermore, in experiments 1 and 2, incubation time of 4 hours was selected because sperm *in vitro* evaluation assays (e.g. sperm capacitation and zona binding tests) use this extended time of incubation (Tsakmakidis et al. 2007, Maravilla-Galván et al. 2009). However, a shorter incubation time of 30

min was used in experiment 3, since the most usual incubation time of *in vitro* capacitation and acrosome reaction protocols in pig studies is 15 – 60 min (Burkin and Miller 2000, Holt et al. 1997).

Experiment 1 - Effect of heparin

A stock solution of heparin in Sperm-TALP medium was prepared at the day of the experiment. Each boar's semen sample was centrifuged at 400g x 10 min and the supernatant was removed. Then, each sample was reconstituted with Sperm-TALP medium in a final concentration of $30x10^6$ spermatozoa/ml. A volume of heparin solution was added in each boar's semen sample in such quantities, so that the final concentration of heparin would be equal to 2, 4, 6, 8 and 10 μg/ml of semen. The five aliquots of different concentrations of heparin and one more without heparin (control) were incubated for 4 hours under *in vitro* conditions (38.5°C, 5% CO_2 in >95% humidified air). At hours 0 and 4, a volume of 20μl from each aliquot was taken for M and CI evaluation.

Experiment 2 - Effect of DMSO

DMSO was added directly in each sperm sample in such quantities, so that its final concentration in diluted semen would be 1, 2, 3 % v/v in the final volume. The three aliquots of different concentrations of DMSO and one more without DMSO (control) were incubated for 4 hours under *in vitro* conditions (38.5°C, 5% CO_2 in >95% humidified air). At hours 0 and 4, a volume of 20μl from each aliquot was taken for M and CI evaluation.

Experiment 3 - Effect of calcium ionophore

A stock solution of calcium ionophore in dimethyl sulfoxide (5Mm) was prepared and stored at -20°C until use. Each boar's semen sample was centrifuged at 400g x 10 min and the supernatant was removed. Then, each sample was reconstituted with Sperm-TALP medium in a final concentration of $30x10^6$ spermatozoa/ml. The proper volume of calcium ionophore was taken from the stock solution and was added to each sample in such quantities, so that the final concentration of A23187 would be equal to 10, 20, 30 μM. The three aliquots of different concentrations of calcium ionophore and one more without A23187, but with the appropriate amount of DMSO as solvent of stock solution (control), were incubated for 30 min under *in vitro* conditions (38.5°C, 5% CO_2 in >95% humidified air). At time 0 and 30 min, a volume of 20μl from each aliquot was taken for CI evaluation.

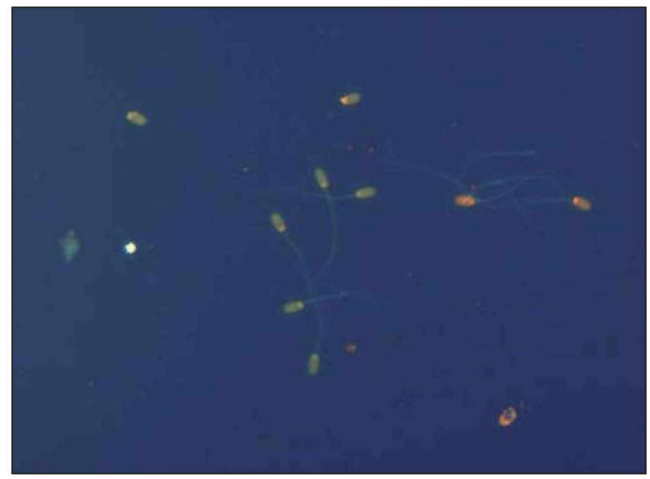

Figure 1. Acridine orange (AO) staining. Sperm heads having compact chromatin structure fluoresce green. Sperm heads having decompacted chromatin fluoresce a spectrum of colors ranging from orange, red to partially or entirely red.

Statistical analysis

Data were analysed according to the statistical procedures described by Petrie and Watson (2006). Furthermore, the SPSS statistical package (SPSS@ 11.01.1Statistical Software Inc., Chicago, IL, USA, 2001) was used for the analysis of variance.

One-way ANOVA models were used to test the effect of semen treatment on sperm traits. If ANOVA showed significant differences among means (main effects), a planned multiple comparison of means was examined by Duncan's multiple range test. Differences were considered significant at $p \leq 0.05$.

Results

Experiment 1 - Effect of heparin

Data analysis showed that CI of boar sperm was not affected significantly by all heparin concentrations tested (table 1). On the other hand, M was increased significantly in samples with heparin at concentrations ranging between 2 and 8 μg/ml compared to the control one ($p<0.05$).

Experiment 2 - Effect of DMSO

DMSO concentration at 1% of semen volume did not affect significantly both evaluated parameters (table 2). A significant increase of CI was notable in medium and high concentration of DMSO treatment groups compared with low DMSO dose and control

Table 1. Effect of heparin (HR) on chromatin instability (CI) and semen motility (M) of boar spermatozoa (Means ± SEM, n=20, experiment 1).

Sperm	HR concentrations (μg/ml, w/v)					
	0	2	4	6	8	10
CI (%)	0.9±0.4[a]	0.7±0.2[a]	0.5±0.1[a]	0.6±0.2[a]	1.1±0.4[a]	0.9±0.2[a]
M (%)	38±2.1[a]	45.75±2.6[b]	49.8±2.7[b]	45.3±2.4[b]	43.9±3.1[ab]	37.8±2.3[a]

Letter n denotes the number of tested ejaculates
Dissimilar letters within rows denote a significant difference ($P < 0.05$).

Table 2. Effect of dimethyl sulfoxide (DMSO) on chromatin instability (CI) and semen motility (M) of boar spermatozoa (Means ± SEM, n=20, experiment 2).

Sperm	DMSO concentration (%, v/v)			
	0	1	2	3
CI (%)*	0.8±0.3[a]	1.1±0.3[a]	2.4±0.2[b]	5.5±1.2[c]
M (%)**	48.4±2.4[a]	46.7±2.3[a]	44.4±2.9[ab]	39.2±2.8[b]

Letter n denotes the number of tested ejaculates
Dissimilar letters within rows denote a significant difference ($P < 0.001$*, < 0.05**).

Table 3. Influence of calcium ionophore (Ca-I) A23187 on chromatin instability (CI) of boar spermatozoa (Means ± SEM, n=20, experiment 3).

Sperm	Ca-I concentrations (μM)			
	0	10	20	30
CI (%)	0.7±0.4[a]	1.5±0.8[ab]	1.6±0.8[ab]	3.7±1.1[b]

Letter n denotes the number of tested ejaculates
Dissimilar letters within rows denote a significant difference ($P < 0.025$).

group ($p<0.05$). Under the influence of 3% DMSO, a significant reduction of M was observed compared to low DMSO and control groups ($p<0.001$).

Experiment 3- Effect of calcium ionophore

As far as the CI is concerned, the statistical analysis of the data showed a significant increase, at the high concentration group compared to control (table 3) ($p<0.025$).

Discussion

Numerous studies have been conducted on culture conditions for the *in vitro* evaluation of sperm function, investigation of the effect of chemicals, toxins or additive substances on male gametes. Usually, during the aforementioned assays, the possible negative effect of the modified concentrations of the medium's components are not taken into account. On the other hand, some chemicals require high dilution rate with the appropriate solvent. The effect of the solvent on routine semen characteristics is usually evaluated. However, its effect on sperm parameters that are not involved in the experimental target of the study (e.g. CI) could be detrimental for spermatozoa. In our effort to contribute to this issue, our study evaluated the changes of two high valuable sperm parameters (M and CI) induced by chemicals, on the efficiency of *in vitro* assays.

Data analysis showed that heparin did not affect CI. However, earlier studies demonstrated that heparin induces decondensation of mammalian sperm nucleus, while different species respond to heparin action differently, e.g. mouse and rat sperm are more stable under heparin treatment than human sperm (Carranco et al. 1983, Jager et al. 1990). According to our experimental conditions, boar sperm was stable to

heparin's decondensation action. These results are in agreement with those of a previous study, which reported that heparin can induce nuclear decondensation of human ejaculated spermatozoa, but not of ejaculated boar spermatozoa (Delgado et al. 1980). Moreover, no effect was observed for M at heparin concentrations of 0, 8 and 10 μg/ml, whilst a significant increase was noticed at concentrations of 2-6 μg/ml. Thus, someone could expect a decreased motility as a physiological response-hyperactivation, with this result being attributed to changes in the total number of motile spermatozoa under *in vitro* conditions of incubation. In addition, taking into account that motility at the control sample was lower than the ones of the 2nd and 3rd experiment after heparin's effect, it is almost similar to the controls of the other experiments. Rodriguez et al. (2011) did not, also, find changes in bovine semen progressive motility after heparin induced capacitation. In agreement with our results, Harshan et al. (2006) reported a significant increase of buffalo bull epididymal semen motility after incubation with heparin (20 μg/ml, 20 min, 37°C).

Moreover, the addition of 1% DMSO had no effect, while the addition of 2% and 3% DMSO increased significantly CI. Semen M was reduced at DMSO concentration of 3%. There is not available data concerning the effect of DMSO on boar spermatozoa chromatin status. An earlier investigation in a different species has shown that the exposure of canine sperm to DMSO (0.6 M) resulted in significant reduction of motility, but it did not affect membrane integrity (Songsasen et al. 2002). Moreover, previous studies reported that DMSO in a Tris-based extender had a deleterious effect on the post-thaw motility and plasma membrane integrity of buffalo sperm (Rasul et al. 2007). Although DMSO has been widely used at *in vitro* experiments as vehicle of numerous chemical substances, no data concerning the possible *in vitro* effect of DMSO on boar sperm motility and chromatin instability are available to our knowledge. Therefore, the present study determines for the first time the toxic effect of DMSO at high concentrations and indicates that its addition, as a solvent but not as a cryoprotectant, in boar sperm for *in vitro* applications should be in concentrations lower than 2% (v/v), since higher concentrations could damage boar sperm. Our motility results are in agreement with the study of Lee et al. (2011). In this study, DMSO was used as cryoprotectant for boar semen freezing and a significant decrease of motility was noticed at DMSO concentrations higher that 5%.

The addition of A23187, at 30 μM concentration, significantly increased CI. Spermatozoa's response to acrosome reaction by A23187 has been used to sperm quality prediction tests. Thereby, Petrunkika et al. (2005), using 1μM of calcium ionophore, found that the assessment of the acrosome reaction in response to ionophore and membrane integrity is a potentially useful parameter for the evaluation of sperm functional state and subfertility in boars, while, Holt et al. (1997) demonstrated that the higher rate of acrosome reaction induced by A23187 is correlated with a smaller litter size. The results of our study support the findings of the above aforementioned studies, since the detrimental effect of A23187 on boar sperm CI was noticed at the high concentration (30 μM), while at the lower tested concentrations it was not toxic for the *in vitro* treatment of boar sperm.

In conclusion, this study demonstrated that the addition of heparin, A23187 and DMSO at concentrations of 8 - 10 μg/ml, 10 μM and 1%, respectively, can be used for *in vitro* handling of boar spermatozoa without any reduction of their motility and chromatin quality. Moreover, the rest of the concentrations of the above chemicals tested here could be responsible for *in vitro* assays' deficiency due to their detrimental effect on spermatozoa's characteristics, relative to their normal function and fertilizing ability.

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