International Journal of Psychosocial Rehabilitation, Vol. 25, Issue 02, 2021
ISSN: 1475-7192

Effect of CNP additive to diluents during capacitation on live percentage and pH of bull epididymal spermatozoa.

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Abstract:

Abattoir male gonads specimens were collected from Al-Shoáalla abattoir from June 2019 until November 2019 in an average of 2 visits per week. This study was designed to evaluate the optimum effect of C-type natriuretic peptide on the caudal epididymal spermatozoa characteristics from a healthy slaughter bull. Dissected and separated the epididymis from the entire testicle made by a small scissor. The caudae were injected with 5-8 ml of the TCM199 media containing 100 IU/ml penicillin-streptomycin and 100 IU/ml Nystatin using an 18 gauge needle attached toa 5-ml syringe then aspirated. After collection of Spermatozoa samples of cauda epididymis were evaluated under the light microscope has individual motility less than 70% of were rejected. The caudal epididymal Spermatozoa samples transported from petri dish to a 10 ml test tube containing 2 ml maturation media (TCM199) that contain 100 IU/ml penicillin-streptomycin and 100 IU/ml Nystatin; the test tube incubated in a 5% CO_2 incubator at 39 C° for 4 hours for sperm maturation, the presence of distal protoplasmic droplet was the criteria of sperm maturation. Then the samples divided into three equal groups control one, low concentration 10^{-7} mol/L, and high concentration 10^{-6} mol/L. It evaluated the effect of C-type natriuretic peptide (CNP) during capacitation on the live percentage and pH of bull epididymal spermatozoa. The live percentage and pH of sperm were analyzed at 5 min, 10 min, 15 min, 30 min, 45 min, 60 min, 120 min, and 180 min for each group. A total of 20 caudal epididymides were studied during the period of the experiment. Evaluation of spermatozoa parameters as a stained smear for dead and alive spermatozoa was conducted in addition to pH; all results were recorded.

The pH of the semen was measured immediately after the recovery of sperms from the epididymal by using a pH litmus paper. A drop of sperms suspension on a glass slide, a drop of Eosin-Negrosin stain was added and mixed. Two smears of each sample were made when each smear dry it was mounted immediately under coverslip using DPX; 200-400 sperms were examined in each smear on a light heated stage microscope at 40x objective power. The final percentage was calculated by taking the average of two smears.

The results of herein study of live percentage in bull epidydimal sperm during the capacitation of control group indicate that the significant changes at the level of (P<0.05) in the control group started within 30 minutes after capacitation while the significant changes starting at level (P<0.05) in low (10^{-7}) mol/L and high (10^{-6}) mol/L concentration with 45 minutes after capacitation to the end of the experiment as well as there were no significant differences between the three studied groups. In contrast, the results of a novel study of pH in bull epidydimal sperm during the capacitation of the control group indicate that the significant changes of pH at the level of (P<0.05) begins within 10 minutes after capacitation while the significant changes starting at level (P<0.05) in low and high concentration with 45 minutes after capacitation to the end of the experiment. At the same time, there were significant differences (P<0.05) between low and high concentration with the control group recorded during the period 45 minutes and lasted until the end of the experiment. The conclusion that there were no changes between control and treated groups in live percentage, and also there was a positive improvement in treated groups in live and pH after capacitation with CNP additive to epididymis sperms.

Keyword: *Live percentage, pH, CNP, bull epidydims sperm.*

Introduction:

Chang (1951) and Austin (1951) in rabbits and rats, respectively, were the first to explain the phenomenon of mammalian spermatozoa needing to wait a period in the female reproductive tract to acquire their fertilizing ability, while Austin (1952) coined the word "capacitation." This is now known as in vivo capacitation. Capacitation of spermatozoa can be accomplished in vitro by using specific media containing the appropriate compounds and pH (Chang, 1951). Several sequential and parallel processes are needed to make the necessary changes. They start as soon as the sperm is expelled, but they last much longer in the female tract or the capacitation medium. Early studies focused solely on in vivo capacitation, but more recent

studies have discovered a wide range of numerous species' capacitation conditions. Imai *et al.* (1977) reported that boar spermatozoa could penetrate zona-free hamster eggs after preincubation in the pig reproductive tract for several hours. This suggested that the inducers found in oviduct fluid are likely to be identical and wide spread across animals.

Fertilization requires a state of readiness known as capacitation. Only capacitated sperm can pass through the acrosome reaction and fertilize the ovum. Capacitation occurs at the sperm head, along with other acrosome reaction preparation elements, while hyperactivation preparation occurs at the sperm tail. Capacitation has recently been divided into fast and slow cases (Salicioni *et al.*, 2007). The triggering of the flagella's vigorous and asymmetric movement is one of the first and earliest occurrences. As soon as the sperm leave the epididymis, these things happen. Changes in sperm movement patterns and their acquisition of the ability to carry out the acrosome reaction by stimulation of a physiological agonist and phosphorylation of tyrosine are examples of slow and late events (Salicioni *et al.*, 2007; Visconti, 2009). Despite this differentiation between quick and slow, early and late, both processes tend to be regulated by broadly similar molecules, e.g., soluble adenylate cyclase (sAC), cyclic adenosine monophosphate (cAMP) (Ren and Xia, 2010).

The process of capacitation is complicatedly controlled by a variety of factors. Albumin removes cholesterol from the sperm plasma membrane first during capacitation. The permeability of the membrane is improved due to this (Langlais and Roberts, 1985; Fujinoki, 2011). Then, through the stimulation of HCO^{3-} (Ho and Suarez, 2001; Fujinoki *et al.*, 2006) and membrane channels (Ren and Xia, 2010), a Ca^{2+} influx occurs, which triggers secondary messenger systems, specifically, a form of sAC that acts as an intracellular signal. (Visconti and Kopf, 1998; Visconti *et al.*, 1999; Fujinoki *et al.*, 2006; McPartlin *et al.*, 2011). Specific protein kinases and phosphates were also activated by sAC (Stoker, 2005; Suzuki *et al.*, 2010). Saleh and Abdul-Ameer (2019) study the effect of heparin on the capacitation of epididymis sperm of ram.

Materials and Methods:

Abattoir male gonads specimens were collected from Al-Shoáalla abattoir west of Baghdad from June 2019 until Novembers 2019 in an average of 2 visits per week. Testes and epididymis of adult bull (60) were obtained from mature local breed bull directly after slaughtering; age is determined. The specimens of male genitalia were transported by the cool box from the abattoir to the Laboratory of Theriogenology / College of Veterinary Medicine /

University of Baghdad. This study was designed to evaluate the optimum effect of C-type natriuretic peptide on the caudal epididymal spermatozoa characteristics from a healthy slaughter bull. Testicles samples with attached epididymis of the adult bull were collected directly at Al-Shoáalla abattoir after slaughtering, kept in a cool box under 4-8 Cº, and transported from the place of collection to the laboratory of Theriogenology in the College of Veterinary Medicine / Baghdad University. Testicle samples in the lab were washed with distils water first, then with normal saline supplement with 100 IU/ ml penicillin and 0.1 mg/ml streptomycin. Dissected and separated the epididymis from the entire testicle by a small scissor. The caudae were injected with 5-8 ml of the TCM199 media containing 100 IU/ml penicillin-streptomycin and 100 IU/ml Nystatin using 18 gauge needle attached to a 5 ml syringe then aspirated. After collection of Spermatozoa samples of cauda epididymis were evaluated under the light microscope has individual motility less than 70% of were rejected. The caudal epididymal spermatozoa samples transported from petri dish to a 10 ml test tube containing 2 ml maturation media (TCM199) that contain 100 IU/ml penicillin-streptomycin and 100 IU/ml Nystatin; the test tube incubated in a 5% CO_2 incubator at 39 Cº for 4 hours for sperm maturation, the presence of distal protoplasmic droplet was the criteria of sperm maturation. Then the samples divided into three equal groups control one, low concentration 10^{-7} mol/L and high concentration 10^{-6} mol/L. It evaluated the effect of C-type natriuretic peptide (CNP) during capacitation on live percentage and pH of bullepididymal spermatozoa. The live percentage and pH of sperm were analyzed at 5 min, 10 min, 15 min, 30 min, 45 min, 60 min, 120 min, and 180 min for each group. A total of 20 caudal epididymides were studied during the period of the experiment. Evaluation of spermatozoa parameters as motility and abnormalities, stained smear for dead and alive spermatozoa were conducted in addition to pH; all results were recorded.

The pH of the semen was measured immediately after the recovery of sperms from epididymal by using a pH litmus paper. This was done according to Carr *et al.* (1985). Drop of sperms suspension on a glass slide, a drop of Eosin-Negrosin stain was added and mixed. Two smears of each sample were made when each smear dry it was mounted immediately under coverslip using DPX; 200-400 sperms were examined in each smears on light heated stage microscope at 40x objective power. The final percentage was calculated by taking the average of two smears. The sperms live percentage was calculated as the following equation: Sperms live percentage% = Number of live sperms / Total number of sperms ×100.

All data obtained were subjected to the Analysis of Variance test (ANOVA). The means ± standard errors of the means were calculated for parameters using (SPSS, 2001), and the significant difference between means was separated using Least Significant Differences test (LSD).

Results:

The observation of live percentage inbull epidydimal sperm (Figure 1) during the capacitation of control group indicate that there was a significant differences at level ($P<0.05$) between 0 time of control group with 30 minutes after capacitation and lasted to the end of experiment (Figure 1). There was a significant differences at level ($P<0.05$) between 5 minutes with 45 minutes after capacitation of control group and persist to the end of experiment (Figure 1). There were a significant differences at level ($P<0.05$) between 10 and 15 minutes and 60 minutes and hereafter after capacitation of control group (Figure 1). There were a significant differences at level ($P<0.05$) between 30 and 45 minutes and 120 and 180 minutes after capacitation of control group (Figure 1). There was a significant differences at level ($P<0.05$) between 60 minutes with 180 minutes after capacitation of control group in the experiment (Figure 1).

The effect of CNP additive to diluents during capacitation on live percentage in low concentration group of bull epidydimal sperm indicated that there were a significant differences at level ($P<0.05$) between 0 and 5 minutes with 45 minutes after capacitation of low concentration group and lasting to the end of experiment (Figure 1). There were a significant differences at level ($P<0.05$) between 10 and 15 minutes with 60 minutes after capacitation in low concentration group and lasted to the end of experiment (Figure 1). There were a significant differences at level ($P<0.05$) between 30 and 45 minutes and 120 and 180 minutes after capacitation in low concentration group (Table 4-22 and Appendix 14). There was a significant differences at level ($P<0.05$) between 60 minutes with 180 minutes after capacitation of low concentration group in the experiment (Figure 1).

The recent result of effect of CNP on live percentage during capacitation in high concentration group of bull epidydimal sperm indicated that there were a significant differences at level ($P<0.05$) between 0 and 5 minutes of high concentration group with 45 minutes after capacitation and lasted to the end of experiment (Figure 1). There were a significant differences at level ($P<0.05$) between 10 and 15 minutes with 60 minutes after capacitation in high concentration group and continue to the end of experiment (Figure 1).

There were a significant differences at level (P<0.05) between 30 and 45 minutes of high concentration group with 120 and 180 minutes after capacitation (Figure 1). There was a significant differences at level (P<0.05) between 60 time of high concentration group with 180 minutes after capacitation in the experiment (Figure 1).

The outcome of this experiment indicated that there were no significant differences between control and treated groups after capacitation on live percentage of bull epidydimal sperm (Figure 1).

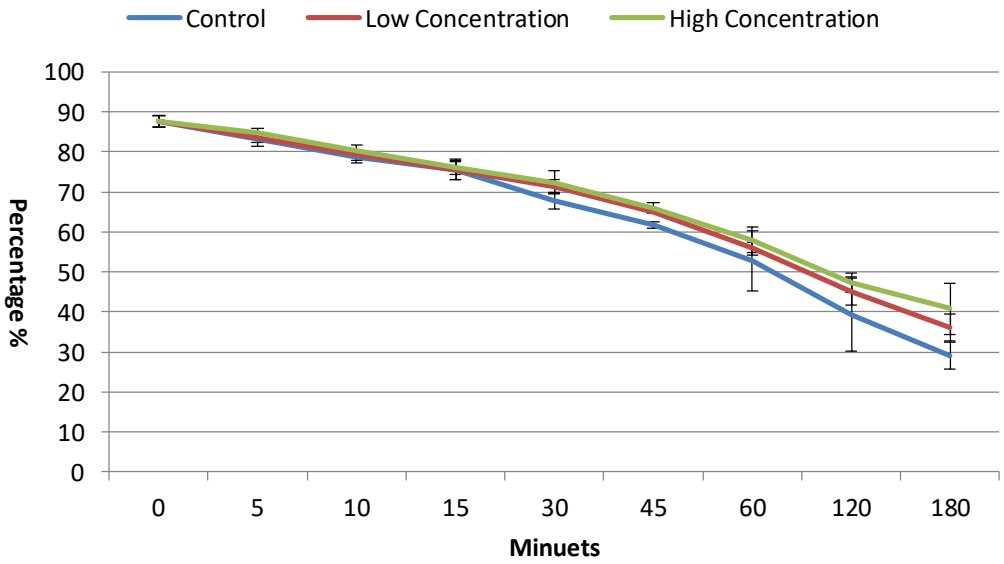

Figure 1: Effect of CNP additive to diluents after capacitation on live percentage (%) of bull epidydimal sperm.

The alteration of pH aftercapacitation the diluted bull epidydimal sperm of control group indicated that there was a significant differences at level (P<0.05) between 0 time of control group with 10 minutes after capacitation and lasted to the end of experiment (Figure 2). There was a significant differences at level (P<0.05) between 5 minutes and 30 minutes after capacitation of control group and lasting to the end of experiment (Figure 2). There was a significant differences at level (P<0.05) between 10 minutes and 45 minutes and hereafter after capacitation of control group (Figure 2). There were a significant differences at level (P<0.05) between 15 and 30 minutes and 60 minutes after capacitation of control group and hereafter to the end of experiment (Figure 2). There was a significant differences at level (P<0.05) between 45 minutes and 120 and 180 minutes after capacitation of control group (Figure 2). There was a significant differences at level (P<0.05) between 60 minutes and 180 minutes after capacitation of control group in the experiment (Figure 2).

The changes of pH alteration after additive of CNP to capacitation media of both low and high concentration group indicated that there was a significant differences at level (P<0.05) between 0 time of low and high concentration group with 45 minutes after capacitation and lasted to the end of experiment (Figure 2). There were a significant differences at level (P<0.05) between 5 and 10 minutes and 60 minutes after capacitation in low and high concentration group and hereafter to the end of experiment (Figure 2). There was a significant differences at level (P<0.05) between 15 minutes with 120 and 180 minutes after capacitation in low and high concentration group (Figure 2). There was a significant differences at level (P<0.05) between 30 minutes and 180 minutes after capacitation of low concentration group in the experiment (Figure 2).

The observation of novel study that there was a significant differences at level (P<0.05) between control and treated groups started from 45 minutes and persist to the end of experiment (Figure 2).

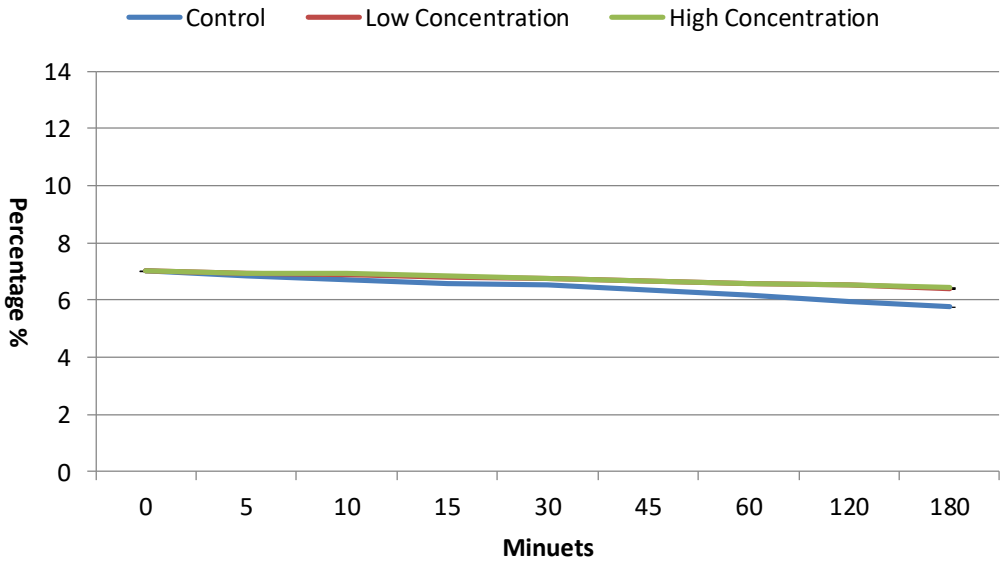

Figure 2: Effect of CNP additive to diluents after capacitation on pH (0-14) of bull epididymal sperm.

Discussion:

Our recent study had an agreement with the result of Patel *et al.*, (2016).

The CNP additive to the capacitation media had no negative effect on live percentage of sperms in two sources of samples. But there is a relative increaser of live percentage in two treatment groups.

A study done by Cheema *et al.* (2015) on natural localization of HBPs incross-bred bull spermatozoa using anti-AZU-1 revealed that these proteins are mainly localized on acrosomal cap.

The changes occurringin spermatozoa during in vivo capacitation beginas soon as they are ejaculated as a result of, among otherreasons, the bicarbonate (HCO_3-) intake, subsequent activation of cAMP synthesis, intracellular pH alkalinisation (pHi), increase in intracellular Ca^{2+} concentration and motility activation (fast capacitation events) (Visconti, 2009). Once inside the female genital tract, other events occurin a sequential and parallel way for an extended period, such as hyperactivation, tyrosine phosphorylationm (Tyr-P) and preparation to undergo acrosome reaction (AR) (slow capacitation events). Both fast and slow events depend on the HCO_3- and Ca^{2+} concentrations, but the slow ones also depend on the presence of cholesterol acceptors like albumin. After maturing as they pass through the epididymis, the spermatozoa are stored in the epididymal cauda in low HCO_3- conditions (~ 4 mmol/L) (Soriano-Úbeda *et al.*, 2019). During ejaculation,the spermatozoa come into contact with the seminal plasma, which contains higher HCO_3- concentrations (~ 20mmol/L) but also decapacitating factors, mainly sperm adhesions from accessory sex glands, which coat and stabilise the sperm surface to prevent premature capacitation. Spermatozoa maturation in the epididymis comprises sequential modifications that have been demonstrated tobe essential for the acquisition of motility and fertility (Soriano-Úbeda *et al.*, 2019).

All biophysiological events in cells involving enzymes, hormones, transmitters, and growth factors are dependent on pH. Any alteration in pH leads to either inhibition of function or deviation of cell function. Biological macromolecules have evolved to perform their function in specific cellular environment, and their dependency on pH for activity and stability reflects the significance of pH (Casey *et al.*, 2010).

Spermcells encounter pH of 7.2-7.4, which become 6.5 in caput epididymis and 6.7-6.8 in cauda epididymis (Bonnie Ng *et al.*, 2018 and Shum *et al.*, 2011). During ejaculation, pH of semen becomes 7.2-7.4, and further after ejaculation into vagina, pH becomes 4.5-7.5, and in cervix, pH increases to 6.5- 7.5 and ultimately in uterus and fallopian tube (7-7.8) (Bonnie Ng *et al.*, 2018 and Shum *et al.*, 2011).

Sperm is the only cell, whose activity is outside the male body, and in the inconsistent chemical milieu of sperm, seminal plasma may have profound effects on sperm quality and pH is one of the most critical factors which determine the semen quality. Spermatozoa are

highly affected by pH. Functions such as motility, viability, capacitation, and acrosome reaction are pH dependent (Zhou *et al.*, 2015).

Mammals exhibit most complex mechanisms to regulate pH in spermatozoa. Past studies have demonstrated that mammalian spermatozoa display complex mechanisms to regulate intracellular pH. The presence of Hv1 channel (proton-gated channel) in mammalian spermatozoa has revealed the importance of H^+ ion in regulating sperm functional parameters (Lishko *et al.*, 2010).

The cytoplasmic pH of sperm could directly be affected by external pH of sperm with complex regulatory mechanism. Sperm intracellular pH exhibits alinear relationship with extracellular pH and studies have also shown that intracellular pH regulates ionic regulation along the sperm membrane (Mishra *et al.*, 2018).

With the reduction in pH from 6.5 to 6, the sperm motility was decreased linearly indicating the potential role of pH in the regulation of sperm motility. Controlled sperm studies keeping viscosity and temperature of follicular fluid constant with a variable pH resulted in alterations in sperm motility and thereby indicated the potential role of pH in regulating sperm motility (Rizvi*et al.*, 2009). pH regulation is also precise, and there may be the existence of other systems likeion channel regulatory systems to regulate pH in spermatozoa (Lishko *et al.*, 2010; Correia *et al.*, 2015 and Lishko and Kirichok, 2010).

pH plays a significant role in regulating sperm motility and fertility competence. That is why spermatozoa have developed dynamic pH regulatory systems by which there is a regulation of intracellular pH. Intracellular pH is affected by extracellular pH and thereby opens many windows of investigation regarding the role of pH in functional significance of spermatozoa (Mishra *et al.*, 2018).

The CNP additive to capacitation media in medium source of semen showed that there were no changes in pH media with time passing. While though in good source of sperms from epidydmis it showed positive effect on keeping pH media during capacitation.

Capacitation includes all post-ejaculation biochemical and physiological changes that enable mammalian spermatozoa to fertilize (Visconti *et al.*, 2011). During capacitation, spermatozoa undergo major physiological changes, such as sperm hyperactivation and the acrosome reaction. Hyperactivation creates the propulsive force for swimming in the viscous oviductal fluid and for penetrating the zona pellucida (Wu *et al.*, 2019). In contrast, the acrosome reaction exposes the membrane components, which is required for the penetration of the zona pellucida and for binding to the egg (Buffone *et al.*, 2012). Zhang

et al. (2005) reported that ANP could induce the acrosome reaction of preincubated giant panda spermatozoa via a PKG pathway. In the present study, CNP was able to not only promote the acrosome reaction but also facilitated sperm hyperactivation, suggesting that it could induce sperm capacitation in vitro.

There is, however, considerable evidence thatintracellular pH (pHi) regulates sperm motility and preservesviability during storage in in vertebrate species, the motility of epididymal rat sperm invarious media depended on the sodium ion concentration and that the Na^+/H^+ - exchange inhibitor, amiloride, would inhibit initiation of motility. Conclusion of a shift in pHi via a Na^+/H^+ - exchange mechanism leads to the initiation of mammalian sperm motility. Mammalian sperm motility might be regulated via changesin pHi because the motility of demembranated, adenosinetriphosphate (ATP)-reactivated bull sperm increasedas the pH of the medium was raised from 6.6 to 7.1. High concentrations of a permanent weak acid (lactic acid) combined with the relatively low pH found within thecauda epididymis may be responsible for depressing pHi and producing the quiescence of sperm within the cauda epididymis (Jones and Bavister, 2000).

In the absence of HCO_{3-} and the presence of other capacitating agents like calcium and albumin, the pHi remained at 6.7–6.8. In the presence of HCO_{3-}, the pHi increased 0.3–0.4 units, confirming that the increase depends exclusively on HCO_{3-}. This increase of around0.3–0.4 units in pHi may seem small, but, it has been demonstrated that an increase of 0.2 units of pH may modify enzymatic activity more than 20-fold (Soriano-Úbeda *et al.*, 2019). It is known that the activity of intra- and extra- cellular enzymes taking part in the cellular metabolism is pH- sensitive.

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