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BIOPHYSICAL AND BIOCHEMICAL CHANGES OF THE WASHED BUFFALO SPERMATOZOA ADDED WITH CAFFEINE

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ABSTRACT

Twelve sexually mature buffalo bulls, were used in the present study. The experimental work was carried out to study the effects of removal of seminal plasma by centrifugation and addition of different concentrations of caffeine (0,2,4 and 8mM/100ml) to the extended cooled buffalo semen with lactose-yolk citrate (LYC) extender on biophysical (percentage of sperm motility, dead spermatozoa, sperm abnormalities and acrosomal damage) and biochemical (aspartate-aminotransferase: AST, alanine-aminotransferase: ALT and alkaline phosphatase: ALP) changes, during chilled storage at 5^0C for up to 6 days and incubation at 37^0C for up to 2 hours. The conception rates of buffalo-cows artificially inseminated with fresh semen and washed cooled semen supplemented without or with 2mM caffeine, were also assessed.

The results showed that, removal of seminal plasma by washing of buffalo semen showed significantly ($P<0.05$) higher the percentage of sperm motility and significantly ($P<0.05$) lower the percentages of dead spermatozoa, sperm abnormalities and acrosomal damage of spermatozoa than the non-washed semen. Supplementation of 2 or 4mM caffeine to the extended cooled semen either washed or non-washed increased significantly ($P<0.05$) the percentage of sperm motility, while decreased significantly ($P<0.05$) the percentages of dead spermatozoa, sperm abnormalities and acrosomal damage of spermatozoa as compared to 8mM caffeine or free-caffeine medium. The storage at 5^0C for up to 6 days or incubation at 37^0C for up to2 hours of the extended cooled buffalo semen either washed or non-

washed decreased significantly (P<0.05) the percentage of sperm motility, while it increased significantly (P<0.05) the percentages of dead spermatozoa, sperm abnormalities and acrosomal damage of spermatozoa. Removal of seminal plasma by washing of buffalo semen the extended decreased significantly (P<0.05) the leakage of AST, ALT and ALP enzymes into the extracellular medium as compared to the non-washed semen. Supplementation of 2, 4, or 8mM caffeine to the extended cooled either washed or non-washed buffalo semen decreased significantly (P<0.05) the amounts of AST, ALT and ALP enzymes released into the extracellular medium as compared to free-caffeine medium. The storage at 5^0C for up to 2 days or incubation at 37^0C for up to 2 hours of the extended cooled buffalo semen either washed or non-washed increased significantly (P<0.05) the amounts of AST, ALT, and ALP released into the extracellular medium. The conception rates of the buffalo-cows artificially inseminated with fresh extended semen with LYC extender or washed cooled semen supplemented with 2mM caffeine was insignificantly higher than the washed cooled semen free-caffeine.

Key words: Buffalo spermatozoa, washed semen,caffeine,enzymes, conception rate.

INTRODUCTION

Long-term preservation of buffalo semen is quite a pressing problem. One of the major constraints of this,has been the lack of appropriate technology for semen processing. Buffalo spermatozoa have been believed to be inherently fragile that upon handling and storage they are subjected to ultrastructural damage and detrimental chemical changes in the molecular structures of their membranes with leakage of enzymes important for fertilization ***(Kakar and Anand, 1984).***

Artificial insemination (AI) in Egypt is still practiced on a very limited scale. The implementation of artificial insemination in buffaloes, using liquid semen has been posing problems in respect of preservation. The extenders used for bull have been commonly used. However, the diluents reported for bull semen should not necessarily apply successfully for buffalo semen, since most of the chemical attributes of semen are known to differ between the two species.

From another point of view,various additives have been incorporated into semen extenders to enhance sperm longevity and fertility ***(Maule, 1962).*** The addition of phosphodiesterase inhibitors which prevent the breakdown of cyclic 3,5 adenosine monophosphate (cAMP) such as caffeine and theophylline, markedly increased respiration and maintained motility of bovine and buffaloes spermatozoa***(Simpson and White,1987, Zeidan, 1994, El-Azab et al., 1998).***

On the other hand,the presence of seminal plasma causes an apparent reduction in glucose uptake by the spermatozoa***(Flipse,1954)***. Decapacitation factors obtained from the seminal plasma of various species inhibit corona penetrating enzyme which is an acrosomal enzyme involved in the passage of fertilizing spermatozoa through the corona radiata surrounding the ovum ***(Ahmad et al., 1996)***. However, limited studies as so far been conducted on the effects of removal of seminal plasma from buffalo semen prior to extension is still somewhat masked.

The present study was planed to define the effects of removal of seminal plasma by washing and caffeine addition on biophysical and biochemical changes of the cooled buffalo semen. Conception rate of buffalo-cows artificially inseminated, was also assessed.

MATERIALS AND METHODS

The experimental work was carried out at Gommaiza Animal Production Research Station, Gommaiza Village, Gharbiya Province, located in the northeastern part of the Nile Delta (31^0N), Animal Production Research Institute, Egypt.

The experimental work was carried out to study the effects of removal of seminal plasma by washing and caffeine addition to the extended cooled buffalo semen with lactose-yolk- citrate extender, on biophysical (semen quality) and biochemical changes (enzymatic activities), during chilled storage at 5^0C for up to 6 days and incubation at 37^0C for up to 2 hours. The conception rates of buffalo-cows artificially inseminated with fresh semen and washed cooled semen without or with 2mM caffeine, were also assessed.

1. Experimental animals:

Twelve sexually mature buffalo bulls at 3 to 5 years of age and 400 to 500 kg average body weight,were used in the present study.They were housed individually under semi-open shed and partially roofed with aspestus forming 4x4 meters. All bulls were healthy and clinically free of external and internal parasites. Palpation of the external genetalia tract showed that they were typically normal.The testicular tone was glandular, all epididymal regions were present and both testes were almost equal in size and moved freely up and down within the scrotal puches. Copulatory patterns for all tested bulls at the beginning of the experiment were judged to be normal.

2. Feeding and management:

During the experimental period,all animals were individually fed coop concentrate mixture according to ***NRC (1978)*** requirements. Dietary allowances were offered twice daily at 07.00a.m. and 16.00 p.m. Clean and fresh water was offered three times daily.

3. Methods of seminal handling:

Semen was collected by means of an artificial vagina and immediately evaluated after collection. Semen was collected from buffalo bulls twice weekly throughout the experimental period by means of an artificial vagina between 08.00 and 10.00 a.m. Two successive ejaculates were obtained from each bull on each day of semen collection. Semen was collected, evaluated, pooled and divided into two equal portions. The first portion was extended with 1ml saline solution (0.9% NaCl) and washed (treated) at 1000*g* Italic for 15 minutes at room temperature. The seminal plasma was removed and the sperm plugs were re-suspended in LYC extender to a volume equal to that of the semen before centrifugation. Washed and non-washed (control) semen were extended with LYC extender in two step. In the first step, washed and non-washed semen were extended with LYC extender at 30^{o} C to half (1semen: 4extender). The

extended semen was cooled to 5^{o}C over 2 hrs.and stored for up to 6 days. After each storage time (0, 1, 2, 4, and 6 days), the washed semen was extended again (second step) with LYC extender added with different concentrations of caffeine (0, 2, 4, and 8mM /100ml) to gave a final extension rate (1 semen: 8 extender) and then incubated at 37^{0}C for 0, 1, and 2 hours. Each of the caffeine solutions was prepared after weighing the requisite amount to obtain the desired molarity, taking into account that 194.19g is equivalent to 1 mole of caffeine (Merck sharpe & Dome). Percentages of each of sperm motility, dead spermatozoa, sperm abnormalities and acrosomal damage of spermatozoa, were evaluated. After each incubation time, washed and non-washed semen were centrifuged at 600RPM for 15 minutes and the supernatant was removed and stored at -20^{0}C until enzymatic assay. Aspartate-aminotransferase (AST), alanine-aminotransferase (ALT) and alkaline phosphatase (ALP) concentrations were estimated to define the best concentration of caffeine maintained of the washed cooled semen quality. The activities of AST and ALT were colourimetric determined using the method described by ***Reitman and Frankel(1957)***.ALP concentration was determined colorimetrically using commercial kits purchased from Bio-Merieux (Marcy L'Eptoile, Charbonnieres, Les Bains, France) according to ***Graham and Pace(1967).*** Percentage of spermatozoa with damaged acrosome was determined by using a Giemsa stain procedure as the method described by ***Watson(1975)***using a phase contrast microscope.

4. Conception rate evaluation:

In the fertility trail, 77 normally cyclic buffalo-cows were used to assess the conception rate. Insemination were carried out using fresh extended, washed cooled free caffeine or supplemented with 2mM caffeine (best level). Buffalo-cows were artificially inseminated with 0.5 ml of semen containing 20x10^{6} motile spermatozoa. Semen was deposited into the uterus by using recto-vaginal insemination technique ***(Salisbury et al., 1978)***. Buffalo-cows were artificially inseminated on the same day if

they were notified to be in heat before 10:00 a.m. Requests received by the center after 10.00a.m. were looked to be served on the following day morning. Each buffalo-cow was received two services with 10-12 hours interval. Conception rates were determined on the basis of pregnancy diagnosis by rectal palpation after 60 days from date of insemination.

Data were statistically analyzed using Least Square Analysis of variance according to ***Snedecor and Cochran (1982)***. Percentage values were transformed to Arc-sin values before being statistically analyzed. Duncan's New multiple range test was used for multiple comparisons ***(Duncan, 1955)***. The conception rate results were analyzed by the Chi-square test.

RESULTS AND DISCUSSION

1. Semen quality:

1.1. Sperm motility (%):

Table 1 showed that, the effect of type of the extended semen (washed and non-washed) on the motility percentages of the cooled buffalo spermatozoa was highly significant ($P<0.05$). The washed semen was significantly($P<0.05$)higher in the percentage of sperm motility than those of the non-washed buffalo semen, during storage at 5^0 C for up to 6 days and incubation at 37^0 C for up to 2 hours. These results are in agreement with those of ***Sahni and Mohan (1990), Goyal et al.(1997),Khan et al.(1998)***and ***El-Kishk(2003)***.These results may be due to the diffusion and removal of the inhibitory factors found in seminal plasma ***(Sengupta et al., 1977)***. ***Al-Somai et al. (1994)*** confirmed that the removal of low molecular weight protein components from seminal plasma was beneficial to sperm survival. ***Moreover,Ahmad et al.(1996)***and ***Khan et al.(1998)*** reported also that low molecular weight component were retained with the spermatozoa and the high molecular weight fraction was removed by centrifugation. The presence of an inhibitory factor for sperm motility in

the seminal plasma and its adverse effect seen more marked in buffalo semen than in cattle semen***(Sahni, 1990)***. The storage of the non-washed buffalo semen in egg yolk extender is problematical owing to the presence in seminal plasma of a phospholipase A (egg yolk coagulating enzyme) produced by the cowper's glands, which catalyzes the hydrolysis of lecithins in egg yolk to fatty acids and lysolecithins,which are toxic to spermatozoa ***(Roca et al., 1997)***. ***Jasko et al. (1991)*** also confirmed that high molecular weight fractions in seminal plasma depress sperm motility percentage,livability and absolute index of livability.The presence of seminal plasma causes an apparent reduction in glucose uptake by spermatozoa ***(Flipse,1954)***.The damaging effect of seminal plasma on spermatozoa is confined to the higher molecular weight fraction of seminal plasma,whereas motility stimulating factor is present in a low molecular weight fraction ***(Baas et al., 1983).***

Supplementation of caffeine to the washed buffalo semen at levels of 2 or 4mM increased significantly ($P<0.05$) the percentage of sperm motility than 8mM or free-caffeine medium, during storage at 5^0C for up to 6 days or incubation at 37^0C for up to 2 hours. The effect of higher level of caffeine (8mM) was detrimental effects on sperm motility. The highest ($P<0.05$) value of the percentage of motility of the cooled buffalo spermatozoa was recorded with caffeine added at a level of 2mM and the lowest ($P<0.05$) value was found with free-caffeine medium. It is interest to notice that, the percentage of motile spermatozoa increased during first day of storage and first hour of incubation and then decreased as the time of storage or incubation increased. These results are in agreement with those of ***Miyamoto and Nishikawa(1979)***who found that when bull semen was diluted with egg-yolk sodium citrate extender and incubated for 6 hours at 37^0C or stored for 7 days at 4^0C, greater stimulation of sperm motility by caffeine appeared at 5.4-18mM or 5.4-13.5mM, respectively. ***Fattouh et al. (1985)*** reported that the optimal increase in the sperm motility of buffalo was recorded with 2mM caffeine rather

than with higher levels (4 or 6 mM). Similar trend was recorded by ***Zeidan (1994)*** in ***Friesian and Abbas (1993)*** in buffalo spermatozoa. These findings may be attributed to the methylxanthines group which acts as a phosphodiestrase inhibitor. The mechanism by which caffeine stimulates sperm motility is thought to involve the inhibition of phosphodiestrase enzyme responsible for breakdown of cAMP with consequent accumulation of cyclic nucleotides, especially cAMP within the sperm cells***(Tash and Means,1983 and Aitken et al.,1983)***.***Lindemann(1978)*** reported also that any substance, such as caffeine when added to semen would stimulate markedly sperm motility through activation of cAMP production. It was evident that, cAMP stimulus sperm motility by direct action on the axoneme of the tail or by indirectly acting on the cell membrane as a secondary messenger ***(Garbers and Kopf, 1980)***.

The advancement of storage or incubation time decreased significantly ($P<0.05$) the motility percentages of either washed or non-washed spermatozoa. Similar trends were reported by ***Ahmad et al. (1996), Roca et al. (1997)*** and ***Zeidan et al. (2004)***. The decline rate in sperm motility became higher as the concentration of caffeine was increased.

1.2. Dead spermatozoa (%):

Table 2 showed that, the effect of type of the extended semen (washed and non-washed) on the percentages of dead spermatozoa was highly significant($P<0.05$).The washed semen was significantly($P<0.05$) lower in the percentage of dead spermatozoa throughout the storage period which lasted, as long as, 6 days and incubation at 37^0C for up to 2 hours as compared with the non-washed semen. These results are in agreement with those of ***Khan et al. (1998)*** and ***El-Kishk (2003)*** in buffalo bull semen. These findings may be due to that the centrifugation and removal of seminal plasma may be prevent the toxic effect of high molecular weight which maintained sperm motility as compared to non-washed semen ***(Ahmad et al., 1996 and Khan et al., 1998).***

The effect of supplementation of caffeine to the washed extended cooled buffalo semen was highly significant ($P<0.05$). Supplementation of caffeine to the washed buffalo semen at levels of 2 or 4mM decreased significantly ($P<0.05$) the percentage of dead spermatozoa than 8mM or free-caffeine medium, during storage at 5^0C for 6 days and incubation at 37^0C for 2 hours. The higher level of caffeine (8mM) increased significantly ($P<0.05$) the percentage of dead spermatozoa. The highest ($P<0.05$) value of the percentages of dead spermatozoa was recorded with free-caffeine medium and the lowest ($P<0.05$) value with 2mM. These results are in agreement with those of ***Abbas (1993)*** and ***El-Kishk (2003)*** in buffalo spermatozoa. These findings may be attributed to that addition of caffeine at concentration of 2 or 4mM/100ml decreased dead of spermatozoa by inhibition of phosphodiestrase enzyme that responsible for breakdown of cAMP and consequently, increased the accumulation of cyclic nucleotides, especially cAMP within the sperm cell. ***Aitken et al. (1983)*** also confirmed that the effect of high concentration of caffeine was toxic on sperm motility, and consequently the percentage of dead spermatozoa was increased.

The advancement of storage or incubation time increased significantly ($P<0.05$) the percentage of dead buffalo spermatozoa either washed or non-washed. Similar trends were reported by ***Zeidan et al. (1998)***.

1.2. Sperm abnormalities (%):

Table 3 showed that, the effect of type of the extended semen (washed or non-washed) on the percentage sperm abnormalities was highly significant ($P<0.05$). The washed semen showed significantly ($P<0.05$) lower in the percentage of sperm abnormalities than non-washed semen, during storage at 5^0C for up to 6 days and incubation at 37^0C for up to 2 hours. These results are in agreement with those of ***Zeidan (1994)*** and ***Goyal et al.1997)***. These results may be due to the removal of toxic substance which causes the damage of the sperm cells and in turn increasing sperm abnormalities.

The effect of supplementation of caffeine to the washed and the non-washed extended cooled buffalo semen was significant (P<0.05). Supplementation of caffeine to the washed cooled buffalo semen at levels of 2or 4mM significantly (P<0.05) decreased the percentage of sperm abnormalities than 8mM or free caffeine medium, during storage at 5^0C for up to 6 days and incubation at 37^0C for up to 2 hours. The highest (P<0.05) value of the percentages of sperm abnormalities was recorded with free-caffeine medium and the lowest (P<0.05) value was found with 2mM caffeine. These results are in agreement with those of ***Mrsny and Meizel (1980) and Zeidan et al. (2004)***.

The advancement of storage or incubation time increased significantly (P<0.01) the percentage of sperm abnormalities of. Similar trends were reported by ***Zeidan et al. (2004).***

1.4. Acrosomal damage of spermatozoa (%):

Table 4 showed that, the effect of type the extended semen (washed and non-washed) on the percentage of acrosomal damage of spermatozoa was highly significant (P<0.05).The washed semen was significantly(P< 0.05)lower in the percentage of spermatozoa with damaged acrosomes than the non-washed semen, during storage at 5^0C for up to 6 days and incubation at 37^0C for up to 2 hours. These results are in agreement with those of ***Goyal et al. (1996)***. These findings may be attributed to that centrifugation removed the toxic components through the drainage of seminal plasma which causes the acrosome damage.

Supplementation of caffeine to the washed cooled buffalo semen was highly significant(P<0.05).Supplementation of caffeine to the washed extended cooled buffalo semen at levels of 2 or 4mM significantly (P<0.05) lower the percentage of sperm acrosomal damage than 8mM or free-caffeine medium, during storage at 5^0C for up to 6 days or incubation at 37^0C for up to 2 hours. The highest (P<0.05) value of the percentage of acrosomal damage of spermatozoa was recorded with free-

caffeine medium and the lowest (P<0.05) value with 2mM caffeine. These results are in agreement with those of ***Zeidan et al. (2004)*** who found that the addition of 6 mM of caffeine to cooled goat semen increased significantly (P<0.05) the percentage of acrosomal integrity of spermatozoa than caffeine medium, during storage at 5^0C for 6 days.

The advancement of storage or incubation time increased significantly (P<0.05) the percentage of acrosomal damage of spermatozoa. Similar trends were reported by ***Roca et al. (1997) and Zeidan et al. (2004).***

2. Enzymatic activities:

Tables 5,6 and 7 showed that, the effect of type of the extended semen (washed and non-washed) on the leakage of aspartate-aminotrans-ferase (AST), alanine-aminotransferase (ALT) and alkaline phosphates (ALP) into the extracellular medium was highly significant (P>0.05). The washed semen significantly (P<0.05) lower the amounts of AST, ALT and ALP enzymes released into the extracellular medium than the non-washed semen, during storage at 5^0C for up to 6 days or incubation at 37^0C for up to 2 hours. These results are in agreement with those of ***Goyal et al. (1996) and EI-Kishk (2003)*** in buffalo spermatozoa. These results may be due to the removal of the inhibitory factors found in seminal plasma ***(Sengupta et al., 1977)*** which causes the cell membrane damage and increased AST, ALT and ALP release ***(Zeidan et al., 2004)***.

Supplementation of caffeine to the washed of the extended cooled buffalo semen was significant (P<0.05) lower the inzymatic activity. Supplementation of the washed cooled buffalo semen with caffeine at levels of 2,4 or 8mM sign-ificantly (P<0.05) decreased the amounts of AST, ALT and ALP enzyme released into the extracellular medium than free caffeine medium throug-hout the storage period which lasted, as long as, 6 days or incubation at 37^0C for up to 2 hours. The highest (P<0.05)value of the amount of AST, ALT and ALP enzyme released into the extracellular medium of the cooled buffalo spermatozoa was

recorded with free-caffeine medium and the lowest ($P<0.05$) value with 2mM caffeine. These results are in agree-ment with those of ***El-Kishk (2003)*** in buffalo bull spermatozoa. Caffeine may able to prevent the increase of the enzyme activity in the extended buffalo semen and consequently, decreased the release of AST,ALT and ALP enzymes into the extracellular medium ***(Zeidan et al., 2004)***.

The advancement of storage at 5^0C or incubation at 37^0C increased significantly($P<0.05$)the amounts of ALP,AST and ALP enzymes released into the extracellular medium when semen was either washed or non-washed. Similar trends were reported by ***El-Kishk (2003) and Zeidan et al. (2004).***

3. Conception rate:

Table 8 showed that,conception rates of the buffalo-cows artificially inseminated with fresh extended semen, extended washed semen and washed extended semen supplemented with 2mM caffeine, were 78.57, 60.87 and 69.23,respectively.The conception was rate was insignificantly decreased as a result of extension and washing of semen. However, no differences were recorded between the fresh extended semen and extended washed semen supplemented with or without 2mM caffeine. Conception rate of buffalo-cows artificially inseminated with extended washed semen supplemented with 2mM caffeine was insignificantly higher than that inseminated with extended washed semen free-caffeine. These results are in agreement with those of ***Cai and Marik (1989), El-Kishk (2003) and Zeidan et al.(2004)***. These results may be due to that caffeine supplementation to the extended washed semen was beneficial to sperm survival in turn increasing conception rate as compared to semen free-caffeine. In addition, ***Dodds and Seidel (1983)*** showed that a higher percentage of cumulus-intact ova was fertilized when 10mM caffeine medium was present in the fertilization medium. ***Similarly, Aitken et al. (1983)*** found a close correlation between spermatozoa movement of human semen and their penetrating ability into cervical mucus.

جدول بالعرض (1)

جدول بالعرض (2)

جدول بالعرض (3)

جدول بالعرض (4)

جدول بالعرض (5)

جدول بالعرض (6)

جدول بالعرض (7)

Table (8): Conception rate of buffalo-cows artificially inseminated with fresh semen and extended washed semen without or with 2mM caffeine.

Type of semen	No. of buffalo-cows inseminated	No. of buffalo-cows conceived	Conception rate
Fresh extended semen	28	22	78.57
Extended washed semen	23	14	60.87
Extended washed semen +2mM caffeine	26	18	69.23

In conclusion, removal of seminal plasma of the buffalo bull semen supplemented with 2 and 4mM caffeine is a practical and improves semen quality and provides effective technique which provides a useful alternative to semen stored in liquid form. Supplementation of such type of semen with 2mM caffeine improved conception rate when used for artificial insemination in buffalo-cows, particularly in those regions of Asia and Africa, where liquid nitrogen may not be available for freezing of semen.

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التغيرات الطبيعية والبيوكيميائية للحيوانات المنوية منزوعة البلازما فى الجاموس والمضاف إليها الكافيين

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تمت هذه الدراسة على عدد 12 طلوقة جاموس ناضجة جنسياً وذلك لمعرفة تأثير نزع بلازما السائل المنوى عن طريق الطرد المركزى مع إضافة مستويات مختلفة من الكافيين (صفر ، 2، 4، 8

ملى مول/100مل) إلى السائل المنوى المخفف والمبرد على التغيرات الطبيعية (النسبة المئوية لحيوية الحيوانات المنوية، الحيوانات المنوية الشاذة وشواذ الاكروسوم) والبيوكيميائية إنزيم (AST، ALP، ALT) أثناء الحفظ بالتبريد على درجة حرارة 5م لمدة 6 أيام والتحضين على درجة 37م لمدة ساعتين. كذلك تم قياس معدل الإخصاب للإناث الجاموس الملقحة إصطناعياً بالسائل المنوى المخفف أو السائل المنوى المبرد بدون إضافة كافيين أو مع إضافة 2 ملى مول كافيين.

أوضحت النتائج أن نزع بلازما السائل المنوى للجاموس أدى إلى زيادة النسبة المئوية لحيوية الحيوانات المنوية مع إنخفاض النسبة المئوية للحيوانات الميتة والحيوانات المنوية الشاذة وشواذ الالكروسوم وكمية إفراز إنزيمات (AST، ALP، ALT) إلى البيئة الخارجية بدرجة معنوية (على مستوى 0.05) مقارنة بالحيوانات المنوية الغير منزوعة البلازما. إضافة 5 أو 4 ملى مول كافيين إلى السائل المنوى المبرد سواء المنزوع أو الغير منزوعة البلازما أدى إلى زيادة النسبة المئوية لحيوية الحيوانات المنوية مع إنخفاض النسبة المئوية للحيوانات المنوية الميتة والحيوانات الشاة وشواذ الاكروسوم بدرجة معنوية على مستوى 0.05 بالمقارنة بإضافة 8 ملى مول كافيين أو الخالية من الكافيين.

إضافة 2,4 أو 8 ملى مول كافيين إلى السائل المبرد سواء المنزوع أو الغير منزوع البلازما أدى إلى إنخفاض كمية إفراز إنزيمات (AST، ALP، ALT) إلى البيئة الخارجية بدرجة معنوية على مستوى 0.05 مقارنة بالسائل المنوى الخالى من الكافيين. حفظ السائل المنوى على درجة حرارة 5م لمدة 6 أيام أو التحضين على درجة حرارة 37م لمدة ساعتين سواء المنزوع أو الغير منزوع البلازما أدى

إنخفاض النسبة المئوية لحيوية الحيوانات النوية مع زيادة النسبة المئوية للحيوانات المنوية الميتة والحيوانات المنوية الشاذة وشواذ الاكروسوم وكمية إفراز أنزيمات (AST، ALP، ALT) إلى البيئة الخارجية بدرجة معنوية (على مستوى 0.05) زيادة معدل الاخصاب بدرجة غير معنوية لإناث الجاموس الملقحة إصطناعياً بالسائل المنوى المخفف، السائل المنوى المبرد والمنزوع البلازما والمضاف إليه 2 مللى مول كافين مقارنة بالسائل المنوى المبرد المنزوع البلازما بدون إضافة كافيين.