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Effect of C-Type Natriuretic Peptide on Ram's Sperms and Ewes Fertility

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Abstract

THIS STUDY estimate the effect of C-Type Natriuretic Peptide (CNP) on quality of ram's epididymal sperms and the rate of fertility in ewes inseminated artificially with CNP-treated and not-treated sperms. Initially, a total of 20 testicular samples were collected, processed to obtain the epididymal sperms, and then divided into two groups; the 1st that treated with the suitable dose of CNP (0.01×10^{-13}) and the 2nd was act as a control (not-treated with CNP). Semen samples of both groups were tested using the turbidimetric analysis. Also, an overall 10 adult ewes were selected, acclimated, subjected to estrus synchronization, and then divided equally into two groups to be inseminated artificially with only epididymal sperms and CNP-treated sperms. The findings of turbidimetric analysis revealed the significant decreasing in values of lag time of CNP-treated sperms (3.11 ± 0.26) when compared to control (6.46 ± 1.48). The results of motility index of both CNP-treated sperms (0.222 ± 0.034) and control (0.247 ± 0.069) were differed insignificantly. Significant elevation in values of velocity ($P<0.011$) was reported in CNP-treated sperms (0.399 ± 0.048) than the control (0.208 ± 0.098). Also, FRMS of CNP-treated sperms (0.028 ± 0.0063) was increased significantly when compared to control (0.019 ± 0.0047). Using the ultrasonography, the findings of artificial insemination recorded that the pregnancy rate after 2 months was 0% (0/5) in ewes inseminated with only epididymal sperms and 40% (2/5) in ewes inseminated with the CNP-treated sperms. In conclusion this represents the first Iraqi study targets the effect of CNP on ram's epididymal sperms and the role of CNP in increasing the fertility rate in ewes. However, changes occur due to excessive exposure to CNP remain unclear recommending moreover studies As well as, the toxicological profile of CNP must be known during utilization of CNP with the semen or as a dietary supplement to increasing the fertility.

Keywords: Sheep fertility, Turbidimetric analysis, Artificial insemination, Ultrasonography, Iraq.

Introduction

Artificial insemination (AI) technology is an assisted reproductive technology that uses a device to inject an appropriate amount of sperm into the female reproductive system [1]. AI is essential for high fertilization rates in lambs, and is access in elderly or injured males and prevents sexually transmitted diseases [2, 3]. Several types of AI are achieved depending on the fertilization method chosen and satisfactory pregnancy rates [4]. Male factors such as ram age, fertility, mass motility and the type of sperm or secretions used should also be considered [5, 6]. Many researchers have demonstrated the relationship of AI to parameters of sperm quality including sperm viability and motility, membrane integrity, sperm DNA fragmentation, sperm nuclear area, and circumference [7-9]. Unfortunately, fertility does not only depend on individual sperm parameters, but also on the fertility of the female, the type of estrus (natural or hormonally regulated estrus), the season and the place of sperm storage [10-13]. Reduced fertility may also result from different methods of sperm processing and preparation for transport and storage [14]. Cryopreservation involves rearranging the lipids that make up the sperm membrane, which

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affects the lipid-lipid and lipid-protein interactions necessary for optimal sperm activity, making frozen and thawed of male sperm less motile compared to fresh sperm. Fresh sperms have better fertility and pregnancy rates in addition to its ability for using fewer sperm; typically, 40 to 80 million sperm per insemination [15, 16].

C-type natriuretic peptide (CNP), a member in natriuretic peptides which originally identified in porcine brain extracts, is a 22 amino acid peptide formed after processing of pre-proKNP by signal peptidase and subsequent cleavage of pro-KNP by purine endoprotease. Two forms of CNP exist in tissues and plasma, CNP-53 and CNP-22, but the protease responsible for converting the extended peptide to the shorter, more abundant form is unknown [17, 18].

Several recent studies have shown that CNP has a direct effect on endothelial and smooth muscle cell mitosis, while simultaneously promoting endothelial growth, thereby promoting wound healing and vascular repair [19, 20]. This dual protective role of CNP was first described in animal models of vein grafting and balloon angioplasty, clearly showing that CNP treatment promotes reendothelialization and reduces dangerous neointimal hyperplasia [21]. Other studies have revealed the increasing of cGMP production after CNP treatment, suggesting the involvement of NPR-B [20].

By comparing the expression of pro CNP and CNP in extracts, Nielson et al. [22] found that the highest peptide concentrations were in male porcine reproductive tissues. Further studies showed that the concentration of CNP in human seminal plasma was higher that of blood plasma [23, 24]. The high expression level of CNP specifically in male reproductive organs indicates the importance of CNP in male reproduction. Therefore, the current study aims to detect the effect CNP on quality of ram's epididymal sperms and the rate of fertility in ewes.

Material and Methods

Ethical approval

This study was authorized and overseen by the Scientific Committee of the Department of Surgery and Obstetrics at the (University of Baghdad, Baghdad, Iraq's College of Veterinary Medicine).

Samples

Twenty testicular samples in total were taken from the adult slaughtered rams at the official abattoirs in Al-Shoula city (Baghdad, Iraq), during April to September (2023). All samples were quickly transferred to the laboratory using a plastic icebox (Fig. 1). Following a wash in distilled water, the testicle samples were added to normal saline supplemented with 100 IU/ml penicillin and 0.1 mg/ml streptomycin. A small scissors was used to dissect and separate the epididymis from the whole testicle, and the caudae were injected with 5-8 ml of TCM199 medium supplemented with 100 IU/ml Nystatin and 100 IU/ml penicillin / streptomycin using gauge 18-needle attached to a 5-ml syringe, and then was aspirated [25].

CNP

Sigma Aldrich prepared CNP vials at 22-25°C, dissolved in distilled water, shaken, and diluted to achieve ideal dosage of 0.01×10-13, minimizing confusion and variation.

Turbidimetric analysis

The lag time, motility index, velocity, and Fraction of rapidly moving sperm (FRMS) were estimated as described by Hasan et al. [26].

Estrus synchronization

Estrus in 10 adult local ewes was synchronized by application the vaginal sponges containing 40 mg of MAP at day 9, and then, the sponges were removed and PMSG was injected at a dose of 500 IU. Then, the study ewes were divided equally into two groups; the G1 that inseminated artificially with only the epididymal sperms, and the G2 that inseminated artificially with the CNP-treated sperms (Fig. 2). Ultrasonography was served to diagnosis the rate of pregnancy (Fig. 3).

Statistical analysis

Values of the study groups are expressed as mean ± standard error (M± SE), and the t test, one way ANOVA, and two way ANOVA in GraphPad Prism software (version 6.0.1) were used to detect significant differences ($P<0.05$) between the values [27].

Results and Discussion

Turbidimetric analysis

In this study, the findings of turbidimetric analysis demonstrated the role of CNP in supporting the activity of CNP-treated sperms as observed in values of lag time that reduced significantly (3.11 ± 0.26) when compared to control (6.46 ± 1.48), (Fig. 4). The results of motility index of both CNP-treated sperms (0.222 ± 0.034) and control (0.247 ± 0.069) were differed insignificantly (Fig. 5). Significant elevation in values of velocity ($P<0.011$) was reported in CNP-treated sperms (0.399 ± 0.048) than the control (0.208 ± 0.098), (Fig. 6). Also, FRMS of CNP-treated sperms (0.028 ± 0.0063) was increased significantly when compared to control (0.019 ± 0.0047), (Fig. 7).

Semen cryopreservation, which entails a variety of cooling and freezing processes, has long been regarded as a useful approach that can be used in a variety of reproductive techniques, including artificial insemination, *in vitro* embryo production, and embryo transfer, as it circumvents time and space constraints [28]. In Iraq, the field studies have focused on the significant influence the quality of thawed semen of bulls in addition to consequences of different rates of success [25, 29-31]; whereas globally, the effect of storage or cooling rates on fertility of semen suspension was studied in different domestic and wild animals such as buck [32], ram [33], and cattle [28].

As a result, various additives have been employed to enhance the sperms' physical characteristics such as arginine [34], caffeine [35], silymarin [36]. In this study, the effect of CNP on cooled semen of rams was studied for first time in Iraq, and the results revealed that the lowered concentration of CNP was revealed the suitable dose of semen suspension could be used for artificial insemination of ewes. Worldwide, a number of studies have performed to detect the effect of CNP on normal human sperm [37], sperm attraction for fertilization [38], regulation of sperm capacitation in genital tract of female rat [39], anti-inflammatory role in rat epididymitis [40], sperm motility and reproduction function of azthenozoospermia in mice [41], and spermatozoa maturation in rats [42]; however, no data were available concerned with the toxicological effect of CNP on semen. Microscopic examination of semen suspension treated with the suitable dose of CNP showed that there were significant decreases in levels of individual motility, live sperms and sperm concentration with advancing the periods of cooling when compared to control, but abnormal sperms were increased more significantly in control compared to CNP.

Estrus synchronization and fertility rate

In this study, the estrus cycle of all study ewes (total No. 10) was successfully synchronized using the vaginal sponges and PMSG. However, the findings of artificial insemination recorded that the pregnancy rate was 0 (0%) in ewes inseminated with the only epididymal sperms and 2 (40%) in ewes inseminated with the CNP-treated sperms (Table 1, Fig. 8).

Studies suggest that mouse nanoparticles (NPs), including CNP, can attract mammalian spermatozoa and increase sperm motility [43, 44]. Xia et al. [41] CNP induces a dose-dependent increase in spermatozoa motility and acrosome reaction, regulating male reproductive function. Kong et al. [38] reported that CNP increases intracellular cGMP and Ca^{+2} levels, inducing sperm accumulation through attraction. Özbek et al. [45] observed the CNP expression in epithelial and smooth muscle cells of the epididymis, suggesting it increases spermatozoa motility via cGMP. Mei et al. [40] the study found that asthenospermia patients' semen samples have lower CNP concentrations compared to normal individuals, improving sperm motility and alleviating cyclophosphamide damage to the reproductive system. Similar findings were recorded by Li et al. [41] who found that CNP alleviates the acute epididymitis injury and decreases invasion and inflammatory reaction of macrophages; suggesting the possibility of using CNP as a potential treatment for epididymitis. Zhao et al. [42] found that CNP plays a role in epididymal sperm maturation as it promotes the acquisition of epididymal sperm fluidity.

Ram spermatozoon is a sensitive cell; it rapidly dies in the room temperature and cryopreservation reduces it fertility potential [46]. Falchi et al. [47] mentioned that fertility cab be reduced as a result of retrogression of morphological and functional properties of spermatozoa including reducing of motilities and declining the sperm survival within the genital tract of female. In a previous study, Foote [48] mentioned that a remarkable lag might be existed among testicular events accountable to alteration in quality of semen and period that alteration is apparent in ejaculated sperms. Subsequently, circumstances must be assumed at a period of semen collection including type of semen collection, degree of sexual preparation and frequency of ejaculation could affect quality of harvested semen. The endogenous antioxidant markers play a role against the ROS production and protect the preserved cells; however, the quantity of these markers may not enough in stress conditions to protecting the cryopreserved or cooled cells causing a significant damage to sperm membranes and reducing recovery of motile sperms [49-53]. Kasimanickam et al. [54] reported that sperm motility parameters including velocity were changed significantly over the storage period with superior results for soy protein after 8 days of cooling. Del Olmo et al. [55] found that insignificant association between sperm parameters and fertility, and that the semen sample collected from a poor fertile rams might yield less velocity and linearity as showed through the motility parameters.

Therefore, several additives have been used to improvement the activity of ram semen such as hyaluronan, cysteamine, taurine, trehalose [56], methionine [57], white and green teas [58], meto-TEMPO I [59], and soybean lecithin [60]. In one study, the authors demonstrated that the using of amino acids (alanine, proline and glutamine) as additives in ram semen play a significant role on lipid peroxidation levels, plasma membrane integrity and sperm motility at pre-freezing and post-thawing [61]. Rather et al. [62] investigate the adding of different antioxidants like taurine, butylated hydroxytoluene and ascorbic acid on ram semen cooled at 4°C for 3 days indicating that the percent of sperm motility was increased with decreasing the morphological abnormalities. Avdatek and Gündoğan [63] concluded the efficacy of amino acids as antioxidants for frozen semen of ram with a high effectiveness of lipoic acid in decreasing DNA damage and oxidative stress. Rateb et al. [64] concluded that the supplementation of omega-3 and melatonin has a superior outcome in maintaining of sperm properties with reducing of lipid peroxidase reaction. Allai et al. [65] overviewed progress in supplements using antioxidant and other materials in improving the fertility rate and quality of ram semen concluding their roles in reducing formation of oxidants in ram semen, maintain its quality and enhance its fertility.

Conclusions

This study, which was conducted for the first time in Iraq, emphasizes the critical function that CNP plays in improving ram sperm, which may provide breeders greater choice when it comes to artificial insemination programs. Also, considerable detrimental effect of cooling rate on sperm DNA and the good effect of CNP in decreasing the DNA fragmentation need to be explored. Additionally, the effects of excessive exogenous CNP exposure are still unclear, and additional supplements are needed to understand the toxicological profile of CNP and its derivatives.

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Conflicts of interest

The author declared that no competing interests.

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TABLE 1. Total results for artificial insemination of ewes with epididymal sperms (G1) and CNP-treated sperms (G2)

Group	Total No.	Estrus synchronization			Pregnancy rate [No. (%)]	No. of foetus
		Type of treatment	Duration	Response [No. (%)]		
G1	5	Vaginal sponge (40 mg)	9 days	5 (100%) B	0 (0%) B	0
		PMSG (500 IU)	After removing sponges			
G2	5	Vaginal sponge (40 mg)	9 days	5 (100%) B	2 (40%) A	3
		PMSG (500 IU)	After removing sponges			(1 Single + 1 twin)

Different vertical large letters refer to significant variation at P<0.05

Fig. 1.The caudal epididymal spermatozoa samples in a Petri-dish

Fig. 2. Artificial insemination of study ewes with CNP-treated and non-treated epididymal sperms

Fig. 3. Ultrasonographic examination of inseminated ewes to diagnosis of pregnancy

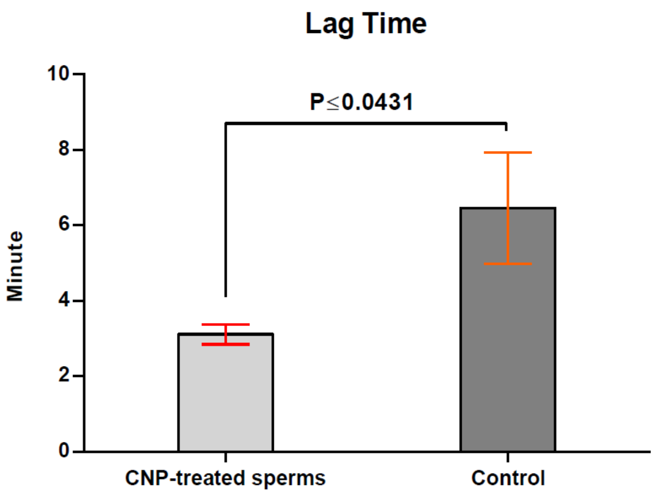

Fig. 4. Turbidimetric analysis of lag time in CNP-treated and non-treated epididymal sperms

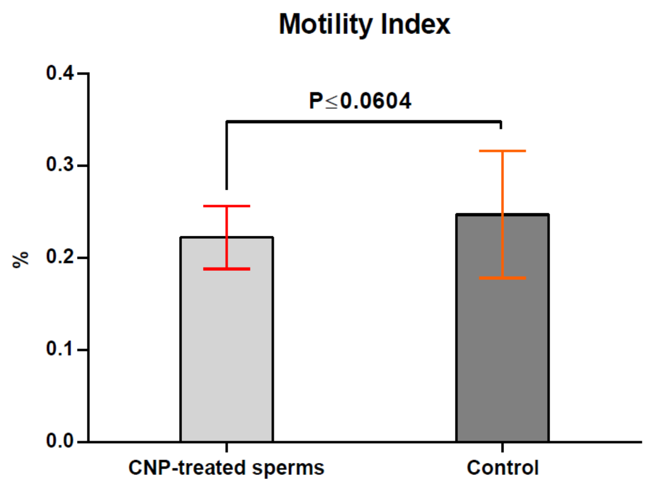

Fig. 5. Turbidimetric analysis of motility index in CNP-treated and non-treated epididymal sperms

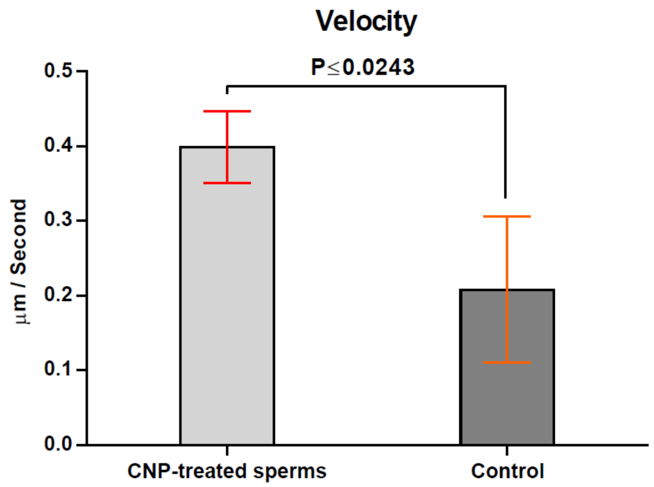

Fig. 6. Turbidimetric analysis of velocity in CNP-treated and non-treated epididymal sperms.

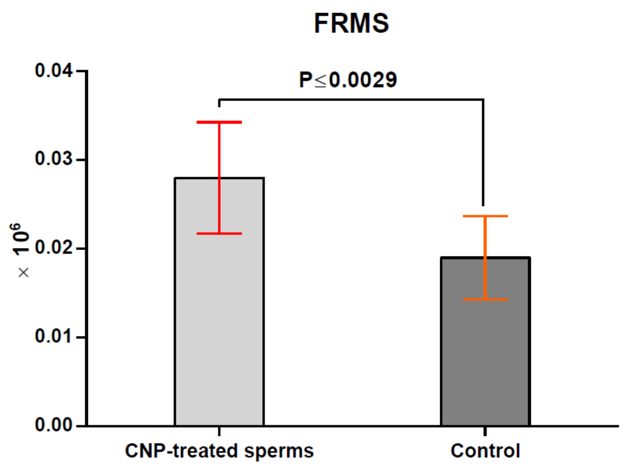

Fig. 7. Turbidimetric analysis of FRMS in CNP-treated and non-treated epididymal sperms

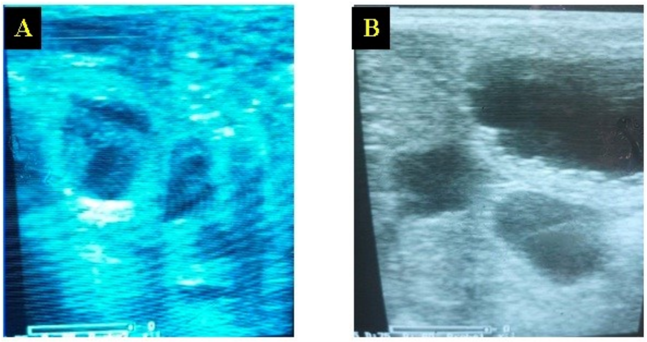

Fig. 8.Ultrasonographic detection of pregnant ewes at 2 months shows the pregnant ewes with single (A), and twin (B) fetuses

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تأثير الببتيد الطبيعي من النوع-ج (CNP) على الحيوانات المنوية للاكباش وخصوبة النعاج

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المخلص

أجريت هذه الدراسة لمعرفة تأثير CNP على جودة الحيوانات المنوية للبربخ ومعدل الخصوبة في النعاج الملقحة صناعيا بالحيوانات المنوية المعالجة وغير المعالجة بالـ CNP. مبدئيا ، تم جمع ٢٠ عينة خصية ومعاملتها للحصول على الحيوانات المنوية من البربخ ومن ثم تم تقسيمها إلى مجموعتين ؛ الأولى تمت معاملتها بالجرعة المناسبة من CNP (١ × ١٠ -١٣) والثانية تمت اعتبارها كمجموعة سيطرة (غير معالجة بـ CNP) . تم اختبار عينات الحيوانات المنوية البربخية لكلا المجموعتين باستخدام تحليل قياس العكر . اضافة الى ذلك ، تم إجماليا اختيار ١٠من النعاج بالغة ومن ثم اقلمتها ومزامنة فترة الشبق فيها ومن ثم تقسيمها بالتساوي إلى مجموعتين ليتم تلقيحها صناعيًا باستخدام الحيوانات المنوية البربخية فقط والحيوانات المنوية المعالجة بالـ CNP. أظهرت نتائج تحليل العكر انخفاضاً معنوياً في قيم زمن التأخر للحيوانات المنوية المعاملة بالـ CNP (٣,١١ ± ٠,٢٦) بالمقارنة مع مجموعة السيطرة (٦,٤٦ ± ١,٤٨) . اختلفت نتائج مؤشر الحركة لكل من الحيوانات المنوية المعالجة بالـ CNP (٠,٢٢٢ ± ٠,٠٣٤) ومجموعة السيطرة (٠,٢٤٧ ± ٠,٠٦٩) بشكل غير معنوي . تمت ملاحظة ارتفـــاع كبـير في قيم السرعة (P <0.011) في الحيوانات المنوية المعالجة بالـ CNP (٠,٣٩٩ ± ٠,٠٤٨) مقارنة بمجموعة التحكم (٠,٢٠٨ ± ٠,٠٩٨). كما أن معدل تحليل الحيوانات المنوية سريعة الحركة (FRMS) للحيوانات المنوية المعالجة بـ CNP (٠,٠٢٨ ± ٠,٠٠٦٣) زاد بشكل ملحوظ بالمقارنة مع مجموعة السيطرة (٠,٠١٩ ± ٠,٠٠٤٧). باستخدام الموجات فوق الصوتية، سجلت نتائج التلقيح الاصطناعي أن معدل الحمل بعد شهرين كان ٠% (٥/٠) في النعاج الملقحة بالحيوانات المنوية المعالجة بالـ CNP و ٤٠% (٥/٢) في النعاج الملقحة بالحيوانات المنوية المعالجة بالـ CNP. في الختام ، فإن نتائج هذه الدراسة تمثل أول دراسة عراقية تستهدف تأثير CNP على الحيوانات المنوية البربخية للكبش ودورها في زيادة معدل الخصوبة في النعاج . ومع ذلك ، فان التغييرات التي يمكن ان تحدث نتيجة التعرض المفرط للـ CNP تبقى غير واضحة وتتطلب مزيدا من الدراسات . اضافة الى ذلك يجب معرفة الملف السمي للـ CNP عند استخدامة مع السائل المنوي او امكانية استخدامه كمكمل غذائي لزيادة الخصوبة.

الكلمات الدالة: خصوبة الأغنام، تحليل العكارة، التلقيح الاصطناعي، الامواج فوق الصوتية، العراق.