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Efficiency of C-type natriuretic peptide on improvement of Iraqi local ram's epididymal sperms

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Abstract

Background: Fertility plays a great role in animal reproduction since high-quality semen improves sheep industry reproduction. The current worldwide data revealed the close relation of C-type natriuretic peptide (CNP) to the reproductive function of rams.

Aims: Evaluation of the effect of CNP on cooled sperms using traditional and molecular assays.

Methods: Totally, of 20 testicular samples were collected, processed to obtain the semen samples, and divided into two parts; one was treated with a suitable dose of CNP, and the other served as a control. Sperm samples of both groups were cooled for 3 days and tested at 0, 24, 48, and 72 hours.

Results: The findings revealed that the suitable dose of CNP-treated sperms was $0.01 \times 10{-}^{13}$. Values of individual motility, live sperms, and sperm concentration were reduced significantly in CNP-24h, CNP-48h, and CNP-72h when compared to control; however, abnormal sperms were increased in both control and CNP groups at 24, 48, and 72 hours when compared to values of 0 hour. Concerning turbidmetric analysis, a significant reduction in values of lag time was observed in CNP when compared to control at all times of cooling intervals. In both CNP and control groups, motility index was decreased at 24, 48, and 72 hours when compared to 0 hour. For velocity, significant increases were shown in CNP compared with control at all cooling intervals. However, values of both groups were increased significantly at 24, 48, and 72 hours times when compared to 0 hour. Fraction of rapidly moving sperm of CNP was elevated at 0 hour and decreased at 24, 48, and 72 hours when compared to control. Expression of the *hNPR-B* gene was reduced gradually in sperms of CNP and control groups at times of cooling intervals.

Conclusion: To the best of our knowledge, this first Iraqi study targets the effect of CNP on epididymal sperms of rams. However, changes that occur after excessive CNP exposure remain unclear, and the toxicological profile of CNP requires furthermore supplements.

Keywords: Sheep fertility, Turbidimetric analysis, MTT assay, CNP, Iraq.

Introduction

Sheep are one of the most common livestock animals in the world, which are raised under different climates, topographic, and management conditions, and served in many countries as a main source of wool, milk, and meat (Shinde and Naqvi, 2015). The fertility of rams was detected to have great economic roles in animal reproduction because the high-quality semen is favorable for the sheep industry and the production of a large number of preferred lambs (Mohapatra and Shinde, 2018; Redden and Thorne, 2020). Although rams can breed at any time of the year, loss of libido and reduced sperm quantity can affect their performance throughout the season of breeding (Maquivar *et al*., 2021). In combination with estrus and ovulation induction/synchronization, artificial insemination is an assisted reproductive technology that was used as a device to inject an appropriate amount of sperm into the female reproductive system to increase the genetic value of animals (Hafez, 2015; Mochida, 2020; Al-Chaabawi *et al*., 2020). Worldwide, artificial insemination in sheep farming should consider the creation of appropriate knowledge suitable for the breed such as ram age, ram fertility, mass motility, and the type of sperm or secretions (Madrigali *et al*., 2021). Natriuretic peptide (NP) is a family of three hormones that correlate structurally and act distinctly and uniquely in the body; atrial NP (ANP) and brain NP (BNP) in the heart, and C-type NP (CNP) (Goetze *et al*., 2020). ANP and BNP are endocrine hormones that help with the regulation of heart structure as well as blood volume and pressure (Nakagawa *et al*., 2019). Comparatively, the mechanism of action of CNP is diverse and researchers in the last decade showed that CNP affects coronary blood flow regulation electrophysiology, vascular integrity, endothelial

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cell and smooth muscle proliferation, angiogenesis, leukocyte activation, and vascular tone (Perez-Ternero *et al*., 2019). In the male reproductive system, several studies showed that testicular function and fertility and regulation of erectile tissue can be regulated by CNP (Hannema *et al*., 2013; Shahin *et al*., 2018; Ückert *et al*., 2019).

In farm animals particularly ram, studies conducted for elucidating the effect of CNP on sperm functions are scarce and need to be supported. Hence, this study aims to estimate the level of CNP toxicity to prepare an effective dose of sperm and detect the influence of CNP on sperm samples cooled for 3 days by the traditional microscopic examination and using the turbidimetric analysis. Real-time polymerase chain reaction (RT-PCR) was applied to detect the genetic expression of the *hNPR-B* gene in sperm samples at different cooling intervals.

Materials and Methods

Samples

Totally, 20 testicular samples were collected from the adult slaughtered rams at the official abattoir of Al-Shoula city (Baghdad, Iraq), and transported as soon as possible using the plastic icebox to the laboratory (Fig. 1). The testicle samples were first washed in distilled water, then in normal saline supplemented with 0.1 mg/ml streptomycin and 100 IU/ml penicillin. Small scissors were used to dissect and separate the epididymis from the whole testicle, and the caudae were injected with 5–8 ml of TCM^{199} 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.

CNP

According to manufacturer instructions (Sigma Aldrich, Germany), the vial of CNP was prepared at room temperature (22°C–25°C), dissolved in distilled water, shacked vigorously, and diluted to obtain different concentrations. Initially, CNP was diluted serially to detect the lowered lethal concentration (LC) of CNP on sperm samples; and then, the LC1, LC50, and LC95 of CNP were re-evaluated and statistically compared. Post selection of the recommended concentration of CNP, the CNP-treated sperms in addition to untreated sperms (control) was subjected to cooling at 3 days and tested at 0 hour (0h), 24 hours (24h), 48 hours (48h), and 72 hours (72h).

Traditional examination

Based on different protocols of sperm examination; morphology, individual motility, viability, sperm concentration, and turbidimetric analysis [lag time, motility index, velocity, and fraction of rapidly moving sperm (FRMS)] were analyzed (AL-Ebady *et al*., 2012; Esteves and Varghese, 2012).

Molecular assay

As described by the manufacturer's instruction of the total RNA Extraction Mini Kit (Favorgen, Taiwan) and the cDNA Synthesis SuperMix kit (Transgen, China), the control and CNP-treated sperms samples preserved in the $TRIzol^{TM}$ reagent were subjected to RNA extraction and cDNA synthesis. Targeting *hNPR-B* gene [(F: 5'-GAA CAC TGC TTC TCG AAT GGA G-3') and (R: 5'-CCA GGT ACC AGC

Fig. 1. Collection and maturation of sperm samples from epididymis.

AGA AGA-3')] and housekeeping gene [(F: 5'-CCG CAT CTT CTT GTG CAG TG-3') and (R: 5'-ACC AGC TTC CCA TTC TCA GC-3')], the qPCR SYBR Green MasterMix Kit (Promega, USA) was served to preparing the mastermix tubes at a final volume of 20 µl. The thermocycler conditions involved 1 cycle of initial denaturation (95°C, 2 minutes), 50 cycles of denaturation (95°C, 15 seconds) as well as annealing and extension (95°C, 1 minute), and 100 cycles of melting curve analysis (95°C, 15 seconds). The final gene expression was calculated with 2^{-DDCT}.

Statistical analysis

The *t-test*, One- and Two-Ways ANOVA in the GraphPad Prism Software (*version 6.0.1*) were served to detect significant variation between the values of different study groups at $p < 0.05$. The study values appeared as Mean ± Standard Errors (M±SE) (Gharban, 2023).

Ethical approval

This study was licensed and supervised by the Scientific Committee of the Department of Surgery and Obstetrics in the College of Veterinary Medicine (University of Baghdad, Baghdad, Iraq).

Results

CNP-toxicity and effective dose of sperm

Significant variation ($p < 0.0001$) between values of LC1 (0.03 pg/ml), LC50 (19.997 pg/ml), and LC95 (2191.964 pg/ml) was seen in the current study; however, the lowered CNP toxicity and suitable dose of sperms suspension treated with CNP was LC1 (0.01 x 10^{-13}) (Table 1).

Sperms quality

Individual motility of sperm suspensions was increased significantly in CNP-0h (75.845 ± 1.43); and reduced significantly in CNP-48h (27.89 ± 2.46) and CNP-72h (2.545 ± 0.35) but not in CNP-24 hours (53.66 ± 2.34) when compared to control groups (Table 2).

Value of live sperms in CNP-0h group (77.5 ± 1.1) was differed insignificantly ($p > 0.05$) when compared to control-0h (82.63 ± 0.89) and control-24h (81.265 ± 0.95) groups; however, gradual significant decreases ($p < 0.05$) were observed in values of CNP-24h (68.6 ± 1.33), CNP-48h (60.78 ± 0.98), and CNP-72h (36.4 ± 1.21) in comparison with those of control-48h (60.78 ± 0.98) and control-72h (49.215 ± 0.93), (Table 3).

Values of abnormal sperms were increased significantly ($p < 0.05$) in both control and CNP groups at the time of 24 (6.29 ± 0.09 and 6.97 ± 0.1, respectively), 48 (11.88 ± 0.21 and 14.81 ± 0.26, respectively), and 72 hours (14.5 ± 0.37 and 15.08 ± 0.43, respectively) when compared to values of CNP-0h (5.15 ± 0.15) and control-0h (4.64 ± 0.09) groups (Table 4, Fig. 2).

Significant decreases ($p < 0.05$) in sperm concentration were started gradually in CNP-24h (21.51 ± 3.05), CNP-48h (10.06 ± 2.87), and CNP-72h (10.89 ± 1.77)

Table 1. Toxicity study of CNP addition and determine the effective dose of LC1, LC50, and LC95 on ram sperms.

Group / Data	LC1	LC50	LC95	*p*-value
CNP concentration	1%	50%	95%	-
Low Lethal (LL)	0.01 c	11.63 b	356.67 a	0.0001
Ultra Lethal (UL)	0.09 c	34.37 b	13467.4 a	0.0001
UL/LLratio	9 b	2.96 c	37.76 a	0.0001

Different horizontal small letters refer to significant variation at $p < 0.05$.

Table 2. CNP addition effect on sperms individual motility percentage of ram sperms during three days of cooling preservation.

Group	Value (%)	*p*-value
Control-0h	59.55 ± 1.96 B	0.013
CNP-0h	75.845 ± 1.43 A	
Control-24h	35.82 ± 1.82 C	
CNP-24h	53.66 ± 2.34 B	
Control-48h	21.933 ± 1.63 D	
CNP-48h	27.89 ± 2.46 D	
Control-72h	5.76 ± 0.73 E	
CNP-72h	2.545 ± 0.35 E	

Different vertical large letters refer to significant variation at $p < 0.05$.

Table 3. CNP addition effect on sperms live percentage of ram sperms during three days of cooling preservation.

Group	Value (%)	*p*-value
Control-0h	82.63 ± 0.89 A	0.029
CNP-0h	77.5 ± 1.1 AB	
Control-24h	81.265 ± 0.95 A	
CNP-24h	68.6 ± 1.33 B	
Control-48h	60.78 ± 0.98 BC	
CNP-48h	53.2 ± 0.86 C	
Control-72h	49.215 ± 0.93 C	
CNP-72h	36.4 ± 1.21 D	

Different vertical large letters refer to significant variation at $p < 0.05$.

Table 4. CNP addition effect on sperms abnormal percentage of rams sperms during three days of cooling preservation.

Group	Value (%)	*p*-value
Control-0h	4.64 ± 0.09 B	0.0046
CNP-0h	5.15 ± 0.15 B	
Control-24h	6.29 ± 0.09 B	
CNP-24h	6.97 ± 0.1 B	
Control-48h	11.88 ± 0.21 A	
CNP-48h	14.81 ± 0.26 A	
Control-72h	14.5 ± 0.37 A	
CNP-72h	15.08 ± 0.43 A	

Different vertical large letters refer to significant variation at $p < 0.05$.

when compared to values of CNP-0h (30.49 ± 3.98) and control-0h (35.41 ± 5.29), (Fig. 3).

Turbidimetric analysis

Regarding the lag time, significant reduction ($p < 0.05$) was observed in CNP-treated sperms when compared to those of control-0 (3.11 ± 0.26 and 6.46 ± 1.48, respectively), 24 (3.86 ± 0.56 and 4.92 ± 0.75, respectively), 48 (7.35±0.68 and 11.74 ± 1.95, respectively), and 72 hours (7.95±1.06 and 16.66 ± 0.79, respectively). However, values of control were decreased at a time of 0 hour but increased at times of 48 and 72 hours; while in CNP 0.01 ´ 10^{-13} group, values of 24, 48, and 72 hours were elevated significantly when compared to 0 hour (Fig. 4).

Although, the percentages of motility index in CNP-treated sperms and control groups varied insignificantly ($p > 0.05$) at 0 hour (0.222 ± 0.034 and 0.247 ± 0.069, respectively); there was a significant reduction ($p < 0.05$) at 24 (0.037 ± 0.003 and 0.072 ± 0.005, respectively), 48 (0.012 ± 0.002 and 0.036 ± 0.0132, respectively), and 72 hour (0.0089 ± 0.0001 and 0.016 ± 0.0011, respectively). In both CNP-treated sperms and control groups, there were significant decreases ($p < 0.05$) in values of motility index at 24, 48, and 72 hours when compared to 0h (Fig. 5).

The velocity of CNP-treated sperms was increased significantly ($p < 0.05$) when compared to control at all cooling intervals; 0 (0.399 ± 0.048 and 0.208 ± 0.098, respectively), 24 (1.286 ± 0.112 and 0.954 ± 0.143, respectively), 48 (0.89 ± 0.075 and 0.53 ± 0.016, respectively), and 72 hours (0.77 ± 0.031 and 0.324 ± 0.073, respectively). However, values of both CNP-treated sperms and control groups were increased significantly at 24, 48, and 72 hours when compared to 0h (Fig. 6).

Although, FRMS was elevated significantly ($p < 0.05$) in CNP-treated sperms at the time of 0 hour (0.028 ± 0.0063) when compared to control (0.019 ± 0.0047), significant decreases ($p < 0.05$) were reported at 24 (0.013 ± 0.002), 48 (0.006 ± 0.0004), and 72 hour (0.002± 0.0005) when compared to control (0.015 ± 0.0054, 0.007 ± 0.0002, and 0.0055± 0.00014, respectively). However, values of both CNP 0.01´10^{-13} and control groups were reduced significantly at 24, 48, and 72 hours when compared to 0h (Fig. 7).

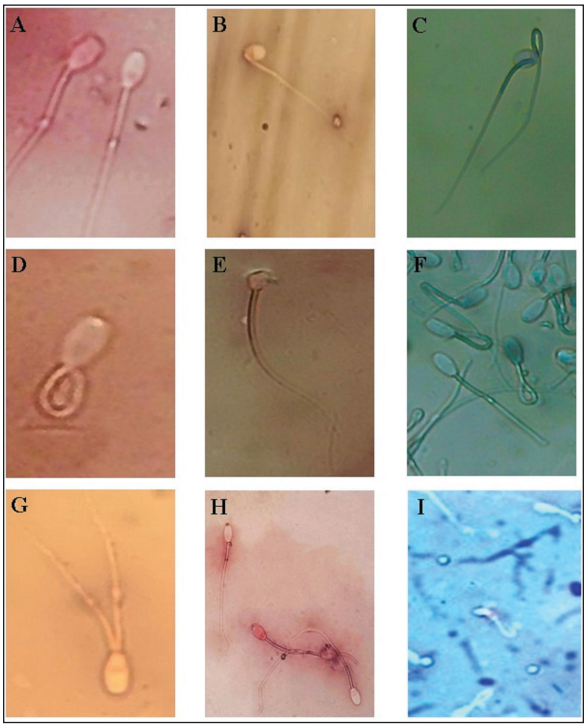

Fig. 2. Microscopic detection of abnormal sperms shows (A): Acrosome defect, (B): Bent neck defect, (C): Double tail, (D): Coiled tail, (E): Irregular head defect, (F): Piriform, (G): Double tail, (H): Plasia sperm, and (I): Tape head.

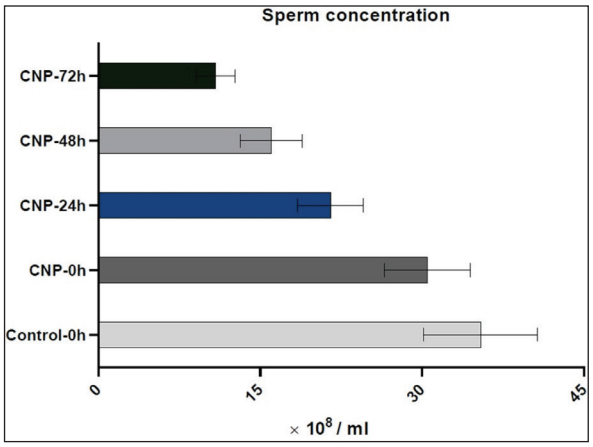

Fig. 3. CNP effect on the concentration of ram sperms during three days of cooling preservation.

Genetic expression of hNPR-B

During 3 days of cooling, the findings of the *hNPR-B* gene were elevated significantly ($p < 0.05$) in CNP-treated sperms at 0 (5.18 ± 0.75), 24 (4.45 ± 0.51), 48 (3.81 ± 0.97), and 72 hours (2.92 ± 0.62) when compared to control groups (1.50 ± 0.50, 1.30 ± 0.42, 1.20 ± 0.25, and 1.10 ± 0.15, respectively). Although significant decreases ($p < 0.05$) in the expression of

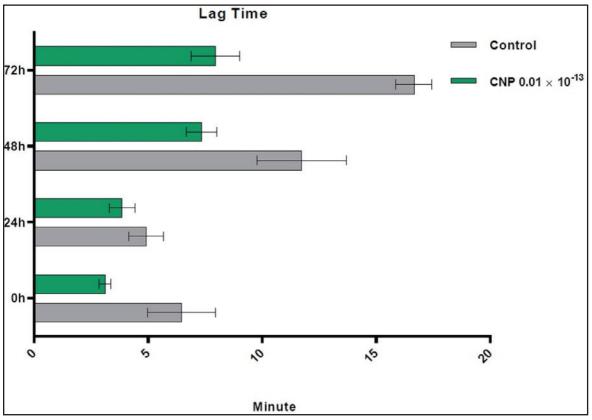

Fig. 4. CNP effect of Turbidimetric analysis of Lag time of ram sperms during three days of cooling preservation.

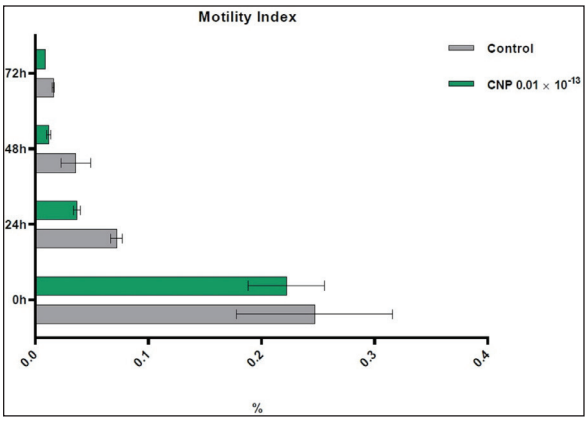

Fig. 5. CNP effect of Turbidimetric analysis of motility index of ram sperms during three days of cooling preservation.

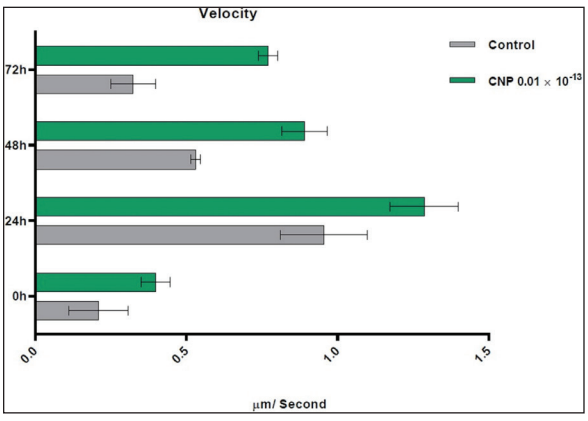

Fig. 6. CNP effect of Turbidimetric analysis of the velocity of ram sperms during three days of cooling preservation.

the *hNPR-B* gene were detected in CNP-treated sperms with advancing days of cooling, the values of this group remain higher than those of control at all times of cooling (Fig. 8).

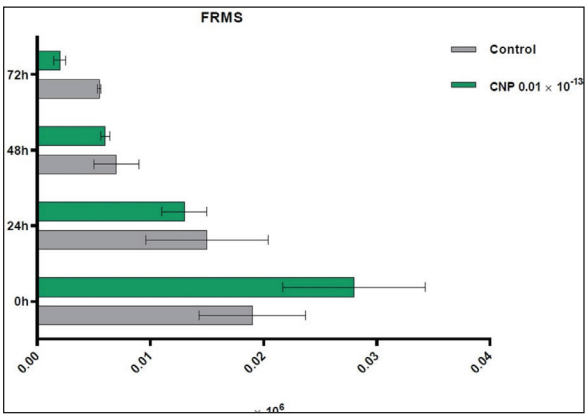

Fig. 7. CNP effect of Turbidimetric analysis of FRMS of ram sperms during three days of cooling preservation.

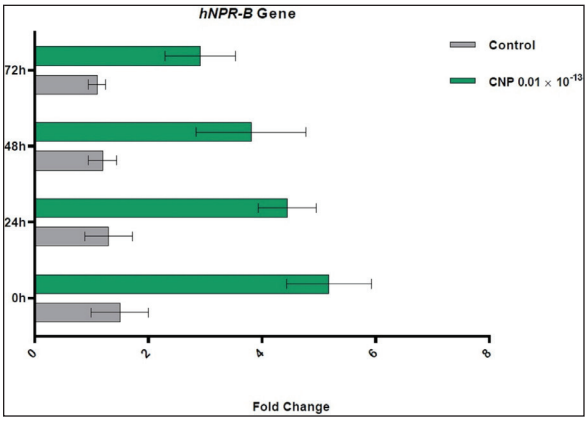

Fig. 8. Expression of *hNPR-B* gene in control and CNP-sperm samples during three days of cooling preservation.

Discussion

For years, semen cryopreservation that comprises different cooling and freezing steps has been a valuable procedure for the application of reproductive technologies such as artificial insemination, *in vitro* embryo production, and embryo transfer since it eliminates the limitations of time and space (Dias *et al*., 2018). In Iraq, the field studies have focused on the significant influence of the quality of thawed semen of bulls with a consequence of variable success rates (Al-Daraji *et al*., 2002; AL-Badry, 2016; Hussain *et al*., 2016; Saleh, 2019; Alhelal and Abdulkareem, 2023); 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 (Ahmad *et al*., 2015), stallion (Cuervo-Arango *et al*., 2015), cattle (Dias *et al*., 2018), donkey (Zhang *et al*., 2018), llama (Zampini *et al*., 2020), and dog (Colombo *et al*., 2022).

Therefore, different additives have been used to improve the physical quality of sperms such as arginine (AL-Ebady *et al*., 2012), caffeine (Shahad *et al*., 2020), and silymarin (Ali *et al*., 2022). In this study, the effect of CNP on cooled sperms of rams was studied for the first time in Iraq, and the results revealed that the lowered concentration of CNP revealed the suitable dose of sperms suspension could be used for artificial insemination of ewes. Worldwide, a number of studies have been performed to detect the effect of CNP on normal human sperm (Xia *et al*., 2016), sperm attraction for fertilization (Kong *et al*., 2017), regulation of sperm capacitation in the genital tract of female rats (Wu *et al*., 2019), anti-inflammatory role in rat epididymitis (Mei *et al*., 2021), sperm motility and reproduction function of azthenozoospermia in mice (Li *et al*., 2022), and spermatozoa maturation in rats (Zhao *et al*., 2023); however, no data were available concerned with the toxicological effect of CNP on sperms. Microscopic examination of sperms suspension treated with the suitable dose of CNP (0.01 ´ 10^{-13}) 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. For the turbidimetric analysis, values of lag time, motility index, velocity, and FRMS showed that the impact of cooling on sperms suspension treated with CNP was lowered in comparison with that detected in sperms samples of control.

Various studies suggested that mouse NPs, including CNP, can exhibit chemoattractant features with the ability to attract mammalian spermatozoa and increase sperm motility (Zamir *et al*., 1993; Bian *et al*., 2012). Xia *et al*. (2016) showed that CNP can induce a significant dose-dependent increase in spermatozoa motility and acrosome reaction, thus, regulating the reproductive function of males. Kong *et al*. (2017) reported that CNP can increase the levels of intracellular cGMP and Ca^{+2} of spermatozoa and induce sperm accumulation by attraction. Özbek *et al*. (2019) observed the CNP expression in the epithelial and smooth muscle cells of epididymis, suggesting that CNP can increase the motility of spermatozoa in epididymis via cGMP. Wu *et al*. (2019) concluded that CNP secreted by the female genital tract might bind to spermatozoa and stimulate successively the intracellular cGMP/PKG signaling by increasing the Ca^{+2} and tyrosine-phosphorylated proteins, promoting hyperactivation and inducing the acrosome reaction which ultimately facilitated sperm capacitation in the genital tract of female rat. Mei *et al*. (2021) showed that the semen samples of asthenospermia patients have a lower concentration of CNP than normal people, with an observable activity in improving the motility of sperm and alleviation of cyclophosphamide damage to sperm motility and reproductive system. Similar findings were recorded by Li *et al*. (2022) 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*. (2023) found that CNP plays a role in epididymal sperm maturation as it promotes the acquisition of epididymal sperm fluidity.

Based on PCR analyses at different times of cooling intervals, the findings of the current study showed that the expression of the *hNPR-B* gene was elevated significantly in CNP samples in comparison with controls. Our findings are in agreement with those demonstrated by other researchers (Ueda *et al*., 2020; Yu *et al*., 2021; Nakagawa and Nishikimi, 2022). NPR-B is a guanylate cyclase-linked receptor that appears to be activated CNP (Pagel-Langenickel *et al*., 2007). Lu and Pan (2017) concluded that CNP can elicit exercise preconditioning-induced protection against tissue injury via the up-regulation of NPR-B receptors. Zhao *et al*. (2023) recorded a significant increase in the expression of *AKAP4*, *Bin1b*, *CD52*, *Dnah17*, and *LDCH* genes in semen samples incubated with CNP; suggesting that, CNP could initiate epididymal motility and regulate the expression of sperm motility and mature-related genes.

Conclusion

This study, done for the first time in Iraq, highlights the important role of CNP in improving the physical properties of ram epididymal sperms while increasing the expression of the *hNPR-B* gene that might play a role in treating growth failure. However, changes that occur after excessive exogenous CNP exposure remain to be clarified, and the toxicological profile of the CNP and its derivatives has required furthermore supplements.

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Conflict of interest

All authors have no conflict of interest to disclose.

Authors' contribution

MSK: Collection and processing of testicular tissues and maturation of sperms. NWZ: Preparation of CNP detection of the level of toxicity at different concentrations. Both authors participate in microscopic examination, turbidimetric analysis, and molecular assaying of gene expression. The final copy of the manuscript was read and approved carefully by both authors.

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Data availability

All data supporting the findings of this study are available within the manuscript.

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