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Effect of in Vitro Maturation Medium Supplementation with Chitosan Nanoparticles on The River Buffalo Cumulus-Oocyte Complexes

Mohamed Elbehary[1], Yousry A Dowidar[1], Abdallah M Ashour[2], Eman M Abd El Fattah[3] and Ahmed Monir[4]*

[1]Biotechnology Department, Faculty of Agriculture, Al-Azhar University, Cairo, Egypt
[2]Animal Production Department, Faculty of Agriculture, Al-Azhar University, Cairo, Egypt
[3]Biotechnology Research Unit, Animal Reproduction Research Institute, Agriculture Research Center (ARC), Giza, Egypt
[4]Artificial Insemination and Embryo Transfer Department, Animal Reproduction Research Institute, Agriculture Research Center (ARC), Giza, Egypt
***Corresponding author:** ahmed.elruby@gmail.com

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ABSTRACT

The present study aimed to investigate the possible effects of chitosan nanoparticles (CNPs) supplementation to the in vitro maturation medium on the expansion of cumulus cells, nuclear maturation, and relative gene expression of Superoxide dismutase-1 (SOD1), B-cell lymphoma-2 Apoptosis Regulator (BCL2), and BCL2 Associated X (BAX) in the River buffalo. Slaughterhouse-derived cumulus-oocyte complexes (COCs) became matured in vitro in the absence (control) or presence of CNPs (10, 25, and 50µg/mL). At the end of maturation, we assessed expansion rates and denuded COCs; then, some replicates were fixed and stained to determine nuclear maturation. Other replicates were vitrified until we acquired enough oocytes, thawed, and assessed gene expression. We concluded that 10µg/mL CNPs significantly increased cumulus cell expansion and nuclear maturation. Whereas 10 and 25µg/mL CNPs non-significantly increased SOD1 relative expression, BCL2/BAX ratio for 10µg/mL CNPs was significantly higher than in control, 25 and 50µg/mL CNPs groups.

Key words: Buffalo; Oocyte; In vitro maturation; Chitosan nanoparticle; Gene expression.

INTRODUCTION

Buffaloes are economically important animals in many Asian, Mediterranean, Latin American, and European countries because they are important source of milk and meat (Ondho et al. 2020; Bertoni et al. 2021). However, the reproductive performance of buffaloes is low due to delayed onset of puberty, silent estrus, poor conception rates, low numbers of Graafian follicles on ovaries, and poor response to hormonal stimulation, which decreases the yield of embryos in embryo transfer practices (Mahesh et al. 2017). This has resulted in increased attention to in vitro embryo production (IVEP) technologies for the rapid proliferation of superior traits in this species(Gasparrini 2013). In vitro environment exposes oocytes and embryos to much higher activities of reactive oxygen species (ROS) compared to physiological in vivo procedures (Gupta et al. 2010). Furthermore, in vitro procedures usually lack enzymatic antioxidants which combat ROS, such as superoxide dismutase, catalase and glutathione peroxidase (Agarwal and Majzoub 2017). Reactive oxygen species have been shown to adversely affect in vitro embryo production (IVEP) through increased membrane selective permeability, enzyme inactivation, DNA fragmentation, mitochondrial enzyme leakage and apoptosis induction (Agarwal and Majzoub 2017).

Chitosan is a glucosamine polymer produced by the de-acetylation of purified chitin (Rampino et al. 2013). It has several biological activities, including its ability to scavenge free radicals (Park et al. 2004). This polymer has been shown to enhance the maturation of porcine oocytes (García et al. 2015). Furthermore, low concentrations of chitosan nanoparticles (CNPs) significantly increased the intracellular Glutathione levels and reduced ROS, which led to significantly higher maturation, cleavage, and blastocysts formation rates (Roy et al. 2021). In cattle, a low concentration of CNPs suppressed the negative effect of linoleic acid (LA) on both the nuclear maturation and cumulus cells expansion. Furthermore, it supressed the detrimental effects of LA on cleavage and blastocyst rates

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(Abdel-Halim 2018). To the best of our knowledge, the possible effects of CNPs on the maturation of buffalo oocytes have not been studied so far. Therefore, aim of the present study was to investigate the effect of supplementation of in vitro maturation medium with chitosan nanoparticles on river buffalo cumulus-oocyte complexes.

MATERIALS AND METHODS

Ethics Approval

All experiments of this study were approved by the Research Committee of the Biotechnology Department, Agriculture Faculty, Al-Azhar University, Egypt, in their 17 February 2021 session.

Chitosan Nanoparticles Preparation

Chitosan nanoparticles were prepared as described by Darwesh et al. (2018). In brief, 14mL of 0.1% tri-sodium polyphosphate (in distilled water) and 35mL of 1% chitosan (in 2.0% acetic acid) were mixed together at room temperature while maintaining mild stirring (550rpm). Then the mixture containing CNPs was subjected to centrifugation for 10min at 10,000rpm. The supernatant was removed; CNPs pellet was washed with distilled water, then ethanol and air dried and stored at 5°C.

For characterization of CNPs, a drop of chitosan (dissolved in distilled water) was placed on the carbon-coated copper grid and air dried at room temperature. Electron micrographs were obtained using a JEOL GEM-1010 transmission electron microscope at 80kV (Amin et al. 2021). The mean diameter for CNPs was 16.7nm (Fig. 1).

The in vitro maturation medium contained M199 (earl salts) supplemented with 25mM sodium bicarbonate, 10% heat-inactivated FCS, 0.2mM sodium pyruvate, 5µg/mL LH, 0.5µg/mL FSH and 1µg/mL estradiol-17β (El-Ruby et al. 2017). The fixative solution used for fixation of oocytes contained acetic acid and ethanol (1:3 v/v). Stain differentiation solution (Aceto-orcein stain, 1%) was composed of acetic acid, distilled water, and glycerol (1:3:1 v/v/v). All vitrification and thawing solutions were prepared according to the protocol described by Attanasio et al. (2010). QIAzol® lysis reagent (QIAGEN, Meryland, USA) was used for the total RNA isolation. Thermo Scientific RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher scientific Inc., Lithuania) was used in the study. Maxima SYBR Green qPCR master mix (2x) (Thermo Fisher Scientific inc., Lithuania) was used for Real-Time PCR. Four gene specific primers were applied in the study. These included SOD1, BCL2, BAX and B actin. The SOD1 had accession number of NM_001290973.1, product length of153bp, with primer sequences of F- GAGAGGCATGTTGGAGACCT and R – TCTGCCCAAGTCATCTGGTT (Khalil et al. 2021). For BCL2, the accession number was XM_025273634.1, product length was110bp, with primer sequences of F- ATGACTTCTCTCGGCGCTAC and R- TGAAGAGCTC CTCCACCAC (Khalil et al. 2021). BAX had accession number of XM_006050927.2, product length 122bp and primer sequences of F- CTTTTGCTTCAGGGTTTCA and R- CGCTTCAGACACTCGCTCA (Khalil et al. 2021). For B actin, accession number was XM_006044278.1, product length was 150bp, the primer sequences were F-GCCCTGGCACCCAGCACAAT and R-GGAGGGGCC GGACTCATCGT (Zhao et al. 2019).

Oocytes Recovery and Maturation

For recovery of oocytes, medium-sized (4-8mm) follicles were aspirated within 2-3 hours after slaughter by an 18-gauge needle (Yousaf and Chohan 2003). Only cumulus-oocyte-complexes (COCs) having evenly granular and homogenous ooplasm and more than two layers of dense cumulus cells were collected. The collected COCs were washed many times in HEBES buffered TCM199, followed by incubation in IVM (10 oocytes/100µL) for 22h at 38.5°C under mineral oil and 5% CO_2 in the air with high humidity (El-Ruby et al. 2017).

Assessment of Cumulus Cell Expansion

Visually, oocyte expansion was classified into three categories: non or low expansion, moderate expansion, and high expansion (Chauhan et al. 1999). For denuding, oocytes were exposed to 0.3% hyaluronidase in PBS and then repeatedly pipetted through a narrow flame-pulled Pasteur pipette, as described by Prentice-Biensch et al. (2012). The denuded and matured oocytes were fixed and stained by Aceto-orcein stain to evaluate their maturation. For this purpose, the protocol described by Prentice-Biensch et al. (2012) was followed. Oocytes with condensed chromatin were considered as mature. Then the matured oocytes were subjected to vitrification and thawing following the procedure of Attanasio et al. (2010).

Total RNA Extraction and First Strand cDNA synthesis

The oocytes were lysed byQIAzol®lysis reagent (QIAGEN, Meryland, USA), as per instructions of the manufacturer. First Strand cDNA Synthesis (Thermo Fisher Scientific Inc., Lithuania) kit was used to generate cDNA from the RNA template immediately after total RNA extraction, as recommended by the manufacturer. The cDNA was stored by deep freeze till use.

Quantitative Real-time PCR

Gene expression levels for SOD1, BCL2, and BAX genes with B-actin as the housekeeping gene was evaluated using the Rotor-GeneTM 6000 Real-Time PCR Thermo cycler (Corbett Life Science, Australia) and primers developed based on cDNA sequences. Maxima SYBR Green qPCR master mix (2x) (Thermo Fisher Scientific inc., Lithuania) was used according to the manufacturer's instructions. The fold difference method $2^{-\Delta\Delta Ct}$ was used to quantify gene expression levels for target genes, as described by Yadav et al. (2013).

Experimental Design and Statistical Analysis

COCs were recovered and matured in the absence (control) or presence of CNPs (10, 25, and 50µg/mL). At the end of maturation, expansion rate was assessed, then oocytes were denuded, then either fixed and stained to assess maturation or vitrified until enough oocytes were acquired for each replicate. The vitrified oocytes were thawed, and the gene expression was assessed. Each experiment was repeated at least three times. Mean values (±SEM) of various parameters for different CNPs treatment groups were computed. The magnitude of variation in cumulus expansion, nuclear maturation and gene

expression for different CNPs treatment groups was assessed using ANOVA and LSD post hoc test (SPSS 23).

RESULTS

Effect of IVM Media Supplementation with CNPs on Cumulus Cell Expansion

As shown in Table 1, supplementation of IVM with 10µg/mL CNPs led to a significant (P<0.05) increase in high cumulus cell expansion when compared to control, 25 and 50µg/mL CNPs groups; where high cumulus cell expansion rates were 73.97±3.64, 48.96±4.19, 57.14±4.20 and 56.83±4.22%, respectively. The differences in high cumulus cell expansion rates among control, 25 and 50µg/mL CNPs groups were non-significant. Moreover, supplementation of IVM with 10µg/mL CNPs also led to a significant (P<0.05) increase in expanded COCs when compared to control (98.63±0.97 Vs. 93.01±2.14%). The rates of expanded COCs of 25 and 50µg/mL supplemented groups were 96.43±1.57 and 94.96±1.86%, respectively.

Table 1: Effect of IVM media supplementation with CNPs on cumulus cell expansion (N=568)

Experimental Groups	High cumulus expansion COCs (%)	Expanded COCs (%)
Control	48.96±4.19a	93.01±2.14a
10µg/mL	73.97±3.64b	98.63±0.97b
25µg/mL	57.14±4.20a	96.43±1.57ab
50µg/mL	56.83±4.22a	94.96±1.86ab

Values (Mean±SEM) with different alphabets within same column indicate significant difference (P<0.05).

Effect of IVM Media Supplementation with CNPs on the Nuclear Maturation

Supplementation of IVM with 10µg/mL CNPs led to a significant (P<0.05) increase in oocyte nuclear maturation rate compared to control; where rates were 84.21±4.21 and 64.10±5.47% for 10µg/mL CNPs and control groups, respectively. Supplementation of IVM with 25 and 50µg/mL CNPs also increased oocyte nuclear maturation rates compared to the control, but the difference was non-significant. Oocyte nuclear maturation rates for 25 and 50µg/mL CNPs and control groups were 73.75±4.95, 74.36±4.98 and 64.10±5.47%, respectively.

Effect of IVM Media Supplementation with CNPs on SOD1, BAX and BCL2 Relative Gene Expression

As shown in Table 2, supplementation of IVM with 10 and 25µg/mL CNPs non-significantly increased SOD1 relative expression when compared to 50µg/mL CNPs and control groups. Values for SOD1 relative expression were 1.25±0.12, 1.30±0.29, 1.03±0.12 and 1.00±0.00 for 10, 25 and 50µg/mL CNPs and control groups, respectively. Regarding BAX relative expression, 10 and 50µg/mL CNPs supplementation significantly (P<0.05) decreased the relative expression when compared to control, where expressions were 0.72±0.00, 0.64±0.27, and 1.00±0.00, respectively. Relative BAX expression of 25µg/mL CNPs supplemented group (0.88±0.15) differed non-significantly from 10µg/mL CNPs and control groups. For BCL2 expression, 10 and 25µg/mL CNPs IVM supplementation significantly (P<0.05) increased the values when compared to control, where expression values were 2.07±0.27, 1.95±0.39, and 1.00±0.00, respectively. Relative BCL2 expression for 50µg/mL CNPs supplemented group was 1.36±0.57, which neither differed significantly from 10 and 25µg/mL CNPs nor the control group. All CNPs supplemented groups (10, 25 and 50µg/mL) had significantly (P<0.05) higher BCL2/BAX ratio when compared to control, the values were 2.89±0.37, 2.19±0.08, 2.14±0.00 and 1.00±0.00, respectively. BCL2/BAX ratio for 10µg/mL CNPs group was the highest when compared with control, 25 and 50µg/mL CNPs supplemented groups (P<0.05).

DISCUSSION

During the in vitro maturation of oocytes, FSH stimulates epidermal growth factor synthesis, which in turn stimulates cumulus cells to secrete hyaluronic acid rich extracellular matrix expanding the cumulus cells. This expansion promotes meiosis resumption by disrupting the cumulus oocyte gap junctions and preventing cAMP/cGMP flow into the oocyte (Sugimura et al. 2018). Therefore, cumulus cell expansion can be used as a marker for assessing the oocyte developmental competence (Han et al. 2006).

Data present in the current study revealed that 10µg/mL CNPs supplementation significantly (P<0.05) enhanced the cumulus cell expansion and oocyte maturation. All groups supplemented with CNPs had non-significant increase of SOD1 expression. BCL2 expression was significantly (P<0.05) increased in 10 and 25µg/mL CNPs supplemented groups. BAX expression was significantly (P<0.05) decreased in 10 and 50µg/mL CNPs supplemented groups. Although All CNPs supplemented groups significantly increased BCL2/BAX ratio compared to control, 10µg/mL CNPs supplemented group showed the highest ratio when compared with control, 25 and 50µg/mL CNPs supplemented groups (P<0.05).

These results agree with those reported by Abdel-Halim (2018), where 10µg/mL CNPs suppressed the detrimental effect of LA on nuclear maturation and cumulus cell expansion. These results are also in line with those of Roy et al. (2021), who found that 25µg/mL CNPs supplementation led to a significant increase in porcine oocyte nuclear maturation. The difference in CNPs dose of the present study and that of Roy et al. (2021) may be attributed to species difference, as the porcine oocytes are more susceptible to lipid peroxidation than bovine due to the presence of more fatty acid contents (McEvoy et al. 2000).

The data in the present study showed that low concentrations of CNPs (10 and 25µg/mL) non-significantly increased the SOD1 expression. SOD1 is a vital gene responsible for the SOD enzyme, which combats oxidative stress, and is the first enzymatic defense against ROS. It protects cells from ROS and produces hydrogen peroxide (H_2O_2) as a by-product, which is then eliminated either by catalase (CAT) or Glutathione Peroxidase (GSH Px) (Wang et al. 2018). So, CNPs supplementation increases SOD1 expression and helps combat ROS, as increase in ROS induces DNA fragmentation (Ashibe et al. 2019), lipid peroxidation, and apoptosis (Tiwari et al. 2016). Furthermore, the study revealed that CNPs also influenced BAX and BCL2 expression. BAX and BCL2 are essential in regulating apoptosis (Yang and Rajamahendran 2002). Kim and Tilly (2004) investigated their role in

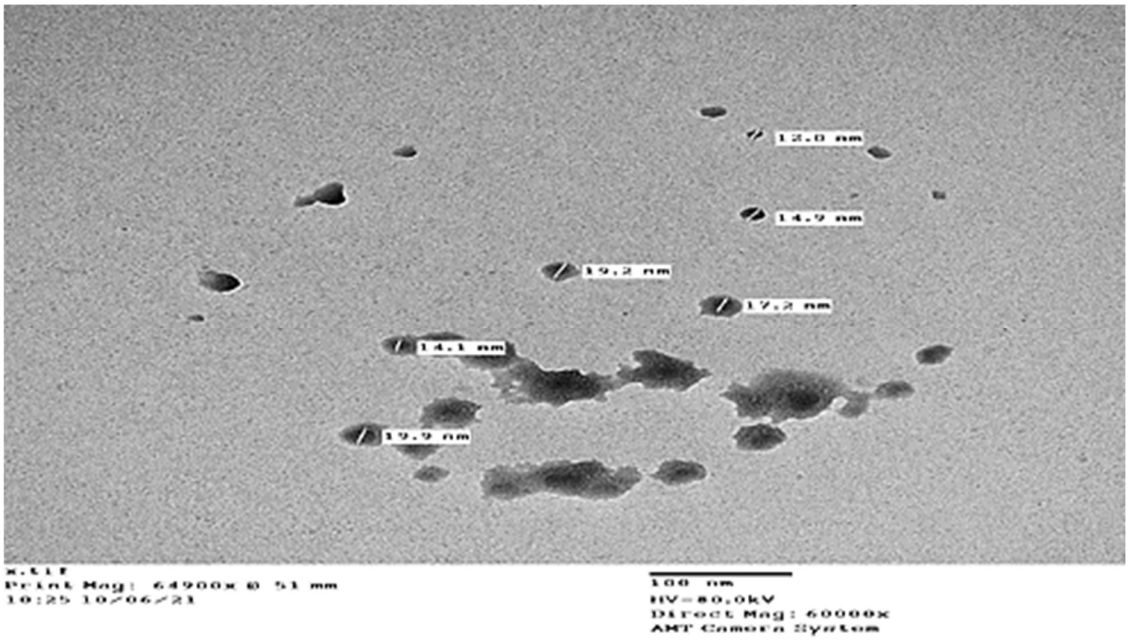

Fig. 1: Transmission electron microscopy image showing CNPs and their diameter in nm.

Table 2: Effect of IVM media supplementation with CNPs on SOD1, BAX and BCL2 relative gene expression

Experimental Groups	SOD1	BAX	BCL2	BCL2/BAX
Control	1.00±0.00	1.00±0.00a	1.00±0.00a	1.00±0.00a
10µg/mL	1.25±0.12	0.72±0.00bc	2.07±0.27b	2.89±0.37c
25µg/mL	1.30±0.29	0.88±0.15ab	1.95±0.39b	2.19±0.08b
50µg/mL	1.03±0.12	0.64±0.27c	1.36±0.57ab	2.14±0.00b

Values (Mean±SEM) with different alphabets within same column indicate significant difference (P<0.05).

oocyte apoptosis, indicating that BAX and BCL2 were the critical factors in initiating or suppressing apoptosis, respectively. According to Yang and Rajamahendran (2002), good grade oocytes and embryos show significantly higher BCL2 expression than BAX expression and vice versa. On the other hand, expression of BAX was much higher than BCL2 expression in poor grade oocytes and embryos. A higher expression of BCL2 than BAX is an indication of healthy embryo. On the other hand, when BAX expression is higher than BCL2, the embryo is of low quality and fragmented. So the favorable impact of 10µg/mL CNPs on oocytes may be explained by its up-regulation of SOD1 and BCL2 and down-regulation of BAX, which in turn enhance oocyte and embryo developmental competence and reduce their apoptosis. The CNPs-induced improvement can be attributed to CNPs antioxidant capability, where CNPs were closely effective as Vitamin C for the protection of macrophages against H_2O_2. Moreover, the CNPs augmented the mRNA expression of SOD and GSH Px (Wen et al. 2013). Chitosan, as bulk material, has free radical scavenging activities (Park et al. 2004). Chitosan significantly lowered free fatty acids and malondialdehyde serum concentrations, elevated SOD, CAT, and GSH Px, resulting in reduced lipid peroxidation (Liu et al. 2008). When exposed to peroxyl radicals, chitosan prevented carbonyl and hydroperoxide synthesis in human serum albumin (Anraku et al. 2008). Moreover, it removed hydroxyl radicals and stopped phosphatidylcholine and linoleate liposome peroxidation (Lee et al. 2018). It prevented the oxidation of hepatotoxic lipids in isoniazid or rifampicin-treated rats (Santhosh et al. 2006). Furthermore, it suppressed glycerol renal oxidative damage either in vitro or in vivo (Wen et al. 2013).

The higher concentrations of CNPs didn't improve the cumulus cells' expansion and oocyte maturation, which is supported by the findings of Roy et al. (2021) and Abdel-Halim (2018). This might have been due to CNPs toxicity when used at high concentrations, as has been reported earlier in porcine oocytes (Roy et al. 2021), bovine oocytes (Abdel-Halim 2018), and zebrafish embryos (Hu et al. 2011).

Conclusion

Supplementation of IVM with low concentration of CNPs (10µg/mL) led to a significant enhancement in cumulus cell expansion and maturation rates. It also significantly increased BCL2/BAX relative gene expression ratio compared to other groups. Furthermore, it non- significantly increased SOD1 relative gene expression. So, further studies are needed to evaluate CNPs impact on cleavage and blastocyst formation rates.

Authors' Contribution

All authors contributed equally in the study.

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