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Effect of Melatonin-loaded Chitosan Nanoparticles (CMN) on Gene Expression of *In vitro* Matured Buffalo Oocyte

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Abstract

This study aimed to evaluate the effect of melatonin and melatonin-loaded chitosan nanoparticle (CMN) supplementation to maturation media on buffalo oocyte maturation rate and relative expression of genes: growth differentiation factor 9 (*GDF9*), bone morphogenetic protein 15 (*BMP15*), B Cell Lymphoma 2 (*BCL2*), Associated X protein (*BAX*) and superoxide dismutase 1 (*SOD1*). Buffalo ovaries were heaved from Al-Mounib slaughterhouse, cumulus-oocyte complexes (COCs) were *in vitro* matured in three different media, TCM-199 medium (control), TCM-199 with melatonin 10^{-9}M, and TCM-199 with CMN 10^{-9}M. The assessment of the nuclear maturation rate was carried out through the presence of the first polar body. In addition, the mature buffalo oocytes were stored on RNA later for genetic analysis of *GDF9*, *BMP15*, *SOD1*, *BCL2*, and *BAX* genes using quantitative real-time PCR (qRT-PCR). The results were reported that buffalo oocytes supplemented with melatonin-loaded chitosan nanoparticle (CMN) or melatonin have a significant effect on nuclear maturation rate 94.04±0.65 and 88.74±0.77 respectively when compared with buffalo oocytes matured with basic media (control) 79.67±1.35. Furthermore, buffalo oocytes supplemented with melatonin-loaded chitosan nanoparticle (CMN) or melatonin showed significant upregulation of *GDF9*, *BMP15*, *SOD1*, and *BCL2* genes and significant downregulation of *BAX* gene when compared with oocyte matured with basic media (control). In conclusion, the results of nuclear maturation rate and relative expression pattern of *GDF9*, *BMP15*, *SOD1*, *BCL2*, and *BAX* reflect that melatonin-loaded chitosan nanoparticle (CMN) and melatonin` may play an important role in the buffalo oocytes developmental competence.

KEYWORDS
Buffalo oocyte, Melatonin-loaded chitosan nanoparticle (CMN), Melatonin, *In vitro* maturation, Relative gene expression.

INTRODUCTION

Reducing reproductive efficiency is one of the critical problems of water buffalo (Tawfik *et al*., 2018). The lower competence of *in vitro* embryo production (IVEP) in buffalo can be enhanced by using high-quality oocytes or by adjusting the system of IVEP (Abd El-Raheem *et al*., 2020). The metabolic alterations in *in vitro* maturation (IVM) media reflected indirectly on buffalo oocyte competence and early embryo development (Abd Ellah *et al*., 2010). The successful *in vitro* embryo production system is principally based on the quality of the oocytes, hence, buffalo oocyte quality is a vital basis of oocyte developmental competence, which is distinct as the capability of the oocyte to mature and extruded the first polar body, get successful fertilization, and develop to the blastocyst stage. Morphological assessment of cumulus-oocyte complex (COC) is based on the number of layers and compactness of cumulus cells, homogeneity of oocyte cytoplasm, and extrusion of the first polar body (Ismail *et al*., 2016). Also, the addition of hormones, elements, serum, and antioxidants to *in vitro* maturation medium enhances the oocyte maturation rate either cytoplasmic or nuclear and decreases the oocyte degeneration (McKenzie *et al*., 2004) which may affect the expression of some antioxidant-related genes, cell marker genes, and anti-apoptosis related genes in buffalo oocytes via some of the biochemical and molecular alterations which enhance the oocyte competence (Sharma *et al*., 2012). Excessive reactive oxygen species (ROS) lead to failure in the maturation of oocytes which may result from low antioxidant defense (Jiao *et al*., 2013). Therefore, the use of antioxidants can overcome and reduce the cell damage caused by ROS which activates some antioxidant enzymes such as superoxide dismutase (SOD) (Fischer *et al*., 2013). So, some researchers used melatonin (N-aceyl-5-methoxytryptamine) supplementation in *in vitro* maturation media which has antioxidant effects on cytoplasmic and nuclear maturation of the porcine oocyte (Yang *et al*., 2020). Moreover, melatonin supplementation enhances the *BMP15* mRNA expression which is related to the developmental competence of bovine oocytes (Kirikin *et al*., 2013). Also, the amphiphilic nature of melatonin helps it to diffuse broadly in diverse subcellular compartments barriers causing a superior anti-apoptotic effect, which prevents nitrosative damage to all macromolecules in all cell compartments (Ramis *et al*., 2015). Furthermore, melatonin act as a cytoprotective agent, which protects the cell genome and cell membrane against damage (Luchetti *et al*., 2010). However, due to some chemical properties of melatonin, including its short half-life and low solubility in water, there are some challenges to using mel-

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atonin (Hoffmeister *et al*., 2012). Therefore, nano-capsules are a promising technology that may rescue the challenges faced using melatonin *in vitro* by enhancing the stability and solubility of melatonin in water (Remião *et al*. 2016).

Nanotechnology has made significant advances in several sectors of research, including biological, medical, and reproductive sciences. As an interesting alternative for drug release, nanotechnology has some advantages such as reducing drug cytotoxicity, increasing bioavailability, enhancing gametes and embryo development, and increasing gamete protection against lipid peroxidation (Barkalina *et al*,. 2014). Also, among many genes, the growth differentiation factor 9 (*GDF9*), and bone morphogenetic protein 15 (*BMP15*) transcripts are considered to be oocyte-specific markers (Pennetier *et al*., 2004). Both factors play important roles in granulosa cell proliferation and buffalo oocyte maturation (Nath *et al*., 2013). *GDF9* and *BMP15* are members of the transforming growth factor-beta superfamily which apply vital actions on oocyte competence. On the other hand, apoptosis is genetically controlled and can be initiated in two major ways, the dead receptor and mitochondrial pathway (Paulini and Melo, 2011). Bcl-2 gene family is considered pro and anti-apoptotic of programmed cell death which saves the mitochondrial integrity, thus preventing the release of apoptotic factors such as cytochrome c (Chakravarthi *et al*., 2015). Furthermore, gonadotropic hormone-induced granulosa cell survival is associated with a decrease in the amount of expression of the *BAX* gene, with a high level of *BAX* indicating granular cell death, follicular atresia, and fetal germ cell apoptosis. (Kirikin *et al*., 2013). Finally, there is a few research work on the study of lipid core nanocapsule melatonin supplementation in *in vitro* maturation media of buffalo oocytes. Therefore, in the focus of the above-detailed studies, the objectives of this study were to study the effect of melatonin and melatonin-loaded chitosan nanoparticle (CMN) supplementation of *in vitro* maturation medium on *in vitro* maturation rate and detect its effect on gene-related for buffalo oocyte developmental competence.

MATERIALS AND METHODS

Chemicals

Unless otherwise specified, all the compounds utilized in this investigation were bought from Sigma-Aldrich , Germany. Pico pure RNA kit free DNase, for mRNA isolation of oocytes and transferable embryo (Thermo Fisher Scientific). QuantiTect Reverse Transcription Kit for cDNA synthesis (QIAGEN). Primers of candidate genes *BMP15*, *GDF9*, *BCL2* and *BAX* (QIAGEN). Chitosan (100-300 KD.) was obtained from Acros-Organic com. Thermo Fisher Scientific New Jersey-US (CAS: 9012-76-4, degree of deacetylation was 90%). Sodium tripolyphosphate (TPP) and melatonin were purchased from Sigma-Aldrich, France. Acetic acid and ethanol were purchased from Elnaser for the chemical industry, in Egypt.

Preparation of melatonin-loaded chitosan nanoparticles (CMN)

According to Dzung *et al*. (2011) synthesized chitosan nanoparticles using the ionic gelation process consuming TPP as an ion crosslinking agent. In brief, 1% chitosan was dissolved in 1% acetic acid solution, on pH 5.5, then filtered. Melatonin was dissolved in 100 µmol/L chitosan solution, and the final volume of TPP solution (0.5 mg/mL) was the third of CS solution volume (1 mg/mL) under magnetic stirring for 1 hour. Then, after half an hour of centrifugation at 10,000 RPM the nanoparticles were produced and finally rinsed with deionized water, lyophilized, and kept at 4 °C.

Characterization of CMN nanoparticles

The morphology of CMN was studied using transmission electron microscopy (TEM) (HT7700; Hitachi, Tokyo, Japan). The zeta potential and the size of the nanoparticle were analyzed, using Particle Sizing Systems, Inc. Santa Barbara, Calif., USA.

Drug Incorporation Determination

The concentration of MTNPs in dichloromethane was diluted 100 times with 30% ethanol. After vigorous stirring and 20 minutes of standing, the water phase was collected. The water phase's absorbance at 278 nm was determined using a UV spectrophotometer. Using the conventional melatonin curve at 278 nm, the melatonin concentration was determined
Encapsulation efficiency= mass of melatonin nanoparticle X 100/ mass of initial melatonin

Collection and in vitro maturation of oocytes

The ovaries of the buffalo were harvested from the Al-Monib slaughterhouse in Cairo Governorate in Egypt and transferred to the lab in a tank filled with normal saline solution (NSS, 0.9% NaCl with100 µg/ml streptomycin, and 100 IU penicillin). Ovaries were cleaned in the lab many times in pre-heated (37 °C) NSS and then maintained at this temperature till aspiration. By 18-gauge needle connected to a sterile syringe filled with 3 ml of phosphate-buffered saline (PBS) + 6 mg/ ml bovine serum albumin F-V + 50 µg/ml gentamicin, oocytes were aspirated from follicles with a diameter of 2 to 8 mm. Following aspiration, follicular fluid was placed in a Falcon tube and left for settling down for 15 minutes at 37 °C water bath. COCs were examined by a stereo microscope at a magnification of 90x and washed three times in an aspiration medium.

According to Kandil *et al*. (1999), buffalo oocyte quality was assessed. Depending on the cumulus investment and equally granulated ooplasm, there were four groups of COCs under a stereomicroscope (90 x) as follows:

Excellent: Oocytes with at least five layers of fully developed cumulus cells (CC) and evenly granulated dark ooplasm.
Good: One to four layers of cumulus cells and evenly granulated dark cytoplasm.
Fair: Oocytes are incompletely encircled by cumulus cells, and the ooplasm has little granulation.
Denuded: Oocytes were covered by zona pellucida and had no cumulus cells.

Good-quality oocytes (excellent and good) with three different maturation regimens: (a) TCM-199 supplemented with fetal calf serum (FCS), follicular stimulating hormone (FSH) (10 µg/ml), pregnant mare serum (PMS) (100 IU/ml), gentamycin (basic media). (b) Basic media + melatonin 10^{-9}M. (c) Basic media + melatonin-loaded chitosan nanoparticle (CMN) 10^{-9}M. Maturation of the oocytes was done in the incubator for 22 h in at humidified environment with 5% CO_2 and 38.5 °C.

According to the extent of cumulus-cell development, the cytoplasmic maturation of buffalo oocytes was evaluated and divided into 4 grades (Kandil *et al*., 1999).

G0: Without expansion.
GI: With slight expansion.
GII: Moderate expansion is moderate.
GIII: Complete expansion.

Oocytes' nuclear maturation was determined by the presence of the 1st polar body (Pb) in the perivitelline space. The Pb was found using an inverted microscope at 200X magnification.

The expansion rate was obtained by dividing the number of oocytes per grade by the total number of oocytes and then the results were multiplied by 100.

The nuclear maturation rate (M II) was calculated by dividing the number of mature oocytes with 1st Pb on the total number of oocytes, and the results were multiplied by 100.

Mature oocytes were denuded completely by repeated pipetting and the nuclear maturation rate was determined (oocyte with first polar body / total number of oocytes). Mature oocytes were stored in RNA later solution at - 80 °C (ultra-deep freeze) until RNA isolation.

RNA extraction and reverse transcription

PicoPureTM RNA Isolation Kit (Arcturus, ThermoFisher, Lithuania) was used to extract mRNA from matured buffalo oocytes. Briefly, oocytes were lysed and adhered to a membrane, where they were washed multiple times before being eluted in 12 µL. RNA concentration was measured using a NanoDrop spectrophotometer (NanoDrop 2000, ThermoFisher, USA). The QuantiTect Reverse Transcription kit (Qiagen, Germany) was used for cDNA synthesis according to the manufacturer's instructions, and the samples were kept at -20 °C.

Real-time Polymerase Chain Reaction (PCR)

All gene transcripts were quantified using real-time quantitative RT-PCR (Quando studio, Applied Biosystems, USA) and Maxima SYBR Green QPCR Master Mix (Thermofisher, Lithuania). According to Livak (2001), primers were customized with the Primer Express program and obtained from GenBank bovine sequences (see Table 1). In a total reaction volume of 25 µL, a primer matrix was composed of 2 µL (50 µg cDNA/µl), 1 µL each of forward (5 mM) and reverse (5 mM) primers, 8.5 L nuclease-free water, and 12.5 µL SYBR Green PCR Master Mix.

In a Quantstudio Real time PCR (qRT-PCR) (Applied Biosystems, USA), triple reactions were completed for each sample. For each primer set, two non-template control samples were added to each plate. Melting curve analysis confirmed the specificity of amplification with each primer set in each assay. The thermal cycler program included 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. For real-time PCR experiments, amounts of mRNAs of interest were compared to the endogenous bovine Glyceraldehyde-3-Phosphate Dehydrogenase (*GAPDH*) gene, and then quantitative analysis was performed using the cycle threshold (CT) method 60, with results reported as fold change compared to the transcript level to the endogenous control.

Experiment design

Ethical approval

This investigation was conducted following standard protocols, with no discomfort or injury to the buffalo. Furthermore, the National Research Centre's Ethics Committee in Cairo, Egypt (NRC, ID: 19/145) accepted the experimental approach.

Studying the effect of melatonin and melatonin-loaded chitosan nanoparticle (CMN) supplementation of *in vitro* maturation media on the maturation rate of buffalo oocytes.

High-quality buffalo oocytes were washed 3 times in PBS and TCM-199 medium then matured in TCM-199 or TCM-199 with melatonin 10^{-9}M or TCM-199 with CMN 10^{-9}M according to its group and incubated in 5% CO_2, 95% humidity at 38.5ºC for 22 h. The evaluation of cytoplasmic and nuclear maturation of buffalos oocytes was carried out according to cumulus oocytes complexes (COCs) cytoplasmic maturation and judged into 4 grades (G0, GI, GII, GIII) (Kandil *et al*., 1999), The presence of the first polar body was the criteria for mature oocyte. After evaluation of cytoplasmic maturation, oocytes were completely decomulated then count the number of oocytes with the first polar body (matured oocytes). Nuclear maturation rate determined (number of oocytes with polar body/ number of total oocytes). Matured oocytes were washed three times with PBS and stored on 10 µL RNA later at -80 °C for gene expression analysis.

Studying the effect of melatonin and melatonin-loaded chitosan nanoparticles (CMN) supplementation in *in vitro* maturation media on gene expression of *in vitro* mature buffalo oocyte.

This experiment was done on 1944 buffalo oocytes which were divided into three groups (control, melatonin 10^{-9}M, and melatonin-loaded chitosan nanoparticle (CMN) 10^{-9}M). The matured oocytes were washed with PBS three times and stored on 10 µL RNA later at -80 °C for gene expression analysis. RNA was extracted from all groups followed by cDNA synthesis as described before. Quantitative analysis of *GAPDH*, *BMP15*, *GDF9*, *BAX*, *BCL2*, and *SOD1* genes as performed using (SYBR Green PCR Master Mix).

Table 1. Details of primers used for quantitative real-time PCR

Gene Name	Primer sequence	Access Gene Bank Number	Annealing tempature	Amplicon size (bp) of product
GAPDH	F: 5-ACCCAGAAGACTGTGGATGG-3 R: 5-ACGCCTGCTTCACCACCTTC-3	BC102589	60	247
BMP15	F: 5-CTGACGCAAGTGGACACCCTA-3 R: 5-GACACACGAAGCGGAGTCGTA-3	AY304484	60	396
GDF9	F: 5-CCTGTCGTTAACAGACTCCTGTC-3 R: 5-CATTTGGCCATGAGGAAGGC-3	FJ529501.2	60	215
BAX	F: 5-TGCAGAGGATGATCGCAGCAGCTG -3 R: 5-CAATGTTCAGCCCATCATGGTC-3	NM_173894.1	60	153
BCL2	F: 5-TCTGCAGGCCTTATGCAAAAC-3 R: 5-TTAATCTCGGCTCGCAACTG-3	AC_000181.1	60	105
SOD1	F: 5-GAGAGGCATGTTGGAGACCT-3 R: 5-CTGCCCAAGTCATCTGGTT-3	XM_006053564	60.15	153

Statistical analysis

The data were presented as mean ± standard erroe (SE). The significance of differences was determined using one-way ANOVA followed by a hoc test. SPSS 16.0.3 was used for statistical analysis.

RESULTS

Morphology and characterization of prepared capsulated melatonin nanoparticle (CMN)

The morphology of the prepared CMN sample was examined using transmission electron microscopy Fig. 1, shows that the prepared particles are semi-spherical in shape with a size distribution between 11 to 22 nm and it also revealed uniformity of the particle distribution throughout the melatonin-loaded chitosan nanoparticles sample. The average particle size is estimated by looking at the minimum and maximum diameter of many particles and we find that the average particle size is up to 30 nm. The efficiency of Encapsulation and Drug Loading in MTNPs, the drug loading of the produced MTNPs was evaluated at 16% with encapsulation efficiency at 34%.

The characterization of melatonin and nano melatonin (Fig. 2) revealed that the intensity-weighted Gaussian distributions analysis means±SD in nano melatonin was 842.2 nm with 0.03 variances while in melatonin was 3738.9 nm with a variance of 2.686. The stability of dispersion (average Zeta potential, Fig. 3) was -29.49

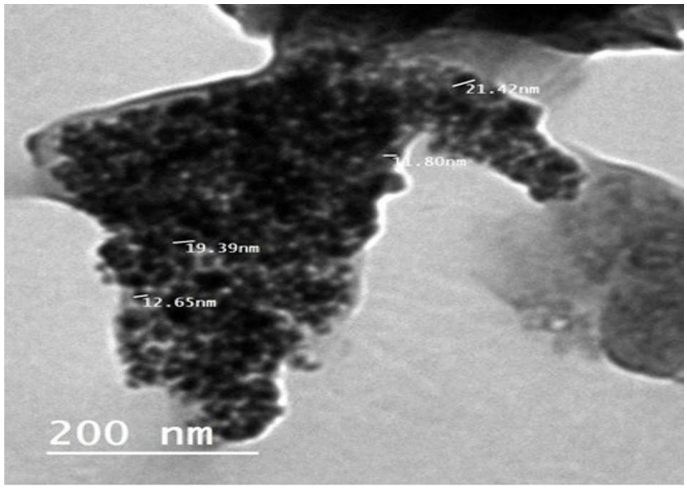

Fig. 1. Transmission electron microscope image of CMN with scale 200 nm at 25 °C.

mV and -21.94 mV in nano melatonin and melatonin respectively..

Studying the effect of melatonin and melatonin-loaded chitosan nanoparticles (CMN) supplementation of in vitro maturation media on the maturation rate of buffalo oocytes

Effect of melatonin and melatonin-loaded chitosan nanoparticles (CMN) supplementation of *in vitro* maturation media on cytoplasmic maturation of *in vitro* matured buffalo oocyte

Table 2 and Fig. 4, shows cumulus expansion GIII (mean

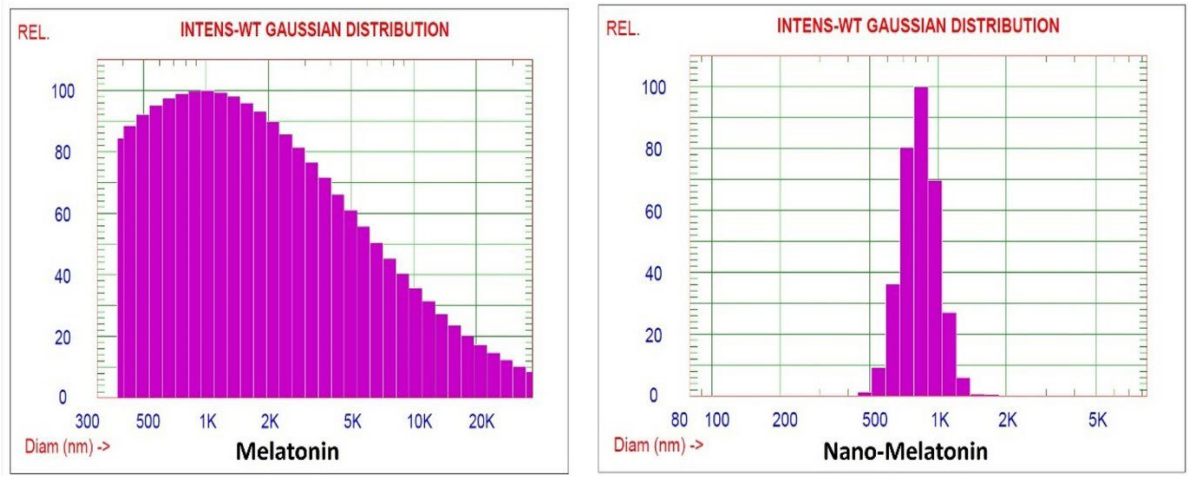

Fig. 2. Intensity weighted Gaussian Distribution Analysis (Solid Particle).

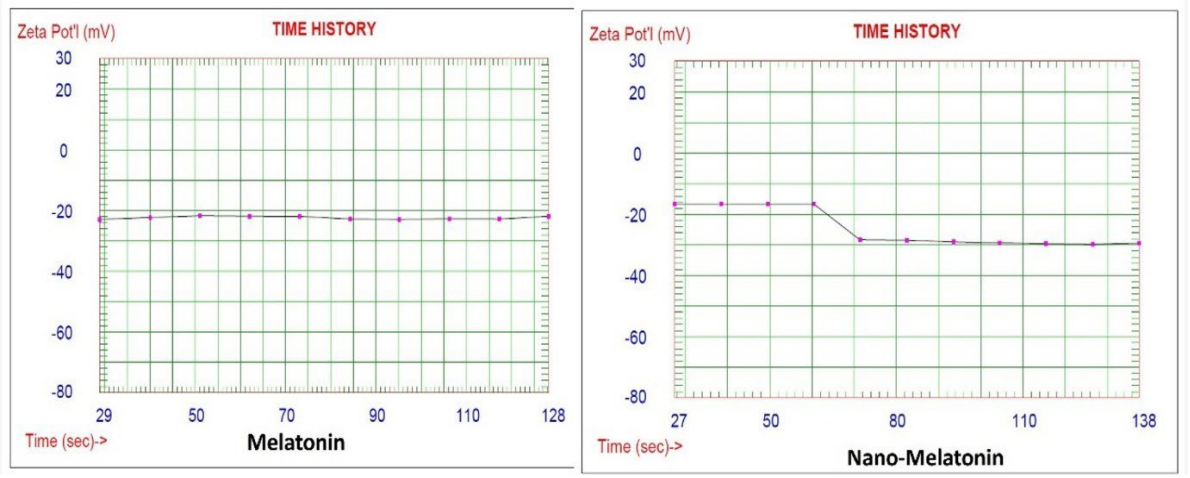

Fig. 3. Average Zeta Potential of melatonin and nano melatonin particles.

Table 2. Effect of melatonin and CMN on cytoplasmic maturation of *in vitro* matured buffalo oocytes.

Media	Oocytes	Oocyte maturation and cumulus cells expansions							
		GIII		GII		GI		G0	
	No.	No.	*Mean%±S.E.	No.	*Mean%±S.E.	No.	*Mean%±S.E.	No.	*Mean%±S.E.
Control	699	308	44.04±0.77^{b}	155	22.16±0.65^{b}	103	14.85±0.78^{a}	133	18.943±0.38^{a}
Melatonin	549	260	47.50±0.41^{b}	154	28.17±0.68^{a}	72	12.32±0.80^{b}	63	12.014±0.79^{b}
CMN	696	358	51.57±0.54^{a}	198	28.51±0.32^{a}	85	12.26±0.34^{b}	55	7.663±0.65^{c}

^{a,b,c}Superscripts to be compared statistically within the same column. Values with different letter superscripts are significantly different (P<0.05).

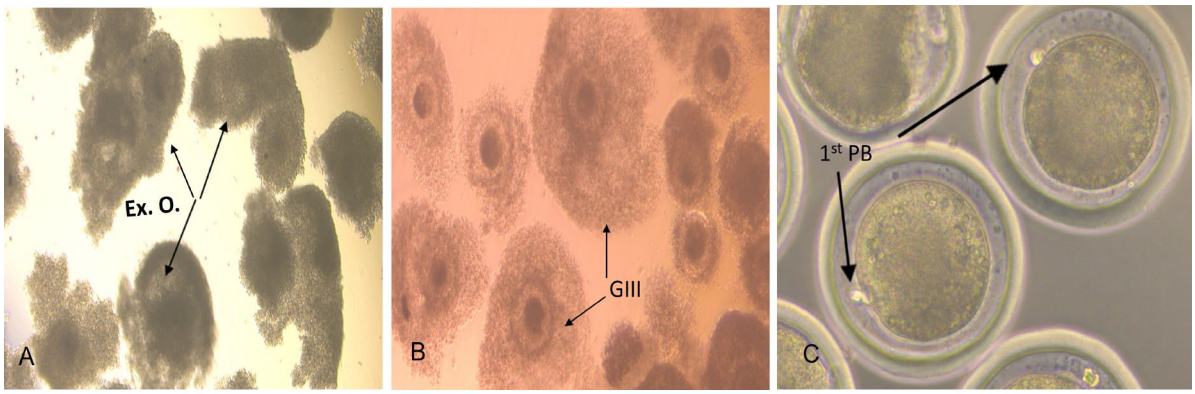

Fig 4. Excellent *in vitro* matured buffalo oocytes using inverted microscope Zeiss.
A) Ex.O.: Excellent quality; B) GIII: Full expansion cumulus oocytes; C) 1st PB: 1st Polar Body.

%±SE) of matured buffalo oocytes supplemented with CMN 10^{-9}M was significantly (P<0.05) increased (51.57±0.54 %) when compared with melatonin 10^{-9}M and control group (47.50±0.41%, 44.04±0.77) respectively. But in GII expansion (mean %±SE) showed a significant (P<0.05) increase of oocyte supplemented with CMN and melatonin (28.51±0.32 %, 28.17±0.68 %) respectively when compared with the control group (22.16±0.65 %). On the other side, the results present a significant (P<0.05) increase in GI expansion (mean %±SE) of oocyte matured in the control group (14.85±0.78 %) when compared with oocyte supplemented with melatonin and CMN (12.32±0.80 %, 12.26±0.34 %) respectively. Finally, results of G0 showed a significant increase of oocyte matured with the control group when compared with the melatonin or CMN group.

Effect of melatonin and melatonin-loaded chitosan nanoparticles (CMN) supplementation of *in vitro* maturation media on nuclear maturation of *in vitro* matured buffalo oocyte

The results in Table 3 and Fig. 4, showed a significant increase in nuclear oocyte maturation (1st polar body) (mean %±SE) for oocytes supplemented with CMN (94.04±0.65 %) when compared with oocytes supplemented with melatonin or the control group (88.74±0.77 %, 79.67±1.35 %) respectively and also, the oocyte supplemented with melatonin showed a highly significant difference than oocyte matured with the control group.

Table 3. Effect of melatonin and CMN on nuclear (1st pb) maturation of *in vitro* matured buffalo oocytes.

Media	NO. of Oocytes	Mature oocyte (1stpb)	
		No.	*Mean%±S.E.
Control	699	556	79.67±1.35^{c}
Melatonin	549	488	88.74±0.77^{b}
CMN	696	656	94.04±0.65^{a}

^{a,b,c}Superscripts to be compared statistically within the same column. Values with different letter superscripts are significantly different (P<0.05).

Studying the effect of melatonin and melatonin-loaded chitosan nanoparticles (CMN) supplementation of in vitro maturation media on gene expression of in vitro matured buffalo oocyte.

Effect of melatonin and melatonin-loaded chitosan nanoparticles (CMN) supplementation on germ cell marker-related genes (*GDF9* and *BMP15*) of *in vitro* matured buffalo oocytes

The present results showed upregulation of *GDF9* gene expression (Fig. 5) of mature oocytes supplemented with melatonin (2.65±1.47) and CMN (3.34±1.69) was significantly higher than *in vitro* matured buffalo oocytes matured with control (1.01±0.23). Also, results of *BMP15* gene expression (Fig. 6) of *in vitro* matured buffalo oocytes in different showed upregulation with significant differences of oocyte supplemented with melatonin (1.83±0.87) and CMN (1.91±1.03) when compared with the control group (1.04±0.36).

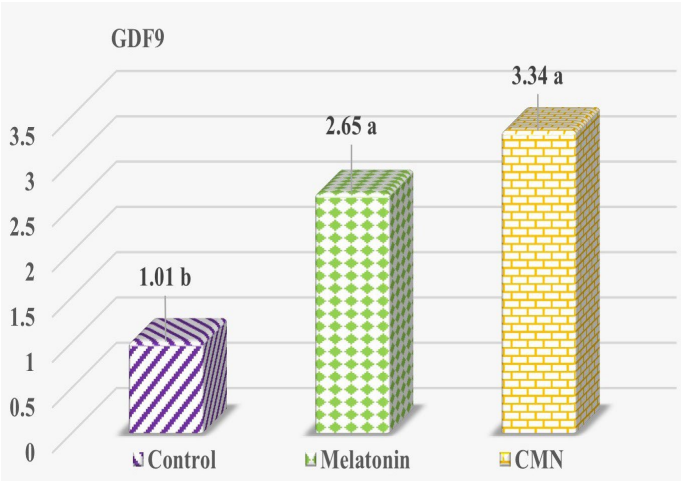

Fig. 5. Effect of melatonin and CMN on *GDF9* gene expression of *in vitro* matured buffalo oocytes.

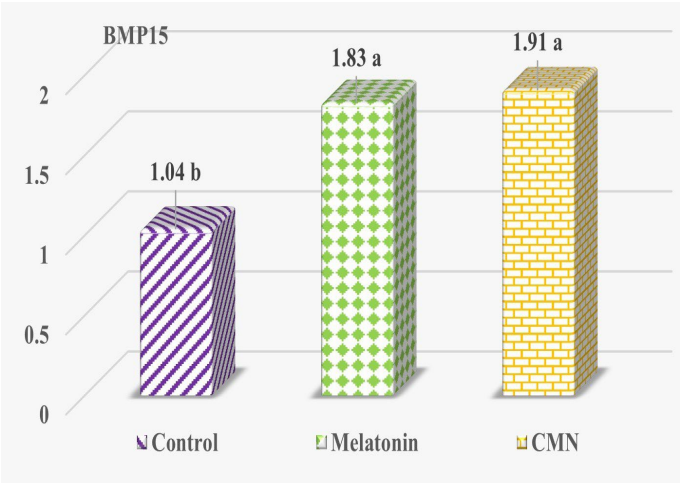

Fig. 6. Effect of melatonin and CMN on *BMP15* gene expression of *in vitro* matured buffalo oocytes.

Effect of melatonin and melatonin-loaded chitosan nanoparticles (CMN) supplementation on apoptosis-related genes (*BCL2* and *BAX*) of *in vitro* matured buffalo oocytes

The present results showed upregulation of *BCL2* gene expression (Fig. 7) of mature oocytes supplemented with melatonin (1.52±1.41) and CMN (2.58±1.03) with a significant effect of CMN group when compared with melatonin or control groups. On the other hand, results of *BAX* gene expression (Fig. 8) of *in vitro* matured buffalo oocytes supplemented with melatonin (0.11±0.45) and CMN (0.05±1.02) showed downregulation with significant differences when compared with the control group (1.02±0.11).

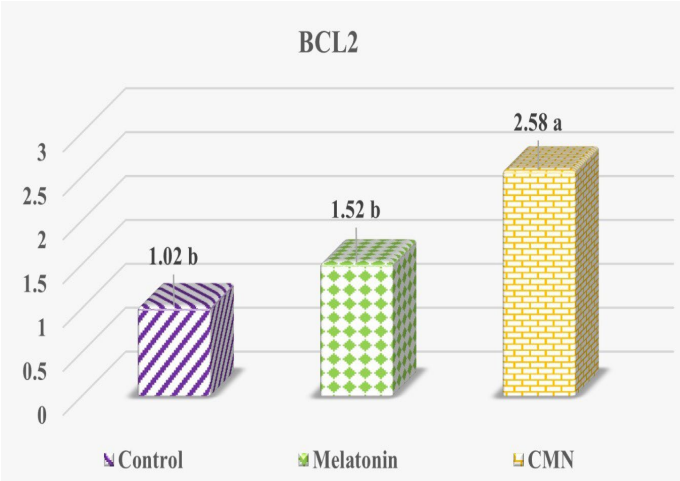

Fig. 7. Effect of melatonin and CMN on *BCL2* gene expression of *in vitro* matured buffalo oocytes.

Effect of melatonin and melatonin-loaded chitosan nanoparticles (CMN) supplementation on *SOD1* as antioxidant-related gene on matured buffalo oocyte

The present results in Fig.ure 9 showed upregulation of *SOD1* gene expression of *in vitro* matured oocyte supplemented with CMN (4.45±0.29) and melatonin (3.71±0.31) with a significant difference when compared with oocyte matured with the control group (1.00±0.05).

DISCUSSION

The present study showed that supplementation of melatonin to IVM medium increases *in vitro* maturation of buffalo oocytes by increasing the cumulus expansion and extrusion of the first polar body, as previously reported by Tian *et al.* (2014) and Zhao *et al.* (2015). Furthermore, The obtained data confirmed that using the encapsulation of melatonin in chitosan nano-particles enhances the favorable properties of melatonin to improve the maturation rate. These results are concomitant to previous work by Remião *et al.* (2016) who encapsulate the melatonin in lipid core nano-capsule (M-LNC).

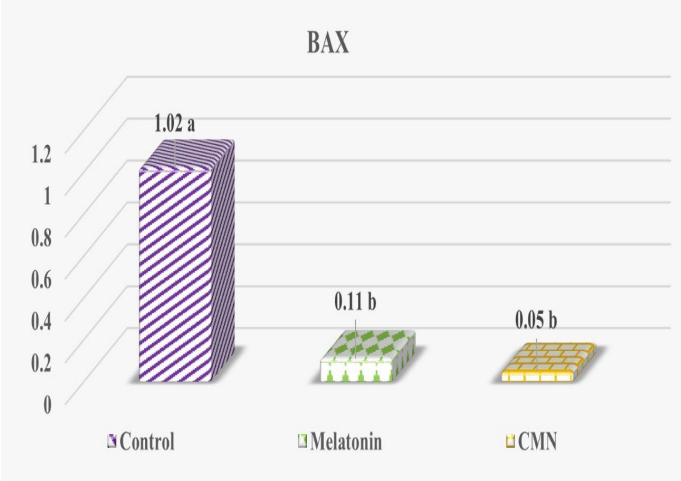

Fig. 8. effect of melatonin and CMN on *BAX* gene expression of *in vitro* matured buffalo oocytes.

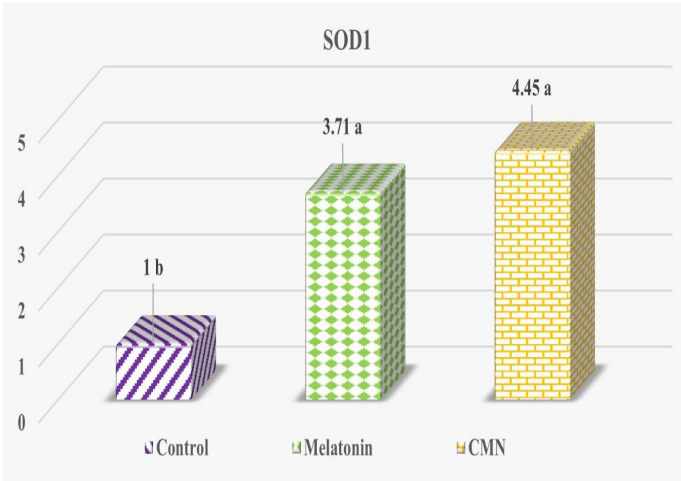

Fig 9. effect of melatonin and CMN on S0D1 gene expression of *in vitro* matured buffalo oocyte.

In this study, there were significant differences ($P < 0.05$) between the percentages of different treatment groups either in nuclear or cytoplasmic maturation for buffalo oocyte supplemented with CMN or melatonin when compared with buffalo oocyte matured with the control group (TCM-199). In the current study, the findings related to the *in vitro* matured buffalo oocytes agree with the results of nuclear maturation reported by Remião *et al.* (2016) who had presented a significant difference between M-LNC group 80.76%±4.54, melatonin group 81.64%±6.69 and control group 63.51%±4.59 of the bovine oocyte. Moreover, results from this study agreed with the results of melatonin reported by El-Raey *et al.* (2011) in cattle who reported a significant difference in nuclear maturation or cytoplasmic maturation when compared with cattle oocyte matured in TCM-199 as a control group. Also, the obtained results on melatonin agree with the results of nuclear and cytoplasmic maturation reported in sheep (Tian *et al.*, 2017), in bovine (Tian *et al.*, 2014), and in porcine (Park *et al.* 2018; Yang *et al.*, 2020) who described a highly significant effect of melatonin supplementation when compared with TCM-199 as basic oocyte maturation media.

This study confirmed that CMN and melatonin treatment upregulate the expression of *GDF9* mRNA and *BMP15* mRNA, with significant effects on buffalo oocytes during IVM. Oocytes showed higher expression of the *GDF9* gene during the maturation and mitosis process (Prochazka *et al*., 2004). Furthermore, the addition of *GDF9* and *BMP15* to maturation media upregulates genes related to oocyte developmental competence (Lin *et al*., 2014).

The present study showed up-regulation on *BMP15* and *GDF9* mRNA expression with a significant difference in *GDF9* mRNA expression between CMN group (3.34±1.69), melatonin (2.65±1.47), and control (1.01±0.23) and this agrees with melatonin result which reported in bovine (Tian *et al*., 2014; Yang *et al*., 2017) and in porcine (Yang *et al*., 2020). *BMP15* relatively gene expression downregulated *in vitro* matured in TCM-199 media when compared with immature buffalo oocytes (Kandil *et al*., 2010), while, in our results, the *BMP15* relative gene expression increased in oocytes matured in addition to melatonin and CMN groups to improve the maturation rate. Melatonin regulates *BMP15* and *GDF9* gene expression in *in vitro*-matured oocytes and improves *in vitro* oocyte maturation in rats (Coelho *et al*., 2020)

In this study, there was a significant difference between the CMN, melatonin, and control groups either in *BAX* or *BCL2* mRNA expression. Supplementation of CMN or melatonin has suppressed the expression of the pro-apoptotic gene (*BAX* mRNA) and stimulated the anti-apoptotic gene (*BCL2* mRNA) in the oocytes during maturation. *BAX* begins the apoptosis via interactions with both chromatin (Geng *et al*., 2010), Bcl-2 can interfere with cytochrome c release and, therefore, inhibit *BAX* activation (Yang *et al*., 2017). LNC-m has potency more than melatonin treatment which enhances the maturation rate and downregulates *BAX* mRNA expression during oocyte maturation in bovine (Remião *et al*., 2016)). Melatonin treatment inhibits the expression of *BAX* mRNA and stimulates the *BCL2* mRNA by activating the MT2 receptor in the granulosa cells of pig oocytes (He *et al*., 2016). Therefore, the downregulation of *BAX* and upregulation of *BCL2* increased the melatonin-induced improvement of buffalo oocyte competence and maturation.

Excessive ROS production is one of the obstacles to *in vitro* embryo production. This ROS may cause the oocyte's apoptosis (He *et al*., 2016). Antioxidants are often added to *in vitro* culture system to reduce the oxidative stress of ROS and enhance oocyte maturation (Wang *et al*., 2014). In this study, we selected melatonin as a powerful antioxidant. Melatonin is a high-level component in follicular fluids (Shi *et al*., 2009). The present study showed up-regulation of *SOD1* mRNA in maturation media supplemented with CMN or melatonin with a significant difference when compared with control media. Remião *et al*. (2016) reported that LNC-m or melatonin enhanced mRNA levels of *SOD1* expression. Also, our result agreed with Yang *et al*. (2017) who reported the *SOD1* expression was upregulated when supplementing the maturation media of bovine oocytes with melatonin.

CONCLUSION

CMN and melatonin at the 10−9 M concentration improve the maturation rate either cytoplasmic or nuclear maturation of buffalo oocyte. CMN is an anti-oxidant and also an anti-apoptotic reagent and affects the expression of several genes which are related to the oocytes' maturation as *GDF9*, *BMP15*, *SOD1*, and *BCL2*. Also, CMN preserves the ATP production which facilitates the oocyte maturation. All these lead to the improvement of buffalo oocyte competence. CMN is superior to using melatonin concerning buffalo oocyte maturation and the regulation of gene expression.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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