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Supplementing *in Vitro* Maturation Medium with Hyaluronic Acid Improves Bovine Oocyte Nuclear Maturation and Expression of Cumulus Expansion Genes

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ABSTRACT

The current study aimed to evaluate the effect of low (MW) hyaluronic acid (HA) supplementation on the outcomes of bovine oocyte in vitro maturation. Cumulus oocyte complexes (COCs), obtained from slaughtered cows (n=193) from the local abattoir, were stained using BCB. The BCB+ oocytes (n = 970) were then divided into two equal groups and cultured in maturation media. One group served as control (without HA supplementation), while the other group was supplemented with 0.002 mM HA. The percentages of early polar body formation, cumulus expansion, and phenotypic morphology of cumulus cells (CCs) were evaluated at three time points: 0, 22, and 44 hrs. As part of the analysis, TNFAIP6, PTX3, and PTGS2 genes associated with cumulus expansion were measured at these timepoints. Compared to the control group, HA supplementation significantly reduced cumulus expansion at 22 and 44 hr. but significantly increased oocyte maturation rate. In addition, with regard to expression of cumulus expansion-related genes, PTX3 and PTGS2 showed significant elevation at 22 hrs. followed by reduction at 44 hrs., TNFAIP6 exhibited a steady, significant increase over time, with the highest expression achieved at 44 hrs. in HA-treated groups. In comparison with the control untreated group, PTGS2 and TNFAIP6 showed significant increases at both 22 hrs. and 44 hrs. post-HA treatment, while PTX3 showed a significant increase only at 22 hrs. Supplementation of *in vitro* oocyte maturation medium with low-molecular-weight HA significantly accelerated nuclear maturation and enhanced the expression of cumulus expansion-related genes in CCs, indicating potential improvement in subsequent IVF outcomes.

1. INTRODUCTION

Cow infertility is one of the major obstacles that hinder the cattle industry in Egypt. Economic losses due to infertility can be substantial, including increased maintenance costs and missed income opportunities, due to fewer calves available for sale. Additionally an extend calving season increase production expenses with smaller calves are weaning the following year [1].

As well as serving as an animal-assisted reproduction technique, in vitro maturation (IVM) of oocytes could prove to be an important treatment for clinical infertility [2]. To emulate natural in vivo maturation, IVM intends to induce immature oocytes from a growing follicle to resume meiosis to enable fertilization through arrest during the second metaphase stage of meiosis [3].

In oocyte IVM techniques, the absence of luteinizing hormone receptors (LHCGRs) on cumulus cells is frequently avoided by using follicular stimulating hormone (FSH). All mammalian species have FSH receptors on their cumulus cells, allowing the hormone to initiate signaling pathways, resulting in resumption of meiosis [4]. Even so, the success of procedures involving in vitro maturation (IVM) of oocytes is still inferior compared to in vivo matured oocytes. It has been hypothesized that the insufficient developmental competence of IVM primate oocytes is due to failure at the timely commencement of embryonic genome activation owing to inadequate cytoplasmic maturation of these oocytes [5]. Consequently, the inability of cumulus cells to provide appropriate signaling may be linked to the failure of IVM settings to offer ideal circumstances for oocytes [6].

Among the main components of cumulus-oocyte complexes (COCs) is the cumulus, consisting of the extracellular matrix and cumulus cells surrounding the oocytes[7]. Mammalian oocyte maturation is accompanied by morphological and physiological alterations in the cumulus cells that surround the oocytes. These changes have direct impacts on oocyte maturity and developmental competence. Cumulus expansion refers to these alterations in cumulus cells[8]. In vitro oocyte maturation success is mostly determined by the quantity of cumulus cell layers, quality of the cumuli, and the extent of expansion of cumulus cells. These factors are also essential for the survival of mature and fertilized oocytes [9,10].

Analysis of the transcriptomic profile of cumulus cells (CCs) allows for noninvasive prediction of oocyte maturation and embryo competence. This is possible because the development of competence in CCs requires bidirectional movement of molecules between CCs and the oocyte [11].Several studies have outlined a molecular technique for predicting embryo quality and pregnancy outcomes by evaluating the gene expression of specific markers associated with cumulus cell expansion [11].

Prostaglandin endoperoxide synthase 2 (PTGS2), tumor necrosis factor alpha-induced protein 6 (TNFAIP6), and pentraxin 3 (PTX3) mRNA expression are positively correlated with accelerated embryonic development. Hence, these genes have been suggested to be cumulus indicators of oocyte developmental competency [12,13]. PTGS2 regulate the production of PGE2 that is essential for cumulus cell expansion and oocytes maturation [14]. PGE2, in turn activates EP2 receptor to induce the expression of specific cumulus expansion genes, such as TNFAIP6, in cumulus cells [15,16].

Moreover, TNFAIP6 exhibits a strong affinity for PTX3. Therefore, the two proteins collaborate to preserve the extracellular matrix [17]. Notably, RNA interference-mediated genetic deletion of PTGS2, PTX3, or TNFAIP6 results in impaired cumulus expansion [18].

It is widely understood that to achieve proper in vitro oocyte maturation, oocytes should be as close to the natural environment as possible during the expansion of cumulus cells [19]. During natural cumulus expansion, glycosaminoglycan (GAG), which is abundant in hyaluronic acid (HA), is produced and released into the extracellular space. GAG serves as a structural element of enlarged cumuli and as a signaling molecule that controls oocyte maturation [7]. To achieve complete expansion of cumulus cells, HA (TNFAIP6 and PTX3) has to be bound to its receptor protein (CD44). Owing to its physicochemical features, HA has a variety of physiological activities, including protein regulation, water distribution and water-binding capacity, in addition to filtration and lubrication effects [20,21]. These data raise an interesting question about the potential value of using extrinsic HA to improve the outcome of IVM. The present study hypothesized that supplementation of culture media with low-molecular-weight HA would improve cumulus cell expansion and oocyte competence during in vitro maturation. To test this hypothesis, the current study examines the effects of HA supplementation on bovine oocyte IVM outcomes, focusing on nuclear maturation rates and the expression profiles of cumulus expansion-related genes.

2. MATERIALS and METHODS

Ethical approval: All procedures and experiments have been approved by the local Bioethical committee of Molecular Biology Research & Studies Institute (IORG0010947-MB-21-45-A). The study was conducted in compliance with relevant guidelines and regulations, and it was reported in accordance with the ARRIVE guidelines [22].

2.1. Oocyte collection

The bovine ovaries were acquired from the Dashlout slaughterhouse in Dairyout, Assiut, between November 2022 and February 2023 and then it was sent to the IVF laboratory. Within 1–2 hours, they were placed in a thermal container filled with 0.9% sodium chloride solution (NaCl) at 37°C. Upon arrival, the ovaries were washed with saline solution .Using a 5-ml syringe attached to an 18-gauge needle, cumulus–oocyte complexes (COCs) were acquired by aspiration from follicles with a diameter of 2–8 mm [23]. Sedimentary COCs were collected and left to settle for five minutes at 37°C in 15 mL conical tubes. After removing the supernatant, Tyrode's Albumin Lactate Pyruvate-HEPES (TALP) medium was used to wash the sediment three times as described elsewhere [24]. To conduct the experiments, we only used COCs that displayed homogeneous cytoplasm and a minimum of three uniform layers of compact cumulus cells [25]**.**

2.2. BCB test

Recovered oocytes were stained with brilliant cresyl blue (BCB) as previously described [26]. The COCs were repeatedly washed in modified Dulbecco's phosphate buffer saline

(DPBS) supplemented with 0.4% bovine serum albumin fraction v (BSA) (OXFORD, INDIA, CAS No. 90604-29-8). The COCs were then incubated for 90 minutes at 38°C in a humidified atmosphere with 5% CO_2 in 100 µl drops of BCB (26 µM in modified PBS) and covered with medical mineral oil. After incubation, COCs were washed twice in modified PBS followed by stereomicroscopic inspection. Accordingly, COCs were classified as BCB+ or BCB- based on the presence of blue color in their cytoplasm (**Figure 1).**

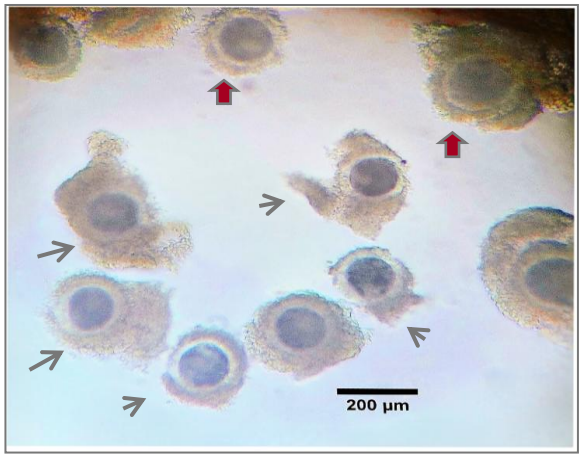

Figure 1. BCB staining of freshly collected bovine COCs. Images taken after BCB incubation for detection of BCB+ (small arrows) and BCB – (big arrows) COCs according to blue color of cytoplasm**.**

2.3. Oocyte in vitro maturation (IVM)

The maturation medium TCM-199 supplemented with L-glutamine (BIO-CHEM, FRANCE, Cat No. 12-109F) was used in the present study after supplementation with 0.02 IU/ml (r-h FSH) recombinant human follicle stimulating hormone (MINAPHARM, EGYPT), 10% (v/v) fetal calf serum (FCS) (Biowest, South America origin), 0.2 mM sodium pyruvate (SDFCL, INDIA), 50 µg/ml gentamycin (Sigma-Aldrich Co., St. Louis, MO, USA), and 1 µg/ml 17β-estradiol (Sigma, USA) as previously described [27]. The classified COCs were washed three times in maturation medium and then incubated after being covered with mineral oil (10-12 oocytes/50 µl droplets of IVM media) for up to 44 hrs. at 38.5°C in an environment of 5% CO2 in a humidified air. The HA group was treated with 0.002 mM (200-400 kDa) hyaluronic acid sodium salt (HA) (Euromedex, France, CAS No. 9067-32-7) dissolved at 4°C before supplementation at the same concentrations as those in the control group.

2.4. Phenotypic evaluation of in vitro maturation of COCs

The cumulus area of the COCs was inspected under an inverted microscope. at 0, 22and 44 hours post incubation. To measure cumulus cell expansion, the difference in average area of all COCs at three time points was calculated as the methods described by Pan *et al*. [28] and Lee *et al*. [29]. This was performed by using ImageJ software (version 1.53e; National Institutes of Health, Bethesda, Maryland, USA). Measurements were standardized for the control group (lacking hyaluronic acid).

2.5. Assessment of oocyte nuclear maturation

A total of 470 oocytes were used for nuclear maturation across HA treated and control groups. In PBS, the COCs were vortexed (at a moderate speed for 20 minutes) until no cumulus cells (CC) remained after 22.44 hours of culture. Following three PBS washes, the oocytes were analyzed under a microscope. As reported by Lee *et al*. [30], Denuded oocytes have been described as immature (not extruding 1st first polar body), a degenerate oocyte (with a damaged oolemma or abnormal cytoplasm), and metaphase II (extruding first polar body).

2.6. Evaluation of cumulus expansion-related gene expression

2.6.1. Sample collection

A total of 30 COCs, with three independent repetitions in each group, were evaluated at three different time points: 0, 22, and 44 hours. To exclude CCs, the COCs were moved to a drop of 50 µl of phosphate-buffered saline (PBS) and subjected to vertexing for 20 minutes at a moderate speed until the CCs were fully isolated. The cellular components were isolated from the eliminated oocyte and subjected to two rounds of centrifugation at 700*g for two minutes each. Following the removal of the supernatant, 5-10 volumes of RNAlater Solution (IND Diagnostic, Inc., Canada, Cat. No. AM7020) added to the cell pellet, which was subsequently stored at -80°C until the extraction of RNA [31].

2.6.2. RNA Extraction and reverse transcription

The total RNA was extracted using RNeasy Micro Kit from (Qiagen, Europe). RNAs were subjected to DNase treatment during extraction to exclude any potential contamination from genomic DNA. The total concentration of the RNA extracted was quantified by using a Nanodrops 2000 spectrophotometer. The absorbance was measured at a wavelength of 260 nanometers. Verification of sample purity was achieved by measuring the A260/A280 nanometer (nm) ratio. Based on the manufacturer's instructions, RNA-to-cDNA reverse transcription kit from Thermo Fisher Scientific (Warrington, UK) was used for the reverse transcription experiment.

2.6.3. Quantitative real-time PCR

The TNFAIP6, PTX3, PTGS2, and β-actin genes were subjected to quantitative RT–PCR using Quant Studio™ 5 Real-Time PCR equipment from Applied Biosystems (USA) Following cDNA synthesis, in duplicate wells.

The primer sets used for the investigated genes listed in Table 1. Primers for cumulus expansion genes (TNFAIP6, PTX3, PTGS2) were designed as described by Huang *et al*. [32], and the β-actin gene was designed as described previously [23]. The PCR mixture in each well included 7.5 µl of Power SYBR® Green PCR Master Mix (Applied Biosystems, UK), 1 µl of each forward and reverse primer for each gene, and 5.5 µl of cDNA, for a final volume of 15 µl. The thermal profiles of the RT–PCR products used for the detection of TNFAIP6, PTX3, PTGS2 and β-actin are shown in Table 2. The

reactions and results have been normalized against values of Ct for β-actin and are presented as the fold change in comparison to the control group as described by [33].

Table 1. List of used primers for the detection of β-actin and selected cumulus expansion genes including the corresponding genes accession numbers

Gene	Primer sequences (Forward/Reverse)	Product size (bp)	Accession No
β-actin	5`-GGCATTCACGAAACTACCTT-3` 5`-CAATCCACACGGAGTACTTG-3`	208	NM_173979
TNFAIP6	5`-TGAAAGATGGGATGCATATTGC-3` 5`-CATTTGGGAAGCCTGGAGATT-3`	101	NM_001007813.2
PTX3	5`-CATGTATGTGAATTTGGACAACGA-3` 5`-GCTTGTCCCACTCGGAGTTC-3`	101	NM_001076259.2
PTGS2	5`-CTTAAACAAGAGCATCCAGAATGG-3` 5`-GCTGTACGTAGTCTTCAATCACAATCT-3`	106	NM_174445.2

Table 2. Thermal profiles of Real-Time PCR that were used for the detection of TNFAIP6, PTX3, PTGS2 and β-actin**.**

Gene		β-actin	TNFAIP6	PTX3	PTGS2
Initial Denaturation	Temp. Time	95°C 5 Min			
Denaturation	Temp. Time	95 °C 40 S	95 °C 40 S	95 °C 30 S	95 °C 40 S
Annealing	Temp. Time	61°C 40 S	54°C 45 S	55°C 40 S	57°C 40 S
Extension	Temp. Time	72 °C 40 S	72 °C 45 S	72 °C 40 S	72 °C 40 S
Cycles			40		

2.7. Statistical analysis

The effects of treatment with low-molecular-weight hyaluronic acid (HA) on the area of cumulus expansion and on the cumulus expansion-related genes were investigated using two-way ANOVA followed by Tukey's multiple comparisons test for determining differences between the two groups at 3 time points. Relative mRNA expression is expressed as a fold change ($2^{-\Delta\Delta CT}$). The oocyte meiotic maturation rates were recorded for each group, before analyzing variance, arcsine transformation is used to maintain variance homogeneity and analysis of variance was performed using the F test. All presented data were subjected at least three biological replicates (n=3). The data were analyzed using GraphPad PRISM 10.2.3 (GraphPad Software, La Jolla, California, USA), and the results are expressed as their mean ± standard error, a significance level of ($P < 0.05$) was used in all analyses.

3. RESULTS

3.1. Expansion of the cumulus oocyte complex (COCS)

The results revealed that there was a statistically significant interaction effect between the time point and type of media on the area of expansion ($F(2, 4) = 28.18$, $P=0.0044$). Tuckey's multiple comparisons test showed that in the HA treated group, the expansion area has been reduced significantly compared with control group at 22 hours ($p<0.01$) and 44 hours ($p=0.0003$). Additionally, the area of expansion within the same group significantly increased at 22 hours and 44 hours. ($p< 0.01$ and $p<0.0001$, respectively) compared to 0 hr. time point and at 44 hr. of compared to 22 hr. ($p= 0.0004$) in the control group. On the other hand, in the HA-treated group, the area of expansion significantly increased at 44 hr. time points compared to both 0 hr. and 22 hr. time points ($p = 0.0006$ and $p< 0.01$, respectively). **(Table 3, Figure *2, 3).***

Table 3. Effect of hyaluronic acid treatment during *in vitro* maturation (IVM) on area of expansion of COCs (mean ± SEM).

Time points		**(area µm^2)**		
		0hr	**22hr**	**44hr**
IVM treatment groups n=30	**Control**	171998 ± 45869 ^{c}	381229 ± 43062 ^{b}	700446 ± 31027 ^{a}
	HA	146104 ± 21776 ^{c}	234011 ± 36809 ^{c}	426665 ± 32796 ^{b}

Three replicates were performed. Values with different superscripts (a-c) are significantly different ($P < 0.05$). **HA**: hyaluronic acid.

3.2. Nuclear maturation rate of oocytes

The results exhibited a significant increase ($P < 0.05$) of nuclear maturation rate in the HA-treated group (88.95 ± 1.259) in comparison to that in the control group (84.38 ± 2.639) (Table 4).

3.3. Expression of cumulus expansion genes in mature COCs:

The results revealed the presence of a statistically significant interaction effect of time and type of media on relative expression levels of TNFAIP6, PTX3 and PTGS2 ($F(2, 12) = 19.49$, $P=0.0002$; $F(2, 12) = 4.364$, $P=0.0376$; and $F(2, 12) = 8.620$, $P=0.0048$, respectively).

3.3.1. Relative TNFAIP6 gene expression.

Tuckey's multiple comparisons test revealed a significantly increased expression at **22** hr. and **44** hr. ($p< 0.01$ and $p<0.0001$, respectively) in the HA-treated group in comparison to the same time points in the control group. Additionally, a significant increase was also

recorded at **22** hr. and **44** hrs. ($p< 0.05$ and $p<0.0001$, respectively) Compared to **0** hr. in HA-treated group **(Table 5, Figure 4).**

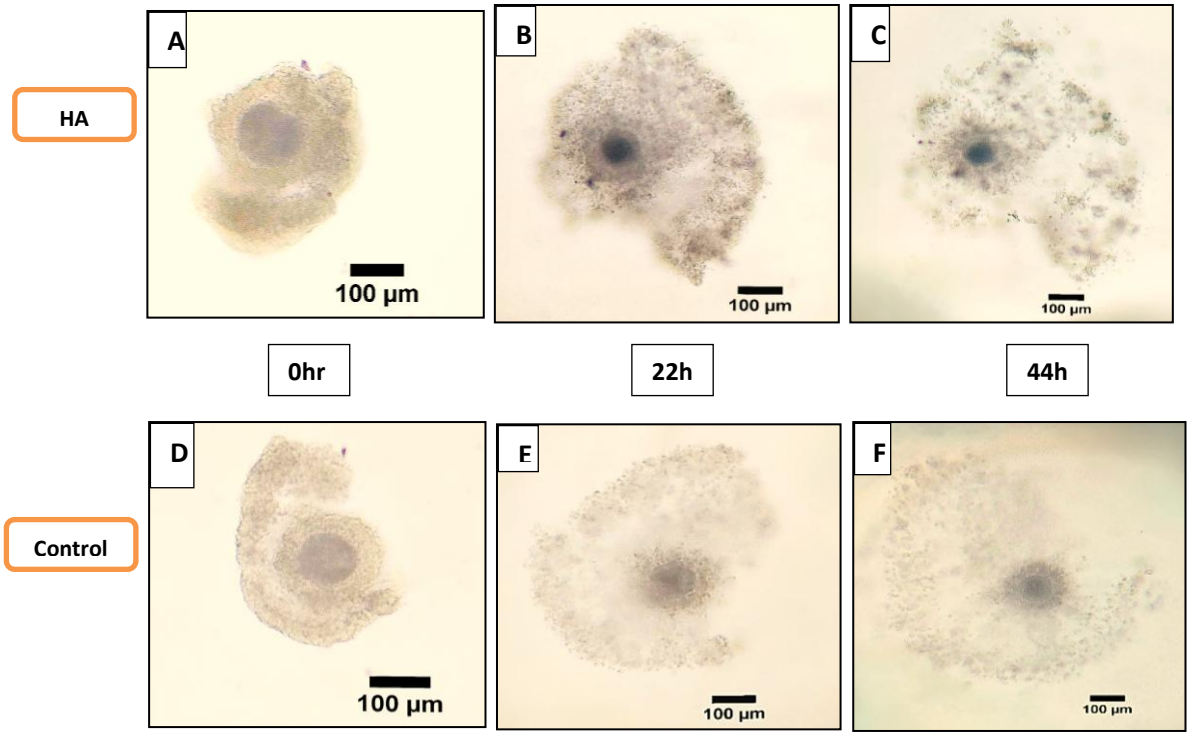

Figure 2. Representative images of the effect of low molecular weight hyaluronic acid during bovine COCs maturation at 0h .22h & 44h. images were taken under inverted microscope (magnification 10X) in order to visualize cumulus expansion pattern. (A-C) Hyaluronic acid group, (D-F) control group.

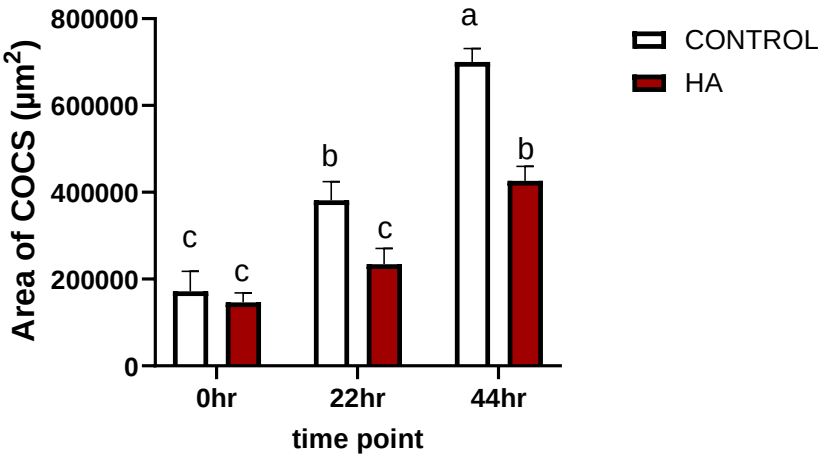

Figure 3. Effect of low molecular weight hyaluronic acid treatment during in vitro maturation (IVM) on area of expansion of COCs at three time point (0hr,22hr,44hr), all data expressed by (mean± SEM). Values with different superscripts(a-c) are significantly different ($P < 0.05$). HA: hyaluronic acid.

Table 4. Nuclear maturation rate in HA-treated group as compared to control group during IVM of BCB+ selected oocytes.

Groups	No. matured oocytes	No. (%) MII oocytes
HA	215	192 (88.95 ± 1.259) [a]
CONTROL	255	218 (84.38 ± 2.639) [b]

Values with different superscripts (a, b) within columns are significantly di*fferent from each other (P < 0.05). HA: hyaluronic acid, MII: metaphase II, Data are expressed as mean ± SEM.*

Table 5. Effect of hyaluronic acid treatment during in vitro maturation (IVM) on relative mRNA expression level **TNFAIP6** of mature COCs at three time point (mean ± SEM).

Time points		TNFAIP6		
		0hr	**22hr**	**44hr**
IVM treatment groups	**Control**	1.042 ± 0.2020 [c]	1.062 ± 0.2685 [c]	1.444 ± 0.3514[c]
	HA	1.070 ± 0.04645 [c]	3.218 ± 0.5971 [b]	7.593 ± 0.9441 [a]

Values with different superscripts (a, b, c) are significantly different (P < 0.05). **HA**: hyaluronic acid.

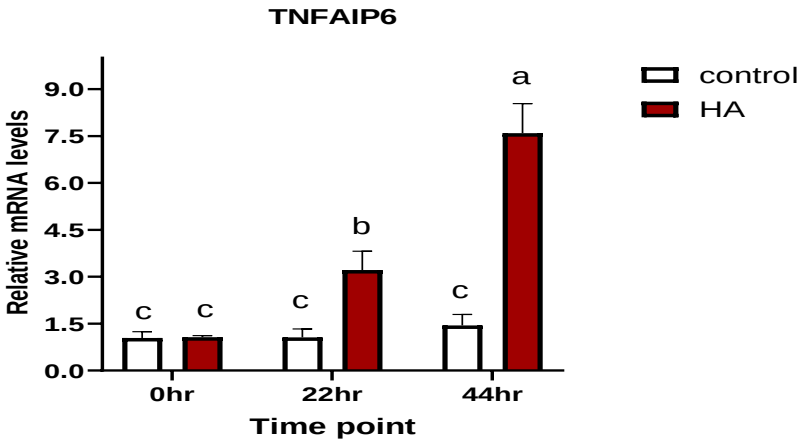

Figure 4. Effect of HA treatment during in vitro maturation (IVM) on relative mRNA expression level TNFAIP6 in cumulus cells of bovine mature COCs at three time point (0hr,22hr,44hr) respectively, Expression levels are shown as relative quantities and were analyzed using real-time polymerase chain reaction. β-Actin was used to normalize gene expression, and mature COCs without HA were used as calibrators, all data expressed by (mean± SEM. Values with different superscripts (a, b, c) are significantly different (P < 0.05). HA: hyaluronic acid.

3.3.2. Relative PTX3 gene expression.

Tuckey's multiple comparisons test showed a significant increase (P=0.0003) at the 22 hr. time point of the HA-treated group when compared to the same time point of control group. Moreover, within the same HA-treated group, PTX3 expression significantly increased ($p< 0.01$) at **22** hr. as compared to 0 hr. time point but was significantly lower ($p<0.05$) at **44** hr. of compared to **22** hr. time points. **(Table 6, Figure 5).**

Table 6. Effect of hyaluronic acid treatment during in vitro maturation (IVM) on relative mRNA expression level **PTX3** of mature COCs at three time point (mean ± SEM).

Time points		PTX3		
		0hr	**22hr**	**44hr**
IVM treatment groups	**Control**	1.111 ± 0.3723 ^{c}	1.180 ± 0.4194 ^{c}	1.434 ± 0.7650 ^{c}
	HA	2.148 ± 0.6357 ^{c}	6.879 ± 1.503 ^{a}	3.730 ± 0.6606 ^{b}

Values with different superscripts (a, b, c) are significantly different (P < 0.05). **HA**: hyaluronic acid.

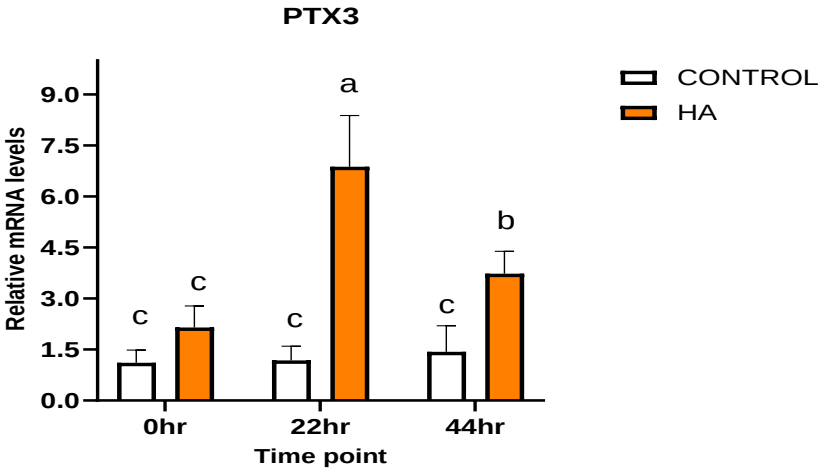

Figure 5. Effect of HA treatment during in vitro maturation (IVM) on relative mRNA expression level PTX3 in cumulus cells of bovine mature COCs at three time point (0hr,22hr,44hr) respectively, Expression levels are shown as relative quantities and were analyzed using real-time polymerase chain reaction. β-Actin was used to normalize gene expression, and mature COCs without HA were used as calibrators, all data expressed by (mean± SEM). Values with different superscripts (a, b, c) are significantly different (P < 0.05). HA: hyaluronic acid.

3.3.3. PTGS2 relative gene expression.

Tuckey's multiple comparisons test indicated that the HA-treated group exhibited increased expression at 22 hr. and 44 hr. time points (P< 0.0001 and p<0.01, respectively) as compared to the control group. In addition, PTGS2 was significantly increased within the same HA-treated group at **22** hr. (p = 0.0003) and **44** hr. (p<0.01) compared to that at 0 hr. time points**. (Table 7, Figure 6)**

Table 7. Effect of hyaluronic acid treatment during in vitro maturation (IVM) on relative mRNA expression level **PTGS2** of mature COCs at three time point (mean ± SEM).

Time points		PTGS2		
		0hr	**22hr**	**44hr**
IVM treatment groups	**Control**	1.219 ± 0.4295 [b]	1.074 ± 0.3013 [b]	0.9797 ± 0.3009 [b]
	HA	1.553 ± 0.2691 [b]	8.602 ± 0.6585 [a]	6.055 ± 1.945 [a]

Values with different superscripts (a, b) are significantly different (P < 0.05). **HA**: hyaluronic acid.

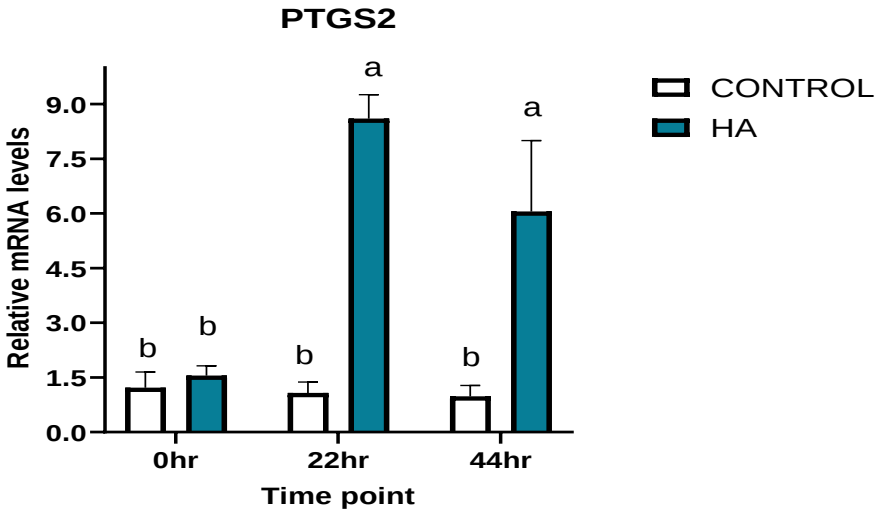

Figure 6. Effect of HA treatment during in vitro maturation (IVM) on relative mRNA expression level PTGS2 in cumulus cells of bovine mature COCs at three time point (0hr,22hr,44hr) respectively, Expression levels are shown as relative quantities and were analyzed using real-time polymerase chain reaction. β-Actin was used to normalize gene expression, and mature COCs without HA were used as calibrators, all data expressed by (mean± SEM). Values with different superscripts (a, b) are significantly different (P < 0.05). HA: hyaluronic acid.

4. DISCUSSION

Incomplete oocyte maturation during IVM is one of the primary factors that reduce the embryo quality [29]. Thus, improving oocyte maturation under in vitro conditions to mimic natural ideal maturation conditions is a long-standing target for many studies [28,29,31,32,34-36]. The present study investigated the effect of supplementation of IVM culture media with low-molecular-weight hyaluronic acid (200-400 kDa) on the oocyte maturation outcomes. The fate of oocyte maturation was evaluated based on the assessment of the area of cumulus expansion in bovine oocytes as well as cumulus expansion-related genes.

Hyaluronic acid was reported to positively affect the growth of blastocysts during IVM and to enhance the meiotic maturation of bovine oocytes without negatively impacting oocyte chromatin integrity [34,37]. It has been reported that HA, which has a 20–200 kDa_molecular weight, participates in biological processes related to the embryonic development, wound healing and ovulation [38]. Conversely, HA with a higher molecular weight (>500 kDa) produces antiangiogenic activity, acting as a natural immunosuppressant [39]. Nonetheless, HA was found in the ovarian tissue and follicular fluid at three molecular weight ranges (<100 kDa, 100–300 kDa, and >300 kDa). Moreover, while optimal expansion of the cumulus layer is essential for the optimal maturation of oocytes [40], achieving complete CC expansion was proven to require the binding of HA proteins (TNFAIP6 and PTX3) as well as its (CD44) receptor protein [41]. A previous study revealed that CD44 receptor signaling can be triggered by binding the small molecular weight (200 kDa) HA [42]. Therefore, the molecular weight of hyaluronic acid (200-400 kDa) which used in the present study was chosen to mimic the environment during in vivo expansion of cumulus cells.

A cumulus expansion intensity measurement is essential for assessing oocyte compatibility for IVF, cloning, and transgenesis. Reviewed by [7]. Interestingly, both the 22 hr. and 44 hr. time points of this study showed that the area of CC expansion was significantly greater in the un-supplemented control group ($p< 0.01$) than the HA group. Similar findings were recorded in mice, where it was suggested that exogenous HA supplementation causes displacement of the endogenous COC matrix HA into the medium, subsequently preventing COC expansion. The HA matrix dissociates within 1-2 days after being produced in vitro. Initially, the procedure is rapid, with approximately 50% of the net loss of hyaluronic acid occurring within 7 hours, between 18 and 25 hours of maturation. The loss of HA from the expanding COC matrix starts at or near the time when cumulus cells stop producing HA. Thus, fertilization rates peak at 20 hours of culture, corresponding to the expansion and retention of HA in the matrix. Fertilization declined after 20 hours and completely disappeared after 40 hours. Although exogenous HA can prevent the assembly of the COC matrix, it does not facilitate the disaggregation process [43].

On the other hand, the present study revealed significantly greater meiotic maturity ($P < 0.05$) in oocytes cultured in HA-supplemented medium than in those cultured in un-supplemented control medium. In addition, regarding CC expansion-related genes, In the present study, we found significant increases in three genes

(TNFAIP6, PTX3, and PTGS2) in the HA-supplemented group at 22 hr. and 44 hr. time point compared to the un-supplemented control group. These genes have been previously assumed to be cumulus markers of oocyte developmental competence that are positively correlated with increased embryo development [12,13].

The HA surrounding an oocyte may act as a protective shield and a reservoir for growth factors. Negatively charged glycosaminoglycans (GAGs) interact with many different chemical compounds, including growth factors. Among the primary mechanisms governing communication interactions between cells are GAG and cytokine interactions, which are mediated by substances secreted or acting locally [44]. Accordingly, granulosa/cumulus cells contain Toll-like receptor (TLR) factors that induce innate immunity in mouse, bovine, and human ovaries, and thus may affect fertility [45]. The innate immune system may therefore play a role in ovulation [46]. TLR family members are expressed in cumulus cells, which can respond to ligands, including fragments of polymeric HA to which hyaluronidases have broken down. This process triggers the expression of inflammation- and innate immune-related genes. PTGS2, TNFAIP6, IL6, and Pdcd1, as well as other cytokines and chemokines, are released from cumulus cells [46]. TLR2 was reported to be an important receptor for HA-mediated responses to ~100–400 kDa HA [47]. Based on these observations, it is believed that the low-molecular-weight HA (200-400 kDa) used in the present study might have stimulated Toll-like receptor (TLR) factors, which in turn increased the expression of PTGS2 and TNFAIP6 in the HA-supplemented group compared to the control group.

In addition, interesting expression profiles of the three CC expansion-related genes within the same group were recorded in the present study. While no significant differences in the expression of any of the studied genes (TNFAIP6, PTX3, PTGS2) were detected between the different time points (0 hr., 22 hr., and 44 hr.) within the control group, the HA-treated group exhibited significant variation in the expression of the 3 genes at 22 hr. and 44 hr. time points as compared to 0 hr. time points. With regard to PTGS2, it was reported that its basal expression in mature bovine COCs increased significantly between 0–6 hr. then decreased between 6–12 hr. and remained unchanged from 12–24 hr. [48]. In addition, another study reported that the maximum expression of PTGS2 in BCB+ COCs was detected at 8 hours after IVM, indicating its participation in extracellular mass (ECM) assembly [49]. In the present study, the brilliant cresyal blue (BCB) test was used to analyze the transcriptomic profiles of oocytes before and after IVM (0, 22, and 44 hrs.). Only BCB+ oocytes were selected because they facilitated selecting of higher-quality oocytes and allowed for the evaluation of the influence of IVM on the transcriptomic profile of oocytes within the same developmental competence. Therefore, the nonsignificant differences in PTGS2 expression at the 3 time points in the control group, as recorded in the present study, are attributed to the failure to detect its maximum expression during the first 6-8 hrs. of IVM, as our recorded time points were at 0, 22 and 44 hrs. of IVM. However, PTGS2 expression was significantly greater in the HA-supplemented group than in the control group from 0 hr. to 22 and 44 hr. time points (7-fold and 4-fold change, respectively), but no significant difference was found between 22 hr. and 44 hr. time points. In other words, the expression level of PTGS2 increased under the influence of HA treatment and remained high throughout the whole incubation period. These findings support the notion that HA treatment could promote oocyte

maturation and increase the competence of COCs. With regard to the TNFAIP6 and PTX3 genes, it was previously reported that they have similar expression profiles in bovine COCs following four different IVM treatments [12]. These findings are in agreement with the current results with regard to the control group, in which no significant differences were recorded between TNFAIP6 and PTX3 at 0, 22 and 44 hr. At the time points, in the HA-supplemented group, the expression of the TNFAIP6 and PTX3 genes differed from each other. TNFAIP6 expression significantly increased from 0 to 22 hr. and after 22 hr. to 44 hr. However, PTX3 expression initially significantly increased beginning at 0 hr. to 22 and then significantly declined at 44 hr. time points. This difference was related to the different effects of HA during the IVM period. The interaction of TNFAIP6 with HA chains is crucial to the subsequent stabilization of expanded cumuli. The binding occurs via the interaction of TNFAIP6 with pentraxin (PTX3), which is produced during the expansion of cumulus into the extracellular matrix and is upregulated by GDF9 [50,51].

In conclusion, the present study revealed significant increases in the expression of cumulus expansion genes (PTGS2, TNFAIP6, PTX3) and the nuclear maturation rate of oocytes in HA-supplemented group when compared to the un-supplemented control group. The expansion area in the HA-supplemented group, on the other hand, was significantly smaller than in the control group. In this way, extracellular HA retention increased in the HA-treated group, which affected the expression of cumulus expansion genes. In addition, such extracellular HA may have a different expansion pattern that decreases the area of COC.

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