Effect of Culture Media, Gonadotropins, Proteins, Growth Factors and Hyaluronic Acid on In-Vitro Maturation of Buffalo Oocytes

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ABSTRACT

This study was carried out to investigate the effect of culture media, gonadotropins, proteins, growth factors and hyaluronic acid (HA) on the IVM rate of buffalo oocytes. Cumulus oocyte complexes were aspirated from ovaries and classified into 4 Grades, excellent and good quality COCs. Oocyte maturation was tested using TCM199, CR1aa or DMEM (Experiment 1); 10 µg/mL FSH + 50 IU/mL eCG, 10 µg/mL FSH or 50 IU/mL eCG (Experiment 2); 10% FCS, 4 µg/mL BSA or 1 µg/mL PVA (Experiment 3). Control group: 10 µg/mL IGF-1 or 10 µg/mL EGF (Experiment 4) or 0, 2.5, 5 and 10 µg HA (Experiment 5). In all experiments, COCs were cultured for 252-24 h, then cytoplasmic and nuclear maturation was conducted. Results indicated that TCM199 achieved the best cytoplasmic and nuclear maturation. TCM199 supplemented with FSH and/or EGF enhanced in-vitro cytoplasmic and nuclear maturation of buffalo oocytes. Adding FCS, using growth factors, or 2.5 µg/mL HA to TCM199 lead to improving maturation rate, the results indicated that in-vitro maturation of buffalo oocytes is improved in TCM-199 supplemented with 50 IU FCS, 10 µg/mL FSH, 10% FCS, 10 µg/mL EGF or 2.5 µg/mL HA.

Keywords: Culture media; Gonadotropins; Protein supplement; Growth factors; Hyaluronic acid.

INTRODUCTION

Buffalo is the backbone of animal resources as the primary source of meat and milk in Egypt (Fahim et al. 2018). However, the reproductive efficiency of buffaloes decreased due to management and biological factors. Assisted reproductive technologies are a promising solution (Warriach et al. 2015). Despite this, the techniques for producing in-vitro buffalo embryos still require more effort (Marin et al. 2019).

Producing matured oocytes with good quality and quantity is an important criterion for fertilization and blastocyst rate (Widayati and Pangestu, 2020). In-vitro oocyte maturation didn’t mimic the same environment as in-vivo, and that caused reducing the quality of oocytes (Abd El-Aziz et al. 2016). This may be due to the many factors affecting in-vitro maturation, such as season of collection, ovarian status, follicle size, oocyte quality, method of recovery, etc. Culture media is one of the important factors that affect oocyte maturation rate and the quality of matured oocytes (Pereira et al. 2019). Tissue culture medium 199 (TCM-199) is widely spread among all IVM labs. (Zhao et al. 2009), whereas Christopher Rosenkranz’s Iaa (CR1aa) culture media was developed for using in-vitro maturation, fertilization, and culture, in addition, it has a simple composition, which makes it easy to produce in large amounts at low cost (Somfai et al. 2010). Adding hormones to IVM media leads to improve maturation rate (Abbasi and Sadrkhanlou, 2000). Basic maturation media of most mammalian oocytes supplemented with follicle stimulating hormone (FSH) is expensive compared with equine chorionic gonadotropin (eCG) (Kouamo and Kharche, 2017). The serum is one of the important elements of IVM media (Chandra et al. 2011, Sreenivas et al. 2014). Fetal calf serum (FCS) contains endotoxins that can inconsistently emerge with oocyte maturation rate and in-vitro embryonic development (McKerann and Bavister, 1992). The global demand for FCS has increased (Sreenivas et al. 2014), and there is a variation between serum batches (Verma et al. 2014). Not forgetting that some FCS are toxic and may be contaminated with fungi, viruses, and mycoplasma (Evans 2014). Replacement of mammal’s serum with other components such as polyvinyl alcohol (PVA) or bovine serum albumin (BSA) in-vitro embryo culture media to treat the problems of serum was a challenge in IVF laps (Watson et al. 2000, Del Collado et al. 2014 Quan et al. 2017). Growth factors promoted oocyte maturation in many species (Arat et al. 2016). Hyaluronic acid (HA) is the major glycosaminoglycans (GAGs) found in follicular and oviduct fluid (Rios et al. 2015). It is produced by cumulus cells during oocyte maturation, it is important for cumulus cell expansion, nuclear maturation, and embryo development. The HA concentration is a critical factor in the success IVM process (Opila et al. 2014).

In-vitro maturation technique developed for buffalo oocytes has tremendously been progressed over decades, but many factors such as maturation media, hormones, supplemented protein, growth factors, HA, oocytes quality, incubation condition, etc. are still influencing their competence and still need to be investigated. Therefore, the focus has been on some factors affecting IVM of buffalo oocytes to obtain a suitable maturation media. This research
aims to examine the effect of the type of IVM medium, supplementation of IVM media with hormones, different protein sources, growth factors, and HA on buffalo oocytes’ maturation rate.

**MATERIALS AND METHODS**

**Collection of ovaries**

This work was executed at the Department of Animal Reproduction and Artificial Insemination, Veterinary Research Institute, National Research Center. For five experiments, buffalo ovaries were collected from a local slaughterhouse (Shubra El-Khaimah, Qualyubia, Egypt) immediately after slaughter, and relocated to the lab in warmed 0.9% sodium chloride salt solution within 2 hours. In Lap, ovaries were rinsed with warmed saline solution three times and once with 70% ethal alcohol.

Collection of ovaries was done by the Department of Animal Reproduction and Artificial Insemination, Veterinary Research Institute, National Research Center.

In all experiments, COCs were matured in 0.5 mL of TCM199 with 50 IU/mL eCG and 10 μg/mL FSH. Aspirated COCs were matured in a sterile 15 mL falcon tube and left in water-bass at 37°C for 15 min to assemble the oocytes at the tube bottom. Then, the sediments were aspirated and put in a 10 cm aseptic Petri dish and PBS was mixed with sediments then examined under a stereomicroscope and rinsed 3 times in PBS and once in culture medium. Then COCs were classified into 4 categories as excellent, good, fair, and denuded according to the method described previously (Abdoon et al. 2018).

**Experimental design**

Five experiments were conducted according to the following experimental design:

<table>
<thead>
<tr>
<th>Exper.</th>
<th>Factors studied</th>
<th>Variable</th>
<th>Additives/medium*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Three types of maturation media</td>
<td>1) TCM-199, 10% FCS, 50 IU/mL eCG and 10 μg/mL FSH for each medium.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2) CR1aa</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3) DMEM</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Gonadotropin hormones</td>
<td>10 μg/mL FSH, 50 IU/mL eCG</td>
<td>TCM-199 with 10% FCS.</td>
</tr>
<tr>
<td>3</td>
<td>Protein supplementation</td>
<td>10% FCS, 50 IU/mL eCG</td>
<td>TCM-199 with 50 IU/mL eCG and 10 μg/mL FSH.</td>
</tr>
<tr>
<td>4</td>
<td>Growth factors</td>
<td>10 μg/mL FSH, 10% FCS, 50 IU/mL eCG, and 10 μg/mL FSH.</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Hyaluronic acid (HA) level</td>
<td>10 μg/mL EGF</td>
<td>TCM-199 with 10% FCS, 10 μg/mL FSH, and 50 IU/mL eCG.</td>
</tr>
</tbody>
</table>

*All media used were supplemented with antibiotics (50 μg/mL gentamycin) DMEM: Dulbecco’s Modified Eagle low glucose medium.

In all experiments, COCs were matured in 0.5 mL of maturation medium in a four-well culture dish (Nunclon, Denmark) in a CO2 incubator for 22 to 24 hours at 38.5°C in humidified air of 5% CO2.

The maturation rate was determined after 22-24h of IVM. Oocyte’s maturation was judged according to The nuclear maturation as indicated by absence (Fig. 1A) or presence of the 1st polar body (Figure 1B), and cytoplasmic maturation by determining the degree of cumulus-cell expansion as follows: grade zero (G0): no cumulus-cell expansion, grade one (G1): with slight cumulus-cell expansion in the outer layer of cumulus-cells, grade two (G2): with moderate cumulus-cell expansion, grade three (G3): with full cumulus-cell expansion (Figure 2).

**RESULTS AND DISCUSSION**

**Experiment 1:**

Influence of culture media on maturation rate of buffalo oocytes.

Results indicated that buffalo oocytes incubated in TCM199 show significantly higher percentage of both...
cytoplasmic in term of the highest oocytes at G3 and nuclear maturation rate (P< 0.05) followed by that incubated in CR1aa, while DMEM medium showed the lowest values (Table 1).

Table 1. Percentage of buffalo oocytes at different grades of cytoplasmic maturation and nuclear maturation rate (mean±SE) as effected by type of maturation media.

<table>
<thead>
<tr>
<th>Media</th>
<th>Classification of cytoplasmic maturation (%)</th>
<th>Nuclear MR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G3</td>
<td>G2</td>
</tr>
<tr>
<td>TCM199</td>
<td>52.27±1.15</td>
<td>17.13±0.51</td>
</tr>
<tr>
<td>CR1aa</td>
<td>44.75±1.26</td>
<td>20.50±1.29</td>
</tr>
<tr>
<td>DMEM</td>
<td>40.50±1.29</td>
<td>20.00±1.29</td>
</tr>
</tbody>
</table>

Overall means within a column with different superscript letters differ significantly (P< 0.05).

Supplementation of TCM199 with eCG with or without FSH achieved significant (P<0.05) increase of full cumulus-cell expansion (G3) percentage and percentage of 1st polar body (Cytoplasmic and nuclear maturation). Regarding the percentage of moderate cumulus-cell expansion (G2). IVM medium supported with combination of FSH and eCG showed significantly (P<0.05) the highest percentage compared with IVM medium with FSH or eCG alone. On the other hand, there is no significant (P>0.05) difference between slight cumulus cell expansions (G1) percentage of buffalo oocytes cultured in IVM medium provided with FSH with or without eCG. Addition of FSH to IVM medium lead significantly (P<0.05) to higher percentage of G0 compared with adding eCG with FSH.

It was reported that FSH stimulates the production of cAMP from cumulus cells to assist the oocyte enter meiotic maturation (Jaffe and Egbert, 2017). Widayati and Pangestu (2020) reported that provide maturation medium with FSH enhanced in-vitro oocyte maturation. Addition of FSH to culture media was important to induce the nuclear and cytoplasmic maturation. Although, FSH is necessary to spontaneous oocyte maturation, it is commonly believed that these hormones enhance oocyte cytoplasmic maturation significantly by adjusting the cumulus cell activities (Da Broi et al. 2018, Tetkova et al. 2019). In study on buffalo oocytes Hegab et al. (2009) found that maturation rate was 80.4% and 73.8% in TCM199 supplemented with 50 μg/mL eCG and 50 μg/mL FSH respectively. Supplementing the maturation media with FSH or eCG enhances subsequent buffalo embryo development stages (Abdoon et al. 2001).

Experiment 3:
Effect of adding protein or PVA to maturation media on maturation rate of buffalo oocytes.

The effect of adding FCS, BSA or PVA to maturation medium on cytoplasmic and nuclear maturation rate of buffalo oocytes are shown in Table 3. Adding FCS as a supplement to IVM medium improved (P< 0.05) the percentage of cytoplasmic and nuclear maturation rate of buffalo oocytes when compared with BSA or PVA. However, there is no significant (P>0.05) difference between moderate cumulus cell (G2) expansion percentage of buffalo oocytes cultured in IVM medium supplemented with FCS, BSA and PVA. Whereas, adding FCS or BSA to IVM medium significantly increased percentage of slight cumulus-cell expansion (G1) compared with adding PVA to maturation medium. Protein presence in IVM media have powerful effect on embryo development (Ali and Sirard, 2002). Also, it has an important role in acceleration formation of pronuclei (Eckert and Niemann, 1995). Albumin chelates heavy metals and participate in pH buffering (Mehta and Kessling, 1990). Also, protein can inhibit the effect of reactive oxygen species (Natsuyama et al. 1993).

Adding FCS to maturation media enhance oocytes maturation compare with BSA in buffalo (Deneke et al. 2013). Similar results were reported in cattle (Sreenivas et al. 2014; del Collado et al. 2015). This may be due to the abundance of nutrients, growth factors, antioxidants in FCS (Sreenivas et al. 2014; Puri et al. 2015). Also, its high content of lipid compared to BSA (del Collado et al. 2015).
This would provide oocyte with energy for growth and development (Prates et al. 2014).

Table 3. Effect of adding protein or PVA to maturation media on cytoplasmic and nuclear maturation of buffalo oocytes.

<table>
<thead>
<tr>
<th>Media</th>
<th>Classification of cytoplasmic maturation (%)</th>
<th>Nuclear MR</th>
<th>1st PB extruded</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G3</td>
<td>G2</td>
<td>G1</td>
</tr>
<tr>
<td>FCS</td>
<td>52.67(a)</td>
<td>17.33</td>
<td>15.00(b)</td>
</tr>
<tr>
<td>BSA</td>
<td>47.67(a)</td>
<td>14.67</td>
<td>10.33(b)</td>
</tr>
<tr>
<td>PVA</td>
<td>27.00(a)</td>
<td>13.67</td>
<td>19.00(b)</td>
</tr>
</tbody>
</table>

#Overall means within a column with different superscript letters differ significantly (P<0.05).
*G0: no cumulus expansion, G1 with slight expansion of the outer layer of cumulus cells. G2 with moderate cumulus cell expansion, and G3 with full cumulus-cell expansion.

- PB: Polar body, MR: maturation rate

Experiment 4: Effect of adding growth factors to maturation medium on maturation rate of buffalo oocytes.

The influence of adding IGF-1 or EGF to maturation medium on cytoplasmic and nuclear maturation of buffalo oocytes are shown in Table 4. Enrichment IVM (TCM-199) medium with EGF increased (P<0.05) percentage of G3 and 1st polar body extruded when compared with IGF-1 or control. While there is no significant difference (P>0.05) difference between moderate cumulus cell expansion (G2) percentage of buffalo oocytes cultured in IVM medium supplemented with or without growth factor. Regarding the percentage of slight cumulus-cell expansion (G1) IVM medium without addition obtained high (P<0.05) percentage compared with IVM medium supported with EGF, there is no significant difference when adding IGF-1 to IVM medium than controlled maturation medium or maturation medium with EGF. Controlled maturation medium or maturation medium supplemented with IGF-1 increased (P<0.05) percentage of G0 compared to add EGF to maturation medium.

Table 4. Effect of adding growth factors to maturation media on cytoplasmic and nuclear maturation of buffalo oocytes.

<table>
<thead>
<tr>
<th>Media</th>
<th>Classification of cytoplasmic maturation (%)</th>
<th>Nuclear MR</th>
<th>1st PB extruded</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G3</td>
<td>G2</td>
<td>G1</td>
</tr>
<tr>
<td>Control</td>
<td>52.67(a)</td>
<td>17.33</td>
<td>15.00(b)</td>
</tr>
<tr>
<td>IGF-1</td>
<td>53.67(a)</td>
<td>20.67</td>
<td>13.67(b)</td>
</tr>
<tr>
<td>EGF</td>
<td>61.67(a)</td>
<td>26.00</td>
<td>7.00(b)</td>
</tr>
</tbody>
</table>

#Overall means within a column with different superscript letters differ significantly (P<0.05).
*G0: no cumulus expansion, G1 with slight expansion of the outer layer of cumulus cells. G2 with moderate cumulus cell expansion, and G3 with full cumulus-cell expansion.

- PB: Polar body, MR: maturation rate

Sirisathien et al. (2002) suggested that EGF act directly on oocytes and/or cumulus cell. Also, addition of EGF to maturation medium for sheep oocytes led to enhance cleaving rate and blastocyst formation (Kelly et al. 2008). However, Vinayak, (2018) showed that adding EGF to maturation medium can improve bovine oocyte maturation.

Yousef et al. (2018) reported that addition of 10-20 ng/mL EGF to IVM medium promotes the maturation of buffalo oocytes. Also, Nagar and Purohit, (2005) mentioned that supplementing the IVM medium with 10 to 100 ng/mL of EGF increased oocytes maturation of goat. EGF is involved in regulating cell proliferation and apoptosis (Chen et al. 2017). IGF-1 and EGF play a valuable role in promoting oocyte maturation and embryo development in many mammalian species (Arat et al. 2016; Yang et al. 2019). In contrast, supplementation of IVM with IGF-1 improve in-vitro maturation in buffalo (Kumar and Purohit, 2004), in yak-cattle crossbred (Yang et al. 2019), in ovine (Kelly et al. 2008) in porcine (Mahanta et al. 2018) and in human (Yu et al. 2012). IGF-1 has multiple functions in cellular metabolism, growth, proliferation, and differentiation (Meiyou et al. 2015). Supplementing IVM medium with IGF-1 enhanced the developmental competence of sheep oocytes compared with control group (Javvaji et al. 2020). Also, Li et al. (2016) reported that enriching the IVM medium with IGF-1 to mouse COCs improve cumulus-cells expansion and oocyte nuclear maturation in mouse. This difference could be due to the dose or culture conditions

Experiment 5 Effect of adding different concentrations of hyaluronic acid to maturation medium on maturation rate of buffalo oocytes.

The influence of adding 2.5, 5.0 or 10 μg/mL hyaluronic acid (HA) to maturation medium on cytoplasmic and nuclear maturation of buffalo oocytes is presented in Table 5. The percentage of full cumulus-cells expansion and nuclear maturation rate was significantly the highest by adding 2.5μg/mL HA to maturation media compared with other groups, while increasing level of HA to 10μg/mL showed the lowest values as compared to control and other levels of HA.

Table 5. Effect of adding different concentrations of hyaluronic acid (HA) to maturation medium on cytoplasmic and nuclear maturation of buffalo oocytes.

<table>
<thead>
<tr>
<th>Media</th>
<th>Classification of cytoplasmic maturation (%)</th>
<th>Nuclear MR</th>
<th>1st PB extruded</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G3</td>
<td>G2</td>
<td>G1</td>
</tr>
<tr>
<td>Control</td>
<td>52.67(a)</td>
<td>17.33</td>
<td>15.00(b)</td>
</tr>
<tr>
<td>10 µg/mL</td>
<td>25.00(a)</td>
<td>20.67</td>
<td>19.67</td>
</tr>
<tr>
<td>5 µg/mL</td>
<td>40.33(a)</td>
<td>28.67</td>
<td>19.33</td>
</tr>
<tr>
<td>2.5 µg/mL</td>
<td>57.67(a)</td>
<td>22.67</td>
<td>12.00</td>
</tr>
</tbody>
</table>

#Overall means within a column with different superscript letters differ significantly (P<0.05).
*G0: no cumulus expansion, G1 with slight expansion of the outer layer of cumulus cells. G2 with moderate cumulus cell expansion, and G3 with full cumulus-cell expansion.

- PB: Polar body, MR: maturation rate

Cumulus-cells expansion is necessary for oocyte maturation (Yokoo et al. 2007) And HA concentration is a critical factor in success IVM process (Opiela et al. 2014).Suppression of HA synthesis during oocyte maturation led to significantly inhibited cumulus-cells expansion (Yokoo et al. 2008) In accordance with the present results, Marei et al., (2012) suggested that low
concentration of HA stimulate oocytes to enter in nuclear maturation. Based on these knowledge’s, it was supposed that the addition of exogenous HA with an adequate concentration could enhance the percentage of oocytes maturation. In this respect, Opiela et al. (2014) found that using oocyte maturation medium containing 0.375 mg/mL or 0.750 mg/mL HA has no significant effect on oocyte nuclear maturation. Also, Opiela et al. (2014) and Corn et al. (2005) mentioned that increasing concentration of HA above 0.07% during IVM may be risky on oocyte maturation. However Marei et al. (2012) showed no significant influence of addition 0, 0.1, 0.5 or 1 mg/mL of HA in maturation media on percentage of bovine oocytes matured.

**CONCLUSION**

The results indicated that in-vitro maturation of buffalo oocytes is improved in TCM-199 supplemented with 50 IU eCG, 10μg/mL FSH, 10% FCS, 10 μg/mL HA or 2.5 μg/mL HA.

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