Effect of in vitro copper supplementation on granulosa cell estradiol synthesis and associated genes

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ABSTRACT

The study was conducted by supplementing cupric chloride dihydrate to modulate the estradiol synthesis in granulosa cells with a hypothesis of possible use of copper to potentiate or partially replace the hormones for estrus induction / estrus synchronization in future studies. In present study copper at three doses (0.1, 0.5 and 1 mM level in culture medium) were tested to observe their effects on in vitro granulosa cell survival, estradiol synthesis and their associated genes of ovarian granulosa cells of goat. There was no effect of copper on the ovarian granulosa cell survival rate. There was a considerable increase in the estradiol level per ml culture medium basis by 6th day of in vitro culture with the second dose of copper i.e. 0.5 mM, but the increase was non-significant (P>0.05). There was no significant effect of copper on estradiol synthesis when expressed on per 30000 cell basis. Effect of copper (0.1 mM and 0.5 mM) on the mRNA expression of genes of aromatase (CYP19A1) and cyclin D2 (CCND2) was estimated. Copper had significantly (P<0.05) increased the mRNA expression of CCND2 and CYP19A1 in ovarian granulosa cells with only one of the two doses tested i.e. 0.5 mM. Hence, copper can be considered as a potential mineral to supplement along with hormones in estrus induction or estrus synchronization protocols to minimize the use of hormones.

Key words: CCND2, CYP19A1 Estrus induction, Goat, mRNA expression.

INTRODUCTION

The growth of ovarian follicles, ovulation and the formation of the corpus luteum are complex processes that involve dramatic changes in granulosa cell function. The role of gonadotropins, regulatory peptides and growth factors in modulation of granulosa cell function is well known (Qadeer, 2006). However, the mechanisms involved in intra-ovarian regulation of follicular development via autocrine and paracrine pathways are not yet well defined. The reproduction of small ruminants like goats and sheep managed under extensive range grazing conditions can be affected by nutrients availability and especially by the mineral content. Imbalance of minerals is associated with reproductive disorders like suppression of estrus, silent estrus, irregular estrous cycle, cystic ovary, poor follicular developments with delayed ovulation, and increase in embryonic mortality and reduced conception rate (Vázquez-Armijo et al., 2011). Less estradiol production is one of the important reasons of impaired reproductive function. Few studies revealed the association of copper and selenium with estradiol production (Basini and Tamanini, 2000; Kendall et al., 2003; Wang et al., 2008; Roychowdhury et al., 2014). Additionally, the effects of minerals in improving fertility was also reported (Wilde, 2006).

Copper (Cu) is essential trace element to maintaining the functioning of living organisms (Yunus et al., 2015). In in vitro studies, it was reported that potential use of Cu as atretic marker and for fertility improvement plans (Bhardwaj and Sharma, 2011). It was also reported that complexes of copper (Cu++) with gonadotropin-releasing hormone (GnRH) are even more effective in the release of luteinizing hormone (LH) than native GnRH (Michaluk and Kochman, 2007). Moreover, Cu-GnRH was more potent in inducing in vivo release of follicle stimulating hormone (FSH) than LH. Noradrenaline is an essential neurotransmitter involved in the secretion of gonadotropin releasing hormone (GnRH) and copper has an important role in the activity of dopamine-amonooxygenase by inducing hydroxylation of dopamine to noradrenaline. FSH and LH are released from anterior pituitary after binding of GnRH with a specific receptor on the gonadotrope cell membrane (Michaluk and Kochman, 2007).

In the ovary, hormones control the development of individual follicles by triggering sequential, dynamic changes in granulosa cell proliferation and gene expression (Pederson, 1970). In ruminants and human, granulosa cells are able to convert theca cell derived androgen to estradiol in presence of aromatase enzyme which is encoded by CYP19A1 gene.

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(Simpson and Devis, 2001). The expression of CYP19A1 mRNA is stimulated by FSH in rats, humans and ruminants (Steinkampf et al., 1987; Fitzpatrick and Richards, 1991; Silva and Price, 2002) by cAMP/protein kinase A (PKA) intracellular second messenger pathway (Conti, 2002). Cellular proliferation and differentiation are fundamental biological processes controlled by extracellular signals that impinge upon cell cycle regulatory machinery and modulate gene expression. During the G1 phase of mammalian cell cycle, D-type cyclins (DI, D2 and D3) regulates the cell cycle. In proliferating cells these D-type cyclins are expressed in overlapping, apparently redundant fashion (Sherr, 1994).

Granulosa cells are steroid secreting and hormone responsive cells under the influence of gonadotropins. Therefore, granulosa cells were employed for studying the steroidogenesis (Grasselli et al., 2001). There have been some sporadic studies which gave some clue on the positive effect of copper and selenium on the in vitro granulosa cell estradiol synthesis (Basini and Tamanini, 2000; Kendall et al., 2003; Roychowdhury et al., 2014). Estradiol concentration was significantly increased (p>0.05) with dietary supplementation of copper in the form of copper sulphate at 8mg/kg of dry matter in cattle (Wang et al., 2008) (p<0.05). Hence, this study was contemplated to explore the effect of copper on granulosa cell proliferation, estradiol synthesis and associated genes.

**MATERIALS AND METHODS**

The experiments were conducted at ICAR-National Institute of Animal Nutrition and Physiology, Bengaluru in 2014-15. All the chemicals used were from Himedia Labs (India) unless otherwise stated.

**Granulosa cell culture:** Granulosa cells were cultured in minimum essential medium as described by Sen et al., (2007) and Gupta et al. (2014) with little modifications as androstenedione was replaced with testosterone and Fetetal Bovine Serum was replaced with steer serum. Culture medium comprised of minimum essential medium supplemented with HEPES (20 mM), Sodium bicarbonate (10 mM) and BSA (0.5% w/v), Non-essential amino acids (1.1 mM), bovine Insulin (1 ng/ml)(Sigma Aldrich), Sodium Selenite (4 ng/ml), Apotransferrin (5 µg/ml) (Sigma Aldrich), Testosterone (104 M), LR3-IGF-1 (2 ng/ml) and Antibiotic antmycotic solution 100 X (10000 U Pencillin, 10mg Streptomycin and 25µg Amphotericin B per ml).

Ovaries from goat of unknown reproductive status were collected from nearby civil slaughter house, Bangalore. They were brought to the laboratory within two hours in an ice box at 4°C containing 0.9% normal saline and Gentamycin (50µg/ml) and washed with chilled normal saline three times. Aspiration medium was taken in 5 ml syringe with 24 gauge needle, and granulosa cells were aspirated from medium sized follicles (3-6 mm) from goat ovaries. Granulosa cells were collected after three times washing with MEM medium by centrifugation at 400 X g for 5 min at 4°C. A 1:5 dilution of Trypan Blue in medium and 1:10 dilution of granulosa cells in medium were mixed to find the viability of cells using automated cell counter (Invitrogen, USA). The viability of the cells ranged between 70-80% at the time of isolation.

After cell counting, it was diluted so that 100,000 live cells were present in 50 µl (2×104 cells/ml). In 96 well plates 150µl of media treated with 0, 0.1, 0.5, and 1 mM copper (Pre-equilibrated in the 37°C CO2 incubator) along with pFSH (10 ng/ml) (Sigma Aldrich: F2293, activity: 1 IU/mg) were added in each well (warmed supplemented MEM) followed by addition of 50 µl cell suspension containing 100,000 live cells to each well. Media was changed on days 2 and 4 of culture by replacing 150 µl of copper supplemented media from each well with fresh 150 µl of media (equilibrated in the 37°C incubator with 5% CO2). On sixth day spent media were stored at -20°C for Hormone assays and cells were preserved for gene expression studies.

**Cell counting:** At the end of the culture, after collecting the spent medium two times, washing with DPBS were performed by centrifugation at 300 X g for 5 min at room temperature. After 2nd washing, 150 µl of solution was removed. Then, trypsinization was performed by adding 50µl of 0.25% trypsin in each well and incubated at 37°C for up to 20 minutes. To neutralize the trypsin digestion, 100 µl of 10% of sterile steer serum was added to each well and mixed well. Viability of cells was determined as described above.

**Radio immuno assay:** Estimation of estradiol in cell culture spent medium was carried out by Radio immuno assay using Beckman Coulter estradiol assay kits. The sensitivity of the assay was 2.5 pg/tube. The intra assay variation and inter assay coefficient of variations of the kits were below 14.4 % and 14.5%.

**RNA Isolation:** Total RNA was isolated from the lysates using the RNeasy mini kit (Qiagen) and DNase treated on column following the manufacturer’s protocol. Total RNA (100 ng/ sample) was then converted into cDNA using the iScript cDNA synthesis kit (Bio-Rad) following the manufacturer’s instructions. Nuclease free water was then added to dilute the cDNA to a total of 40 µl.

**Standardization of primers concentration by real time PCR:** Standardization of concentration of primer was done by testing different concentration of primers at 1.5, 2.5, 5, 7.5 and 10 pM concentration with FAST SYBR Master Mix kit (Applied Bioscience, USA). On the basis of melt curve, the particular concentration of primer was selected for real time PCR. Standardization was done for CYP19A1, RPS18 (house keeping gene) and CCND2.

**Q-PCR:** Quantitative PCR was performed using duplicate 10 µl reactions containing 5 µl of SyBrGreen (dye) qPCR Super Mix (Applied bioscience, USA), CYP19A1 as 7.5 pM, CCND2
as 2.5 pM and RPS18 as 5 pM of forward and reverse primer (1 µl each), 1 µl of cDNA with 3.75 ml of nuclease free water. The amount of transcripts present for CCND2 and CYP19A1 gene in the treated and control cells was normalized to RPS18 (housekeeping gene). The PCR condition and primer sequences are mentioned in Table 1 and Table 2, respectively.

**Statistical analysis:** The data was analysed using Graphpad PRISM software. The differences between means of different groups of an experiment were analysed by one way ANOVA followed by Tukey’s Multiple comparison test. The percentage values of cell recovery rates were converted to arcsine values before statistical analysis. Significance was determined at P<0.05.

**RESULTS AND DISCUSSION**

In the present study, caprine granulosa cells were cultured to find out the effect of copper on cell proliferation / survival rate and estradiol synthesis and their associated genes. Copper in the form of CuCl$_2$·2H$_2$O was used at the dose rates of 0.1, 0.5 and 1 mM levels which is equivalent to 17, 85 and 170µg of CuCl$_2$·2H$_2$O/ml, respectively. Only the first two doses of copper were tested for their effects on gene expression for the following reason. There was no significant difference among the three doses of copper in terms of estradiol production on 30000 cell basis. The gene that code for aromatase enzyme i.e. CYP19A1 was chosen for gene expression studies because of importance of aromatase in the estradiol synthesis. Cyclin D is responsible for cell proliferation and hence the study of expression of its gene i.e. CCND2 would give insight into the granulosa cell proliferation in the present study.

**Effect on ovarian granulosa cell survival:** Cupric Chloride (CuCl$_2$·2H$_2$O) was supplemented to ovarian granulosa cell culture medium at different doses i.e. 0.1, 0.5 and 1 mM concentrations. Complete clumping of granulosa cells was noticed in the cell cultures by sixth day of culture (Fig.1). There was no significant difference (P>0.05) in the recovery rates/ survival rate of granulosa cells among different dose groups of copper (Fig. 2). The mean cell recovery rates were in the range of 29-33% in different groups of copper supplementation. There was a minor and non-significant (P>0.05) gradual increase in the cell survival rate from the control group up to the dose of 0.5mM, but there was a non-

![Fig 1: Clumping of ovarian granulosa cells on the sixth day of in vitro culture](image)

**Fig 1:** Clumping of ovarian granulosa cells on the sixth day of *in vitro* culture

![Fig 2: Cell recovery (%) after six days of in vitro culture of ovarian granulosa cells with different doses of copper (mM).](image)

**Fig 2:** Cell recovery (%) after six days of *in vitro* culture of ovarian granulosa cells with different doses of copper (mM).

Values of different superscripts did not differ significantly (P>0.05)

### Table 1: qPCR conditions.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Cycle(s)</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage-I</td>
<td>1</td>
<td>95°C</td>
<td>20 sec</td>
</tr>
<tr>
<td>Stage-II</td>
<td>40</td>
<td>95°C &amp; 60°C</td>
<td>95°C-3sec, 60°C-30sec</td>
</tr>
<tr>
<td>Stage-III</td>
<td>1</td>
<td>95°C &amp; 60°C</td>
<td>95°C for 15sec, 60°C for 30sec, 95°C for 15sec</td>
</tr>
</tbody>
</table>

### Table 2: Gene transcripts, primer sequences and resulting fragment size

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence(5'→3')</th>
<th>Accession no.</th>
<th>Productsize (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPS 18</td>
<td>F: GTGGTTGGAGGAAGCAGACA R: TGATCACGGTCCACCTCATC</td>
<td>NM_001285747.1</td>
<td>95</td>
</tr>
<tr>
<td>CYP19A1</td>
<td>F: GCCTCCTCTCTCAAACCAGACA R: TCGTGCGGAAATCTATGCGAT</td>
<td>XM_005680985.1</td>
<td>166</td>
</tr>
<tr>
<td>CCND2</td>
<td>F: GGCAAGTTGAAATGGAACCTGG R: CCGGTACATGGCACAATTGTGAAG</td>
<td>NM_001033614.2</td>
<td>161</td>
</tr>
</tbody>
</table>
significant decrease there after i.e. with the dose of 1.0 mM of copper. The granulosa cells will undergo a natural onset of apoptosis when cultured in serum-free conditions (Yang and Rajamahendran, 2000). FSH has been reported to reduce apoptosis in cultured granulosa cells (Tilly and Tilly, 1995; Yang and Rajamahendran, 2000). The apoptosis in follicles was also shown to be inhibited by super oxide dismutase and catalase (Tilly and Tilly, 1995). It has been reported that there is threefold increase in catalase activity in presence of FSH in cultured granulosa cells which prevent cellular apoptosis and increase estradiol production (Behl and Pandey, 2002). In present study copper was used at different doses in cultured granulosa cells and the percentage cell survival was studied. It has shown that copper has non-significant effect on the cell survival rate at 0.1 and 0.5 mM doses, but the dose of 1 mM copper caused a non-significant decrease in the cell survival rate which may be due to reduced catalase and superoxide dismutase activity. The antioxidant enzyme activity in future studies may give better idea about the decrease in cell recovery with 1 mM dose.

**Effect on estradiol synthesis:** Cupric Chloride (CuCl$_2$,2H$_2$O) was supplemented to ovarian granulosa cell culture medium at different doses i.e. 0.1, 0.5 and 1 mM concentrations. There was a significant (P<0.05) difference among different dose groups of copper in the estradiol levels (Table 3). The mean estradiol levels were in the range of 75 to 130pg/ml (Fig 3) and in terms of 30000 cell basis, the estradiol levels were in the range of 73 to 117pg / 30000 cells (Fig 4). There was a slight and non-significant (P>0.05) increase in the estradiol production on per ml medium basis up to the dose of 0.5mM, but with the highest dose of 1.0 mM, there was a significant drop in the synthesis of estradiol compared to the lower doses. Though there was a slight drop in the estradiol synthesis on 30000 cell basis with the doses of 0.1 and 1.0 mM of copper compared to control group, it was not significant (P>0.05). In a study made on bovine granulosa cells in vitro (Kendall et al., 2003) copper had no significant effect on estradiol production when it was tested at the concentrations of 0.05, 0.51, 5.16 and 51.6µg/ml in presence of 1 ng/ml of ovine FSH. However, when it was tested @ 516 µg/ml concentration, it reduced the estradiol concentration significantly. Hence, in the present study, the doses were chosen as 0.1mM, 0.5mM and 1mM, which are equivalent to 17 µg, 85 µg and 170µg of CuCl$_2$,2H$_2$O /ml. In terms of absolute copper concentration, they were equivalent to 6.3 µg of copper /ml, 31.5 µg of copper /ml and 63 µg of copper /ml, respectively. However, there are no previous studies on the effect of copper on *in vitro* granulosa cell estradiol synthesis. In the present study, there was a considerable increase in the estradiol levels when CuCl$_2$,2H$_2$O was supplemented at 0.5mM, which can be a guiding value for selecting the doses to be used in live animals for supplementing the hormonal action in thesuper-ovulation or estrus induction or estrus synchronization protocols. Use of the minerals to partially replace the hormones in these assisted reproductive technologies will minimize the use of hormones in animals that will abrogate the negative effects of hormonal resistance and increase the acceptance of

<table>
<thead>
<tr>
<th>Dose (mM)</th>
<th>Estradiol(pg/ml) Mean ±SEM</th>
<th>Estradiol(pg/30000 cells) Mean ±SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (Control)</td>
<td>112.9±14.76</td>
<td>115.3 ±15.07*</td>
</tr>
<tr>
<td>0.1</td>
<td>121.1±15.47</td>
<td>112.2±14.33*</td>
</tr>
<tr>
<td>0.5</td>
<td>130.4±19.84</td>
<td>117.0±17.80*</td>
</tr>
<tr>
<td>1.0</td>
<td>75.2±9.65</td>
<td>73.7±9.46*</td>
</tr>
</tbody>
</table>

SEM- Standard error mean

Values in a column with different superscripts differ significantly (P<0.05)
meat from the animals that are treated with no or less amounts of hormones. The LH-induced differentiation of bovine theca cells in vitro was prevented by thiomolybdates and copper supplementation could ameliorate this effect. So, copper has an important role in the normal pattern of ovarian steroidogenesis. There are no reports available on the effect of copper on in vitro estradiol production in goats. However, Wang et al. (2008) reported significantly increased estradiol level in Simmental heifer when they were supplemented with copper sulphate at 8mg/kg of dry matter of feed.

When ovine FSH was incorporated @ 10ng/ml in ovarian granulosa cell cultures of goat, it could not increase the estradiol production, but when supplemented at 100ng or 200ng/ml, it could significantly increase the estradiol levels (Behl and Pandey, 2002). In the present study, FSH was added along with testosterone, Insulin and IGF in culture medium to trigger the production of at least basal levels of estradiol in order to facilitate the study of effect of minerals on estradiol production.

Effect on mRNA expression: Effect of copper on mRNA expression of CCND2 and CYP19A1 was studied in ovarian granulosa cells after harvesting them after 6 days of in vitro culture. There was a significant (P<0.05) increase in the mRNA expression of CYP19A1 when copper was supplemented at 0.5mM concentration into the ovarian granulosa cell culture of goats. But there was a non-significant (P>0.05) increase in the CYP19A1 mRNA expression when copper was supplemented at 0.1mM level (Fig 5). There was no significant effect of copper on mRNA expression of CCND2 in ovarian granulosa cells of goat when cultured for six days in presence of different doses of copper. However, there was a non-significant (P>0.005) but considerable increase when copper was added to the ovarian granulosa cell culture (Fig 6).

There are no studies conducted on the effect of copper on mRNA expression of CCND2 or CYP19A1 in ovarian granulosa cells. However, Roychowdhury et al. (2014) tested the effects of copper sulphate on porcine granulosa cell survival rates, Insulin like growth factor-I levels and the gene expression of cyclin B1 gene. They opined that copper can interfere in the pathways of porcine granulosa cell proliferation through hormonal and intracellular peptide cyclin B1. In the present study, though there was a non-significant increase in the cyclin D2 gene (CCND2) gene expression, but it was a considerable increase when copper was added to the ovarian granulosa cell culture. The results of the present study indicated that copper had positive effect on estradiol synthesis and associated gene expression in ovarian granulosa cells of goat.

CONCLUSION

Repeated use of hormones for estrus induction and synchronization may induce hormone resistance in animals. Use of minerals to potentiate the action of hormone in these reproduction technologies in the future studies can minimize the use of hormones. Copper at the dose of 0.5 mM had considerably increased the estradiol production and significantly increased the aromatase gene expression (increase of mRNA expression of CYP19A1). Hence copper can be considered as potential agents that can be supplemented along with hormones for estrus induction or estrus synchronization, which needs to be studied in live animals. There is a further scope for studies in this direction in which the copper dependent enzymes like Lysyl oxidase can be estimated in the granulosa cell cultures to probe the effect of copper further. The estimation of enzymes like protein kinase A, Protein kinase B or their mRNA expression may throw more light on the mechanism of action of copper in stimulating the estradiol synthesis.
REFERENCES


