



Influence of curcumin and carbazole on ovine ovarian preantral follicle and granulosa cell functions

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Curcumin is a polyphenolic substance produced from the rhizome of the herb *Curcuma longa* (Mishra and Mishra 2018). Curcumin's antioxidant (Jakubczyk *et al.* 2020), anti-inflammatory (Ueki *et al.* 2013), anti-apoptotic (Geng *et al.* 2017), and antibacterial (Mun *et al.* 2013) properties have also garnered considerable attention. Curcumin has also been shown to stimulate ovarian function by increasing folliculogenesis and decreasing apoptosis in murine ovarian cells (Aktas *et al.* 2012). Similarly, other significant compound that have a substantial influence on female reproductive functions is carbazole (an alkaloid present in leaves of *Murraya koengii*, roots of *Glycomis pentaphylla*, and *Clausena heptaphylla*; Shanthini *et al.* 2020). The aim of this study was to determine the effect of curcumin and carbazole at various doses on the activities of the ovine preantral follicle and granulosa cell.

Sheep ovaries were collected from the civil slaughter house, Bengaluru. The ovaries were collected and transported in 0.9% warm (37°C) normal saline supplemented with gentamicin (50 µg/ml). Follicular fluid was aspirated from the surface follicles of the ovary and suspended in the medium (TCM-199 + 0.3% BSA) according to the methodology described by Nandi *et al.* (2017, 2018). Granulosa cells [(0.8–1) × 10⁵/well] were cultured for 2 days with a standard culture medium containing TCM-199 + HEPES (20 mM) + L-glutamine (3 mM) + bovine serum albumin (1%) + insulin-transferrin-selenium (1%) + gentamicin (50 mg/ml) in a CO₂ incubator (38.5°C with 5% CO₂ in air + 90–95% relative humidity). Total 6 groups + control [Control, T1 (1 µM), T2 (5 µM), T3 (10 µM), T4 (25 µM), T5 (50 µM), T6 (100 µM)] were maintained each with 6 replicates of bioactive compounds. Curcumin and carbazole treatment groups were treated separately in separate trials for GC culture. Cultured GC's were then harvested (after 2 days) and viability of the cells were determined by trypan blue exclusion test (Nandi *et al.* 2018). Viable granulosa cells were counted further by using

haemocytometer and cell number increment (10⁵) was determined for each bioactive compound separately. Similarly, preantral follicles (PF) with definite follicular outline and visible oocyte were isolated from the above collected ovaries as per the method described by Nandi *et al.* 2017. PF specifically measuring 250 to 450 µm and having more than 4 layers of granulosa cells (Large PF) were isolated and viability was checked with 0.04% trypan blue staining technique (Nandi *et al.* 2017). Viable PF were cultured as per the procedure described by Nandi *et al.* (2017) in the standard PF culture media containing MEM supplemented with BSA (0.3%), glutamine (2 mM), sodium pyruvate (0.23 mM), hypoxanthine (2 mM), insulin-selenium-transferin (1%), gentamicin (50 µg/ml) and FSH-P (7 µg/ml; biological potency = 7 IU/mg; F2293; LH 24 ≤ 1%). Total 6 groups + control [Control, T1 (1 µM), T2 (5 µM), T3 (10 µM), T4 (25 µM), T5 (50 µM), T6 (100 µM)] were maintained each with 6 replicates of bioactive compounds. Large PF were cultured for 7 days and the media was changed every alternate day (day 1, 3, 5). The preantral follicles' growth rate, viability rate, and survival rates were calculated as described by Nandi *et al.* (2017).

Growth rate of follicle (µm/day) = (Final diameter (µm) of follicle as observed on day 7 – Initial diameter (µm) of follicle) / Days of culture

Viability rate (%) = (Number of follicles that were not stained (viable) / Follicle cultured) × 100

Survival rate (%) = (No. of viable follicles at the end of culture / no of viable follicles put into culture) × 100.

Furthermore, the release of estradiol (pg/ml) in granulosa cell culture medium on day 5 was examined using enzyme-linked immunosorbent assay kits (Diagnostics Biochemicals Pvt. Inc., Ontario, Canada). The data were statistically analyzed using a one-way ANOVA followed by the Tukey's test. At the 5% level of significance (P 0.05), the significance or non-significant of differences between mean values was determined.

The survival rate of the PF cultured in the media containing 10 µM (74.3 ± 1.5%) and 25 µM (76.3 ± 1.4%) curcumin was significantly higher than those obtained in lower doses. Besides that, further increment of the levels

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of curcumin did not improve the survival rate. Moreover, the survival rate was decreased at the highest level, i.e. 100 μM dose of curcumin ($64.7 \pm 2.1\%$) which was similar to those obtained in control ($62.6 \pm 1.3\%$) and lower doses of curcumin. Similarly, the 25 μM dosage ($16.1 \pm 0.9 \mu\text{m/day}$) of curcumin significantly increased the growth rate of cultured PF compared to control ($7.6 \pm 0.6 \mu\text{m/day}$) and lower doses. Further increments in curcumin doses, on the other hand, greatly reduced the PF's growth rate. The reason attributed for the PF growth and survival rate by addition of curcumin at specific dose to the culture media might be due to upregulation of the expression of genes and/or pathways involving folliculogenesis (Hendarto and Widjati 2018, Yan *et al.* 2018). The obtained results are in accordance with the Hendarto and Widjati (2018) where the addition of curcumin @ 20 μM to the bovine COCs culture has improved the expression levels of GDF-9 and KitL genes which subsequently improved the folliculogenesis. Compared to the control and lower doses of curcumin, the granulosa cell number and estradiol production were significantly higher @ 50 μM dose of curcumin. Moreover, increasing the curcumin dose did not result in a significant improvement in the number of granulosa cells. The increment in granulosa cell number caused by the addition of curcumin at a specific dose could be due to curcumin's positive effect on proliferation and steroidogenesis in the granulosa cells of the ovaries, as evidenced by Kadasi *et al.* (2017) who found that adding curcumin @ 10 μM stimulated progesterone and testosterone levels in the granulosa cells of the ovaries. Vashist *et al.* (2018), on the other hand, found that adding 50 μM curcumin to buffalo granulosa cells reduced cell viability. Curcumin @ 50 μM dosages (24 h and 48 h) and 10 μM doses (72 h) were found to be cytotoxic in the human granulosa cells by Moreira-Pinto *et al.* (2019). Shi *et al.* (2006), on the other hand, found that this action was beneficial in human ovarian cancer cells, as curcumin in the regular diet reduced the proliferation of human ovarian cancer cells. Increasing curcumin levels, viz. 100 μM significantly reduced estradiol synthesis. Curcumin's phytoestrogenic effect, where it can exert its effects on the hypothalamo-hypophyseal-ovarian axis via interacting with the endocrine system, could be the basis for estradiol stimulation (Bachmeier *et al.* 2010).

The survival rate of the PF cultured in 25 μM ($74.3 \pm 1.3\%$) carbazole added media was significantly higher than that of the PF cultured in lower doses ($62.2 \pm 1.7\%$). Aside from the preceding, increasing carbazole levels resulted in a decrease in the PF survival rate. The reduction was most apparent at higher carbazole dose, viz. 100 μM level ($44.6 \pm 1.4\%$). Compared to the control and lower doses, the granulosa cell number increment was significantly higher @ 50 μM carbazole levels. The number of granulosa cells did not increase as the carbazole levels were increased further. Furthermore, at higher carbazole dose a significant reduction in granulosa cell number was observed. There is no significance between

the groups with regards to the estradiol production. There is a scarcity of literature on the culture of preantral follicles and granulosa cells in relation to carbazole alkaloids. The majority of research on the effects of plant-derived alkaloids has focused on apoptosis in cancer cells.

In conclusions, curcumin was found to be effective for PF survival and growth rate at 10 and 25 μM concentrations, respectively. Curcumin was also successful in increasing granulosa cell number and estradiol production at 50 μM doses. Carbazole was shown to be effective at 25 μM doses for improving PF survival, growth, and granulosa cell number increment. The different carbazole doses, on the other hand, have no effect on estradiol production. Curcumin and carbazole had a hormetic effect on the sheep ovarian preantral follicle, granulosa cells, and estradiol production (Martel *et al.* 2019).

SUMMARY

The present study was undertaken to study the effect of plant bioactive compounds curcumin and carbazole on sheep ovarian functions. In the present study, both the bioactive compounds were tested at different levels (Control, T1-1 μM , T2-5 μM , T3-10 μM , T4- 25 μM , T5-50 μM , T6-100 μM) on preantral follicle (PF) growth rate, survival rate (6 days culture), granulosa cell (GC) number increment (2 days culture) and estradiol production (5 days GC culture spent media). Curcumin had shown a significantly higher PF survival rate (%), i.e. 74.3 ± 1.5 , 76.3 ± 1.4 at 10 and 25 μM levels respectively. Similarly, higher PF growth rates ($\mu\text{m per day}$), i.e. 16.1 ± 0.9 was observed at 50 μM levels. Similarly, curcumin was effective @ 50 μM level to increase the granulosa cell number as well as estradiol production with a mean granulosa cell number ($\times 10^5$) and estradiol production (pg) values of 1.55 ± 0.04 and 85.3 ± 3.3 respectively. Likewise, carbazole was effective at the level of 25 μM to increase the PF growth rate ($\mu\text{m per day}$), survival rate (%) with mean values of 74.3 ± 1.3 , 12.1 ± 0.9 . Similarly, carbazole was effective at 50 μM dose levels in the granulosa cell number increment ($\times 10^5$) with a mean value of 1.57 ± 0.02 . No significant change in estradiol production was observed in carbazole treated group.

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REFERENCES

- Aktas C, Kanter M and Kocak Z. 2012. Antiapoptotic and proliferative activity of curcumin on ovarian follicles in mice exposed to whole body ionizing radiation. *Toxicology and Industrial Health* **28**: 852–63.
- Augustyniak A, Bartosz G, Cipak A, Duburs G, Horakova L, Luczaj W and Zarkovic N. 2010. Natural and synthetic antioxidants: An updated overview. *Free Radical Research* **44**: 1216–62.
- Bachmeier B E, Mirisola V, Romeo F, Generoso L, Esposito A,

- Dell'Eva R, Blengio F, Killian P H, Albini A and Pfeffer U. 2010. Reference profile correlation reveals estrogen-like transcriptional activity of curcumin. *Cellular Physiology and Biochemistry* **26**: 471–48.
- Geng X, Hong Q, Wang W, Zheng W, Li O, Cai G, Chen X and Wu D. 2017. Biological membrane-packed mesenchymal stem cells treat acute kidney disease by ameliorating mitochondrial-related apoptosis. *Scientific Reports* **24**: 7.
- Hendarto H M W and Widjati W. 2018. Curcumin improves growth factors expression of bovine cumulus-oocyte complexes cultured in peritoneal fluid of women with endometriosis. *International Journal of Reproductive BioMedicine* **16**: 775–82.
- Iman V, Mohan S and Abdelwahab S L. 2016. Drug design, development and therapy dove press anticancer and anti-inflammatory activities of girinimbine isolated from *Murraya koenigii*. *Drug Design Develoepement and Therapy*. **11**: 103–21.
- Jakubczyk K, Druzga A, Katarzyna J and Zydecka K S. 2020. Antioxidant potential of curcumin—A meta analysis of randomized clinical trials. *Antioxidants* **9**: 1092.
- Kádasi A, Maruniaková N, Štochmalová A, Bauer M, Grossmann R, Harrath AH, Kolesárová A and Sirotkin A V. 2017. Direct effect of curcumin on porcine ovarian cell functions. *Animal Reproduction Science* **182**: 77–83.
- Martel J, Ojcius D M, Ko Y F, Ke P Y, Wu C Y, Peng H H and Young J D. 2019. Hormetic effects of phytochemicals on health and longevity. *Trends in Endocrinology and Metabolism* **30**: 335–46.
- Mishra J and Mishra K. 2018. *Curcuma* sp. The nature's souvenir for high altitude illness *Management of High Altitude Pathophysiology*, Elsevier.
- Mohan K and Jeyachandran R. 2012. Alkaloids as anticancer agents. *Annals of Phytomedicine* **1**: 46–53.
- Moreira-Pinto B, Costa L, Fonseca B M and Rebelo I. 2019. Dissimilar effects of curcumin on human granulosa cells: Beyond its anti-oxidative role. *Reproductive Toxicology* **95**: 51–58.
- Mun S H, Joung D K, Kim Y S, Kang O H, Kim S B, Seo Y S, Kim Y C, Lee D S, Shin D W, Kweon K T and Kwon D Y. 2013. Synergistic antibacterial effect of curcumin against methicillin-resistant *Staphylococcus aureus*. *Phytomedicine* **15**: 714–18.
- Nandi S, Tripathi S K, Gupta P S P and Mondal S. 2017. Effect of metabolic stressors on survival and growth of *in vitro* cultured ovine preantral follicles and enclosed oocytes. *Theriogenology* **104**: 80–86.
- Nandi S, Tripathi S K, Gupta P S P and Mondal S. 2018. Nutritional and metabolic stressors on ovine oocyte development and granulosa cell functions *in vitro*. *Cell Stress and Chaperones* **23**: 357–71.
- Shanthini N G, Sheela T and Begum S. 2020. Extraction, fractionation, isolation and characterization of a carbazole alkaloid mukonicine from the leaves of *Murraya koenigii*. *Journal of Pharmacognosy and Phytochemistry* **9**(5): 1161–63.
- Shi M, Cai Q, Yao L, Mao Y, Ming Y and Ouyang G. 2006. Antiproliferation and apoptosis induced by curcumin in human ovarian cancer cells. *Cell Biology International* **30**: 221–26.
- Ueki M, Ueno M, Morishita J and Maekawa N. 2013. Curcumin ameliorates cisplatin-induced nephro-toxicity by inhibiting renal inflammation in mice. *Journal of Bioscience and Bioenergy* **115**: 547–51.
- Vashisht M, Rani P, Sunita, Onteru S K and Singh D. 2018. Curcumin primed exosomes reverses LPS-induced pro-inflammatory gene expression in buffalo granulosa cells. *Journal of Cell Biochemistry* **119**: 1488–1500.
- Yan Z, Dai Y, Fu H, Zheng Y, Bao D, Yin Y, Chen Q, Nie X, Hao Q, Hou D and Cui Y. 2018. Curcumin exerts a protective effect against premature ovarian failure in mice. *Journal of Molecular Endocrinology* **60**: 261–71.