Indian Journal of Animal Sciences **88** (11): 1272–1274, November 2018/Short communication

Effect of different doses of Wnt signal inhibitor (IWR) on estradiol synthesis of ovarian granulosa cells in goat

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Received: 14 March 2018; Accepted: 2 July 2018

Key words: Estradiol synthesis, Inhibitor of Wnt response (IWR), Ovarian granulosa cell, Wnt signal

There are 3 major pathways that operate in the estradiol synthesis i.e. protein kinase A (PKA), protein kinase B and Wnt signal. The signalling molecule Wnt stands for Wingless-related integration site. The name Wnt is a portmanteau (word derived after blending two words) of int1 gene (an oncogene present in many species of animals) and Wg gene (gene for wingless character in *Drosophila*, which is same as int1 gene of other species). Among the 3 important pathways of estradiol synthesis, the Wnt signal pathway is the least studied pathway in domestic animals. The pathways of estradiol synthesis in ovarian granulosa cells fallows crosstalking between different pathways and complex mechanisms that are still not fully understood. The Wnt signalling pathway is conserved in various species from worms like flat worms to mammals, and plays important roles in development, cellular proliferation and differentiation (Gifford 2015). The molecular mechanisms by which the Wnt signal regulates cellular functions are becoming increasingly well understood. There has been a growing interest on the role of Wnt signalling in the ovarian granulosa cell estradiol synthesis (Boyer *et al.* 2010). The positive role of Wnt signal in estradiol synthesis of murine granulosa cells is well established. There are few sporadic studies on the role of Wnt signal in the estradiol synthesis in domestic animals (Castnon *et al.* 2013, Gupta *et al.* 2014). Lee *et al.* (2016) indicated that Wnt /β-catenin signalling pathway activation is required for proliferation of chicken primordial germ cells *in vitro*.

Gupta *et al.* (2014) used inhibitor of Wnt response (IWR) to study the role of Wnt signal in estradiol synthesis of cattle. But the role of Wnt signal in the estradiol synthesis in goats has not been studied yet. But before studying the role of Wnt signal by using IWR, we need to standardize the dose of IWR to get the maximal inhibitory effect on estradiol synthesis. Hence, this study was undertaken to know the effective dose of IWR for inhibiting the estradiol synthesis in ovarian granulosa cells of goat.

Granulosa cells were isolated from the medium size follicles (ovaries of abattoir origin) and 6-day serum free granulosa cell culture was performed as previously described (Sen *et al.* 2007). Details of granulosa cell recovery and percentage of live cells at the time of recovery are given in Table 1. The chemicals were procured from Sigma, USA. Granulosa cells were routinely counted with automated cell counter of Invitrogen Inc. But intermittently the concentration was also counter checked with Haemocytometer. The culture method is described below in brief. After cell counting, cell suspension was diluted so that 100,000 live cells are present in 50 µl (2×10^6 cells/ml) culture droplet in 96 well culture plate (Eppendorf, Germany). The experiment consisted of 4 different treatment groups, viz. culture medium with DMSO (1 µl/ml) (Control group) or medium containing 0.1, 1.0 or 10 µM of IWR (dissolved in DMSO) (Groups 2, 3 and 4, respectively) for 6 days. Porcine FSH (sigma) was added @ 10 ng/ml to all the groups to induce the estradiol synthesis. The cultured medium consisted of MEMα containing insulin (1 ng/ml), transferrin (5 µg/ml), IGF1 (2 ng/ml), sodium selenite (4 ng/ml), androstenedione (10^{-6} M), penicillin (100 IU/ml), streptomycin (0.1 mg/ml) and fungizone (0.625 µl/ml).

In 96 well plates, 150 µl of media (pre-equilibrated in CO_2 incubator at 37°C) were added in each well (warmed supplemented MEMα as per the groups mentioned above) 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 media from each well with fresh 150 µl of media (equilibrated in the incubator 37°C). On the sixth day of culture, media was removed and stored at –20°C until analysis for estradiol concentrations and the cells were washed, trypsinized and counted as previously described (Sen *et al.* 2007). Briefly, the spent media were stored at –20°C in 1.5 ml tubes for hormone assays. Two time washing with DPBS were performed by centrifugation at 300 × g for 5 min at room temperature. After second washing, 150 µl of solution was removed. Trypsinization was performed by adding 50 µl of 0.25% trypsin in each well and incubated at 37°C for up to

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Table 1. Percentage of live cells at the time of isolation from abattoir derived goat ovaries.

Trial no.	Total cells per ovary (in lakh)	Live cells per ovary (in lakh)	Live cell %
1	3.60	3.00	81.0
2	0.48	0.38	82.0
4	1.00	0.80	80.0
7	0.75	0.67	89.0
8	2.60	2.00	75.0
9	3.40	2.40	70.5
10	1.90	1.50	80.5
11	0.92	0.68	75.0
12	1.36	1.06	78.0
13	1.29	0.90	71.0
14	1.58	1.20	77.0
15	2.30	1.81	79.0

20 min. To neutralize the trypsin digestion, 100 µl of 10% of sterile steer serum was added to each well and mixed well. For checking the viability of cells, trypan blue method was used. The cell suspension was centrifuged at 1,000 g for 5 min and the cell pellet was snap frozen and preserved at –85°C. Estimation of estradiol in cell culture spent medium that was harvested at the end of 6-day cell culture was carried out by enzyme linked immune assay (ELISA) kits (Calbiotech, USA). The sensitivity of the assay was 3.9 pg/ml. The intra-assay and inter-assay coefficient of variations of the kits were below 10%.

Statistical analysis: Results were expressed as means±SEM. A value of $P<0.05$ was considered statistically significant. The statistical software Graph Pad Prism (San Diego, USA) was used for analyzing the data. All percentage values were subjected to Arcsine transformation before the statistical analysis. The difference in the cell recovery rate among different groups of treatments and the difference in the estradiol synthesis among different groups of treatments was analyzed by ANOVA and post-ANOVA test of Tukey Multiple comparison test.

At the time of isolation of ovarian granulosa cells, there were more than 70% of live granulosa cells available for *in vitro* culture (Table 1). There was no significant difference in the number of cells recovered at the end of the 6-day culture period. The recovery rate was 30 to 40% in different groups. The second dose of IWR, i.e. 1 µM concentration had maximal inhibitor effect on estradiol synthesis (Fig. 2). The results are in conformity with the results obtained by Gupta *et al.* (2014), wherein the same dose of IWR was found to be the most effective in inhibiting the estradiol levels in bovine granulosa cells. The inhibition of estradiol synthesis by IWR indicated the positive role of Wnt signal in the estradiol synthesis of ovarian granulosa cells in goat. This is the first report on the role of Wnt signal in the estradiol synthesis for this species. In cattle, Castanon *et al.* (2013) and Gupta *et al.* (2014) made similar observation. There is no report on the role of Wnt signal in the estradiol synthesis in other species of domestic animals. However, the positive role of Wnt signal in rodents was established by Boyer *et al.* (2010).

WNTs are secreted extracellular signalling molecules that transduce their signals by binding to G protein-coupled receptors of the frizzled (FZD) family. They control diverse developmental processes such as cell fate specification, cell proliferation, cell differentiation and apoptosis. Wnt proteins are a family of cysteine rich glycoproteins that play a role during development and also in cancer. WNTs elicit their action through 3 different pathways, viz. the canonical Wnt/ β-catenin pathway, the non-canonical planar cell polarity pathway and the Wnt/Ca2 pathway. Wnt was reported to stimulate the estradiol synthesis and granulosa cell proliferation using canonical pathway (Boyer *et al.* 2010, Wang *et al.* 2010). Beta-catenin (or β-catenin) is the main component in the canonical pathway that can be targeted to study the effects of Wnt signaling.

Two novel classes of small molecules, i.e. inhibitors of Wnt production (IWP) and inhibitors of Wnt response (IWR) were discovered by Chen *et al.* (2009) after a high stringency cell-based screening strategy to identify small molecule antagonists of the Wnt/β-catenin pathway from a 200,000 compound synthetic chemical library. Though these compounds were invented for the chemotherapy of cancer, they paved a way for chemical genetic approach for studying Wnt pathway responses, which includes estradiol synthesis. IWR inhibits Wnt signaling by stabilizing AXIN, thereby stimulating the destruction of β-catenin. Gupta *et al.* (2014) successfully used IWR to find out the role of Wnt signaling in the estradiol synthesis of cattle, and, hence, in our studies also, the same inhibitor of Wnt was used to study the role of Wnt in the estradiol synthesis of ovarian granulosa cells in goat/buffalo.

The present study indicated the positive role of Wnt signal in the estradiol synthesis of ovarian granulosa cells in goat. The dose of 0.1 µM was found to be effective for inhibiting the estradiol synthesis in the ovarian granulosa cells of goat. These findings would help in further elaborate

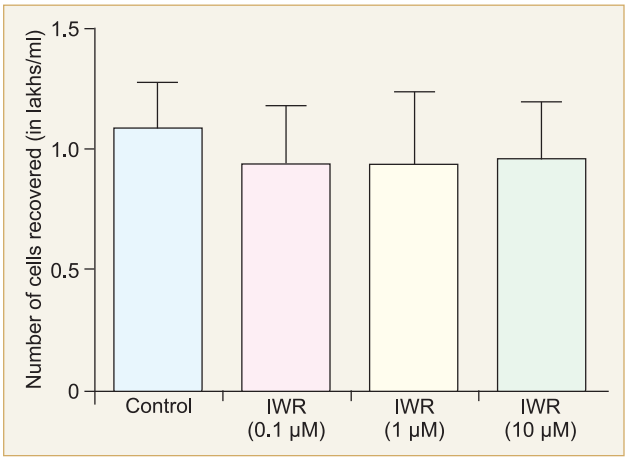

Fig. 1. Effect of different doses of IWR on recovery rate of ovarian granulosa cells of goat *in vitro* at the end of six day culture. There was no significant difference ($P>0.05$) among different groups.

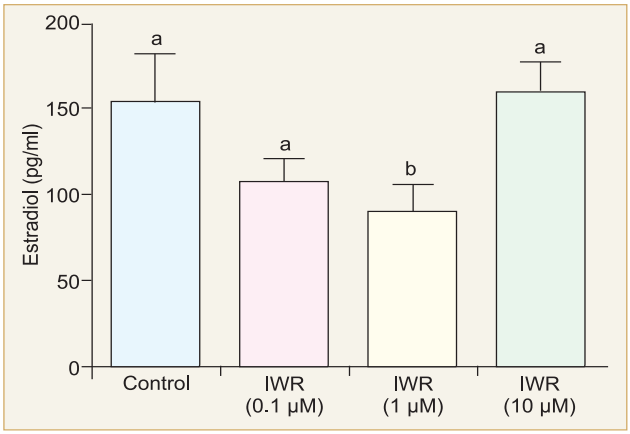

Fig. 2. Effect of different doses of IWR on estradiol synthesis of caprine ovarian granulosa cells *in vitro*. Columns with different superscripts differ significantly (P<0.05).

studies on the role of Wnt signal in the estradiol synthesis of goat as well as other domestic animals.

SUMMARY

Studies on the role of Wnt signal in the estradiol synthesis in domestic animals are scarce, though it is one of the important signalling pathways that is responsible for estradiol synthesis. Using chemical inhibitors of Wnt signal like inhibitor of Wnt response (IWR) is one of the methods to study the role of Wnt signal. In this study, the effective dose of IWR to inhibit the estradiol synthesis in ovarian granulosa cells of goat was determined. Granulosa cells were isolated from abattoir derived caprine ovaries. They were cultured in the presence of 3 different doses of IWR, i.e. 0.1, 1 and 10 µM for 6 days in the culture medium comprising minimum essential medium along with other necessary components (insulin (1 ng/ml), transferrin (5 µg/ml), IGF1 (2 ng/ml), sodium selenite (4 ng/ml), androstenedione (10^{-6} M), penicillin (100 IU/ml), streptomycin (0.1 mg/ml) and fungizone (0.625 µl/ml)) along with follicle stimulating hormone (porcine FSH @ 10 ng/ml). There was no significant difference among different groups in the granulosa cell recovery rate at the end of the 6 day culture. The IWR dose of 1 µM resulted in significantly more inhibition of estradiol synthesis in the cultured ovarian granulosa cells. This study indicated that the Wnt inhibitor IWR can be used @ 1 µM concentration for elaborate studies to explore the role of Wnt signal in the estradiol synthesis in goats. It also indicated that Wnt signal has a positive effect in estradiol synthesis of goats.

ACKNOWLEDGEMENTS

The work was carried out with the support of Department of Biotechnology under the Project No. BT/PR9024/AAquote/1/558/2013.

REFERENCES

Boyer A, Goff A K and Boerboom D. 2010. Wnt signaling in ovarian follicle biology and tumorigenesis. *Trends in Endocrinology and Metabolism* **21**(1): 25–32.

Castanon B I, Stapp A D, Gifford C A, Spicer L J, Hallford D M and Hernandez J A. 2013. Follicle-stimulating hormone regulation of estradiol production: possible involvement of Wnt2 and β-catenin in bovine granulosa cells. *Journal of Animal Science* **90:** 3789–97.

Chen B, Dodge M E, Tang W, Lu J, Ma Z and Fan C W. 2009. Small molecule mediated disruption of Wnt-dependent signalling in tissue regeneration and cancer. *Nature Chemical Biology* **5**(2): 100–107.

Gifford J A H. 2015. The role of Wnt signaling in adult ovarian folliculogenesis. *Reproduction* **150**(4): R137–48.

Gupta P S P, Folger J K, Rajput S K, Lv L, Yao J, Ireland J J and Smith G W. 2014. Regulation and regulatory role of Wnt signalling in potentiating FSH action during bovine dominant follicle selection. *PLoS ONE* **9**: e100201.

Lee H C, Lim S and Han J Y. 2016. Wnt/β-catenin signaling pathway activation is required for proliferation of chicken primordial germ cells *in vitro*. *Scientific Reports* **6**: 34510.

Sen A, Bettegowda A, Jimenez-Krassel F, Ireland J J and Smith G W. 2007. Cocaine- and amphetamine-regulated transcript regulation of follicle-stimulating hormone signal transduction in bovine granulosa cells. *Endocrinology* **148**: 4400–10.

Wang H X, Tony Y L and Kidder G M. 2010. Wnt2 regulates proliferation of mouse granulosa cells through beta catenin. *Biology of Reproduction* **82**(5): 865–75.