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Synergistic effects of insulin-like growth factor ii (IGF-II) and leutinizing hormone (LH) on steroid hormone secretion by cultured bovine granulosa cells

O F SMITH[1], A A OGUNSOLA[2], A O LADOKUN[3] and A AJADI R[4]

University of Agriculture, Abeokuta, Nigeria

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ABSTRACT

The synergistic effects of insulin-like growth factor II (IGF-II) and leutinizing hormone (LH) and the effect of IGF-II alone on production of progesterone (P_4) and estradiol (E_2) by bovine granulosa cells (GC) cultured *in vitro* were determined in this study. Bovine granulosa cells obtained from ovaries of slaughtered cows were cultured for 4 days in carbon (IV) dioxide (CO_2) incubator at 37°C, 5% CO_2 in atmospheric air and 100% relative humidity, using tissue culture medium199 (TCM 199) as culture medium. The medium was changed on the second day and testosterone added to serve as substrate for estadiol production. Insulin growth factor II (IGF-II) alone, or IGF-II + LH were added into the culture medium at 0, 0.1, 1.0, 10, 50, and 100 (ng/ml) levels of inclusion. Concentrations of progesterone and estradiol 17β produced were measured by radioimmunoassay. The results obtained showed that IGF-II alone and IGF-II + LH had significant effects on progesterone and estradiol produced by cultured granulosa cells. The result also revealed higher values of progesterone (4.78 ng/ml) and estradiol 17β (9.68 pg/ml) when IGF-II (10 ng/ml) and LH (50 ng/ml) were included together in the culture medium as compared with progesterone value (1.84 ng/ml) and estradiol 17β value (3.57 pg/ml) when IGF-II alone was included in TCM-199. From the result obtained, it can be concluded that IGF-II (10 ng/ml) + LH (50ng/ml) could be added to TCM-199 to give better synergistic effects on bovine granulosa progesterone and estradiol production as compared to addition of IGF-II (50ng/ml) alone.

Key words: Bovine granulosa cells, IGF-II, LH

The growth, differentiation, and steroidogenic capabilities of granulosa cells have most often been examined by culturing the cells in the presence of hormones and growth factors (Thomas *et al.* 2003, Sutton *et al.* 2003, Spicer *et al.* 2004, Park *et al.* 2004, Qadeer 2006). The interaction between gonadotropins and IGF-II on granulosa cell stereiodogenesis have been studied to some extent in bovine theca cells (Spicer and Echternkamp 1995, Spicer *et al.* 2002, 2004) and in rats (Hsueh *et al.* 1984, Adashi *et al.* 1990, Adashi 1995). Also, the pioneering experiments of Falck (1959) demonstrated that both theca and granulosa cells are essential for follicular estradiol secretion, and the classic studies of Greep (1942), Fevold (1941) and Lostroh and Johnson (1966) indicated that both LH and FSH are required for follicular growth and development. Theca cells secrete androgen and its production is stimulated by LH (Fortune and Armstrong 1977), but they appear to possess little or no ability to aromatize the androgen to estradiol (Fortune and Armstrong 1978). In contrast, granulosa cells can convert exogenous androgen to estradiol (Dorrington *et al.* 1975, Fortune and Armstrong 1977, Erickson and Hsueh 1978), but they seem incapable of synthesizing androgens (Fortune and Armstrong 1977). Because granulosa cells do produce progesterone *in vitro*, it appears that they lack the enzyme necessary to continue the steroidogenic pathway to androgens. Granulosa cells from immature, hypophysectomized rats lack aromatase, but the enzyme can be induced in these cells by treatment *in vitro* or *in vivo* with FSH, but not LH (Dorrington *et al.* 1975, Armstrong and Papkoff 1976). Taken together, these results provide evidence for the two-cell, two-gonadotropin model for estradiol production (Fortune and Quirk 1988). However, granulosa cells of developing rat follicles acquire LH receptors as they differentiate during the estrous cycle (Uilenbroek and Richards 1979), and they also acquire LH receptors *in vitro* following treatment with LH in culture (Nimrod *et al.* 1977, Erickson *et al.* 1979). As the granulosa cells of ovulatory follicles acquire LH receptors during the rat estrous cycle, they become capable of producing progesterone and of aromatizing exogenous androgen to estradiol in response to either LH or FSH (Fortune and Hilbert

Present address: [1,2,3]Department of Animal Physiology (e-mail[1]: smithof@live.com), [4]Department of Veterinary Surgery, University of Agriculture, Abeokuta, Nigeria

1986, Quirk *et al.* 1986). Experiments with bovine theca and granulosa cells, isolated from proestrous follicles provided evidence that in this species also both theca and granulosa cells are necessary for follicular estradiol production. Bovine theca cells secrete androgen *in vitro*, primarily in the form of androstenedione, and its production is stimulated by LH, but not by FSH (Fortune 1986). Granulosa cells secrete progesterone, and both LH and FSH increase its production (Fortune 1984). However, granulosa cells appear incapable of converting progestins to estradiol, because they secrete estradiol only when an aromatizable androgen is included in the culture medium (Fortune and Hansel 1979, Fortune 1984). Consequently, this study aimed to evaluate the effects of preincubated granulosa cells of ovaries obtained from local abbatoir with insulin growth factor II (IGF-II) and leutinizing hormone (LH) on steroid hormones production for future *in vitro* maturation and fertilization studies.

MATERIALS AND METHODS

Collection of the ovaries: Ten pairs of ovaries from cows of unknown reproductive history were collected from the local abattoirs. The ovaries were kept in 0.9% normal saline containing 100 mg/ml streptomycin sulfate and 100IU/ml penicillin-G sodium in a thermosflask at 37°C and were transported to the laboratory within 1 h after slaughter. In the laboratory, high level of sterility was maintained throughout the experiment.

Preparation of culture media: The medium used for the granulosa cells culture was the modified M-199 with Earle's salts. The medium was in powdery form in bottles of 9.5 g and was diluted in 1 litre of de-ionized water. Furthermore, 3.36 mM of $NaHCO_3$ was added to each of the 1L TCM-199 solution. Also, 1% bovine serum albumin (BSA) was added to half of the TCM-199 while 5% fetal calf serum (FCS) was added to 47.5 ml from the remaining part of TCM-199 while 0.8% of antibiotics (containing penicillin, streptomycin, and amphotericin) was also added to each half of the medium, after which 0.2 µm membrane filter was used to filter both media for sterility. All constituents of medium were aseptically prepared in the laboratory.

Methodology: The ovaries were washed in fresh sterile physiological saline (without antibiotics) to remove blood and other contaminants. On the surface of the ovaries were several small follicles, within the size 1–5 mm and few medium size follicles of 8 mm. There was no visible corpus luteum on the ovaries. The ovaries were dried lightly with sterile paper towels before granulosa cells recovery from the follicles. All the visible follicles were aspirated into the culture medium, TCM-199. Granulosa cells from the visible follicles were collected by aspiration using 18 inch needle and 50 ml syringe, and were washed in serum-free medium. Granulosa cells were separated from follicular fluid by centrifuging at 3000 rpm for 10 min. Cell pellets were mixed thoroughly with the culture medium and aliquoted at 50 µl/ml per well into 24-well micro plates containing 450 µl of medium per well. Cultures were incubated at 37°C in a 95% air (i.e. 5% CO_2 atmosphere), and 100% relative humidity. The CO_2 incubator was continuously powered with electricity throughout the experiment. Medium was changed after 2 days. To obtain optimal attachment, cells were maintained in the presence of 10% fetal calf serum for the first 48 h of culture. After this time, incubations continued in serum medium with hormones and IGF addition. Hormonal and IGF-II treatments were maintained for 2 days. Testosterone (10 ng/ml each) was added as substrate for estradiol production to the culture medium at the third day of the culture. The experiment was terminated on the fourth day.

Data collection: At the termination of the experiment, cultured medium samples were collected for determination of estradiol and progesterone concentration (as a measure of steroidogenic capacity of cells) by radioimmunoassay method. Estradiol-17β was assayed in maturation media (Abraham *et al.* 1972) by radioimmunoassay using a commercially-available kit.

Experimental design: The experimental design included: (1) Supplementation of additives, i.e. (a) addition of the combination of IGF-II and LH; (b) IGF-II alone, and (2) varied levels of inclusion: (a) 0ng/ml, (b) 0.1ng/ml, (c) 1ng/ml, (d) 10ng/ml, (e) 50ng/ml, and (f) 100ng/ml. Each treatment was replicated 3 times and randomly distributed in the incubator.

Statistical analysis: Data collected in this study was subjected to analysis of variance (ANOVA). Significant means were separated using Duncan multiple range test (Duncan 1955). All data were analyzed using Systat Analytical Computer Package.

RESULTS AND DISCUSSION

IGF-II effects on bovine granulosa progesterone and estradiol production *in vitro*. Table 1. showed the effects of IGF II alone on bovine granulosa progesterone and estradiol production *in vitro*. The lowest progesterone concentration value of 0.78 ng/ml was obtained at 0 ng/ml level while the highest concentration of progesterone (1.87 ng/ml) was recorded at 10 ng/ml level of IGF II inclusion which was not significantly different from the value obtained at 50 ng/ml level of IGF II inclusion (1.84 ng/ml). It was noticed that increasing the level of inclusion of IGF II from 50 ng/ml to 100 ng/ml showed a depressed significant effect of 1.51ng/ml progesterone concentration. Meanwhile, in the results of IGF II effects on estradiol production, showed similar pattern at varying inclusion levels to that obtained in progesterone.

In vitro: *Synergistic effects of IGF II with LH on bovine granulosa progesterone production:* IGF II inclusion level at 10 ng/ml together with LH inclusion level at 50 ng/ml recorded the highest value (4.78±0.12 ng/ml) of progesterone as shown in Table 2. There were no significant differences in progesterone values among LH inclusion levels at 10 ng/

Table 1. Effects of IGF II on bovine granulosa progesterone and estradiol production *in vitro*

IGF II inclusion level (ng/ml)	Progesterone (ng/ml)	Estradiol17ß (pg/ml)
0	0.78^{d}±0.06	1.13^{d}±0.12
0.1	1.03^{c}±0.06	1.49^{c}±0.12
1.0	1.41^{b}±0.06	3.63^{a}±0.12
10	1.87^{a}±0.06	3.67^{a}±0.12
50	1.84^{a}±0.06	3.57^{a}±0.12
100	1.51^{b}±0.06	3.08^{b}±0.12

a,b,c,d means in the column with different superscript are significant (P<0.05).

Table 2. Synergistic effects of IGF II and LH on bovine granulosa progesterone and estradiol production *in vitro*

IGF II inclusion level (ng/ml)	LH inclusion level (ng/ml)	Estradiol (pg/ml)	Progesterone 17ß (ng/ml)
10	0	3.08^{e} ± 0.25	1.45^{d} ± 0.12
10	0.1	3.83^{d} ± 0.25	2.28^{c} ± 0.12
10	1.0	8.09^{c} ± 0.25	3.50^{b} ± 0.12
10	10	9.36^{ab} ± 0.25	4.56^{a} ± 0.12
10	50	9.68^{a} ± 0.25	4.78^{a} ± 0.12
10	100	8.87^{b} ± 0.25	4.48^{a} ± 0.12

a,b,c,d,e means in the column with different superscript are significant (P<0.05).

ml, 50 ng/ml and 100 ng/ml with 10 ng/ml IGF II. Zero ng/ml LH inclusion with IGF II (10 ng/ml inclusion) recorded the least value of progesterone while there was significant difference among LH inclusion levels 0 ng/ml (1.45 ng/ml progesterone), 0.1 ng/ml (2.28 ng/ml progesterone) and 1.0 ng/ml (3.50 ng/ml progesterone), and 10 ng/ml (4.56 ng/ml progesterone).

In vitro: *Combined IGF II and LH effects on bovine granulosa estradiol production:* Table 2 showed the combined effects of IGF II and LH on bovine granulosa estradiol production *in vitro*. There was significant difference in the values of estradiol recorded among all the LH inclusion levels with 10 ng/ml IGF II. LH inclusion level 50 ng/ml + IGF II recorded the highest value (9.38 pg/ml) of estradiol which was significantly different from the second highest value (9.36 pg/ml) recorded by 10 ng/ml LH inclusion level with IGF II. As in the case of others, 0 ng/ml LH with IGF II (i.e. no inclusion of LH and IGF II) recorded the least value of estradiol, while there was gradual increase in the values until LH inclusion level reached 50 ng/ml when depression in the values of estradiol sets in.

In the past, an incredible effort has been made to understand the mechanism underlying follicle growth and differentiation and several *in vitro* systems have been developed. The protocols developed in experimental models are highly valuable in agricultural applications to increase the number and the quality of ova available for *in vitro* fertilization (Canipari 2000). It has been shown that follicle stimulating hormone (FSH) is essential for the growth of preantral follicles and for graafian morphogenesis (Spears *et al.* 1998). FSH alone is able to sustain follicle development and antral formation, however, it was further shown that the contemporary presence of LH significantly augments the percentage of follicles presenting large antral cavities and increases progesterone production (Cortvrindt *et al.* 1998, Smitz *et al.* 1998). Also, *in vivo*, LH is the physiological signal that triggers resumption of meiotic maturation and all changes necessary in the follicle to obtain a fertilizable gamete. However, in addition to gonadotropins, a number of other factors were reported to affect oocyte maturation. Granulosa and cumulus cells secrete a wide variety of growth factors that may either amplify or attenuate gonadotropins action in the ovary. These include: insulin-like growth factors (IGF-I and II) (Feng *et al.* 1988, Lorenzo *et al.* 1996), epidermal growth factor (Lonergan *et al.* 1996, Smitz *et al.* 1998) etc. Several of these factors have been used together with gonadotropins to find the ideal conditions for follicle growth *in vitro.* In the present study, results obtained show that IGF-II alone or in combination with LH increased both estradiol and progesterone production by granulosa cells at certain levels of inclusion. The result further showed that as the level of inclusion increases (IGF-II alone or IGF-II + LH combination) the amount of progesterone and estradiol equally increases. However, at 50 ng/ml inclusion of IGF-II alone or IGF-II + LH combination, the maximum amount of progesterone and estradiol is reached. The amount of the steroids declines when at 100 ng/ml of IGF-II alone or IGF-II + LH combination. Previous evidence that characterized interaction between gonadotrophins and IGFII on granulosa cell steroidogenesis and mitogenesis in cattle also showed that aromatase activity in granulosa cells from large follicles was much more sensitive to the effects of IGF-II than aromatase activity in the granulosa cells from small follicles, and that gonadotropins and IGF-II synergized to induce steroidogenesis in bovine granulosa cells. This synergism likely involves IGF-induced upregulation of the gonadotropin receptors, as shown in this study and in previous studies (Zhou *et al.* 1997). It may be included that there is a need for supplementation of hormones and growth factors into culture medium for a successful bovine *in vitro* maturation or *in vitro* fertilization studies.

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