Endocrine, *vol. 12, no. 1, 41–45, February 2000* 0969–711X/00/12/1:41–45/$11.25

Novel Studies on Influence of Gonadotropins and Insulin-like Growth Factor-I on Growth of Cumulus Oophorus in the Rat

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In the final developmental stage of a Graafian follicle, there are two functionally distinct types of granulosa cells: the cumulus cells (CCs) and the mural granulosa cells (MGCs). Previous studies focused on follicle-stimulating hormone (FSH) and insulin-like growth factor I (IGF-I) interactions in MGCs. Our goal was to study these interactions in CC proliferation. Immature rats received in vivo treatments of either saline, equine chorionic gonadotropin (eCG) with high FSH activity, an IGF-I analog (LR^3-IGF-I) with poor binding to IGF-I binding proteins, or a combination of both hormones. CCs from each in vivo treatment were then cultured and treated in vitro with either saline, FSH, or IGF-I. CCs proliferation were assessed by measurement of ^{3}H-thymidine incorporation. Prior in vivo treatment with eCG resulted in the highest proliferative activity of CCs when combined with FSH in vitro treatment. In vivo treatment with LR^3-IGF-I had no effect on CC replication. CC replication was higher in FSH in vitro treatment than of IGF-I. The combination of eCG and LR^3-IGF-I was the only in vivo treatment to stimulate higher CC proliferation with IGF-I in vitro treatment. This study suggests that FSH does not act through IGF-I, a mechanism previously proposed.

Key Words: Insulin-like growth factor-I; follicle-stimulating hormone; granulosa cells; cumulus cells; IGF-I analog.

Introduction

As the Graafian follicle matures, new layers of granulosa cells are formed concentrically around the oocyte. However, as the follicular fluid increases, the granulosa cells become specialized into two groups: (1) the cumulus cells (CCs) or cumulus oophorus, which surround the oocyte; and (2) the mural granulosa cells (MGCs), which form the rest of the granulosa cells *(1)*. We have already shown in a bovine model *(2,3)* that the two subgroups behave quite differently. For example, the CCs replicate at a much faster rate than the MGCs. In the case of MGCs, gonadotropins and insulin-like growth factor-I (IGF-I) have an additive stimulatory effect on cell proliferation. By contrast, in a similar setup for CCs, IGF-I stops the stimulatory effect of gonadotropins *(2)*. We later showed that this phenomenon was related to different destinies of these two subgroups. Mucification is only seen in CCs, and this differentiation results in the prevention of proliferation *(3)*. Maturation of the oocyte is highly dependent on its surrounding granulosa cells, the CCs. This communication is achieved through gap junctions and paracrine signaling factors. In turn, oocytes are thought to influence the specialization of CCs by materials secreted and transmitted to the CCs. This area has recently been reviewed *(4,5)*.

In the present study, we attempted to simulate physiological variables in order to study the two main factors responsible for granulosa cell proliferation: gonadotropins and IGF-I. The in vivo actions of gonadotropins is recognized as the essential factor in the final stages of folliculogenesis. IGF-I, its receptor, and binding proteins are all present in the follicle. The receptor for IGF-I has been clearly demonstrated on MGC *(6)*. Follicle-stimulating hormone (FSH) stimulated the production of IGF-I while decreasing the production of IGF-I-binding proteins (IGF-I-BPs). Knockout mice for IGF-I gene do not develop mature follicles; this was reviewed by Adashi*(6)*. We therefore conducted specific studies of these factors on the growth of CCs, which has never been studied in the rat before. We have conducted such studies in the bovine model *(2,3)* and noted marked difference in the response of CCs vs MGCs. It is not possible to study the physiological role of IGF-I by parenteral administration because this growth factor rapidly binds to very abundant IGF-I-BPs and is inactivated. Therefore, we utilized an analog of IGF-I, Long Arg^3-IGF-I (LR^3-IGF-I), which binds very poorly to IGF-I-BPs and therefore has been demonstrated to have physiological effects *(7,8)*.

Received October 27, 1999; Revised December 2, 1999; Accepted December 2, 1999.

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Table 1
Effects of In Vitro Treatment of Saline on ^{3}H-Thymidine Incorporation on Cultured Oocyte-CC Complexes of Immature Rats Treated with eCG, LR^3-IGF-I, eCG + LR^3-IGF-I, or Saline

In vivo treatment	^{3}H-thymidine incorporation (dpm/CC) (mean ± SEM) In vitro treatment in saline[a]
Saline (n = 12)	463 ± 53[a]
eCG (n = 12)	383 ± 76[a]
LR^3-IGF-I (n = 12)	538 ± 81[a]
eCG + LR^3-IGF-I (n = 12)	302 ± 60[a]

Values with the same superscript are not significantly different ($p > 0.05$).

Table 2
Effects of In Vitro Treatment of FSH on ^{3}H-Thymidine Incorporation on Cultured Oocyte-CC Complexes of Immature Rats Treated with eCG, LR^3-IGF-I, eCG + LR^3-IGF-I, or Saline

In vivo treatment	^{3}H-thymidine incorporation (dpm/CC) (mean ± SEM) In vitro treatment in FSH (5 ng/mL)
Saline (n = 12)	826 ± 69[a]
eCG (n = 12)	1257 ± 175[b]
LR^3-IGF-I (n = 12)	787 ± 60[a]
eCG + LR^3-IGF-I (n = 12)	1196 ± 168[b]

Values with a different superscript are significantly different ($p < 0.001$). The eCG group is significantly higher than the saline and LR^3-IGF-I groups ($p < 0.001$). The eCG + LR^{3}IGF-I group is significantly higher than the saline and LR^3-IGF-1 groups ($p < 0.001$).

Results

Table 1 shows the effect on ^{3}H-thymidine incorporation of four different in vivo treatments: control (saline), equine chorionic gonadotropin (eCG), LR3-IGF-I, or a combination of eCG with LR^3-IGF-I. No in vitro hormonal addition was made in this group. It can be seen that prior treatment with eCG or LR^3-IGF-I or a combination of both made no difference in thymidine incorporation of CCs in subsequent in vitro culture. Table 2 shows the effect of the four treatment groups when CCs were subsequently cultured in a medium containing FSH (5 ng/mL) in vitro. These results differ from the previously mentioned ones in that in vivo treatment with eCG (gonadotropins) resulted in enhanced response to in vitro culturing with FSH at a statistically significant level ($p < 0.001$). Table 3 shows the situation when the in vitro treatment was IGF-I for the four in vivo treatments. There was no effect of IGF-I (10 ng/mL) on

Table 3
Effects of In Vitro Treatment of IGF-I on ^{3}H-Thymidine Incorporation on Cultured Oocyte-Complexes of Immature Rats Treated with eCG, LR^3-IGF-I, eCG + LR^3-IGF-I, or Saline

In vivo treatment	^{3}H-thymidine incorporation (dpm/CC)(mean ± SEM) In vitro treatment in IGF-I (10 ng/mL)[a]
Saline (n = 12)	573 ± 78[a]
eCG (n = 12)	473 ± 48[a]
LR^3-IGF-I (n = 12)	568 ± 72[a]
eCG + LR^3-IGF-I (n = 12)	504 ± 45[a]

Values with the same superscript are not significantly different ($p > 0.05$).

Table 4
Effects of In Vitro Treatment of FSH, IGF-1, or Saline on ^{3}H-Thymidine Incorporation on Cultured Oocyte-CC Complexes of Immature Rats Treated In Vivo with Saline

In vitro treatment	^{3}H-thymidine incorporation (dpm/CC)(mean ± SEM) In vivo treatment in Saline
Saline (n = 12)	463 ± 53[a]
FSH (n = 12)	826 ± 69[b]
IGF-I (n = 12)	573 ± 78[a]

Values with a different superscript are significantly different ($p < 0.005$).

thymidine incorporation of CC. Table 4 compares the in vitro culture with saline (control), FSH, or IGF-I in the groups with no in vivo treatment, in which there was a statistically significant effect with FSH ($p < 0.005$). IGF-I results were higher than those for saline but not statistically significant. Tables 5–7 demonstrate the effect of in vitro treatment with FSH or IGF-I when the animals received the three different in vivo treatments with gonadotropins (eCG), IGF-I (as an analog), or the two combined, respectively. In all these, FSH had a statistically significant positive effect whereas IGF-I seems to have had a stimulatory effect that does not reach statistical significance. The exception was for the combination treatment of eCG and LR^3-IGF-I where a significant effect of IGF-I in vitro on ^{3}H-thymidine incorporation was observed compared to the saline group (Table 7). Figure 1 shows schematically the relationship of all the variables investigated.

Discussion

Cumulus oophorus (CCs) is now recognized to be composed of a specialized variety of granulosa cells that

Table 5
Effects of In Vitro Treatment of Either FSH, IGF-1, or Saline on ^{3}H-Thymidine Incorporation on Cultured Oocyte- CC Complexes of Immature Rats Treated In Vivo with eCG

In vitro treatment	^{3}H-thymidine incorporation (dpm/CC) (mean ± SEM) In vivo treatment with eCG
Saline (n = 12)	383 ± 76^a
FSH (n = 12)	1257 ± 175^b
IGF-I (n = 12)	473 ± 92^a

Values with a different superscript are significantly different ($p < 0.0001$).

Table 6
Effects of In Vitro Treatment of FSH, IGF-1, or Saline on ^{3}H-Thymidine Incorporation on Cultured 0ocyte-CC Complexes of Immature Rats Treated In Vivo with LR^3-IGF-I

In vitro treatment	^{3}H-thymidine incorporation (dpm/CC)(mean ± SEM) In vivo treatment with LR^3-IGF-I
Saline (n = 12)	538 ± 81^a
FSH (n = 12)	787 ± 60^b
IGF-I (n = 12)	568 ± 72^a

Values with a different superscript are significantly different ($p < 0.0001$).

Table 7
Effects of In Vitro Treatment of FSH, IGF-1, or Saline on ^{3}H-Thymidine Incorporation on Cultured Oocyte-CC complexes of Immature Rats Treated In Vivo with eCG + LR^3-IGF-I

In vitro treatment	^{3}H-thymidine incorporation (dpm/CC)(mean ± SEM) In vivo treatment with eCG + LR^3-IGF-I
Saline (n = 12)	302 ± 60^a
FSH (n = 12)	1196 ± 168^b
IGF-I (n = 12)	504 ± 45^a

Values with a different superscript are significantly different ($p < 0.01$).

influence the development of oocytes at the same time that they are influenced by the oocytes. In most, if not all, previous studies in the experimental animals utilized (rats), MGCs were investigated. We feel that an understanding of the physiology of CCs is of more than scientific curiosity. A thorough knowledge of reproduction may help us solve three vital problems facing humanity: population control, production of proteins (for an ever-increasing world popula-

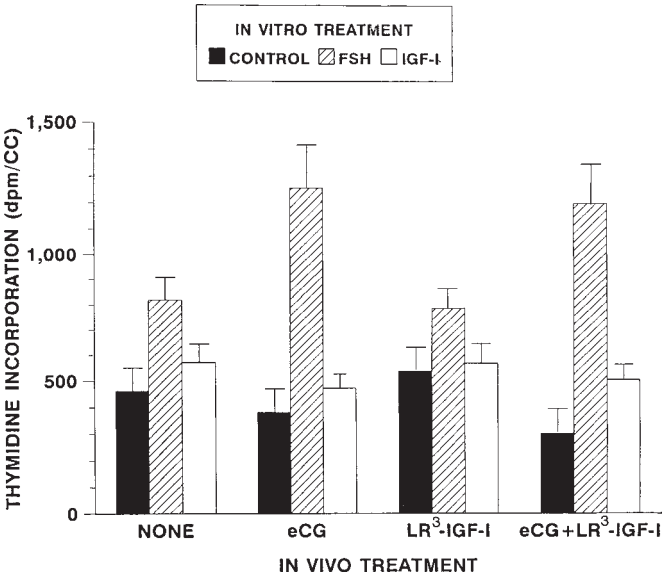

Fig. 1. Thymidine incorporation of oocyte-cumulus complexes (CCs) from rats treated in vivo with eCG; an IGF-I analog; LR^3-IGF-I; both; or saline. Cultured CCs were treated in vitro with FSH (5 ng/mL), IGF-I (10 ng/mL), or saline before being challenged with ^{3}H-thymidine for measurement of replication. Mean values of thymidine incorporation are reported per each CC. Treatment with FSH in vitro increased thymidine incorporation over all in vivo treatments ($p < 0.05$). Treatment with IGF-I in vitro increased DNA synthesis in eCG + LR^3-IGF-I-treated rats ($p < 0.01$). , Control; , FSH; , IGF-I.

tion), and treatment of infertility (one in six couples). CCs have been shown to play a very important role. For example, it has been demonstrated in humans that oocytes with a reduced diameter of the oocyte cumulus complex have a lowered potential for fertilization and embryonic development *(9)*.

Gonadotropins and IGF-I have emerged as the major factors in the development of the Graafian follicle and maturation of the oocyte. It has been postulated that gonadotropins may act via IGF-I. However, Adashi *(6)* has pointed out that this suggestion is inferential, and we must await more studies. Our group, in a series of studies, has attempted to create physiological circumstances to evaluate the role of gonadotropins and IGF-I in oogenesis and folliculogenesis *(2,3)*. In the experiments reported herein, we have shown that prior treatment with gonadotropins make the CCs more responsive to FSH (Table 2). This is an important point to recognize and may well be owing to induction of FSH receptor activity by prior treatment with FSH. In the current study, we have shown a much higher replication rate in response to FSH vs IGF-I. Also, in vivo treatment with FSH-containing eCG had an effect on CC replication that IGF-I (analog) did not (Table 2). Therefore, in the context of our study, FSH does not seem to act via IGF-I, a concept that was being entertained previously *(6)*. In a previous study, when we investigated the dose response of IGF-I in CC proliferation in the rat, we showed a modest but statistically significant positive replication response

(unpublished data). In the current study, in all subgroups, IGF-I had a higher stimulatory effect than control, although statistically significant values were reached only when the animals were previously treated with a combination of eCG and IGF-I analog (Tables 4–7). Note that the eCG and FSH we used can have impurities, and in future experiments we plan to use specific recombinant products. Because LR^3-IGF-I binds very poorly to IGF-I-BPs, we made certain that increased levels of this cytokine were achieved at the ovarian level. Indeed, we have previously demonstrated a statistically significant stimulatory effect of this substance in folliculogenesis in the rat model *(10)*. Therefore, with respect to CCs, IGF-I has a more minor cell replication effect whereas FSH (independently) has a more major effect.

Efforts are being made to mature human oocytes in vitro. Previously we demonstrated the beneficial effect of gonadotropins in this respect *(11,12)*. We have considered the addition of IGF-I to oocyte culture of immature oocytes. However, the current study suggests that in the context of oocyte cumulus maturation, IGF-I may not have a major role.

In conclusion, we have demonstrated, for the first time, in a physiological setup the relationship of gonadotropins and IGF-I in proliferation of CCs in the most commonly used experimental model: the rat. Using an IGF-I analog (which binds very poorly with IGF-I-BPs), we have demonstrated, for the first time, the in vivo effect of increasing the availability of IGF-I to CCs. We have shown that it is the gonadotropins that sensitize the CCs to respond more vigorously to FSH in the process of cell replication, not IGF-I. Also, we have demonstrated that prior in vivo (clinical) treatment with a combination of gonadotropins and IGF-I analog resulted in CCs being more responsive to IGF-I in vitro (compared to control). Furthermore, we have shown that the role of IGF-I is inferior to FSH as a stimulator of CC replication. Finally, we have shown that both FSH and IGF-I are independent of each other and that these physiological studies do not support the notion that FSH acts via IGF-I. It is probable that each follicle, according to its maturity and differentiation of its CC type and MGCs, has a dynamic and varying pattern of response to FSH, IGF-I, and a number of other factors. Note however, that we did not actually study the peripheral interaction of IGF-I with its receptor, IGF-I-BPs, and proteinases affecting the binding proteins. These are areas for future research.

Materials and Methods

Animals and In Vivo Treatments

Immature Sprague-Dawley rats were purchased at 20 d of age (Charles River, St. Constant, Canada) and were housed in an animal care facility, maintained on a 14-h light/10-h dark light cycle. The animals were provided with food and water ad libitum in accordance with the institutional animal care guidelines. The experimental design is shown in Fig. 2.

At 24 d of age, rats were stratified by body weight into four groups with equal mean body weight. They were then randomized to receive continuous sc infusion of an IGF-I analog, (LR^3-IGF-I),(Gro-Pep Pty, Adelaide, Australia) or saline as control at a rate of 1 mg/(kg•d) with Alzet Mini-osmotic pumps (Alza, Vacavile, CA). This infusion rate was selected on the basis of previous dose-response studies in rats employing stimulation of growth and anabolic responses as end points of biological efficacy *(7,8)*. Infusion of LR^3-IGF-I was continued until rats were sacrificed at d 29 of age.

At 27 d of age, rats were randomized to receive either a sc injection of 15 IU of eCG(Ayerst, Montreal, Canada) or saline alone. The injection was given at 9:00 AM, and the rats were sacrificed 48 h later, which is the equivalent of proestrus for eCG-treated rats.

Rats were weighed immediately before sacrifice by cervical dislocation, and their reproductive tracts were removed under aseptic conditions. Ovaries were dissected from the oviducts and adherent adipose and connective tissues, weighed, and placed in HEPES-buffered tissue culture medium 199 (HTCM-199; Sigma, St. Louis, MO) for recovery of CCs.

Cell Culture and In Vitro Treatments

Surface follicles (approx 30 follicles per rat) on ovaries were punctured with a hypodermic needle, and the ovaries were gently compressed with forceps and agitated in the medium to release CCs. Pooled CCs from six rats on each in vivo treatment were rinsed through two successive washes in HTCM-199 and a final rinse in bicarbonate-buffered medium (BTCM-199; Sigma). Ten to fifteen CCs were then transferred at random to each well of a 96-well tissue culture plate (Nalge Nunc, Napierville, IL) containing BTCM-199. Each culture will receive one of the following in vitro treatments: 5 ng/mL of ovine FSH (NIDDK-oFSH-17), 10 ng/mL of IGF-I (Boehringer Mannheim, Laval, Canada), or media alone (control). After the addition of the in vitro treatments, CCs were incubated for 20 h in a humidified atmosphere of 5% CO_2 in air, at 37.5°C in a water-jacketed tissue culture incubator (Forma Scientific, Marietta, OH). At the end of the incubation period, 1 μCi (20 Ci/mmol) of ^{3}H-thymidine (ICN Biochemicals, Irvine, CA) was added to each well, and cultures continued for a further 8 h. Cells were then harvested with a Skatron cell harvester, and the filter disks containing the cells were air-dried. The incorporated radioactivity was counted with a liquid scintillation spectrometer for measurement of ^{3}H-thymidine incorporation as an index of DNA synthesis.

Each experiment was repeated 4 times using 6 rats per in vivo treatment, and 3 in vitro replicates per each in vitro hormone treatment (total of 12 observations per each in vitro hormone treatment). Responses of CCs to the various in vitro treatments (FSH, IGF-I, and saline) were compared in LR^3IGF-I, eCG, eCG + LR^3-IGF-I,

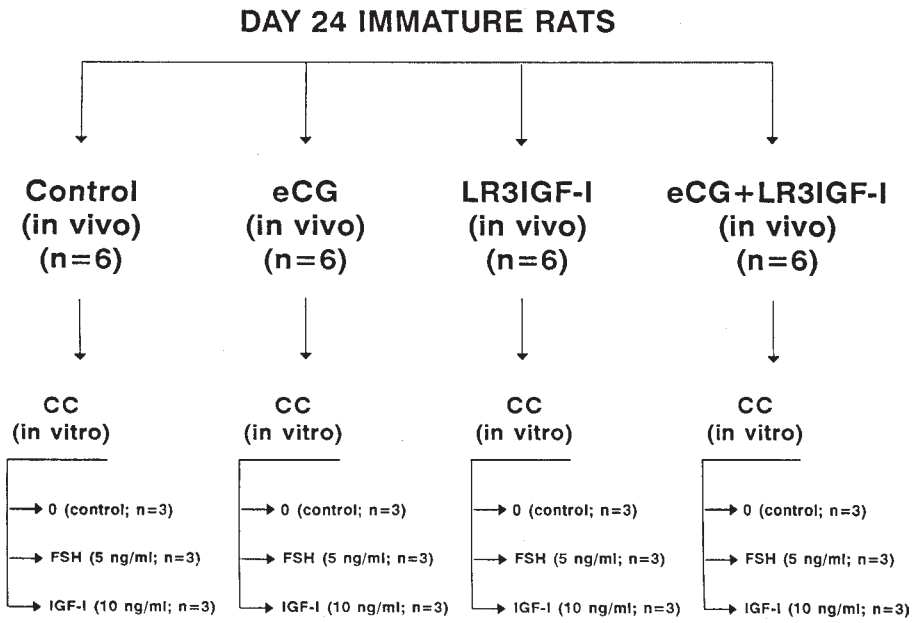

Fig. 2. Diagram of the experimental design of this study. Rats were treated in vivo with eCG; an IGF-I analog; LR^3-IGF-I; a combination of eCG and LR^3-IGF-I; or saline alone. Oocyte-cumulus complexes (CCs) were cultured with FSH, IGF-I, or saline before being challenged with ^{3}H-thymidine for measurement of replication. This experiment was repeated four times.

and saline-treated rats by analysis of variance. The results were reported as disintegrations per minute per each oocyte cumulus complex.

Acknowledgments

We express our sincere thanks to Prof. D.T. Armstrong from the Department of OB/GYN, University of Western Ontario, London, Ontario, Canada, for support and guidance; and to Anne Rouleau and Gerry Barbe for technical assistance. We are also grateful to Serono, Canada, for financial support.

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