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Novel Demonstration of a Physiologic/Pharmacologic Role of Insulin-Like Growth Factor-1 in Ovulation in Rats and Action on Cumulus Oophorus

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Gonadotropins are well known to be the most important stimulus for ovarian follicular development. More recently, there is indirect evidence that insulin-like growth factor-1 (IGF-1) is also a very important autocrine/paracrine factor in folliculogenesis. We had access to an analog of IGF-1, LR^{3}IGF-1, which binds very poorly to IGF-1 binding proteins and therefore was shown by previous investigators to have biologic effects. We studied rats that were superovulated with the use of gonadotropins. We showed that the addition of LR^{3}IGF-1 by infusion further increased the ovulation rate (statistically significant) and increased the ovarian weight in two of three strains of rats. We demonstrated that infusion of LR^{3}IGF-1 or injection of equine chorionic gonadotropin or a combination of these two were associated with oocytes with reduced number of cumulus cells (statistically significant). We conducted an experiment to determine whether in vitro culturing with varying dosage of IGF-1 may stimulate cumulus cell replication to improve the quality of oocyte cumulus complex. IGF-1 did not show any change in this respect. Through these physiologic (pharmacologic) studies, we have shown that IGF-1 (analog) can further increase the ovulation rate induced by gonadotropins.

Key Words: LR^3 IGF1; IGF1; gonadotrophins; ovulation; cumulus oophorus.

Introduction

The gonadotropins, particularly follicle-stimulating hormone (FSH), are undoubtedly the major in vivo factor in follicular development and ovulation *(1)*. However, when gonadotropins are added to granulosa cells in vitro, the effects are rather modest *(2,3)*. When insulin-like growth factor-1 (IGF-1) is added in vitro, the proliferative effects on granulosa cells and steroidogenesis are dramatic in most species *(4,5)*. It has been shown that granulosa cells are the site of IGF-1 production, reception, and actions *(6,7)*. Pioneering work by Baker et al. *(8)* using knockout repletion techniques has further established the importance of IGF-1 in oogenesis and ovulation. They reported that both sexes of adult mice homozygous for a targeted mutation of the IGF-1 gene, encoding IGF-1, are infertile dwarfs. The female mutants failed to ovulate even after administration of gonadotropins, which was apparently the primary cause of their infertility.

All the aforementioned studies point to a very important role for IGF-1 in oogenesis, and more extensive studies are needed to establish the physiologic and pharmacologic role of IGF-1. In this context, the interaction of IGF-1 with gonadotropins must be sorted out because FSH and IGF-1 are emerging as the major endocrine/paracrine factors in ovulation. There is a problem with the use of IGF-1 clinically in that it rapidly binds to its binding proteins, when administered parenterally. Therefore, it is not possible to deliver IGF-1 to the site of action, and physiologic and pharmacologic studies are not feasible. We have had access to an analog of IGF-1 (LR^{3}IGF-1) that binds very poorly to the binding proteins. It was demonstrated that parenteral administration of this analog produced significant biologic effects expected of IGF-1 *(9,10)*. In this article, we demonstrate, for the first time, the physiologic/pharmacologic role of IGF-1 and its place as an adjunct to FSH in the induction of superovulation. The latter is now extensively used in human infertility treatment and has a potential in farm animals to increase protein production (to feed an ever-growing world population).

Results

Effect of LR^{3}IGF-1 and Equine Chorionic Gonadotropin In Vivo on Ovarian Weight—Experiment 1

Tables 1 and 2 summarize the design and results, respectively, from experiment 1. No statistically significant effect

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Table 1
Design of Experiment 1

	Sprague-Dawley rats				Dark Agouti rats		Long-Evans rats	
	1	2	3	4	5	6	7	8
Number of rats	12	12	12	12	12	12	12	12
Infusion from d 24–29	Saline	LR^3IGF-1	Saline	LR^3IGF-1	Saline	LR^3IGF-1	Saline	LR^3IGF-1
Injection on d 27	Saline	Saline	eCG	eCG	eCG	eCG	eCG	eCG
Rat sacrifice on d 29	All	All	All	All	All	All	All	All

Table 2
Effects of LR^3IGF-1 Infusion and eCG Injection on Body and Wet Ovarian Weights of Prepubertal Rats (Combined Observation)[a]

Rat strain	Treatment subgroup	In vivo treatment (n = 12)		Body weight (g)	Ovarian weight (mg)
		LR^3IGF-1	eCG		
Sprague-Dawley	1	None	None	89 ± 2*	0.2 ± 1.8*
	2	1 mg/d	None	96 ± 2*	23.6 ± 0.6*
	3	None	15 IU	90 ± 1*	43.2 ± 8.1†
	4	1 mg/d	15 IU	91 ± 3*	56.8 ± 10.8†
Dark Agouti	5	None	15 IU	72 ± 1*	47.4 ± 1.8*
	6	1 mg/d	15 IU	76 ± 3*	59.2 ± 1.7†
Long-Evans	7	None	15 IU	100 ± 5*	52.5 ± 11.0*
	8	1 mg/d	15 IU	104 ± 2*	89.6 ± 7.6†

[a]Values are mean ± SEM. Values that do not share a common superscript within columns differ within rat strains ($p < 0.05$).

was noted on the body weight. As expected, treatment with equine chorionic gonadotropin (eCG) alone increased ovarian weight ($p < 0.01$). Treatment with LR^3IGF-1 alone had no effect on body and ovarian weights within the same rat strain. In the group that was treated with eCG, the addition of LR^3IGF-1 resulted in a weight increase in the ovaries in all three strains, and this reached a statistically significant level in the Dark Agouti and Long-Evans strains but not in the Sprague-Dawley strain.

Effect of LR^3IGF-1 and eCG In Vivo on Ovulation Rate and Cumulus Cell Numbers—Experiment 2

Tables 3–5 summarize the design and results from experiment 2. When four treatment regimens were compared (control, LR^3IGF-1, eCG, and eCG plus LR^3IGF-1), the body weights did not show any change. The ovarian weight showed a statistically significant increase related to treatment with eCG. In the group treated with eCG, the addition of LR^3IGF-1 resulted in an average ovarian weight of 58.9 vs 50.0 mg for those rats not treated with LR^3IGF-1 that was not statistically significant. The ovulation rate as judged by the number of oocytes found in the oviducts per animal showed an increase with the use of eCG (1.4 ± 3.5 with no treatment vs 22.1 ± 3.5 for eCG). LR^3IGF-1 did not influence the ovulation rate when no eCG was used (1.4 ± 3.5 [control] vs 1.3 ± 3.5 for LR^3IGF-1 treatment group). When rats were treated with eCG, the addition of LR^3IGF-1 resulted in a further increase in the ovulation rate (35.4 ± 3.7 for eCG + LR^3IGF-1 vs 22.1 ± 3.5 for eCG, no LR^3IGF-1. The differences were statistically significant ($p < 0.01$).

Table 3
Design of Experiment 2

	Treatment subgroup			
	A	B	C	D
Number of rats	14	14	14	14
Infusion d 24–30	Saline	LR^3IGF-1	Saline	LR^3IGF-1
Injection on d 27	Saline	Saline	eCG	eCG
Injection of d 29	hCG	hCG	hCG	hCG
Rat sacrifice on d 30	All	All	All	All

The average number of cumulus cells per oocyte cumulus complexes (OCCs) of the control group was 16,340 ± 1980 (SEM) (Table 5). Treatment with LR^3IGF-1 halved this to 8370 ± 2560, which was statistically significant ($p < 0.01$). Administration of eCG alone or in combination with LR^3IGF-1 resulted in a reduction in cumulus cells in comparison to control ($p < 0.0001$). The lowest value was seen with combination of eCG and LR^3IGF-1. However, the values among treatment groups LR^3IGF-1 alone (8370), eCG alone (5810), and eCG ± LR^3IGF-1 (5,030) did not reveal a statistically significant difference.

Table 4
Effects of LR^3IGF-1 and eCG
on Body and Wet Ovarian Weights and Ovulation Rate in Sprague-Dawley Rats[a]

In vivo treatment	Treatment subgroup	Rat number (n)	Body weight (g)	Ovarian weight (mg)	Ovulation rate
Saline	A	14	87.3 ± 8.8*	24.8 ± 2.7*	1.4 ± 0.7*
LR^3IGF-1	B	14	90.2 ± 9.8*	32.0 ± 4.4*	1.3 ± 0.8*
eCG	C	14	83.7 ± 6.6*	50.0 ± 8.9†	22.1 ± 2.8†
eCG + LR^3IGF-1	D	14	90.9 ± 6.4*	58.9 ± 9.2†	35.4 ± 7.5‡

[a]This experiment was done in three replicates. Values are mean ± SEM. Values that do not share a common superscript within columns differ ($p < 0.01$).

Table 5
Effects of LR^3IGF-1 and eCG
on Ovulation Rate and Number of Cumulus Cells per Ovulated OCCs in Sprague-Dawley Rats[a]

In vivo treatment	Treatment subgroup	Rat number (n)	Number of OCCs (range)	Number of cumulus per OCC (range)
Saline	A	14	1.4 ± 0.7* (0–9)[b]	16,340 ± 6000* (6944–31,250)
LR^3IGF-1	B	14	1.3 ± 0.80* (0–9)[b]	8370 ± 2500† (4429–11,250)
eCG	C	14	22.1 ± 2.8† (0–38)[b]	5810 ± 864† (2731–12,813)
eCG + LR^3IGF-1	D	14	35.4 ± 7.5‡ (15–102)[b]	5030 ± 511† (3550–10,167)

[a]This experiment was done in three replicates. Values are mean ± SEM. Values that do not share a common superscript symbol within columns differ ($p < 0.01$).

[b]In the saline group, 9 rats did not ovulate; in the LR^3IGF-1 group, 11 rats did not ovulate; in the eCG group, 1 rat did not ovulate, and in the eCG + LR^3IGF-1 group, all rats ovulated.

Table 6
Design of Experiment 3[a]

	Treatment subgroup									
	1	2	3	4	5	6	7	8	9	10
In vivo d 27			Saline					15 IU eCG		
In vivo 18–20 h later			hCG					hCG		
No. of rats			10					10		
Day 29 sacrifice			All					All		
In vitro culture with IGF-1; dose (ng/mL)	0	6.2	12.5	25	50	0	6.2	12.5	25	50
No. of replicates	4	4	4	4	4	4	4	4	4	4

[a]The whole experiment was repeated three times, i.e., 12 analyses per each of the subgroups.

Effect of IGF-1 In Vitro on Cumulus Cell Proliferation—Experiment 3

Tables 6 and 7 show the design and results of experiment 3. Previous in vivo treatment with eCG resulted in more cumulus cell (CC) replication of oocyte cumulus cell complexes (OCC) in vitro compared to when no eCG was given in vivo. This difference was statistically significant ($p < 0.05$). The addition of varying doses of IGF-1 to the culture medium did not make any difference to CC replication.

Discussion

We have demonstrated in vivo, for the first time, that IGF-1 may be used as an adjunct to FSH to improve the superovulatory response to FSH. The role for IGF-1 was highly predicted by previous in vitro studies and null mutation studies *(8,11)*. Growth factors as pharmacologic agents are now being rapidly developed and utilized in medicine and veterinary practice. For example, the granulocyte colony-stimulating factor is utilized to stimulate production of

Table 7
Effect of eCG Treatment In Vivo on ^{3}H-Thymidine Incorporation in Cumulus Cells from Hooded Wistar Rats During Culture in Varying Concentrations of IGF-1

In vitro IGF-1 (ng/mL)	No. of observations	No. of oocytes in each replicate	Hormone treatment in vivo	
			None[a]	15 IU eCG[b]
0	12	10	653 ± 163[c]	1460 ± 365
6.25	12	10	684 ± 153	1376 ± 344
12.5	12	10	763 ± 190	1388 ± 347
25	12	10	567 ± 142	875 ± 219
50	12	10	836 ± 209	1251 ± 313

[a] Values in this column showed no difference statistically.
[b] Values in this column did not show a statistically significant difference.
[c] ^{3}H-thymidine incorporation: dpm/OOC ± SEM; values for eCG-treated rats were significantly different from untreated rats ($p < 0.05$).

granulocytes in patients undergoing chemotherapy for malignant diseases *(12)*. With the availability of an analog of IGF-1, which is not "mopped up" by binding proteins, we have proven the feasibility of the clinical use of IGF-1. In addition, we have confirmed the suspected interaction of IGF-1 with gonadotropins in a clinical (in vivo) setting. The work of investigators including Liu et al. *(11)* and Baker et al. *(8)* on IGF-1 mutants demonstrated the vital role of IGF-1 and its binding protein in many aspects of embryogenesis and survival including reproduction. However, these ingenious technologies needed to be followed by physiologic and pharmacologic studies to evaluate the interaction of IGF-1 with other major players of oogenesis and ovulation, especially FSH, which is the significant component of gonadotropins, and luteinizing hormone which is the significant component of human chorionic gonadotrophin (hCG).

We have also been interested in the specific role of gonadotropins and IGF-1 in cumulus cell proliferation and differentiation *(5,13)*. It is now becoming clear that two types of granulosa cells, cumulus cells and mural granulosa cells, have distinct and different roles and interactions with gonadotropins and IGF-1. In the present study, we demonstrated that IGF-1 or gonadotropins cause a reduction in the number of cumulus cells per oocyte. Our group and others *(14)* have previously made similar observations. We demonstrated, in the context of human in vitro fertilization, that a reduced number of cumulus cells is related to lower fertilization and 2–4 cell stage embryo development *(15)*. This has led to the concept that superovulation, whether with FSH or combined FSH and IGF-1, may reduce the quality of OCCs. An increased number of oocytes obtained by the use of FSH or a combination of FSH and IGF-1 may not be proportionately related to more viable embryos.

What is the mechanism of reduced number of cumulus cells when IGF-1 analog was added to FSH in vivo? This could be related to the fact that a combination of IGF-1 and FSH promoted more of the small OCCs to reach ovulation. Alternatively, IGF-1 may have had a negative effect on cumulus cell proliferation. Finally, IGF-1 may have had a positive effect on cumulus cell replication but more occytes with much fewer cumulus cells were stimulated to reach ovulation. Although IGF-1 stimulated cumulus cell proliferation, the number of cumulus cells was still low because these OCCs started with much fewer cells. We therefore conducted experiment 3 in vitro to evaluate the effect of IGF-1 in proliferation of cumulus cells. Table 7 shows that varying dosage of IGF-1 at this stage of OCC development had no significant effect on cumulus cell proliferation (although the values for the dose 25 μg/mL appeared lower than all others). Note that we used actual IGF-1 in these studies, and, therefore, involvement of IGF-1-binding proteins was not ruled out. However, that we did not see any reponse with increasing dose may mean that IGF-1 is not effective in this situation.

In previous studies in the bovine model, we ascertained that mucification of cumulus cells is an indication of cumulus cells being mature and not replicating *(13)*. The OCCs may be unresponsive to any agent with respect to further proliferation. Therefore, we may have to modify the stimulation with gonadotropins (with or without IGF-1 analog) so that cumulus cells proliferate more before they undergo maturation. This may enable us to have better quality of OCCs.

Note that we did not estimate the blood glucose values in our studies. However, the metabolic effects of LR^{3}IGF-1 have been studied in two previous publications *(9,10)*.

Interestingly, the Sprague-Dawley strain of rats did not show an increase in ovarian weight in response to LR^{3}IGF-1 whereas the other two strains, Dark Agouti and Long-Evans, did. These strains mature somewhat differently in time, which may account for the difference in ovarian weight.

In conclusion, we have demonstrated a physiologic/pharmacologic interaction between gonadotropins and IGF-1 in a clinical (in vivo) setting, for the first time confirming what was suspected from in vitro studies. We have also made the novel observation that in a clinical setting, the combined stimulatory effect of gonadotropins and IGF-1 causes a reduction in cumulus cells per oocyte, which may be a detrimental factor for fertilization and embryogenesis. In vitro use of IGF-1 did not increase cumulus cell replication. Production of an analog of IGF-1 has permitted us to make the novel prediction that IGF-1 may be entering the realm of therapeutics, as some other growth factors have done.

Materials and Methods

Animals

Sprague-Dawley, Long-Evans, Dark Agouti, and Hooded Wistar prepubertal rats (Charles Rivers, St. Constant, Que-

bec) were housed at the animal care facilities of the University of Western Ontario. Rats were maintained on a 14-h light:10-h dark cycle and had access to food and water ad libitum in accordance with the institutional animal care guidelines. Sprague-Dawley, Long-Evans, and Dark Agouti rats were utilized to study the response of these animals with different maturation rates. Hooded Wistar rats were used simply because of availability at the time.

Pump

An infusion pump (Alzet miniosmotic pump; Alza, Vacavile, CA) was used to deliver LR^3IGF-1 or the control material, saline. Infusion was through an sc route.

Treatments

IGF-1 was purchased from Boehringer Mahnheim (Laval, Canada).

LR^3IGF-1

The IGF-1 analog was LR^3IGF-1 obtained from Gro-Pep-Pty (Adelaide, Australia). The dosage used was 1 mg/kg of body weight daily administered subcutaneously and continuously. The dosage chosen was on the basis of previous dosage-response studies in rats employing stimulation of growth and anabolic responses as end points *(9,10)*.

Equine Chorionic Gonadotropin

eCG was obtained from Ayerst (Montreal, Canada), and the dose was 15 IU injected subcutaneously on d 27.

Human Chorionic Gonadotropin

hCG was obtained from Ayerst (Montreal, Canada). The dose was 15 IU administered intraperitoneally at 56 h after the injection of eCG.

Experiment 1

The design of experiment 1 is depicted in Table 1. There were eight subgroups. Group 1 was Sprague-Dawley rats that received saline infusion from d 24 to 29 and had no injection of eCG. Group 2 was Sprague-Dawley rats that received LR^3IGF-1 infusion from d 24 to 29 but did not receive eCG. Group 3 was Sprague-Dawley rats that received saline infusion and on d 27 eCG. Group 4 was Sprague-Dawley rats that received LR^3IGF-1 infusion and eCG injection. Groups 5 and 6 were the same treatments as groups 3 and 4 but were Dark Agouti rats. Groups 7 and 8 received the same treatments as groups 3 and 4 but were Long-Evans rats. Groups 1–8 each consisted of 12 rats with 12 sets of data for analysis.

Experiment 2

The design of experiment 2 is shown in Table 3. Only Sprague-Dawley strain was used for these experiments. Three replicates were done consisting of 4, 4, and 6 animals, i.e., 14 sets of animals and 14 sets of data for statistical analysis in each subgroup. Subgroup A received sc saline infusion from d 24 to 30 and one ip injection of hCG on d 29. Group B received sc infusion of saline and one sc injection of eCG at d 27 and one ip injection of hCG 56 h later. Group C received saline infusion, eCG, and hCG. Group D received LR^3IGF-1 infusion, eCG, and hCG. All animals were sacrificed on d 30.

Weight Measurement

Body weight and ovarian weight were done just after sacrificing the animals, which was d 29 for the first and d 30 for the second experiment. Wet weight measurements were done for the ovaries. Body weights were measured intact immediately after the animals were sacrificed.

Ovulation Rate

The second experiment was designed so that ovulation had taken place. Oviductal ampullae were examined under a dissecting microscope and punctured to release OCCs into 100 µL of phosphate-buffered saline (PBS). Fifty microliters of 1% hyaluronidase solution (Sigma, St. Louis, MO) was added to disperse cumulus cells counted with a hemocytometer. The resulting cumulus cell numbers were calculated per OCC, based on the number of oocytes obtained from each rat.

Experiment 3

The design of experiment 3 is shown in Table 6. Rats were given saline (control) or eCG on d 27 to induce superovulation. They all received an injection of hCG 18–20 h later. The animals were sacrificed on d 29 by cervical dislocation, a time interval during which follicles would not have ruptured yet. The reproductive tracts were removed under aseptic conditions. Ovaries were dissected from oviducts, and adherent tissues were placed in HEPES-buffered tissue culture medium 199 (HTCM 199; Sigma) for recovery of follicular oocytes. While viewing under a dissecting microscope, surface follicles (approx 20–30 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 OCCs. These OCCs were rinsed through two successive washes in HTCM-99 and a final rinse in bicarbonate buffered tissue culture medium (BTMC 199; Sigma). Ten OCCs were then transferred to the wells of a 96-well tissue culture plate (Nunc, Napierville, IL) containing BTCM199, to which varying doses of IGF-1 were added to achieve appropriate concentrations of 6.2, 12.5, 25, and 50 ng/mL of IGF-1. The volumes were adjusted to 0.25 mL/well.

Cell Cultures

Ninety-six-well tissue culture plates containing OCCs in BTCM199 and various IGF-1 concentrations were incubated for 18–20 h in a humidified atmosphere of 5% CO_2 in air, at 37.5°C in a water-jacketed tissue culture incubator (Forma, Marietta, OH). At the end of this period, 4 µCi of ^{3}H-thymidine (20 Ci/mmol) (ICN, Irvine CA) was added to each well and the cultures continued for a further 6–8 h.

Cells were then harvested with a Skatron cell harvester. Filter disks containing the air-dried cells were counted for radioactivity with a liquid scintillation spectrometer.

Statistical Analyses

Experiment 1 was done in one replicate using 12 rats per treatment. Experiment 2 was done in three replicates using, respectively, 4, 4, and 6 rats per treatment, for a total of 14 rats per treatment for the experiment. An analysis of variance was performed for treatment effects (eCG and LR^3IGF-1) on ovulation rate, number of cells per OCC, and wet ovarian and body weights *(16)*. Experiment 3 was done on four analyses per study and was replicated three times; that is, studies were on the basis of 12 observations per each subgroup.

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