Effects of IGF-I bioavailability on bovine preantral follicular development *in vitro*

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Abstract

The aim of this study was to determine the effect of regulation of IGF-I bioavailability on preantral follicle development *in vitro*. Bovine preantral follicles were cultured for 6 days in serum-free medium with increasing doses of Long R3 (LR3) IGF-I (an analog with low affinity for IGF-binding proteins (IGFBPs)), or human recombinant IGF-I (hrIGF-I). Follicle diameter and estradiol production were measured every second day. On day 6, ratios of oocyte/follicle diameter and oocyte morphology were assessed by histological examination, and IGFBP-2 and -3 were detected by immunocytochemistry and *in situ* hybridization respectively. Both types of IGF-I increased follicle diameter in a dose-dependent manner ($P < 0.05$) and increased estradiol production over control levels ($P < 0.05$). However, follicles treated with LR3 IGF-I and the highest concentration of hrIGF-I (1000 ng/ml) had smaller oocyte/follicle ratios, and increased oocyte degeneration, compared with controls or follicles treated with physiological concentrations of hrIGF-I ($P < 0.05$). IGFBPs were detected in cultured preantral follicles, indicating a requirement for regulation of IGF bioavailability during the early stages of follicular development. Specifically, IGFBP-3 mRNA was found to be expressed in oocytes, and IGFBP-2 immunoreactivity was detected in oocytes and granulosa cells of cultured follicles. In summary, the regulation of IGF-I bioavailability by IGFBPs is necessary for the co-ordination of oocyte and follicle development *in vitro*.

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Introduction

Ovarian follicular development is regulated by both endocrine and intraovarian mechanisms which co-ordinate somatic cell proliferation and differentiation. This regulation occurs at various levels, with a constantly shifting contribution by endocrine, autocrine, and paracrine factors. The intraovarian insulin-like growth factor (IGF) autocrine/paracrine system regulates ovarian follicular development and steroidogenesis, either alone or in synergy with gonadotropins (Giudice 1992). There is also increasing evidence that IGF-I stimulates both proliferation and differentiation of ruminant granulosa cells *in vitro* (Campbell *et al.* 1996, Gutierrez *et al.* 1997a) and in rodents (Adashi *et al.* 1997). However, there are differences in temporal and spatial production of the IGF-I between rodents and domestic species, which suggest different mechanisms of action (Leeuwenberg *et al.* 1995, Yuan *et al.* 1996, Gutierrez *et al.* 1997b, Wandji *et al.* 1998, Schams *et al.* 1999, Armstrong *et al.* 2000).

The bioactivity of IGFs is controlled by their association with a family of specific IGF-binding proteins (IGFBPs), which are found in association with the extracellular matrix (ECM) and cell membranes, as well as in follicular fluid. Six IGFBPs have been identified, and these proteins can either inhibit or facilitate IGF action, depending on post-translational changes such as phosphorylation, proteolytic degradation or binding to the ECM (Monget & Bondy 2000). The IGF system has been well characterized in bovine antral follicles (Armstrong *et al.* 1998, 2000), but there are fewer studies on the effects of IGFs during the earlier stages of follicular development. To date, the expression of mRNAs encoding IGFBP-2 to -5 have been found in bovine follicles, and expression of IGFBP-2, -4, and -5 in ovine follicles (Armstrong & Webb 1997, Webb & Armstrong 1998). IGFBP-2 and -4 mRNA expression in non-atretic bovine antral follicles is confined to granulosa and theca tissue respectively (Armstrong *et al.* 1998). During follicular development and atresia, there are distinct changes in the temporal and spatial

expression of these proteins *in vivo*. For example, during the development of follicular dominance, intrafollicular amounts of IGFBP-2, -4, and -5 are decreased, whereas during atresia, the levels of these proteins are increased (Austin *et al.* 2001). In addition, proteolysis of IGFBP-4 and -5 in follicular fluid has been shown to have implications for follicular selection and dominance in cattle (Rivera & Fortune 2003). The importance of the IGF system in determining follicular fate in dominant follicles and stage-specific effects have been studied extensively (refer to Fortune *et al.* 2004). A cell-specific pattern of components of the IGF system and gonadotropin receptor gene expression has also been demonstrated in bovine cumulus-oocyte complexes during oocyte maturation (Nuttinck *et al.* 2004). Thus, changes in the expression of IGFBPs, coupled with changes in the activity of IGFBP-specific proteases, provide a mechanism to regulate the bioavailability of IGFs during folliculogenesis (Armstrong *et al.* 1998).

Little is known about the role of the IGF system during the development of preantral follicles. However, the IGF-I binding has been demonstrated in bovine preantral follicles (Wandji *et al.* 1992), and type 1 IGF receptor mRNA has been detected in bovine oocytes, granulosa, and theca cells from the preantral stage (Armstrong *et al.* 2000). In addition, IGFBP-2 mRNA has been detected in granulosa cells and oocytes, and IGFBP-3 mRNA in oocytes of bovine preantral follicles (Armstrong *et al.* 2002).

LR3 IGF-I, a synthetic IGF-I analog, which is characterized by more than a 1000-fold reduced affinity for IGFBPs (Francis *et al.* 1992), is widely used in follicle and granulosa cell culture systems. This factor has been previously reported to increase the growth of bovine preantral follicles in a long-term culture (Gutierrez *et al.* 2000). In contrast, our work has shown that LR3 IGF-I, at a concentration of 10 ng/ml, does not increase follicular growth during a 6-day culture period, and has hypothesized that the follicles have not differentiated sufficiently *in vitro* to respond appropriately to this potent form of IGF-I (McCaffery *et al.* 2000). Moreover, the same study demonstrated that LR3 IGF-I was detrimental to oocyte development during early folliculogenesis *in vitro* (McCaffery *et al.* 2000). More recent work from our laboratory has demonstrated that during antral follicle development, the effects of IGF-I on granulosa cell proliferation and differentiation and oocyte health are dose and stage dependent (Walters *et al.* 2006). In addition, IGF-I plays a role in regulating its own bioavailability by influencing the expression of IGFBP-2 in a stage-dependent manner (Walters *et al.* 2006).

Taken together, the previous studies suggest that the stage of development is likely to be a key in determining the role of IGF-I in oocyte and follicular development. The aims of the present study were to determine the effect of sequestration of IGF-I by IGFBPs during the

preantral stage of follicular development. In order to investigate this, bovine preantral follicles were cultured in the presence of increasing concentrations of recombinant IGF-I, or the analog LR3 IGF-I. Since these distinct forms of IGF-I differ only in their affinity for IGFBPs, any differential effects can be attributed to regulation by IGFBPs produced within the follicle. It is hypothesized that sequestration of IGF-I by IGFBPs during the preantral stage of follicular development will be beneficial to oocyte growth and survival.

Materials and Methods

Preantral follicle isolation

Preantral follicles ($144.8 \pm 0.6 \mu\text{m}$) were isolated from bovine ovaries as described previously (Thomas *et al.* 2001).

Preantral follicle culture

For the control group, preantral follicles ($n=36$ follicles) were cultured individually in 96-well plates (Bibby Sterilin Ltd, Stone, Staffs, UK) in 250 μl culture medium (McCoy's 5a medium with bicarbonate supplemented with HEPES (20 mM), BSA (0.1%), L-glutamine (3 mM), penicillin (100 IU/ml), streptomycin (0.1 mg/ml), transferrin (2.5 $\mu\text{g/ml}$), selenium (4 ng/ml), androstenedione (10^{-7} M), insulin (10 ng/ml) and L-ascorbic acid, sodium salt (50 $\mu\text{g/ml}$), all obtained from Sigma Chemicals. For the treatment groups, either LR3 IGF-I (Groppe Pty Ltd, Adelaide, SA, Australia) or human recombinant IGF-I (hrIGF-I, Sigma) was added to the control medium. The concentrations of LR3 IGF-I were 5 ng/ml ($n=36$), 10 ng/ml ($n=34$), and 50 ng/ml ($n=35$). The concentrations of hrIGF-I were 10 ng/ml ($n=34$), 50 ng/ml ($n=34$), 100 ng/ml ($n=34$), and 1000 ng/ml ($n=36$). Plates were incubated for 6 days in a sterile humidified air atmosphere with 5% CO_2 at 37 °C. Each set of cultures ($n=6$) took place under identical conditions. Follicle diameters were measured using a crossed micrometer (basement membrane to basement membrane) under the dissection microscope on days 0, 2, 4, and 6, and half of the medium was replenished every second day.

Detection of estradiol in culture medium using a delayed enhancement lanthanide fluorometric immunoassay (DELFI)

Medium from follicles cultured in control medium, or in the presence of LR3 IGF-I (5, 10, 50 ng/ml) or hrIGF-I (10, 50, 100 ng/ml, 1000 ng/ml; $n=18$ per treatment group) was analyzed for estradiol content, as a marker for follicular differentiation. Estradiol was biotinylated by standard procedures using 17 β -estradiol-3-(O-carboxy) methylether and EZ-Link biotin hydrazide (Pierce, Warriner, Chester, UK). Nunc-Immuno Maxisorp

96-well plates were coated with donkey anti-sheep serum by incubating overnight at 4 °C in the presence of donkey anti-sheep serum (250 µg/ml) made up in carbonate buffer pH 9.6 (100 µl/well). The primary antibody was raised in sheep against 17β-estradiol 6-(O-carboxymethyl)-oxime: BSA (Webb *et al.* 1985).

Biotinylated estradiol, follicle conditioned medium, estradiol standards, and a 1:50 000 dilution of primary antibody, made up in 200 µl of assay buffer, was added to the precoated wells of the microtitre plate. The assay buffer consisted of Tris buffer (50 mmol/l; pH 7.8) supplemented with sodium chloride (150 mmol/l), bovine gamma globulin (1%, w/v), Tween-20 (0.01%; v/v), thimerosal (0.0008%; w/v), and diethylenetriamine penta-acetic acid (0.1 moles/l). After incubating the plates overnight at 4 °C they were washed (4×) in a wash buffer consisting of Tris buffer (50 mmol/l; pH 7.8) supplemented with sodium chloride (150 mmol/l), Tween-20 (0.01%; v/v) and thimerosal (0.0008%; w/v) before adding 100 µl of assay buffer containing 100 ng/ml europium labeled streptavidin (Perkin–Elmer Life Sciences, Waltham, MA, USA) followed by incubation at room temperature for 1 h with shaking. The plates were washed 4× in wash buffer before addition of 200 µl of DELFIA enhancement solution (Perkin–Elmer Life Sciences) to each well of the microtitre plate, and incubated for a further 5 min with shaking at room temperature. The plates were analyzed on a Victor 2 Multilabel Counter (Perkin–Elmer Life Sciences) by time-resolved fluorimetry. The emission and excitation wavelengths were 615 and 340 nm respectively with a time delay of 400 µs. The inter- and intra-assay coefficients of variation were 13.2 and 9.6% respectively. The minimum detectable level was 8.5 pg estradiol per well.

Histological assessment of cultured follicles

At the end of the culture period, follicles were fixed overnight in Bouin's solution and dehydrated in ethanol. Absolute ethanol was replaced with cedar wood oil for a minimum of 24 h, then the oil was cleared from the follicles using toluene for 30 min. Follicles were embedded in paraffin wax (60 °C), with changes every hour for 4 h to remove all traces of toluene. The samples were sectioned (6 µm) and mounted on gelatin-coated slides, and allowed to dry overnight at 37 °C before staining with hematoxylin and eosin.

Histological measurements and observations were made under the light microscope with a crossed micrometer (Graticules Ltd, London, UK). The section containing the oocyte nucleolus, or if this was absent, the largest cross-section of the oocyte was used for observations and measurements. An oocyte was classed as degenerate if it was misshapen or contained no germinal vesicle. An oocyte was classed as healthy if it had normal morphology with an intact germinal vesicle.

Histological observations were made on day 6 of culture in order to determine the mean ratio of oocyte/follicle diameter and oocyte morphological status for each treatment group. Preantral (153.4 ± 4.8 µm; $n=19$), and early antral follicles (234.7 ± 10.8 µm; $n=15$) isolated and fixed on day 0 were analyzed as *in vivo* comparisons. The sample numbers for the cultured follicles were: control ($n=25$); hrIGF-I (10 ng/ml, $n=25$; 50 ng/ml, $n=30$; 100 ng/ml, $n=26$; 1000 ng/ml, $n=28$); LR3 IGF-I (5 ng/ml, $n=29$; 10 ng/ml, $n=27$; 50 ng/ml, $n=24$).

Detection of IGFBP-3 mRNA expression in cultured follicles

In order to detect IGFBP-3 in cultured follicles, a proportion of follicles fixed on day 6 of culture ($n=10$ follicles) were randomly selected, fixed in 4% paraformaldehyde, embedded in paraffin wax and sectioned (6 µm). Sections were then mounted onto slides. Sections were dehydrated, fixed, and probed with ³⁵S labeled IGFBP-3 riboprobes according to the method described by Armstrong *et al.* (2002). The bovine IGFBP-3 probe corresponded to position 611–968 of a bovine IGFBP-3 cDNA (Armstrong *et al.* 2002). After the final high stringency wash, the sections were dipped in autoradiographic K2 photographic emulsion (Ilford, Limited, Mobberley, Cheshire, UK) and exposed for 6 weeks at 4 °C. Sections were then developed (Kodak D-19) and fixed (Ilford Hypam fixer) before staining in hematoxylin and eosin. The sections were mounted in DPX mountant before microscopic examination.

Detection of IGFBP-2 immunoreactivity in cultured follicles

A proportion of follicles fixed on day 6 of culture ($n=10$ follicles) was randomly selected and used for detection of IGFBP-2 immunoreactivity using the method described by Armstrong *et al.* (1998), with some modifications. Follicles were fixed in 4% paraformaldehyde, embedded in paraffin wax and sectioned (6 µm). Sections were then mounted onto slides. Antigen retrieval was performed by placing sections in 0.01 M citrate buffer (10 min microwave (800 W), 20 min at room temperature, 0.19% citric acid (anhydrous) in distilled H₂O, pH 6). Sections were washed in PBS (2× 5 min), and endogenous peroxidase was blocked by placing sections in 3% hydrogen peroxide for 10 min, followed by washing (2× 5 min, PBS). The rabbit anti-bovine IGFBP-2 antiserum (Upstate Biotechnology Incorporated, Lake Placid, NY, USA) was diluted 1:500 vol/vol before use. After probing with primary antibody, the sections were washed and stained using a goat anti-rabbit IgG labeled with horseradish peroxidase (Dako Laboratories, Ely, UK). Non-specific binding was by replacing primary antibody with normal rabbit serum.

Statistical analyses

Mean follicle diameters and estradiol production at the end of the culture period were compared between experimental groups using a one-way ANOVA, followed by Tukey's multiple comparison test (Graphpad Prism, San Diego, CA, USA). The mean ratios of oocyte diameter/follicle diameter for each treatment on day 6 of culture were also compared using an ANOVA followed by Tukey's multiple comparison test. In addition, the number of degenerate oocytes as a proportion of the total number of oocytes were compared between treatment groups using 2-proportions analysis (Minitab, State College, PA, USA).

Results

Follicle growth

Follicles were cultured for 6 days in control medium, or in medium containing hrIGF-I (10 ng/ml, 50 ng/ml, 100 ng/ml, 1000 ng/ml) or LR3 IGF-I (5 ng/ml, 10 ng/ml, 50 ng/ml). There was significant follicle growth from day 0 to 6 in the presence and absence of hrIGF-I (Fig. 1A) and LR3 IGF-I (Fig. 1B; $P < 0.05$). At the end of culture, follicle diameter was increased over control levels by hrIGF-I (50 and 1000 ng/ml; $P < 0.05$).

Estradiol production

Medium from follicles cultured in control medium, or in the presence of LR3 IGF-I (5, 10, 50 ng/ml) or hrIGF-I (10, 50, 100 ng/ml, 1000 ng/ml) was analyzed for estradiol concentration. During the 6-day culture period, there was significant ($P < 0.05$) estradiol production by follicles in the presence and the absence of hrIGF-I (Fig. 1D) and LR3 IGF-I (Fig. 1D). At the end of culture, estradiol levels were increased over control levels by hrIGF-I (50 ng/ml; $P < 0.05$).

Co-ordination of oocyte and follicle growth

Histological observations were made on days 0 and 6 of culture in order to determine the mean ratio of oocyte/follicle diameter for each treatment group. Mean ratios of oocyte/follicle diameter are represented in Fig. 2A. A significant difference in the ratio of oocyte/follicle diameter between groups was detected by ANOVA ($P < 0.01$). Follicles treated with all concentrations of LR3 IGF-I and high concentrations of hrIGF-I (1000 ng/ml) had significantly lower oocyte/follicle ratios than day 0 preantral and early antral follicles, day 6 control follicles, or follicles treated with 10 or 50 ng/ml hrIGF-I ($P < 0.05$).

Oocyte degeneration

From histological observations on day 6 of culture, it was determined that a higher proportion of follicles treated with all concentrations of LR3 IGF-I and the highest concentration of hrIGF-I (1000 ng/ml) contained degenerate oocytes compared with all other treatment groups on day 6 ($P < 0.05$). This data is represented in Fig. 2B as the mean percentage of follicles containing degenerate oocytes for each treatment group. Examples of morphologically normal and degenerate oocytes are represented in Fig. 3.

Localization of IGFBP-3 mRNA and IGFBP-2 immunoreactivity in cultured preantral follicles

mRNA encoding IGFBP-3 was detected in oocytes and granulosa cells of preantral follicles *in vitro*, with the oocyte signal represented in Fig. 4A and C. IGFBP-2 immunoreactivity was localized to oocytes and granulosa cells of cultured preantral follicles (Fig. 4E). Negative controls for IGFBP-3 and -2 are also represented in Fig. 4 (B, D and F respectively).

Figure 1 (A) Growth of bovine preantral follicles in control medium (crosses) or in the presence of hrIGF-I (10 ng/ml (open squares), 50 ng/ml (triangles), 100 ng/ml (closed circles), 1000 ng/ml (open circles)). (B) Growth of bovine preantral follicles in control medium (crosses) or in the presence of LR3 IGF-I (5 ng/ml (open squares), 10 ng/ml (triangles), 50 ng/ml (closed circles)). Values are mean $\pm$ S.E.M. (C) Estradiol production by bovine preantral follicles cultured in control medium (crosses), or in the presence of hrIGF-I (10 ng/ml (open squares), 50 ng/ml (triangles), 100 ng/ml (closed circles), 1000 ng/ml (open circles)). (D) Estradiol production by bovine preantral follicles cultured in control medium (crosses), or in the presence of LR3 IGF-I (5 ng/ml (open squares), 10 ng/ml (triangles), 50 ng/ml (closed circles)). Values are mean $\pm$ S.E.M.

Figure 2 (A) Mean ratio of bovine oocyte/follicle diameter on day 0 (*in vivo* controls: preantral and early antral) and day 6 of culture. Values are mean $\pm$ S.E.M. (B) Mean percentage of follicles containing degenerate oocytes on day 6 of culture. Different superscript letters denote significant difference.

Discussion

It has recently been shown that the concentration of IGF-I as well as the stage of development is important for determining the effects of this factor on oocyte and follicular development in bovine antral follicles (Walters *et al.* 2006). Moreover, LR3 IGF-I, which has a low affinity for IGFBPs, was found to exert a negative effect on bovine oocyte growth within preantral follicles *in vitro* (McCaffery *et al.* 2000). Here, we have confirmed that during the preantral stage, LR3 IGF-I reduces oocyte/follicle ratio and does not maintain normal oocyte morphology *in vitro*. In contrast, when follicles were treated with physiological concentrations of recombinant IGF-I, which associates with IGFBPs, oocyte/follicle ratios and oocyte health were restored to levels comparable with controls. Since these distinct forms of IGF-I differ only in their affinity for IGFBPs, any differential effects can be attributed to regulation by IGFBPs produced within the follicle. Using a dose-response experiment, we have shown that at high concentrations, recombinant IGF-I exerts similar negative effects on oocyte development as LR3 IGF-I. Thus, the regulation of IGF-I bioavailability by IGFBPs is necessary for the co-ordination of early oocyte and follicular development.

Within bovine antral follicles, there is evidence of a complete intrafollicular IGF system (Armstrong *et al.* 1998, 2000) in which follicles express IGF ligand and mediation of IGF action, and bioavailability occurs through receptors and binding proteins. The expression

of mRNA encoding IGF-I and -II is developmentally regulated in a species-specific manner (Armstrong & Webb 1997, Webb & Armstrong 1998). Unlike rodents, mRNA encoding IGF-I has not been detected in bovine follicles at any stage of development, and since IGF-II mRNA expression is restricted to thecal tissue of antral follicles (Armstrong *et al.* 2000), it might be hypothesized that IGFs play a limited role in preantral follicle development. However, immunoreactive type 1 IGF receptor has been visualized in oocytes and granulosa cells of primordial, preantral, and antral human follicles (Qu *et al.* 2000), and in bovine oocytes from the preantral stage (Armstrong *et al.* 2000), which suggests a role for IGFs, probably acting through endocrine mechanisms, during early follicle and oocyte development. It has recently been shown that the

Figure 3 Histological sections representing (A) a morphologically normal oocyte from a follicle cultured with hrIGF-I, with an intact germinal vesicle (arrow) and (B) a degenerating oocyte from a follicle cultured with LR3 IGF-I. Bar = 25 μ m.

Figure 4 Light (A and B) and dark (C and D) field illumination of sections of cultured bovine preantral follicles probed with antisense (A and C) and sense (B and D) insulin-like growth factor binding protein (IGFBP)-3 RNA. IGFBP-3 mRNA expression is present in the oocyte (long arrow) and granulosa cells (short arrow). Bar = 15 μ m. (E) Cultured bovine preantral follicle incubated with an antibody raised against recombinant IGFBP-2, showing positive staining (dark brown) in the oocyte and granulosa cells (arrows), (F) bovine preantral follicle incubated with normal rabbit serum. Bar = 50 μ m.

concentration of IGF-I as well as the stage of development is important for determining the effects of this factor on oocyte and follicular development in bovine antral follicles (Walters *et al.* 2006). For example, high concentrations of recombinant IGF-I stimulated proliferation and differentiation of antral follicles during a 6-day culture. However, in contrast to our present findings in preantral follicles, treatment with high concentrations of IGF-I did not have a detrimental effect on oocyte health within the antral follicles, and actually resulted in a decrease in oocyte degeneration in follicles within the 281–380 μ m size range (Walters *et al.* 2006). This finding, along with the results of the current study, suggests that preantral and antral follicles differentially respond to high concentrations of IGF-I and that controlled access to IGF-I is crucial for the proper co-ordination of early oocyte and follicular development.

In the present study, IGFBPs were detected in cultured preantral follicles, indicating a requirement for regulation of IGF bioavailability during the early stages of follicular development *in vitro*. The presence of IGFBP-2 and -3 (protein and mRNA respectively) in the oocyte, as well as in the granulosa cells, provides insight into the role of the germ cell in orchestrating its own development, as well as that of the somatic cells, by regulating access to growth factors. In fact, it is well established that the oocyte plays a crucial role in somatic cell development and function, as well as *vice versa* (Vanderhyden *et al.* 1992, Eppig *et al.* 2002). IGFBP-2 protein expression in antral follicles has been shown to be regulated by IGF-I in a dose-dependent manner (Walters *et al.* 2006), with larger antral follicles (281–380 μ m) showing increased expression of IGFBP-2 protein in the presence of high concentrations of IGF-I (Walters *et al.* 2006). These results suggest that the granulosa cells at this stage of development have differentiated sufficiently to respond to IGF-I by increasing expression of IGFBP-2 (Walters *et al.* 2006). Interestingly, these follicles have a decreased incidence of oocyte degeneration in the presence of high concentrations of IGF-I *in vitro*. In the same study, smaller antral follicles responded to high concentrations of IGF-I by decreasing expression of IGFBP-2 protein, and there was no protective effect of IGF-I on oocyte health (Walters *et al.* 2006). These results suggest that the ability of the follicle to control IGF-I bioavailability is stage dependent and this ability is correlated with the role of IGF-I in oocyte development. In the current study, the levels of IGFBP proteins were not quantified, although it is possible that IGFBP expression was decreased in the presence of high concentrations of IGF-I, and this may be responsible for the observed effects on oocyte development. This possibility requires further investigation.

Using IGF-I analogs with different binding protein affinities, we can suggest that regulation of IGF-I by IGFBPs within preantral follicles is important for obtaining normal oocyte growth patterns *in vitro*. Since this control mechanism is removed when follicles are treated with LR3 IGF-I, or high concentrations of recombinant IGF-I, inappropriate stimulation of the oocyte by IGF-I may occur during culture. A previous *in vivo* study in cattle has shown a correlation between decreased follicular IGFBP-2 and -4 mRNA expression and reduced oocyte developmental competence, suggesting that an inappropriate increase in IGF bioavailability may be detrimental to oocyte development (Armstrong *et al.* 2001). These findings are in agreement with the data presented in the current study. It should also be noted that high concentrations of IGF-I, as well as acting via specific IGF receptors, can also bind to insulin receptors within the follicle (Giudice 1992). Insulin receptors have been detected in oocytes, granulosa, and theca cells of human preantral follicles

(Samoto *et al.* 1993), and also in bovine follicles (Armstrong *et al.* 2001). Therefore, this would provide a further mechanism, in addition to lack of regulation by binding proteins, for over-stimulation of the oocyte in the presence of the highest concentration of recombinant IGF-I.

The precise role of IGFs in the regulation of oocyte development during early folliculogenesis is unclear. IGF-I stimulates nuclear maturation of cumulus-enclosed oocytes (Gomez *et al.* 1993, Yoshimura *et al.* 1996, Pawshe *et al.* 1998), and improves early embryonic development (Pawshe *et al.* 1998). Moreover, IGF-I knockout mice have reduced glucose transporter expression, which is thought to be essential for oocyte maturation and successful ovulation (Zhou *et al.* 2000). Since factors which accumulate in the oocyte may have a long-lasting effect on subsequent development, the IGF-I is likely to exert an effect on oocyte maturation in early follicle stages by binding to the cell surface receptors long before oocyte competence has been achieved.

Development of preantral follicles from domestic animals or humans to a stage where the oocyte can be matured *in vitro* will require a long-term culture period, and account should be taken of the changing needs of the follicular unit during development. Due to the protracted nature of preantral follicle development in large mammals, culture systems should be developed to accelerate oocyte growth, without inducing inappropriate follicular differentiation. A recent study has reported that recombinant IGF-I increased bovine preantral follicle and oocyte diameter during serum-free culture (Itoh *et al.* 2002). By contrast, in a long-term culture system, LR3 IGF-I promoted bovine preantral follicle growth and antrum formation, but oocyte diameter was not increased (Gutierrez *et al.* 2000). Taken together with the current study, it is evident that IGF-I has differential effects on oocyte and follicle development, and that the regulation by IGFBPs is beneficial for early oocyte development. We have shown that the regulation of IGF-I by IGFBPs stimulates follicle growth, although not all concentrations of recombinant IGF-I increased follicle diameter over control levels. However, by comparison of oocyte/follicle ratios, we have pinpointed a marked difference between recombinant and LR3 IGF-I in terms of co-ordination of oocyte and follicular growth. We were also able to determine that the patterns of oocyte/follicle growth obtained during culture with LR3 IGF-I were not comparable with normal *in vivo* development. Since appropriate co-ordination of oocyte and follicle development is essential for normal oocyte growth and acquisition of developmental competence, the present study suggests that the IGF system is likely to be involved in this process. The differential effects of the IGF-I analogs on the oocyte did not correlate with estradiol production, a marker of follicular differentiation, therefore more research is required to determine

the mechanism(s) of IGF-I action on oocyte development. Since the type 1 IGF receptor is present in oocytes within bovine preantral follicles (Armstrong *et al.* 2000), removal of the regulatory influence of IGFBPs *in vitro* may allow a direct over-stimulation of the oocyte by IGF-I.

In conclusion, this study demonstrates a role for the IGF system in regulating the early stages of oocyte and follicle development. This regulation appears to be crucial for appropriate co-ordination of oocyte and follicle growth, thus preservation of these physiological control mechanisms will be important during future follicle culture regimes.

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