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# COMPARISON OF LEPTIN LEVELS IN SERUM AND FOLLICULAR FLUID DURING THE OESTROUS CYCLE IN COWS

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Leptin is mainly synthesised in white adipose tissue. Besides its effects on body weight and metabolic homeostasis, leptin also has effects on puberty, sexual maturation and reproduction. In this study the relationship between leptin, IGF-1, oestradiol ($E_2$) and progesterone levels were investigated in serum and follicular fluid from cows. This study included 72 healthy, Brown Swiss cows aged 4–5 years. Samples from the jugular vein and follicular fluids were collected. Phases of the oestrus cycle of cows were classified according to their serum progesterone levels (< 3.18 nmol/l, follicular phase and the others as luteal phase). Follicles were grouped as large (≥ 8 mm) or small (< 8 mm). Leptin, IGF-1, oestradiol and progesterone levels were measured from serum and follicular fluid. Leptin concentrations were found to be significantly higher in luteal-phase follicular fluid of small follicles ($P < 0.05$). These were classified as atretic follicles. There was a positive correlation between serum and follicular fluid leptin levels in the luteal phase. Serum leptin was found to have a positive correlation with follicular fluid progesterone level ($P = 0.01$) in the preovulatory follicles. The present study shows that there is a relationship between the concentration of leptin in follicular fluid and atresia in small follicles.

**Key words:** Leptin, reproduction, folliculogenesis, cow

Leptin, the product of the obese (ob) gene, is primarily synthesised by adipose tissue and has an important role in the regulation of food intake and body weight. As a satiety hormone, leptin concentration can change in response to calorie intake and can suppress appetite, increase metabolic rate, and regulate weight gain and fat deposition (Zhang et al., 1994). In addition to its effects on body weight and metabolic homeostasis, leptin has an important role in puberty,

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sexual maturation and reproduction (Ahima et al., 1996). Previous studies reported that intraperitoneal leptin injection restored sterility in genetically obese male (Mounzih et al., 1997) and female mice (Chehab et al., 1996). Furthermore, normal prepubertal female mice that were injected with leptin showed earlier maturation of the reproductive tract than controls (Chehab et al., 1997). It is thought that leptin and steroid hormones have a relationship with each other (Spicer and Francisco, 1997, 1998; Brann et al., 2002). Recent data on healthy women showed higher leptin levels during the luteal phase of spontaneous menstrual cycles which correlated with LH levels (Riad-Gabriel et al., 1998; Cella et al., 2000). This may indicate a relationship between leptin and follicle development. Although these reports suggest that leptin has a positive effect on reproduction, other studies revealed a negative effect of high levels of leptin. Cell culture studies demonstrated that leptin caused a dose-dependent inhibition of the IGF-1 augmentation of FSH-stimulated oestradiol production in rat (Zachow and Magoffin, 1997), bovine (Spicer and Francisco, 1997, 1998) and human follicle cells (Agarwal et al., 1999). Therefore, it is thought that there may be a relationship between leptin, IGF-1 and gonadal steroids.

In the present study, we hypothesised that leptin may have some other effects on follicle development besides its central effect on the female reproductive system. Our aim was to define the relationship between leptin, IGF-1, oestradiol and progesterone concentrations during the follicular and luteal phases of the oestrous cycle of the cow.

## Materials and methods

### *Animals and experimental procedures*

Jugular vein blood and ovaries of 72 healthy, Brown Swiss dairy cattle aged 4–5 years old were collected immediately following slaughter at a nearby commercial abattoir and transported to the laboratory within 1 h. Diameters of the follicles on the ovaries were measured and follicular fluid was aspirated with a syringe and hypodermic needle. Serum and follicular fluid samples were stored at –80 °C following centrifugation at 3000 rpm for 10 min (Echternkamp et al., 1994; Agarwal et al., 1999).

### *Analysis of hormones*

Concentrations of leptin (Multi-species leptin RIA kit, Linco Research, Inc., St Charles, MO) and total IGF-1 (Biosource IGF-1-D-RIA-CT kit cat # 30 158 60) were quantified by RIA. Oestradiol (E2) and progesterone concentrations were measured by chemiluminescence (Immulite 2000 Estradiol and Immulite 2000 Progesterone commercial kits by DPC, Los Angeles, CA) in the serum and follicular fluid. Due to the higher values of oestradiol and progesterone, the

follicular fluids were diluted 1:100 before the assay. Sensitivities of the assay were 1.0 ng/ml, 0.4 ng/ml, 15 pg/ml, 0.2 ng/ml for leptin, IGF-1, $E_2$, progesterone, respectively. The intra- and inter-assay coefficients of variation were 3.1% and 7.8% for leptin, 3.9% and 5% for IGF-1, 6.4% and 7.8% for $E_2$ and 6.3% and 9.1% for progesterone, respectively, in follicular fluid and 3.1% and 7.8% for leptin, 3.9% and 5% for IGF-1, 9.9% and 16% for $E_2$ and 14% and 17% for progesterone, respectively, in serum. In luteal phase assays, the intra- and inter-assay coefficients of variation of $E_2$ measurement in follicular fluid and progesterone measurement in serum were 4.3% and 6.7% for $E_2$ and 6.7% and 9.5% for progesterone, respectively.

*Classification of follicles*

Cows with serum progesterone levels below 3.8 nmol/l were classified as being in the follicular phase and those with levels above 3.8 nmol/l in the luteal phase (Mariana et al., 1991). Follicles were classified according to their diameters as large (≥ 8 mm) or small (< 8 mm). The large follicles were grouped as oestradiol-active (EA) ($E_2$ > 183.5 nmol/l) and oestradiol-inactive (EI) ($E_2$ < 183.5 nmol/l) (Echternkamp et al., 1994; Spicer and Francisco, 1997). As a result, follicles were grouped as stated below:

*Group 1:* All follicles taken during the follicular phase in the cows
- 1.1. Large follicles (≥ 8 mm)
  - i. Large and oestradiol-active (EA) follicles
  - ii. Large and oestradiol-inactive (EI) follicles
- 1.2 Small follicles (< 8 mm)

*Group 2:* All follicles taken during the luteal phase in the cows
- 2.1. Large follicles (≥ 8 mm)
  - i. Large and oestradiol-active (EA) follicles
  - ii. Large and oestradiol-inactive (EI) follicles
- 2.2. Small follicles (< 8 mm)

*Statistical analysis*

Differences between group means (± S.E.M.) were analysed by Student's *t*-test or Mann-Whitney U test. Correlation between parameters were analysed by Spearman's or Pearson's correlation test. Statistical significance was determined as $P \leq 0.05$. Probabilities between $P \geq 0.06$ and $P < 0.1$ were defined as approaching significance.

## Results

Mean serum leptin concentration was not different between the follicular and the luteal phase (0.33 ± 0.02 nmol and 0.37 ± 0.03 nmol/l, respectively; $P > 0.05$).

IGF-1 concentrations were significantly higher in the follicular fluid taken during the luteal phase compared with the follicular fluid of follicular-phase cows ($P < 0.01$) (Table 1). IGF-1 concentrations were significantly higher in the follicular fluid of large follicles during the luteal phase compared with the follicular fluid of the large follicles of follicular-phase cows ($P < 0.05$).

In the luteal phase, the mean leptin concentration of the follicular fluid was not different from that found in the follicular phase ($P = 0.5$ NS) (Table 1).

**Table 1**

Comparison of follicular fluids obtained in the luteal and in the follicular phase

| | Follicular phase (n = 69) | Luteal phase (n = 92) | P |
|---|---|---|---|
| Oestradiol (nmol/l) | 406.7 ± 98.0 | 203.3 ± 4.4 | 0.06 |
| Progesterone (nmol/l) | 724.7 ± 114.7 | 1175.6 ± 217.1 | 0.07 |
| Leptin (nmol/l) | 0.29 ± 0.03 | 0.31 ± 0.02 | 0.56 |
| IGF-1 (nmol/l) | 32.4 ± 2.4 | 43.8 ± 3.8 | 0.01** |

Data are shown as means ± S.E.M. **$P < 0.01$

Follicle size was found to be significantly higher in the follicular fluid of EA follicles as compared to EI follicles during the follicular phase ($P < 0.001$; Table 2). In the EI follicles, progesterone and leptin concentrations were higher than in EA follicles of the follicular phase ($P < 0.05$). However, the difference of means between two groups was not statistically significant ($P = 0.46$ NS; Table 2). In the luteal phase, progesterone concentrations of the EI follicles were significantly higher than those of the EA follicles ($P < 0.02$; Table 2).

Oestradiol levels were significantly higher in the follicular fluid of large follicles compared to small follicles both during the follicular and the luteal phase ($P < 0.04$ and $P = 0.05$, respectively; Table 3). Leptin levels were significantly higher in the follicular fluid of small follicles compared to large follicles of the luteal phase ($P < 0.04$; Table 3). A positive correlation ($r = 0.3$, $P < 0.001$) was found between follicle size and oestradiol concentration in all follicular fluids.

Leptin levels in the follicular fluid were not significantly different from those found in the serum in both the follicular and the luteal phase. A significant correlation between serum and follicular fluid leptin levels was found for the luteal phase ($r = 0.6$, $P < 0.001$). A positive correlation was found between serum and follicular fluid IGF-1 concentration both in the luteal and the follicular phase ($r = 0.3$, $P = 0.001$ and $r = 0.6$, $P = 0.001$, respectively). In addition, a positive correlation ($r = 0.5$, $P = 0.01$) was found between serum leptin and follicular fluid progesterone concentration in the EA follicles during the follicular phase, whilst a negative correlation was seen between leptin and IGF-1 concentration in follicular fluid (approaching significance, $r = 0.3$, $P = 0.10$). Furthermore, there was a sig-

**Table 2**

Comparison of follicular fluid in EA and EI follicles

| | Follicular phase | | | Luteal phase | | |
|---|---|---|---|---|---|---|
| | EA (n = 23) | EI (n = 29) | P | EA (n = 24) | EI (n = 41) | P |
| Diameter (mm) | 12.7 ± 0.5 | 10.3 ± 0.4 | 0.001*** | 11.6 ± 0.4 | 11.1 ± 0.4 | 0.16 |
| Oestradiol (nmol/l) | 987.6 ± 217.3 | 64.9 ± 9.9 | 0.001*** | 584.0 ± 109.7 | 48.0 ± 6.9 | 0.001*** |
| Progesterone (nmol/l) | 402.5 ± 93.8 | 997.5 ± 229.5 | 0.02* | 933.3 ± 585.4 | 1286.3 ± 234.6 | 0.02* |
| Leptin (nmol/l) | 0.28 ± 0.03 | 0.33 ± 0.06 | 0.46 | 0.32 ± 0.04 | 0.28 ± 0.03 | 0.29 |
| IGF-1 (nmol/l) | 35.5 ± 4.4 | 31.6 ± 3.8 | 0.51 | 51.5 ± 10.3 | 41.8 ± 4.8 | 0.66 |

Data are shown as means ± S.E.M. *P < 0.05; ***P < 0.001; EA = oestradiol-active; EI = oestradiol-inactive

**Table 3**

Comparison of follicular fluid in large (≥ 8 mm) and small (< 8 mm) follicles

| | Follicular phase | | | Luteal phase | | |
|---|---|---|---|---|---|---|
| | Large (n = 55) | Small (n = 14) | P | Large (n = 65) | Small (n = 27) | P |
| s | 11.3 ± 0.3 | 5.9 ± 0.3 | 0.001*** | 11.3 ± 0.3 | 4.8 ± 0.2 | 0.001*** |
| Oestradiol (nmol/l) | 440.5 ± 113.4 | 60.9 ± 24.9 | 0.04* | 236.0 ± 49.9 | 63.1 ± 20.1 | 0.05* |
| Progesterone (nmol/l) | 712.6 ± 138.0 | 649.0 ± 129.1 | 0.31 | 1121.9 ± 251.8 | 1238.6 ± 381.6 | 0.26 |
| Leptin (nmol/l) | 0.34 ± 0.04 | 0.28 ± 0.03 | 0.95 | 0.29 ± 0.02 | 0.36 ± 0.03 | 0.04* |
| IGF-1 (nmol/l) | 30.5 ± 2.7 | 31.7 ± 5.2 | 0.59 | 43.6 ± 4.7 | 44.2 ± 6.5 | 0.91 |

Data are shown as means ± S.E.M. *P < 0.05; ***P < 0.001

nificant negative correlation between follicular fluid oestradiol and progesterone concentrations in the EI follicles in both the luteal and the follicular phase ($r = 0.4$, $P = 0.01$ and $r = 0.3$, $P < 0.05$, respectively).

## Discussion

Ovarian function in cows is regulated by the interactions of systemic (pituitary gonadotropins) and local (ovarian steroid, peptide and protein hormones) feedback mechanisms leading to two or three follicular waves in one oestrous cycle. During the progression of any wave, one of the follicles becomes dominant whilst the others undergo atresia, leaving only the dominant follicle to develop under the influence of the follicular phase. It has been reported that as the follicle increases in diameter, aromatase activity of the granulosa cells also increase and thus more oestradiol is produced (Ginther et al., 1996).

In this study, a positive correlation ($r = 0.3$, $P < 0.01$) was observed between follicle diameter and follicular fluid oestradiol content. These results suggest that there is a relationship between follicular growth and oestradiol levels and are in accordance with the known increase in aromatase activity with follicle development (Ginther et al., 1996). It is known that dominant follicles produce oestradiol and inhibin that reduce the FSH released from the adenohypophysis, thus terminating the FSH available for other follicles to develop (Kastelic, 1994). In addition, it is thought that some local intraovarian compounds play a role in the atresia of follicles (Webb and Armstrong, 1998). Furthermore, there is a significant decrease in oestradiol and an increase in progesterone content of atretic follicles; the former is seen in parallel with the reduction of aromatase activity of granulosa cells (Mariana et al., 1991; Stevenson, 1997).

In this study, in both follicular- and luteal-phase follicular fluid, progesterone concentrations of oestradiol-inactive (EI) follicles were found to be higher than those of oestradiol-active (EA) follicles (Table 2). A negative correlation ($r = –0.4$, $P < 0.01$ and $r = –0.3$, $P < 0.03$) was observed between oestradiol and progesterone concentrations of oestradiol-inactive follicles in both groups. The oestradiol concentrations in small follicles of both the follicular and the luteal phase were significantly lower than those of large follicles, while the progesterone concentrations were not significantly higher in small follicles than in large follicles (Table 3). These results support the hypothesis that small-diameter follicles that are EI and have high progesterone in both the follicular and the luteal phase are atretic.

The effects of leptin on the reproductive system are not fully understood in either humans or animals. Exogenous leptin was shown to restore fertility in obese-infertile ob/ob mice (Chehab et al., 1996; Mounzih et al., 1997) and caused earlier onset of sexual cycles in prepubertal mice (Chehab et al., 1997).

Some studies reported that leptin had an inhibitory effect on synergistic action of IGF-1 on FSH-induced oestradiol synthesis but such levels of the leptin can be regarded as being above the physiological range (Zachow and Magoffin, 1997; Agarwal et al., 1999).

In this study, luteal-phase large follicles had significantly lower leptin concentrations than luteal-phase small follicles. Also, lower oestradiol and higher progesterone concentrations were detected in smaller follicles, which is an indicator of ongoing atresia (Table 3, Fig. 1). Moreover, in the follicular phase, leptin concentrations were higher in oestradiol-inactive (EI) follicles, which are also considered as atretic, than in oestradiol-active (EA) follicles, but the difference was not statistically significant. Besides, oestradiol concentrations were significantly lower and progesterone concentrations were significantly higher in EI follicles as compared to EA follicles (Table 2). The fact that leptin concentrations are higher in atretic follicles, and that leptin reduces steroidogenesis in granulosa and theca cells (Zachow and Magoffin, 1997; Spicer and Francisco, 1997, 1998; Agarwal et al., 1999; Spicer et al., 2000) suggests that leptin might be a cytokine that plays a role in follicular atresia, which is also consistent with the theory that unknown intraovarian factors (e.g. limited bioavailability of IGF-1) are responsible for atresia (Webb and Armstrong, 1998).

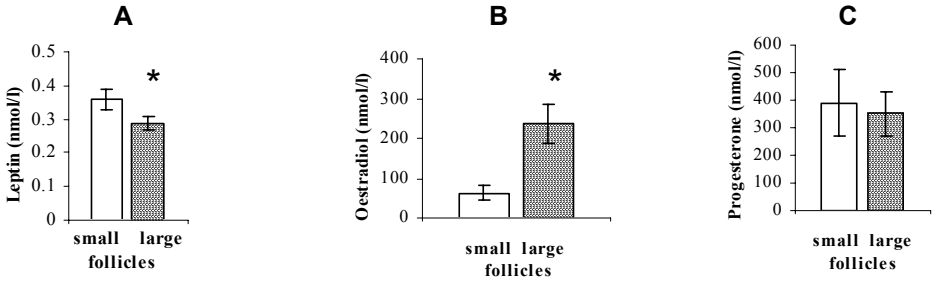



*Fig. 1.* Comparison of leptin (A), oestradiol (B) and progesterone (C) concentrations between small and large follicles during the luteal phase. $^{*}P < 0.05$: significance of difference between small and large follicles

Leptin plays a role in the central regulation of reproduction. It is known that in healthy cyclic women serum leptin concentrations differentiate, increase and peak in luteal phase (Riad-Gabriel et al., 1998; Cella et al., 2000). On the contrary, in this study it was observed that serum and follicular fluid leptin concentrations were not significantly higher in cows that were in the luteal phase compared to those in the follicular phase. However, in all follicles of luteal-phase cows there was a positive correlation between follicular fluid and serum leptin concentrations. No study on either humans or animals has investigated the relationship between follicular fluid leptin concentrations and follicle diameter.

Some recent studies (Delavaud et al., 2000; Delavaud et al., 2002) have revealed that a newly developed bovine-specific RIA is more reliable for assaying ruminant leptin. These authors found lower values using the multispecies commercial leptin RIA kit due to the lower sensitivity of the antibody; they also declared that, despite the differences in absolute values observed in the homologous and heterologous assays, the physiological variation in plasma leptin was generally similar (Delavaud et al., 2000). In addition, Bellmann et al. (2004) and Wegner et al. (2001) used the multispecies leptin kit in cattle as a valid assay. Of course, the results of the present study could be strengthened by a more specific assay; but this would not change the direction of the results.

IGF-1 is a factor that plays an important role in folliculogenesis and is also produced by the dominant follicle. *In vitro* studies have revealed that IGF-1 supports gonadotropin-induced steroidogenesis in both theca and granulosa cells and stimulates follicular cell proliferation (Lackey et al., 1999). Echternkamp et al. (1994) demonstrated a relationship between oestradiol and total IGF-1 in dominant follicles of cows. Beg et al. (2002) found a relationship between oestradiol and free IGF-1 in dominant follicles of cows and showed that IGF-1 concentrations were higher than Insulin-like Growth Factor Binding Proteins (IGFBP) in follicles. In this study, total concentrations of IGF-1 in oestradiol-active follicular-phase follicles, which are considered preovulatory follicles, were not found to be different from those of oestradiol-inactive follicles. In accordance with this, De La Sota et al. (1996) did not find any difference in IGF-1 levels between dominant and subordinated tertiary follicles. They stated that IGF-1 concentrations in follicles may be stable, while IGFBP may have a selective link to IGF-1, thus reducing the potential trophic effect of IGF-1 on follicular growth. De La Sota et al. (1996) commented that IGFBP, being low in the EA dominant follicle, meant that more free IGF-1 was needed to potentiate the effect of FSH, which may result in an increase of aromatase activity and androgen biosynthesis. Limited bioavailability of IGF-1 might be an important intraovarian factor responsible for atresia.

In this study, while oestradiol was high in dominant follicles of all groups, no correlation was detected between follicle oestradiol and follicle IGF-1. It may be that both free IGF-1 and IGFBP concentrations are required to fully examine the relationship between oestradiol and IGF-1. Echternkamp et al. (1994) and De La Sota et al. (1996) showed that IGFBP was lower in large follicles than in small follicles, suggesting that biologically active free IGF-1 plays an important role in follicle growth. These studies indicate the importance of IGF-1 in the developing follicles. In this study no relationship was found between follicle diameter and IGF-1 levels. This might be due to that fact that we measured total IGF-1 (both free and bound IGF-1).

In this study, IGF-1 levels in large follicles were found to be higher in luteal-phase cows than in follicular-phase cows. These data do not indicate that

large follicles of follicular-phase cows are under less influence of IGF-1. It was stated (Monniaux and Pisselet, 1992) that large follicles could be stimulated with less IGF-1, as granulosa cells of large follicles contained 10–15 times more IGF-1 receptors than small follicles (Spicer et al., 1994) and large follicles in sheep had lower concentrations of IGF-1 (Khalid and Haresign, 1996).

Previous studies using cell cultures have not been able to elucidate the mechanism by which leptin weakens the effect of IGF-1. We found a negative correlation (as approaching significance $r = –0.35$, $P = 0.10$) between leptin and IGF-1 levels in the preovulatory follicles. Measurement of both free IGF-1 and IGFBP in a larger number of follicles may give a better understanding of the relationship.

To our knowledge, this is the first report to investigate the relationship between progesterone and leptin in follicular-phase EA follicles. In this study, a positive correlation was found between serum leptin and follicle progesterone in follicular-phase EA follicles. It is known that in the final stages of preovulatory development theca cells begin to synthesise progesterone, thus progesterone levels rise in the follicular fluid (Stevenson, 1997). However, the effect of this rise of progesterone before ovulation on follicle growth and/or ovulation is not clear.

In conclusion, the positive relationship of progesterone and leptin concentrations may be an indicator of follicular atresia in small follicles both in the luteal and the follicular phase in cows. The relationship between leptin and progesterone in the dominant follicle requires further investigation but it might be that leptin has an important role in other stages of follicular development.

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