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**Original article**

# Potential leukocyte attractants in the bovine peri-ovulatory ovary

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**Summary** — This study investigated interrelationships between the bovine ovarian cycle and white blood cells and tested the hypothesis that the ovary produces collagen-like materials with leukocyte attractant activity. We examined the *in vitro* secretion of leukocyte attractant activity by peri-ovulatory ovarian tissues and evaluated the leukocyte attractant potential of some ovarian biochemicals. Fluid from mature ovarian follicles and medium conditioned by follicular tissue, early luteal tissue or granulosa cells had significant attractant activity. The activity could be removed by protein precipitation but not by collagenase. Collagenase also failed to alter the electrophoretic profile of the samples. Collagenase (800 IU/ml), ascorbic acid (10–1 000 μg/ml) and $CaCl_2$ (50–560 μg/ml) had significant leukocyte attractant effects. Native collagen types I and IV (100–1 000 μg/ml) had fewer expressed attractant activities, which were unaffected by collagenase pre-treatment. The attractant activity of collagenase itself was removed by protein precipitation. Our observations suggest: (1) that follicular and luteal tissues produce leukocyte attractant(s); (2) that granulosa cells contribute to the secretion of this material; (3) that the principal ovarian attractants are neither the native collagen types I or IV nor their collagenase-releasable fragments; and (4) that collagenase, ascorbic acid and $Ca^{2+}$ are strong candidates as attractant constituents of ovarian secretions.

**bovine ovary / follicular fluid / polymorphonuclear leukocyte / collagenase / ascorbic acid / calcium / granulosa**

**Résumé — Substances leuco-attractives potentielles dans l'ovaire péri-ovulatoire bovin.** *Cette étude porte sur les relations possibles entre le cycle ovarien et les globules blancs. Notre hypothèse de travail est que l'ovaire produit des substances attractives pour les leucocytes. Nous avons examiné la sécrétion* in vitro *de substances attractives par des tissus ovariens péri-ovulatoires. Le fluide de follicules murs et du milieu conditionné par du tissu folliculaire, du tissu de corps jaune ou des cellules de la granulosa ont une activité attractive significative. L'activité est éliminée par la précipitation des protéines mais non par la collagénase. La collagénase ne modifie pas le profil électrophorétique des échantillons. La collagénase (800 μg/ml), l'acide ascorbique (10–1 000 μg/ml) et le chlorure de calcium*

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*(56–560 µg/ml) ont des effets attractifs significatifs. Le collagène natif de type I et IV (100–1 000 µg/ml) montre des niveaux d'activité inférieurs, non modifiés par un prétraitement à la collagénase. L'activité attractive de la collagénase est supprimée par la précipitation des protéines. Nos observations suggèrent que : i) les tissus folliculaires et lutaux produisent des substances leuco-attractives ; ii) les cellules de la granulosa contribuent à leur sécrétion ; iii) les principaux attractifs ovariens ne sont ni le collagène type I ou IV, ni leurs fragments produits par la collagénase ; iv) la collagénase, l'acide ascorbique et $Ca^{2+}$ seraient des substances attractives des tissus ovariens.*

***ovaire bovin / liquide folliculaire / lymphocyte / polymorphonucléaire / collagénase / acide ascorbique / calcium / granulosa***

## INTRODUCTION

White blood cells can play an important role in the regulation of ovarian function. Leukocytes migrate to the ovary, especially during the periovulatory period, and are able to regulate steroidogenesis, ovulation, luteinization and the remodelling of follicular tissue. They act through the production or transport of cytokines, gonadotropins, proteolytic and angiogenic factors, biogenic amines, prostaglandins and ascorbic acid (Stern and Coulam, 1992; Norman and Brannstrom, 1994).

The factors that attract the white blood cells to the ovary are unknown. Leukocyte attractant activity has been demonstrated in bovine, ovine and human follicular fluid (Seow *et al,* 1988; Norman and Brannstrom, 1994) and in medium conditioned by ovine luteal and follicular tissue (Murdoch, 1987; Murdoch and McCormick, 1989; Murdoch and Steadman, 1991). The activity has been ascribed to molecules of low molecular weight (100 kDa; Seow *et al,* 1988; 15–16 amino acids: Murdoch and McCormick, 1989). The active material from ovine tissue has peptide characteristics similar to collagen (Murdoch and McCormick, 1993), and collagenase digestion of collagen type I releases fragments with leukocyte attractant activity (Malone *et al,* 1991). Other substances with known activity include formulated oligopeptides produced by capillary endothelium (Caterina and Devrotes, 1991), some cytokines (Norman and Brannstrom, 1994), derivatives of arachidonic acid (Snyderman and Goetzl, 1981; Murdoch, 1987) and chorionic gonadotropin (Reinisch *et al,* 1994).

The aims of our investigation were: i) to determine the cellular origin of leukocyte attractant activity in the periovulatory bovine ovary; ii) to test the hypothesis that this is attributable to collagen or collagen fragments; and iii) to evaluate the leukocyte attractant activity of other ovary-related molecules.

## MATERIALS AND METHODS

All chemicals used in the experiments were of analytical, molecular or cell culture grade and obtained from Sigma (Saint Louis, USA) unless otherwise stated.

### *Follicular fluid collection and granulosa culture*

Ovaries from Holstein cows, 2–4 years of age, were collected at a local slaughterhouse and the stage of the estrous cycle was determined by cells and follicular fluid which were aspirated from non-atretic follicles, 10–15 mm in diameter, and separated by centrifugation (10 min, 200 *g*). Follicular fluid from several animals was pooled, aliquoted, and stored at –20°C for use in all further experiments. Granulosa cells were washed 3 times in medium consisting of DMEM/Ham's F-12 1:1 mixture supplemented with gentamicin (50 µg/ml, Pharmachim, Sophia, Bulgaria), insulin (5 µg/ml, Lečiva, Prague, Czech Republic), BSA

(1 mg/ml, fraction V), transferrin (5 µg/ml) and selenium (5 ng/ml). The cell suspension was aliquoted into 24-chamber plate wells (Sarstedt, Vienna, Austria) at $10^7$ cells/ml (determined by hemocytometer) and cultured for 4 d at 37°C under 5% $CO_2$ in humidified air. Each day, medium from a number of wells was removed and stored at –20°C to await analysis.

### *Follicular and luteal tissue incubation*

Follicles and early (1–2 d post-ovulation) corpora lutea were isolated from ovaries, dissected from adherent tissue and cut into pieces (approx 1 x 15 mm, 75–100 mg). The pieces (8 per group) were washed 3 times and incubated for 20 h as described above. The conditioned medium was collected and stored at –20°C. Medium incubated without tissue was used as control.

### *Leukocyte preparation*

All leukocytes used in these experiments were obtained from the same group of 3 mature Dorset ewes. They were isolated from whole heparinized blood by centrifugation (700 *g*, 30 min) through a discontinuous Percoll gradient comprising densities of 1.065, 1.072 and 1.082 g/l. Erythrocytes and platelets concentrated at the bottom and top of the gradient respectively. The majority of mononuclear cells (monocytes and lymphocytes) accumulated at the upper interface, whilst granulocytes (neutrophils, eosinophils, basophils) accumulated at the lower interface. The latter fraction was carefully aspirated, washed 3 times by centrifugation (200 *g*, 10 min), resuspended, and brought to a final concentration of approximately $10^8$ cells/ml. Viability by Trypan blue exclusion was 95–99%.

### *Leukocyte migration assay*

The migration of leukocytes in response to the test substances was assessed by a modification of the method of Murdoch (1987). Briefly, 3 ml of Ham F-12 medium containing 0.5% agarose and 0.5% BSA at 55°C was pipetted onto microscope slides and allowed to solidify at room temperature for 1 h. Parallel rows of 3 circular wells, 2 mm diameter and 2 mm apart, were cut in the gel by means of needle tubing mounted in a Perspex frame. Five microlitres of granulocyte suspension were pipetted into the central well, whilst control medium and the experimental sample were pipetted into flanking wells. Slides were incubated for 2 h in a humidified 5% $CO_2$ atmosphere at 37°C. Cells migrated over the slide surface below the agarose, with greater movement in the direction of the well containing a leukocyte attractant. After incubation, cells were fixed to the slide by immersion in 10% buffered formalin for 1 h. The agarose was carefully removed by inverting the slide, and the slide stained by Wright–Giemsa stain. The maximal linear distance of leukocyte migration from the border of the wells towards the experimental and control wells were measured by microscope eyepiece micrometer.

### *Other treatments*

Collagenase types II and VII (Sigma, 800 IU/ml), ascorbic acid (0.1–1 000 µg/ml), lactic acid (Gibco, 100 µg/ml), glucose (Gibco, 100 µg/ml), $CaCl_2$ (Gibco, 56 and 560 µg/ml), EDTA (Gibco, 146 and 1 460 mg/ml) were dissolved in Ham's F12 medium immediately before the experiments. Collagen types I and IV from human placenta were dissolved in Ham's F12 medium acidified by 10 N HCl, neutralized by 10 N NaOH and brought to 1 mg/ml. In some experiments, samples of culture and incubation media were treated with metaphosphoric or trichloroacetic acid (3%, 4°C overnight), to precipitate proteins. After centrifugation (700 *g*, 10 min), the supernatant was collected and neutralized by 10 N NaOH. In other experiments, conditioned media and collagens were treated with collagenases in the presence of $CaCl_2$ (56 µg/ml) for 0.5–2 h at 37.5°C. The reaction was inhibited by bringing the samples to 4°C.

Follicular fluid, conditioned medium and collagens, before and after acid precipitation or treatment with collagenase, were subjected to polyacrylamide gel electrophoresis (6% gel at 120 V) with sodium dodecyl sulphate (SDS-PAGE) under non-reducing conditions (Laemmli, 1970). Samples (20 µl) were dissolved in loading buffer consisting of 0.3 M Tris, 20% SDS and 50% glycerol with bromophenol blue.

### *Statistical analysis*

Each experiment was repeated 2–4 times and typical data is presented. The indicated rates of leukocyte migration towards control and sample wells are the average of 15–18 wells ± SEM. The significance of differences between the groups was assessed according to the Mann–Whitney non-parametric test.

## RESULTS

Neutrophils and eosinophils showed a net migration towards bovine follicular fluid and towards medium conditioned follicles, corpora lutea or a 4 d culture of granulosa cells (fig 1). No migratory response of basophils, lymphocytes or monocytes were noted. Follicular fluid had a dose-dependent attractive effect on neutrophils and eosinophils (fig 2) and was used (at 100%) as a positive control in further experiments. The attractant activity of medium from granulosa cell culture increased up to day 3 of culture and tended to decline thereafter (fig 3).

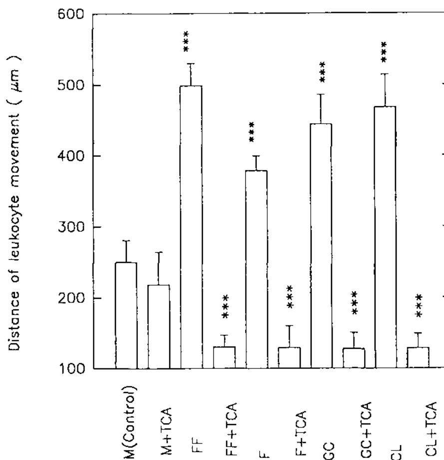


**Fig 1.** Leukocyte attracting activity of bovine follicular fluid (FF) and of medium not conditioned (M) and conditioned by cultured ovarian follicles (F), corpus luteum (CL) and granulosa cells (GC) before and after treatment with trichloroacetic acid (+ TCA). Values are means ± SEM, *** $p <$ 0.001 *versus* control (medium without additions).

Acid pre-treatment of the above samples to precipitate proteins (confirmed by PAGE, fig 4) resulted in the disappearance of their attractant properties (fig 1). Pre-treatment with collagenases did not change their electrophoretic profile (not shown) or their leukocyte attractant activity (fig 5).

Collagen types I and IV possessed a low but significant leukocyte attractant activity (fig 5). Collagenase treatments digested both collagens (data of PAGE, not shown) but did not influence their attractant properties (fig 5). Collagenase itself had a significant attractant effect in the presence of $Ca^{2+}$ ions (fig 5). The treatment of both collagens and collagenase by metaphosphoric acid removed their attractant activity (not shown).

Since both follicular fluid and the collagenase solutions contained high (millimolar) concentrations of cations, the leukocyte attractant activity of $CaCl_2$ was studied. It possessed potent activity which could be prevented by chelation with EDTA. Neither

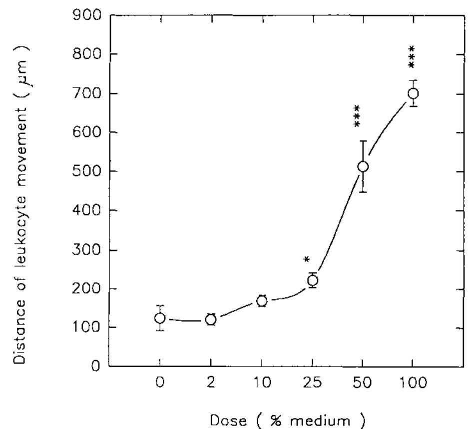


**Fig 2.** Leukocyte attracting activity of bovine follicular fluid. Values are means ± SEM, * $p < 0.05$, *** $p < 0.001$ *versus* control (medium without additions).

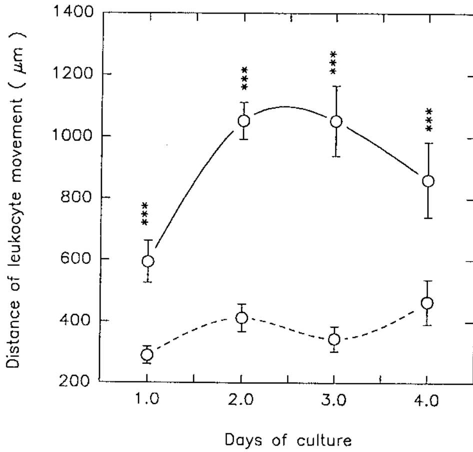


**Fig 3.** Leukocyte attracting activity of the medium cultured with (—) and without (- - -) bovine granulosa cells. Values are means ± SEM, *** $p < 0.001$ between the control and conditioned medium.

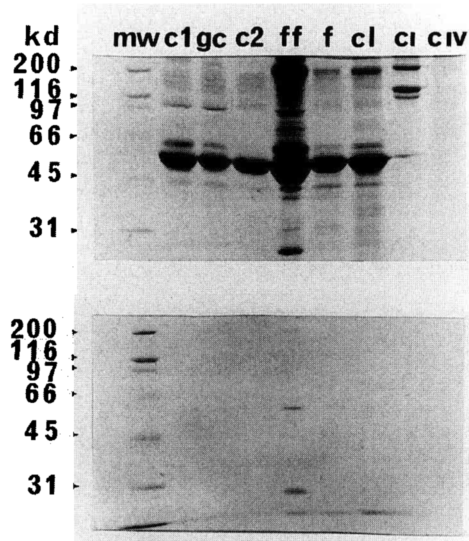


**Fig 4.** PAGE of the follicular fluid (FF), collagens type I (CI) and IY (CIY) and of the medium conditioned by granulosa cells (GC), ovarian follicles (F) and corpus luteum (CL) before (top) and after (bottom) treatment with trichloroacetic acid. MW: indicator of molecular weight (not treated with acid); $C_1$ and $C_2$: control media (cultured without cells) for GC and F and CL, respectively.

$CaCl_2$ supplementation nor EDTA chelation affected the attractant activity of follicular fluid (fig 6).

Ascorbic acid had a dose-dependent attractant activity (fig 7) with an $ED_{50}$ of 22 μg/ml and maximal effect at 100 μg/ml. Similar concentrations of lactic acid and glucose had no influence on leukocyte movement (fig 8). The attractant activity of ascorbic acid was unaffected by addition of $CaCl_2$ or EDTA (not shown).

## DISCUSSION

Our results confirm previous reports (Murdoch, 1987; Seow *et al*, 1988; Murdoch and Steadman, 1991; Murdoch and McCormick, 1993) that pre-ovulatory follicular fluid and the media conditioned by follicular and luteal tissues contain a leukocyte attractant. The demonstration of attractant activity in media from granulosa cell culture provides the first evidence that it is produced endogenously by granulosa cells. The results of our further experiments suggest that several different biochemical components within the ovary may be involved in the peri-ovulatory attraction of leukocytes.

The removal of activity from follicular fluid and conditioned media by acid precipitation suggests that proteins are responsible for a significant fraction of the attractant effect. Low molecular weight collagen-like peptides have been proposed as the active component of conditioned medium/follicular fluid (Murdoch and McCormick, 1993). Collagens have been detected in follicular fluid (Christiane *et al*, 1988), synthesized by developing follicles and corpora lutea, and turned over during the remodelling of peri-ovulatory tissues (Luck and Zhao, 1993; Luck, 1994; Luck *et al*, 1994). In the present

experiments, collagen type I (the principle luteal collagen; Luck and Zhao, 1993) and type IV (a component of the follicular basement membrane; Bertolussi *et al*, 1989; Luck and Zhao, 1993) were able to attract leukocytes although the effects were relatively small, even at high concentrations of collagen. Treatment with collagenase did not increase attractant activity beyond the background, suggesting that such a treatment does not release small active molecules fragments of the type previously described. Collagenase treatment of follicular fluid had no visible effect on the electrophoretic pro-

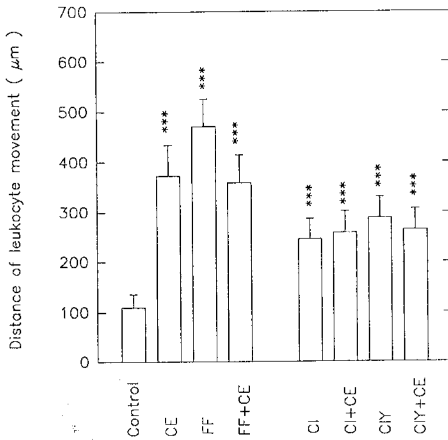


**Fig 5.** Leukocyte attracting activity of follicular fluid (FF), collagens types I (CI) and IY (CIY) before and after treatment with collagenase (CE). Symbols as in figure 1.

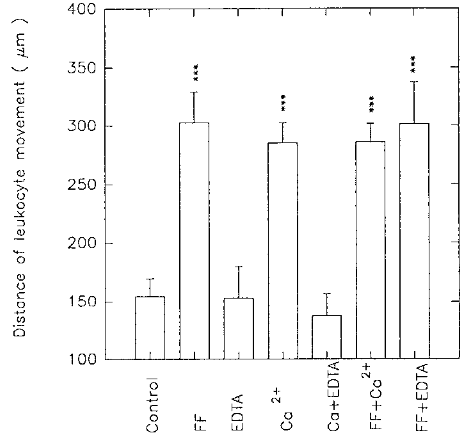


**Fig 6.** Leukocyte attracting activity of bovine follicular fluid (FF), $CaCl_2$ ($Ca^{2+}$), EDTA and their combinations. Symbols as in figure 1.

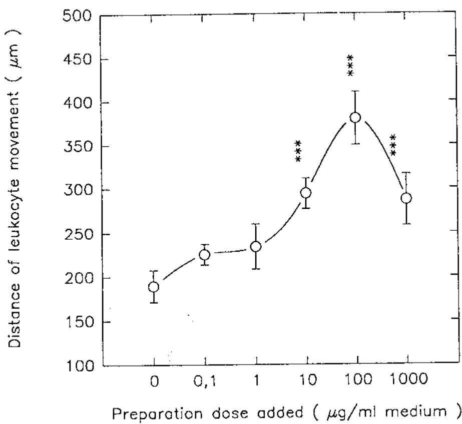


**Fig 7.** Leukocyte attracting activity of ascorbic acid. Symbols as in figure 1.

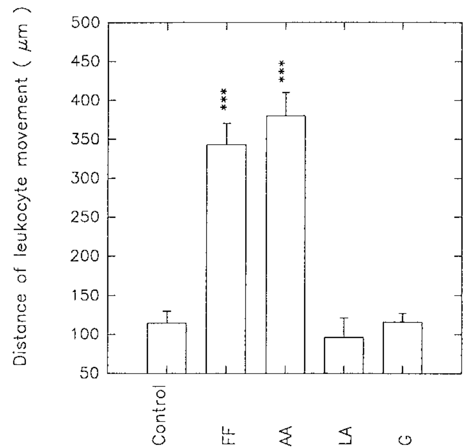


**Fig 8.** Leukocyte attracting activity of follicular fluid (FF), ascorbic acid (AA), lactic acid (LA) and glucose (G). Symbols as in figure 1.

file of the material. In any case, the known poor solubility of intact collagen indicates that it could account for only a small proportion of the protein in follicular fluid and tissue-conditioned media. We therefore conclude that intact collagen makes a minor, if any, contribution to the total attractant activity. The identity of the small molecular weight fragments reported previously remains uncertain.

The expression of a strong attractant activity by collagenase itself is of interest since metalloproteinases are an important component of the proteolytic cascade activated during the ovulatory process (Luck, 1994). The cellular origin of the collagenase in the follicle wall is uncertain but it is produced by ovarian luteal cells (Gerland *et al*, 1994). Although the proteolytic activity of metalloproteinases is cation-dependent, the leukocyte attracting property of our collagenase was calcium-independent as demonstrated by its undiminished activity in the presence of EDTA.

Calcium ions are known to be involved in the mechanism of leukocyte movement and to facilitate their chemoattraction to other molecules (Snyderman and Goetzl, 1981; Nath and Gallin, 1986; Caterina and Devrotes, 1991). In the present experiments, $Ca^{2+}$ were found to be attractive in themselves. Since neither the cells nor the gel used in these experiments was likely to have been calcium free, this movement suggests that leukocytes are sensitive to a concentration gradient. The physiological significance of this effect in relation to the ovary is not certain, but $Ca^{2+}$ released from damaged cells during the inflammation-like process of ovulation may be effective in attracting leukocytes to the luteinizing follicle (Espey, 1980).

The leukocyte attractant properties of ascorbic acid were tested because this material is actively accumulated by the follicle and corpus luteum (Luck and Zhao, 1993; Jeyasseelan *et al*, 1994). Ascorbate is a co-factor in collagen biosynthesis (Pinnell, 1985) and in the production of steroid and protein hormones (Padh, 1991; Goralczyk *et al*, 1992), and promotes the luteinization of granulosa cells (Luck and Jungclas, 1987, 1988). Leukocytes can accumulate high concentrations of ascorbic acid (Bergsten *et al*, 1990) and may be its principle transport mechanism (Washko *et al*, 1993). Although leukocyte chemotaxis is known to be facilitated by ascorbate (Johnston and Huang, 1991; Verstraeten *et al*, 1991), our observations show that ascorbic acid is itself a potent attractant for leukocytes. Neither glucose nor lactic acid, which have similar physico-chemical characteristics to ascorbic acid, were attractive to the cells. The dose–response range for attraction to ascorbate was very similar to that previously observed in the stimulation of bovine granulosa cells (Luck and Jungclas, 1987) and covers the concentrations found in bovine (Wise, 1987) and human (Jeyasseelan *et al*, 1994) follicular fluid.

In summary, our experiments demonstrate that ovarian granulosa cells produce substance(s) which attract leukocytes and which may partially account for the attractant activity of pre-ovulatory follicular fluid. The principal ovarian attractant is probably proteinaceous but is unlikely to be intact collagen, despite the ability of the latter to attract leukocytes. Collagenase relased during the ovulatory process may be an additional candidate. Ovarian attractant activity may also reside with non-proteinaceous materials including ascorbic acid and $Ca^{2+}$ ions. We conclude that a complex of substances, accumulated and released during the follicular-luteal cycle, may account for the attraction of leukocytes to the ovary.

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