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SPONTANEOUSLY ARISING DISEASE

Altered Expression of Pro-inflammatory Cytokines in Ovarian Follicles of Cows with Cystic Ovarian Disease

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Summary

A growing body of evidence suggests that ovulation shares many of the features of an inflammatory reaction and that cytokines play many diverse and important roles in reproductive biology. The aim of this study was to examine the expression of the pro-inflammatory cytokines interleukin (IL)-1α, IL-6 and tumour necrosis factor (TNF)-α in ovarian cells from cows with cystic ovarian disease (COD) as compared with that in ovarian structures from regularly cycling cows. Expression of genes encoding IL-1α, IL-6 and TNF-α was detected by real-time polymerase chain reaction in follicular cells from ovaries from healthy cows and cows with COD with no significant differences. However, immunohistochemistry showed increased expression of IL-1α, IL-6 and TNF-α in cystic follicles, suggesting that this expression may be related to the persistence of follicular cysts. The effect of COD was evident for IL-1α and TNF-α, and a follicular structure–disease interaction was observed in the expression of all the cytokines evaluated. Thus, altered expression of these proinflammatory cytokines may be related to ovulation failure and development of follicular cysts.

Keywords: cow; cytokine; immunohistochemistry; ovarian cyst

Introduction

Ovulation, which is induced by the pituitary release of luteinizing hormone (LH), involves many cell types. The ovulatory process includes changes in gene expression in theca cells, granulosa cells, oocytes and invading leucocytes, which are the main cellular components of the follicle (Espey and Richards, 2002; Brännström *et al*., 2010).

Several features of mammalian ovulation indicate that this process is similar to an inflammatory response and many cytokines have been linked to this process by demonstration of their expression within the ovary after stimulation by LH (Espey, 1994; Espey *et al*., 2004; Brännström *et al*., 2010). Most of these ovulation-associated cytokines are produced by activated macrophages and are also expressed in granulosa and theca cells (Espey *et al*., 2004; Sirotkin, 2011). The production of cytokines in the ovary and their influence on some ovarian processes suggests that cytokines could be important autocrine, paracrine or endocrine regulators of ovarian function (Sirotkin, 2011; Smolikova *et al*., 2012; Sheldon *et al*., 2014).

One of the mediators of inflammation is the interleukin (IL)-1 system, which consists of two different pro-inflammatory cytokines, IL-1α and IL-1β (Dinarello, 1998). The IL-1 system has several sites of synthesis in the ovary and IL-1-like bioactivity has been reported in human, equine and porcine

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follicular fluid at the time of ovulation (Hurwitz *et al.*, 1992; Terranova and Rice, 1997). IL-1 may be involved in several ovulation-associated events such as the synthesis of proteases, regulation of plasminogen activator activity and prostaglandin and nitric oxide production. IL-1 also regulates ovarian steroidogenesis (Gérard *et al.*, 2004).

IL-6 is a regulator of inflammation and immunity, which may represent a physiological link between the endocrine and immune systems, and is a modulator of ovarian function (Buyalos *et al.*, 1992). IL-6 is produced in rat, porcine and bovine granulosa cells, which suggests a potential role for this cytokine in the autocrine and/or paracrine regulation of ovarian function (Alpizar and Spicer, 1993; Gorospe and Spangelo, 1993). Immature rat granulosa cells have been reported to secrete IL-6 *in vitro* and a role for this cytokine as a potential regulator of steroidogenesis was suggested by the finding that follicle stimulating hormone (FSH) inhibits its release and that IL-6 inhibits FSH-induced progesterone synthesis (Gorospe and Spangelo, 1993). IL-6 is also produced by human granulosa cells in pre-ovulatory follicles at the time of ovulation (Kawasaki *et al.*, 2003).

Tumour necrosis factor (TNF)-α is a mediator of the immediate–early response and can promote ovarian cell proliferation. In undifferentiated ovarian cells, TNF inhibits steroidogenesis, while in differentiated cells, it stimulates progesterone synthesis (Terranova and Rice, 1997; Bornstein *et al.*, 2004).

Cystic ovarian disease (COD), one of the most important causes of reproductive failure in cattle, causes economic losses to the dairy industry because it increases the calving to conception interval (Garverick, 1997). COD is the most frequently occurring ovarian disorder associated with impaired ovulation and incidence ranges from 1 to 30% depending on herd and breed conditions (Peter, 2004; Silvia *et al.*, 2005; Vanholder *et al.*, 2006; Rizzo *et al.*, 2011; Cattaneo *et al.*, 2014). The pathogenesis and mechanisms of cyst formation are not fully understood, but it is accepted that the main component of the aetiopathogenesis of COD is altered function of the hypothalamic–pituitary–ovarian axis (Silvia *et al.*, 2002). However, the persistence of follicles over time is related to an intra-ovarian component.

Many studies have evaluated the presence of different cytokines in the normal ovary, but how these cytokines are altered during ovarian dysfunction has not been elucidated fully. Considering the multiple factors involved in ovulation, we hypothesized that the alteration of one or more components in this process may contribute to the pathogenesis of COD in cows. Comparison of gene and protein expression may aid in understanding additional causes of COD and will be vital in understanding the process of anovulation and cyst formation. Because the ovary is a site of inflammatory reactions and ovarian cells could represent sources and targets of cytokines, the aim of this study was to examine IL-1α, IL-6 and TNF-α expression in somatic ovarian cells from healthy cows and cows with spontaneously arising COD.

Materials and Methods

All procedures were evaluated and approved by the Institutional Ethics and Security Committee of the Facultad de Ciencias Veterinarias, Universidad Nacional del Litoral, Santa Fe, Argentina (protocol number: 84/11) and were consistent with the *Guide for the Care and Use of Agricultural Animals in Research and Teaching* (2010).

Collection and Preparation of Tissues

Samples were collected from local dairy farms. Twenty healthy multiparous (66.2 ± 25.4 month old; 2.8 ± 1.3 lactations) Holstein cows, at least 45 days after calving (55.3 ± 9.1 days in milk) and with high yield (mean 26.7 ± 9.2 kg of milk per day at diagnosis), were assigned to the control group ($n = 20$). Oestrous cycles were synchronized using the Ovsynch protocol as follows: animals were injected with a gonadotropin-releasing hormone (GnRH) analogue (buserelin acetate; Gonaxal®; Biogénesis-Bagó, Buenos Aires, Argentina, 10 μg/animal) on day 0, a prostaglandin $F2_{\alpha}$ analogue (D + Cloprostenol, Enzaprost D-C®, Biogénesis-Bagó, 150 μg/animal) on day 7 and a GnRH analogue (Gonaxal®, 10 μg/animal) on day 9. The time of ovulation was monitored by transrectal ultrasonography using a real-time B-mode scanner equipped with a 5 MHz linear array transrectal transducer (Honda HS101V, Tokyo, Japan) and was designated as day 1 of the oestrous cycle, because ovulation occurs 24–32 h after the second injection of GnRH (Pursley *et al.*, 1995). Follicular development was monitored daily by ultrasonography to obtain samples of normal growing follicles (approximately day 18) when the dominant follicle reached a diameter >10 mm. Spontaneous follicular cysts were identified from Holstein cows at local dairy farms as one follicular structure of 20 mm in diameter, persisting for at least 10 days in the absence of a corpus luteum (Bartolome *et al.*, 2005; Vanholder *et al.*, 2006). Twenty multiparous (64.8 ± 24.9 month olds; 3.3 ± 1.5 lactations) Holstein cows, at least 45 d after calving (65.9 ± 27.8 days in milk) and with high yield (mean 29.7 ± 6.2 kg of milk per day at diagnosis), with COD were selected. Diagnosis and

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