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Characterization of Cytoskeletal Proteins in Follicular Structures of Cows with Cystic Ovarian Disease

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Summary

The distribution of intermediate filaments (vimentin, cytokeratins, desmin) and microfilaments (α-smooth muscle actin and muscle specific actin) was studied immunohistochemically in bovine ovaries, with and without cystic ovarian disease. The immunohistochemically stained area (IHCSA), was quantified by image analysis, to evaluate the expression of these cytoskeletal proteins in the follicular wall of healthy antral, atretic, and cystic follicles. The granulosa cell layer of cystic follicles and atretic follicles had a significantly larger IHCSA for vimentin than did healthy antral follicles. Cytokeratins reacted lightly in the granulosa cells of antral follicles of normal ovaries, whereas granulosa cells of atretic and cystic follicles showed significantly higher IHCSA values. Immunohistochemical localization of desmin, muscle specific actin, and α-smooth muscle actin was restricted to the theca externa. This study supports earlier suggestions that strongly positive reactions with vimentin and cytokeratin antibodies observed in the granulosa cells of cystic follicles are due to the reorganization that occurs in the follicle during the process of cystic development, and are associated with changes in the expression of cytoskeletal proteins that are essential to proper cellular functioning.

Keywords: cattle; cystic ovary; follicular cysts; ovarian disease

Introduction

Cystic ovarian disease (COD) is one of the most common causes of reproductive failure in cattle (Peter, 2004). The incidence of COD in dairy cattle has been reported to range from 5.6% to 18.8% (Peter, 2004; Silvia *et al.*, 2005). This estimate may be too low, as more than 60% of cows that develop COD before the first post-partum ovulation recover spontaneously and remain undetected (Peter, 2004). A cystic follicle, in cattle, has been defined as an anovulatory follicle-like structure (more than 20 mm in diameter) that may persist in the ovary (usually for more than 10 days), with or without a corpus luteum (CL) (Silvia *et al.*, 2002; Peter, 2004).

The mechanisms that lead to the development of follicular cysts have been the object of speculation and research for many years (Wiltbank *et al.*, 2003), but are still poorly understood. It is believed that the disease has a multifactorial aetiology (Peter, 2004). Follicular cysts appear to be caused by an endocrine imbalance in the hypothalamo-hypophyseal-gonadal axis (Lopez-Diaz and Bosu, 1992; Hamilton *et al.*, 1995). Abnormal production of luteinizing hormone (LH) was reported in cows with cystic follicles by Hamilton *et al.* (1995). Since LH largely affects the function of the granulosa and theca interna layers (Voss and Fortune, 1993), altered steroidogenesis may occur in the cystic follicles (Isobe *et al.*, 2003). By contrast, although many studies have outlined the dynamics of follicular growth, our understanding of the cellular and molecular

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changes that occur within the ovarian follicle leading to anovulation is still incomplete. Cellular changes may be in the form of an aberrant production of growth factors by the granulosa cells, inappropriate secretion of extracellular matrix (ECM) proteins, or changes in the cytoskeletal proteins (Peter *et al.*, 1995; Salvetti *et al.*, 2003, 2004).

The cytoskeleton is composed of three types of high molecular weight polymers, namely microtubules, microfilaments, and intermediate filaments. Microtubules and microfilaments are homogeneous and ubiquitous structures (Feuilloley and Vaudry, 1996), whereas intermediate filaments represent a heterogeneous family of fibres whose expression depends upon the level of differentiation of the cells (Goldman *et al.*, 1990). In exocrine (Sinha and Wagner, 1987) and endocrine cells (Ravindra and Grosvenor, 1990), which secrete their products by exocytosis, microtubules and microfilaments play a role in both the traffic of secretory granules in the cytoplasm and the fusion of the vesicles with the plasma membrane. Although the presence of vesicles has been observed in steroidogenic cells, it is generally accepted that steroids are released immediately after synthesis, by diffusion through the plasma membrane (Bomsel *et al.*, 1986). However, during the last decade, a number of studies have demonstrated that pharmacological agents that induce either disruption or stabilization of cytoskeletal fibres strongly affect steroid hormone secretion (Chen *et al.*, 1994). This factor is particularly important in the changes in steroidogenesis that may take place during cystogenesis (Isobe *et al.*, 2003).

Cytoskeletal proteins have been extensively examined in the ovarian cells of many species, including laboratory animals, farm animals, and human beings (Selstam *et al.*, 1993; van den Hurk *et al.*, 1995; Khan-Dawood *et al.*, 1996; Löffler *et al.*, 2000; Marettova and Maretta, 2002). However, the changes that occur in pathological situations, such as the development of ovarian cysts, have been studied only in experimental models. Thus, in induced follicular cysts in rats, structural and functional changes occur during cystogenesis, and these may be associated with changes in the expression of cytoskeletal proteins (Salvetti *et al.*, 2004).

It has been hypothesized that cytoskeletal proteins contribute to the structural integrity of cells (Schliwa and Van Blerkom, 1981) and participate in cell-to-cell binding and in differentiation events (Luna and Hitt, 1992). The purpose of this study was to evaluate the role of intermediate filament and microfilament proteins during cystogenesis in cows, by examining the pattern of expression in normal and cystic bovine ovaries.

Materials and Methods

Collection and Preparation of Tissues

Ovaries with ($n = 12$) or without ($n = 12$) cystic follicles were collected at a local abattoir in the city of Rafaela (Santa Fe, Argentina), within 20 min of death, from mixed breeds of cows, assessed visually as non-pregnant.

Macroscopical abnormalities, other than ovarian cysts, were not observed in the reproductive tract of any of the cows. Follicular cysts were diagnosed when the follicles were more than 20 mm in diameter, in the absence of a functional CL in either right or left ovary (Brown *et al.*, 1982). The cystic follicles used in this study showed no signs of luteinization.

Ovaries without cystic follicles were used for the observation of antral and atretic follicles. The stage of the oestrous cycle was defined by macroscopical observation of the ovaries (colour, consistency, CL stage, number and size of follicles) and the uterus (colour, consistency and mucus) (Berisha *et al.*, 2004).

The ovaries were dissected, sectioned, and fixed in 10% buffered formalin for 6 h at 4 °C and washed in phosphate-buffered saline (PBS). For light microscopy, fixed tissues were dehydrated in an ascending series of ethanol concentrations, cleared in xylene, and embedded in paraffin wax. Serial sections (5 μm), were mounted on 3-aminopropyl triethoxysilane (Sigma, St Louis, MO, USA)-coated slides and dried for 24 h at 37 °C (Ortega *et al.*, 2004).

Classification of Follicles

Follicles were classified macroscopically and microscopically according to the criteria listed in the *Nomina Histologica* (1994). Only follicles that appeared healthy (i.e., well vascularized and with a transparent follicular wall and fluid) and whose diameter was >5 mm were used and classified as antral. The selection of this follicle-size category was based on the reported gonadotropin dependence and changes in the expression of steroidogenic enzymes and LH receptor mRNAs. Xu *et al.* (1995) showed that at this size follicle growth will be halted if follicle-stimulating hormone (FSH) is suppressed. The atretic follicles were sub-classified as obliterative or cystic, based on the descriptions in Table 1.

Cystic follicles were initially classified microscopically into types 1, 2 or 3, on the basis of their granulosa cell layer structure (Isobe and Yoshimura, 2000); however, they were later considered to be a single group, due to lack of differences between the types in respect of the parameters studied (preliminary data not published). At least 20 follicles of each type were examined, except in the case of cystic follicles, of which 12 were analysed.