proposed suggesting that gonadotropin releasing hormone (GnRH) and gonadotropin are implicated in the pathogenesis of ovarian cysts. Actually, some authors attribute the disease to alterations in synthesis and release of GnRH [6,7], luteinizing hormone (LH) [7-9] or even to a receptor deficiency at the pituitary level [10,11].

2-D PAGE, originally described by O'Farrell [12] in 1975, is the method of choice for the separation of cell proteins, where proteins are separated in two sequential steps. This technique is an important proteomics tool, using which many protein spots can be visualized, resulting in a global view of a proteome's state [13]. Furthermore, recent developments in 2-D PAGE and mass spectrometry technologies have enabled quantitative analysis of differential proteomics, such as a comparison between normal and disease status, allowing identification of protein markers to characterize a specific physiological or pathological cell or tissue state [14,15] which have been used in the fields of diagnosis and biomarker identification of animal and human diseases [16,17].

As described above, research on BOFC has focused on endocrinological conditions [6,8], but there are few research reports on proteins in the FF of BOFC. Although Mortarino et al. [18] published one short research report about making a 2-D PAGE map of proteins expressed in the FF of BOFC, they used a 2-D PAGE process using FF which had not been depleted of abundant proteins such as albumin or immunoglobulin G. Therefore, it was thought that they overlooked spots of minor, but important proteins. Muranaka et al. [19] also examined protein in FF; the content of total protein in the FF of BOFC was significantly lower than that of the FF of normal follicles.

This study was designed to determine any substantially increased proteins in the FF from BOFC using a differential proteomics technique. The results help to clarify the pathology and etiology of BOCF and will contribute to the discovery of BOFC biomarkers.

Methods

Experimental design

This study consisted of the two following experimental phases; 1) examination of protein sample preparations from FF, and 2) assessment of increased proteins in the FF of BOFC by 2-D PAGE.

Experiment 1: Examination protein sample preparation

Cystic follicles were collected from dairy cows at a local slaughterhouse. FF was aspirated carefully with a 20 mL syringe, and centrifuged at 10,000 × g at 4°C for 30 minutes to eliminate cells and other insoluble materials, and stored at -30°C until processing for protein sample preparation.

Three types of FF protein sample were prepared in order to find an appropriate sample preparation method for 2-D PAGE.

Type A: Non-treatment

Type B: Deplete impurities (salts, lipids, detergent or nucleic acid)

Type C: Deplete both abundant proteins (albumin and IgG) and impurities

A detailed sample preparation method for depletion of both abundant proteins and impurities for Type C is shown in the following section on "Sample preparation". Non-depleted FF was used as Type A. Deplete impurities from FF were used as Type B. Samples depleted of both abundant proteins (albumin and IgG) and impurities were used as Type C. These samples were subjected to 2-D PAGE and silver stain gel images were compared visually.

Details of sample preparation, 2-D PAGE and silver staining are shown in the latter parts of this section.

Experiment 2: Assessment of increased proteins in the FF of BOFC

Ovaries with (n = 4) or without (n = 3) cystic follicles were used in this experiment. A follicular cyst was diagnosed when the follicle was greater than 25 mm in diameter in the absence of a functional corpus luteum in both the right and left ovaries [1]. Follicles of about 10 mm in diameter from ovaries without cystic follicles were used as large normal follicles. About 300 – 1000 μL of FF was aspirated carefully from each follicle with a syringe. After measureming progesterone and estradiol-17β in FF, FFs from cystic follicles were stored individually and those from normal follicles were pooled.

Each follicle was fixed in formalin after aspiration of FF, processed for histological examination and stained with hematoxylin and eosin using a standard method.

The FF protein samples were prepared by the Type C method (depleted of both abundant proteins (albumin and IgG) and impurities). Each sample from cystic follicles with pooled control samples was simultaneously processed for 2-D PAGE twice and gels were visualized by silver staining. The stained gels were scanned using a desktop scanner and scanned images from cystic and normal FFs were analyzed with PDQuest software version 7.1 (Bio-Rad Laboratories Inc., Hercules, USA). Expressions of protein spots on the images from cystic and normal FFs that were processed for 2-D PAGE simultaneously were compared, and a overall comparison of the results from 4 cystic follicle images was made.