2-D PAGE was examined in the present study in order to obtain satisfactory 2-D PAGE images for differential proteomics. The quality of the 2-D PAGE images was improved and the number of visualized spots increased by removing abundant proteins, such as albumin and IgG from FF, as well as other body fluids, serum [22,23], cerebrospinal fluid [24] and bile [25], and by removing impediments, such as salts, nucleic acid and lipids, for the isoelectric focusing process [26] in this study. Concomitant to the removal of albumin and IgG, there was a significant enhancement in the staining intensity of several lower abundance protein spots. This indicates that 2-D PAGE using refined FF is a useful experimental tool for research on the physiology of FF in the ovaries of farm and experimental animals.

The sample ovarian follicles used in this study were examined morphologically and endocrinologically. The diameters of cystic follicles were greater than 25 mm by macroscopic observation; the granulosa cell layers were already exfoliated and the theca internals of some cystic follicles were thinner than those of healthy follicles. These observations show that the follicles examined in this study were late stage cystic follicles [1,27-29]. Non-dominant regressing cystic follicles showed lower estradiol-17β concentrations and higher progesterone concentrations compare to normal follicles or dominant cystic follicles, so that the ratio of estradiol-17β/progesterone of non-dominant regressing cystic follicles was below 1 [30]. The ratios of estradiol-17β/progesterone of three of four cystic follicles in this study were below 1 and these values are consistent with the histological examination, i.e. that these follicles were late stage cystic follicles.

This study was designed to identify the specific proteins in the FF of BOFC in order to help clarify the pathology of BOFC using 2-D PAGE. We found 8 additional protein spots on the gel from the cystic follicles compared with the normal control, and these proteins were identified as BMFA, EAF, MeS, VEGF-receptor, GAPDH, HSP70, BLG and SD using the MOLDI-TOF MS technique.

Heat shock proteins (HSPs) are encoded by genes whose expression is substantial during stressful conditions, such as heat shock, inhibition of energy metabolism, exposure to heavy metals, oxidative stress, and inflammation [31,32]. Under these conditions, HSPs increase cell survival by protecting and disaggregating stress-labile proteins. Under no-stress conditions, HSPs have multiple housekeeping functions, such as folding and translocating newly synthesized proteins [32,33]. It was confirmed that HSP70 was clearly expressed in the granulosa cells of human ovaries in vitro [34], and was synthesized by oocytes and cumulus cells in cows [35], and by granulosa cells within follicles in rats [36]. HSPs play an important role in fertilization and early embryonic development [37-39]. Recently, the ability to inhibit apoptosis has become widely recognized as a function of HSP70, and this ability may contribute to its protective effect against cell death [39-42]. HSP70 inhibits apoptosis and this effect may be ascribed to its neutralizing interactions with several proapoptotic effectors [40]. Isobe and Yoshimura [43] reported that in the theca interna of bovine ovarian follicles, a high frequency of apoptosis was noted in the early cystic follicles, whereas this frequency decreased in late cystic follicles. They concluded that the decrease in the rate of cell apoptosis may be responsible for the delay in follicular regression, and that control of apoptosis may be essential for reducing the incidence of cystic follicles. This observation is consistent with our result that HSP70, which decreases apoptotic cell death, was increased in the FF of cystic follicles. Peter [3] emphasized that understanding the mechanisms that regulate granulosa cell growth, differentiation and apoptosis is of great clinical importance because aberrant signalling in any of these pathways is likely to be related to in cystic ovaries. HSP70 might be the key protein related to apoptosis in granulosa cells. It is thought that cows with follicular cysts are exposed to various kinds of stress such as oxidative stress [29], negative energy balance, poor liver function and low circulating insulin-like growth factor-I [44]. Although the relationship between these stresses and the increase in HSP70 in FF in this study is unclear, it is speculated that over-expression of HSP70 decreases apoptosis in the theca interna and leads to delayed regression of cystic follicles which had prevented ovulation due to hormonal imbalance.

VEGF is known as a hypoxia-inducible mitogen for endothelial cells [45], and is also referred to as a vascular permeability factor [46], affecting vasodilatation and capillary permeability and stimulating endothelial cell growth and angiogenesis in vivo. VEGF and its receptors have been found in the ovaries of numerous species including bovine, ovine, porcine and monkey ovaries, and may be important regulators of ovarian angiogenesis [47]. In humans, VEGF is implicated in the etiology of serious reproductive disorders such as polycystic ovary syndrome, and in this syndrome, elevated VEGF may interact with its receptors, such as Flk-1/KDR, in the affected ovaries, preventing granulosa cell apoptosis and the resultant follicle atresia, thus contributing to the growth and persistence of a large number of follicles [45]. Isobe et al. [48] reported that the numbers of positive vessels and areas with von Willebrand factor, an indicator of endothelial damage, were significantly reduced in the theca of follicles in association with the cyst development. This suggested that fewer degenerative changes in the vasculature occur in BOFC, which allows a consistent blood supply and may result in maintaining follicular tissue sta-