—Brief Note—

Ammonia Concentration in Porcine Ovarian Developing Follicles

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Abstract: *An experiment was conducted to investigate pH, osmolality, and the concentration of ammonia, total protein, glutamine and glutamic acid in follicular fluid at different developmental stages (<2, 3–4 and 5–6 mm in diameter) and serum of porcine. The concentrations of ammonia and total protein content were determined with the catalyzed indophenols reaction and the Bradford assay method and read on a spectrophotometer set at 625 and 595 nm, respectively. Glutamine and glutamic acid concentrations were determined by HPLC. The pH value was lower (P<0.05) but osmolality was higher (P<0.05) in follicular fluid than in serum. The concentration of ammonia was lower (P<0.05) in follicular fluid than in serum. On the other hand, glutamine and glutamic acid concentrations were higher (P<0.05) in the follicular fluid than in serum. The pH increased and osmolality decreased with increasing follicle size, and protein content was almost similar in small, medium and large follicular fluid. Ammonia, glutamine and glutamic acid concentrations decreased (P<0.001) as follicular size increased. During early follicular development ammonia and amino acids were synthesized for high metabolic breakdown of protein and gradually decreased due to metabolism of ammonia and glutamic acid to glutamate.*

Key words: *Ammonia, Glutamine, Glutamic acid, Follicular fluid, Serum*

Introduction

The growing follicle undergoes a transformation from a solid mass of cells enclosing the ovum to a fluid-filled follicle containing a centrally located antrum into which projects a column of cells, the cumulus oophorus, continuous with the membrane granulose. The ovum, surrounded by the corona radiata, sits attached to the cumulus oophorus and is positioned eccentrically within the antrum [1]. Initially small fluid-filled areas, appear among the multiplying granulosa cells, and they become confluent giving rise to a single fluid-filled antrum. The composition of the follicular fluid changes as the follicle matures and the antral volume increases. Young antral follicles contain a primary fluid largely composed of proteoglycans. The proteoglycans become diluted by a gradual influx of fluid derived from the plasma enriched by steroids and minute amounts of proteins synthesized by the theca interna and granulosa cells [2, 3].

Ammonia concentrations in bovine follicular fluid are greatest in small follicles and decrease as follicle diameter increases, indicating a dynamic pattern of ammonia concentration in developing follicles and immature oocytes. Follicular cells develop in a microenvironment that contains concentrations of ammonia greater than those in the microenvironments of most somatic cells [4]. Living organisms excrete the excess nitrogen resulting from the metabolic breakdown of amino acids. Many aquatic animals simply excrete ammonia.

Glutamine can be converted to glutamic acid, and subsequently to α-ketoglutarate, which is an essential intermediate in a variety of metabolic reactions, such as, the synthesis of urea from ammonia via the urea cycle synthesis of amino acids, as well as the synthesis of nitrogenous bases for nucleic acid synthesis. Although, ammonia concentrations have been determined in bovine and human follicular fluids [5, 6], there are as yet no published reports on ammonia and amino acid concentration of porcine follicular fluid. Considering this and also the role of glutamin and glutamic acid in detoxification and utilization of

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ammonia produced during follicular development, the present experiment investigated the change in pH, osmolality, and the concentration of total protein, ammonia, glutamine and glutamic acid in porcine follicular fluid.

Materials and Methods

Sample collection and preparation

A total of 20 ovaries from 10 matured Large White sows (6 months of age) were collected from the local slaughterhouse just after slaughter, and brought to the laboratory within 1–2 hours in chilled 0.9% (w/v) NaCl solution. The ovaries were dissected, freed from other tissues, rinsed thoroughly in 0.9% (w/v) NaCl solution and blotted dry with paper towel. Three categories of follicles (<2, 3–4 and 5–6 mm in diameter) were selected and follicular materials were harvested by aspiration using an 18 gauge needle attached to a 15-ml disposable syringe. Oocytes and all other tissue debris were separated by centrifugation at 800 × g for 10 minutes, and the supernatant was collected as follicular fluid for analysis. Follicular fluid samples were collected from 20 follicles of each category. Blood samples were collected from the same animals, and for comparison with bovine serum, blood samples were collected from 10 Japanese Black cows kept on a farm of Shinshu University. Blood samples were centrifuged at 800 × g for 10 minutes and analyzed as early as possible to determine the pH, osmolality and the concentration of total protein, ammonia, glutamine and glutamic acid.

The pH and osmolality evaluation

The pH and osmolality values of follicular fluid and serum samples were measured by a pH meter (HM-30S, Tokyo TOA Electronics Ltd. Japan) and a Micro Osmometer (Model 3300, Advanced Instruments Inc. USA), respectively.

Protein and ammonia determination

Total protein content was assessed by the Bradford Assay Method [7] with the standard curves, R^2=0.9851, Y=0.0697x ± 0.0519. The method of ammonia determination was based on a two-step reaction. Two milliliters of sodium pentacyanonitrosylferrate (III) dihydrate (Nacalai Tesque Inc. Kyoto. Japan), were added to 10 μl of each sample of porcine follicular fluid and 15 μl each of porcine and bovine serum samples, then 2 ml of sodium hydroxide solution (Wako pure Chemical Industries Ltd. Japan) and sodium hypochlorite solution (Nacalai Tesque Inc. Kyoto, Japan) were added and finally distilled water was added to make the mixture up to 5 ml. The mixtures were vortexed and kept at room temperature for about 30 minutes to maximize blue color production. Following the catalyzed indophenols reaction, the absorbances of the samples were measured and read on a spectrophotometer (Model DU 640; Beckman Instruments Inc., Fullerton, CA, USA) set at 625 nm. The procedure was performed ten times for the analysis. The results were calculated from a standard curve, ranging from 0 to 0.02 × 10^{-3} μg/ml, and converted to micromolar values using a coefficient A=1,000 × (17.03 g) L^{-1}. Standard curves were obtained using regression analysis. The R^2 value was always calculated as a quantity measure and its mean value from 5 standard curves (R^2=0.9997, Y=4.95x ± 0.0005).

Glutamine and glutamic acid determination

To remove the protein content from the follicular fluid and porcine serum samples, an equal volume (1 ml) of methanol was added to the samples, and centrifuged at 2,000 × g for 30 minutes. Then the lower part was filtered, and the filtrates were collected as prepared samples. One hundred microliters of 100 mM phenylisotiociante (acetonitrile solution) and 100 μl of 1 M triethylamine (acetonitrile solution) were added to 200 μl of each sample. The sample solutions were allowed to stand at room temperate for 1 hour, and then 400 μl hexane was added to each solution and vortexed. After separating, the hexane layers were membrane filtered (0.45 μm) and 4 μl of each filtrate sample was injected into a high performance liquid chromatography (Shimadzu Co. Ltd., Kyoto, Japan). To determine the concentration of glutamine and glutamic acid in porcine follicular fluid and serum, STR ODS-II (150 mm × 4.6 m i.d; Shimadzu Co. Ltd., Kyoto, Japan) was used at 40°C temperature. The mobile phases consisted of 10 mM sodium phosphate buffer (pH 7.0) and acetonitrile solution at a rate of 95:5 respectively. The detector was set at 254 nm wavelength for maximum absorption. The results were calculated from a standard curve ranging from 0 to 0.02 × 10^{-3} μg/ml, as described previously. To get average results for the study, this analysis was repeated three times. Standard curves were for glutamine, R^2=0.9997, Y=5.95x ± 0.0003 and glutamic acid, R^2=0.9995, Y=5.99x ± 0.0002.

Statistical analysis

All data are expressed as mean ± SEM. Statistical analysis was performed using the Fisher's protected

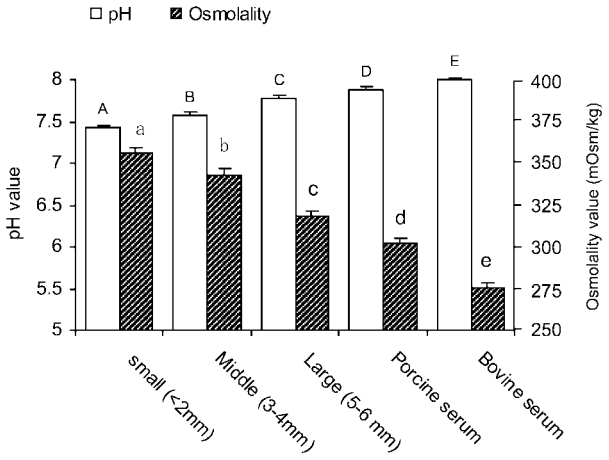

Fig. 1. pH and osmolality values in follicular fluid of different size follicles and serum (porcine & bovine). $^{A–E}$ and $^{a–e}$: Ratios with different letters are significantly different (P<0.05) for pH and osmolality values. Values are the mean ± SEM of ten replicates.

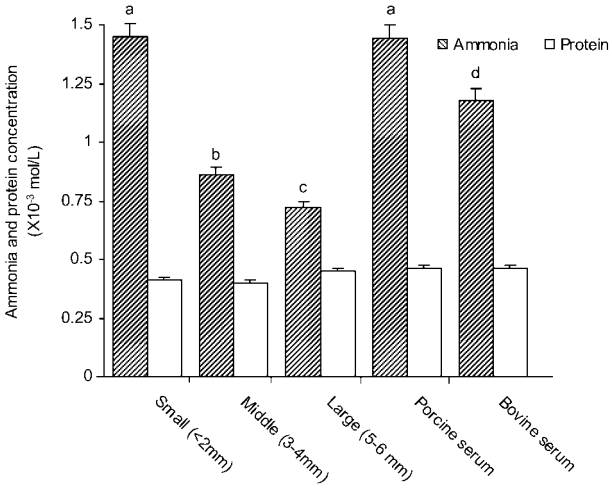

Fig. 2. Ammonia and protein concentration in follicular fluid of different size follicles and serum (porcine & bovine). $^{a–d}$: Ratios with different letters are significantly different (P<0.05). Values are the mean ± SEM of ten replicates.

least significant difference (FPLSD) method. The NCSS (Number Cruncher Statistical System) Version 5.01 computer software package was used for all statistical analyses. Differences were considered significant at P<0.05.

Results

The pH and osmolality values of follicular fluid and serum are shown in Fig. 1. The pH value was lower (P<0.05) but the osmolality was higher in follicular fluid than in serum. The pH value increased and osmolality decreased significantly (P<0.05) as follicular size increased. The concentrations of ammonia and total protein are shown in Fig. 2. The mean value of ammonia concentration in follicular fluid, 1.01 ± 0.11 μM, was significantly (P<0.05) lower than that of serum, 1.44 ± 0.01 μM. It was determined with significantly higher concentration (P<0.001) in the follicular fluid of smaller follicles (<2 mm in diameter) compared with larger follicles (5–6 mm in diameter). Ammonia concentrations in follicular fluid were 1.45 ± 0.04, 0.86 ± 0.02 and 0.72 ± 0.01 μM in small, medium (3–4 mm in diameter) and large follicles, respectively. Ammonia concentration in the follicular fluid decreased significantly (P<0.05) as the follicle size increased, but total protein content did not differ (P>0.05) between follicular fluid, 0.43 ± 0.03 μM, and serum, 0.46 ± 0.01 μM: small follicles, 0.41 ± 0.002 μM; medium follicles, 0.40 ± 0.01 μM; and large follicles, 0.45 ± 0.002 μM.

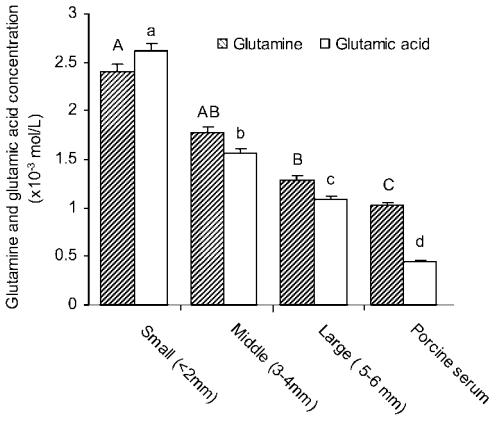

Fig. 3. Glutamine and glutamic acid concentrations in follicular fluid of different size follicles and porcine serum. $^{A–C}$ and $^{a–d}$: Ratios with different letters are significantly different (P<0.05) for glutamine and glutamic acid. Values are the mean ± SEM of three replicates.

The pH, osmolality, total protein content and ammonia concentration was significantly (P<0.05) different between porcine serum and bovine serum.

Glutamine and glutamic acid concentrations in follicular fluid and porcine serum are shown in Fig. 3. The concentrations of both glutamine and glutamic acid in follicular fluid, 2.45 ± 0.35 and 2.66 ± 0.05 μM, respectively, were significantly (P<0.05) higher than

those in porcine serum, 1.02 ± 0.05 and 0.44 ± 0.03 μM. These amino acids were greater ($P<0.001$) in the follicular fluid of smaller follicles than the fluid of larger follicles. Glutamine and glutamic acid concentrations in follicular fluid were 2.41 ± 0.35 and 2.61 ± 0.04 μM in small follicles, 1.78 ± 0.15 and 1.56 ± 0.02 μM in medium follicles and 1.29 ± 0.10 and 1.09 ± 0.02 μM in large follicles, respectively, and their concentrations decreased significantly ($P<0.05$) as follicle size increased.

Discussion

In this experiment, pH increased but osmolality decreased as follicular size increased. The concentrations of ammonia in porcine follicular fluid were found to be greatest in small sized follicles and decreased as follicle diameter increased, indicating a dynamic pattern of ammonia concentration in developing follicles. Hammon *et al.* [5] also observed ammonia concentration was greater in the bovine follicular fluid from smaller follicles compared with larger follicles and its concentration decreased as follicle size increased. Milner [4] stated that immature oocytes and follicular cells develop in a microenvironment which contains ammonia in greater concentrations than those in the microenvironments of most somatic cells. The ammonia concentration in bovine follicular fluid, 366 μM [5], was remarkably higher than that seen in porcine follicular fluid, but in bovine serum, 1.18 μM, it was significantly lower than that seen in porcine serum. The greater amount of ammonia observed in bovine follicular fluid [5] may be produced when bovine consume excess rumen degradable protien (RDP) or feed supplemented with excess urea [8–10]. Comparatively more ammonia is produced in bovine (ruminant) through hydrolysis of urea by urease, secreted from ruminal microflora, but due to the absence of these microflora in porcine (not a ruminant) they are unable to hydrolyse urea to ammonia [8]. Total protein content was almost the same in porcine serum and bovine serum. Whereas elevated systemic concentration of ammonia/ ammonium compound (pka=9.24) or urea in ruminants reduces embryo survivability by disrupting the follicular, oviductal and/or uterine environments [10–13].

In this study, it was observed that both glutamine and glutamic acid in follicular fluid decreased as follicule size increased. During early follicular development, a large amount of protein is needed. In order to synthesize protein, higher metabolic activities occur in oocytes [14]. At that time, amino acids, different nitrogenous substances, precursors of amino acids are accumulated in the follicular fluid from the circulation [8]. Thus, higher concentrations of glutamine and glutamic acid might be found in smaller follicles. Gardner and Lane [15] have proposed that ammonia may mediate its adverse effects through decreasing the concentration of α-ketoglutarate by promoting its conversion to glutamic acid. Glutamic acid may be involved in the fixation of ammonium via its conversion to glutamine, catalyzed by glutamine synthetase [16], as observed during systemic hyper-ammonaemia [17]. Quantitatively, glutamic acid disappearance was affected by ammonia. Glutamic acid decreased with increasing ammonium concentration, providing an alternative source of α-ketocarboxylic acid to that supplied exogenously [8]. The concentration of ammonia, glutamine and glutamic acid decreased as follicle size increased. The lower concentrations of ammonia, glutamine and glutamic acid in larger follicles may be a result of attenuation by the rapid accumulation of fluid in the antrum during the later stages of follicular development, as proposed by Ginther *et al.* and Godsen *et al.* [18, 19]. It is also possible that ammonia uptake by granulosa cells is inhibited by elevated potassium concentrations in the follicular fluid of small follicles [4]. Martinelle and Häggström [20] reported that active intracellular transport of ammonium ions occurs via transport proteins like the Na^+/K^+-ATPase and the Na^+ K^+ $2Cl^-$ co-transporter. Furthermore, they showed that excess potassium ions inhibited intracellular ammonium ion transport due to competitive binding to the transport proteins. There is evidence that potassium concentrations are elevated in follicular fluid from small follicles [19].

In conclusion, ammonia was synthesized due to high metabolic breakdown of protein and nitrogenous substances, while follicles developed from small to large. The oocytes grow in an environment of moderately decreasing ammonia, glutamine and glutamic acid concentrations due to attenuation of ammonia and utilization of these amino acids to increase oocyte mass and produce granulosa cells with increasing follicular fluid.

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