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Cloning of *GJA1* (connexin43) and its expression in canine ovarian follicles throughout the estrous cycle

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Abstract

GJA1 (also known as connexin43 or Cx43) is the most abundant gap junction protein in mammalian tissues including the ovary. Here, it facilitates intercellular communication among granulosa cells and growing oocytes, thereby connecting the developing gamete to the hormonal axis as well as to the essential network of supporting granulosa cells. To date, the pattern of follicular *GJA1* expression has not yet been defined for canines, a species with unique reproductive physiology including delays in follicle development, ovulation, oocyte maturation and fertilization. Here, we report the complete mRNA sequence for canine *GJA1* and identify not only increases ($P < 0.05$) in *GJA1* mRNA expression in follicles at the secondary stage and larger, but also differences in expression levels between estrous cycle stages in both secondary and antral stage follicles. Expression of *GJA1* mRNA in secondary follicles during proestrus was higher than in anestrus or estrus ($P < 0.01$), and at diestrus ($P < 0.10$). Antral follicles obtained during estrus expressed lower levels of *GJA1* mRNA than any other cycle stage ($P < 0.01$). GJA1 mRNA expression in primary and large antral follicles was similar across the estrous cycle. Despite the extensive length of the canine estrous cycle as compared with that of other mammals, the *GJA1* mRNA and protein expression profiles are not significantly different from those reported for other species and suggests that mechanisms regulating *GJA1* transcription are not likely to contribute to the extended delays in follicle and oocyte development in the dog.

Keywords: GJA1; Connexin43; Gap junctions; Canine; Ovarian follicle; Estrous cycle; Oocyte; IVM; *In vitro* maturation

1. Results and discussion

Maturation and ovulation of an oocyte competent to undergo fertilization and embryonic development involves a highly coordinated, interdependent relationship between the oocyte and its surrounding somatic support cells. This dynamic system reflects a fine balance between meiotic arresting factors and others that stimulate cell cycle progression and cytoplasmic maturation secreted by both cell types. Exchange of these factors can be mediated by paracrine, autocrine, and/or gap junctional pathways that allow bi-directional diffusion of regulatory and informational molecules between somatic cells contacting each other and/or the oocyte (Eppig, 1982). Regulation of gap junction intercellular communication (GJIC) within the ovarian follicle prior to ovulation is complex and appears to involve gonadotrophins, steroid hormones, growth factors, second messengers and ions which can affect the gating of these channels (Granot and Dekel, 2002; Bolamba et al., 2002; Bolamba et al., 2003). This mode of intercellular communication begins during primordial follicle formation and

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continues throughout folliculogenesis until just prior to ovulation (Eppig, 1982; Mitchell and Burghardt, 1986; Eppig, 1991; Eppig et al., 1997; Grazul-Bilska et al., 1997; Goodenough et al., 1999; Kidder and Mhawi, 2002).

GJA1 (Connexin43, Cx43) is the most abundant connexin in the mammalian ovary; specifically in the somatic granulosa support cells at all stages of follicle development (primordial to ovulatory) during which time *GJA1* gene expression exhibits a pronounced increase concurrent with follicle growth and maturation beginning at the secondary stage (Grazul-Bilska et al., 1997; Lampe and Lau, 2000; Nuttinck et al., 2000; Perez-Armendariz et al., 2003; Teilmann, 2005). This correlates with cytoplasmic maturation and maintenance of meiotic arrest of the developing oocyte in antral follicles until the preovulatory gonadotropic surge (Goodenough et al., 1999; Nuttinck et al., 2000; Gittens et al., 2003; Perez-Armendariz et al., 2003). Uncoupling of granulosa–oocyte gap junctions just prior to ovulation is thought to occur at the LH surge and initiates meiotic maturation by eliminating exposure to meiotic inhibiting factors, which also signifies the completion of cytoplasmic maturation for most mammalian species. Atretic follicles and late corpora lutea (CL) also have decreased levels of both *GJA1* mRNA and protein indicating diminishing communication between cell types (Mayerhofer and Garfield, 1995; Grazul-Bilska et al., 1998; Nuttinck et al., 2000; Acosta and Miyamoto, 2004). Homozygous mutation of *GJA1* causes early neonatal death due to abnormal cardiac morphology, and severe pathophysiologic anomalies in a number of organs, including reduced germ cell numbers in fetal gonads and disrupted gametogenesis in both males and females (Reaume et al., 1995; Juneja et al., 1999; Gittens et al., 2003).

The process of follicle development and oocyte maturation in canines is poorly understood compared to that in other mammalian species. Primordial follicle development is not completed until ~15–17 days after birth, and follicular growth of primary follicles occurs at ~4–5.5 months of age at sexual maturity (Anderson and Simpson, 1973). Canids exhibit varied lengths for each cycle stage between bitches, have a prolonged inter-estrus phase (~6 months) between fertile periods, ovulate immature oocytes (dictyate stage) ~36–48 h after the LH surge which mature in the oviduct roughly 2 days postovulation, and have functional corpora lutea during diestrus lasting ~60 days (Farstad, 2000). This hallmark in canine reproductive physiology is in sharp contrast to that of most other domestic mammalian species wherein ovulation of mature, metaphase II oocytes normally occurs ~30 h postLH surge. Furthermore, canine oocytes can remain viable for as long as 6–8 days postovulation (Concannon and Verstegen, 2005). Hence, the degree of developmental competence of the canine oocyte prior to or at ovulation is unclear and therefore reproductive technologies including *in vitro* maturation and fertilization are not adequately developed.

There is little information regarding connexin expression or the functional status of gap junctions within canine ovarian follicles and/or cumulus–oocyte complexes (COC). Cx43 protein was identified in cumulus and corona radiata cells of the Blue fox during *in vitro* maturation (IVM) experiments and showed a reduction, but not complete uncoupling of GJIC in corona cells after 72 h IVM (Srsen et al., 1998). This is a dramatic difference compared to *in vivo* matured oocytes of the Blue fox or for IVM bovine oocytes where gap junctions are completely disconnected from the oocyte, suggesting the corona radiata cells may regulate the resumption of meiosis during *in vitro*, but not *in vivo* oocyte maturation in this species (Hyttel et al., 1990). Functional gap junction channels were reported in an experiment using microinjected Lucifer yellow into the oocyte cytoplasm of cumulus–oocyte complexes (COCs) of adult bitches during late proestrus, which diffused into the cumulus cells, whereas no dye transfer was detected into oocytes of bitches in anestrus (Luvoni et al., 2001). This observation suggests a functional difference in GJIC between the two estrous cycle stages that may be hormonally influenced. While these studies provide some insight to GJIC in canine COC *in vitro*, more detailed research is necessary to further characterize gap junctions (i.e., localization, expression, and functionality of specific connexins) in the canine ovary during follicle development. In light of the unusual features of the canine estrous cycle and process of oocyte maturation, it is of interest to characterize the distribution of the most abundant gap junction protein known to be present in ovarian tissues during follicular development and also to discern differences in *GJA1* mRNA expression between several stages of the estrous cycle.

1.1. Sequence of canine Cx43

PCR primers were designed based on mRNA sequence homology between bovine, human and mouse, and used in a reverse transcription polymerase chain reaction (RT-PCR). Sequence analysis of the PCR products resulted in a 1251 base gene sequence (1149 base full length cds) that encodes the 382 amino acids for canine *GJA1*. This sequence is catalogued in GenBank as Accession No. AY462223. Sequence comparisons of the obtained PCR product to those published in GenBank showed 93% homology with bovine, 92% with human and pig, and 87% with mouse cDNA coding sequences. Sequence conservation was strongest in coding regions that define conserved protein domains common to all connexins. Considering that canines are commonly used as subjects for laboratory research including cardiovascular studies, these data provide a canine-specific gene sequence for additional molecular and genetic studies.

1.2. GJA1expression analysis in canine tissues

Canine-specific PCR primers designed against the sequence qualitatively confirmed the presence of *GJA1* transcripts in multiple canine tissues expected to express *GJA1* (Fig. 1). Expression was verified by RT-PCR using methods listed above.

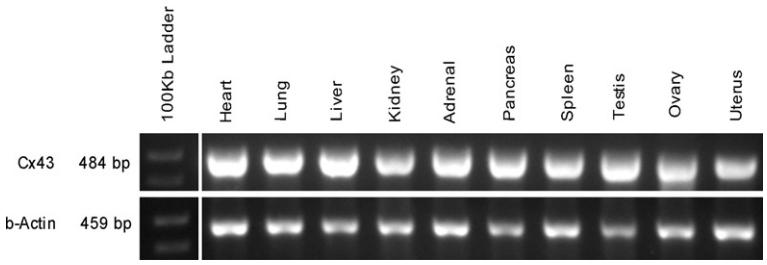

Fig. 1. Qualitative expression analysis of canine *GJA1* in various canine tissue types indicating its presence where expected using bovine β-actin as a positive control.

1.3. Real-time PCR of canine ovarian follicles

GJA1 mRNA expression increased in follicles at the secondary stage and larger ($P < 0.05$) compared to primary follicle expression levels, and differences in expression levels were detected between estrous cycle stages in both secondary and antral follicles (Figs. 2 and 3). Specifically, secondary follicles obtained during proestrus showed higher expression levels than in anestrus or estrus ($P < 0.01$), and at diestrus ($P < 0.10$). Both *GJA1* mRNA expression and protein levels increase in early and mid-phases of corpora lutea of several species, and decrease as progesterone levels taper with luteolysis (Johnson et al., 1999; Grazul-Bilska et al., 1997; Itahana et al., 1996; and Mayerhofer and Garfield, 1995). In the dog, progesterone production from the CL gradually increases prior to ovulation, peaks ~15–30 days postovulation, followed by a gradual decline that continues for 5–6 weeks. Therefore, the statistical finding of $P < 0.10$ for secondary follicles obtained during diestrus is representative of a trend with statistical significance. Antral follicles obtained during estrus expressed lower ($P < 0.01$) levels of *GJA1* mRNA than any other cycle stage. No significant differences were detected among estrous cycle stages for either primary or large antral follicles. The pattern of expression is also consistent with the estrogen profile throughout the cycle in that *GJA1* mRNA expression increases as estrogen increases, and then sharply declines during estrus, as do estrogen levels in the bitch just prior to ovulation (Concannon et al., 1989). Nonetheless, these data are consistent with those reported for the mRNA expression and protein localization patterns in other species (rat, pig, cow, mouse, and sheep) wherein the increase in GJA1 protein and mRNA levels are concomitant with follicular growth in follicles beyond the secondary stage, and believed to be a key developmental turning point in oocyte cytoplasmic development and maturation (Wiesen and Midgley, 1993, 1994; Itahana et al., 1996; Grazul-Bilska et al., 1998; Johnson et al., 1999; Nuttinck et al., 2000; Melton et al., 2001; Wright et al., 2001; Granot et al., 2002; Teilmann, 2005). Furthermore, these data represent the first report of *GJA1* mRNA expression within the canine ovary.

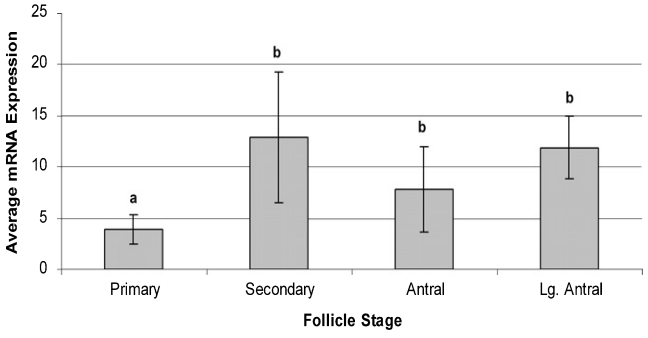

Fig. 2. Average expression of canine *GJA1* mRNA in primary, secondary, antral and large antral ovarian follicles of adult bitches regardless of estrous cycle stage. Error bars represent means ± standard deviation.

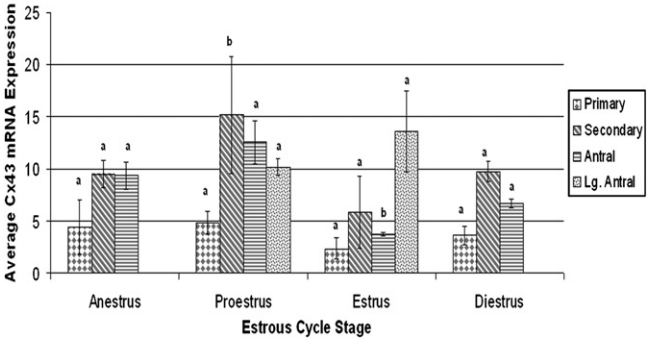

Fig. 3. Average expression of canine *GJA1* mRNA in primary, secondary, antral and large antral ovarian follicles of adult bitches within each estrous cycle stage. Error bars represent means ± standard deviation.

Due to the variability in length of each estrous cycle stage between bitches and the availability of ovaries obtained from local veterinary clinics, it was impossible to consistently collect tissues from animals on the exact day of any given cycle stage. However, because each cycle stage is lengthy compared to most other mammals, and can be characterized both morphologically and/or cytologically, classification for each cycle stage is relatively straightforward. Therefore, it should be noted that the degree of variability within each estrous cycle stage is unknown with respect to a specific collection day for any given cycle stage. Furthermore, considering that these experiments utilized donated tissues from local veterinary clinics and not laboratory animals in a controlled environment, it was not possible to control the breed type or estrous cycle stage of the animals used for this study. Thus, the distribution of animals at any given cycle stage are not equal, but represent the most frequent stage at which bitches are presented for ovariohysterectomy at the facilities used in this study.

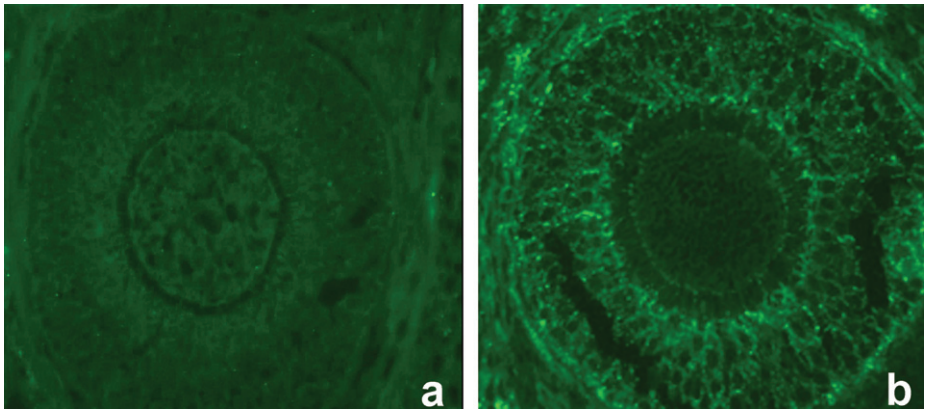

Fig. 4. Immunolocalization of GJA1 protein in frozen sections of a secondary follicle of the canine ovary during estrus. (a) Control sections were labeled with non-specific mouse IgG. (b) Immunfluorescent localization of GJA1 protein using a mouse IgG against rat Cx43. Bright green fluorescence indicates positive staining. The width of each field is 320 μm.

1.4. Protein localization of GJA1 in canine ovarian follicles

Preliminary experiments of qualitative immuno-cytochemical localization of GJA1 protein in secondary follicles in the canine ovary revealed characteristic punctate staining patterns that were localized to the theca, mural granulosa cells and also in the surrounding granulosa-cumulus cell borders. Notably, more intense staining was observed in the corona radiata cells immediately surrounding the oocyte, as well as in *trans*-zonal projections and at the vitelline membrane than in other parts of the follicle (Fig. 4). This observation is consistent with localization patterns reported for other mammalian species, with exception of the abundant staining at the vitelline membrane previously reported in the Blue fox (Grazul-Bilska et al., 1997; Srsen et al., 1998; Lampe and Lau, 2000; Perez-Armendariz et al., 2003). Further studies are required in order to determine if GJA1 protein expressed in the vitelline membrane serves a functional role such as control of meiotic resumption as indicated by Srsen et al., 1998, and also to determine differences among and between follicle size and estrous cycle stage. Regardless, this immuno-cytochemical analysis of GJA1 in the dog suggests that the protein is translated and appears localized in a manner similar to that reported for other mammals (Itahana et al., 1996; Grazul-Bilska et al., 1998; Johnson et al., 1999; Nuttinck et al., 2000; Melton et al., 2001; Wright et al., 2001; Granot et al., 2002; Teilmann, 2005).

2. Experimental procedures

2.1. Sequence of canine GJA1

Total RNA was isolated from canine adult uterus previously snap frozen in liquid nitrogen using a Dounce homogenizer (Kontes, Vineland, NJ) and the Trizol (Gibco, Carlsbad, CA) reagent, and then subjected to semi-quantitative RT-PCR to confirm *GJA1* mRNA expression. Briefly, primers were designed against conserved regions of the bovine and human *GJA1* mRNA (GenBank Accession Nos. J05535 and NM000165, respectively) coding sequences and the resulting PCR products were gel purified using the QIAEX II Agarose Gel Extraction system (Qiagen, Valencia, CA), and the PCR products were sequenced using an ABI 377XL Genetic Analyzer at the Texas A&M Core Sequencing Facility.

2.2. Tissue collection, RNA isolation, and RT-PCR for expression analysis

Canine tissues from major organs (heart, lung, liver, kidney, adrenal, pancreas, and spleen) were harvested from laboratory research animals at euthanasia ($n = 2$) through a university tissue-sharing program using protocols approved by the Texas A&M University Institutional Animal Care and Use Committee. Reproductive tissues were harvested from local veterinary clinics during routine sterilization ($n = 6$ animals) that would have otherwise been discarded. All tissues were immediately snap frozen in liquid nitrogen upon collection, and stored at −80 °C until use. A Dounce homogenizer (Kontes, Vineland, NJ) was used for tissue disruption. RNA was isolated from each tissue using the RNA-STAT-60 system (Tel-Test Inc., Friendswood, TX), and 5 μg of total RNA seeded into a reverse transcription reaction utilizing the Enhanced Avian Reverse Transcriptase system (Sigma–Aldrich, St. Louis, MO). A 100 ng sample of cDNA was subjected to PCR amplification using the Clontech Advantage GC2 PCR kit (BD Biosciences, Palo Alto, CA) with primers designed against the canine *GJA1* coding sequence (GenBank AY462223). The primer pair used was 5′ GAGCACGGCAAGGTGAAAAT and 3′ CAGTTTGC TCACTTGCTTG, producing a 484 bp PCR product. A separate reaction amplifying β-actin was used as a positive control. PCR products were resolved on a 2% agarose gel, stained with ethidium bromide and imaged using Alphaimager 2000 software.

2.3. Ovary tissue collection and follicle isolation

Reproductive tracts from normal, client-owned bitches aged 6 months to 5 years of various breeds or breed crosses were collected after routine ovariohysterectomy at private veterinary clinics and transported to the laboratory at 4 °C in physiological saline solution (PSS) supplemented with a 1% solution of 10,000 U penicillin G sodium ml^{-1} and 10,000 μg streptomycin sulphate (P/S) ml^{-1} (Gibco) (Table 1). Both ovaries were removed from the reproductive tract, washed free from blood in fresh PSS, and estrous cycle stage was determined according to ovarian morphology as previously described (Hewitt, 1997). Intact ovarian follicles (primary, secondary, antral, and large antral; described below) were collected by enzymatic tissue dissociation in a mixture of 5 mg/ml Collagenase Type I (Sigma–Aldrich, St. Louis, MO) and 1.0 mg/ml Dispase II (Roche Biochemical, Indianapolis, IN) diluted in TL Hepes medium supplemented with 3 mg/ml PVA for 1–2 h at 37 °C and then briefly vortexed to dislodge surrounding ovarian tissue. The enzymatic reaction was stopped by the

Table 1
Representation of the number of animals used and the number of pools used in real-time PCR analysis of *GJA1*

Stage	Primary		Secondary		Antral		Large antral	
	Animals	Pools	Animals	Pools	Animals	Pools	Animals	Pools
Anestrus	2	2	5	4	2	2	0	0
Proestrus	5	3	16	9	9	3	6	2
Estrus	2	2	4	4	2	2	2	2
Diestrus	8	4	18	8	9	4	0	0

addition of two volumes of fresh TL Hepes medium and the digestion mixture transferred to a sterile, plastic grid dish for searching. Whole follicles were gently pipetted to further remove residual ovarian and thecal tissue to reveal an intact, basement membrane that encased the entire follicular contents (mural granulosa, cumulus granulosa, and oocyte). Follicles were subsequently washed three times in fresh PSS, and separated according to follicle stage. Briefly, follicles were considered primary when diameter is ~35 μm > 100 μm and oocyte surrounded by one layer of cuboidal granulosa cells; secondary when diameter is ~100 μm > 800 μm, the oocyte is surrounded by two or more layers of granulosa cells, and may include early antrum formation; antral follicles when diameter is 800 μm > 2 mm and oocyte diameter is ~100 μm; and large antral (tertiary) follicles when diameter is >2 mm and oocyte is >100 μm. Poly-ovular follicles were excluded from this study. Follicles were pooled on day of collection according to size and stage, and consisted of at least 10 follicles in any given pool. Follicles were then lysed in ~25 μl of guanidinium thiocyanate (GITC) with 1% BME (β-mercaptoethanol) for 20 min on ice and stored at −80 °C until assayed.

2.4. RNA isolation, reverse transcription and real-time PCR of canine ovarian follicles

Total RNA was isolated from canine ovarian follicles using the Trizol extraction method (Invitrogen, Carlsbad, CA), DNAseI treated, and reverse transcribed using 1 μg total RNA from each sample with the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). In cases where only two animals are represented for a given follicle size or stage, samples were collected on separate dates and analyzed individually (not pooled) to account for an independent run.

Canine-specific primers were designed based on the canine nucleotide sequence for *GJA1* (AY462223), and also for canine β-actin (AF021873) for normalization. The canine primer sequence used was 5′ AGAAAGA GGAGGAGCTCAAAGTTG and the 3′ sequence was TTCAATCTG CTTCAACTGCATGT producing a 71 bp amplicon. The β-actin primer sequence was 5′ TCACGGAGCGTGGCTACAG and the 3′ sequence was TCCTTGATGTCACGCACGAT producing a 64 bp amplicon. Reactions were prepared using 24 μl of a master mix (comprised of 12.5 μl of 2× SYBR Green Master Mix (Applied Biosystems, Foster City, CA), 1.25 μl each of a 1:10 dilution of each 100 μM forward and reverse primer, and 9 μl of RNAse free water) for each sample analyzed, and 100ng of sample RNA. Each sample was run in duplicate for both target genes on the same plate to measure gene expression using the ABI7900 sequence detection system (Applied Biosystems, Foster City, CA).

2.5. Protein localization of Cx43 in canine ovarian follicles

Canine ovaries were collected from local veterinary clinics at routine ovariohysterectomy ($n = 3$ anestrus; 1 proestrus; 2 estrus; 2 diestrus). Ovaries were removed from the reproductive tract, washed free from blood in physiologic saline solution, categorized according to estrous cycle stage as described by Hewitt (1997), dissected into ~5 mm sections, embedded in O.C.T Tissue-Tek and snap frozen in liquid nitrogen. All samples were stored at −80 °C until sectioning for immunochemistry.

Frozen sections (8 μm) of ovarian tissue were cut with a cryotome and mounted on Superfrost/Plus microscope slides (Fisher Scientific, Pittsburgh, PA). Sections were fixed in −20 °C methanol for 10 min, permeabilized with 0.3% Tween-20 in 0.02 M PBS, and then blocked in antibody dilution buffer (2 parts of 0.02 M PBS, 1.0% BSA, 0.3% Tween-20 [pH 8.0] and one part glycerol) containing 5% normal goat serum for 1 h at room temperature. Sections were rinsed in PBS and incubated overnight at 4 °C with 2.5 μg/ml mouse IgG against rat Cx43 (Zymed, San Francisco, CA). Following three rinses in PBS for 10 min each, sections were incubated with fluorescein-conjugated goat anti-mouse IgG (Zymed, San Francisco, CA) for 1 h at room temperature and again washed in PBS three times for 10 min each. Control sections were processed in the same way with the exception that 2.5 μg/ml of purified, non-specific mouse IgG was used in place of IgG generated against rat Cx43.

Sections were then overlaid with a coverslip and an Prolong antifade mounting reagent (Molecular Probes, Eugene, OR), viewed with a Ziess Axioplan2 microscope (Carl Zeiss Inc., Thornwood, NY) fitted with a Hamamatsu chilled 3CCD color camera (Hamamatsu Corporation, Bridgewater, NJ). Digital images were captured using Adobe Photoshop 5.0 (Adobe Systems, Seattle, WA) and Apple PowerMac G3 computer (Apple Computer, Cupertino,CA).

2.6. Statistical analysis

For real-time PCR, the ct values for *GJA1* were normalized against those for β-actin, averaged among duplicate runs, and converted to expression level using Microsoft excel 2000. Analysis of variance (ANOVA) was employed to statistically compare expression levels between estrous cycle stages and follicle sizes, and significant differences were further evaluated using the Tukey/Kramer post hoc test for multiple comparisons using StatView 5.0.1. Protein localization of GJA1 was a qualitative analysis.

References

Acosta, T.J., Miyamoto, A., 2004. Vascular control of ovarian function: ovulation, corpus luteum formation and regression. Anim. Reprod. Sci., 127–140.

Anderson, A.C., Simpson, M.E., 1973. The Ovary and Reproductive Cycle of the Dog (Beagle). Geron-X, Inc., Los Altos, CA.

Bolamba, D., Floyd, A.A., McGlone, J.J., Lee, V.H., 2002. Epidermal growth factor enhances expression of connexin 43 protein in cultured porcine preantral follicles. Biol. Reprod. 67, 154–160.

Bolamba, D., Patino, R., Yoshizaki, G., Thomas, P., 2003. Changes in homologous and heterologous gap junction contacts during maturation-inducing hormone-dependent meiotic resumption in ovarian follicles of Atlantic croaker. Gen. Comp. Endocrinol. 131 (3), 291–295.

Concannon P.W., Verstegen J., 2005. Some unique aspects of canine and feline female reproduction important in veterinary practice. Proc World Sm. Anim. Vet. Assoc.

Concannon, P.W., McCann, J.P., Temple, M., 1989. Biology and endocrinology of ovulation, pregnancy and parturition in the dog. J. Reprod. Fertil. Suppl. 39, 3–25.

Eppig, J.J., 1982. The relationship between cumulus cell-oocyte coupling, oocyte meiotic maturation, and cumulus expansion. Dev. Biol. 89, 268–272.

Eppig, J.J., 1991. Intercommunication between mammalian oocytes and companion somatic cells. BioEssays 13 (11), 569–574.

Eppig, J.J., Chesnel, F., Hirao, Y., O'Brien, M.J., Pendola, F.L., Watanabe, S., Wigglesworth, K., 1997. Oocyte control of granulosa cell development: how and why. Hum. Reprod. 2 (2), 127–132.

Farstad, W., 2000. Assisted reproductive technology in canid species. Theriogenology 53 (1), 175–186.

Gittens, J.E.I., Mhawi, A.A., Lidington, D., Ouellette, Y., Kidder, G.M., 2003. Functional analysis of gap junctions in ovarian granulosa cells: distinct role for connexin 43 in early stages of folliculogenesis. Am. J. Physiol., Cell. Physiol. 284, C880–C887.

Goodenough D.A., Simon A.M., Paul D.L., 1999. Gap junctional intercellular communication in the mouse ovarian follicle. Wiley, Chichester (Novartis Foundation Symposium 219), pp. 226–240.

Granot, I., Dekel, N., 2002. The ovarian gap junction protein connexin43:regulation by gonadotrpins. Trends Endocrinol. Metab. 13 (7), 310–313.

Granot, I., Bechor, E., Barash, A., Dekel, N., 2002. Connexin43 in rat oocytes: developmental modulation of its phosphorylation. Biol. Reprod. 66, 568–573.

Grazul-Bilska, A.T., Reynolds, L.P., Redmer, D.A., 1997. Gap junctions in the ovaries. Biol. Reprod. 57, 947–957.

Grazul-Bilska, A.T., Redmer, D.A., Bilski, J.J., Jablonka-Shariff, A., Doraiswamy, V., Reynolds, L.P., 1998. Gap junctional proteins connexin 26, 32, and 43 in sheep ovaries throughout the estrous cycle. Endocrine 8 (3), 269–279.

Hewitt D.A., 1997. Oocyte maturation and fertilization in the bitch; the use of *in vitro* culture. A Ph.D. Thesis, The Royal Veterinary College, University of London.

Hyttel, P., Farstad, W., Mondain-Monval, M., Bakke Lajord, K., Smith, A.J., 1990. Structural aspects of oocyte maturation in the blue fox (*Alopex lagopus*). Anat. Embryol. 181, 325–331.

Itahana, K., Morikazu, Y., Takeya, T., 1996. Differential expression of four connexin genes, Cx-26, Cx-30.3, Cx-32, and Cx-43, in the porcine ovarian follicle. Endocrinology 137 (11), 5036–5044.

Johnson, M.L., Redmer, D.A., Reynolds, L.P., Grazul-Bilska, A.T., 1999. Expression of gap junctional proteins connexin 43, 32, and 26 throughout follicular development and atresia in cows. Endocrine 10 (1), 43–51.

Juneja, S.C., Barr, K.J., Enders, G.C., Kidder, G.M., 1999. Defects in the germ line and gonads of mice lacking connexin 43. Biol. Reprod. 60, 1263–1270.

Kidder, G.M., Mhawi, A.A., 2002. Gap junctions and ovarian folliculogenesis. Reproduction 123, 613–620.

Lampe, P.D., Lau, A.F., 2000. Regulation of gap junctions by phosphorylation of connexins. Arch. Biochem. Biophys. 384 (2), 205–215.

Luvoni, G.C., Luciano, A.M., Modina, S., Gandolfi, F., 2001. Influence of different stages of the oestrous cycle on cumulus-oocyte communications in canine oocytes: effects on the efficiency of *in vitro* maturation. J. Reprod. Fertil. Suppl. 57, 141–146.

Mayerhofer, A., Garfield, R.E., 1995. Immunocytochemical analysis of the expression of gap junction protein connexin 43 in the rat ovary. Mol. Reprod. Dev. 41, 331–338.

Melton, C.M., Zaunbrecher, G.M., Yoshizaki, G., Patino, R., Whisnant, S., Rendon, A., Lee, V.L., 2001. Expression of connexin43 mRNA and protein in developing follicles of prepubertal porcine ovaries. Comp. Biochem. Physiol. B 130, 43–55.

Mitchell, P.A., Burghardt, R.C., 1986. The ontogeny of nexuses (gap junctions) in the ovary of the fetal mouse. Anat. Rec. 214 (3), 283–288.

Nuttinck, F., Peynot, N., Humblot, P., Massip, A., Dessy, F., Flechon, J.E., 2000. Comparative immunohistochemical distribution of connexin 37 and connexin 43 throughout folliculogenesis in the bovine ovary. Mol. Reprod. Dev. 57, 60–66.

Perez-Armendariz, E.M., Saez, J.C., Bravo-Moreno, J.F., Lopez-Olmo, V., Enders, G.C., Villalpando, I., 2003. Connexin43 is expressed in mouse fetal ovary. Anat. Rec. 271A (2), 360–367.

Reaume, A.G., DeSousa, P.A., Kulkarni, S., Langille, B.L., Zhu, D., Davies, T.C., Juneja, S.C., Kidder, G.M., Roussant, J., 1995. Cardiac malformation in neonatal mice lacking connexin 43. Science 267, 1831–1834.

Srsen, V., Kalous, J., Nagyova, E., Sutovsky, P., King, W.A., Motlik, J., 1998. Effects of follicle-stimulating hormone, bovine somatotrophin and okadaic acid on cumulus expansion and nuclear maturation of Blue fox (*Alopex lagopus*) oocytes *in vitro*. Zygote 6, 299–309.

Teilmann, S.C., 2005. Differential expression and localization of connexin-37 and connexin-43 in follicles of different stages in the 4-week old mouse ovary. Mol. Cell. Endocrinol. 234, 27–35.

Wiesen, J.F., Midgley Jr., A.R., 1993. Changes in expression of connexin 43 gap junction messenger ribonucleic acid and protein during ovarian follicular growth. Endocrinology 133 (2), 741–746.

Wiesen, J.F., Midgley Jr., A.R., 1994. Expression of connexin43 gap junction messenger ribonucleic acid and protein during follicular atresia. Biol. Reprod. 50, 336–348.

Wright, C.S., Becker, D.L., Lin, J.S., Warner, A.E., Hardey, K., 2001. Stage-specific and differential expression of gap junctions in the mouse ovary: connexin-specific roles in follicular regulation. Reproduction 121, 77–88.