Characterization of follicles in girls and young women with Turner syndrome who underwent ovarian tissue cryopreservation

Running title: Ovarian follicles and Turner syndrome

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Capsule: Ovarian tissue cryopreservation (OTC) may be considered in girls and young women with Turner syndrome, however the benefit may be limited to a highly selected group of TS mosaic patients.
Abstract

Objective: To characterize ovarian follicles of girls and young women with Turner syndrome (TS) who underwent ovarian tissue cryopreservation (OTC).

Design: Retrospective case-control study.

Setting: University hospital.

Patients/Animals: 15 girls and young women with TS aged 5-22 years at OTC were included together with 42 control girls and young women aged 1-25 years who underwent OTC due to cancer.

Intervention(s): None

Main Outcome Measure(s): Follicle density (follicles/mm³), morphology and health were assessed in ovarian cortex biopsies from TS patients and compared to control ovaries. Hormone concentrations were measured in blood samples and follicle fluid samples. Immature cumulus oocyte complexes (COCs) were obtained and matured in vitro.

Results: Follicles were found in 60% (9/15) of the biopsies from TS ovaries. In 78% (7/9) of the ovaries with follicles, the follicle density was within the 95% CI of the control group. There was a high rate of abnormal follicle morphology. 6 follicle specific proteins were expressed similarly in TS and control ovaries. However, markers of apoptosis and zona pellucida were found to be abnormal in TS. TS follicle fluid from small antral follicles had lower concentrations of estrogen and testosterone and higher concentrations of AMH than controls (p=0.036, 0.001, 0.005, respectively). 31 COCs were collected from one patient and cultured for 48 hours in vitro, resulting in 5 MII oocytes (maturation rate 16%, degeneration rate 19%).

Conclusion: The benefits of OTC may be limited to a highly selected group of TS mosaic patients in whom a sizeable pool of normal follicles is present at OTC.

Keywords: Turner syndrome, follicle density, ovarian tissue cryopreservation
**Introduction**

Turner syndrome (TS) is caused by the absence of one of the two X chromosomes in all cells or a proportion of cells, affecting approximately 1:2000 Caucasian girls (1). The most common karyotype is 45,X (47%), followed by different mosaicism, most commonly 45,X/46,XX (17%). The main reproductive effect of TS is Primary Ovarian Insufficiency (POI) (2,3). Although menarche occurs spontaneously in 15-30% of TS girls, the prevalence of natural pregnancy is as little as 2-7% (2–6).

Two previous studies have reported the presence of ovarian follicles in 15/47 (7) and in 8/9 (8) adolescent TS patients. Mosaicism and spontaneous menarche were predictive for the presence of follicles, consistent with pregnancy being most prevalent in women with mosaic TS (2,5), and ovarian tissue cryopreservation (OTC) has been suggested as an option for fertility preservation (7). Predictors for the presence of ovaries without follicles in TS girls include karyotype 45,X, low serum AMH, high serum FSH, and absent menarche or puberty (7,8). Oocyte donation is the only way for TS patients with POI to conceive, but pregnancies carry a substantial risk to mother and fetus (6,9,10).

The aim of this study was to characterize the number and morphology of follicles in girls and young women with TS, who underwent OTC. We have also evaluated follicle and oocyte function to assess the potential for future fertility restoration in order to evaluate whether or not to perform OTC in this group of patients.

**Material and Methods**

*Patients and ovarian tissue cryopreservation*

A total of 15 girls and young women with TS aged 5.0-22.4 years (mean age 15.4 years) were included together with 42 control girls and young women aged 1.5-25.5 years (mean age 15.2 years). The control group were referred to the Danish program for fertility preservation by OTC because of a cancer diagnosis and have not revived any gonadotoxic treatment before OTC. Patients from both groups were only included if an ovarian cortex biopsy was spared for histology in connection with OTC. All patients underwent OTC between the years 2002 and 2016. Follicle densities in the control girls with cancer below the age of 18 years has previously been published (11). One additional ovarian biopsy from a girl with Fanconi anemia was included for immunohistochemical (IHC) staining. During the preparation of ovarian tissue, two patients presented with visible antral follicles on the ovarian surface from which follicle
fluid from a total of 8 small antral follicles was collected. In one of these patients additionally a total of 31 cumulus oocyte complexes (COCs) were obtained from the medulla tissue. Patient characteristics are given in Table 1; there were 7 in the Danish cohort, 5 from UK and 3 from Australia. All controls were age matched patients having OTC for conditions other than TS in Denmark. The ovarian cortex was prepared as previously described for slow-freezing (12,13) and stored in liquid nitrogen. Additionally, one small piece of cortex (≤ 2x2x1 mm) was obtained for histological examination. The OTC schemes were approved by the ethics committee of Copenhagen and Frederiksberg (H-2-2001-044) and Lothian Health (ref 06/S1103/26). The storage and collection of patient data were approved by the Ministry of Health (J. no. 30-1372) and by the Danish authorities to comply with European Union tissue directives. All participants, or parents for younger patients, gave informed consent in writing.

**Tissue processing**

Tissues from Edinburgh (subjects 1, 3, 9, 13, and 14) were fixed in 10% neutral buffered formalin (NBF); tissues from Melbourne (subjects 7, 8, and 11) were fixed in 4% paraformaldehyde, whereas the remaining tissues from Copenhagen were fixed in Bouin’s solution. In Edinburgh and Melbourne 5 or 6 µm sections of paraffin embedded human ovarian cortex were prepared, de-waxed and stained with haematoxylin and eosin for estimation of follicle density (14). In Copenhagen, 30 µm sections were stained with periodic-acid Schiff and Mayer’s reagents for further estimation of follicle density, 5 µm sections were processed for IHC staining.

**Immunohistochemical staining**

Sections were de-paraffinated in xylene, rehydrated in ethanol followed by antigen retrieval in either 10 mM sodium citrate, pH 6 or 10 mM Tris, pH 9. Retrieval was not required for zona pellucida protein 1 and 2 (ZP 1 and ZP 2) (15). Endogenous activity was inhibited using 1.5% peroxidase, followed by inhibition of nonspecific binding with 1% bovine serum albumin (BSA) (Sigma Aldrich, Copenhagen, Denmark). Sections were incubated with primary antibodies overnight at 4ºC except for ZP protein antibodies, which were incubated at 37ºC for 1 hour; details of antibodies and conditions are given in Supp. Table 1. Secondary antibody used was rabbit-anti-mouse-HRP (Dako, Glostrup, Denmark) and visualised with 3,3′-diaminobenzidine tetrahydrochloride (DAB+ Substrate Chromogen System, Dako). Both Universal negative control serum® (BioCare Medical, CA, USA) and antibody dilution buffer
was used in place of primary antibody as negative controls and showed no staining (Supp. Fig. 1). An Apop Tag Plus Peroxidase In Situ Apoptosis Detection Kit (Millipore, North Ryde, Australia) detecting apoptosis terminal deoxynucleotidyl transferase mediated dUTP Nick End Labeling (TUNEL) was also included.

Follicle density

Two methods were used to estimate the non-growing follicle density in the ovarian cortex. In Copenhagen, the follicle density was estimated in 30 µm section using a mathematical model described by Schmidt and colleagues (16). In brief, this model was based on the fraction of sections, the mean primordial follicle diameter, and a correction factor (α) to account for the possibility of counting the same follicle more than once. Since the mean diameter of a primordial follicle is 44 µm (17) and the sections were 30 µm, there was a possibility to count the same follicle two or three times (16). In Edinburgh and Melbourne follicle density was measured in 5 µm sections by McLaughlin and colleagues (14). In brief, all tissues sections were examined for the presence of follicles. To avoid overcounting, follicles were only assessed when the nucleolus was observed. The follicle density was determined by dividing the total number of follicles in the biopsy by the volume of tissue analyzed. To evaluate if the two methods of data collection were comparable a predictive model was used (14), which combine an age-related normative model for follicle population in the human ovary (18) and an age-related normative model for the volume of the human ovary (19). Comparison of data obtained by the two methods used shows good agreement using the predictive model (14).

Hormone assays

Follicle fluid was aspirated using a 29 gauge syringe from small antral follicles (< 7.0 mm in diameter) during preparation of ovarian tissue for cryopreservation. Estradiol, testosterone, AMH, and inhibin-B concentrations were measured in follicle fluid (after appropriate dilution) using commercially available enzyme-linked immunosorbent assay (ELISA) kits. Estradiol and testosterone were measured with Nova Tech ELISA assays (DNOV003, DNOV002, Aalborg, Denmark), AMH and inhibin-B were measured with the UltraSensitive AMH/MIS and inhibin-B ELISA kit (AL-105, AL-107, Ansh Labs, Webster, TX, USA) (20).

Western blot
Western blot analyses were performed according to the manufacturer’s instructions (Invitrogen provided by Thermo Fischer, Hvidovre, Denmark). In brief, follicle fluid proteins were separated on a NuPAGE 4-12% Bis-Tris mini gel, the proteins were subsequently blotted to a PVDF membrane (Thermo Fischer). The membrane was blocked in 5% skimmed milk and incubated with primary AMH antibody (AMH 39/48, Ansh Labs, TX, USA) overnight at 4°C and subsequently with secondary horseradish peroxidase-conjugated goat-anti-mouse antibody (Sigma Aldrich, Brøndby, Denmark) for 1 hour at room temperature. Signal was detected with Pierce™ SuperSignal West Femto Substrate (Termo Fisher) and visualized with the DNR MicroChemi 4.2 bio-imaging system. We have previously validated the specificity of the AMH antibody used by blocking with surplus recombinant AMH, whereafter all AMH related bands disappeared (21).

*In vitro maturation of COCs*

Immature COCs from small antral follicles were collected from the surplus medulla tissue from one patient with mosaic TS and cultured in IVM medium (Origio, Måløv, Denmark) supplemented with FSH and LH and overlaid with liquid paraffin at 37°C in 5% CO₂ humidified incubator for 48 hours as previously described (22). Cumulus cells were removed after 48 hours and the developmental stage of the denuded oocytes was evaluated using an inverted microscope and classified as either: ‘GV’, with a distinct germinal vesicle; ‘MI’, no germinal vesicle and no polar body; ‘MII’, one polar body extruded.

*Statistics*

Data on follicle density from the Danish girls below the age of 18 years have previously been published (11). An adjusted model including the new data has more normally distributed residuals (84% of a perfect Gaussian distribution), and hence is expected to have lower generalization error when assessing densities from subjects not used to derive the model. Statistical analysis was performed using GraphPad Prism 6.07 program (GraphPad Software, Inc., CA, USA) and Microsoft Excel version 14.6, with linear regression to evaluate follicle density against age and Mann-Whitney U-test to compare the hormone concentrations in follicular fluid. Significance was defined as p< 0.05.
Results

A total of 15 girls and young women with TS aged 5.0-22.4 years old were included in this study. Three cases were postpubertal (i.e. >18 years old). Moreover, 11 of the 15 cases were diagnosed with mosaic TS.

Follicle density

Follicles were found in 60% (9/15) of the biopsies from TS ovaries - 8 girls with a mosaic karyotype and one 45,X, who was aged only 5 years and the youngest in the series (Table 1). No follicles were found in 2 patients with menopausal FSH levels (ages 14 and 17), in 2 with mildly elevated FSH levels (aged 17 and 22), nor in a further 2 patients (aged 8 and 14) with undetectable AMH levels. All patients with follicles had a detectable AMH level and/or FSH <10I U/L (except one on whom no hormone data were available).

While follicle density was below age-matched mean for most TS patients, it was within the 95% CI for controls in 78% (7/9) of the cases, in 22% (2/9) it was below the 95% CI (Fig. 1). Including both TS patients with and without follicles (n=15), no correlation between follicle density and age was found (p>0.1). When follicle densities from the present study were combined with previously published TS data including both patients with and without follicles (n=23) (8), no correlation between follicle density and age was found (p>0.1) (Fig. 1). The cortex tissues were fixed in either 4% paraformaldehyde or Bouins solution before hisological examination and theoretically this difference in fixation may impact the follicle densities, however we find this very unlikely.

Follicle morphology and immunohistochemistry (IHC)

Most (6/9) ovaries showed a high rate of abnormal follicle morphology. The follicle morphology of three subjects are illustrated in Fig. 2. The major abnormalities observed in primordial follicles were misshapen, vacuolated oocytes and an incomplete layer of granulosa cells surrounding the oocyte (Fig. 2A) leading to irregular oocyte shape and partial lack of connection to the basal lamina and stromal cells. This also manifested in di-oocytic follicles (Fig. 2B). In many follicles the granulosa cells were swollen and did not have the flattened appearance normally observed in primordial follicles. Nuclear material within some oocytes was diffuse or pale suggesting an absence of the germinal vesicle membrane (Fig. 2A). Empty and degenerating follicles were often seen (Fig. 2A,B,D). Granulosa cell invasion of the oocyte of primary follicles were occasionally seen (Fig. 2E) together with shrunken granulosa cells
and contracted ooplasm (Fig. 2F). In some subjects, normal morphology was detected in the majority of follicles (Fig 2G,H,I), as in control ovarian tissue.

The presence of 6 granulosa cell or oocyte specific proteins (20,23) were detected by IHC in three TS ovaries (subjects 1, 6 and 12) and one age-matched control (Supp. Fig. 2). TUNEL staining (subject 8) showed some areas with healthy follicles (Supp. Fig. 3C), however there were other areas of poor stromal integrity with most follicles having only an occasional healthy granulosa cell and evidence of apoptosis in the oocyte (Supp. Fig. 3D). High levels of ZP 1 and 2 staining were observed within the oocytes and scattered throughout the cortical stromal tissue, which may indicate residual ZP proteins from eliminated follicles and has been observed previously following xenografting of normal ovarian cortex (Gook, unpublished) (Supp. Fig 3A,B). Normal very low levels of ZP 1 expression was detected in 43% (83/193) of the primordial follicles and was elevated in 57% (110/193), which suggests atresia. Normal ZP 2 expression was detected in 23% (45/199) of the follicles. The proportion of morphologically normal follicles was estimated to be 7% from TUNEL staining.

**Hormone concentrations in follicle fluid**

Hormone concentrations were measured in 8 follicles obtained from two girls with mosaic TS (subject 4 and 12): none of the other girls had small antral follicles that could be aspirated. The mean diameter ±SEM of the follicles was 5.0 mm ±0.4 (range: 3.4-6.7 mm) and the mean concentrations in follicle fluids were: estradiol 19 ±9 nmol/L; testosterone 132 ±23 nmol/L; AMH 2,941 ± 587 ng/ml; and inhibin-B 81 ±15 ng/ml, respectively (Supp. Table 2; Fig. 3A). The concentrations of estrogen and testosterone in follicular fluid from girls with TS were significantly lower and AMH higher than the concentrations in follicular fluid from size-matched (3.4-5.9 mm) follicles from age-matched controls (24) (p=0.036, 0.001, 0.005, respectively; Supp. Table 2). No differences in inhibin-B concentrations between TS and normal follicle fluids were found (p>0.1). All 6 follicle fluids from subject 12 was analysed with western blot detecting for AMH and compared to 6 follicle fluids obtained from control size matched follicles and no differences in AMH processing was detected (Fig. 3B).

**In vitro maturation of oocytes**

COCs (n=31) were cultured from one mosaic girl (subject 12). After 48 hours of culture, 6 had degenerated, 13 remain at the germinal vesicle (GV) stage, while 12 had resumed meiosis (7 at MI and 5 at the MII stage), resulting in a maturation rate of 16% and degeneration rate of
Although this outcome is only from one patient, this is a lower maturation rate and higher degeneration rate than that previously reported by our group for COC from young women (<20 years) with normal ovaries (55%, 4% respectively) (22).

**Discussion**

To our knowledge, this study is the first to characterize follicles and explore the IVM potential of oocytes from girls and young women with TS in comparison to age-matched controls. Follicles were detected in the ovarian cortex in 9 of 15 patients with TS, and the presence of follicles was associated with detectable serum AMH and normal FSH levels. Follicle density was within the 95% CI of normal age-matched girls and young women in 7 of these 9 patients. TS patients originated in Denmark, UK, and Australia, whereas all control patients were Danish, and we cannot rule out that the evaluated ovarian parameters would have been different in a cohort originated in UK or Australia, though we find it unlikely. All except one had mosaic TS, confirming observations from Borgström and colleagues (7) and consistent with women with this karyotype having a higher chance of conceiving (2,5,7,9). While follicles were detected in the youngest patient included, a 5-year-old with 45X karyotype, primordial follicle morphology was abnormal, with most follicles having an incomplete granulosa cell layer, oocyte vacuoles or collapse, or absent oocyte (empty follicles).

The diversity of follicle morphology between patients further complicated the prediction of who may benefit from OTC. In some cases, follicle morphology was similar to normal whereas in others, a high proportion and range of abnormalities were seen. This is supported by increased expression of ZP proteins (normally very low in healthy primordial follicles and elevated in atretic follicles (25)) and DNA fragmentation detected in one TS patient. This may indicate limited potential for later fertility in this TS patient. Empty follicles have been observed in human ovarian cortex cultured in the presence of an inhibitor of mTOR (26) and were detected in ovaries analysed in all 3 centres negating any effect of different fixation methods. However, all 6 glycoproteins related to oocyte growth and follicle health that were evaluated were detected, suggesting that at least a proportion of TS follicles may be normal and functional.

From one 18-year-old girl with TS, immature COCs were aspirated and matured in vitro. This demonstrates that TS oocytes can develop to the MII stage and may possess fertility potential. This is consistent with a case study that reported oocyte retrieval and maturation to MII in a
young woman with mosaic TS (27), with 65% of the oocytes obtained having a normal karyotype. While the karyotype was not assessed here, these findings suggest that immature oocytes can be collected from the medulla tissue in connection with OTC and that these oocytes could be an additional source for fertility preservation in mosaic TS, although the maturation rate appeared lower than with oocytes from normal women.

We also identified that hormone concentrations in follicle fluid from small antral follicles from TS ovaries were strikingly different from the concentrations found in size-matched normal follicles, with low concentrations of estradiol and testosterone, and markedly higher AMH. Inhibin-B concentrations appeared normal. The low testosterone concentration may reflect abnormal theca cell function, and may impact follicle development including granulosa cell proliferation, which is reflected in the low estradiol concentrations. AMH is predominantly present during follicle development until follicular selection for dominance (20). There is a strong negative relationship between follicle fluid AMH and estradiol in normal women (28), which appears to be exaggerated in TS. These high AMH concentrations together with very low steroid concentration in TS follicles may reflect abnormal function of the somatic cells of the follicle, and additionally through impairment of the regulation of folliculogenesis, contribute to the accelerated follicle loss in TS confirmed here. Western blot was used to evaluate the processing of AMH in TS follicle fluids and non TS (normal) follicle fluids from size matched follicles. No difference in AMH processing was detected between TS and normal, suggesting that the processing of AMH in TS patients are similar to normal.

Although it is now well recognized that transplanted frozen/thawed ovarian tissue can restore fertility no one has, to our knowledge, transplanted ovarian tissue to a woman with TS, despite OTC being reported (7–9,27,29,30). Thus, it remains to be demonstrated whether transplanted frozen/thawed ovarian tissue from TS girls/adolescents has the capacity to restore fertility. The rationale behind OTC in TS patients is different from other medical indications like chemotherapy. In case of cancer, the ovarian tissue has been exposed to no or a discrete injury before OTC. This contrast with TS ovarian tissue, which itself has a limited life expectancy and auto-grafted TS tissue will therefore have a limited survivability, why the benefit of OTC in TS patients may be limited. Further, maternal risks, including mortality during pregnancy in TS are very high, largely due to cardiovascular risks (9,10), which has to be taken into account when considering auto-grafting in TS patients. The cardiovascular risks may reflect the
underlying connective tissue abnormalities present in TS (31), which may also be relevant to the stromal tissue and somatic cells of the ovary. In vitro maturation and surrogacy may also be considered an option for TS patients. A recent review suggested that TS patients should be evaluated in early childhood to allow them to benefit from fertility preservation options (32), which is important to these patients and their parents (33).

Conclusions

The present analysis showed that even where follicles are present in girls with mosaic TS, many of these follicles may show abnormalities that are likely to limit their potential for later development and to support fertility. However, normal follicles were also present. Therefore, it appears reasonable to consider OTC for fertility preservation as an option for highly selected adolescent patients with mosaic TS where endocrine assessment does not indicate POI, and if other health issues do not preclude pregnancy. Oocytes from TS medulla tissue may also provide an additional fertility option. However, it is important to note that transplantation of frozen/thawed ovarian tissue has not yet been performed in women with TS, and it remains to be seen whether the procedure can restore fertility.

Author’s roles

LSM designed the project, wrote the paper, did IHC staining, figures, and tables. KC wrote the paper, measured follicle density, did IHC staining and tables. RAA wrote the paper, recruited patients, responsible for the cryopreservation of ovarian tissue (UK). EET and MMcL analysed ovarian tissue and measured follicle densities (UK). TWK did the statistical analysis. SGK cryopreserved ovarian tissue and did histological analysis (Denmark). DAG wrote the paper, recruited patients, measured follicle density, cultured oocytes, and did IHC staining, responsible for the cryopreservation of ovarian tissue (Australia). EE recruited patients and did the ovariectomies (Denmark). CYA designed the project and wrote the paper.

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References


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**Figure legends**

**Fig. 1.** Follicle density in normal ovaries (triangles) and in girls with TS (filled circles) plotted against age. Only TS tissues with follicles detected (9/15) are included. Previously published data on follicle density in girls with TS (n=5) are included (8) (open circles).

**Fig. 2.** Ovarian morphology in tissue from 3 girls with TS. (A,B,C) Subject 8. (A) Primordial follicles with missing granulosa cells (arrows), some follicles had diffuse or pale nuclear material suggesting absence of germinal vesicle membrane (arrowheads); (B) fused follicles (arrows); (C) follicles with normal granulosa cells (arrows), scale bar: 100 µm. (D,E,F) Subject 1. (D) Primordial follicles with collapsed oocytes (arrows) and empty follicle (arrowhead); (E) granulosa cell invasion of the oocyte of a primary follicle (arrow); (F) primary to secondary transition, shrunk granulosa cells and contracted ooplasm, scale bar: 50 µm. (G,H,I) Subject 12. (G, H) Normal morphology in the majority of primordial follicles with flat granulosa cells (arrows) and primary/intermediate follicles with cuboidal granulosa cells (arrow heads); (I) tertiary follicle, scale bar: 50 µm.

**Fig. 3.** (A) Concentration of AMH and inhibin-B in follicle fluids (FF) (red circles) obtained from two girls with TS aged 13.5 and 17.8 years (subject 4 and 12) and in age-matched controls (grey triangles). AMH concentrations in these TS follicles are extremely high, whereas the inhibin-B concentration is similar to age-matched controls. (B) Detection of AMH in AMH standard (Std.) (lane 1), FF from TS patient subject 12 (lane 2-7), and in size matched follicles from different normal women (lane 8-13). Lane 7 was loaded with less FF than the remaining because the sample was used up, which explain the weaker/no bands seen. Arrows indicate AMH cleavage fragments. The blots show no difference in the composition of AMH forms in TS and normal FF.

**Supp. Fig. 1.** Negative controls. (A,B) TS Mosaic, 14.4 years; (C,D) TS Mosaic, 17.8 years; (E,F) Control, 10.5 years. (A,C,E) Primary antibody preplaced with antibody dilution buffer (1% BSA in PBS). (B,D,F) Primary antibody preplaced with Universal Negative Control serum®.

**Supp. Fig. 2.** Expression of 6 proteins important for follicular growth: pro-region of Anti-Müllerian hormone (proAMH), growth/differentiation factor 9 (GDF9), bone morphogenetic protein 15 (BMP15), insulin-like growth factor-binding protein 4 (IGF BPB4), pregnancy-
associated plasma protein A (PAPP-A), stanniocalcin 2 (STC2) in ovarian cortical tissue from three TS patients aged 5.0, 14.4 and 17.8 years and one control aged 10.5 years.

**Supp Fig. 3.** Expression of zona pellucida proteins (ZP 1 and ZP 2) and apoptotic marker (TUNEL) in a TS ovary aged 14.8 years (subject 8). (A) ZP 1 protein was detected in a pattern resembling normal follicles (arrows) and in some follicles aberrant staining was observed (arrowhead). (B) Low expression of ZP 2 was observed in healthy looking primordial follicles (arrows), while an apparent increased staining was observed in other follicles. (C) Stromal cells, oocytes (arrows) and granulosa cells in this area were predominantly TUNEL negative (green staining), though TUNEL was observed in some granulosa cells (arrowhead). (D) Single stranded DNA (TUNEL positive) was in other areas observed in some oocytes and granulosa cells (arrows). Cells of the stromal tissue also had evidence of single strand DNA (weak brown staining). Dotted boxes indicate enlarged areas. Scale bar: 100 µm and 50 µm on magnifications.