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1 **Development of molecular markers for zebrafish (*Danio rerio*) ovarian follicle growth**
2 **assessment following *in-vitro* culture in cryopreservation studies**

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13

14 **Abstract**

15 Development of *in vitro* culture protocol for early stage ovarian follicles of zebrafish is
16 important since cryopreserved early stage ovarian follicles would need to be matured *in vitro*
17 following cryopreservation before they can be fertilised. Development of molecular markers
18 for zebrafish (*Danio rerio*) ovarian follicle growth assessment following *in vitro* culture of
19 early stage zebrafish ovarian follicles in ovarian tissue fragments is reported here for the first
20 time although some work has been reported for *in vitro* culture of isolated early stage
21 zebrafish ovarian follicles. The main aim of the present study was to develop molecular
22 markers in an optimised *in vitro* culture protocol for stage I and stage II zebrafish ovarian
23 follicles in ovarian tissue fragments. The effect of concentration of the hormones human
24 chorionic gonadotropin and follicle stimulating hormones, and additives such as Foetal
25 Bovine Serum and Bovine Serum Albumin were studied. The results showed that early stage
26 zebrafish ovarian fragments containing stage I and stage II follicles which are cultured *in*
27 *vitro* for 24 h in 20% FBS and 100mIU/ml FSH in 90% L-15 medium at 28°C can grow to
28 the size of stage II and stage III ovarian follicles respectively. More importantly the follicle
29 growth from stage I to stage II and from stage II to stage III were confirmed using molecular
30 markers such as *cyp19a1a* (also known as *P450aromA*) and *vtg1* genes respectively.
31 However, no follicle growth was observed following cryopreservation and *in vitro* culture.

32 **Keywords:** Zebrafish, ovarian follicle, *in-vitro* maturation, tissue fragments, molecular
33 marker

34

35 **1 Introduction**
36

37 Due to environmental factors and various human activities, there have been sharp increases of
38 threatened or endangered aquatic species in the last 10 years [16]. Cryopreservation of
39 gametes of aquatic species plays an important role in preserving the genetic heritage of these
40 species and the development of cryobanks allows storage of the genetic materials for
41 unlimited periods. Cryopreservation of fish reproductive materials also has important
42 applications in biomedicine and aquaculture. Fish sperm cryopreservation of many species
43 has been achieved, but cryopreservation of fish oocytes and embryos has not been fully
44 achieved although some limited successes have been reported [11, 13, 17]. Maternal genome
45 cryopreservation is important as it preserves the mitochondrial DNA and mRNAs that
46 determine the early stages of embryonic development [38]. Cryopreservation of immature
47 fish oocytes offers several advantages over embryos due to their smaller size, lower water
48 content and the absence of a fully formed chorion. However our previous studies showed that
49 the membrane permeability of larger sized late stage oocytes is lower than early stages [15,
50 46] and late stage oocytes are also more sensitive to chilling injury [39].

51 Ovarian tissue cryopreservation has been considered to be a viable alternative to
52 cryopreservation of oocytes or embryos in human [35] and is also proven to be promising in
53 fish species [11, 23, 24]. Culturing of oocytes or ovarian follicles in ovarian tissues offers
54 several advantages as the ovarian follicles remain in their natural three-dimensional structure
55 where they are likely to be protected from physical stress and damage [1]. Although *in vitro*
56 maturation methods have been reported for late stage III zebrafish oocytes [36, 38], studies
57 on *in vitro* maturation of isolated early stage zebrafish ovarian follicles have been relatively
58 limited [24, 38] and ovarian follicle growth following *in vitro* culture has mainly been
59 assessed by measuring the size change of the follicles. In this study the development of
60 molecular markers for zebrafish (*Danio rerio*) ovarian follicle growth assessment following
61 optimising *in vitro* culture protocol of early stage zebrafish ovarian follicles in ovarian tissue
62 fragments is reported here for the first time.

63 Adult zebrafish have asynchronous ovaries, containing follicles of all stages of development
64 [5]. Unlike the mammalian oocytes, the zebrafish oocytes are relatively large in size. The
65 zebrafish ovary consists of a thin epithelium, and each follicle containing an oocyte
66 surrounded by inner granulosa cell layer and an outer theca layer [42]. Follicle development
67 in the zebrafish ovary is broadly divided into the growth stage and the maturation stage which
68 are synchronized by hormones [5]. According to Selman et al [37] the development of
69 zebrafish oocytes is divided into five stages. Stage I (the primary growth stage with follicle
70 diameter of 7-140 µm), Stage II (cortical alveolus stage with follicle diameter of
71 approximately 140 - 340 µm), Stage III (vitellogenesis stage with follicle diameter of 340-
72 690 µm), Stage IV (oocyte maturation stage with follicle diameter of 690-730 µm), and Stage
73 V (mature egg with diameter ranges from 730-750 µm).

74 In teleost two different gonadotropins, follicle-stimulating hormone (FSH) and luteinizing
75 hormone (LH) contribute to follicle development [5]. The pituitary secretes FSH and LH
76 which acts upon the gonads, stimulating their growth and production of eggs or sperms, and
77 synthesis of gonadal hormones [28]. The growth stage is controlled by FSH and the
78 maturation stage by LH [31] though little is known about the physiological roles of FSH and
79 LH in teleosts *in vitro* culture.

80 It has been reported that supplementation of culture medium with Foetal Bovine Serum (FBS)
81 may enhance cell growth in follicles [7]. FBS has been previously used in different fish cell
82 culture experiments at different concentrations and it has been shown to increase the cellular
83 growth rate when combined with fish muscle or ovary extracts using L15 medium [12,18].
84 Seki et al [36] reported that Bovine Serum Albumin (BSA) was effective for the cytoplasmic
85 maturation of late stage III (0.65-0.69 mm in diameter) zebrafish oocytes.

86 Tsai et.al [38] reported *in-vitro* culture with hCG treatment increased the follicular diameter
87 from isolated stage II follicles to stage III follicles. It is also known that FSH acts in early
88 folliculogenesis and is essential for adequate development up to the vitellogenesis stage [19].
89 The presence of FSH receptors in granulosa cells suggests that FSH can promote follicular
90 development and growth [3, 20]. An *in-vivo* study on salmonoids has shown that FSH is
91 important in stimulating vitellogenin uptake by the oocytes [40]. Treatment with FSH
92 significantly increases the follicular diameter in most of mammalian *in-vitro* cultures [33, 34].
93 Since fish gonadotropins are not easily available, hormones from mammalian sources have
94 been commonly used as the alternative in various studies in fish [19].

95 The main aim of the present study was to develop greater insight into *in vitro* culture
96 condition for zebrafish ovarian tissue fragments containing stage I and stage II follicles as
97 there has been no report on use of molecular markers for assessing stage I and II zebrafish
98 follicles development in ovarian tissues following *in vitro* culture. *In vitro* culture of ovarian
99 tissue procedure was investigated using growth supplements (FBS and BSA) and hCG and
100 FSH. The viability was assessed using Trypan Blue (TB) staining and the follicle growth was
101 measured using confocal microscopy. In order to further assess the growth of the ovarian
102 follicles after 24h culture, the expression of *cyp19a1a* and *vtg1* genes were studied. Although
103 *vtg1-7* are expressed predominantly in the liver of female fish, *vtg1* and *2* are expressed in
104 ovary, *vtg1* is a known biomarker for estrogenicity in developing zebrafish [29]. *cyp19a1a*
105 gene is also known to be widely expressed in zebrafish ovary, the *cyp19a1a* mRNA levels are
106 increased in the pre-vitellogenic follicles during oocytes growth and the levels are decreased
107 drastically at the mature stages [14]. Ovarian follicles growth and viability was also assessed
108 following cryopreservation and *in vitro* culture.

109

110 ## 2 Material and Methods

111 Adult zebrafish were sourced from local aquatic centres and maintained in the fish culture
112 laboratories at the University of Bedfordshire. Fishes were kept in filtered and aerated 40L
113 tanks at 27°C±2°C (pH 7.2-8) with a light/dark cycle of 12/12h. Fish were fed three times a
114 day with 'Tetramin' (Tetra, Germany) dry fish flake food and fresh brine shrimp. All fish
115 handling protocols used in this study were approved by the UK Home Office and the Ethics
116 Committee at the University of Bedfordshire.

117 ### *2.1 Development of in-vitro culture protocol*

118 Experiments were conducted on tissue fragments containing stage I and stage II ovarian
119 follicles. To obtain ovarian tissue fragments, adult zebrafish were anaesthetized with a lethal
120 dose of tricaine (0.6mg/ml for 5-10mins), and ovaries were removed after decapitation and
121 immersed immediately in 90% Leibovitz-15 (L-15) medium at pH 9. The ovarian tissue

fragments containing stage I and stage II follicles were carefully dissected from the ovaries and were cut into thin slices (2.3mm) using syringe needles. The ovarian tissue pieces were flattened and stretched until stage I and stage II follicles were clearly visible. After dissection, the ovarian fragments were washed three times in L-15 medium and then were randomly distributed in wells of 6-well plates containing L-15 medium. Ovarian fragment dissections were carried out within 20 min at the room temperature. Experiments were conducted on tissue fragments of 0.35-0.45mm in length and 2.3mm in thickness containing stage I and stage II ovarian follicles. Ovarian fragments were washed three times in washing medium (0.01M PBS, 400μg/ml gentamycin, 200 U/ml penicillin and 2.5mg/ml amphotericine B) before culture. To develop *in- vitro* culture procedures for zebrafish ovarian tissue fragments, the effect of FBS, BSA, hCG and FSH (all from Sigma) were studied. The control ovarian tissue fragments were cultured in 90% L-15 medium (pH 9) and the treated tissue fragments were cultured in 90% L-15 medium containing different concentrations of FBS, BSA, hCG and FSH for 24h at 28°C. Each ovarian fragment was individually cultured in a 6-well culture plate. After 24h *in-vitro* culture, ovarian follicle growth was assessed by measuring follicle diameters in bright field with a confocal microscope (Leica TCS SP5). Ovarian follicle viability was also assessed after culture using trypan blue staining. Ovarian tissue fragments were incubated in 0.2% trypan blue for 3-5min at the room temperature and then washed with 90% L-15 medium before they were assessed under a light microscope. Stained follicles were considered non-viable and unstained follicles were considered viable. For all experiments, three replicates were used for each treatment and experiments were repeated three times.

2.1.1 Effect of FBS on early stage ovarian follicle growth and viability within the tissue fragment

Ovarian fragments were prepared for culture by washing three times in washing medium (0.01M PBS, 400μg/ml gentamycin, 200U/ml penicillin and 2.5mg/ml amphotericine B). Ovarian fragments containing stage I and stage II follicles were placed in 1.5ml of 90% L-15 medium (pH 9) containing 10, 20, 25% FBS for 24h at 28° C in 6-well tissue culture plates. The culture medium was freshly made and filter sterilized. One piece of ovarian fragment was individually cultured. Control ovarian fragments were incubated in 90% L-15 medium (pH 9). After *in vitro* culture, ovarian follicle growth within the fragment was assessed by measuring the diameter in bright field with confocal microscope. Ovarian follicle viability was also assessed after culture using trypan blue staining.

2.1.2 Effect of BSA on early stage ovarian follicle growth and viability within the tissue fragment

Ovarian fragments were prepared for culture as described above. Ovarian fragments containing stage I and stage II follicles were placed in 1.5ml of 90% L-15 medium (pH 9) containing 0.5% BSA [36] for 24h at 28°C in 6-well tissue culture plates.

2.1.3 Effect of hCG on early stage ovarian follicle growth and viability within the tissue fragment

Ovarian fragments were prepared for culture as described above. Ovarian fragments containing stage I and stage II follicles were placed in 1.5ml of 90% L-15 medium (pH 9) containing 10IU/ml hCG for 24h at 28° C in 6-well tissue culture plates.

164 *2.1.4 Effect of FSH on early stage ovarian follicle growth and viability within the tissue*
165 *fragment*

166 Ovarian fragments were prepared for culture as described above. Ovarian fragments
167 containing stage I and stage II follicles were placed in 1.5ml of 90% L-15 medium (pH 9)
168 containing 10, 20, 30 and 40mIU/ml FSH for 24h at 28° C in 6-well tissue culture plates.

169 The culture methods and assessment methods described in the above paragraph were used in
170 these experiments.

171 *2.2 Growth assessment after in-vitro culture using molecular markers*

172 Ovarian follicles were collected from zebrafish ovaries and were placed in 90% L-15
173 medium. Ovarian follicles of different stages (I, II) were separated using syringe needles.

174 For *in vitro* cultured samples; the ovarian tissue fragments were cultured in 90% L-15
175 medium (pH 9.0) with 20% feotal bovine serum (FBS) and 100mIU/ml follicle stimulating
176 hormone (FSH) for 24h at 28°C. Each ovarian fragment was individually cultured in a 6-well
177 culture plate.

178 *2.2.1 RNA extraction and DNase treatment*

179 Total RNA was extracted from ovarian follicles using the trizol method (Invitrogen, UK).
180 This was followed by the DNAse treatment step to remove any genomic DNA contamination.
181 The quantity and purity of each RNA was checked for quantity and purity using a
182 Biophotometer (Eppendorf, UK) at 260 and 280 nm.

183 *2.2.2 Reverse transcription*

184 Aliquots of total RNA (1µg) were transcribed using the precision qScript Reverse
185 Transcription Kit (Primer design Ltd, UK). For the conventional PCR undiluted cDNA was
186 used in subsequent steps; and for the real time PCR cDNA was diluted 1:2 in molecular
187 biology grade water and stored at -80°C.

188 *2.2.3 PCR analysis*

189 The PCR reactions consisted of NH_4 PCR buffer (Bioline, UK), 200µM dNTP (Bioline),
190 1.5mM $MgCl_2$ (Bioline), 2U BIOTAQ^{TM} DNA polymerase (Bioline), 0.5 µM each primer
191 (Table 1), 1µg RNA template and PCR RNAse free water. The conditions for PCR were
192 initial denaturation at 94°C for 5min, 40 cycles of amplification at 94°C for 30s, annealing
193 temperature (Table 1) for 30s and the extension step at 72°C for 10min. The size of PCR
194 products was analised on 2% agarose gels.

195

196

197

198

199

Table 1

Information of gene name, accession ID and primer sequences inclusing annealing temperature and product size

Gene name	Accession ID	Forward/reverse primer (5'-3')	Annealing temp.(ºC)	Amplicon size (bp)
cyp19a1a	AF226620.1	F:CAGACTGGACTGGCTGCACAAGAA R:TGTCTGGAGCCGCGATCACCAT	59	221
vtg1	NM_22.4	F:ACTACCAACTGGCTGCTTAC R:ACCATCGGCACAGATCTTC	60	100
EF1-α (*eef1a1l1)*	NM_131263.1	F:CTGGAGGCCAGCTCAAACAT R:ATCAAGAAGAGTAGTACCGCTAGCATTAC	60	87

2.2.4 Real time PCR

The standards for real time PCR of *vtg1* and *cyp19a1a* along with housekeeping gene *EF1*-α were produced using conventional PCR. The DNA was isolated from excised bands using the EZNA Gel extraction kit (Omaega Bio-Tek, VWR, UK) according to the manufacturer's instructions. The isolated DNA was quantified using a Biophotometer (Eppendorf, UK) at 260nm and diluted to 2ng/µl followed by 10-fold serial dilutions to generate standards for real time PCR.

Real time PCR was performed on a RotorGene 6000 cycler (Corbett Research, UK) to quantify the expression level of *cyp19a1a* and *vtg1*. The reaction tubes contained 7.5µl of sensimix 2X reaction buffer (contained heat activated DNA polymerase, ultrapure dNTPs, $MgCl_2$ SYBR® Green), 333nm of Primers (Table 1) and 2µl of cDNA sample, made up to 15µl with PCR water. The reaction conditions were 1cycle at 95°C for 10min, followed by 50 cycles at 95°C for 10s, the appropriate annealing temperature (Table 1) for 15s and at 72°C for 15s. Data were acquired on the FAM/SYBR channel at the end of each extension step. Relative gene expression levels were calculated using the standard curve quantification methods with kinetic PCR efficiency correction used in the RotorGene software. Gene expression was relative to time zero and normalised by housekeeping gene *EF1- α*. *EF1- α* were used for this study as these genes were shown to have the highest stability during zebrafish studies [22].

$$\text{Relative quantification} = \frac{(E_{\text{target}})^{\Delta \text{CP(target)(control-sample)}}}{(E_{\text{ref}})^{\Delta \text{ CP (ref)(control-sample)}}}$$

Where *E* is the real time PCR efficiency and ΔCP is the crossing point difference between the unknown sample and the control sample.

2.3 Cryopreservation and in vitro culture of ovarian follicles in tissue fragments

Two cryoprotectants were used in these experiments: methanol and ethanol. The no observed effect concentrations (NOECs) for methanol and ethanol for stage I and stage II follicles within

241 the follicles were identified as 2M in the previous experiments, therefore 2M were used in the
242 controlled slow cooling experiments. 2M methanol and 2M ethanol was made up in 90% L-15
243 medium. The ovarian tissue fragments were exposed to cryoprotectant solutions for 30 min at
244 room temperature and then were loaded into 0.5ml plastic straws before placing in a
245 programmable cooler. Ovarian tissue fragments incubated in cryoprotectant-free 90% L-15
246 medium were used as controls. The following cooling protocols were used: cooling at 2°C/min
247 from 20°C to seeding temperature (−7.5°C for 2M), manual seeding and held for 15 min,
248 freezing from seeding temperature to −40°C at 4°C/min and from −40°C to −80°C at 10°C/min
249 and hold for 10 min, samples were then plunged in liquid nitrogen at −196 °C and held for at least
250 10 min. Samples were thawed using a water bath at 28°C. Removal of cryoprotectant was
251 conducted in four-step (1M methanol, 0.5M methanol and 0.25M methanol in 90% L-15 medium,
252 2.5 min for each step).
253
254 The tissue fragments were cultured after freeze-thawing, the ovarian fragments were washed
255 twice in 90% L-15 medium (pH 9) and were prepared for culture by washing three times in
256 the washing medium (0.01M PBS, 400µg/ml gentamycin, 200 U/ml penicillin and 2.5mg/ml
257 amphotericine B). One fragment was cultured in 1.5ml of 90% L-15 medium (pH 9)
258 containing 100mIU/ml FSH and 20% FBS in 6 welled plates for 24h. Ovarian follicle growth
259 within the fragment was assessed by measuring the diameter with an ocular micrometer under
260 microscope. Ovarian follicles viability was also assessed using trypan blue staining. Three
261 replicates were used for each experiment. The experiments were repeated at least three times.
262

263 *2.4 Statistical Analysis*

264 Statistical analysis was carried out using SPSS (SPSS for windows version 16.0) and
265 Microsoft Excel. The normality and homogeneity of the variance was tested. Comparisons
266 were made by one-way ANOVA, where difference was found. Tukey's post hoc test was
267 carried out to establish which samples were significantly different. All data were expressed as
268 mean ± SEM across the three replicates and *P* values of less than 0.05 were considered to be
269 significant.

270

271 **3 Results**

272 *3.1 Development of in-vitro culture method for zebrafish ovarian tissue fragments*

273 *3.1.1 Effect of BSA on early stage ovarian follicle growth and viability within the tissue*
274 *fragment*

275 The growth and viability of stage I and stage II ovarian follicles within the ovarian tissue
276 fragment after culturing in various concentrations (0.125, 0.25 and 0.5%) of BSA in 90% L-
277 15 for 24 h culture at 28°C are shown in Fig 1. The results showed that there were no
278 significant increases in diameter in stage I and stage II follicles when cultured with BSA for
279 24h compared to the controls (no BSA) at 0h and 24h (Fig 1a). Results obtained by TB
280 staining (Fig 1b) showed that 0.125% BSA exposure did not compromise follicle membrane
281 integrity but membrane integrity was significantly compromised when 0.25% or 0.5% BSA
282 was used.

283 *3.1.2 Effect of FBS on early stage ovarian follicle growth and viability within the tissue*
284 *fragment*

285 The growth and viability of stage I and stage II ovarian follicles within the ovarian tissue
286 fragment after culturing in various concentrations (10, 20 and 25%) of FBS in 90% L-15 for
287 24 h culture at 28°C are shown in Fig 2a. The results showed that the diameter of stage I and
288 stage II ovarian follicle significantly increased in samples cultured with 20% FBS. There
289 were no significant differences in follicle diameters between control and follicles cultured
290 with 10% or 25% FBS ($P>0.05$). The viability of ovarian follicles following culturing in
291 different concentrations of FBS is shown in Fig 2b. The results indicated that 10 and 20%
292 FBS treatment did not result in any significant change of membrane integrity when compared
293 to the control group but the treatment with 25% FBS decreased the viability of the stage II
294 follicles significantly following 24h of culture. Hence 20% FBS was compared with other
295 growth factors in the subsequent experiment.

296 *3.1.3 Effect of hCG on early stage ovarian follicle growth and viability within the tissue*
297 *fragment*

298 The growth and viability of stage I and stage II ovarian follicles in ovarian tissue fragments
299 after culturing in 10IUhCG along with 0.125% BSA and 20% FBS in 90% L-15 for 24h at
300 28°C are shown in Fig 3a. Whilst there were no significant increases in follicle diameter
301 between controls (0h) and the treated groups for stage I and stage II follicles, membrane
302 integrity was not compromised when hCG with 20% FBS was used although membrane
303 integrity was significantly compromised when hCG with 0.125% BSA was used (Fig 3b).

304 *3.1.4 Effect of FSH on early stage ovarian follicle growth and viability within the tissue*
305 *fragment*

306 The growth of stage I and stage II ovarian follicles within the ovarian tissue fragment after
307 culturing in various concentrations (40, 60, 80, 100 and 120mIU/ml) of FSH in 90% L-15 for
308 24h culture at 28°C are shown in Fig 4a. The results showed that the diameter of stage I and
309 stage II ovarian follicles increased in samples cultured with 100mIU/ml FSH. Hence
310 100mIU/ml FSH was used in the subsequent experiment.

311 The growth of stage I and stage II ovarian follicles in ovarian tissue fragments after culturing
312 in 100mIU/ml FSH along with 0.125% BSA in comparison to 100mIU/ml FSH with 20%
313 FBS in 90% L-15 for 24h culture at 28°C are shown in Fig 4b. The results showed that
314 treatment with FSH and 20% FBS increased the diameter in both stage I and stage II ovarian
315 follicles in fragments. The results also showed that membrane integrity was not compromised
316 for follicles incubated in FSH with 20% FBS when compared to the control groups following
317 24h of culturing at 28°C (Fig 4c).

318 *3.2 Growth assessment for stage I and stage II zebrafish ovarian follicles after in vitro*
319 *culture using molecular markes*

320 Gene expression studies were performed for *cyp19a1a* and *vtg1* genes. Stage I, II and III
321 ovarian follicles were collected and subjected to RNA extraction, cDNA synthesis and PCR.
322 PCR product was analysed using agarose gel electrophoresis. Housekeeping gene *EF1*-α was

323 used as control. In order to assess level at stage I, II and III ovarian follicles quantitative
324 analysis was carried out. Fig 5a showed that the expression of *cyp19a1a* gene was higher in
325 stage II compared to stage I and stage III ovarian follicles. Fig 5b showed that the expression
326 of *vtg1* gene was higher in stage III when compared to stage I and stage II ovarian follicles.
327 This is the first study on the expression of *cyp19a1a* and *vtg1* genes involved in the follicle
328 development in the zebrafish ovarian fragments *in vitro* studies. The results showed that these
329 genes can be used to distinguish different stages of the follicle growth and confirm
330 morphological differences of ovarian follicle during development.

331 Fig 6 shows that after 24 h *in-vitro* culture, the level of expression of *cyp19a1a* gene in stage
332 I follicles was of the same level expressed in stage II follicles prior to culture (Fig 6a). This
333 indicates that stage I follicles developed to stage II follicles following culture. Similarly the
334 level of expression of *vtg1* gene in stage II follicles showed the same level of expression of
335 stage III follicles prior to culture, indicating stage II follicles developed to stage III follicles
336 following *in vitro* culture in 90% L-15 medium with 100mIU/ml FSH and 20% FBS for 24h
337 (Fig 6b). The results showed that *cyp19a1a* and *vtg1* genes can be used as usefull markers to
338 assess the growth of early stages of zebrafish ovarian follicles *in vitro*.

339 *3.3 Ovarian follicle viability following cryopreservation and in vitro culture*
340
341 The results showed that stage I and stage II follicles from the freeze-thawed group did not
342 show any increase in diameter when measured at different time points following *in vitro*
343 culture (0h, 2h, 6h and 24h) (Fig 7a, b). The results obtained from TB staining assessing the
344 membrane integrity of the cryopreserved stage I and stage II follicles indicated a significant
345 decrease when compared to the unfrozen controls (Fig 7c).
346

347 **4 Discussion**

348 *4.1 Effect of media supplements on ovarian follicle growth in tissue fragment*

349 Results from the present study showed that after 24h of *in-vitro* culture, early stage zebrafish
350 ovarian follicles grew bigger in size when ovarian fragments were incubated in 20% FBS
351 when compared to the other concentrations. FBS has been shown as an effective growth
352 factor in most fish culture and has been reported to increase the cellular growth rate [12]. The
353 present study has also shown that FBS is also effective in promoting early stage ovarian
354 follicle growth *in vitro*. Studies undertaken by Otala et. al [32] on human ovarian tissues
355 indicated that culture medium supplemented with FBS preserved the integrity of the oocyte
356 granulosa-stroma interaction, which is important for the development of early stage follicles.

357 Although Seki et al [36] reported that BSA was effective for the cytoplasmic maturation of
358 zebrafish oocytes in later stage zebrafish oocytes, the present study showed that the benefit of
359 using BSA in culture medium for early stage zebrafish ovarian follicles is very limited. In *in-*
360 *vitro* studies conducted on human ovarian tissue also showed that incubation with albumin
361 induced cell death [27]. Newton et al. [27] has also reported that FBS increased murine
362 oocytes development when compared to those cultured in BSA.

4.2 Effect of gonadotropins on the ovarian tissue fragments

Gonadotropins are well characterised in fish species. FSH and LH are expressed differently during the reproductive cycle. The level of FSH is high in oocytes growth stage and LH is high in the maturation stage [1, 18, 43]. Since fish gonadotropins are not readily available, hormones from mammalian sources have been commonly used as the alternatives in various studies in fish [38].

The results obtained in the present study showed that the exposure to 10IU/ml hCG did not aid follicle growth when compared with controls. hCG acts as an effective inducer of oocyte maturation in several teleost. The promotion of oocyte maturation by hCG in larger oocytes (eg stage III zebrafish oocytes) has been well documented in teleost. hCG has been confirmed to stimulate maturation of the gonads of several fish species and stimulates steroid production in vitellogenic and full grown ovarian follicle [47]. Studies with hCG treatment have also shown that hCG stimulates the growth in later stage ovarian follicles in human [6]. However, the results from the recent study indicated that hCG was not effective in stimulating zebrafish follicle growth at early stages, this is also in agreement with the study undertaken by Wu et al. [45] that stage I and stage II zebrafish follicles did not respond to 1IUhCG treatment, unless they are larger than 0.52mm (stage III). We used a different concentration of hCG in the present study since it was proven to be effective for stage III ovarian follicles [38].

The results obtained from ovarian follicles cultured in 100mIU/ml FSH in the present study showed that the follicle sizes increased following 24h of culture confirming the important role of FSH in promoting ovarian follicle growth. FSH is involved in early folliculogenesis [15], the fact that FSH treatment increases the number of preantral and small antral follicles in mouse supports that follicular growth up to antrum formation is controlled by FSH [44]. In salmonids, it has been proposed that FSH is likely to be important for promoting follicle growth in the ovary [41]. Furthermore, Meduri et. al [26] has also reported that FSH receptors appear during early stage ovarian follicle development. FSH is essential for the differentiation of granulosa cells and it regulates the transzonal connection between the oocytes and surrounding granulosa cells. This study suggested for the first time that FSH is also effective in promoting early stage zebrafish ovarian follicle growth *in vitro*.

4.3 Growth Assessment for stage I and stage II zebrafish ovarian follicles after in vitro culture using molecular marker

The expression of *cyp19a1a* and *vtg1* in early stage zebrafish follicles is reported in the present study for the first time. Our study demonstrated that the expression of *cyp19a1a* gene was higher in stage II ovarian follicles when compared to stage III ovarian follicles. Although studies on the expression of *cyp19a1a* in late stage zebrafish ovarian follicles have been carried out, eg Ings *et al.* [14] reported that the expression of *cyp19a1a* peaked in zebrafish previtellogenic follicles and dropped off to almost non-detectable levels in maturing follicle, expression was not measured in earlier developmental stages. Kumar *et al.* [18] also showed that *cyp19a1a* expression decreased as follicles matured in the channel catfish. Other studies have found that the expression of *cyp19a1a* peaks during mid-vitellogenesis with a drop in expression during maturation in medaka [8], tilapia [4], salmonids [30], artificially matured Japanese eels [25] and red seabream [10]. Studies have suggested increased *cyp19a1a* and its mRNA expression is associated with increased enzyme activity during vitellogenesis [9].

406 The present study showed that the expression of *vtg1* gene was higher in stage III follicles
407 when compared to stage I and stage II ovarian follicles. *vtgs* are the most abundant proteins in
408 the mature teleost oocytes. In late stage zebrafish oocytes, large amounts of *vtgs* in their large
409 forms were observed [47]. The variability of protein and mRNA levels defines the specific
410 maturation stage. Until recently, liver was assumed to be the main site for *vtg* synthesis in
411 teleost. Levi *et al.* [21] showed that *vtgs* are expressed and synthesized in the intestine and
412 ovary in addition to liver, however synthesis in the liver is much higher than in other tissues.
413 The mRNA expression reflects the *in vivo* gene expression in zebrafish, in which
414 maturational competence was acquired through *in vitro* gonadotropin stimulation [2].

415 Results from the present study showed that after 24 h *in-vitro* culture, the level of expression
416 of *cyp19a1a* gene on in stage I follicles showed the level of expression of stage II follicles
417 obtained prior to culture and the level of expression of *vtg1* gene in stage II follicles showed
418 the level of expression of stage III follicles obtained prior to culture. These results
419 demonstrated that stage I follicles developed to stage II follicles and stage II follicles
420 developed to stage III follicles respectively following culture in 100mIU/ml FSH with 20%
421 FBS for 24h at 28C. *cyp19a1a* and *vtg1* genes have proved to be simple and sensitive
422 markers for assessing the growth of the developing zebrafish ovarian follicles *in vitro* and can
423 be used as markers to assess the growth of follicles from stage I to stage II and from stage II
424 to stage III. The present gene expression study is focussed on mRNA levels of the target
425 genes, and neither the corresponding proteins levels nor the functionality were assessed.
426 Further studies are needed in these areas.

427 4.4 *Ovarian follicle viability following cryopreservation and in vitro culture*
428
429 When the cryopreserved ovarian tissue fragment were cultured using the protocol developed
430 in the present study, the results showed that the ovarian follicles within the fragment did not
431 show any growth after the *in-vitro* treatment. The results also showed that the ovarian
432 follicles viability was significantly lower than those of the controls after 24h culture. These
433 results are in agreement with the previous information on cryopreserved ovarian tissue
434 fragments which showed damage in the membrane integrity and metabolic activity of the
435 cryopreserved tissues (38). The cryopreserved ovarian follicles within the fragments did not
436 show any growth, possibly due to the damage of thecal and granulosa cells by intracellular
437 ice formation. Further studies are required on the effect of cryopreservation procedures and
438 the impact of intracellular ice formation on early stage ovarian follicles.
439

440 **5 Conclusion**

441 In conclusion, an effective protocol has been developed in the present study for early stage
442 zebrafish ovarian follicle development *in vitro*. Stage I and stage II zebrafish ovarian follicles
443 developed to stage II and stage III respectively following culture in 100mIU/ml FSH with 20%
444 FBS for 24h at 28°C. *cyp19a1a* and *vtg1* genes expressions were studied in early stage
445 zebrafish ovarian follicles for the first time and they were proven to be effective markers in
446 distinguishing the growth patterns in early stage ovarian follicles and confirm the structural
447 difference in terms of the ovarian follicle development. However, no follicle growth was
448 observed following cryopreservation and *in vitro* culture.

449

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451 impartiality of the research reported

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453

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595 1a 1b

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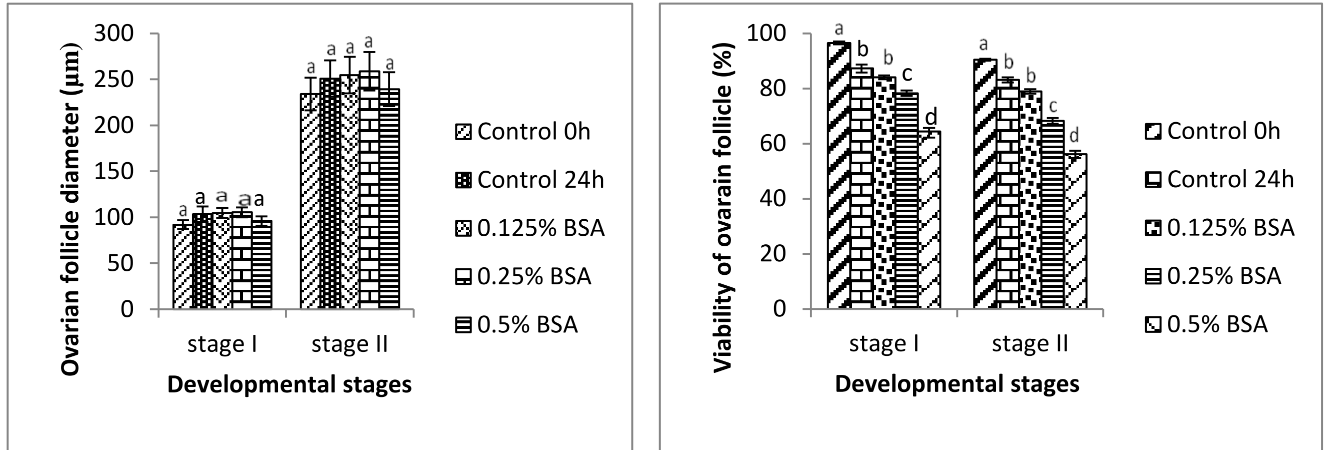

599

600 Fig 1: The growth (1a) and viability (1b) of stage I and stage II ovarian follicles within the
601 ovarian tissue fragments in 0.125, 0.25 and 0.5% BSA made up in 90% L-15 medium after 24
602 h culture at 28°C. Follicles before culture were used as controls (0h). Error bars represent
603 Standard Errors of the Mean. Groups with no common superscript differ significantly from
604 one another (*P<0.05*).

605

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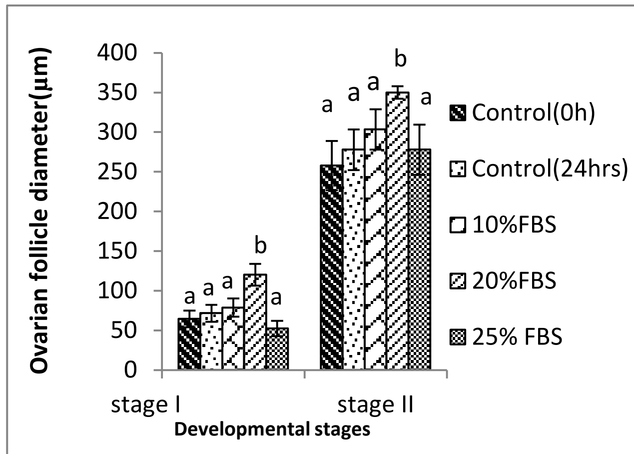
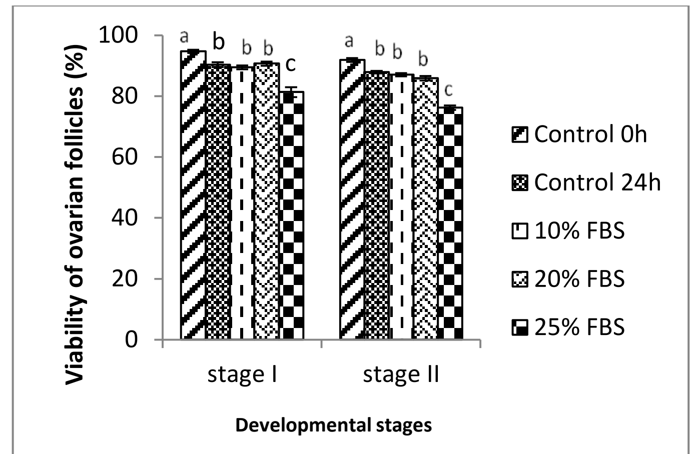
607 2a

2b





609

610 Fig 2: The growth (2a) and viability (2b) of stage I and stage II ovarian follicles within the
611 ovarian tissue fragments in 10, 20 and 25% FBS made up in 90% L-15 medium after 24 h
612 culture at 28°C. Follicles cultured in 90% L-15 medium were used as controls (0h and 24h).
613 Error bars represent Standard Errors of the Mean. Groups with no common superscript differ
614 significantly from one other (*P<0.05*).

615

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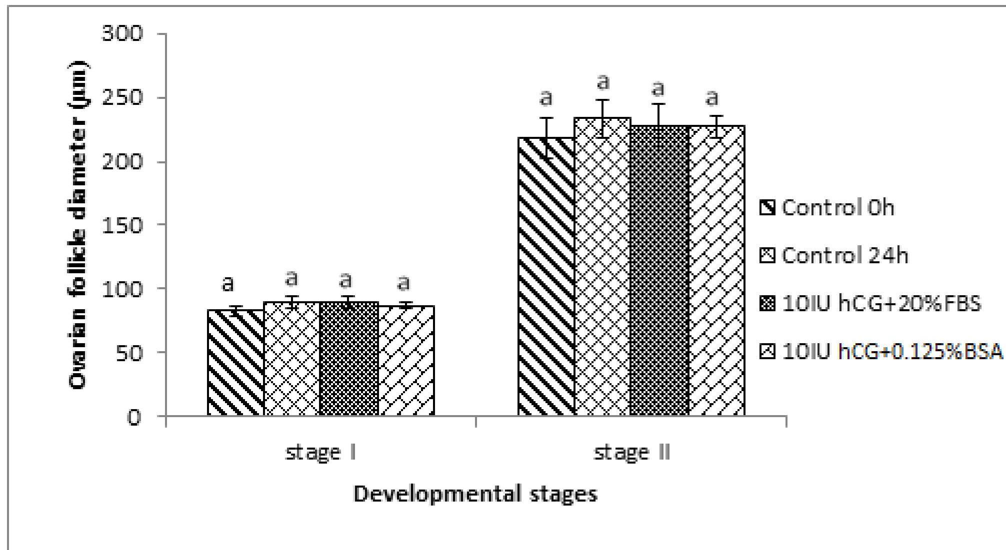
617 3a



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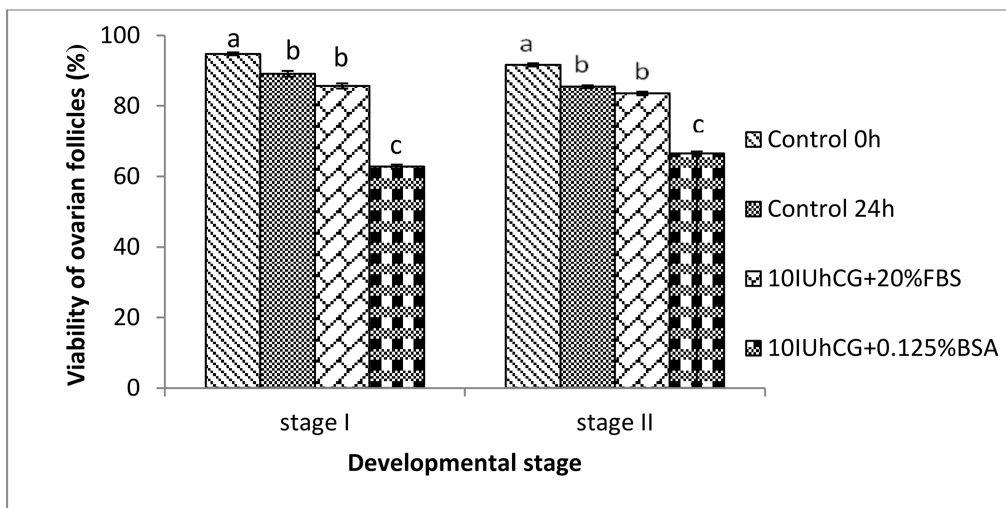
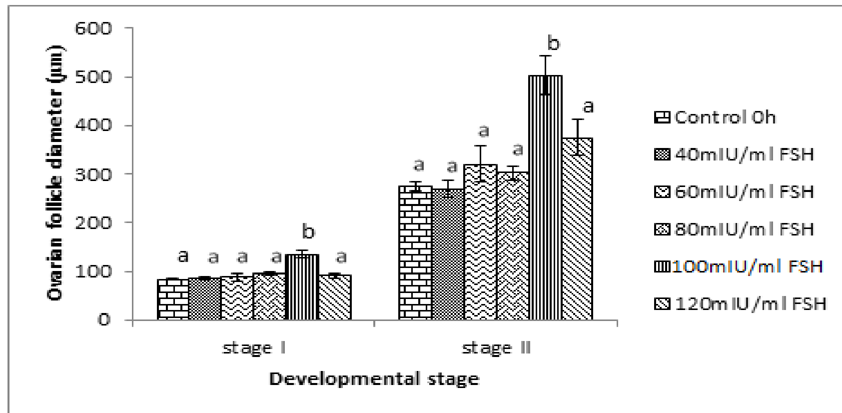


620

621 Fig 3: The effect of 10IU/ml hCG with 20% FBS and 0.125% BSA on stage I and II ovarian
622 follicles growth (3a) and viability (3b) within the ovarian tissue fragments in 90% L-15
623 medium at pH 9.0 after 24h culture at 28°C. Follicles cultured in 90% L-15 medium were
624 used as controls (0h and 24h). Error bars represent Standard Errors of the Mean. Groups with
625 no common superscript differ significantly from one other (*P<0.05*).

626

627 4a



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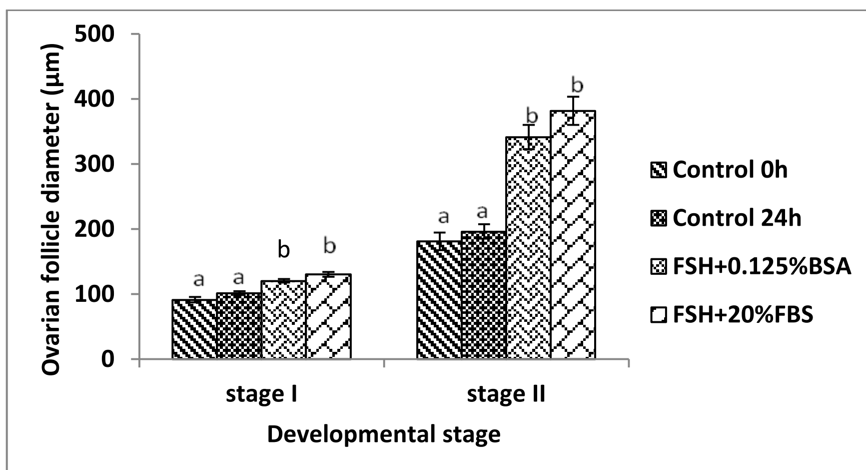

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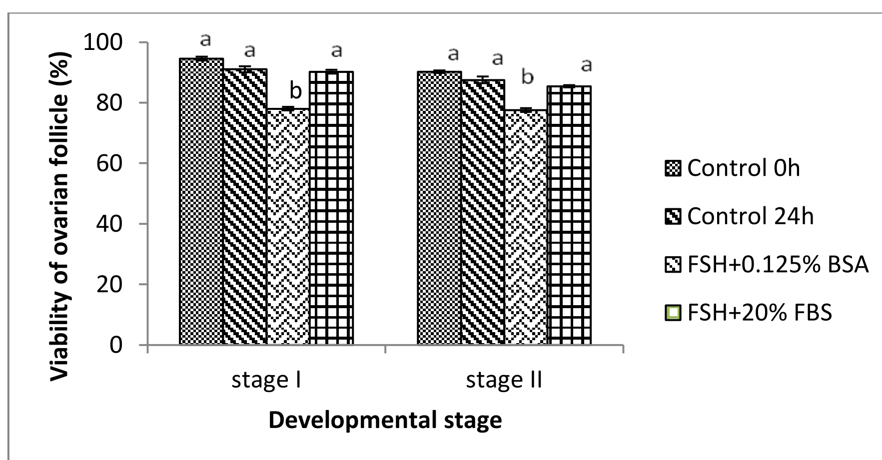

632

633 Fig 4: The effect of FSH (40, 60, 80, 100 and 120 mIU/ml) (4a), 100mIU/ml FSH with 0.125%
634 BSA and 20% FBS on stage I and II ovarian follicles growth (4b) and viability (4c) within
635 the ovarian tissue fragments in 90% L-15 medium at pH 9.0 after 24h culture at 28°C.
636 Follicles cultured in 90% L-15 medium were used as controls (0h). Error bars represent
637 Standard Errors of the Mean. Groups labelled with different letters differ significantly from
638 one another ($P<0.05$).

639

640 5a 5b

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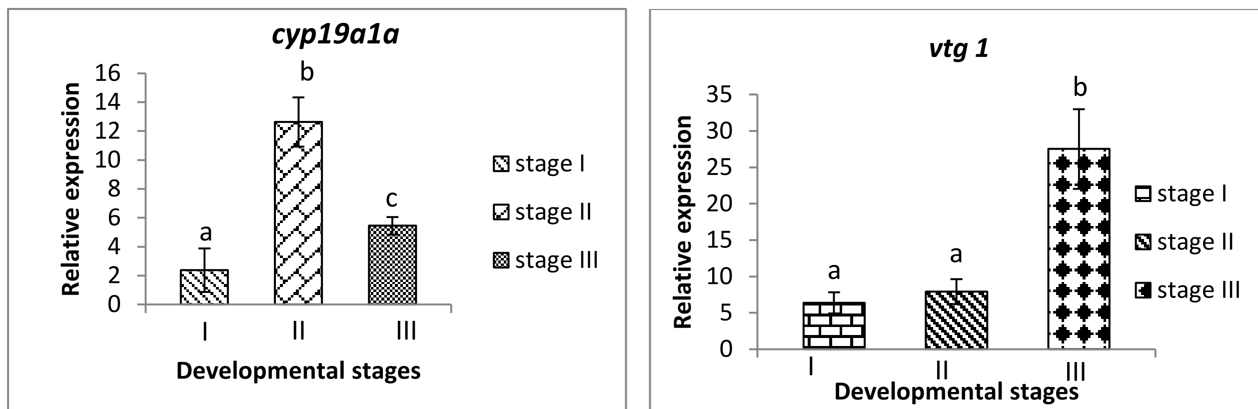
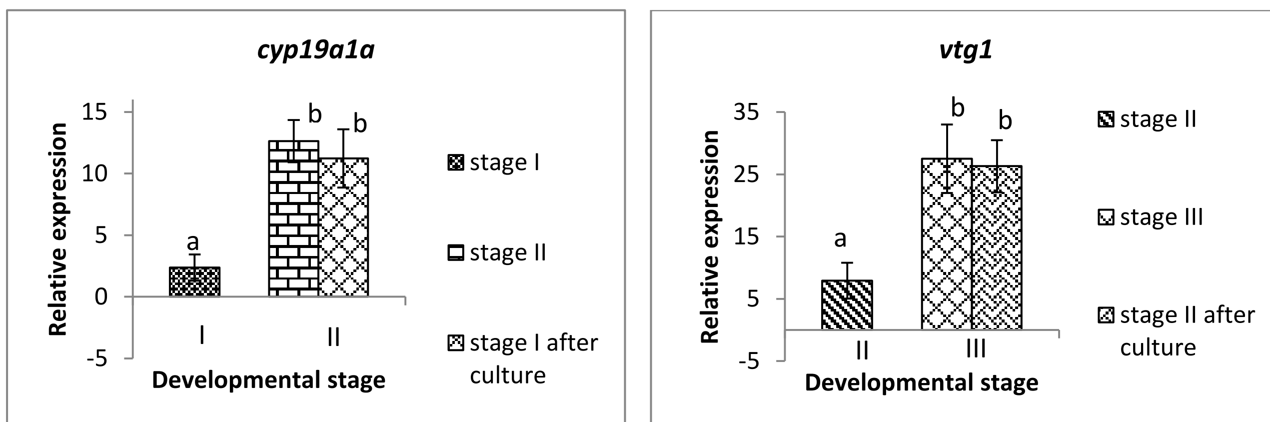


643

644 Fig 5: Relative mRNA expression of *cyp19a1a* gene (5a) and *vtg1* gene (5b) in zebrafish
645 ovarian follicles. The values are normalised to *EF1-a*. Values represent the mean SEM of
646 relative expression as determined by RT-PCR. Different letters indicate significant
647 differences.

648 6a 6b

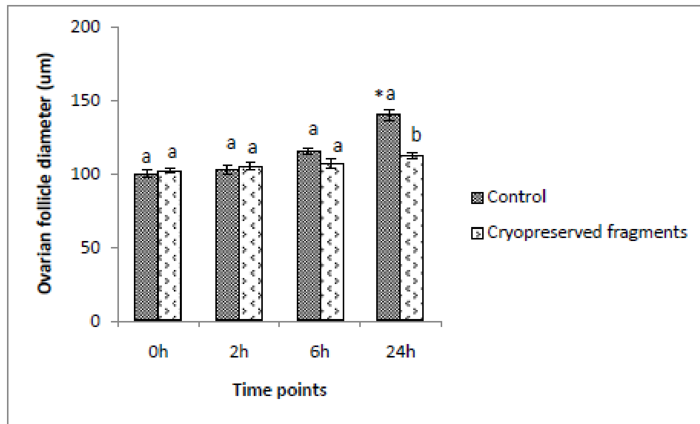


650

651 Fig 6: Relative mRNA expression of *cyp19a1a* gene (6a) and *vtg1* (6b) after 24h *in vitro*
652 *culture* at 28°C. The culture medium contained 90% L-15 medium with 100mIU/ml FSH and
653 20% FBS. It shows the growth from stage I to stage II follicles and from stage II to stage III
654 follicles respectively. The values are normalized to *EF1*-α. Values represent the mean SEM
655 of relative expression as determined by RT-PCR. Different letters indicate significant
656 differences.

657

658 7a



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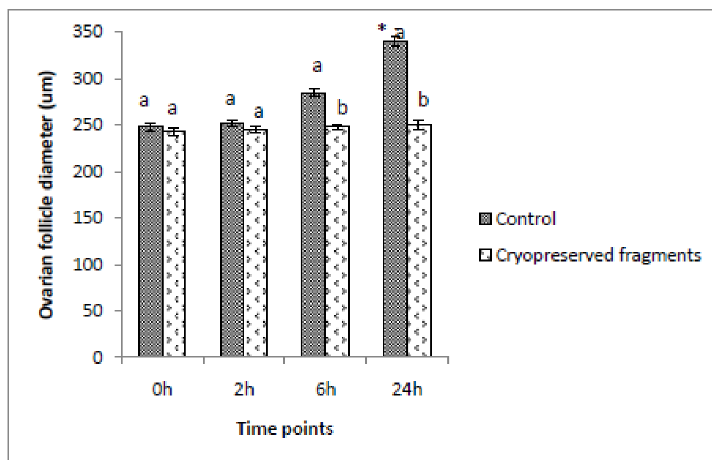

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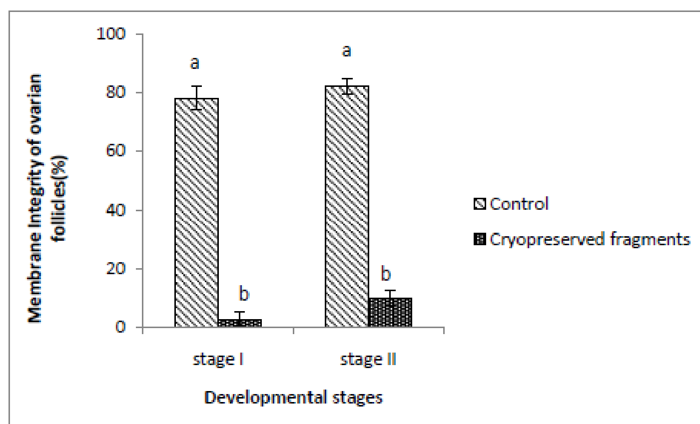
662 7c



663

664

665 Figure 7: The growth (7a,b) and viability (7c) of stage I (a) and stage II (b) ovarian follicles within the
666 ovarian fragments after freeze-thawing and culturing in 90% L-15 medium (pH 9) containing
667 100mIU/ml FSH with 20% FBS for 24hr at 28°C. The tissue fragments were incubated in 2M
668 methanol in 90% L-15 medium+20% FBS for 30min at room temperature and then frozen to −196°C
669 at post-seeding cooling rate 4°C/min. Cryoprotectant was removed in four steps. The diameters of
670 ovarian follicles were measured with an ocular micrometer under microscope. The viability was
671 assessed by TB staining. Error bars represent standard errors of the mean. Different letters indicate
672 significant differences between the control and cryopreserved groups ($p<0.05$) and * indicate the
673 significant difference between the time points.

674