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IGF1 stimulates differentiation of primary follicles and their growth in ovarian explants of zebrafish (*Danio rerio)* cultured *in vitro*

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The present study is an attempt to elucidate the involvement of insulin-like growth factor (IGF1) in the differentiation and growth of primary follicles in ovarian explant cultures of zebrafish. Ovaries from adult females were cultured in triplicate sets/treatment group for 15 days at 22°C in the laboratory. Culture medium was supplemented with either insulin (1 ng/mL) or IGF1 (1 ng/mL) or insulin + IGF1 (Experiment 1) or 0.1 or 1.0 or 10 ng/mL of IGF1 (Experiment 2). Ovaries cultured in medium alone served as controls and those fixed at the beginning of the culture as initial controls. Experiments were repeated. On the 16th day ovarian explants were fixed in Bouin's fluid and processed for paraffin embedding, sections (3 μm) were cut and stained with hematoxylin-eosin. Follicles were classified into 6 stages and atretic follicles (AF). Previtellogenic, vitellogenic and total follicle number was calculated. At the start of the culture, ovaries contained all stages of growing and degenerating follicles. In *in vitro* cultured control ovaries, vitellogenic follicles underwent atresia, while, primary follicles remained unaffected. Insulin or insulin + IGF1 treated ovaries did not differ significantly while IGF1 exposed ovarian explants had greater ($P < 0.05$) number of primary follicles compared to controls. IGF1 also caused an increase in the number and growth of primary follicles in a dose dependent manner although; cultures were not supplemented with gonadotrophic hormones. Results suggest that locally derived intra-ovarian IGF1 may have a role in the differentiation and growth of primary follicles in zebrafish ovary.

Keywords. *Danio rerio*; differentiation; *in vitro* culture; insulin-like growth factor 1; ovarian explants; primary follicle

1. Introduction

Reproduction in fishes is profoundly influenced by environmental cues, the actions of which are mediated via systemic neuroendocrine, endocrine, paracrine and autocrine signals that in turn modulate the events of gamete formation and release (Reinecke 2010; Lubzens *et al.* 2010). In teleosts, oogonial stem cells originate from ovarian epithelium and hormones from pituitary and ovary orchestrate follicle development, growth, and maturation (Selman *et al.* 1993; Clelland and Peng 2009; Nagahama and Yamashita 2008; Nakamura *et al.* 2011; Shoae 2012). Nevertheless, recent investigations on fish ovary emphasize the pivotal role of locally-derived growth factors in ovarian physiology by fine-tuning the actions of gonadotropins and steroid hormones by regulating their receptors *in situ* (Clelland and Peng 2009; Reinecke 2010; Nelson and Kraak 2010a, b; Shoae 2012; Das *et al.* 2013; Mukherjee *et al.* 2014; Zhang *et al.* 2015; Das *et al.* 2016a, b).

Zebrafish (*Danio rerio)* primarily emerged as an ideal model to study vertebrate development, is now being intensely employed in biomedical research. This fish attains sexual maturity in 3 months with persistent oogonia in adult ovary, consequently, a female produces fertilizable eggs year-round through asynchronous oogenesis and folliculogenesis (Clelland and Peng 2009). Hormonal regulation of follicular growth and maturation are well understood, i.e. follicle stimulating hormone (FSH) and luteinizing hormone (LH) from hypophysis stimulate vitellogenesis and oocyte maturation respectively through their specific cognate receptors expressed on somatic follicle cells (Nagahama *et al.* 1995; Nagahama and Yamashita 2008; Zhang *et al.* 2015). However, regulatory mechanisms underlying the differentiation and growth of primary follicles are poorly understood and need elucidation (Clelland and Peng 2009).

Recent findings on gene expression profiles of zebrafish ovary indicate that a variety of growth factors and their receptors viz, β cellulin, epidermal growth factor-EGF (Tse and Ge 2009, 2010), insulin like growth factors-IGF1, IGF2 and IGF3 (Maures *et al.* 2002; Schlueter *et al.* 2007; Reinecke 2010; Nelson and Kraak 2010a, b; Irwin and Kraak 2012; Li *et al.* 2015; Zhou *et al.* 2016), members of transforming growth factor (TGF-β) super family such as, activin, inhibin growth differentiation factor-GDF9, TGFβ1, and

bone morphogenetic proteins (BMP7 and BMP15), TGF ∝ (Pang and Ge 2002; Peng *et al.* 2009; Li and Ge 2011, 2013), platelet-derived growth factor A-PDGF-A (Liu *et al.* 2002; Liu and Ge 2007), vascular endothelial growth factor-VEGF/vascular permeability factor (Geva and Jaff 2000), follistatin (Wang and Ge 2004), pituitary adenylate cyclase activating polypeptides-PACAPs (Zhou *et al.* 2011), *Kit* ligand (Yao and Ge 2010), insulin receptors *insra* and *insrb* (Das *et al.* 2016b) are expressed and characterized in different cellular components of ovary providing an evidence for their local bioavailability and role (s) in ovarian physiology (Clelland and Peng 2009; Das *et al.* 2016a, b).

The present investigation is an attempt to understand the involvement of insulin/insulin like growth factor (IGF1) signaling if any, in the differentiation, early development and growth of follicles in adult zebrafish ovary. The study has utilized *in vitro* ovarian explant cultures as an experimental model and quantitative analysis of follicles under various stages of development and growth (follicular kinetics) to address the question.

2. Materials and methods

2.1 *Animals*

Adult (body size: 27 ± 1 mm; body mass: 160 ± 15 mg) female zebrafish (wild type) procured from Aquastar, Chennai, were acclimated to the laboratory conditions (temperature, 26 ± 1°C and photoperiod, 11.30L: 12.30D) for 4 weeks before use. Fish were fed twice/day on commercial pellets and or *Artemia nauplius ad libitum* (Rajapurohit and Pancharatna 2007). Permission to work on zebrafish was obtained from Animal Ethical committee, CPCSEA, India, under Institutional Registration # 639/GO/02/a/CPCSEA).

2.2 *Initial controls*

Ovaries dissected from adult females (n = 5) at the start of the experiment in order to ascertain follicle composition at the start of the culture, served as initial controls.

2.3 *In vitro ovarian cultures*

All the glassware and surgical instruments used for the *in vitro* culture were cleaned and autoclaved at 15 lb for 20 min, prior to use. Adult gravid female zebrafish were euthanized by hypothermia (Wilson *et al.* 2009) and ovaries were excised and collected in zebrafish ringer solution (Nagahama *et al.* 1995). Left and right ovaries were then placed separately in two glass culture tubes containing 1 mL of culture medium (Miura *et al.* 1991). One of the ovaries was exposed to the treatment and the other ovary of the same animal was exposed to culture medium alone that served as control. Each treatment group consisted triplicate set of ovaries. The experiments were repeated in order to ascertain the consistency of the results.

2.4 *Culture medium*

Culture medium was composed of Leibovitz (L-15), 1.7 mM, Proline, 0.1 mM, Aspartic acid, 0.1 mM Glutamic acid, 0.5% Bovine Serum Albumin fraction-V, 50 µg/L retinol, and 10 mM HEPES, adjusted to pH 7.4 (Miura *et al.* 1991, Lokman *et al.* 2007). The medium along with supplements was replaced daily and the cultures were checked for contamination under a stereo zoom in the culture chamber. All ovarian explant cultures were maintained under aseptic conditions at 22°C on a clean bench of laminar air flow for 15 days. On the 16th day, cultures were terminated and ovaries were fixed in Bouin's fluid.

2.5 *Experimental design*

Treatment Groups	Experiment-I	Experiment-II
1.	Initial Controls (At the beginning of culture)	Initial Controls
2.	Controls (Culture medium alone)	Controls
3.	Insulin (1 ng/mL)	IGF1 (0.1 ng/mL)
4.	Insulin like growth factors 1 (IGF1 1 ng/mL)	IGF1 (1 ng/mL)
5.	Insulin (1 ng/mL) + IGF1 (1 ng/mL)	IGF1 (10 ng/mL)

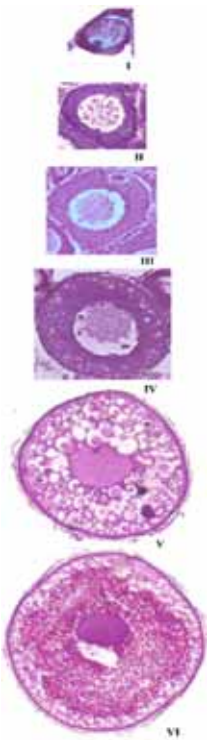
2.6 *Hormones, growth factors and chemicals*

Insulin (porcine) and human recombinant IGF1 were procured from Sigma-Aldrich, USA. Culture medium supplements, viz. HEPES, aspartic acid, glutamic acid retinol, and chemicals used for histology were purchased from Hi media, India.

2.7 *Tissue preparation for microscopy*

Bouin's fixed ovaries were processed for paraffin embedding; serial sections of 3 µm thick were cut using a semi-automated microtome (Leica RM2255) and stained with hematoxylin and eosin. Quantification of growing and degenerating follicles was made using a bright-field research microscope (Olympus) fitted with a digital camera (ProgRress, Capture, Jenoptik, Germany) for photomicrography.

Table 1. Staging of growing follicles of *D. rerio*

Stage	Size range (μm)	Characteristics	Images
I	< 125	Smallest individual previtellogenic follicles	
II	126–250	Previtellogenic follicles in perinucleolar stage	
III	251–375	Largest previtellogenic follicles	
IV	376–500	Follicles at peripheral cortical alveolar stage	
V	501–625	Follicles with yolk filled alveoli distributed throughout ooplasm	
VI	> 625	Vitellogenic follicles	
Atretic Follicles		Follicles exhibiting signs of degeneration	

2.8 *Classification and Quantification of Ovarian follicles*

Counting of follicles belonging to stage I–VI was made from serial histological sections using a microscope fitted with an ocular micrometer. The classification adapted for categorizing developing follicles was basically similar to that used by Selman *et al.* (1993) with a modification. Previtellogenic primary follicles were classified into 3 stages (I–III), based on their size, as the focus of the investigation was on this class of follicles, stage IV–VI constituted vitellogenic follicles, viz. stage IV follicles were with peripheral cortical alveoli (mark the beginning of vitellogenesis), stage V comprised follicles with yolk filled alveoli distributed throughout the ooplasm and stage VI included all vitellogenic follicles packed with yolk granules (table 1). Initially, the diameter of follicles (n = 200) belonging each stage was measured randomly and the arithmetic means were calculated, which were divided by the thickness of the section (3 μm) to get mean frequency (MF) of each follicle appearing in consecutive serial sections. All the follicles present in the cross section were enumerated from alternate serial sections, and sum of follicle count of each stage was then divided by the mean frequency of corresponding stage and multiplied by two to get the actual number of follicles belonging to that stage. Follicle counting was performed independently by second and third authors (SSN and BBG) in order to tally and confirm the readings.

2.9 *Statistical analysis*

The data were analysed using a non-parametric Mann-Whitney statistical test for small sample size (as n = 3 in the present study) to assess the significant difference between the sample medians of follicle counts of treated and corresponding control ovaries. The results were judged significant at $P < 0.05$ (Campbell 1989).

3. Results

3.1 *Initial controls*

Initial control ovary contained all the stages of normal follicles under growth and atretic follicles (figure 1A). A total of 1736–1884 follicles were present per single ovary with a mean value of 1804 ± 79 (figure 2). Stage-wise differential quantification indicated that stage I follicles amounted 61%, stage II—27%, stage III—1.64%, stage IV—0.74%, stage V—0.65%, stage VI—0.14% and AF formed 8.58% of total follicle count (figure 2).

3.2 *Ovaries cultured in vitro*

3.2.1 *Controls:* Histology of the control ovaries revealed that all the larger vitellogenic follicles underwent atresia and a few medium sized follicles were still in the process of degeneration and the total number of follicles per ovary was reduced drastically to 564 ± 116 on an average (figures 1B and 2). Most of the small previtellogenic follicles were unaffected and remained healthy (figure 1). Stage I, II and III follicles formed 60–63%, 26–28% and 1–2% of total follicle count respectively (figure 2). Small vitellogenic follicles amounted <1% and atretic follicles 10–30% (figure 2).

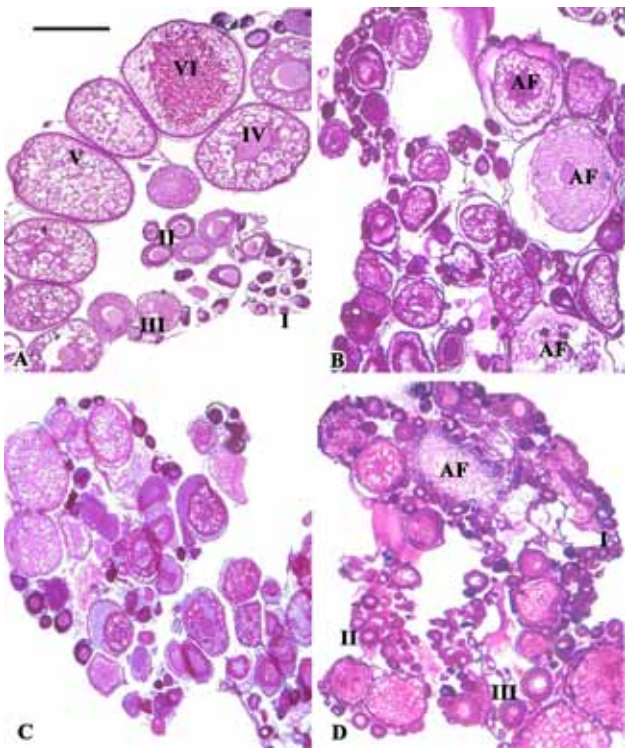


Figure 1. Histological cross sections of ovary of adult zebrafish (Hematoxylin eosin). Cross section of a portion of initial control ovary (**A**); *in vitro* cultured (15 days) control ovary (**B**); *in vitro* cultured ovary with supplementation of 1 ng/mL insulin (**C**); and ovary cultured in presence of 10 ng IGF1 (**D**). (Scale bar = 500 µm). Note the distribution of stage I follicles along ovarian epithelium and growing primary follicles towards the interior of the ovary.

3.2.2 *Effects of Insulin, IGF1 and Insulin + IGF1 on the formation and growth of follicles in ovarian explants cultured in vitro:* Ovarian explants cultured *in vitro* with supplementation of insulin contained a total of 435 ± 152 follicles, of which 64.5% were at the previtellogenic stage. 2.08% were vitellogenic and 33.29% of follicles were under degeneration; these values did not vary significantly from those recorded for corresponding controls (figures 1C and 2). While, in IGF1 treated ovarian explants, stage I, stage II, stage III and total number previtellogenic follicles were greater ($P < 0.05$) numerically compared to those of corresponding controls but the number of vitellogenic and atretic follicles remained unchanged (figures 2 and 3). In insulin + IGF1 supplemented group the follicle composition of explants remained comparable to those of controls and of insulin group (figures 2 and 3).

3.2.3 *Effects of different doses of IGF1 on the formation and growth of primary follicles in ovarian explants cultured in vitro:* Effects of different doses of IGF1 on the follicle counts of *in vitro* cultured ovaries in comparison with those of controls are shown in figures 3 and 4. The data indicate that IGF1 caused a dose-dependent numerical increase in stage I, stage II, stage III and total previtellogenic follicles and also total follicle count of ovary over those of control values. On the contrary, stage IV, V and VI vitellogenic follicles were reduced significantly (figure 3). Atretic follicles number was lower compared to controls (figures 1D, 3 and 4).

4. Discussion

IGF signalling is a highly conserved system in vertebrate evolution and adult gonads of all vertebrates are known to express abundantly both ligands and receptors of IGFs hinting their role in reproduction (Roith *et al.* 1993; Perrot *et al.* 2000; Maures *et al.* 2002; Schlueter *et al.* 2007; Werner *et al.* 2008). In mammals, IGFs are considered as intra-ovarian key regulators for follicle development and growth (Spicer 2004; Silva *et al.* 2009; Juengel *et al.* 2010). Although the involvement of IGFs in fish reproduction is relatively less understood compared to mammals, the former are known to express multiple copies of IGF genes (Shoae 2012). The expression of all three forms, IGF1, IGF2 and IGF3 and their receptors are documented in the fish ovary (Reinecke 2010; Nelson and Kraak 2010a, b; Sanchez and Smitz 2012; Li *et al.* 2015). IGF1 peptide and receptors are

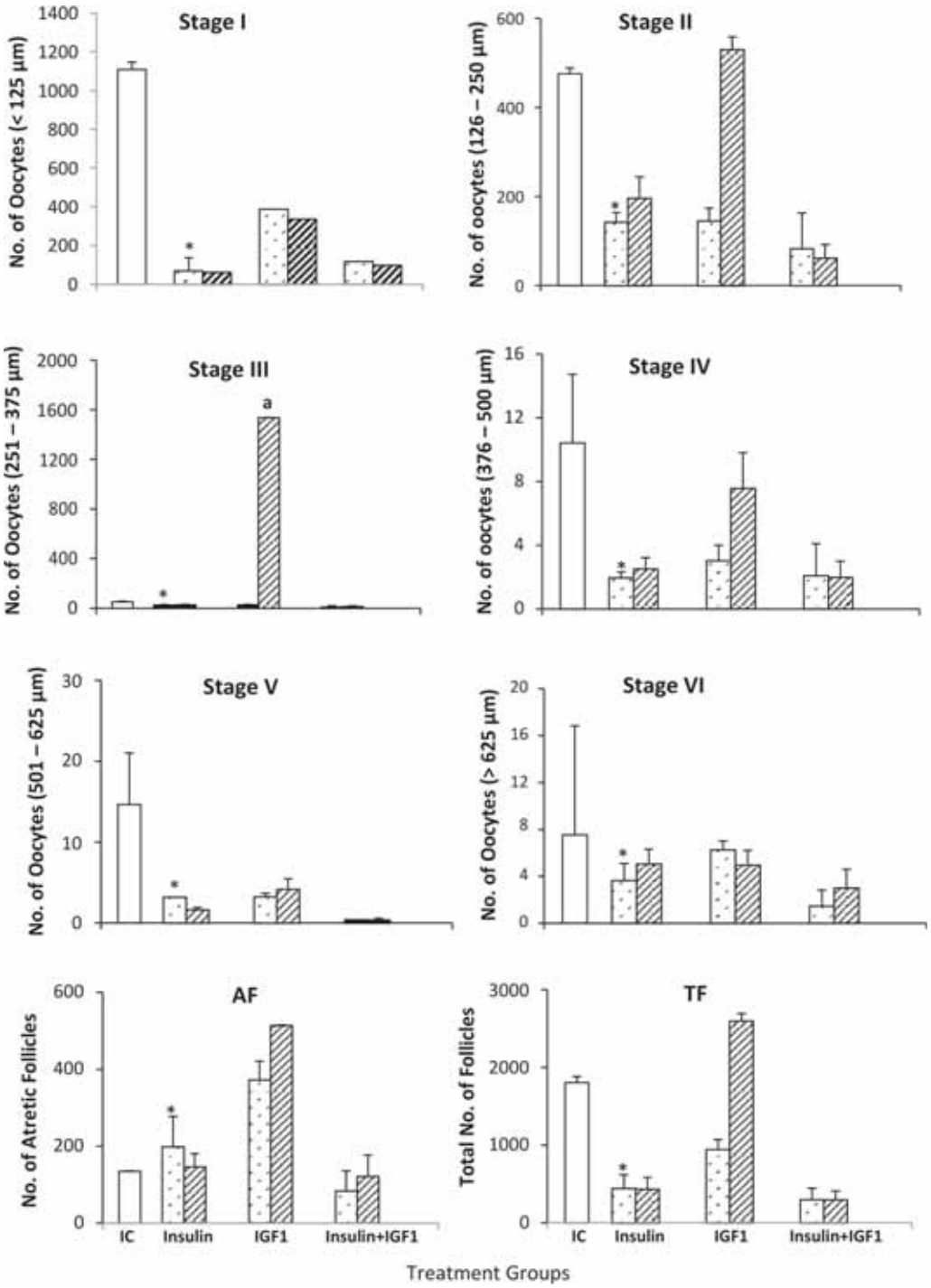


Figure 2. Frequency distribution (%) of stage I-VI follicles in the initial control and in *in vitro* cultured ovarian explants exposed to insulin (I), insulin like growth factor 1 (IGF1), insulin + IGF1, in comparison with controls (ovarian explants cultured in medium alone). Values are mean ± standard errors. * = significant compared to initial controls; 'a' = significant compared to controls. AF = atretic follicles; TF = total number of follicles.

reported in young developing primary ovarian follicles of tilapia, *Oreochromis niloticus, O. mosambicus,* gilthead seabream (*Sparus aurata*) and european seabass (*Dicentrachus labarx*) (Perrot *et al.* 2000; Berishvili *et al.* 2006; Garcia-Lopez *et al.* 2011). Likewise, IGF1 mRNA and peptides are localized in granulosa and thecal cells of the

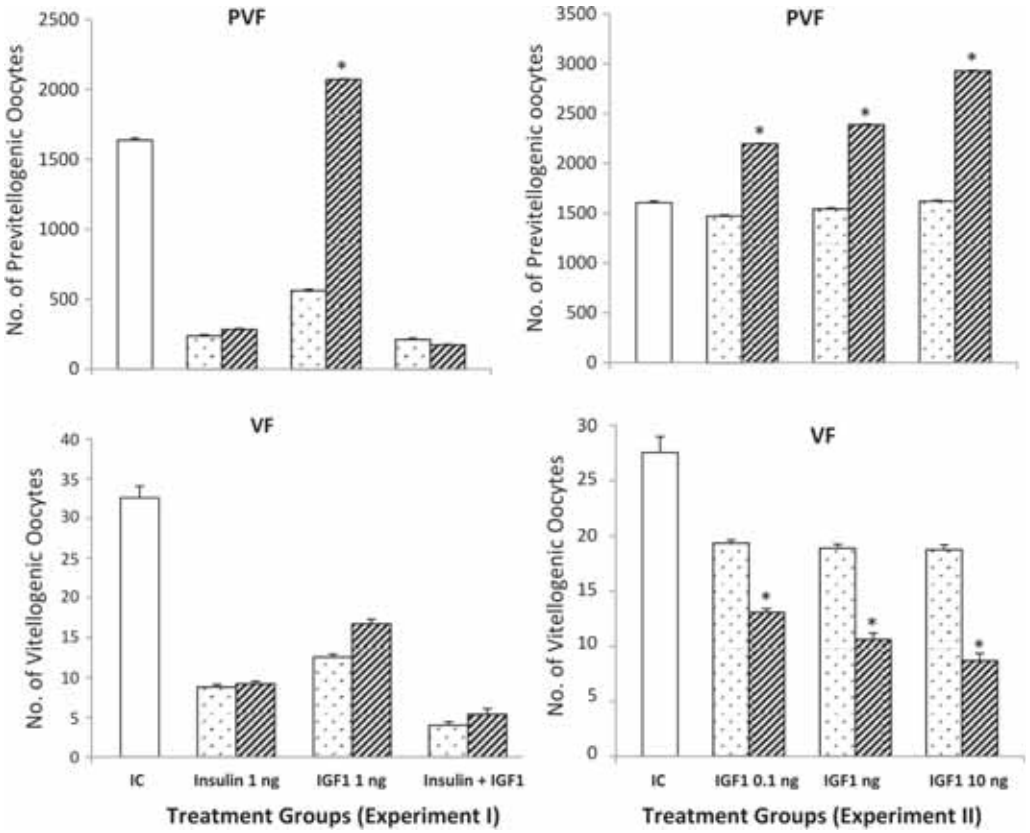


Figure 3. Frequency distribution of previtellogenic (PVF) and vitellogenic follicles (VF) in the initial control and *in vitro c*ultured ovarian explants exposed to insulin (I), insulin like growth factor 1 (IGF1), insulin + IGF1, in comparison with controls (ovarian explants cultured in culture medium alone) (Experiment I) and in ovarian explants exposed to graded concentrations of IGF1 (Experiment II). Values are mean ± standard errors. * = significant compared to corresponding controls.

ovary of red seabream, *Pagrus major* (Kagawa *et al.* 1995), gilthead seabream and tilapia species (Schmid *et al.* 1999; Perrot *et al.* 2000; Berishvili *et al.* 2006).

In zebrafish, IGF1, IGF2 and IGF3 peptides and receptors are detected in the ovary (Pang and Ge 2002; Nelson and Kraak 2010a, b; Irwin 2011; Shoae 2012; Irwin and Kraak 2012; Li *et al.* 2015). The role(s) of IGF system in the ovarian development of zebrafish is recently reported by Nelson and Kraak (2010a). In this fish, the IGF1system is differentially regulated by hormones and functions both in autocrine and paracrine manner to control intraovarian events (Nelson and Kraak 2010b, Irwin 2011). Likewise, insulin receptor sub types *insra* and *insrb* are expressed in midvitellogenic and fully grown follicles of zebrafish indicating their role of insulin in later stages of follicle growth and maturation (Das *et al.* 2016b).

In the present investigation involvement of insulin signalling, in the differentiation and growth of early primary follicles if any, was studied using systemic-free ovarian explant culture models supplemented with insulin (hormone) and IGF1 (growth factor) individually and in combination. The protocol used for ovarian explant culture was basically similar to that described earlier for eel ovary by Miura et al, (1991), Lokman et al, (2007) and Miura and Miura (2008) with slight modifications, i.e. (i) Culture medium was replaced daily in order to eliminate the possibility of contamination and provide the continuous supply of the nutrients for the growing follicles and (ii) Leibovitz L15 medium was supplemented with a buffering component HEPES that eliminated the necessity of CO2 gas circulation (Leibovitz 1963; Good *et al.* 1966). Temperature used was slightly lower than system temperature to avoid contamination as reported by earlier workers (Miura *et al.* 1991, 2002; Lokman *et al.* 2007; Miura and Miura 2008; Johnston 2013). Further, as the emphasis was to study the regulatory mechanisms involved in the production and growth of previtellogenic primary follicles, maintenance of vitellogenic follicles in ovarian explants was not addressed, therefore cultures were not supplemented with either vitellogenin or gonadotropin. Also, while, classifying follicles, a special emphasis was to primary follicles and they were

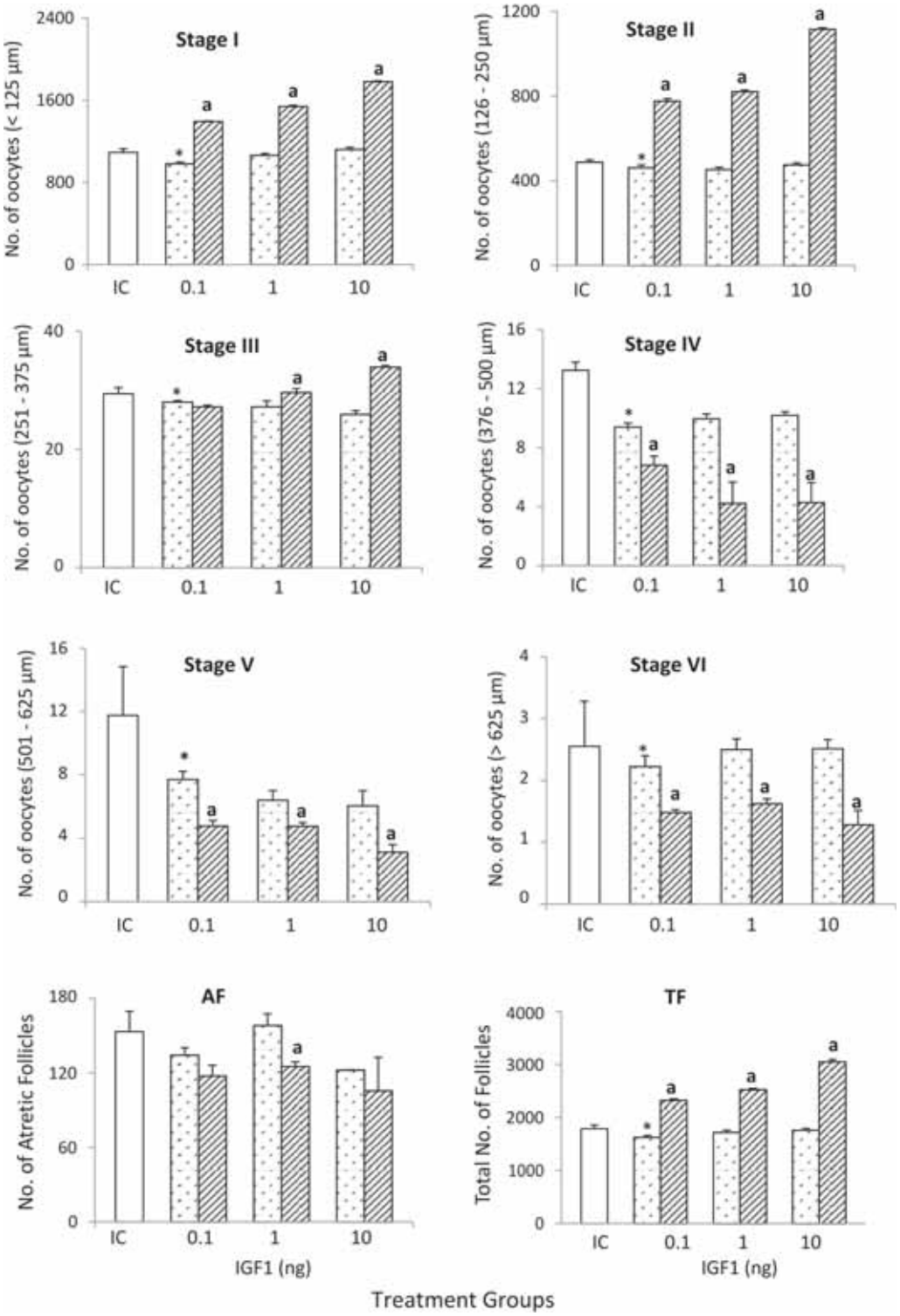


Figure 4. Frequency distribution (%) of follicles Stage I-VI in the initial control ovary and *in vitro* cultured ovarian explants exposed to 0.1 ng/mL, 1 ng/mL and 10 ng/mL insulin like growth factor 1 (IGF1) with corresponding controls (ovarian explants cultured in culture medium alone). AF = atretic follicles; TF = total number of follicles. Values are mean ± standard errors. * = significant compared to initial controls; 'a' = significant compared to controls.

categorized into 3 stages (I–III) based on their size that caused a slight deviation in the method from the standard classification/staging of ovarian follicles of zebrafish used by earlier workers (Selman *et al.* 1993).

The findings of the present study reveal that insulin individually and in combination with IGF1 did not cause any significant change in the number or size of the previtellogenic follicles in cultured ovarian explants. Interestingly, IGF1 alone induced the formation of previtellogenic follicles *de novo* and caused their growth to large previtellogenic/yolk vesicle stage (that marks the onset of vitellogenesis) in a dose-dependent manner in spite of the fact that the cultures were out of systemic influence and culture medium was not supplemented with gonadotropins. These results clearly indicate the following: (i) production and early growth of primary follicles is not obligatory to gonadotropins, (ii) insulin may not be involved in the process, (iii) locally derived IGF1 seems to have a role in the differentiation of primary follicles, (iv) as the ovaries have potential to produce steroid hormones (androgens/estrogens) locally, they may also have a regulatory influence, (v) if so; the regulatory actions of local hormones might be potentiated/synergized by intraovarian IGF1. Earlier studies on eel (*Anguilla australis*) ovary report that IGF1 and a nonaromatizable androgen 11 ketotestosterone increased the size of previtellogenic follicles in the ovarian explants cultured *in vitro* (Lokman *et al.* 2007). Similarly, in coho salmon *Onchorhynchus kisutch*, IGF1 stimulated the growth of late previtellogenic follicles in long term (21 days) cultured ovaries (Forsgren and Young 2012). In the *in vitro* cultured ovarian fragments of atlantic cod, *Gadus morhua,* androgens 11 ketotestosterone and testosterone increased the proportion of advanced staged previtellogenic follicles (Kortner *et al.* 2008; Kortner *et al.* 2009). IGF1 is reported to be involved in the early stages of oogenesis in the salmon, based on plasma dynamics studies (Campbell *et al.* 2006). In mammals (eutherian and marsupials) IGF1 is an important regulator of ovarian follicle development (Juengel *et al.* 2010). Supplementation of growth factors including IGF1 promotes meiosis in *in vitro* cultured mouse fetal ovaries (Lyrakou *et al.* 2002). In summary, the results of the present study direct that intra-ovarian IGF1 signalling is involved in regulation of the differentiation of primary follicles and their growth in the adult ovary of zebrafish.

5. Conclusion

The intra-ovarian IGF1 may be involved either directly or indirectly in the differentiation of primary follicles *de novo* and their growth in zebrafish ovary.

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