Morphological and molecular effects of cortisol and ACTH on zebrafish stage I and II follicles

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Abstract

Oogenesis in zebrafish (Danio rerio) is controlled by the hypothalamus–pituitary–gonadal axis and reproductive hormones. In addition, an interference of stress hormones is known with reproductive biology. In the presented work, we aimed to explore the hypothesis that cortisol (Cort) and ACTH may affect early oogenesis in zebrafish, given the presence of the specific receptors for glucocorticoids and ACTH in the zebrafish ovary. Follicles at stages I and II were exposed in vitro to 1 μM Cort and ACTH for 48 h, then ultrastructural and molecular effects were analyzed. The comet assay demonstrated increased tail moments for Cort and ACTH treatment indicative of DNA damage. The mRNA expression of apoptotic genes (bax, bcl-2) was not altered by both treatments, but Cort increased significantly the expression of the ACTH receptor (mc2r). Cort stimulated the presence of the endoplasmic reticulum, predominantly at stage II, while ACTH induced a strong vacuolization. Viability of oocytes was not affected by both treatments and fluorescent staining (monodansylcadaverine/acridine orange) indicated a reduced quantity of autophagosomes for ACTH, and lower presence of nucleic acids in ooplasm for Cort and ACTH. Concluding, different responses were observed for stress hormones on early stages of zebrafish oocytes, which suggest a role for both hormones in the stress-mediated adverse effects on female gametogenesis.


Introduction

Five stages of oocyte development have been described in the oogenesis of zebrafish: primary growth phase (stage I), cortical alveoli stage (II), vitellogenic stages III and IV, and mature stage V (Selman et al. 1993). The oocyte stages can be distinguished by size (stage I: <0.14 mm; stage II: 0.14–0.34 mm; stage III: 0.34–0.69 mm; stage IV: 0.69–0.73 mm; stage V: >0.73 mm), which increases in particular at stage II due to the appearance of cortical alveoli, and at stages III and IV due to the incorporation of hepatic-exported vitello-genin. The oogenesis is regulated at a higher level through the hypothalamus–pituitary–gonadal (HPG) axis by secretion of gonadotropin hormones, the follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Feist & Schreck 1996, Clelland & Peng 2009). Gonadotropins regulate the growth, differentiation and the steroidogenesis in the ovary depending on the oocyte stages. In contrast to the huge amount of information about endocrine and paracrine regulation of oogenesis in the oocyte stages III–V (Pang & Ge 2002a,b, Clelland & Peng 2009), little is known on the mechanisms regulating the development of the early oocyte stages (I and II). This is surprising, since it is known that important steps take place in the primary growth phase, which involves the inclusion of maternal RNA, the beginning lipid accumulation and synthesis, or the formation of the follicular cell layers (Urbatzka et al. 2011).

In addition to the role of reproductive hormones in the regulation of oogenesis (Sousa et al. 2015), other hormones may have the potential to exert some effects on this process. For instance, it has been suggested that an interaction exists between growth and the reproductive system, with larger fish of the same age possessing advanced oocyte stages (Campbell et al. 2006). Furthermore, a crosstalk between the reproductive HPG axis and the stress axis, hypothalamus–pituitary–adrenal (HPA) axis is known, with suppression effects of stress hormones on either the gonadotropins in the pituitary (Milla et al. 2009), steroidogenesis in the gonads (Alsop et al. 2009) or gonad size (Carragher & Sumpter 1990). Other adverse effects on female reproductive performance in teleost species comprised follicle atresia,
declined progeny quality or lowered vitellogenesis (Milla et al. 2009). Again the majority of effects were studied on later oocyte stages, focussing on oocyte maturation and ovulation (Milla et al. 2009). Generally, the stress response is mediated by the secretion of adrenocorticotropic hormone (ACTH) by the pituitary gland. ACTH stimulates the corticosteroid synthesis in the interrenal cells of the head kidney leading to the cortisol (Cort) release into the blood during a stress response (Mommsen et al. 1999, Fuzzen et al. 2011). Plasma ACTH level in rainbow trout were about 120 pg/ml (Aluru & Vijayan 2008) and in tilapia 40–50 pg/ml (Balm et al. 1995). Plasma level of Cort ranges from 1 to 400 ng/ml in teleost fish varying between species and reproductive status (Milla et al. 2009); 40–60 ng/ml were reported in zebrafish (Felix et al. 2013). Furthermore, gonads of teleost fish may have the capacity of corticosteroidogenesis (Milla et al. 2009), and receptors for glucocorticoids are present in the gonads. In zebrafish, the ACTH specific receptor, melanocortin-2 receptor (mc2r), was identified in male and female gonads, and ACTH suppressed the human choriogonadotropin stimulated E2 secretion (Alsop et al. 2009). Two isoforms of the glucocorticoid receptors (alpha and beta) were identified in zebrafish. GRα is the main receptor binding dexamethasone and Cort, while GRβ is a splice variant that inhibits GRα (Schaaf et al. 2008). The available data about the presence and functionality of the receptors provide first indications that stress hormones might have a particular role during oogenesis in teleost fish.

In the presented work, we aimed to explore the hypothesis that Cort and ACTH may affect the early oogenesis in zebrafish, given the presence of the specific receptors for glucocorticoids and ACTH in the zebrafish ovary. Therefore, stage I and II follicles were cultured in a recently established in vitro system (Sousa et al. 2014) and incubated for 48 h with Cort and ACTH at a concentration of 1 μM. Transmission electron microscopy (TEM) was employed to study effects of the stress hormones on the ultrastructural morphology of the oocytes. The induction of DNA damage, indicative of oocyte apoptosis, was analyzed by the Comet assay. Results were complemented by real-time PCR quantification of pro-apoptotic (bax) and anti-apoptotic (bcl-2) genes, glucocorticoid (nr3c1) and melanocortin receptor 2 (mc2r), and by fluorescent staining of autophagosomes and nucleic acids.

Materials and methods

An approval by an ethics committee was not necessary for the presented work, since chosen in vitro procedures are not considered animal experimentation according to the EC Directive 86/609/EEC for animal experiments.

Isolation of ovarian follicles and in vitro exposure experiment

The oocytes for the in vitro culture were prepared from 6-month-old females of zebrafish as previously described in full detail (Sousa et al. 2014), using either oocyte fragments or follicles. Briefly, the follicles were removed and cleaned in a PBS solution with 1% penicillin and streptomycin (Sigma–Aldrich) and 0.1% amphotericin B (Sigma–Aldrich). Follicles were carefully separated by needles, smooth pipetting and gentle agitation in EDTA 0.1 M for two rounds of 15 min, and then sorted by size.

The final concentration of hydrocortisone (synonym of cortisol (Cort); Sigma–Aldrich) or ACTH (rat; NHP, National Hormone and Peptide Program, CA, USA) was 1 μM for hormones, and the solvent control group contained 0.1% of ethanol (EtOH). The chosen concentration of stress hormones was in the range of other studies that induced physiological effects in exposed teleost ovarian follicles in vitro (goldfish, carp, snapper: 2.7 μM Cort, Pankhurst et al. 1995; zebrafish: 0.5 μM Cort, 1.5 IU/ml ACTH (4 μM ACTH), Alsop et al. 2009). The exposure lasted for 2 days (48 h) and the medium was changed every 24 h of exposure. A 48 h exposure regime was chosen from previous work demonstrating morphological effects in zebrafish follicles in vitro (Sousa et al. 2015). The exposure was performed in 24 well-plates using either 20–25 stage I follicles, 20–25 stage II follicles or 2–3 ovarian fragments per well. Six biological replicates (n = 6) were performed per each treatment group and for both culture methods using each six individual female zebrafish. After the exposure, fragments of ovaries were processed for electron microscopy observation, and follicles were used to perform the comet assay, real-time PCR, viability assay and fluorescence staining in the conditions described in the following paragraph.

Viability of exposed follicles were analysed by trypan blue staining, which is based on membrane integrity. Final concentration of trypan blue was 0.1% in PBS; live oocytes showed only membrane staining, while dead oocytes incorporate trypan blue and stained blue. Viability was assessed using various concentrations of Cort and ACTH (1 μM to 10 nM) and was not different between EtOH and Cort or ACTH treatment groups of all tested concentrations respectively. Follicles displayed an average of 80% viability, which is in concordance with the recently published in vitro culture procedure for zebrafish follicles (Sousa et al. 2014).

Electron microscopy

The processing of the samples for TEM can be found in detail as previously described (Sousa et al. 2014). For stage I, the following parameters were studied: mitochondria in the ooplasm, germinal vesicle reticulation, Golgi apparatus, zona pellucida development, and perinucleus. For stage II, the following parameters were evaluated: mitochondria agglomerate in the ooplasm, mitochondria dispersed, cortical alveoli, rough endoplasmic reticulum (rER) (Meijide et al. 2005) around the cortical alveoli, rER in the ooplasm, smooth endoplasmic reticulum (sER) in the ooplasm, Golgi apparatus, and zona pellucida development. Three qualitative classes were defined.
for these parameters: absent, present, abundant (Sousa et al. 2015). The frequency of observations was classified into four different percentage distributions (+, 0–25%; ++, 25–50%; ++++, 50–75%; ++++, 75–100%).

**Alkaline Comet assay**

Frosted microscope slides (VWR, Carnaxide, Portugal) were covered with 1% normal melting point agarose (NMA) in PBS and dried overnight at room temperature (Belpaeme et al. 1998, Lacaze et al. 2010). Follicles of stages I and II were collected and mixed in 1% low melting point agarose (LMA). The mixture was loaded on the slide (over the NMA layer) and covered with a coverslip. A third layer of LMA was added on top and slides were lysed in freshly made alkaline solution (2.5 M NaCl; 100 mM Na₂ EDTA; 10 mM Tris-HCl, pH 10; 1% Triton X-100; 10% DMSO) at 4°C overnight. After lysis, the DNA unwinding was performed by submergence of the slides in alkaline solution (1 mM EDTA and 300 mM NaOH, pH 13) for 40 min in ice-cold conditions, before electrophoresis was conducted at 20 V and 300 mA for 30 min. Slides were neutralized three times for 5 min with a neutralization buffer (Tris-HCl pH 7.5) and stained with 0.02% ethidium bromide for 15 min. After cleaning the slides with ultrapure water, they were observed under UV light with an inverted fluorescent microscope Olympus IX71 and photographed by an Olympus microscope (Olympus, IX71) and photographed by an Olympus DP71 camera. The freeware Comet Score (freeware from TriTek, Summerduck, VA, USA) was used for scoring the comets including the PCR efficiencies according to Pfaffl (2001).

**Fluorescence staining**

Exposed follicles to Cort and ACTH (as previously described) were stained with a specific dye for autophagosomes (monodansylcadaverine (MDC)) and with acridine orange (AO) indicative of acidic vesicles (red emission) or cytoplasmic nucleic acids (green emission). Oocytes were incubated for 6 h in the final concentration of 0.3 mM MDC or 10 μg/ml AO, fixed for 2 h in 4% formaldehyde and washed in PBS several times before mounted with fluoromount (Sigma) on viewing chambers. Slides were viewed on a fluorescent microscope (Olympus, IX71) and photographed by an Olympus DP71 camera.

<table>
<thead>
<tr>
<th>Gene name (GenBank identifier)</th>
<th>Forward primer 5′–3′</th>
<th>Reverse primer 5′–3′</th>
<th>Amplification efficiency (%)</th>
<th>Annealing temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melanocortin receptor 2, mc2r (AY161848)</td>
<td>AATCTCCCCTCCAGCATCC</td>
<td>ATCACGCCCAACTACCC</td>
<td>89.2</td>
<td>55</td>
</tr>
<tr>
<td>Glucocorticoid receptor α, nr3c1 (EF436284)</td>
<td>ATTACCTGTGTGTCGGGCGGCACAC</td>
<td>GGAGTGGACTGTGCTGGATTACC</td>
<td>85.4</td>
<td>59</td>
</tr>
<tr>
<td>bcl-2 associated X protein, bax (AF231015)</td>
<td>TGCCCTGCGCCTTGCATCC</td>
<td>GTCCCATCCACCTGTCC</td>
<td>91.5</td>
<td>55</td>
</tr>
<tr>
<td>B-cell lymphoma 2, bcl-2 (NM_001030253)</td>
<td>AATCTTCTCAACTCTG</td>
<td>ATCTTTCTATTCCTCC</td>
<td>82.7</td>
<td>55</td>
</tr>
<tr>
<td>Elongation factor 1 α, eif1a (AY422992)</td>
<td>ATCCGCTGTGGTAATGTGGTGAGCAGTGTGGCAATCC</td>
<td>92.8</td>
<td>59</td>
<td></td>
</tr>
<tr>
<td>Ribosomal protein L8, rpl8 (BC065432)</td>
<td>ATAGTCTCGTGTCTGGAGGAG</td>
<td>TCGGAGGTGTGGGAATAACCG</td>
<td>90.6</td>
<td>59</td>
</tr>
</tbody>
</table>

Table 1 Sequences, amplification efficiencies, and annealing temperature of the primers used for real-time PCR in zebrafish (*Danio rerio*) oocytes.
Statistics

Statistical analysis was performed using GraphPad Prism version 5.00 for Windows, GraphPad Software (San Diego, CA, USA). For all data, first the normality and equal variances were tested (Kolmogorov–Smirnov, Barthlett’s test). If conditions of ANOVA were fulfilled, one-way ANOVA was performed followed by Tukey’s post hoc test. If conditions were not met, non-parametric tests were performed. The Kruskall–Wallis test was performed followed by Dunn’s multiple comparison post-hoc test. In all analysed data, differences of \( P < 0.05 \) were regarded as statistically significant.

Results

Transmission electron microscopy

Different responses were obtained with the stress hormones studied. Stage I follicles exposed to Cort revealed a higher proliferation of sER than in the control group (Table 2). Observations of stage I follicles exposed to ACTH revealed no alterations compared to the control.

At stage II, Cort strongly stimulated the presence of rER in the ooplasm in a form similar to annulate lamellae (Fig. 1) and this result was consistent in all observations (Table 2). ACTH induced a prominent vacuolization in the ooplasm of almost all follicles observed at stage II (Fig. 1). The strong vesicle formation impeded frequently the observation of other ultrastructural parameters.

Comet assay

The follicles from the exposure experiment were employed to analyse the induction of DNA damage by the Cort and ACTH. The analyses were performed separately for stages I and II (Fig. 2) and the damage was more evident in stage II than in stage I. In stage I, Cort, ACTH and \( H_2O_2 \) (as positive control) induced a two-, 2.5- and fivefold more elevated tail moment compared to the control, respectively. For stage II follicles, Cort, ACTH and \( H_2O_2 \) elevated the tail moment six-, six- and 11-fold compared to the control respectively. Cort and ACTH induced a significantly higher DNA damage, visible as increased tail moment, compared to the control in stages I and II (\( P < 0.05 \)).

mRNA expression

The mRNA expression coding for the receptor of ACTH (mc2r) and of glucocorticoids (nr3c1) were analysed in stage I and II follicles. Cort, but not ACTH, induced an increased mRNA level of mc2r in stage II follicles (\( P < 0.05 \)) compared to the solvent control (Fig. 3). The mRNA expression of nr3c1 was not changed in stage II follicles. The mRNA expression of a pro-apoptotic (bax) and an anti-apoptotic gene (bcl-2) did not reveal any alterations of the transcript level of both genes in response to Cort and ACTH treatment in stage II follicles (Fig. 3). No alterations were observed for all analyzed genes in stage I follicles (data not shown).

Table 2 Frequency distribution of the ultrastructural observations of zebrafish follicles exposed to stress hormones.

<table>
<thead>
<tr>
<th>Stage</th>
<th>sER</th>
<th>rER</th>
<th>Vacuolization</th>
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<tr>
<td></td>
<td>Absent</td>
<td>Present</td>
<td>Abundant</td>
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<td>Stage I</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cort (1 µM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACTH (1 µM)</td>
<td></td>
<td></td>
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<td></td>
<td></td>
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</table>

The frequency of observations was classified into four different percentage distributions (++, 0–25%; +++, 25–50%; ++++, 50–75%; +++++, 75–100%). EtOH, solvent control; Cort, Cortisol; ACTH, adrenocorticotropic hormone; sER, smooth endocrine reticulum; rER, rough endocrine reticulum.

Figure 1 Representative images of TEM of follicles at stage II exposed to stress hormones. EtOH – solvent control, 0.1% ethanol; Cort – 1 µM Cortisol; ACTH – 1 µM adrenocorticotropic hormone. The left images show the periphery of the oocytes and the right images the ooplasm. Mit, mitochondria; oo, ooplasm; sER, smooth endocrine reticulum; rER, rough endocrine reticulum; CA, cortical alveoli; zp, zona pellucida; T, Thecal cell; G, granulosa cell; *, vacuoles.
Fluorescent staining

The MDC staining demonstrated lower fluorescence intensity in the stage II oocytes from the ACTH treatment group \( (P!0.05) \) compared to the control, while no difference was present in the Cort treatment group (Fig. 4). AO staining with red emission (indicative of acidic vesicle) was not observed in the ooplasm of stage I and II follicles. AO staining with green emission (indicative of nucleic acids) was reduced in both Cort and ACTH treated follicles of stages I and II compared to the control \( (P!0.05) \). Only data for stage II are presented (Fig. 4).

Discussion

Although it is generally accepted that stress hormones affect the reproductive physiology of female teleost fish, especially during oocyte maturation, ovulation (Milla et al. 2009) or disrupting normal steroidogenesis (Carragher et al. 1989, Carragher & Sumpter 1990), few information exist about the potential effects of stress hormones on the development of teleost oocytes at the early oocyte stages.

The comet assay is a widely used technique that detects single or double-strand breaks and therewith genotoxic effects of compounds or apoptosis (Tice et al. 2000, Liao et al. 2009). Studies using the comet assay detected that steroids, in particular estradiol, synthetic (xeno)oeostrogens (Yared et al. 2002, Djelic & Anderson 2003, Petridis et al. 2009) or thyroid hormone and T3 (Djelic & Anderson 2003), can be genotoxic agents, as they induce the formation of reactive oxygen species. No works have been published so far evaluating the effects of stress hormones on DNA damage in oocytes. In the present work, the exposure of zebrafish follicles at stages I and II revealed that both stress hormones, Cort and ACTH, induced a significant increase in the tail moments in both oocyte stages, which is indicative of increased DNA damage. In accordance to our data, Cort and catcholamines induced DNA damage in the fibroblast murine cell line 3T3 (Flint et al. 2007). Despite some studies relating the comet assay with apoptosis (Chiang et al. 2010), the alkaline comet assay cannot elucidate which type of DNA damage is occurring, single-strand or double-strand breaks (Chourcoun et al. 2001, Roser et al. 2001, Collins 2004).

Glucocorticoids have been shown to induce genes that are involved in the stimulation of apoptosis (Koller 2000, Sapolsky et al. 2000), especially in studies involving immune cells, but also in germline cells (Baum et al. 2005). Glucocorticoids activate the glucocorticoid receptors that in turn can activate apoptosis via genomic and non-genomic actions.
were studied, which belong to the intrinsic, mitochondrial dysfunction was hypothesized (Aluru & Vijayan 2008).

Role for ACTH signalling in stress-mediated reproductive function in gonads is still unknown. An auto-regulation by ACTH was described in rainbow trout and a zebrafish gonads (Alsop & Vijayan 2008). Our best knowledge, we present for the first time evidence for an effect of ACTH on the ultrastructural morphology of oocytes, instead of the classical pathway of action via the intercellular cells to induce Cort.

Regarding the ultrastructural morphology, oocytes had very different responses to the exposure of stress hormones Cort and ACTH. Cort increased the sER development in oocytes stage I, but especially induced a strong proliferation of rER in stage II oocytes. In contrast, a high vacuolization was observed in the oocytes exposed to ACTH, especially at stage II. A stimulating effect of Cort on the proliferation of endoplasmic reticulum in stage I and II oocytes, as observed in our data, was not described yet in other studies with oocytes. However, in the thymus of chicken embryo (Chan 1991) and in rat hepatocytes, the endoplasmic reticulum was more developed and increased in size and complexity in response to Cort (Chan 1991, Pilichos et al. 2005). Further investigations are needed to analyze whether the increase of endoplasmic reticulum by Cort may be a sign of stimulated early oogenesis. In contrast to the effects of Cort, the ultrastructural analyses in oocytes exposed to ACTH revealed a strong vacuolization in the ooplasm. Similar results were described from an exposure experiment in rats, where ACTH induced hemorrhage, a strong vacuolization and a higher number of apoptotic cells in the adrenal gland in a dose-dependent manner (Burkhardt et al. 2011). Interestingly, an increase of nucleosomes (histone-complexed DNA fragments) in the blood was observed in the same study (Burkhardt et al. 2011), which is in agreement to our observation of an increased DNA damage. In an attempt to characterize the induced vacuolization by ACTH, fluorescence staining was applied using MDC as specific dye for autophagosomes (Biederbick et al. 1995, Escobar et al. 2010) and acridine orange for acidic compartments (red emission) or nucleic acids (green emission) (Okuthe 2013, Notaro et al. 2014). ACTH treatment led to a significantly reduced incorporation of MDC into the ooplasm of stage II oocytes, which could be indicative of a lower presence of autophagosomes or an increased removal rate. Both stress hormones reduced significantly the green fluorescence intensity of stage I and II follicles, which demonstrated a reduced presence of nucleic acids in the ooplasm (DNA & RNA). Cytoplasmic nucleic acid level in zebrafish oocytes are mostly derived from RNA-containing free ribosomes associated with the endoplasmic reticulum and therefore a proxy for protein synthesis (Okuthe 2013). Hence, reduced AO staining in Cort and ACTH exposure could be related to a reduced protein synthesis at the endoplasmic reticulum. Viability analysis demonstrated that the vacuolization was not connected to an alteration of viability, and therefore was not a sign of oocyte degradation or follicular atresia. To our best knowledge, we present for the first time evidence for an effect of ACTH on the ultrastructural morphology of oocytes, instead of the classical pathway of acting via the intercellular cells to induce Cort.

Conclusion

ACTH and Cort both induced DNA damage in follicle stages I and II as revealed by the Comet assay. Cort increased significantly the mRNA expression of mc2r in stage II follicles and stimulated the presence of the endoplasmic reticulum in stages I and II. In contrast, a high level of oocyte vacuolization was observed in response to the ACTH treatment in stage II follicles, which did not influence their viability. Fluorescent staining revealed a reduced quantity of autophagosomes for ACTH, and a reduced content of nucleic acids in the

Figure 4: Analysis of fluorescence staining of stage II follicles. Monodansylcadaverine (MDC) stains specifically autophagosomes and acridine orange (AO) the nucleic acids (RNA and DNA): EtOH – solvent control, 0.1% ethanol; Cort = 1 μM Cortisol; ACTH = 1 μM adrenocorticotropic hormone.
ooplasm for both stress hormones. The data presented here demonstrated different responses of Cort and ACTH exposure on zebrafish follicles, which suggest a role for both hormones in the stress-mediated adverse effects on female gametogenesis.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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