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.

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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 vitellogenin. 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 adrenocorticotrophic 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 (Mommensen *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 chorionic gonadotropin stimulated E₂ secretion (Alsop *et al.* 2009) in zebrafish follicles. Two isoforms of the glucocorticoid receptors (alpha and beta) were identified in zebrafish. GR α is the main receptor binding dexamethasone and Cort, while GR β is a splice variant that inhibits GR α (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 μ M. 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; NHPP, National Hormone and Peptide Program, CA, USA) was 1 μ M 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 μ M Cort, Pankhurst *et al.* 1995; zebrafish: 0.5 μ M Cort, 1.5 I.U./ml ACTH (4 μ M 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 μ M 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 perinuclear. 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 submersion 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 DP71 camera. The freeware Comet Score (freeware from TriTek, Summerduck, VA, USA) was used for scoring the comets and the results were analysed for the 'tail moment', which is the product of the percentage of DNA in the tail with the length of the comet. Hydrogen peroxide (H₂O₂) was used as positive control.

mRNA expression

Total RNA of stage I and II follicles, respectively, was extracted with the Nucleospin RNA XS kit (Macherey-Nagel, Dueren, Germany) including an on-column DNase digestion step according to the manufacturer instructions. RNA content was measured with the Qubit fluorometer (Quant-it RNA BR assay kit) and 90 ng of total RNA was transcribed into cDNA in a 10 µl reaction by using the iScript cDNA synthesis kit (Bio-Rad)

(Sousa *et al.* 2015). Real-time PCR assays were developed to determine the mRNA expression of melanocortin receptor 2 (*mc2r*), glucocorticoid receptor α (*nr3c1*), bcl-2 associated X protein (*bax*), B-cell lymphoma 2 (*bcl-2*), elongation factor 1 α (*ef1a*) and ribosomal protein L8 (*rpL8*) in oocytes. Quantitative PCR primers were designed with the software Beacon Designer (PREMIER Biosoft International) to span an intron sequence; primer sequences and amplification efficiencies derived from standard curves of cDNA dilution series are given in Table 1. The PCR efficiencies of the assays ranged between 82.7 and 92.8%. Two µl of a 1:10 dilution of the original cDNA sample was added to a reaction mixture containing 1 × iQ SYBR Green Supermix (Bio-Rad) and 200 nM of each primer, making a total volume of 20 µl per reaction. The PCR profile had the following conditions: 95 °C of initial denaturation for 5 min; 95 °C for 20 s; annealing temperature for 20 s; and 72 °C extension 20 s for 40 cycles. No template controls were included in each run and melting curves were generated to confirm the specificity of the assays.

The expression of target mRNA was normalized using a multiple reference gene approach in order to avoid quantification bias (Urbatzka *et al.* 2013) according to the Bestkeeper software (Pfaffl *et al.* 2004). The mRNA expression of *ef1a* and *rpL8* and their amplification efficiencies were used to calculate the Bestkeeper normalization factor, which was steadily expressed under the chosen experimental settings. Relative gene expression was then calculated with the $\Delta\Delta C_t$ method 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 1 Sequences, amplification efficiencies, and annealing temperature of the primers used for real-time PCR in zebrafish (*Danio rerio*) oocytes.

Gene name (GenBank identifier)	Forward primer 5'–3'	Reverse primer 5'–3'	Amplification efficiency (%)	Annealing temperature (°C)
Melanocortin receptor 2, mc2r (AY161848)	AATCTCCCTCCAGCATCC	ATCACAGCCACAATCACC	89.2	55
Glucocorticoid receptor α , nr3c1 (EF436284)	ATTACCTGTGTGCTGGGCGAAAC	GGAATGGACTGCTGCTGGATTACC	85.4	59
bcl-2 associated X protein, bax (AF231015)	TGCCTGTGCGCTTGTCATC	GTCCCATCCACCCTGTTCC	91.5	55
B-cell lymphoma 2, bcl-2 (NM_001030253)	AATCCTCTCCAACTCTG	ATCCTTTCTATTTCATCTCC	82.7	55
Elongation factor 1 α , ef1a (AY422992)	ATCCGTCGTGGTAATGTGG	TGAGCAGTGTGGCAATCC	92.8	59
Ribosomal protein L8, rpL8 (BC065432)	ATAGTCTGCTGTCTGGAGGAG	TCGGGATTGTGGGAAATAACG	90.6	59

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

Stage I	sER			Stage II	rER			Vacuolization		
	Absent	Present	Abundant		Absent	Present	Abundant	Absent	Present	Abundant
EtOH (0.1%)	+	++	++	EtOH (0.1%)	+++	++	—	++++	—	—
Cort (1 µM)	—	—	++++	Cort (1 µM)	—	—	++++	+++	+	—
ACTH (1 µM)	+	+++	+	ACTH (1 µM)	++	+	—	—	+	++++

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, adrenocorticotrophic hormone; sER, smooth endocrine reticulum; rER, rough endocrine reticulum.

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, Bartlett'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 Kruskal–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).

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 adrenocorticotrophic hormone. The left images show the periphery of the oocytes and the right images show 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.

Figure 2 Comet assay of zebrafish follicles exposed to stress hormones. (A) Stage I, total number of oocytes > 142; (B) Stage II, total number of oocytes > 73. The assay was replicated with six individual females, and 20–30 stage I oocytes and 10–15 stage II oocytes were used from each female per treatment group. EtOH – solvent control, 0.1% ethanol; Cort – 1 μ M Cortisol; ACTH – 1 μ M adrenocorticotrophic hormone. Different letters represent significant differences at $P < 0.05$.

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)estrogens (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 catecholamines 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 (Choucroun *et al.* 2001, Roser *et al.* 2001, Collins 2004).

Glucocorticoids have been shown to induce genes that are involved in the stimulation of apoptosis (Kofler 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

Figure 3 Expression of mRNA of ACTH receptor (*mc2r*) and glucocorticoid receptor α (*nr3c1*) in follicles of stage II exposed to the different stress hormones. The mRNA expression was normalized with the geometrical mean of *ef1a* and *rpl8* derived by the Bestkeeper approach, and represent the values of six individual females. Different letters represent significant differences at $P < 0.05$; NS, not significant. EtOH – solvent control, 0.1% ethanol; Cort – 1 μ M Cortisol; ACTH – 1 μ M adrenocorticotrophic hormone; rel mRNA expression – relative mRNA expression vs the solvent control EtOH.

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 μ M Cortisol; ACTH – 1 μ M adrenocorticotrophic hormone.

(Schlossmacher *et al.* 2011). In contrast, glucocorticoids served as potent inhibitors of apoptosis in primary rat and human granulosa cells (Sasson & Amsterdam 2003). In our study, the mRNA expression of apoptosis related genes, *bax* (pro-apoptotic) and *bcl-2* (anti-apoptotic) were studied, which belong to the intrinsic, mitochondrial apoptosis pathway (Schlossmacher *et al.* 2011). However, both genes did not change in the stage I or II follicles exposed to Cort and ACTH compared to the solvent control. This way, apoptosis did not seem to be induced by the stress hormones, at least on the transcriptional level.

Furthermore, the mRNA expression of glucocorticoid receptors were analyzed, and the exposure of oocytes to Cort at stage II increased significantly the mRNA expression of *mc2r*, an effect that was not observed for ACTH (Fig. 3). During the embryo development of zebrafish, a parallel increase of Cort and *mc2r* was observed, which suggested a regulation of the mRNA expression of *mc2r* by Cort (Alsop & Vijayan 2008). Our data support such a regulation of *mc2r* by Cort. The *mc2r* is highly expressed in the adrenal cortex (interrenal tissue in fishes) and when bound with ACTH leads to glucocorticoid biosynthesis (Webb & Clark 2010). The presence of *mc2r* was detected in trout (Aluru & Vijayan 2008) and zebrafish gonads (Alsop *et al.* 2009), but the function in gonads is still unknown. An auto-regulation of *mc2r* by ACTH was described in rainbow trout and a role for ACTH signalling in stress-mediated reproductive dysfunction was hypothesized (Aluru & Vijayan 2008).

Regarding the ultrastructural morphology, oocytes had very different responses to the exposure of the 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 interrenal 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

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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