Effect of Maternal Estradiol Hormone on Gene Expression in Zebrafish (*Danio rerio*) Embryos and its Clinical Implications

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ABSTRACT: Maternal steroid hormone, estradiol plays significant role in fertilized eggs by performing proper embryo development. The study was performed to observe the genetic effects of estradiol by using reverse transcriptase polymerase chain reaction. A total three steroid responsive genes were analysed namely *anx2a*, *ccng1* and *hmox1*. The *ef1a* gene was used as reference to normalize data. The transcripts of *anx2a* were down regulated in E2 0.01 treated embryos at 8 hpf when compared to contol and E2 0.1 treated embryos. The *ccng1* transcripts for E2 0.1 and E2 0.01 and E2 treated embryos were equally and strongly expressed. There was also no up or down-regulation when analysed with *hmox1* gene. So the results suggest that different estradiol concentrations can alter specific genetic expression at 8 hpf embryonic state and clinically it could be used as an effective gene therapy. However, extensive studies should be conducted to assess its beneficial effects on human being.

Key words: Gene expression, Estradiol hormone, Zebrafish, Clinical implications

INTRODUCTION

In teleost fish like other oviparous vertebrates, the yolk of ovulated eggs contain significant amount of liposoluble hormones such as thyroid, steroid and retinoid etc. These hormones are taken up from either maternal circulation or follicular envelopes.[1,2] This store of maternal hormones may accomplish the regulatory requirements of larvae for growth, development, osmoregulation, stress responses and other physiological functions prior to the functional development of their own endocrine glands.[3]

Experiments on some fish species have shown that hormones especially maternal steroid hormones play important role in larval development and thus may affect egg quality.[4] For example, several sex steroids have been reported to be present in fertilized eggs of tilapia and salmonids, cortisol in Mozambique tilapia (*Oreochromis mossambicus*) and testosterone in medaka (*Oryzias latipes*).[5-8] In addition, dynamic changes in cortisol content profile were reported in tilapia and zebrafish, showing a steady decline from fertilization until hatching in both species.[9,10]

Estradiol or 17β-estradiol is an important sex hormone. Estradiol is abbreviated E2 as it has two hydroxyl groups in its molecular structure. E2 has not only a critical impact on reproductive and sexual functioning, but also affects other organs, including the bones. So the studies was undertaken to investigate the genetic effects of estradiol on fertilized zebrafish eggs.

MATERIALS AND METHODS

Breeding and treatment. Natural breeding was performed to obtain fertilized eggs with 28.5°C water temperature and with a photoperiod of 14 h light and 10 h dark. Immediately after fertilization, fertilized eggs were pooled in estradiol (0.1 mg/l and 0.01 mg/l), and abs EtOH (1 mg/l) solutions for 2 h for development and fixation up to 8 hours post

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fertilization (hpf). After 2 h of treatment, the estradiol and EtOH treatment solution was poured out and eggs were gently washed 5-fold in fish water to eliminate any trace of estradiol and ethanol.

RNA isolation. To evaluate gene expression, total RNA was extracted from pools of about 60 embryos at 8 hpf developmental stages using TRIZOL reagent, according to the manufacturer's instructions (Invitrogen, Milan, Italy). The experimental phase of extraction and manipulation was carried out under a chemical hood using sterile glassware or sterilized at 200°C in oven for the entire night. The isolated RNA samples were stored at -80°C until future use.

RNA and cDNA samples, obtained from embryos and PCR **(**Polymerase Chain Reaction) reactions were analysed by agarose (Fisher Molecular Biology, USA) gel electrophoresis. This was carried out until the marker dye (bromophenol blue, added to the sample prior to loading) reaches the end of the gel. The nucleic acids in the gel are visualised by staining with the intercalating dye gel red and examined under ultraviolet light.

Quantification of total RNA. The concentration of total RNA was measured by NanoDrop Spectrophotometer (Celbio, Milan, Italy), which allows an assessment of the state of purity or protein contamination. The concentration of 1.5 µl solution of nucleic acid was determined by measuring the absorbance at 260 nm. An A_{260} of 1.0 is equivalent to a concentration of 50 µg/ml for double-stranded DNA, or 40 µg/ml for single-stranded DNA or RNA. The A_{260}/A_{280} ratio should be 1.8 for pure DNA and 2.0 for pure RNA preparations.[11]

Reverse transcription of RNA. After quantification of extracted RNA, reverse transcription was performed using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) protocol (Invitrogen), according to the manufacturer's instructions. M-MLV RT uses single-stranded RNA or DNA in the presence of a primer to synthesize a complementary DNA strand.

The M-MLV RT PCR is a two-step process. Briefly, 2 µg of the purified total RNA was mixed with random hexamer and pure water to a final volume of 10 µl, denatured by incubation at 70ºC for 5 min and then placed on ice for 5 min. Then, 15 µl of cDNA synthesis Buffer (5X), dNTP Mix (10 mM), M-MLV reverse transcriptase enzyme and H_2O were added to each sample. The samples were incubated in a PCR machine at 25°C for 10 min, 50°C for 50 min and 70°C for 15 min. Then, the cDNA reactions were either stored at -20ºC or used for PCR immediately.

Touchdown PCR. Total cDNA (200 ng) was amplified by PCR containing 4µl of ImProm-II™ 5X Reaction Buffer, 2.4 µl of $MgCl_2$, 1 µl of dNTP mix, 0.5 µl of Recombinant RNasin® Ribonuclease Inhibitor, 0.5 µl of the respective primers, 1 µl ImProm-II™ Reverse Transcriptase and ultrapure water to a final volume of 10 µl. The melting point of the primer sets the upper limit on annealing temperature. The annealing temperature of the initial cycle is 5-10°C above the melting temperature of the primers and the temperature is gradually reduced by 1°C for each following cycle down to the lowest melting temperature value of the primer. PCR products for each gene of interest were sequenced to confirm amplicon identity. The list of primers are shown in Table 1.

Table 1. List of primers

Primer	Orientation	Sequence	Position	Accession number
ef1a-F	Sense	5′-GACAAGAGAACCATCGAG-3′	+177→+194	NM_131263
ef1a-R	Antisense	5′-CCTCAAACTCACCGACAC-3′	+447→+430	
ccng1-F	Sense	5′-GATTGAGGATCAGCACGAG-3′	+804→+822	NM_199481
ccng1-R	Antisense	5′-CAGTTATGGGCACTCAACAC-3′	+1099→+1080	
anx2a-F	Sense	5′ -GCACAGATGTGAAGTGCTG-3′	+646→+664	NM_181761
anx2a-R	Antisense	5′ -CAGTCGTCTCCATTGCAC-3′	+1059→+1042	
hmox1-F	Sense	5′- CCACACACCGATATGCAC -3′	+582→+599	NM_00127516
hmox1-R	Antisense	5′ -CAACGTGATGCCCACTCC-3′	+1030→+1013	

RESULTS AND DISCUSSIONS

The quality of RNA was identified by running the extracted RNA samples through agarose gel electrophoresis by indicating two bands: an upper 28S rRNA and a lower 18S rRNA (Figure 1). So the quality of extracted RNA was found good due to the absence of additional bands or smear.

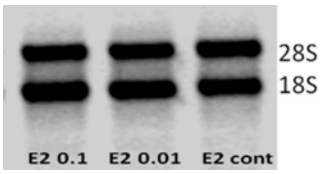

Figure 1. Separation of total RNA on denaturing gel electrophoresis followed by gel red staining.

The quantification results of extracted RNA is shown in table 2 where E2 0.01 and E2 0.1 received higher concentration 746.47 and 574.99 ng/µl respectively. But lower concentration observed 314.23 ng/µl in E2 control sample. Samples E2 0.01 and E2 control obtained pure quality RNA because A_{260}/A_{280} was greater than 2.[11]

To determine the effects of estradiol on gene expression during first developmental stage, a total three genes were selected namely *annexin A2a* (*anx2a*), *cyclin G1* (*ccng1*) and *heme oxygenase* (*decycling*)*1* (*hmox1*). The touchdown PCR analysis revealed that the transcripts of *anx2a* were poorly expressed in E2 0.01 treated embryos when compared to control and E2 0.1 treated embryos as shown in Figure 2. But there was no *anx2a* transcripts different between E2 0.1 and control embryos. It may be assumed that biological mechanisms can influence the regulation of anx2a transcripts with changing the amount of E2. This messenger was up-regulated when embryos were knocked down with MO2-esr2a.[12] *anx2a* plays vital role as calcium ion binding, calcium-dependent phospholipid binding and cytoskeletal protein binding.[12-14] So these binding capacities could be reduced by down-regulating *anx2a* gene expression. *ccng1* transcripts for all treatments were strongly and equally expressed at 8 hpf stage embryos (Figure 3). The transcript of cyclin G1 (ccng1), that encodes a protein with intrinsic growth inhibitory activity, was found to be up-regulated at 48 hpf when knocked down with MO2-esr2a.[12,15]

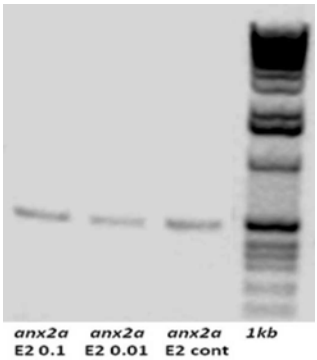

Figure 2. Expression of *anx2a* transcripts during 8 hpf developmental stage of zebrafish.

Table 2. Quantification of extracted RNA by NanoDrop Spectrophotometer.

Sample ID	ng/µl	A_{260}	A_{260}/A_{280}
E2 0.1	574.99	14.3	1.97
E2 0.01	746.47	18.6	2.04
E2 cont.	314.23	7.8	2.04

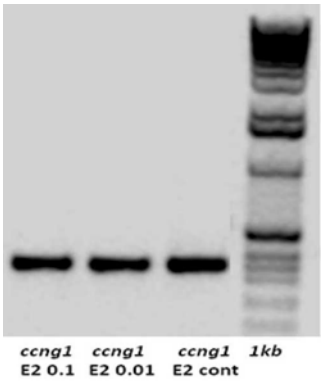

Figure 3. Expression of *ccng1* transcripts during 8 hpf developmental stage of zebrafish.

Finally, the expression of heme oxygenase (decycling)1 (*hmox1*) transcripts were analysed to determine the effects of estradiol. *Hmox1* encodes a protein that in mammals, catabolizes heme to biliverdin, carbon monoxide, and free iron and is involved in iron homeostasis.[16] In zebrafish, this gene is expressed in the extraembryonic yolk syncytial layer, lens, and a small population of blood cells.[17] There was no up or down-regulation when

analysed with *hmox1* gene (Figure 4). Significant down-regulation of *hmox1* transcripts by RT-PCR at 8 and 48 hpf developmental stages when knocked down with MO2-esr2a.[12] Estradiol has been attached to the expansion and progression of cancers such as ovarian cancer and endometrial cancer. Estradiol effects target tissues by interacting with estrogen receptors. These estrogen receptors are involved in gene expression.[18] When hormone binds to the estrogen receptors, it possibly causes damage to the DNA, increase in cell proliferation and DNA replication.[19]

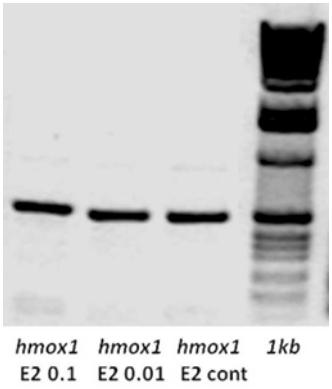

Figure 4. Expression of *hmox1* transcripts during 8 hpf developmental stage of zebrafish.

The present report reveals that oocyte estradiol concentration can change genetic expression at different developmental stages in zebrafish. So a powerful regulatory control linked to maternal experience in terms of estradiol can be directly transmitted into lineage before their actual sensing of surrounding environmental cues. In this way estradiol transcripts in oocyte may expect an epigenetic plasticity in zebrafish development, as a maternal contribution to the adaptive value of the progeny.

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