Bangladesh Pharmaceutical Journal ***17****(1): 55-61, 2014*

Expression Analysis of Glucocorticoid Hormone During Early Developmental Stage of Zebrafish (*Danio rerio*) its Therapeutic Implications

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Abstract

Glucocorticoid is prescribed for the treatment of many human diseases. The work was performed to investigate the role of glucocorticoid hormone on the gene expression during zebrafish (*Danio rerio*) embryogenesis. Microarray hybridization technique was used to analyse dys-regulation of different gene expression at 12 hours post fertilization (hpf) when single cell fertilized eggs were treated with glucocorticoid hormone. The expression of 149 genes were affected, with 143 up-regulated and 6 down-regulated. The results suggest that glucocorticoid may be also used in therapeutic approach to change genetic expression at early developmental stage.

Key words: Glucocorticoid hormone, Expression, Zebrafish.

Introduction

In fish, like other vertebrates, the cellular responses allied with cortisol signaling are thought to be mediated by a glucocorticoid receptor (GR). Cortisol is also known to play an important role in modulating a vast array of physiological processes, including organ development, protein and fat metabolism, carbohydrate homeostasis, immune response, neural activity, memory, behaviour and bone formation in both unstressed and stressed animals (Mommsen *et al*., 1999; Barnes, 2006; De Kloet *et al*., 2005 ; Wang, 2005; Migliaccio *et al*., 2007). Since cortisol exerts different effects on the various organs of the body, it is likely that the functional targets of GR are different in each tissue and these tissue-specific mechanisms need to be elucidated in order to understand the functional genomics of the stress response (Phuc Le *et al*., 2005; Vegiopoulos and Herzig, 2007).

Mammalian studies using microarrays and bioinformatics tools have identified that GR is responsible for gene regulatory networks in the liver of mice treated with dexamethasone. Some genes are directly linked to GR signaling, while other genes are regulated indirectly by interaction of GR with other transcription factors, including estrogen receptor (ER) and CCAAT/enhancer binding protein beta (C/EBPB) (Aluru and Vijayan, 2007). Due to their various actions, glucocorticoids have become fundamental therapeutic agent. As a class of compounds including synthetic analogues, they are among the most prescribed drugs in the world. Clinically, glucocorticoids are widely prescribed in the treatment of chronic autoimmune/inflammatory and allergic diseases, such as asthma, inflammatory bowel disease, rheumatoid arthritis, and skin disorders (Schaff *et al*., 2009).

Studies on several fish species have shown that maternal steroid hormones may be involved in the early development of the offspring. For example, sex steroid hormones were found in the eggs of coho salmon (*Oncorhynchus kisutch*) (Feist *et al*., 1990) and testosterone in medaka (*Oryzias latipes*) (Iwamatsu *et al*., 2006). On the basis of these findings, the present study was undertaken to discover the effect of glucocorticoid hormone on the gene expression of zebrafish at 12 hours post-fertilization (hpf) embryos as well as to establish its therapeutic implications.

Materials and methods

Fertilized zebrafish eggs were pooled and incubated in cortisol treatment solution and control solution for 2 h for development and fixation at 12 hpf. The concentration of cortisol treatment solution was 13.8 μM while the control solution contained 2 ml abs. ethanol/ 1 L fish water.

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After treatment, the eggs were gently washed at least 5-fold in water to eliminate any trace of cortisol and ethanol. Finally the eggs were frozen in liquid nitrogen at 12 hpf to perform molecular analyses.

RNA extraction: Total RNA extraction of cortisol-treated and control embryos using Trizol reagent was performed according to the manufacturer's instructions (Invitrogen, Milan, Italy). The extracted RNA samples were stored at -80°C until future use.

Extracted RNA samples, obtained from embryos were analyzed by agarose (Fisher Molecular Biology, USA) gel electrophoresis for quality check. The concentration of total RNA was quantified by NanoDrop Spectrophotometer (Celbio, Milan, Italy).

LiCl precipitation: The key requirement for microarray hybridization is highly pure RNA, in which A_{260}/A_{230} must be higher than 2.2 and A_{260}/A_{280} should be higher than 2. Extracted RNA was purified by LiCl precipitation. For this purpose, RNA samples were heated at 40°C for 6 min and centrifuged at 12,000 *g* for 15 min at 4°C. Then, 9.5 µl of 4 M LiCl was added to each tube, which was left overnight at 4°C for precipitation. It was then centrifuged at 12,000 *g* for 20 min at 4°C and 200 µl of 75% EtOH were added to the pellet for washing. The mixture was again centrifuged at 12,000 *g* for 20 min at 4°C and the supernatant discarded. Finally, the RNA was re-quantified by NanoDrop Spectrophotometer.

RNA quality control: After LiCl precipitation, RNA quality assessment and quantification were performed by using Agilent 2100 bio-analyzer and RNA LabChip. The bio-analyzer software automatically generates the ratio of the 18S to 28S ribosomal subunits. This ratio plays an important role in determining the level of sample degradation in gel electrophoresis. The Agilent technologies have also introduced a new tool for RNA quality assessment through RNA Integrity Number (RIN). The RIN software algorithm allows the classification of eukaryotic total RNA, based on a numbering system from 1 to 10, with 1 being the most degraded profile and 10 being the most intact one. Only the samples having RIN value greater than 8 were used to perform microarray hybridization.

Two-color Microarray hybridization: Two-Color Microarray-based gene expression analysis (Agilent Technologies, Santa Clara, CA) was performed for transcript quantification in embryos treated with cortisol at 12 hpf. The analysis was performed at CRIBI, Italy, using Agilent Whole Zebrafish Genome Oligo Microarrays 4x44K slide. The experimental design is shown in Table 1.

Agilent's Two-Color Microarray-based Gene Expression Analysis used cyanine 3- and cyanine 5-labeled targets to measure gene expression in control and treated samples.

The Agilent Feature Extraction Software (FES) was used to read out and process the microarray image files. The software determines feature intensities and ratios (including background subtraction and normalization), rejects outliers and calculates statistical confidences (*P* values). Subsequently, the genes were classified into functional groups using gene ontology and analyzed individually at the site "Entrez Gene" (http://www.ncbi. nlm. nih.gov/gene).

SAM 3.0 (Significance Analysis of Microarrays) statistical program was used to analyse expression of up- and down-regulated genes using 1% False Discovery Rate (FDR), which points out statistically significant up- and down-regulated genes from the set of a microarray experiment.

Results and Discussion

RNA quality control: RNA quality was confirmed by analysing RIN using Agilent 2100 bio-analyzer. RIN was assessed on the base of the presence or absence of degradation products in the entire electrophoretic trace of the RNA sample. In this way, interpretation of an electropherogram was facilitated, comparison of samples was enabled and repeatability of experiments was ensured. The results of single analyzed sample were described as an electropherogram and a virtual image of an internal standard agarose gel. Excellent quality of RNA was assessed when the bands of 28S and 18S rRNAs were well separated and their correspondent peaks were in good evidence. RNA samples used in this experiment obtained RIN values 9.6, 10 and 9.7 for control samples and 9.5, 10 and 8.6 for cortisol treated samples. From this result it is clear that RNA quality was good and could be used for further analysis because RIN value greater than 8 is acceptable for microarray hybridization(Schroeder *et al.*, 2006 ; Thompson, *et al.*, 2007).

cRNA quantification and incorporation of cyanine: The hybridyzation experiment was carried out according to the experimental design whereas the cRNA quantification and incorporation of Cy3 (green) and Cy5 (red) of each sample is shown in Table 2. Treated samples were labelled with Cy5 and the control with Cy3.

Table 1. The experimental design of microarray analysis. Cy3= Control and Cy5=Treated.

Experiments	Cy3	Cy5
Array 1_1	1.1 Control 12 hpf	1.1 Cortisol-treated 12 hpf
Array 1_2	1.2 Control 12 hpf	1.1 Cortisol-treated 12 hpf
Array 1_3	1.3 Control 12 hpf	1.1 Cortisol-treated 12 hpf

Scanning and microarray data analysis: Hybridization expression data were collected by scanning the signal intensities of the corresponding spots on the array by dedicated fluorescence Agilent's DNA microarray scanner. The spatial distribution of significantly up- and down-regulated features for array 1_1, array 1_2 and array 1_3 is shown in Figure 1, where red spot indicates that the fluorescence intensity of the Cy5 signal is higher than that of Cy3, which means that the corresponding gene is over-expressed. Green spots indicate that the fluorescence intensity is higher in the control sample than treatment sample, which means that the corresponding gene is down-regulated.

Table 2. Quantification of cRNA and incorporation rates of cyanine Cy3 and Cy5 for control and treatment samples, respectively.

Samples	Labeling	Concentration cRNA (ng/µl)	Volume (µl)	Dye (picomol/µl)	Incorporation rate (pmol/µg)
1.1 Control 12 hpf	Cy3	411	30	7.3	17.8
1.2 Control 12 hpf	Cy3	362	30	3.2	8.8
1.3 Control 12 hpf	Cy3	293	30	4.7	16.4
1.1 Cortisol-treated 12 hpf	Cy5	248	30	5.1	12.5
1.1 Cortisol- treated 12 hpf	Cy5	407	30	4.7	13.4
1.1 Cortisol- treated 12 hpf	Cy5	350	30	2.5	8.3

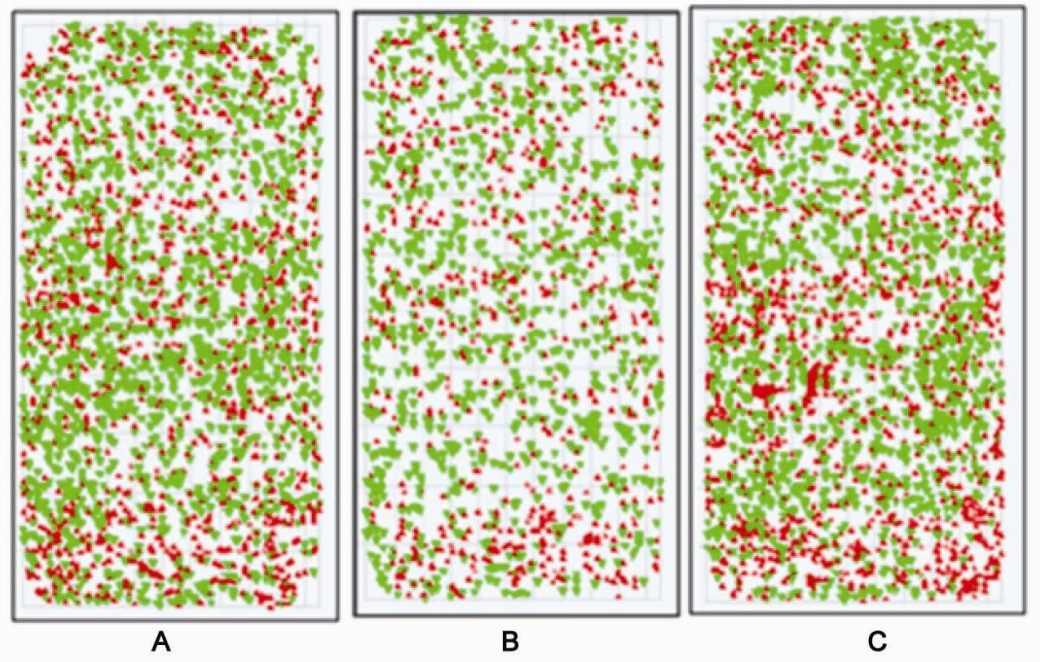

Figure 1 Spatial distribution of significantly up- and down-regulated features. A: Array1_1, B: Array 1_2, C: Array 1_3.

Image processing was performed using Agilent's Feature Extraction Software (FES) and the normalization of data was performed automatically. This software offers, among other features, the possibility to visualize the results of the data analysis in a log ratio versus log processed signal scatter plot (Figure 2).

Finally, SAM 3.0 (Significance Analysis of Microarrays) statistical program was used to analyze the up- and down-regulated genes due to cortisol treatment. Statistical analysis indicates the up-regulation of 143 genes down-regulation of 6 genes. The analysis was performed from public databases (National Centre of Biotechnology Information; NCBI, USA) for specific gene description. The annotations used were derived from Gene Ontology (GO), which provides information on molecular function, as well as from various pathway resources for information on involvement in biological signaling pathways. A descriptions of up- and down-regulated genes at 12 hpf with their functional groups and not annotated genes are shown in Tables 3 and 4, respectively.

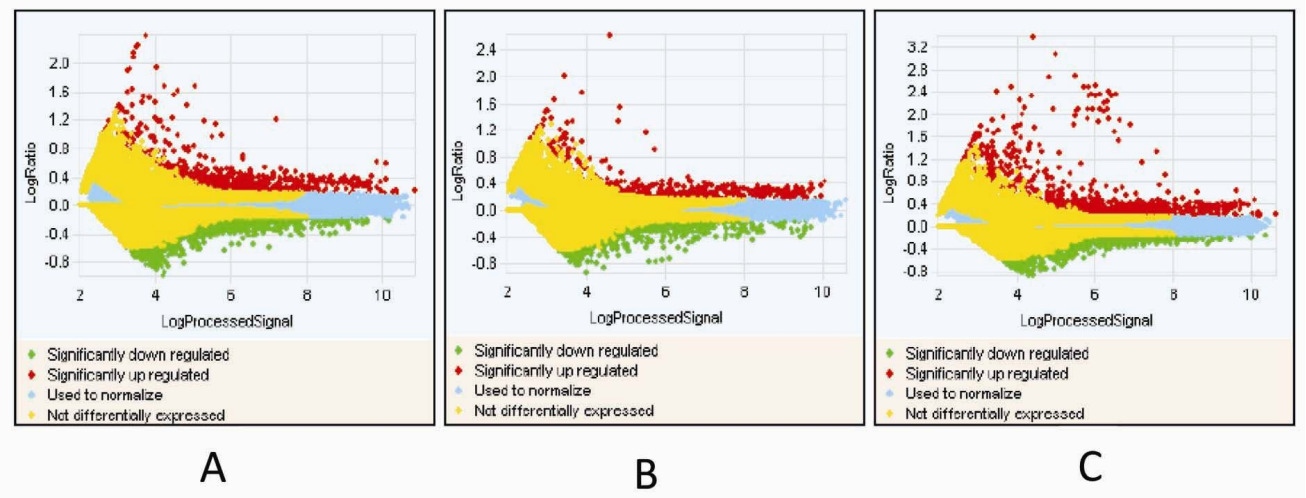

Figure 2 Scatter plot of log ratio versus log processed signal intensities. Red spot: significantly up-regulated genes ($P < 0.05$). Green spot: significantly down-regulated genes ($P < 0.05$). Yellow spot: Not differentially expressed genes. Blue spot: Genes used to normalize. A: Array1_1, B: Array 1_2, C: Array 1_3.

Bioactivity of cortisol, either maternal or exogenous, is expected in zebrafish embryos because the mRNA encoding its main cognate receptor, GR, is the most abundant among maternal transcripts encoding nuclear and membrane steroid receptors inovulated oocytes (Pikulkaew *et al.*, 2010). Its translation into protein to act as GR-cortisol complex on gene targets is to be assumed in the embryo, because the activation of the GR signaling pathway is essential for mesoderm formation in zebrafish (Nesan *et al.*, 2012). At this stage, cortisol appears to influence the rates of zygotic transcription with a prevalent up-regulation and a minor down-regulation. By knocking down glucocorticoid receptor mRNA, 114 and 37 transcripts were up- and down-regulated respectively which is the agreement to our result (Pikulkaew *et al.*, 2011). In addition, intelectin, TLR-5M and TLR-5S mRNA transcripts were poorly and highly expressed at different embryonic stages when oocytes immersed 3 h prior to fertilization in cortisol-enriched ovarian fluid at either 100 ng/ ml or 1000 ng /ml (Li *et al.*, 2011).

Glucocorticoids are also used for the treatment of certain cancer like Hodgkin's lymphoma, multiple myeloma and acute lymphoblastic leukemia. Glucocorticoids can be moderately engaged to glucocorticoid-induced apoptosis due to its anti-inflammatory and antineoplastic actions (Rhen and Cidlowski, 2005). After binding with glucocorticoid receptor, this hormone can upregulate proapoptotic genes and downregulate antiapoptotic genes (Mok *et al.*, 1999). So maternal glucocorticoid dosage should be regarded as the fundamental integral sensor of the

Table 3. Up- (↑) and down-regulated genes at 12 hpf with their specific functions and descriptions.

Genes	Descriptions	12 hpf	
		Up	Down
mrpl20	mitochondrial ribosomal protein L20	↑	-
sepsecs	Sep (O-phosphoserine) tRNA:Sec (selenocysteine) tRNA synthase	↑	-
vasp	vasodilator-stimulated phosphoprotein	↑	-
sec14l1	SEC14-like 1 (*S. cerevisiae*)	↑	-
plp1a	proteolipid protein 1a	↑	-
atpif1	ATPase inhibitory factor 1	↑	-
tbl3	transducin (beta)-like 3	↑	-
commd1	copper metabolism (Murr1) domain containing 1	↑	-
mybpc2b	myosin binding protein C, fast type b	↑	-
acot9.2	acyl-CoA thioesterase 9.2	↑	-
dscam	down syndrome cell adhesion molecule	↑	-
rbp4	retinol binding protein 4, plasma	↑	-
slc30a4	solute carrier family 30 (zinc transporter), member 4	↑	-
kpna3	karyopherin (importin) alpha 3	↑	-
atp2b2	ATPase, Ca++ transporting, plasma membrane 2	↑	-
dscr3	down syndrome critical region gene 3	↑	-
mfsd7	major facilitator superfamily domain containing 7	↑	-
Snx16	sorting nexin 16	↑	-
lppr5b	lipid phosphate phosphatase-related protein type 5b	↑	-
hhip	hedgehog interacting protein	↑	-
apaf1	apoptotic protease activating factor 1	↑	-
myoc	myocilin	↑	-
dgcr8	DiGeorge syndrome critical region gene 8	↑	-
parn	poly(A)-specific ribonuclease (deadenylation nuclease)	↑	-
foxf1	forkhead box F1	↑	-
mef2ca	myocyte enhancer factor 2ca	↑	-
ldb1a	LIM-domain binding factor 1a	↑	-
znf367	zinc finger protein 367	↑	-
nfe2l3	nuclear factor (erythroid-derived 2)-like 3	↑	-
ets2	v-ets erythroblastosis virus E26 oncogene homolog 2 (avian)	↑	-
hmga1b	high mobility group AT-hook 1b	↑	-
il7r	interleukin 7 receptor	↑	-
pde8a	phosphodiesterase 8A	↑	-
bckdha	branched chain keto acid dehydrogenase E1, alpha polypeptide	-	↓
tcp1	t-complex polypeptide 1	-	↓
march8	membrane-associated ring finger (C3HC4) 8	↑	-
lrrc4c	leucine rich repeat containing 4C	↑	-
ate1	arginyltransferase 1	↑	-
aplp	amyloid beta (A4) precursor-like protein	↑	-
sh2b1	SH2B adaptor protein 1	↑	-
arl3	ADP-ribosylation factor-like 3	↑	-
ripply3	ripply3	↑	-
slit3	slit (Drosophila) homolog 3	↑	-
ankfy1	ankyrin repeat and FYVE domain containing 1	↑	-
crfb8	cytokine receptor family member b8	↑	-
rgs5b	regulator of G-protein signaling 5b	↑	-
oxsr1b	oxidative-stress responsive 1b	↑	-
adck1	aarF domain containing kinase 1	↑	-
spag1b	sperm associated antigen 1b	↑	-
tbk1	TANK-binding kinase 1	↑	-
zdhhc5a	zinc finger, DHHC-type containing 5a	↑	-
trim35-24	tripartite motif containing 35-24	↑	-

stressor load on the mother's life during oogenesis while the cortisol deposit would act as an additional clinical factor in the maternal programming of embryo development.

Table 4. Not annotated up and down-regulated genes at 12 hpf.

Name of genes	12 hpf	
	Up	Down
zgc:114123	↑	-
zgc:66337	↑	-
si:dkey-165a24.4	↑	-
zgc:114175	↑	-
si:dkey-11e23.5	↑	-
im:6903943	↑	-
si:dke-21k24.2	↑	-
zgc:77816	↑	-
zgc:112992	-	↓
zgc:171485	↑	-
zgc:153845	↑	-
si:dkey-71l1.1	↑	-
zgc:153893	↑	-
si:dkeyp-22b2.2	↑	-
zgc:64022	↑	-
zgc:73144	↑	-
zgc:122979	↑	-
zgc:73359	↑	-
zgc:153031	↑	-
si:dkey-21k24.2	↑	-
zgc:165519	↑	-
zgc:110655	↑	-
zgc:136758	↑	-
zgc:113983	↑	-

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