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Original Research Article

Effect of Chronic Unpredictable Stress on Behaviour and Apoptosis in Zebrafish Heart

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Abstract

Apoptosis plays an important role in the pathogenesis of cardiovascular diseases. Zebrafish has emerged as a promising animal model for studying stress related disorders. To evaluate the effect of chronic unpredictable stress (CUS) exposure on anxiety behaviour and apoptosis in zebrafish heart.320 adult zebrafish of both sexes were used. They were divided into Control and CUS group of 160 fish each. CUS groups were exposed to CUS for 15 days. The anxiety behaviour was assessed using novel tank and light/dark preference test. The effect of stress induced apoptosis in cardiac myocytes was assessed using qRT-PCR and cortisol levels were evaluated using ELISA. Novel tank showed a significant decrease ($p<0.05$) in average duration, transitions and time spent in upper tank, and a significant increase ($p<0.05$) in the latency to reach the upper portion of the tank, freezing duration and erratic movement in stressed zebrafish. In light/dark preference test, CUS exposed zebrafish spent more time in the light compartment. A significant increase ($p<0.05$) in cortisol level and *crf* expression and decrease in *gr* expression ($p<0.05$) was observed in stressed zebrafish. A significant increase ($p<0.05$) in *p53*, *noxa, tnfα*, caspase3 expression and decrease ($p<0.05$) in of *bcl2* expression was seen in heart of the CUS group. Exposure of zebrafish to chronic unpredictable stress has induced anxiety like behaviour. The increased expression of *p53*, *noxa, tnfα*, *caspase3* and decreased expression of *bcl2* indicates the onset of apoptosis in zebrafish heart.

Keywords: Chronic unpredictable stress, Zebrafish, Cardiac myocytes, Anxiety, Apoptosis.

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Introduction

Globally, cardiovascular disease is a leading cause of death[1] and has increased the mortality in India with >80% of death[2]. Stress plays a major role in the pathophysiological processes associated with cardiovascular system[3]. Stress is generally defined as the state of threatened homeostasis or disharmony[4]. To bring back the homeostatic conditions complex range of responses are activated which are known as the stress response[5]. The key

effector of stress response is through hypothalamic pituitary adrenal (HPA) axis, the hypothalamic secretion of corticotropin-releasing factor (CRF) stimulates adrenocorticotropic hormone (ACTH) synthesis from the anterior pituitary, which in turn regulates the synthesis of glucocorticoids in the adrenal cortex. Glucocorticoids are a class of steroids hormone that are essential for the organism to survive[6]. Glucocorticoids are involved in many physiological processes such as metabolism, cardiovascular regulation, immune response, electrolyte homeostasis, growth, reproduction and behaviour[5,7,8].

Oxidative stress is the cytopathological consequences of an imbalance between the endogenous reactive oxidative species (ROS) and pro-oxidative enzymes. ROS appears to be the principle mediator for cardiomyocytes dysfunction in both apoptosis and necrosis[9,10]. Apoptosis is a naturally occurring cell death, and it is essential for development and homeostasis for multicellular organisms. When apoptosis proliferate, adverse biological consequences take place[11]. Apoptosis plays a major role in cardiovascular diseases[12] and can be triggered by various stimulus including DNA damage, intracellular damage, toxins and extracellular signals[13]. Apoptosis can be stimulated by two different pathways: a) Intrinsic pathway (mitochondrial pathway)-via release of cytochrome C from the mitochondria, which activates different caspases signals[14] and b) Extrinsic pathway (death domain pathway) - via the signal from outside of the cell. After the activation of different intermediate molecules by signaling cascade, both the pathways meet up at the final caspase activation step and commonly lead to cleavage of different proteins[15].

Zebrafish is an outstanding model for genetic, embryonic and physiological studies[16]. Zebrafish has also been used to study the mechanism leading to human cardiac diseases and model to human congenital cardiac diseases[17], stress and anxiety[18]. Chronic unpredictable stress (CUS) was reported to be one of the most relevant stress paradigms in rodents. One of the most clinically relevant stress paradigms in rodents mimics a number of behavioural characteristics observed in patients with anxiety, depression and mood disorder[19]. In recent years, zebrafish said to be the powerful vertebral model to study apoptosis[20] and a potential in vivo model to study cell death[20,21]. Accordingly, the present study was designed to study the effect of chronic unpredictable stress in intrinsic and extrinsic pathway of apoptosis in zebrafish heart. The anxiety behaviour and gene expressions of apoptotic genes were carried out in zebrafish heart.

Materials and methods:

Animal and housing:

A total of 320 adult wild type zebrafish of both sexes were obtained from a commercial fish supplier. The fish were acclimatized to the laboratory conditions by maintaining them at 28±2ºC, 14/10h light/dark cycle. They were fed twice a day with commercial flakes and live shrimp provided with constant aeration[22]. The pH of the water is maintained between 7- 8. The fish were segregated into two groups – control and CUS induced group with 160 fish in each. All the protocols were approved by the Institutional Animal Ethical Committee.

Chronic unpredictable stress protocol:

Succeeding a two-week adaptation period, the 160 fish were subjected to a range of chronic stressors such as restrain stress, predator stress, low water level stress (dorsal body exposure), over - crowding stress, chasing stress, cold stress and heating stress. The fish were exposed to one of the abovementioned stressors twice a day for a period of 15 days (Table 1).

Administration of CUS:

Restraint stress: Each fish was restrained in a 2ml micro centrifuge tube with perforations at both the ends to allow free flow of water (Duration 90 min). Predator stress: The fish were alarmed by a predator fish (*Archocentrus nigrofasciatus)* in close vicinity but avoiding direct contact (Duration 50 min). Both predator fish and zebrafish were kept in a small tank separated by a glass partition. Low water level stress: The water was drained in the housing tanks to expose the animals' dorsal body surface (Duration 2 min). Overcrowding stress: 250ml beaker crowded with 10 fishes/150 ml of water (Duration 50 min). Chasing stress: Racing the animals using a net (Duration 8 min). Cold Stress: Exposing the animals to 23º C (Duration 30 min). Heating Stress: Heating the tank for 33º C (Duration 30 min) [19, 23, 24]. Time and sequence of stressors were altered on daily basis to prevent habituation and to promote unpredictability. A control group was also retained in the same room provided with ideal conditions for a period of 15 days. Despite stressful conditions, no extreme harm was caused to the animals nor abnormal number of deaths witnessed.

Behavioural analysis:

Novel tank test:

Behavioural testing was performed using the novel tank diving test, representing a 1.5-L trapezoidal tank (15.2 cm height × 7.1 cm width × 27.9 cm top length × 22.5 cm bottom length) maximally filled with aquarium-treated water. Novel tanks rested on a level surface and were divided into two equal horizontal portions, marked by a dividing line on the outside walls. Behavioural testing occurred between 10.00 and 17.00 h. Once each fish was individually transferred to a novel tank, its swimming behaviour was recorded. The following behavioural endpoints: latency to reach the upper half (top) of the tank, time spent in the top, number of transitions (entries) to the top, number of erratic movements, and freezing durations were calculated. Erratic movements were defined as sharp changes in direction and/or velocity and repeated rapid darting behaviours. Freezing was defined as a total absence of movement, except for the gills and eyes, for 2s or longer. Collectively, a reduction in exploration (i.e., longer latency to reach the top half, fewer entries to the top, more freezing) or elevated erratic movements represent behavioural profiles indicative of high stress and anxiety[25,26]. We also calculated the average top entry duration (total time spent in top divided by the number of entries), as additional endpoints reflecting the level of zebrafish anxiety[27].

Light and dark preference tank:

Fifteen fish in each group was tested randomly in light and dark preference tank. The preference tank was 2L rectangular tank divided into three chambers. In the centre of the tank, there was a start box that opened into both chambers. Both the light and dark sides are opened to the fish. The fish is tested individually for a time period of 15 minutes by video recording[28,29]. Both the control group and the CUS exposed were examined and the latency spent in dark and light portion of the tank were assessed.

Measurement of cortisol:

Briefly, individual body samples obtained from experimental and control fishes were homogenized in 1 mL of ice-cold 1×PBS buffer. Samples were transferred to glass extract tubes and cortisol was extracted twice with 5 mL of diethyl ether (Fisher Scientific, USA). After ether evaporation, the cortisol was reconstituted in 1 mL of 1× PBS. To quantify cortisol concentrations, ELISA was performed using a cortisol assay kit (Cayman Chemicals, India). ELISA plates were measured in a Megallan plate reader using the manufacturer's software.

Table 1: Chronic unpredictable stress protocol

		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Week 1	Morning	RS	CS	PS	HS	CS	RS	PS
	Evening	HS	OCS	C	LWLS	C	LWLS	HS
Week 2	Morning	LWLS	PS	OCS	HS	CS	RS	PS
	Evening	C	CS	RS	LWLS	C	OCS	HS

C- Chasing stress, OCS- overcrowding stress, LWLS- low water level stress, RS-Restrain stress, PS-predator stress, CS- cold stress, HS-Heat stress

Table 2: Forward and reverse primer sequences

GENE		PRIMER SEQUENCE
βactin	Forward Reverse	5'-CGA GCA GGA GAT GGG AAC C-3' 5'-CAA CGG AAA CGC TCA TTG C-3'
crf	Forward Reverse	5'-CCG CCG TAT GAA TGA TAG AGC-3' 5'- GAT GGA AAG TGA TGA CAG TG-3'
gr	Forward Reverse	5'-AAC ATG CTG TGT TTC GCT CC-3' 5'-CTG CAA GCA TTT CGG GAA AC-3'
p53	Forward Reverse	5'-GGG CAA TCA GCG AGC AAA-3' 5'-ACT GAC CTT CCT GAG TCT CCA-3'
noxa	Forward Reverse	5'-CGA ACC TGT GAC AGA AAC TTG-3' 5'-CTG CGC GCA CTC TAC TAC A-3'
bcl2	Forward Reverse	5'-AGG AAA ATG GAG GTT GGG ATG-3' 5'-TGT TAG GTA TGA AAA CGG GTG GA-3'
tnfα	Forward Reverse	5'-ACC AGG CCT TTT CTT CAG GT-3' 5'-TGC CCA GTC TGT CCT TCT-3'
caspase3	Forward Reverse	5'-CCG CTG CCC ATC ACT A-3' 5'-ATC CTT TCA CGA CCA TCT-3'

Gene expression:
mRNA extraction and cDNA synthesis:

After 14 days of stress, the zebrafish were cryoanaesthetized and euthanized in 24 hours after the CUS protocol[30]. By means of the established protocol, the heart was dissected and removed under dissection microscope[31]. The heart samples from 40 zebrafish were pooled. The total RNA was extracted from pooled adult zebrafish heart using Trizol Reagent (Sigma- Aldrich, India) in accordance with the kit's manual (Invitrogen). The purity of the RNA was spectrophotometrically quantified. cDNA synthesis - cDNA was synthesized from the isolated RNA by reverse transcription (iScript cDNA Synthesis Kit, BIO–RAD). The primers were designed in primer blast of NCBI. βactin was used as a control. The set of primers used are mentioned in (Table 2).

Gene expression analysis by qRT-PCR:

Quantitative Real time (qRT) PCR analysis were done for the genes that code for crf and gr, molecular markers of stress related disorders. qRT – PCR was also performed for the apoptotic genes p53,noxa, bcl2,tnfα and caspase3. All qRT– PCR reactions were executed in CFX96 BIORAD Real -Time PCR using SYBR green master mix plus for SYBR assay. qRT – PCR was achieved in triplicate using gene specific primers. Thermal profiles for βactin, crf, p53, bcl2, caspase3 in qRT – PCR: initial

denaturation at 95ºC for 5 min, 40 cycles of 95ºC for 10 sec for denaturing, 1 min annealing step at 60ºC and a final 30 sec extension at 72ºC. Similarly, thermal profiles for gr and noxa, tnfα: initial denaturation at 95 ºC for 5 min, 40 cycles of 94 ºC for 1 min for denaturing, 1 min annealing step at 62ºC and a final 1 min extension at 72ºC. The results were expressed as relative expression levels. The relative abundance of gene expression was quantified by normalization to βactin levels. The data was computed by the 2-ΔΔCT method [32].

Results:

Measurement of cortisol and expression of stress markers:

Corticosterone level in the CUS exposed zebrafish was significantly higher compared with control zebrafish. As a confirmation of stress, the expression of *crf* and *gr* were detected by qRT-PCR. The gene expression of *crf* showed significant increase ($p<0.05$) and *gr* expression showed a significant decrease ($p<0.05$) in chronic unpredictable stress induced group when compared to the control group [24]. Data are expressed as bar diagram with mean and standard deviation as shown in (Figure 1).

Novel tank test:

In the CUS exposed group, zebrafish exhibited a significant decrease ($p<0.05$) in time spent in the upper portion of the tank, transitions to top and average entry duration when compared to the control group. In the CUS exposed group, there was an increased latency ($p<0.05$) to the upper portion of the tank, erratic movements and freezing duration compared to the control group. Data are expressed as bar diagram with mean and standard deviation as shown in (Figure 2).

Light and dark preference tank:

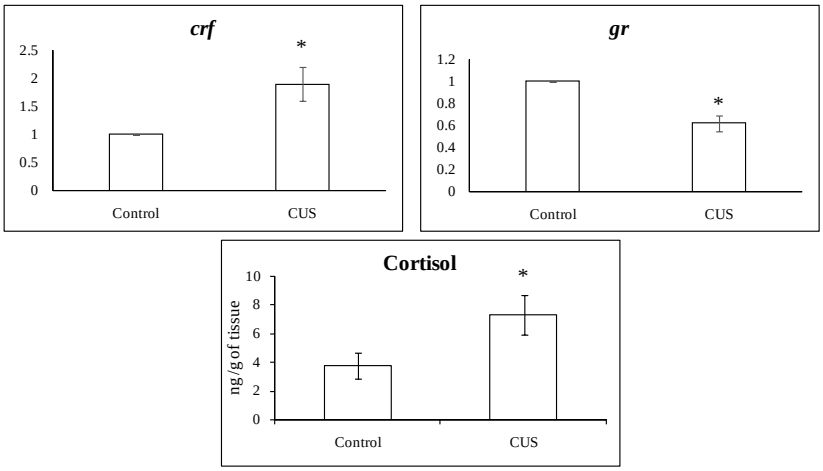
In the CUS exposed group, zebrafish showed a significant increase ($p<0.05$) in the time spent in light and significant decrease ($p<0.05$) in the time spent in dark when compared to the control group. Data are expressed as bar diagram with mean and standard deviation as shown in (Figure 3).

Expression of apoptotic genes:

As a confirmation of apoptosis induced by *p53*, the gene expression levels of *p53*, *noxa*, *bcl2* and *caspase3* were detected in both control and CUS exposed group. Substantial alterations were seen in the gene expression of apoptotic genes. The gene expression levels of *p53*, *caspase3*, *tnfα* and *noxa* presented a significant increase ($p<0.05$) in CUS exposed group compared with control group. A significant decrease ($p<0.05$) in *bcl2* was observed in CUS exposed group compared with control.

Data are expressed as bar diagram with mean and standard deviation as shown in (Figure 4). Apoptosis occurs in the cell by two pathways namely intrinsic mediated by *p53*[33] and extrinsic pathway mediated by *p53* and *tnfα*[34]. The heart of the zebrafish exposed to CUS has showed a significant increase in the expression of *p53* gene which mediates both intrinsic and extrinsic apoptotic pathway. In normal cells, the *p53* protein level is low. DNA damage and other stress signals may trigger the increase of *p53* proteins, which have major functions: growth arrest, DNA repair and apoptosis. The noxa gene which is involved in *p53* mediated apoptosis also showed a significant increase in CUS exposed fish compared with control fishes. The anti-apoptotic gene *bcl-2* plays an important role in promoting cellular survival and inhibiting the actions of pro-apoptotic proteins. The decrease in the expression of *bcl-2* observed in this study upon exposure to CUS is an indication of the progression of apoptosis in the zebrafish heart. *tnfα* is a cell signaling protein which induces the death signaling in the cell in response to DNA damage. Increased expression of *tnfα* in exposure to

CUS is an indication of apoptosis. Since *caspase3* is a common apoptotic pathway for both intrinsic and extrinsic pathway. Increase expression of caspase3 in the zebrafish heart after CUS indicates the central role of *caspase3* in the execution of cell apoptosis.



(p<0.05 is considered significant* indicates significance compared with control.)

Figure 1: Expression of crf and gr gene expression in control and chronic unpredictable stress exposed group.

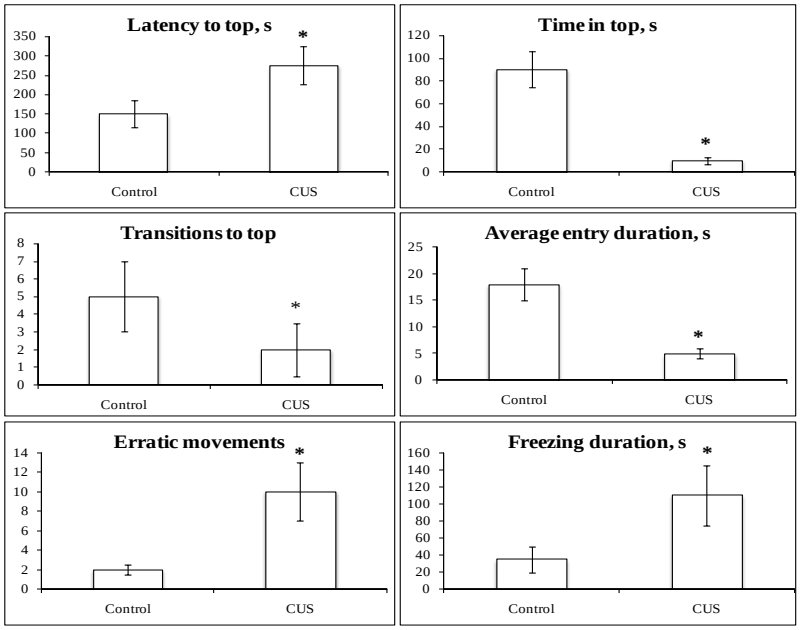


Figure 2: Novel tank: p<0.05 is considered significant* indicates significance compared with control.

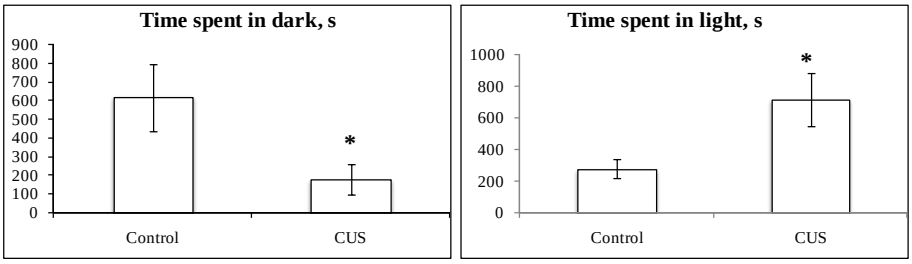


Figure 3: Light / Dark preference test: p<0.05 is considered significant* indicates significance compared with control.

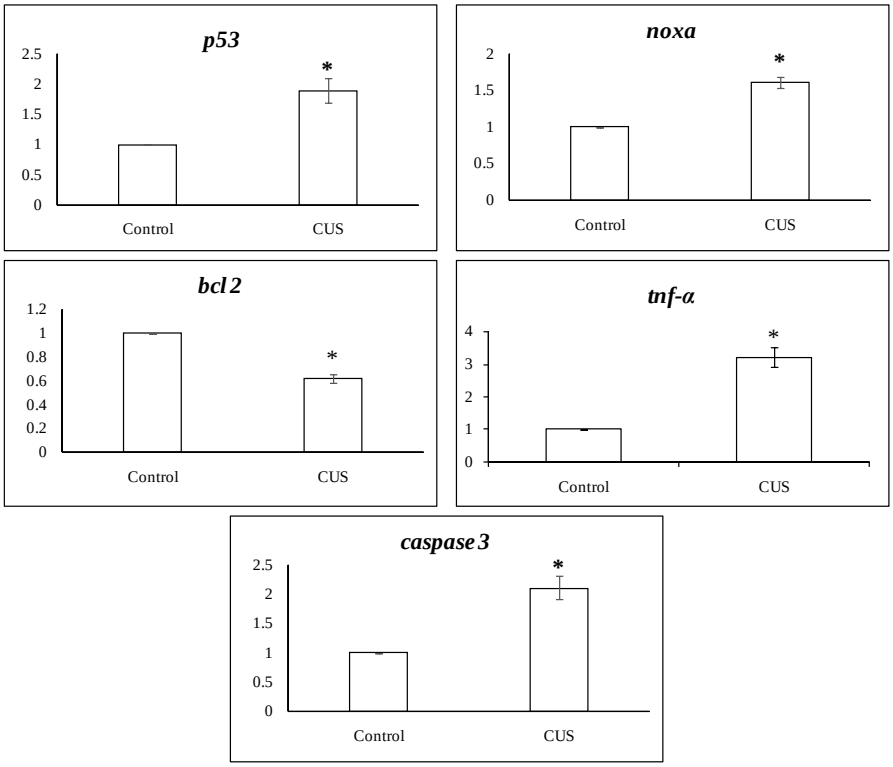


Figure 4: Expression of intrinsic gene bcl2, extrinsic apoptotic genestnfα andnoxa, p53 and caspase3 genes in zebrafish heart were seen using qRT PCT: p<0.05 is considered significant* indicates significance compared with control.

Discussion:

As a response to the chronic unpredictable stress, persistent activation of HPA axis would have caused the increased secretion of CRF, shown by the increased expression of *crf* gene. The decreased expression of *gr* gene may be due to the feedback mechanism caused by the down regulation of receptors[35,36,37]. This is well correlated with the increase in cortisol observed in the present study. A significant rise in serum cortisol levels after acute restraint stress in male rats[38] after applying the acute restraint stress for 90 min. Various studies have shown similar results[39] highlighted positive effects of glucocorticoids at both cellular and behavioural levels. However, they concluded that severity of the stressor was of central importance. The study done by Pinnock et al[40] revealed that animals exposed to single-stress (one period of 1-hour of acute restraint stress) showed increase in CRF levels. Trunk cortisol determination is a valuable indicator of

stress in zebrafish. The stress response is related with HPA activation through increase of CRF and cortisol release[41]. As proposed by Selye[42], chronic stress response over activates the HPA axis, which promptly develops the state of exhaustion which leads to dysregulation of stress mediators and even causes death. As expected, due to chronic unpredictable stress there was elevated level of cortisol[23,25]. In activation of apoptosis in zebrafish heart through the induction of chronic unpredictable stress, the key effectors for the stimulation of stress is through the activation of hypothalamic pituitary adrenal (HPA) axis to produce CRF and subsequent release of glucocorticoids. CRF is the key modulator of stress[36,43]. Generally, glucocorticoid modulates the HPA axis by inhibiting its activation through delayed feedback mechanism and involves in genomic alteration.

Cardiovascular disease stays as an escalating cause of death in many developing countries[10]. Stress has a foremost effect on cardiovascular system though it has a varied pathology. Stress plays a major role in vulnerability and progress in cardiovascular diseases. Stress has a deleterious effect on the cardiovascular system[44]. When there is increased effect of stress, there is an imbalance between the reactive oxidative species and the antioxidants. Increased release of reactive oxidative species is the principle mediator of cardiac myocytes dysfunction through apoptosis and necrosis[9]. According to the recent study reports, apoptosis plays a key role in many cardiovascular diseases such as the acute myocardial infarction[45], end stage heart failure[46] dilated cardiomyopathy[47], atherosclerosis[48] and myocarditis[49].

After chronic unpredictable stress paradigm, zebrafish was exposed to novel tank and light and dark preference test to analyse the behavioural alteration in both control and stress groups simultaneously. Novel tank and light and dark preference test are considered to be the characteristic test for anxiety. In novel tank test, zebrafish exposed to CUS showed a longer latency to reach the top of the tank and the time spent in the upper portion of the tank transition to top and the average duration was reduced. It has also been evident that there was an elevated erratic movement and freezing duration. While in the control group, zebrafish was explorative[29,27]. Light and dark preference test were also used to assess anxiety in fish. Zebrafish naturally have a preference to stay in dark environment rather than to be in light environment. This form of discrimination in zebrafish is due to aversion to danger[50]. CUS exposed zebrafish was showed elevated time spent in the light environment than in the dark environment[19]. Based on the results of novel tank test and light and dark preference test, CUS exposed zebrafish provides evidence that they are anxietic.

Chronic stress response in the cardiac myocytes causes apoptosis. Apoptosis is mediated through two major pathways intrinsic or mitochondrial pathway and extrinsic or death domain pathway[15]. *p53* is one of the extensively studied tumor suppressor gene. It acts to integrate multiple stress signals in a series of quantitative and qualitative events of response[51,52]. In this way, when chronic unpredictable stress was induced, this would have caused DNA damage in *p53* mediated apoptosis and gets triggered[53].

p53 mediated apoptosis activates intrinsic pathway, the *p53* may induces noxa in response to DNA damage[54,55]. noxa is the transcriptional target of tumor suppressor *p53*[56]. noxa upregulation causes mitochondrial permeabilization which can mediate apoptosis[57]. As *noxa*, *p53* may induce permeabilization in the outer membrane of mitochondria, which may cause the inhibition of anti-apoptotic *bcl2*[51,58]. Due to the permeabilization of mitochondria there is a release of cytochrome C, which associates with *apaf1* and caspases cascade[57,59] and

there is activation of *caspase3* and which induces cell death[56] in cardiomyocytes. From the above mentioned evidences, it may be concluded that, mitochondrial pathway may be activated by *p53* involving caspases cascade leading to apoptosis.

p53 also mediates the extrinsic pathway, through the induction of transmembrane protein genes which are encoding for tnfα and there forms a death inducing signaling complex. It may promote the activation of caspases cascade and it may upregulate *caspase3* which in turn induces apoptosis in cardiomyocytes[34,60]. Both mitochondrial pathwayas well as the death domain pathway converges at *caspase3* which cleaves the inhibitor of the caspase, activating deoxyribonucleases and leads to apoptosis in the cardiomyocytes[61]. The present study gives an idea that chronic unpredictable stress causes apoptosis in the cardiomyocytes of zebrafish leading to various cardiac disorders.

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