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Forced Swim Stress Does Not Induce Structural Chromosomal Aberrations in Rat Bone Marrow

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Abstract: Anything that poses a challenge or a threat to the well-being is a stress. Understanding the genetic material and cellular response of rats threatened with repeated swim stress provides insights that can influence human healthy. The aim of the present study was to assess the genetical damage and cytological changes caused by exposure of the test organism (Rattus rattus) to forced swim stress. For this purpose, animals have been submerged in water path 15 min daily for 2 weeks. Following that we performed a Micronuclei (MN) test using MNNCE (Micronucleated Normocromatic Erythrocytes) and MNPCE (Micronucleated Polychromatic Erythrocytes), NDI (Nuclear Division Index) and cytological parameters using NDCI (Nuclear Division Cytotoxicity Index), necrotic and apoptotic cells in rat's bone marrow samples. Results showed that there was a slightly but not significant increase in the frequency of micronucleated as well as in cytological parameters in bone marrow cells. In light of these results if the time-influence taken account forced swim stress may be considered unsafe suffering for a long time.

Key words: Submergence stress, micronucleus, NDI, NDCI, toxicity, chromosomal aberrations

INTRODUCTION

Most existing animal models for stress involve the simultaneous application of physical and psychological stress factors. Stress models including chronic mild stress (Li *et al*., 2003; Papp *et al*., 2002; Lei and Tejani-Butt, 2010), acute or chronic restraint (O'Mahony *et al*., 2011), acute or chronic inescapable stress (Storey *et al*., 2006), unpredictable chronic stress (Herman *et al*., 1995), variable chronic stress (Katz *et al*., 1981), acute electric foot-shock (Yoshioka *et al*., 1995) and communication box for hours or 7 days (Liu *et al*., 1999) have been commonly used to explore the relationship between psychological stress and its effects. Chronic mild stress includes food and water deprivation, paired housing, soiled cage, backward tilting of cages and stroboscopic illumination in darkness. Water and food deprivation isolation, flashing light, forced swim, restraint and cold are used in variable chronic stress (Liu *et al*., 1999). The communication box is a very good psychological stress model but cruel to rats that are exposed repetitively to electric foot shock. Most of the other existing stress models involve the simultaneous application of both physical and psychological stressors. Moreover, physical stress can influence the effects of psychological stress.

Psychological stress leads to various changes including clinical depression, cardiovascular disease, cancer as well as impaired spatial learning and memory (Cohen *et al*., 2007). But the relationship between the chromosomal aberration and the forced swim stress has not been completely examined.

Thus, the aim of this study was to provide a model fill some of the void in the evaluation of the toxicity potential of forced swim *in vivo* using micronucleus, NDI (Nuclear Division Index) and NDCI (Nuclear Division Cytotoxicity Index).

The micronucleus test is an *in vivo* and *in vitro* short-time screening test (Heddle, 1973; Schmid, 1975) is widely used to detect genotoxic effects (Villarini *et al*., 1998). It is one of the simple, reliable, cheap and rapid screening system for both clastogenic effects (chromosome breakage, formation of a centric fragments) and an eugenic (chromosome lagging and effects on spindle) (Heddle *et al*., 1993, 2011). Clastogenic and an eugenic agents affect the spindle apparatus which can be differentiated on basis of the relative induced micronucleus sizes or with the presence of kinetochores. Micronuclei formation can occur in any nucleated and dividing tissue of any species (Heddle, 1973).

MNi are morphologically identical to but smaller than nuclei. They also have the following characteristics: the diameter of MNi in human lymphocytes usually varies between 1/16th and 1/3rd of the mean diameter of the main nuclei which corresponds to 1/256th and 1/9th of the area of one of the main nuclei in a BN cell, respectively. MNi are non-refractile and they can therefore be readily

distinguished from artefact such as staining particles. MNi are not linked or connected to the main nuclei. MNi may touch but not overlap the main nuclei and the micronuclear boundary should be distinguishable from the nuclear boundary. MNi usually have the same staining intensity as the main nuclei but occasionally staining may be more intense (Fenech, 2000).

Nuclear Division Index (NDI) is useful parameters for comparing the mitogenic response of lymphocytes and cytostatic effects of agents examined in the assay. NDI is often calculated using the equations (Eastmond and Tucker, 1989; Fenech 2000):

$$NDI = \frac{M1 + 2(M2) + 3(M3) + 4(M4)}{N}$$

Where:

M1-M4 = The number of cells with 1-4 nuclei
N = The total number of viable cells scored

A more accurate assessment of nuclear division status is obtained if necrotic and apoptotic cells are included in the total number of cells scored because at higher toxic doses of chemicals tested one can expect a very large proportion of cells becoming non-viable. It is therefore, important to note that both binucleate ratio and the NDI are overestimated if necrotic and apoptotic cells are not included when scoring cells. A more accurate estimate of nuclear division status and cell division kinetics can be obtained using the following modified equation which takes account of viable as well as necrotic and apoptotic cells:

$$NDCI = \frac{(Ap + Nec + M1 + 2(M2) + 3(M3) + 4(M4))}{iV^*}$$

Where:

NDCI = Nuclear division cytotoxicity index
Ap = Number of apoptotic cells
Nec = Number of necrotic cells
M1-M4 = Number of viable cells with 1-4 nuclei
N* = Total number of cells scored (viable and non-viable)

Necrosis and apoptosis is important for the accurate description of mechanism of action and measurement of cellular sensitivity to a chemical, stress or radiation (Fenech, 2000).

Cells showing chromatin condensation with intact cytoplasmic and nuclear boundaries or cells exhibiting nuclear fragmentation into smaller nuclear bodies within an intact cytoplasm/cytoplasmic membrane are classified as apoptotic. Cells exhibiting a pale cytoplasm with numerous vacuoles and damaged cytoplasmic membrane with a fairly intact nucleus or cells exhibiting loss of cytoplasm and damaged/irregular nuclear membrane with a partially intact nuclear structure are classified as necrotic (Fenech, 2000).

In the light of these data and information, the experiment has been designed to assess the toxicity impact of forced swim on rat's bone marrow cells.

MATERIALS AND METHODS

Animal treatment *in vivo* test: Experiments were performed with female Wistar rats obtained from the animal house at King Khalid University, Department of Biological Sciences (weight range, 220-240 g) that were kept at controlled environmental conditions at room temperature (22±2°C) and under 12 h light/dark cycles. For the time-course experiment, rats were divided at random into 5 groups, five female albino rats animals in each group and the exposure time to forced swim was 30 days. In preliminary assay, group one was forced to swim 3 times daily for 5 min each while second group was forced to swim 10 min each last group was forced to swim 15 min each.

The positive control group was injected by single dose Cyclophosphamide and the exposure time was 24 h. Animals were sacrificed by cervical dislocation after the different treatments.

Test and positive control: Cyclophosphamide was obtained from Sigma Chemical Company.

Micronucleus assay: At the end of the experimental period, animals (Rats from each of the treatment groups and the positive control group) were sacrificed by cervical dislocation at the noon of the next day after the last injection. Both the femora were removed and cleaned with gauze by removing all the adhering muscle and tissue and subjected to micronucleus assay. The bone marrow was flushed out from both femurs using 1 mL of RPMI 1640 medium (bone marrow cells were pooled from both femurs of each animal) and centrifuged at 1000 rpm for 10 min. The cell were washed twice with Phosphate Buffered Saline (PBS) followed by centrifugation at 1000 rpm for 10 min. The supernatant was removed by aspiration and the cells were fixed in cold 3:1 methanol:acetic acid. Slides were prepared by dropping portions of the pellet on slides then air-dried for 20 min. Slides were stained with 5% solution of Giemsa in 0.01 M phosphate buffer at pH 7.4 according to the method described by Schmid (1975) with slight modifications. Two bone marrow smears per animal were prepared.

Genotoxicity scoring: The micronucleus frequency (expressed as percent micronucleated cells), NDI, NDCI, necrotic and apoptotic cells were determined by analyzing the number of micronucleated PCEs from at least 1000 PCEs per preparation (Fenech, 2000).

Cytotoxicity scoring: The PCE:NCE ratio, necrotic and apoptotic cells were also calculated to evaluate the cytotoxic effect of forced swim stress by scoring the number of PCEs and NCEs in 1000 cells per animal (Ouanes *et al*., 2003).

Statistical analysis: Statistical analysis were performed using anova single factor was considered to indicate statistical significance.

RESULTS AND DISCUSSION

Part 1: Clastogenic effect of forced swim was the main purpose of this part of the study. Micronucleus test revealed that there was no significant induction of micronucleus in Wister rats bone marrow. Comparing results showed that control group and the experimental group have almost same value of MNE. Whereas the positive control showed more significant comparing with experimental and negative groups (Table 1). The microscopic investigation of micronuclei also not showed a variation in their shapes and number per cell as shown in (Table 1). The micronucleus type (M1) was found in all groups while M2, M3 and M4 types were not present in all groups including negative control. Although, it was very slightly in positive control (Table 1 and Fig. 1, 2).

Part 2 (cytotoxicity of forced swim stress): In this part of the study, we tried to asses cytotoxic effect of forced swim stress by scoring necrotic and apoptotic cells. The microscopic investigation of necrotic and apoptotic cells also showed very rare variation in all treated groups, apoptotic and necrotic cells were slightly increased depending on the stress time as summarized in Table 2 and Fig. 2).

Stress is a famous word refers to agents occur inside the cell and have many different effects such as oxidative stress (Cechella *et al*., 2014), forced swim produces signs

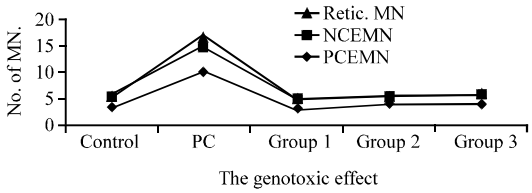

Fig. 1: The ratio of micronucleus formation after forced swim stress in bone marrow cells in different groups. Retic: Reticulocytes; NCE: Normochomatophyl; PCE: Polychromatophyl; PC: Positive Control; Retic. MN: 0.2, 2.3, 0.3, 0.2, 0.5; NCEMN: 2, 4.6, 2, 1.6, 1.5 and PCEMN: 3.4, 10.4, 3.1, 4, 4.1

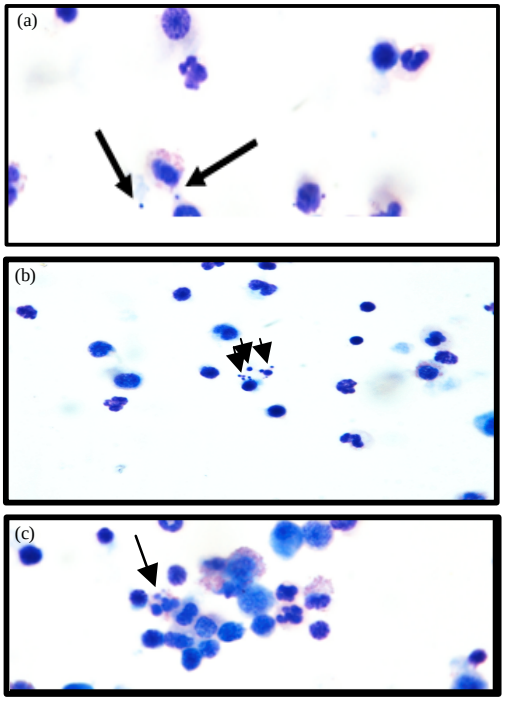

Fig. 2: a) M1 (one micronuclei per cell); b) M4 (four micronucleus per cell) and c) apoptotic cell were observed in different groups, cells were stained by Giemsa and May-Grunwald stain and photographed by Nikon microscope 40X

Table 1: Genotoxicity results for all experiment groups

Genotoxicity results of stress (Control+stressed groups)					Comparing to control	
Bone marrow	Total MN.	Min. MN.	Max. MN.	Abundant MN. type	f	F_{crit}
Control	15	1	5	M1	-	-
Positive (Cp)	42	6	12	M1	2.00	2.59
Group 1	19	0	4	M1	118.00	6.59
Group 2	13	1	5	M1	40.57	6.59
Group 3	17	1	6	M1	28.79	6.59

Table 2: Cytotoxicity results for all experiment groups

Cytotoxicity results of forced swim stress (Control+stressed groups)						Comparing to control	
Bone marrow	Nec.	Apop.	NCE	PCE	Ratio of PCE/Total	f	F_{crit}
Control	1	0	601.6	398.4	0.3984	-	-
Positive (Cp)	0	1	624.0	376.0	0.3760	1147.1	9.55
Group 1	0	0	617.0	383.0	0.3830	2407.2	9.55
Group 2	1	0	659.5	340.5	0.3405	179.42	9.55
Group 3	1	0	714.8	285.2	0.2852	50.680	9.55

Nec.: Necrotic cells; Apop.: Apoptotic cells; NCE: Normochomatophyl; PCE: Polychromatophyl; PC: Positive Control

and symptoms typical of other stress (Lin *et al*., 2014). As well as most of the studies say exercise is considered to be a safe medicine to attenuate inflammation and cellular senescence (Cechella *et al*., 2014). But her the point that should confirmed is swim for fun does not look like swim for keep alive. Because swim for fun has very low stress potential while swim to keep a live full of fear leads to different type of stress (Yoshioka *et al*., 1995). This study was designed to explore the relationship between this type of stress (fear stress) and the formation of micronucleus or apoptosis. Although, micronucleus and apoptosis look like to be different parameters but many studies were confirmed that micronucleus and apoptosis are very related to each other, once a cell has made the decision to proliferate, rather than to senesces or differentiate, it is a vital that undamaged DNA is replicated because if DNA damage is substantial, its replication can lead to chromosome loose or rearrangement. Thus, there is a major check point in late G1 phase that is activated by damaged DNA. At this check point, cells are prevented from entering S phase, although there is debate over whether cells are able to repair DNA and go on to enter S phase or whether they are permanently arrested in a state resembling senescence or do activate Caspases and go on apoptosis (Di Leonardo *et al*., 1994; Dulic *et al*., 1994; Donehower, 2014; Shaltiel *et al*., 2014).

The results do confirmed the previous observations, regarding the relationship between the ratio of apoptotic cells and the ratio of micronucleated cells. Demonstrated that no significant differences in cytotoxic and genotoxic responses to the forced swim test. Results also re-stressed the association between genotoxic and cytotoxic responses to the swim test.

In addition to that experimental variables such as body weight and water depth could not significantly modify the outcome of micronucleus testing. Also, it is very clear that forced swim is not carry high rescue to effect the genetic material in bone marrow cells.

CONCLUSION

In the light of all these results and previous research, we recommend that it may need more specific research to identify and explore the mechanism of action of forced swim stress.

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