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Research Article

Restraint Stress-Induced Micronuclei and Lymphocyte Alteration in Albino Rat

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Abstract

Aim: In this investigation restraint stress of 3 hours/day for 6 days has been given to evaluate the mutagenicity on bone marrow cells of male rat, *Rattus norvegicus*.

Main Methods: Micronucleus test (MNT) has increasingly been used to detect the genotoxic effects of physical and chemical stress, and its frequency considered to reflect the genotoxic damage to cells, mainly the chromosomes. Besides, morphologically altered lymphocyte is taken as an index of cytotoxicity. Both parameters were used in the present study by physical restraint stress for genotoxic and cytotoxic endpoints by *in vivo* assay on bone marrow cells of Wistar albino rat, *R. norvegicus*. Cytogenetic preparations were made by Giemsa-staining technique.

Key Findings: Increase in micronuclei (MN) and altered cells (lymphocytes) frequencies were significantly higher than control. The mean frequency of MN 3.73 ± 0.3 and altered cells 7.41 ± 1.21 were significant as compared to their control mean value 0.34 ± 0.1 and 2.90 ± 0.9, respectively. A positive time- and stress-response was observed in sampling.

Significance: The restraint stress was found to be genotoxic as well as cytotoxic in *R. norvegicus*. Further suitability of the adopted parameters for the screening of the genotoxicity by restraint stress is required on long term exposures.

Keywords: *Restraint Stress; Bone Marrow; Micronucleus; Lymphocyte Alterations; Immune System*

Introduction

Evidences suggest that behavioral factors such as stress can influence, and/or even cause a wide range of diseases [1]. It is now well established that stress influences the immune system [2,3]. Stress is conceived as any physical, psychological and/or environmental stimulus which disturbs physiological homeostasis and may result in widespread changes in a variety of biological systems [4]. Restraint stress is an appropriate and well-known method to induce chronic physical and emotional stress [5].

Accumulating evidence indicates that stress can stimulate numerous pathways leading to an increased production of oxidants [6]. Intensive stress has detrimental effects on organism by causing cellular and tissue injury [7]. It results in the generation of oxidative stress which has been implicated in a variety of pathophysiological states [8]. Recent studies clearly indicate that psychogenic stress in rats induces damage on both chromosomal and molecular levels [9,10].

It is also associated with strong psychological and immune function changes [11]. So, one major pathway by which stress may be related to genotoxicity is change in immune reactivity during and after exposure to stress because psychological stress can modulate

cell-mediated immunity by suppressing lymphocyte proliferation and natural killer (NK) activation, lowering the number of CD4+ cells in the peripheral blood and altering CD4/CD8 T cell ratios [12,13]. Thus, stress affects the immune system by exerting profound effects on lymphocyte trafficking and localization of different subsets [14,15]. It has been hypothesized that stress may influence immune function by inducing damage to nuclear material within an immune cell and/or alter the capacity of immune cells to repair DNA damage [16]. It is further reported that stress induces deoxyribose nucleic acid (DNA) damage which results in chromosomal damage in bone marrow cells and increased unscheduled DNA synthesis in lymphocytes [9,10]. In another study the reduction in lymphocyte count was also noticed in rats exposed to restraint stress [17].

Micronucleus assay in mammalian (rodent) system has been widely used in determining oxidative stress ability to induce chromosomal damage using bone marrow cells of rats/mice, according to the International Conference on Harmonization (ICH) as standard *in vivo* genotoxicity assay [18]. Only few studies have focused attention on the mechanism of micronuclei (MN) formation by restraint stress whereas numerous other studies have revealed that exhausting physical activity and severe environmental and/or psychological stress have strong suppressive effects on the immune system [19]. Such suppression of the immune system has significant implications for disease susceptibility and progression. Investigations in both humans and animals have revealed that stress could promote tumor development [20], autoimmunity [21] and infectious diseases by influencing the onset, course, and outcome of the pathological processes [22]. Interestingly, acute psychological stressors and moderate physical exercise transiently enhance immune responses [23]. Acute restraint stress of 2hrs could considerably enhance delayed-type hypersensitivity reaction in a rodent model [14]. In addition, acute stress has also been shown to increase antibody production [24].

When a bone-marrow erythroblast develops into a reticulocyte or a polychromatic erythrocyte, then nucleus is expelled out and any micronucleus that has been formed remains in the otherwise enucleated cytoplasm and is thus readily recognizable [25,26]. There are several studies on production of micronuclei induced by using regular industrial chemicals and agricultural pesticides in plants and animals [27-30] but there is no report on restraint stress-induced MN in BMCs on male rat, *R. norvegicus.* The present study was design therefore to assess the effects of restraint stress on the micronucleus frequency in polychromatic erythrocytes (PCEs) and alteration in lymphocytes on bone marrow cells of rats. To the best of our knowledge this is the first study which involves evaluation of the MN frequencies and lymphocyte alterations on bone marrow cells of rats subjected to restraint stress.

Materials and Methods

Animals and housing

Adult male albino rats (200 ± 50 gm) were obtained from Central Animal House of J.N Medical College, A.M.U, Aligarh. The animals kept in polypropylene cages, were housed under standard conditions (30 ± 2°C, light: dark = 1:1 h cycle) and maintained on standard pellet diet and water *ad libitum*. The rats belonging to either of groups were sacrificed in compliance with the ethical regulations formulated by the Ethical Committee (Registration No. 401/CPCSEA) of the University.

Treatment

Animals were divided into two groups and each group consisted of six animals. The arrangements of groups were as follows: First group treated as control and second was of restraint stress (3h/day for 6 consecutive days). The animals were restrained [31]. for given duration in a wire meshed cage at a fixed time.

Micronucleus test

The micronucleus test was carried out following the procedure of Schmid [25].

Sample collection and slide preparation

Animals were sacrificed on 7th day and femurs were dissected out immediately. Bone marrows were flushed out with 1 ml of fetal bovine serum (FBS) in a tube as a fine suspension and centrifuged at 1,000 rpm for 5 minutes. The suspension was smeared on a clean glass

slide. The slides were dried overnight and fixed in 100% methanol for 5 min and stained with Giemsa. Slides were cleared in xylene and permanently mounted by DPX [25]. Best slide was analyzed for scoring and photographed. In each group a maximum of 6000 cells were examined at a magnification of 40 X and 100 X.

Statistical analysis

Statistical evaluation was done by applying Student's t-test, and values were considered significant at $P < 0.05$.

Results

Rats were treated with restraint stress of 3 hours/day for six days consecutively. MN frequency of bone marrow cells in stressed conditions has been presented in table 1. The counts were used to calculate mean frequency and standard deviation (SD).

Treatment	Exposure (Hrs/day)	Number of animal (n)	Total scored cells	PCEs	Total no. of MNPCEs	Mean frequency of MN $\bar{x}$ (%) ±SD
Control	3	6	6000	1775	6	0.34 ± 0.1
RS	3	6	6000	1126	26	3.73 ± 0.3

Table 1: *Micronuclei induction in bone marrow cells treated in vivo by restraint stress.*

**Significantly higher than the respective control value (t-test, P < 0.05).*

According to obtained data, a significantly high value of MN induction occurred in PCEs after 6 days exposure of restraint stress as compared to control. The mean frequency of MN by using 3 hours/day for 6 days of post-administration was recorded to be 3.75 ± 0.3 and 0.34 ± 0.1 of control group (Table 1; Figure 1; $P < 0.05$). Figure 2 indicates the location and existence of MN in PCEs. Distribution studies of MN revealed one micronucleus per cell in most of the observations, though some cells may have two to three per MN (Figure 2). Importance of sampling times to coincide with the maximum response of MN induction and altered cells were authenticated by the samplings followed in our studies. The time- and stress-dependent increase was clearly shown in this experimental study of restraint stress of 6 days exposure for 3 hours, consecutively.

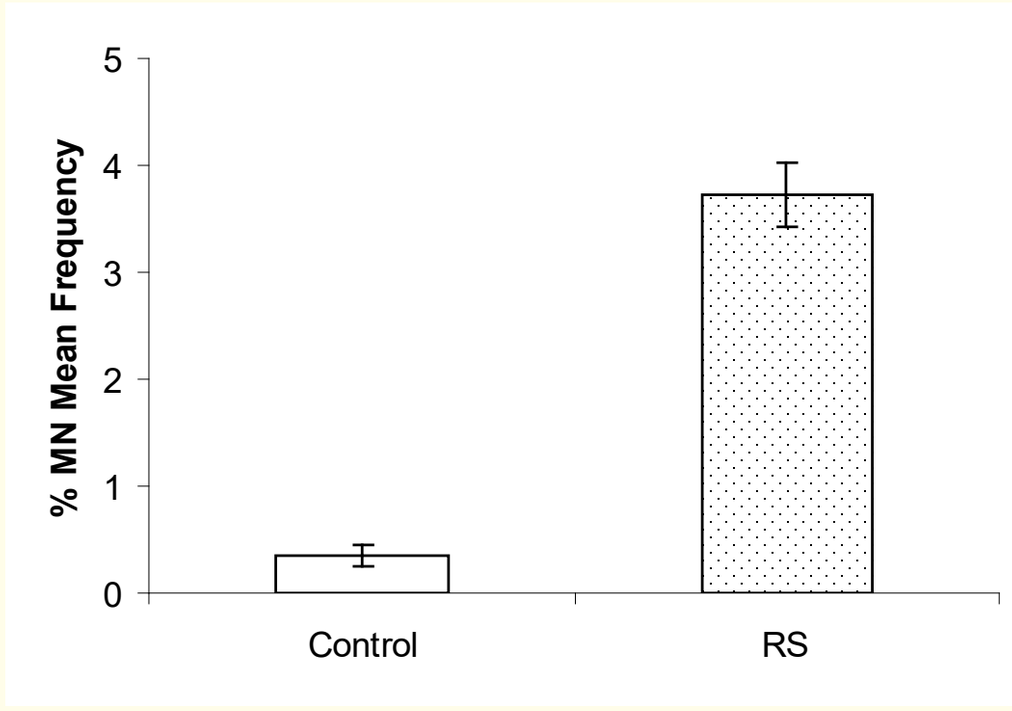

Figure 1: *Bar diagram showing micronuclei induction by restraint stress of 6 days exposure on Rattus norvegicus.*

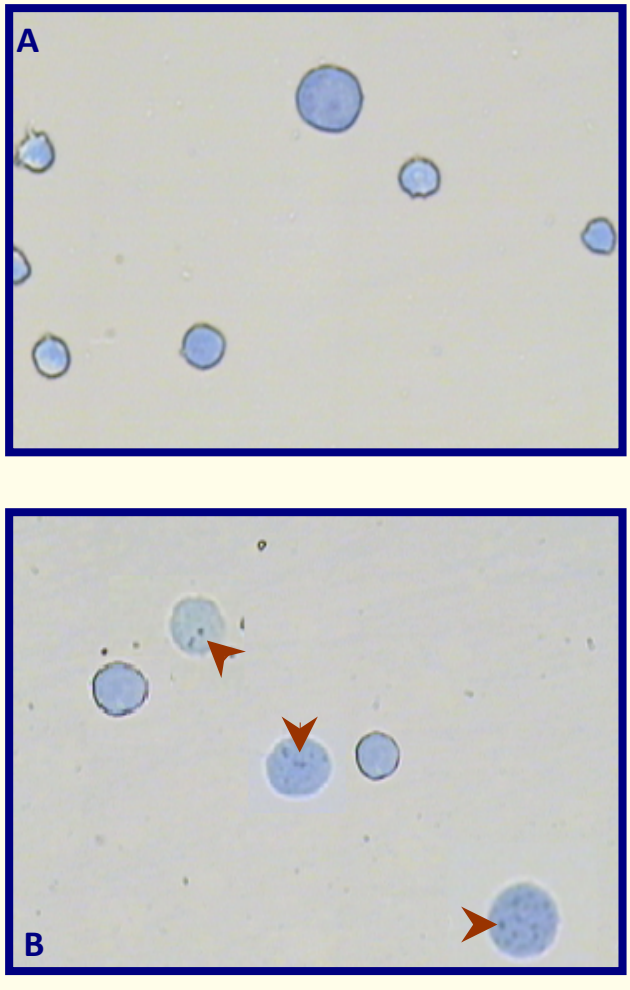

Figure 2: *Photomicrograph showing the normal plate of polychromatic erythrocytes (A) and micronuclei induction in polychromatic erythrocytes (B) by restraint stress of 6 days exposure on R. norvegicus.*

A summary of altered lymphocytes is shown in table 2. Alteration in the BMCs was higher and significant ($P < 0.05$) as compared to control. In most of the altered lymphocytes, the nuclear material was expelled out by the effect of restraint stress. The mean frequency of altered lymphocytes by restraint stress was 7.41 ± 1.21 as compared to 2.90 ± 0.9 of control group (Table 2); Figure 3; $P < 0.05$). In figure 4 the position of altered cells was indicated, in which nuclear material was expelled out, systemically.

Treatment	Exposure (Hrs/day)	Number of animal (n)	Total scored cells	Total no. of (AC) cells	Mean frequency of MN $\bar{x}$ (%) ±SD
Control	3	6	7500	218	2.90 ± 0.9
RS	3	6	7500	556	7.41 ± 1.21*

Table 2: *Alteration in the bone marrow cells treated in vivo by restraint stress on Rattus norvegicus.*

**Significantly higher than the respective control (t-test, $P < 0.05$).*

AL: Altered Lymphocytes.

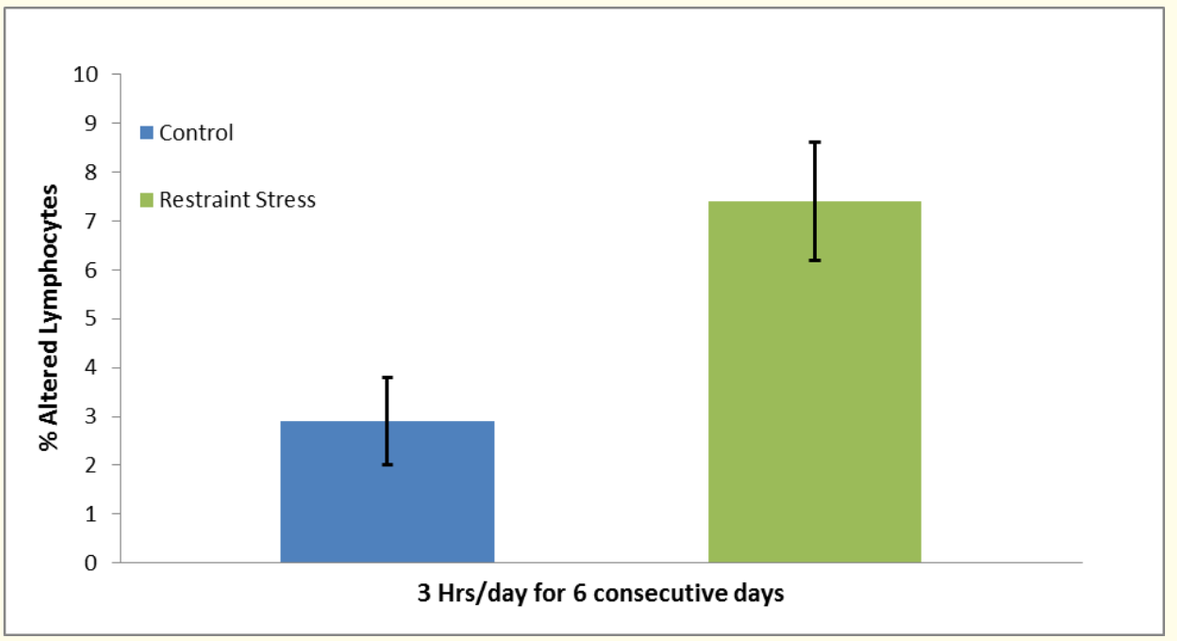

Figure 3: *Histogram showing lymphocyte alterations in bone marrow cells due to restraint stress of 6 days exposure on R. norvegicus.*

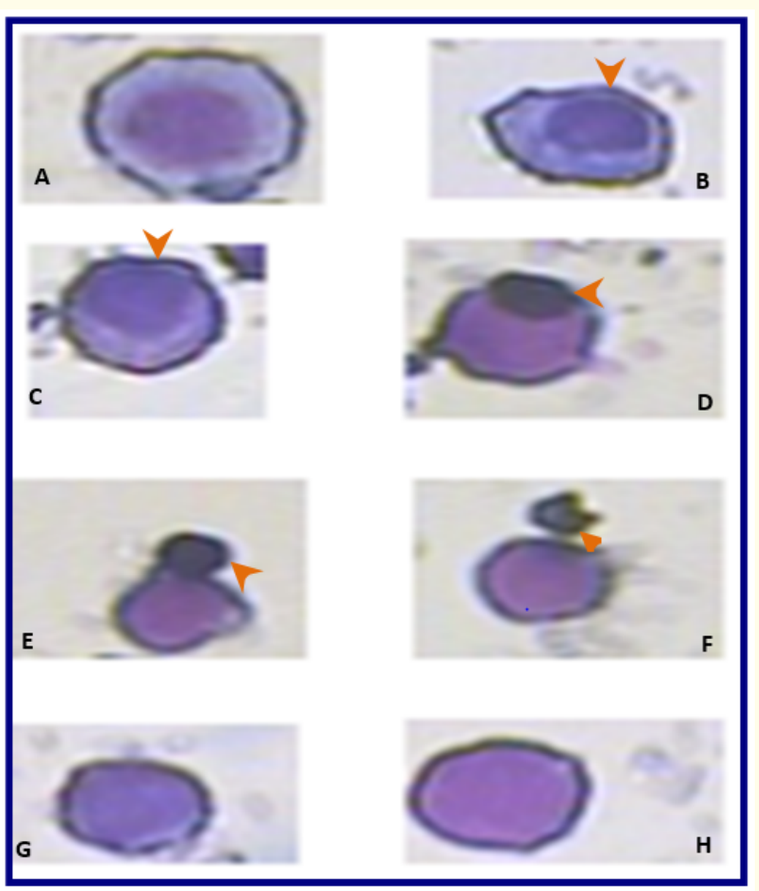

Figure 4: *Photomicrograph showing the normal lymphocyte (A); altered lymphocytes in which nuclear material expelled out from the cell by the damaged periphery (C-F) nuclear material completely removed from the cells (G and H) due to the restraint stress of 6 days exposure in lymphocytes on R. norvegicus.*

Discussion

Restraint stress-induced effects in mammalian systems has been extensively exploited for the last few decades but its effects on mammalian bone marrow cells of *R. norvegicus* is still scarce. The significant cytogenetic damage in BMCs was leading to micronucleus induction among the rats when treated with restraint stress for 3 hours per day for six days, consecutively (Table 1). In the present study, after treating *R. norvegicus* with restraint stress, a time- and stress-dependent increase of MN formation in PCEs were recorded. Our data demonstrated that restraint stress can produce mutagenicity which may damage the chromosomal DNA as indicated by micronucleus formation and lymphocytes alteration. The MN values were higher (3.73 ± 0.3) and showed the significance at $P < 0.05$ level when Student's t-test was applied.

Stress showed increase in chromosomal aberrations (CAs) and sister chromatid exchange (SCEs) in bone marrow cells (BMCs). The increase, to differing degrees, CAs and SCEs induced by the exposure to a variety of stressors, such as cold and warm water stress, white nose, and continuous or intermittent foot shock, demonstrated the general phenomenon of stress. The chromosomal damage can occur in as short a time as 2 hours and endure for at least 25 hours following exposure to stress. Furthermore, the detection of stress-induced damage by means of Unscheduled DNA Synthesis (UDS) extended these observations to the molecular level and to yet another cell type, leucocytes [10]. While in the present investigation restraint stress showed its positive effectiveness on PCEs and lymphocyte of bone marrow cells of rats, significantly.

A focus on the role of stress in disease has led to the development of the field of psychoneuroimmunology. Intensive research in this field in recent years has substantiated that there are physiological and molecular, as well as anatomical connections between the Central Nervous System (CNS), and the endocrine and immune system [32,33] which has clearly indicated by a report of Riley [34]. While on the other hand, research has also demonstrated that psychological factors, such as stress, contribute to the predisposition, onset, and course of several illnesses, such as depression, infections, rheumatoid arthritis, coronary heart disease, and other cancer in humans, and to herpes simplex, poliomyelitis, coxsackie B, polyoma, and induction and growth of Walker carcinoma and Ehrlich ascites in animals [35,36]. The micro nucleated polychromatic erythrocyte (MNPCE) frequency in rats without the added stress of tube restraint were consistently higher than those of rats with the added stress of tube restraint [38] but in our findings, it was different from above studies due to 6 days exposure of restraint stress for 3hrs/day consecutively which showed the effectiveness on PCEs and lymphocytes.

The stress-induced reduction in lymphocyte count was attributed to redistribution and trapping of lymphocytes in lymph nodes [38]. Under normal conditions in rats, the neutrophil count is less than that of lymphocyte [17] and, there is an inverse relation between these two cells both in basal and stressed conditions. Thus, the stress induced increase in neutrophil count could be secondary to reduction in lymphocyte count. Several clinical investigations have shown that stress can cause quantitative and/or qualitative alterations in human platelets [39,40]. The circulating platelet count is essentially regulated by production in the bone marrow, destruction and accumulation in the spleen, and intravascular consumption [41]. There is also some evidence to suggest that platelet adhesiveness and/or agreeability may transiently increase following exercise and acute mental stress [39,40]. In laboratory animals, however, studies concerning the relation between stress and platelets have been comparatively few, although some investigators have reported that some special forms of stress such as hypoxia [42], magnetic fields [43] and hypothermia [44] lead to thrombocytopenia. Thus, the relation between stress and platelets is complicated and the effects of severity and duration of stress species differences still remain to be elucidated.

The possible reason of these findings may be of restraint stress-induced oxidative stress can modulate protein and nucleic acids as earlier reported by our team which reacts with metabolic activities during that stress-time because without involving the metabolic machinery it cannot interact with DNA [30]. In the present study, we evaluated micronucleus incidence in BMCs on *R. norvegicus* by restraint stress and examined the data which was higher and significant ($P < 0.05$). In our findings, the ejaculation of nuclear material of lymphocytes on bone marrow may be due DNA damage by restraint stress. Restraint stress acts as precursor to genotoxic agents (pre-

carcinogens, pre-mutagens) which have no genotoxic properties per se and are converted into ultimate genotoxic agents by metabolism in susceptible organisms in pre-carcinogens, such metabolic products have generally been found to be more effective than the present in carcinogenic potency and range of tissue affected. Further investigation is required to define the potential causes for these different results. Our findings also observed micronuclei induction after restraint stress and supported these studies for the damaging effects of restraint stress in bone marrow cells (BMCs). Though, various changes in the immune system have been shown to be associated with stress, the exact mechanisms responsible for stress-modulated immune response remains to be elucidated.

In this investigation we concluded that restraint stress can induce the mutagenicity as micronuclei in the BMCs of *R. norvegicus*. Further, the nuclear material expelled out by restraint stress also suggests that restraint stress can also disturb the cytogenesis in the BMCs of *R. norvegicus* that are attributable to cytotoxic and genotoxic effects of the restraint stress.

Conflict of Interest Statement

None.

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