Effect of Restraint Stress on the Adrenal Cortex of Prepubertal Male Albino Rat: A Histological and Immunohistochemical Study

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Abstract

Background: The adrenal gland is the foremost stress responsive organ. This study was done to demonstrate the restraint stress-induced histological and immunohistochemical changes occurring in the adrenal cortex during the prepubertal stage of development.

Material and Methods: Forty male prepubertal albino rats (3 weeks old) of Wistar strain were included. The rats were divided into two groups; the control group and the stressed group. The stressed group was subjected to restraint stress for 14 days. The glands were subjected to light microscopic examination using Hx&E, Masson’s trichrome stains, and immunohistochemical examination using Ki67 and followed by statistical analysis of the mean area percent of fibrosis and optical density of immunoreactivity.

Results: The stress–induced changes were in the form of appearance of areas of areas of congestion and degeneration, increase in the mean area percent of collagen as well as appearance of immunoreactive nuclei. These findings were absent in the control group.

Conclusion: Stress caused modifications in the adrenal cortex structure and proliferative activity. This proliferative characteristic makes the adrenal gland a model system for the study of tissue renewal to accommodate needs.


Introduction

The response of an organism to stressful stimuli whether physical, metabolic, endotoxic or psychological, is characterized by the activation of autonomic and neuroendocrine system responses. The major neuroendocrine response mediating stress adaptation is activation of the hypothalamo–pituitary-adrenal (HPA) axis, with stimulation of CRH from the hypothalamus, leading to stimulation of pituitary ACTH secretion with the adrenal cortex representing the end organ and releasing glucocorticoids essential for stress adaptation [1].

Stressors like restraint results in increased mRNA expression levels of the ACTH receptor in the adrenal glands. This is accompanied by increased plasma CRH levels, suggesting an increased ACTH responsiveness in the stressed rats. These changes that occur in the adrenal glands in response to stress exposure enable the organism to mount adequate glucocorticoid responses to better cope with challenging situations [2].

Aim of work:

The aim of the present work was to demonstrate the histological, as well as the immunohistochemical changes occurring in the adrenal cortex following exposure to restraint stress in the prepubertal male albino rats.

Material and Methods

This study was done between April 2014 and March 2016. Forty prepubertal (3 weeks old) male albino rats of Wistar strain were used in this study. The animals were obtained from Kasr El-Ainy animal house. The animals were categorized into two groups; a control group and a stressed group.

The experiment was conducted according to the guidelines of the laboratory animal committee at Kasr El-Ainy. All animals were kept under standard laboratory conditions.
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The control animals were housed in standard cages (41x28x19cm).

Stress was conducted by placing each animal in plastic restrainers, the size of which was 6cm diameter x20cm long for 2 hours daily for 14 days. Restrainers were perforated at each end to allow ventilation and avoid overheating. During the stress protocol, animals could breathe, urinate and defecate without difficulty [4].

To reduce variance in the physiological parameters due to daily rhythms, all animals were sacrificed by cervical decapitation at the same time point in the circadian cycle, between 9:00 and 11:00 am.

The adrenal glands were dissected from the surrounding fat and subjected to the following:

**Paraffin technique [5]:**

The adrenal glands were dissected, fixed in 10% buffered formalin, and paraffin blocks were prepared. Sections of 5µm thickness were cut and stained with Hematoxylin and Eosin (Hx&E) stain [6] and Masson’s Trichrome stain [7].

**Immunohistochemical examination [8]:**

The Ki 67 antigen (a cell division marker) was used to detect cell proliferation in the adrenal cortex. Immunohistochemistry for Ki67 antigen detects cells that are in late G1, S, G2 and M but not G0 phases of the cell cycle [9].

1- Adrenal glands were dissected, fixed and sectioned as described above but mounted on Polysine slides.
2- Sections were dewaxed and washed in ethanol.
3- Endogenous peroxidase activity was blocked by immersing sections in 3% hydrogen peroxide in methanol for 30 minutes in the dark at room temperature.
4- Sections were then rehydrated in 70% aqueous ethanol (2-5min) and washed in phosphate buffered saline (PBS; 2-5min).
5- To unmask antigenic sites, sections were microwaved at full power for 4-5min in 0.01M sodium citrate buffer, pH 6.0,39 and then cooled and washed in PBS (5min).
6- Sections were treated with blocking serum for 30min.
7- Sections were then treated with primary anti Ki67 antibody for 2 hours, rinsed then washed in TBS (2-5min).
8- Biotinylated secondary antibody was applied for 30min.
9- Sections were washed in TBS (2-5min), treated with avidin biotin-horseradish peroxidase solution for 30min at room temperature and washed in TBS (2-5min).
10- Sections were finally incubated for 3min with 3,3’-diaminobenzidine solution (DAB isopac, Sigma D9015), comprising 25µl DAB (2mg/ml) + 5.9mls 0.2 MTBS + 1 µl 30% H2O2.
11- Sections were rinsed in water to stop the reaction, which produces a stable, dark brown insoluble precipitate at sites of antibody localization.

12- After lightly counterstaining with hematoxylin, slides were rinsed, dehydrated through graded ethanol, cleared in xylene and mounted under coverslips with DPX mounting medium.

The cells were considered positive for the immunohistochemical expression of the Ki 67 antigen when the nuclei are stained with a brownish color [10].

**1- The area percent of collagen fibers in Masson’s Trichrome stained sections:**

The image analyzer was first calibrated automatically to convert the measurement units (pixels) produced by the image analyzer program into actual micrometer units. Using the measuring field menu; the area, area percentage and standard measuring frame of a standard area were chosen. In each chosen field adrenal gland tissue was enclosed inside the standard measuring frame and then the connective tissue area was masked by a blue binary color to be measured. The measurements were done under magnification 400 in ten non–overlapping fields from each age group. The degree of color was fixed for all sections (Fig. 1).

**2- Optical density in Ki 67 stained sections:**

Estimation of the proliferating cells in the adrenal cortex was done through measurement of optical density of the immunoreactivity in the Ki 67 stained sections (Fig. 2).

**Statistical results:**

The measurements and counts were tabulated and subjected for statistical analysis using SPSS system.
Results

Control group:

1- Light microscopic results:

Examination of haematoxylin and eosin stained sections of the adrenal cortex of the prepubertal control albino rats revealed that the adrenal cortex exhibited three distinct zones ZG, ZF and ZR. ZG lied immediately beneath the capsule (Fig. 3). It consisted of arcades of rounded cells with acidophilic cytoplasm and rounded to oval nuclei (Fig. 4). The ZF lied between ZG and ZR and it appeared as regular columns of oval cells (Fig. 3). The ZF cells exhibited rounded nuclei with occasional binucleation. The ZF cells were separated by blood sinusoids lined by endothelial cells (Fig. 4). The innermost ZR was arranged as intermingled cords and network of cells (Fig. 3).

Examination of Masson’s trichrome stained sections showed that the gland was covered by a thin fibrous connective tissue capsule with a fine network of delicate collagen fibers separating the cells (Fig. 5).

2- Immunohistochemical results:

Ki 67 immunostaining revealed absence of immunoreactivity in the adrenal cortex of this group (Fig. 6).

Stressed group:

1- Light microscopic results:

Examination of haematoxylin and eosin stained adrenal cortex of the prepubertal stressed albino rats showed areas of degeneration with pale staining nuclei in the ZG and ZF. Congested blood capillaries were also observed in the ZF and ZR (Fig. 7).

Examination of Masson’s trichrome stained sections showed areas of thickened collagen bundle among the congested cells of the ZF (Fig. 8).

2- Immunohistochemical results:

Ki 67 immunostaining demonstrated multiple scattered immunoreactive nuclei all over the adrenal cortex (Fig. 9).
Fig. (4): A micrograph of a section in the adrenal cortex of the prepubertal control group showing the ZG formed of clusters of cells with acidophilic cytoplasm (dotted arrow) and rounded to oval nuclei. The ZF cells appear columnar with rounded nuclei (short arrows) and occasional binucleation (long arrows). The ZF cells are separated by blood sinusoids lined by endothelial cells (arrowheads). (Hx&E x200).

Fig. (5): A micrograph of a section in the adrenal cortex of the prepubertal control group demonstrating a thin fibrous connective tissue capsule (C) with a fine network of delicate collagen fibers (arrows) separating the cells. (Masson’s trichrome x150).

Fig. (6): A micrograph of a section in the adrenal cortex of the prepubertal control group showing absence of immunoreactivity. (Ki 67 x200).

Fig. (7): A micrograph of a section in the adrenal cortex of the prepubertal stressed group showing areas of degeneration with pale staining nuclei in the ZG (short arrows) and ZF (long arrows). Congested blood capillaries (arrowheads) in the ZF and ZR are also observed. (Hx&E x200).

Fig. (8): A micrograph of a section in the adrenal cortex of the prepubertal stressed group showing an area of thickened collagen bundle (long arrow) in the ZF. Congested blood capillaries (short arrows) are also observed in the ZF and ZR. (Masson’s trichrome x150).

Fig. (9): A micrograph of a section in the capsule (C), ZG and ZF of the adrenal cortex of the prepubertal stressed group showing multiple scattered immunoreactive nuclei (arrows). (Ki 67 x200).
Statistical results:

1- **Area percent of collagen:**

   The mean area percent of collagen increased in the stressed rats of the when compared with their controls, but this increase was found to be statistically non-significant ($p>0.05$).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Area of collagen (Mean ± SD)%</th>
<th>$p$-value</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>23±1.6</td>
<td>0.072 (NS)</td>
</tr>
<tr>
<td>Stressed</td>
<td>24.9±1.1</td>
<td></td>
</tr>
</tbody>
</table>

   NS: Non significant ($p>0.05$).

2- **Optical density of Ki 67 immunoreactivity:**

   The control group showed absence of immunoreactivity while the optical density of the stressed group was found to be 0.74.

**Discussion**

The process of experimental stress induction on animals have been described in response to various types of physical and psychological stressors such as restraint stress, cold stress, heat stress, noise stress, crowding stress, water deprivation, electric shock or water immersion [11].

Among all the above listed methods of stress induction, the present study chose restraint as a stress model because restraint is the method that imposes the least pain, suffering or distress to the animal.

Histological examination of the prepubertal stressed rats revealed multiple congested blood capillaries in the ZF and ZR as well as areas of degeneration in the ZG and ZF. Similar observations were reported by [12] who investigated the effect of stress on the pituitary and adrenal glands of prepubertal rats and found hyperemia and degeneration in both glands.

Since the adrenal tissue is a cell renewal system with dynamic structural changes, the homeostasis of the adrenal cortex during stress is considered to be accomplished by cell proliferation [13]. With this principle, the present study evaluated the immunoreactivity of Ki67 in an attempt to further elucidate the intra-adrenal response to stress. Immunoreactivity of Ki67 was quantitatively evaluated by optical density and expressed by mean ± SD.

In the present study, immunoreactivity was detected only in the stressed groups while all the control groups showed no evidence of immunoreactive nuclei. This finding comes in accordance with [14] who pointed out that cellular turnover in the adrenal cortex occurs only to accommodate physiological needs or in response to experimental manipulations.

The increase in the area percent of fibrosis in the present study was found to be statistically non-significant. This could be explained by [15] who pointed out that rats show a period of decreased responsiveness to noxious stimuli during the first few weeks of life. The nature of this impairment could be due to immaturity of the HPA axis including immaturity of the neural pathways and the neurotransmitter inputs.

**Conclusion:**

Stress exposure can exert direct effect on both morphology as well as the functionality of the adrenal glands. A remarkable change was the appearance of proliferating cells in response to stress.

**References**

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