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Full Length Research Paper

Unbiased stereological and histological study of silymarin effects on hamster adrenocortical structure in response to an exogenous glucocorticoid

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Adrenal cortex is an essential portion for life and its function can be affected by many chemical agents and drugs. In this investigation, effect of silymarin, a flavonoid, on adrenocortical structure of male dexamethasone (Dexa) treated hamsters were studied by histological and unbiased stereological techniques. The results showed that the adrenal gland mass of Dexa treated hamsters was significantly decreased in comparison to animals that received Dexa with silymarin (Sily). It was also obtained that there are no significant difference in the zona glomerulosa (ZG) volume and cell number among examined groups. The volume of the cortex, zona fasciculata (ZF) and zona reticularis (ZR) and cell number of these regions were significantly reduced in Dexa treated hamsters compared to controls (P<0.05), whereas in Dexa + Sily treated animals, this reduction was not observed. Histological results showed that tissue structure of ZF and ZR was same in all groups except Dexa treated animals. In the hamster that received Dexa, a sever hyperemia was observed in the adrenal cortex. Finally it can be concluded that silymarin seem to be a suitable protective drug for side effect of glucocorticoid therapy in adrenal glands.

Key words: Adrenal cortex, dexamethasone, hamster, glucocorticoid, silymarin, unbiased stereology.

INTRODUCTION

Pivotal role and key function of the adrenal gland in different stages of life caused to it is called a biological clock (Pawlikowski, 2005). The adrenal gland consists of two ontogenetically, structurally and functionally distinct endocrine tissues, the cortex and the medulla. The medullary cells are of neurodermal origin, whereas the cortical ones are of mesodermal origin (Mitani et al., 1999). The adrenal cortex is necessary to life in a variety of essential factors and its hormones influence numerous essential processes (Banks, 1993; Leeson et al., 1988). Adrenal is one of the most common endocrine organs affected by chemically induced lesions (Ribelin, 1984). It is especially important to understand the structure and function of the adrenal gland to correctly interpret the significance and mechanisms of drug-induced lesions (Rosol et al., 2001). On the other hand, adrenal insufficiency may be difficult to differentiate from other same conditions, such as chronic fatigue syndrome and depression. So, investigation of adrenal biology appears to be very important (Schimmer and Parker, 2001).

Glucocorticoid hormones influence the activity of al-most every cell in the body; they modulate the expression of approximately ten percent of human genes (Buckingham, 2006). Glucocorticoids and a variety of synthetic glucocorticoid agonists are able to control carbohydrate, protein and lipid metabolism and to regulate immune and cardiovascular functions. But in therapeutic concentrations, glucocorticoids are strongly immunosuppressive and anti-inflammatory, which has made them one of the most frequently prescribed drugs worldwide (Schmidt et al., 2004) and glucocorticoid therapy is the most common cause of adrenal failure (Cunha et al., 2004). Furthermore, it has been observed that dexamethasone treatment caused rapid adrenal atrophy (Mughal et al., 2004).

Silymarin is a mixture of polyphenolic flavonoids ex

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racted from fruits and seeds of milk thistle (Silybum maria-num [L.] Gaertn) (Greenlee et al., 2007; Katiyar et al., 2005). Extracts of S. marianum have been used in the treatment of liver diseases for more than 2000 years. It has been established that its effects results from a strong antioxidant activity (Kummer et al., 2001). Pharmacological studies indicated that there is no known LD50 for silymarin in laboratory animals (Lahiri-Chatterjee et al., 1999). Its lack of toxicity even when used in high doses broadens silymarin's potential for therapeutic use (Katiyar, 2002; Manna et al., 1999). Nowadays, silymarin became an ingredient of phyto-pharmaceuticals used often in some supportive therapy (Mansour et al., 2006). The present study was conducted to elucidate the effects of silymarin on adrenal cortex response to an exogenous glucocorticoid.

MATERIALS AND METHODS

Animals and experimental procedures

Twenty young adult male golden hamsters (*Mesocricetus auratus*) are used in this study. All hamsters were acclimatized to laboratory conditions in a room temperature maintain at 22 ± 1°C and a relative humidity range of 40-75% with a regular 12 h light:12 h dark cycle. The time of lights on and lights off were 7:30 AM and 7:30 PM respectively. They were kept for one week before use and fed a commercial pellated diet with the addition of fresh vegetables and water ad libitum during experimentation. At the beginning of the experimental period, the animals were randomly allocated to the following four groups, each consisting of five animals:

1. Control group that were given no drug.
2. Dexamethasone group (Dexa) which treated by 7 mg/kg daily.
3. Silymarin group (Sily) which treated by 100 mg/kg daily.
4. Dexamethasone and silymarin (Dexa + Sily) group which treated by 7 mg/kg dexamethasone and 100 mg/kg silymarin daily by IP injection. All injections were done at 10:00 am for 7 days by intraperitonealy (IP) injection. Then hamsters were weighted and two to three animals were housed in per cage. Animals were conducted in accordance with Iranian Humane Care and Ethical Animal Welfare.

Histological studies

One day after the final injection, the hamsters were euthanized and the body weight was recorded. Then the abdominal cavity was opened and the bilateral adrenal glands were quickly removed, trimmed of fat and connective tissue, weighed and fixed in buffered formalin 10%. The samples were processed by routine and standard paraffin embedding and serially sectioned in 5 µ thickness. Tissue sections were deparaffinized and stained with haematoxylin and eosin (H&E) (Bancroft and Gamble, 2002). At least microscopic slides were used for evaluation of their general morphology, histological description and unbiased stereological studies.

Stereological studies

A systematic uniform random strategy was used to obtain first section and sampling period in each block. Then, every tenth section of the series was saved, stained with haematoxylin and eosin and used for morphometric evaluation. Stereological analysis of sections and adrenocortical components was performed using Weibel's multipurpose test grid M42 by a point counting technique.

Ten fields of each adrenal zona were counted in a single section from each adrenal gland. Relative adrenal mass was also calculated as a percent of body mass. The total volume of adrenal gland, adrenal cortex and different cortical zone were estimated by Cavalieri's point-counting principle (Fabricius et al., 2007; Howard and Reed, 1998). Although tissue shrinkage occur during processing of the tissue, but it has been supposed that tissue shrinkage is the same in all experimental groups. To obtain an estimate of cell number per adrenal cortical zone, two equatorial sections of each gland were chosen and 30 test areas of zona glomerulosa and 50 test areas of zona fasciculata and zona reticularis were estimated at a high magnification with Weibel's multipurpose test grid M42. Since adrenocortical cells are mononucleic, numerical density of nuclei corresponds to the number of cells; hence the average number of adrenocortical cells in each zona was calculated from the number of nuclear profiles of parenchymal cortical cells (Milosevic et al., 2005; Plecas-Solarovic et al., 2003). Total number of adrenocortical cells in each zona was estimated from the formula: N = mean Nv Vref, where "Vref" is the volume of each zona that estimated with the Cavalieri's method. Mean Nv = - h, where "-" is the total number of nuclei that are counted in all of the dissectors; "h" is height of the dissector and " " is sum of frame associated points hitting reference space (Davanlou and Smith, 2004; Howard and Reed, 1998).

Statistical analysis

Statistical analysis was performed using the SPSS software package version 13.0. All results are expressed as the mean ± SEM. Variation within groups was indicated by the coefficient of variation (CV) while the average coefficient of error (CE) was used to estimate the precision of the measurements (Fabricius et al., 2007). The CE was calculated according to the formulas in Gundersen et al. (1999) and Davanlou and Smith (2007). Data were assessed for normality using the one-sample Kolmogorov-Smirnov test. Statistical comparison among treatment groups was made by one-way analysis of variance (ANOVA) with the least significant difference (LSD) test as a post hoc test to evaluate the means. A t-test was employed to determine statically significant differences between left and right adrenal glands. AP values less than 0.05 were considered statistically significant (Petrie and Watson, 2006).

RESULTS

As Table 1 shows the adrenal gland consisted about 0.024% of body weight in an adult male hamster. The absolute and relative adrenal gland mass of Dexa treated hamsters were significantly decreased whereas this reduction was not observed in Dexa + Sily treated animals in comparison to controls (Table 1). There were no significant differences between left and right adrenal glands in morphological parameters and histological features ($p > 0.05$).

Histological investigation of adrenal gland revealed that the parenchyma of the cortex consists of polygonal cells, which comprise great bulk of the adrenal cortex. In Zona Glomerulosa (ZG), the cells were small and possess few vacuoles and more organized into small arcs supported by connective tissue and capillaries. Cell structure and size of ZG were same in all experimental groups and no marked change was detected in the morphological structure of this region. The results of the stereological study of cortical zones showed that there are no differences in the volume of the ZG among various groups (Table 2).

Table 1. Body weight, absolute and relative weight of adrenal gland in different groups.

	Control	Dexa	Sily	Dexa + Sily
Body weight (g)	83.04 ± 6.64	77.17 ± 5.29	83.10 ± 2.77	74.66 ± 5.70
Absolute adrenal weight (mg)				
Left adrenal	9.94 ± 0.36^{a*}	8.00 ± 0.35^{b}	9.74 ± 0.35^{ac}	9.00 ± 0.07^{c}
Right adrenal	9.96 ± 0.29^{a}	7.82 ± 0.36^{b}	9.48 ± 0.45^{ac}	8.78 ± 0.11^{bc}
Total	19.90 ± 0.64^{a}	15.82 ± 0.72^{b}	19.22 ± 0.79^{ac}	17.78 ± 0.18^{c}
Relative adrenal weight (%)	0.024 ± 0.001^{a}	0.021 ± 0.001^{b}	0.023 ± 0.001^{a}	0.024 ± 0.002^{a}

* The means in rows with different superscripts are significantly different ($p< 0.05$).

Table 2. Estimated volume of adrenocortical zona in the adrenal glands (mm^3).

	Control	Dexa	Sily	Dexa + Sily
Cortex	7.03 ± 0.17^{a}	5.21 ± 0.22^{b}	6.78 ± 0.21^{a}	6.15 ± 0.04^{c}
CV	0.07	0.13	0.09	0.02
CE	0.03	0.04	0.03	0.03
Zona glomerulosa	1.26 ± 0.02	1.22 ± 0.01	1.25 ± 0.01	1.23 ± 0.01
CV	0.06	0.03	0.05	0.03
CE	0.03	0.04	0.03	0.03
Zona fasciculata	4.73 ± 0.12^{a}	3.22 ± 0.19^{b}	4.52 ± 0.17^{a}	3.98 ± 0.04^{c}
CV	0.08	0.18	0.12	0.03
CE	0.04	0.04	0.04	0.03
Zona reticularis	1.04 ± 0.02^{a}	0.77 ± 0.03^{b}	1.01 ± 0.03^{a}	0.94 ± 0.01^{c}
CV	0.06	0.12	0.09	0.02
CE	0.03	0.04	0.03	0.03

*The means in rows with different superscripts are significantly different ($p<0.05$).

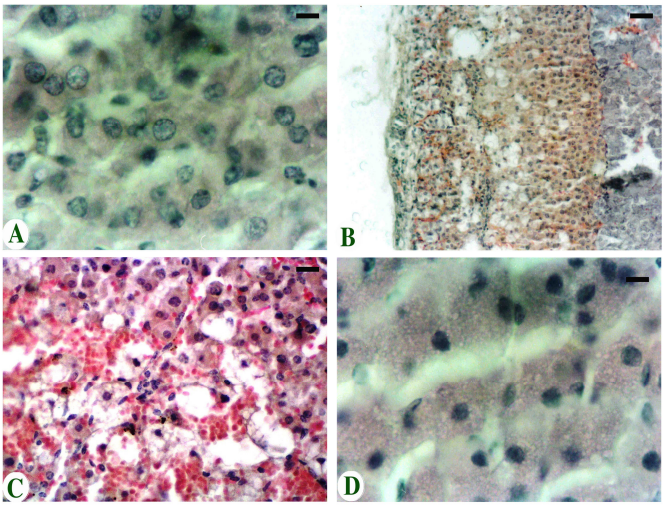

Figure 1. Light micrographs of adrenal gland, stained with H&E. (A) Part of ZF in normal hamsters, showing large and polyhedral cells filled with lipid droplets. Scale bar, 20 µm. (B) Adrenocortical zones in Dexa treated hamsters, reduction in volume of cortex, cell shrinkage, nucleus condensation and a sever hyperemia is noticeable. Scale bar, 200 µm. (C) ZF in Dexa treated hamsters, cell shrinkage, decrease of cytoplasmic lipid droplet and a sever hyperemia is noticeable. Scale bar, 50 µm. (D) Part of ZF in Dexa + Sily treated hamsters, showing that silymarin has decreased dexamethasone effects on ZF cells. Scalebar, 20 µm

The results were also obtained that there is no significant difference in number of ZG cells among examined groups (Table 3).

The Zona Fasciculata (ZF) was widest zone of the cortex and composed of uniformly large, polyhedral and vacuolated cells with large, light and vesicular nuclei that arranged in radial columns. The cytoplasm of ZF cells contained a moderate number of lipid droplets (Figure 1A). The zona reticularis (ZR) was an irregular network of anastomosing cell cords associated with extensive vascular sinuses. However the cells of this region have morphologic features similar to those of the ZF, but they were smaller and contained fewer lipid droplets. Histological results showed that tissue structure of ZF and ZR was same in all groups except Dexa treated animals. In the hamster that received Dexa, a sever hyperemia were observed in the adrenal cortex and cell shrinkage and nucleus condensation were noticeable in ZF and ZR of these animals (Figures 1B and 1C), whereas Silymarin could protect adrenocortical cells and decrease these changes (Figure 1D).

Table 2 illustrates that the volume of cortex, as well as the volume of the ZF and ZR was significantly reduced in Dexa treated hamsters ($P<0.05$). In the animals that received Dexa, a significant decreasing was also found in the number of ZF and ZR cells compared to controls (Table 3). As Figure 2 shows, the relative volume of cortex, ZF and ZR

Table 3. Number of adrenocortical cells ($\times 10^6$) in the adrenal glands.

	Control	Dexa	Sily	Dexa + Sily
Zona glomerulosa	1.07 ± 0.02	1.04 ± 0.01	1.06 ± 0.01	1.05 ± 0.01
CV	0.06	0.02	0.05	0.02
CE	0.03	0.02	0.03	0.02
Zona fasciculata	2.09 ± 0.04[a]	1.46 ± 0.06[b]	2.07 ± 0.05[a]	1.92 ± 0.02[c]
CV	0.06	0.14	0.07	0.03
CE	0.03	0.03	0.03	0.03
Zona reticularis	0.74 ± 0.01[a]	0.54 ± 0.02[b]	0.73 ± 0.02[a]	0.67 ± 0.00[c]
CV	0.05	0.13	0.09	0.02
CE	0.03	0.03	0.03	0.03

*The means in rows with different superscripts are significantly different (P<0.05)

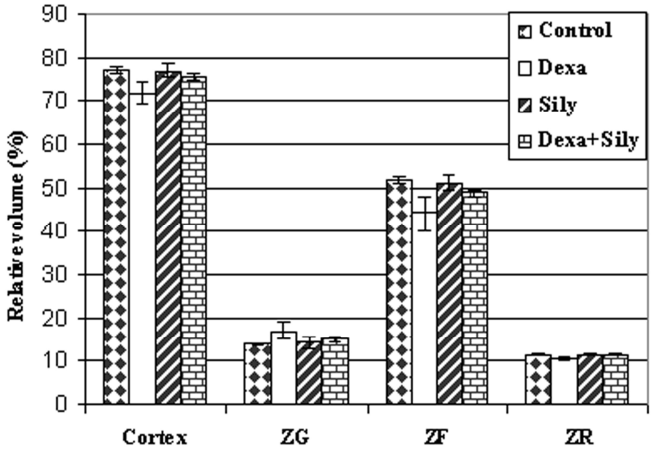

Figure 2. Relative volume of cortex, Zona Glomerulosa (ZG), Zona Fasciculata (ZF) and Zona Reticularis (ZR) in the adrenal glands of hamster.

were decreased in Dexa treated animals, while ZG relative volume increased in comparison to other groups.

DISCUSSION

Previous studies showed that the hamster is a potentially useful animal model for the study of adrenal biology in relation to human physiology. Hamsters differ from other rodents, such as the rat and the mouse, and like humans; cortisol is their major glucocorticoid that produce in adrenal gland (Brière et al., 1997). Adrenal cortex of hamsters like other mammals consists of three regions including ZG, ZF and ZR. However there is no difficult in separately identifying of ZG cells from ZF cells but distinguishing of boundary between ZF and ZR was some difficult, hence the size of the cells and their arrangement were applied for distinction of different zones (Reaven et al., 1988).

The results of present experiment explained several points. First, Dexa injection induced a significant reduction of absolute and relative adrenal mass due to reduction of cortical mass specially that of ZF. This was expected because glucocorticoid synthesis is performed mostly in the ZF that it is the largest part of the cortex (Koko et al., 2004). Histological and stereological results of this study revealed that Dexa injection provoked a depletion of lipid droplets from the ZF cells and also a reduction in the ZF volume and the cell number of this zone. This reduction was also weakly observed in ZR cells, which represent the innermost layer adjacent to the medulla. Many evidence indicated that cells of both ZF and ZR are under control of adrenocorticotrophic hormone (ACTH), an anterior pituitary hormone, and its actions are mediated via its interaction with specific receptors on the cell surface of adrenocortical cells (Mesiano and Jaffe, 1997).

It is well known that ACTH affects growth and endocrine function in the adrenal cortex and ZF cells secret glucocorticoids under the regulation of ACTH (Mitani et al., 2003). The release of ACTH is influenced by a wide variety of internal and external factors such as stressors and exogenous glucocorticoids (Hullinger and Andrisani, 2006). Circulating levels of glucocorticoids exert a negative feedback influence upon the hypothalamus and pars distalis (Banks, 1993). Dexamethasone suppresses the hypothalamic-pituitary-adrenal (HPA) axis (Cunha et al., 2004; Nolan and Levy, 2001). Mughal et al. (2004) and Lesnieska et al. (1992) reported that dexamethasone treatment result in rapid adrenal atrophy. It is also demonstrated that dexamethasone treatment of pregnant rhesus monkeys during late gestation causes atrophy of the fetal zone of the adrenal (Mesiano and Jaffe, 1997).

Stereological data of this investigation obtained that the cell number of ZF and ZR reduced following Dexa administration. This phenomenon could be explained by the induction of programmed cell death. Glucocorticoids (especially dexamethasone) are known to induce apoptosis and reduce proliferation in a variety of different cells (Ranta et al., 2006; Schmidt et al., 2004). Different studies showed that apoptosis was increased in the adrenal cortex after administration of glucocorticoids by suppression of ACTH secretion (Kiess and Gallaher, 1998). Glucocorticoids have been conferred to induce oxidative stress and the release of mitochondrial cytochrome c (Hegardt et al., 2003; Tonomura et al., 2003). On the

other hand, glucocorticoids have been proposed to suppress prosurvival factors such as nuclear factor-κB (Amsterdam et al., 2002). Thus, although glucocorticoids are widely used therapeutic tools for many causes, but it must be administrated carefully (Ranta et al., 2006). However results have been indicated an increase in relative volume of ZG in Dexa treated hamsters compared to other groups, but this increasing of relative volume of ZG due to reduction of total volume of adrenal gland.

The second important point of this experiment was silymarin effect on adrenal structure of Dexa treated animals. As stereological studies show, there was no different in the relative adrenal weight of animals that received dexamethasone accompanied by silymarin. Silymarin partially prevented from histological changes and reduction of ZF and ZR volume and cell number of these zones following dexamethasone administration and produce a partial blockade/recovery. However, the molecular bases of cytoprotective effects of silymarin is yet unknown; it might be related to its antioxidant activity and capable of scavenging both free radicals and reactive oxygen species (Abrol et al., 2005; Kohno et al., 2005; Křen and Walterová, 2005).

The known modes of action of silymarin or silybin include both direct antioxidant activity mediated through scavenging of free radicals, and modulations of antioxidant and inflammatory enzymes (Zhao et al., 2000), inhibition of mitogenic and cell survival signaling or modulations of cell-cycle regulators (Singh et al., 2002). Silymarin can also interact directly with cell membrane components to prevent any abnormalities in the content of lipid fraction (Pradhan and Girish, 2006). It has been established that anti-inflammatory effects of silymarin might be related to the inhibition of the transcription factor NF-κB, which regulates the expression of various genes involved in the inflammatory process, in cytoprotection and carcinogenesis. It has also been hypothesized that silymarin may act by modulating the activation of regulating substances of the cellular cycle (Fraschini et al., 2002).

Manna et al. (1999) studied the effect of silymarin on NF-κB activation induced by various inflammatory agents. Silymarin blocked TNF-α-induced activation of NF-κB in a dose- and time-dependent manner. It has been also reported that the application of silymarin significantly reduced apoptosis (Fraschini et al., 2002; Křen and Walterová, 2005). Silymarin suppresses activation of caspases in various cell types following TNFα treatment.

Recent reports indicated that NF-κB protects cells from undergoing apoptosis. Furthermore, it has been also showed that the inhibition of apoptosis was mediated through suppression of caspases activation and silymarin may suppress caspases through its antioxidant activity (Manna et al., 1999). In addition, silymarin treatment caused a change in the ratio of Bax/Bcl-2 in a manner that favors apoptosis. It also induced the cytochrome c release, activation of caspase-3 and caspase-9 and cleavage of poly (ADP-ribose) polymerase (Katiyar et al., 2005).

At least, however recognition of cytoprotective effects of silymarin on the adrenal gland require further investigation in detail, but on the basis of the results obtained throughout this study, it can be concluded that silymarin seem to be a suitable protective drug for side effect of glucocorticoid therapy.

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