Effects of Immobilization Stress on Kidneys of Wistar Male Rats: A Morphometrical and Stereological Analysis

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Introduction

Stress is characterized by increased activity in all tissues. It is a reactive process that triggers organic and behavioral responses related to a set of physiological changes. These changes have as a central pattern the hyperfunction of the adrenal gland due to increased release of glucocorticoids (GC) from its cortex [Selye, 1946; Cima et al., 2004]. This is why stress is frequently referred to as a physiological exposure to an excess of GC.

GC enables an organism to meet energetic demands and strengthens the general defense mechanism by adapting it to the stressor stimulus. However, prolonged stress has a destructive effect on tissues since an excess of GC is able to inhibit several body activities and negatively influences cellular proliferation and differentiation [Rosmond and Björntorp, 2000]. Thus, several studies have been developed to verify how prolonged stress and excess GC in different maturation phases can affect the organism and health.

It has been demonstrated that early exposure to GC can accelerate or delay the functional maturation of organs depending on its amount and exposure time [Hook et al., 1975; Meyer, 1985]. Prenatal pharmacological or physiological exposure to excess GC alters the fetal development by inducing a metabolic and endocrine imbalance in various organs, including the kidney [Hook et
al., 1975; Krishtal and Gozhenko, 1989; Chen et al., 2004]. Singh et al. [2007] observed that maternal GC treatment results in a nephron deficit and development of hypertension in the offspring of rats, suggesting increased risk of hypertension and other diseases in adult life [Seckl, 2004]. They suggested it may have important implications for women experiencing significant stress during pregnancy.

On the other hand, some studies have demonstrated that postnatal exposure to prolonged stress and excess GC is also able to affect the anatomy and physiology of some organs [Krishtal and Gozhenko, 1989; Cortez et al., 2003]. Krishtal and Gozhenko [1989] showed that both stress and GC elevate the urinary excretion of hydrogen ions, and Hu et al. [2000] observed a significant increase in adrenal and kidney weight in repeated swimming-stressed animals when compared to controls.

There is not much literature about the effects of the different kinds of chronic stress on kidney morphology, and further studies are required to clarify the role of renal structural changes induced by stress in the pathogenesis of renal disease. It is possible that prepubertal chronic immobilization stress leads to increased GC release and that this hormone causes a negative effect on renal tissue morphology. Thus, the aim of the present study was to verify the morphological changes induced by prepubertal immobilization stress in the kidney of rats by using stereological methods.

**Material and Methods**

Fifteen 4-week-old Wistar male rats were included in this study. The animals were kept with their mothers until the third week of life. Only male pups were used in the study. The rats were kept in a room with controlled temperature (25 ± 1°C) with an artificial dark-light cycle (lights on from 7:00 a.m. to 7:00 p.m.) and were fed standard rat food and water ad libitum. The rats were weighed weekly until the day of death. All experiments were done according to Brazilian law for the scientific use of animals, and this project was formally approved by the local ethics committee.

The rats were randomly assigned to two groups: control (n = 7) and stressed (n = 8). The control group was maintained under standard conditions until 9 weeks of age. The stressed group was submitted to stress stimuli daily until 9 weeks of age.

Stress stimuli were performed by immobilization in a rigid opaque plastic cylinder that restrained the movements of the rats [Retana-Marquez et al., 2003]. The cylinders, with different diameters and length, were adjusted weekly depending on the animal’s weight. The animals did not experience any pain and were contained for 2 h daily from the 28th to the 63rd day of life. Adrenal mass index (weight of adrenal/body weight) and testosterone serum concentration (measured by radioimmunoassay) were used to assess the physiologic efficacy of the stress stimulus [Bauer et al., 2001; Hardy et al., 2005]. On the 64th day of life, under deep anesthesia, the blood was collected by heart puncture and the serum was separated by centrifugation and used for creatinine and testosterone determination. The rats were killed by an anesthetic overdose and the kidneys and adrenal glands were dissected, cleared of adipose tissue and weighed.

The volume of the right kidneys was estimated by the water displacement method, [Scherle, 1970]. The kidneys were then sectioned frontally, fixed in 10% formaldehyde and routinely processed for paraffin embedding. Serial 5-μm sections of the entire kidney were obtained and stained with hematoxylin and eosin. The cortical/medullary ratio was estimated by using the Cavalieri principle [Cruz-Orive and Weibel, 1990] and the absolute cortical volume was calculated by multiplying the cortical/medullary ratio by the renal volume.

The left kidneys were sectioned sagittally into small fragments, fixed in 10% formaldehyde, routinely processed for paraffin embedding, sectioned in thicknesses of 5 μm and stained with hematoxylin and eosin. From each animal, 26 histological fields obtained from different sections of the kidney cortex were acquired with a digital camera coupled to a microscope. A transparent M42 test-system was adjusted on the monitor and the glomerular volume density was estimated by the point-counting technique [Aguila et al., 2005]. The volume-weighted mean glomerular volume was estimated with the point-sampled intercepts method [Gundersen and Jensen, 1985], analyzing 50 glomeruli per animal.

The estimation of the total number of glomeruli per kidney was calculated by multiplying the cortical volume by the glomerular volume density, divided by the volume-weighted mean glomerular volume [Aguila et al., 2005]. Additionally, the number of glomeruli corrected for kidney weight was calculated by dividing the total number of glomeruli of one kidney by the mean kidney weight of each animal [Aguila et al., 2005].

Student’s t test was used for mean comparisons. In all cases, significance was set at a probability value of 0.05. All analyses were performed using GraphPad Prism software.

**Results**

Increased adrenal mass index and decreased serum testosterone levels confirmed the effectiveness of the stressor stimuli used for this research. In fact, such alterations have been shown to be related to chronic stress and high concentrations of GC [Welch, 1993; Porges et al., 1994; Almeida et al., 1998; Dallman et al., 2003; Kavitha et al., 2006]. For these parameters, significant differences were observed between the two groups (table 1). In the group of stressed rats, the adrenal mass index was 15% higher and serum testosterone level was 57% lower than in the control group.

During our experiment, the stressed rats presented less body weight gain when compared to the controls. This difference increased and became statistically significant in the second week of the experiment (6th week of
The impact of stress stimuli on kidney biometric and stereological parameters, as well as on serum creatinine levels, can be observed in Table 2. Also, one may note that the mean values of kidney weight, kidney volume and kidney volume index were significantly lower in the stressed group by 15, 31.9 and 21.6%, respectively (p < 0.0001). No statistically significant difference was found in the cortical/medullar ratio. The glomerular volume density in the stressed group was significantly less than that of controls (2.1%, p = 0.0022); nevertheless, we found no significant difference between the groups concerning values of volume-weighted mean glomerular volume (p > 0.05).

Figure 2 shows the number of glomeruli per kidney. The number of glomeruli corrected for kidney weight was significantly less (by 45%) in stressed animals when compared to controls (p < 0.0001), even when the kidney weight was corrected (36%; p = 0.0003). Despite the reduced number of glomeruli per kidney in stressed rats, no significant difference in serum creatinine levels was found.

Table 1. Stress stimuli impact on adrenal mass index and testosterone serum concentrations

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Stressed</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adrenal mass index, g/kg</td>
<td>0.1245 ± 0.005</td>
<td>0.1432 ± 0.006</td>
<td>0.026</td>
</tr>
<tr>
<td>Testosterone concentration, ng/ml</td>
<td>0.1925 ± 0.045</td>
<td>0.0820 ± 0.007</td>
<td>0.028</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE.

Table 2. Stress stimuli impact on final weight and kidney biometric and stereological values

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Stressed</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final weight, g</td>
<td>231.3 ± 6.26</td>
<td>200.5 ± 3.58</td>
<td>0.0007</td>
</tr>
<tr>
<td>Kidney weight, g</td>
<td>0.9093 ± 0.90</td>
<td>0.7725 ± 0.01</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Kidney volume, ml</td>
<td>1.163 ± 0.03</td>
<td>0.7913 ± 0.02</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Kidney volume index, ml/kg</td>
<td>5.030 ± 0.08</td>
<td>3.942 ± 0.09</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Cortical volume, ml</td>
<td>0.6893 ± 0.03</td>
<td>0.4584 ± 0.01</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Vv[Glom], %</td>
<td>7.483 ± 0.34</td>
<td>5.405 ± 0.40</td>
<td>0.0022</td>
</tr>
<tr>
<td>VWGV, 10^4 μl</td>
<td>147.1 ± 5.51</td>
<td>131.4 ± 5.95</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Serum creatinine level, mg/dl</td>
<td>0.300 ± 0.00</td>
<td>0.287 ± 0.01</td>
<td>&gt;0.05</td>
</tr>
</tbody>
</table>


Fig. 1. Body weight gain of rats from the beginning of stress stimuli (4th week of life) until the end of the experiment (9th week of life). Note that stressed animals (represented by squares) had lower gain than control animals (represented by circles). A statistical difference (*) between the groups was noted from the 6th week of life until the end of the experiment.
Discussion

Our results on body weight gain in rats submitted to immobilization stress (table 2, fig. 1) are similar to those reported by Hu et al. [2000], who also found a significant reduction in total body weight (approximately 25%) in rats submitted to repeated stress as compared to controls. We also observed lower kidney volume and kidney volume index in stressed rats than controls (table 2), although the cortical/medullar ratio did not vary significantly.

Prepubertal immobilization stress caused a reduction of 45% in the number of glomeruli per kidney (fig. 2) and 36% for the value corrected for kidney weight (fig. 3) when compared to controls. Nephrotic potential in prenatal and neonatal exposure to high levels of GC has already been demonstrated. Singh et al. [2007] showed that maternal treatment resulted in a nephron deficit of 21% in male offspring, and Chen et al. [2004] reported changes in renal proximal tubular cells. Two mechanisms have been indicated as playing a major role in glomerular alteration in these cases: the suppression of renal mitotic activity in the cortical nephrogenic zone and changes in glomerular proteoglycan expression [Celsi et al., 1999; Kuroda et al., 2002; Ortiz et al., 2003].

The exact mechanism behind the reduction in the number of glomeruli by prolonged stress in prepubescent rats is still not clear, but it is known that early exposure to excess GC can exert negative effects on cell prolifera-

Fig. 2. Total number of glomeruli per kidney of control rats and chronically stressed animals. Note that stressed animals had a significant decrease in the number of nephrons (mean ± SEM; \* \( p < 0.0001 \)).

Fig. 3. Number of glomeruli corrected for kidney weight of control rats and chronically stressed animals. Note that stressed animals had a significant reduction in this parameter (mean ± SEM; \* \( p < 0.0003 \)).
cially in the prepubertal phase, can contribute to the progression of chronic kidney disease and high rates of hypertension in these groups of people [Hoy et al., 2008; Bruce et al., 2009].

It is important to consider that in humans, glomerular number falls with age, and the relationship between the loss of glomeruli with age and hypertension has been discussed by several authors. A lower glomerular number and larger mean glomerular volumes in hypertensive patients in comparison to nonhypertensive people has been demonstrated, providing evidence that a low glomerular number is a determining factor for hypertension and renal disease [Hoy et al., 2008].

Despite the reduced number of glomeruli per kidney in stressed rats, we did not find a difference in serum creatinine level (table 2). This does not disagree with results found in the literature.

Cunningham et al. [1985] observed that after 5 days of exposure to cold (5°C), rats exhibit hyperphagia, hypermetabolism and increased glucose oxidation, as well as a significant increase of the urinary excretion of GC and urea nitrogen. Creatinine excretion, however, did not vary. On the other hand, biochemical analysis of blood from rats sacrificed 4–8 h after the Cosmos-1667 flight revealed an increase of creatinine associated with a significant increase of GC. However, the authors [Popova et al., 1988] attributed the increase in the creatinine concentrations to the specific effects of microgravity related to musculoskeletal or fluid-electrolyte change. In addition, an increased urinary cortisol/creatinine ratio in the domestic dog submitted to acute psychological stress was observed by Rooney et al. [2007], but this increased ratio may have been due to the stress-induced elevation in serum GC level and/or the decrease of serum creatinine.

Based on observations of Cunningham et al. [1985], we can speculate that the unchanged serum creatinine level following chronic stress might result from the increase in hepatic gluconeogenesis by increased protein intake rather than increased catabolism of extrahepatic proteins (in particular, muscle proteins) since hyperphagia normally follows the serum GC increase.

The absence of statistical difference in serum creatinine levels suggests that glomerular change due to prepubertal stress cannot be diagnosed in the routine clinical evaluation of renal function until adult age, when renal insufficiency would occur.

In conclusion, morphometric and stereological analysis demonstrated that chronic stress before puberty causes an important reduction in the number of nephrons in rats without raising serum creatinine. Unchanged creatinine levels might result from physiological adaptation after being under the stressor stimulus for a long duration (from the 18th to the 63rd day of life) since the adaptation to the stressor is the aim of biological stress. The increase in gluconeogenesis by increased protein intake rather than increased protein catabolism can be considered as the more relevant adaptive mechanism for this case. However, the morphological alterations may have serious implications predisposing individuals to renal disease and hypertension in adult life.

Acknowledgements

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Benchimol de Souza et al.


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