Effects of chronic prepubertal stress on serum level of kisspeptin and histopathological changes in the kidney of the male rat

Hamed Akbari1, Mehrnoosh Maalhagh3, Abdolreza Sotoodeh Jahromi4, Mohammad Yasin Karami5, Mehrnaz Mehrabani6 & Mohammad Hadi Nematollahi1,7*

1Department of Biochemistry, School of Medicine, Kerman University of Medical Sciences, Kerman, Iran
2Herbal and Traditional Medicines Research Center, Kerman University of Medical Sciences, Kerman, Iran
3Department of Ophthalmology, Student Research Committee, Shiraz University of Medical Sciences, Shiraz, Iran
4Research Center for Non-Communicable Diseases, Jahrom University of Medical Sciences, Jahrom, Iran
5Department of General Surgery, Student Research Committee, Shiraz University of Medical Sciences, Shiraz, Iran
6Physiology Research Center, Institute of Basic and Clinical Physiology Sciences, Kerman University of Medical Sciences, Kerman, Iran
7Research Center for Hydatid Disease in Iran, Kerman University of Medical Sciences, Kerman, Iran.

Chronic stress through excess levels of glucocorticoids (GC) can initiate processes that finally may lead to pathological changes in some tissues. It has been demonstrated that level of kisspeptin, an important mediator in the reproductive system and kidney morphogenesis, is also decreased during chronic stress. In the current study, we postulated that prepubertal chronic stress through decreasing levels of kisspeptin and its function can mediate histopathological changes in the kidneys. This is an experimental study done on 45 immature healthy male Wistar rats (22-24 days old) without any symptoms of puberty. Rats were divided randomly into three groups: Pretest, control and stressed. Immobilization stress was applied 2 h for 10 consecutive days and serum level of cortisol, testosterone, and kisspeptin were measured and compared between groups. Morphometric and histopathologic changes of the kidneys also were evaluated at the end of the experiment. Mean serum level of kisspeptin in pretest group, control group and stressed group were 0.03±0.009, 90.60±4.882 and 15.50±3.774 pg/mL, respectively. The level of kisspeptin and testosterone was significantly diminished in the stressed group compared to control (P <0.001). Results of this study did not show any significant morphometric and histopathologic changes between the groups. In conclusion, chronic prepubertal immobilization stress has a profound impact on kisspeptin level, however, doesn’t have any significant alterations in the kidney histomorphology.

Keywords: Kisspeptin, Cortisol, Glucocorticoids, Immobilization stress, Testosterone

All organisms need to maintain a complex dynamic equilibrium called homeostasis to survive. However, it may be disrupted by internal or external stressors. In such situations, homeostasis could be re-established by a series of appropriate physiological and behavioral changes in many organs including the kidneys1-3. However, if stress overcomes, it can lead to cell and tissue injuries as well as the onset of diseases4-6. Renal consequences including high-pressure levels and the presence of calculi in the urinary system are one of the destructive responses to stressful condition7. Stressors involve a long list of potential adverse forces, which can be emotional or physical. Immobilization (IMO) of rats is an effective psycho-emotional stressor, which has been employed as a model of stress8.

Hormones associated with stress protect the body in a short run and promote adaptation (allostasis). However, many investigations have demonstrated that chronic stress conditions are associated with allostatic overload9,10. A hallmark of the stress response is the activation of the autonomic nervous system and hypothalamic-pituitary-adrenal (HPA) axis10. The HPA axis is an important pathway activated by both acute and chronic stress. Glucocorticoids (GCs) such as cortisol is one of the major effectors of the HPA pathway and they adapt organisms to the stressor stimulus while meeting energetic demands and supporting the general defense mechanism. Early exposure to GC can accelerate or postpone the functional maturation of organs depending on its amount and exposure time11-13. It has been shown that prolonged stress due to excessive GC production inhibits several body activities and negatively influences cellular proliferation and differentiation14. Prenatal pharmacological or physiological exposure to
excessive GC affects the fetal development by disturbing the metabolic and endocrine balance in some organs, including kidneys.15,16 Moreover, some studies have confirmed that postnatal exposure to prolonged stress and excessive GC is also able to affect the anatomy and physiology of some organs.17. Hu et al. observed a significant decrease in body weight gain but increase in adrenal and kidney weight among repeated swimming stressed animals compared to the controls.18 In addition, it was shown that chronic stress, decrease body weight gain, kidney volume, kidney volume index, glomerular volume and glomerular density.19

Stress has profound inhibitory effects on reproductive function through the suppression of pulsatile gonadotrophin-releasing hormone (GnRH) and secretion of luteinising hormone (LH)20. Recently, kisspeptin (formerly known as metastin) and its G-protein-coupled receptor 54 (Kiss-1r) have been shown to play a pivotal role in the central control of the hypothalamic-pituitary-gonadal (HPG) axis.21. KISS-1 encoded by KISS-1 gene in humans was originally identified as a suppressor of metastasis in human malignant melanoma.22. Kisspeptin-54 is cleaved from the 145-amino acid precursor polypeptide encoded by KISS-1 gene and cleaved further to smaller fragments of 14, 13, and 10 amino acids, all sharing the same C-terminal decapeptide RFamide (arginine-amidated phenylalanine) sequence.22. Kiss-1 receptor G-protein-coupled receptor-54 (GPR54) is a multifunctional receptor, which plays critical roles in puberty development, vasoconstriction, and metastasis suppression.23. It was shown that kisspeptin administration to prepubertal female rats and monkeys could result in advanced puberty onset.24. It was shown that the reduced Kiss-1r expression may be a contributing factor in the stress-related suppression of LH secretion.25. Moreover, stress could reduce the expression of KISS-1 gene through increasing GC.26

Bone morphogenetic protein-7 (BMP7) is required for kidney organogenesis. Deletion of GPR54 decreases BMP7 expression and Smad1 phosphorylation in the developing kidney; such data support the role of KISS-1/GPR54 in embryonic kidney branching morphogenesis and glomerular development.27,28 Nevertheless, the role of KISS-1/GPR54 signaling in postnatal kidney growth is yet obscure. Little is known about non-specific changes in the kidney structure among the animals exposed to chronic or repetitive stress. In this context, the present experiments had two aims. Foremost, we measured stress-induced kisspeptin, testosterone, and cortisol level responses in the rats throughout pubertal development and, second, we investigated whether chronic stress could change the anatomy and morphology of kidney in prepubertal rat following exposure to a chronic stressor.

Materials and Methods

Animals

Forty-five Wistar male rats (22-24 days old) were included in this survey. The rats were maintained under standard conditions (12 h dark/light cycle, room temperature at 23±1°C, humidity at 45%). The study was accepted by Ethical Committee of Jahrom University of Medical Sciences and was performed in accordance with its guideline base on the National Institutes of Health Principles of Laboratory Animal Care (NIH publication no. 85-23, revised 1985). They were randomly divided into the following three groups consisting of: pretest group (I), killed on the first day of the study to measure the baseline of hormonal level, control group (unstressed) (II), maintained under standard conditions and freely moving, and stressed group (III) subject to repeated 2 h immobilization for 10 consecutive days.

Preputial separation

The prepubertal male rat was separated by measuring balano-preputial, considered as the external markers of the action of sex steroids. These markers are signs of the proper activation of all levels of the HPG axis and reliable external signs of puberty onset.27

Chronic immobilization stress

Stress was induced in the rats through immobilization in a rigid plastic cylinder that restrained the movements of the rats.28 The restrainer, with different diameters and length, was adjusted weekly depending on the animals’ growth. The animals did not experience any discomfort and were contained for 2 h daily in the morning (08:00 to 10:00 a.m.) from the 28th to 38th day of life. At the end of the experiment, animals were decapitated without anesthesia. Blood was collected and serum was separated by centrifugation and used for hormone assay. Kidneys were wholly removed and fixed in the formalin solution. The kidneys were cut in half longitudinally, placed in paraffin blocks, and cut in thin (5 μM) slices. Staining was done using hematoxylin–eosin (HE)
technique. Each specimen was analyzed using a light microscope.

**Hormone assay**

Blood was collected into the tubes containing EDTA centrifuged 4000 x g for 5 min at 4°C to separate plasma and the plasma samples were stored at −70°C until assayed. Kisspeptin level was measured by ELISA (Rat kisspeptin, Cat. No: MBS073437, MyBioSource). Testosterone level was measured by a rat testosterone ELISA kit (Rat Testosterone, Free (FT) ELISA Kit Cat. No: MBS005579, MyBioSource). Cortisol level was measured by a rat cortisol ELISA kit (Rat cortisol, Cat. No: MBS701698, MyBioSource). All assays were performed according to manufacturer's instructions. Testosterone and cortisol serum concentrations were used to assess the physiological efficacy of the stress stimulus.

**Morphometric and histopathologic evaluations of kidneys**

The kidney mass index was estimated as follows: weight of kidney/body weight. To calculate the number of glomeruli of the 6-week-old kidney, the average of five fields high-performance field (HPF) was chosen randomly in the cortex layer of a section as a glomerular number/HPF/section. A calibrated micrometer eyepiece was used to measure the thickness of the medulla and cortex for cortex/medulla ratio. For histopathologic evaluation of crescent formation, cellularity, inflammation, and hyaline membrane were observed.

**Statistical analysis**

Student’s t-test and one-way ANOVA analysis were used for mean comparisons. In all the cases, significance was set at the probability value of 0.05 and all the analyses were performed using SPSS 14 software.

**Results**

**Body weight, kidney weight and kidney mass index**

The results showed that stress stimuli had a significant effect on body weight gain. In group III, the body weight gain was significantly less than group II (P <0.001). Also, the mass kidney index and kidney weight were reduced significantly. Data were shown in Table 1.

**Hormonal changes**

Our results of hormonal changes were rational, as expected. With the induction of stress in group III, the level of kisspeptin was significantly diminished compared to group II (P <0.001) and the cortisol level was significantly raised in group III compared to group II, which showed the stress stimuli were appropriate for the experimentation. The level of testosterone was significantly decreased in group III compared to group II and was meaningfully higher in group II compared to the prepubertal level of testosterone in group I. Fig. 1 presents the results of hormonal change during the study.

<table>
<thead>
<tr>
<th>N</th>
<th>Control</th>
<th>Stressed</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Final body weight (g)</td>
<td>153±9.6</td>
<td>151±7.7</td>
</tr>
<tr>
<td></td>
<td>Kidney weight (g)</td>
<td>0.92±0.22</td>
<td>0.73±0.21</td>
</tr>
<tr>
<td></td>
<td>Kidney mass index</td>
<td>0.57±0.04</td>
<td>0.48±0.05</td>
</tr>
</tbody>
</table>

Fig. 1—Effects of chronic immobilization stress on (A) cortisol; (B) testosterone; and (C) kisspeptin levels. [Significant differences in serum level of cortisol, testosterone and kisspeptin was shown between control and stressed groups. Star shows significant difference (P <0.001)]
Morphometric and histopathologic evaluations
No remarkable differences were found in glomerular number and cortex/medulla ratio between control and stressed group. Histopathological analysis indicated that all the specimens obtained from rats show almost identical histological appearances. Thus, we did not have any significant histopathological changes. The following results were obtained after histological examination: lack of crescent formation, no significant difference between cellularity and inflammation, and no significant difference in the hyaline membrane.

Discussion
There are many emotional or physical threats named stress that challenges the organism’s life. In such situations, acute stress may protect the living organism29,30. On the contrary, chronic stress can initiate processes that lead to pathological changes in some tissues31. It is well documented that excess levels of GC as a consequence of chronic stress is responsible for such destructive changes32. To date, little is known about GC-mediated mechanisms in chronic stress. It also has been demonstrated that level of kisspeptin, an important reproductive hormone, is decreased during chronic stress33.

The development of the mammalian kidney initiates at the embryonic day through a series of communications between the Wolffian duct, the ureteric bud and the metanephric mesenchyme34. Bone morphogenetic proteins (BMPs), multifunctional cytokines of transforming growth factor, play important roles in ureteric bud development, ureteric bud branching, tubule maintenance and nephrogenesis. Thus, BMP7 is required for proper kidney formation. It was shown deficiency of BMP7 causes a halt in kidney development35-37. Yi et al.38 showed that GPR54 (Kiss-1 receptor) plays a role in kidney-branching morphogenesis and glomerular development, clarifying the biological functions of KISS-1/GPR54 in kidney development. Activation of GPR54 by KISS-1 leads to activation of transcriptions factors that bind to the BMP7 promoter and regulate BMP7 expression. BMP7 has been proved that has a critical role in kidney branching morphogenesis and glomerular advancement23. But there is no study on the effect of KISS-1/GPR54 on kidney morphology in prepubertal and adult. Since kisspeptin plays an important role in kidney development as well as the onset of puberty23, based on previous observations, we postulated that chronic stress through decreasing levels of kisspeptin and its function can mediate histopathological changes in the kidneys.

In the present study, repeated immobilization of rats, as a stressful procedure, was used for induction of chronic stress in male Wistar rats. The other reports also employed this model for successful induction of chronic stress38,39. Current results have shown that stress was induced successfully and hormonal changes are rational as expected, so that kisspeptin was decreased significantly in the stressed group (Gr. III) compared to control group (Gr. II) (P <0.001) and the cortisol level also remarkably was elevated in group III compared to group II. These results indicated that the stress stimulus was satisfying to experiment. Furthermore, the level of testosterone was determined to measure the pubertal delay. Results showed that the level of testosterone significantly was decreased in group III and elevated in group II compared to the prepubertal level of testosterone in group I. During our experiment, the stressed rats (group III) presented less body weight gain when compared to the controls. This difference increased and became statistically significant in the second week of the experiment. It was speculated that the reduction of testosterone level is associated with preventing further weight gain and pubertal delay in group III. The results of pretest group showed that the animals were in prepubertal state and confirm our default.

In agreement with our results, it was shown that stressed animal showed a significant decrease in total body weight gain and had at least 2-3 fold higher levels of plasma corticosterone as compared to control unstressed animals32. In another study, stress related GC elevation apparently inhibit expression of KISS-1 gene so that in mice lacking the GC receptor in neuron containing kisspeptin, no inhibition occur33. Moreover, it was shown that chronic immobilization stress could stably and drastically reduce plasma testosterone levels in hamsters40.

In addition to hormonal changes, the current study was also evaluated stress related anatomical and histopathological changes in the kidneys of rats. Some researchers have demonstrated a relationship between postnatal prolonged exposure to stress and anatomical and physiological changes in some organs. For instance, Stubbe and colleagues showed that supranormal exposure to glucocorticoid can alter structural and functional of kidney medullary permanently41. In another study, Hu et al.18 observed a significant increase in adrenal and kidney weight in rats under repeated
swimming stress when compared to controls. Benchimol et al.\textsuperscript{19} have confirmed that prepubertal chronic immobilization stress leads to increased GC release and this hormone causes a negative effect on renal tissue morphology.

There are many studies that show various histopathological changes in different tissues under immobilization stress\textsuperscript{19,42-44}. In the current study, despite a significant reduction in mean values of kidney weight, no significant differences were found in histopathological parameters between stressed group and control group. The possible reason for histopathological changes may relate to the age of animals in this study. There are some reports that display different response to stress related to age and it is well-known that stress-induced reactions of the HPA axis in the aged are greater than those in younger adults\textsuperscript{45}. However, in our study, the rats were young. It is also reported that chronic stress could remarkably decrease kidney weight\textsuperscript{19}. Further, similar to our result, Benchimol et al.\textsuperscript{19} reported that cortical/medulla ratio of kidneys was not affected during stress induction compared to control group. However, on the contrary to our results, they showed a significant difference between the numbers of glomeruli per kidney of the stressed group in comparison to control group. This discrepancy may be related to differences between stress times of two studies so that in Benchimol’s report stress stimuli were performed over five weeks by immobilization and in the current study, stress stimuli were induced during 10 days. Furthermore, Foilb et al.\textsuperscript{46} have shown that acute and chronic stress induces mild or moderate histopathological changes including cellular damage and interstitial edema in kidneys of adult male rats. This discrepancy may be contributed to the differences between stress-related responses observed before and after the puberty\textsuperscript{46}. Also, unchanged histopathological morphology might result from physiological adaptation after being under the stress stimulus for a long duration. It has been previously reported that general adaptation is defined as the sum of all non-specific, systemic reactions of the body which arise upon long continued exposure to stress and adaptation to the stressor is the aim of biological stress\textsuperscript{47}. Despite all above-mentioned challenges, we suggest more investigation for getting additional information about the destructive effects of stress on tissues.

**Conclusion**

In conclusion, under the conditions of the present study chronic immobilization stress decreases kisspeptin and testosterone level and increases the cortisol level. However, morphometric results demonstrated that chronic stress before puberty has no significant effect on the number of glomeruli per kidney, cortex/medulla ratio and also histopathologic findings.

**Acknowledgement**

This study was supported by a grant from Jahrom University of Medical Sciences, Jahrom, Iran.

**References**


44 Murthy KD, George MC, Ramasamy P & Mustapha ZA, Housing under the pyramid reduces susceptibility of hippocampal CA3 pyramidal neurons to prenatal stress in the developing rat offspring. *Indian J Exp Biol*, 51 (2013) 1070.

