The Influence of Exposure to Stress of Pregnant Rats on the Adrenal Gland Structure of their Offspring. An Unbiased Stereological Study

by *Ali Noorafshan*, Mohammad Karimipoor, Soghra Bahmanpoor, Farzaneh Dehghani*

School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran

Summary

Many factors may interact with normal differentiation and growth of tissues and cells. Stress might be experienced during pregnancy and it has been shown that stress may cause low birth weight. The effect of prenatal manipulations on the HPA axis has been focused on physiological and biochemical alteration of the adrenal gland. A stereological examination of the influences of prenatal stress on the structure of the developing adrenal gland of one day and 21 day-old rats has now been performed.

In this study experiments were conducted to test the hypothesis that exposure to restraint stress during pregnancy in rat results in structural changes in the developing adrenal gland of their pups.

Female rats were exposed to restraint stress from the first day of pregnancy throughout gestation. Male offspring of stressed rats (PS= experimental) and of unstressed mothers (C= control) who were one day and 21 days of age were selected. Their body weight (BW), crown-rump length (CRL), biparietal diameter (BPD), volume of the gland and the cortical layers and medulla were estimated using stereological methods.

The results showed that the prenatal stress led to a decrease in BW, but CRL and BPD remained unchanged. Also, a significant increase in volume of the adrenal gland and cortical layers in one day and 21 day-old offspring were observed. The volume of the medulla of the adrenal gland of neonate rats remained unchanged but the volume of the medulla in 21 day-old rats was decreased.

Therefore, it can be concluded that prenatal stress alters the structure of the developing adrenal gland.

Introduction

Many factors may interact with normal differentiation and growth of tissues and cells. Stress might be experienced during the pregnancy and it has been shown that it may cause low birth weight. In response to a challenge or threat, the organism reacts by simulating the hypothalamic-pituitary-adrenal (HPA) axis and sympathetic nervous system. Several studies have shown that the degree of activation of the HPA axis and the duration of response can be altered permanently by gestational stress (*Weinstock et al., 1998*). Despite numerous studies on stress that have pointed out that the human or animal adrenal gland is always involved in adults (*Pellegrini et al., 1998; Rubin et al., 1996; Engeland et al., 1975*), the effect of prenatal manipulations on the HPA axis have mainly focused on physiological and biochemical alterations of the adrenal gland (*Bauer et al., 2001; Takahashi et al., 1998; Sapolsky et al., 1984; Cratty et al., 1995; Brown & Gray 1988*). However a stereological examination of the influence of prenatal stress on the structure of the developing adrenal gland of one day and 21 day-old rats has not been performed. Therefore the aim of this study was to determine the changes in volume of the adrenal gland, cortex, cortical zones and medulla of the rats which experienced prenatal stress (PS= experimental group) and the control (C) rats, just one day and 21 days after birth; the latter is the time when the three layers of the rat adrenal cortex could be differentiated from each other.

*Correspondence: Ali Noorafshan, Associate Professor, PhD, Anatomy Dept., School of Medicine, Shiraz University of Medical sciences, Zand Ave., Shiraz, Iran, Fax: +98-711-2304372, E-mail: noora@sums.ac.ir

Materials and Methods

Housing conditions. Thirty female inbred adult Sprague-Dawley rats weighing 240±20 g were obtained from the Laboratory Animals Center of Shiraz University of Medical Sciences, (Shiraz, Iran) and were treated according to the standard directive. They were singly housed in cages with chopped wood bedding. Fourteen days after arrival, in the presence of male inbred Sprague-Dawley rats weighing 400±30 g and observing of vaginal plug or microscopic examination of their vaginal smear (if there was an uncertainty for mating), the mating was confirmed. Then female rats were randomly assigned to experimental and control groups. Each group included 15 rats that were individually housed in their breeding cages. Animals were allowed *ad libitum* access to standard rat chow (produced by the Laboratory Animal Center of Shiraz University of Medical Sciences) and water throughout the experiment. They were maintained on a constant 12-hour light/dark cycle with a relative temperature of 22± 2°C and at 60-80% relative humidity.

Prenatal stress. Stress was performed each day of pregnancy from day 1 until delivery of the experimental (PS) rats. Pregnant females were individually restrained for 45 minutes, three times a day during the light phase in plastic transparent cylinders (7-cm diameter, 19-cm long) (*Lemaire et al., 2000*). Control pregnant females were left undisturbed in their home cages. Just after birth, one male neonate from each mother was selected randomly and the other offspring were raised by their biological mothers until weaning (21 days after birth). At this time, the three layers of the suprarenal gland were well identifiable microscopically and one 21day-old male rat from each mother was selected.

Histological procedure. Offspring were anesthetized, weighed and their CRL and BPD were measured. The suprarenal glands were removed and immersed in neutral buffered formaldehyde. After embedding the tissues in paraffin, complete serial sections (5 micrometer thickness) were cut and stained with Heidenhain's azan.

Stereological study. The Cavalieri method (*Mouton, 2002; Gundersen et al., 1988a; Gundersen et al., 1988b*) was used as an estimator of gland volume. Thus, twelve sections were selected using a systematic sampling design and a random start for stereological estimations. Each sampled section was analyzed using a video-microscopy system made up of a microscope (E-200, Nikon, Japan) linked to a video camera (SONY, Japan, SSC Dc 18P), a P4 PC computer, and a LG monitor (795 FT plus) to determine the parameters. By means of stereology software designed at our lab, the stereological probe (points) was superimposed upon the images of the tissue sections viewed on the monitor. The volume was estimated using the following formula:

$V(total) = \sum P.(a/p).d$

Where the "V (total)" was the gland volume, "$\sum P$" was the sum of the points falling on the section profile, "a/p" was the area associated with each point at the level of tissue, and "d" was the distance between the sections sampled. Volume density, "Vv", (*Mouton, 2002; Gundersen, et al., 1988a; Gundersen et al., 1988b*) of the cortex and medulla of the gland of one day old rats, and zona glomerulosa (ZG), zona fasciculata (ZF), zona reticularis (ZR) and medulla of the 21 day-old rats were estimated using point-counting and the following formula:

$Vv = P(layer)/P(ref)$

Where "P(layer)" and "P(ref)" were the number of test points falling on the layer profile and on the reference space respectively.

The absolute amount of the layers was estimated by multiplying the density by the volume of the gland to prevent the "reference trap" (*Mouton, 2002; Gundersen et al., 1988a; Gundersen et al., 1988b*).

Results

Body weight, CRL and PBD lengths.

As it appears from Table 1 and 2, a significant decrease in body weight of one day old (~9%) (p<0.001) and 21day-old (~8.5%) (p<0.05) experimental rats (PS) was seen but the CRL and BPD lengths were not altered.

Table 1. Mean ± standard deviation and 95% confidence interval (CI) of the body weight (BW) (g), crown-rump length (CRL) (cm) and bi-parietal diameter (BPD) (cm), in one day old control and experimental rats

Groups	BPD	CRL	BW
Control	0.64±0.13	5.95±0.56	7.45±1.03
CI	0.57-0.70	5.66-6.23	6.93-7.98
Experimental	0.61±0.09	5.82±0.46	6.76±0.31*
CI	0.56-0.66	5.58-6.05	6.60-6.91
* p<0.001 experimental vs. control			

Table 2. Mean ± standard deviation and 95% confidence interval (CI) of the body weight (BW) (g), bi-parietal diameter (BPD) (cm) and crown- rump length (CRL) (cm) in 21 day-old control and experimental rats

Groups	BPD	CRL	BW
Control	1.42±0.03	10.65±0.38	43.86±4.47
CI	1.40-1.44	10.46-10.85	41.59-46.12
Experimental	1.41±0.02	10.59±0.43	40.12±5.09*
CI	1.40-1.43	10.37-10.80	37.54-42.69
* p<0.05 experimental vs. control			

Table 3. Mean ± standard deviation and 95% confidence interval (CI) of the total volume (TV) (mm^3) of the adrenal gland, volume density (Vv) (%) and absolute volume (AV) (mm^3) of the cortex (c) and medulla (m) in one day old control and experimental rats

Group	AVm	AVc	Vvm	Vvc	TV
Control	0.008±0.002	0.084±0.02	9.61±1.64	90.47±1.64	0.094±0.02
CI	0.007-0.009	0.073-0.095	8.78-10.45	89.61-91.33	0.08-0.1
Experimental	0.008±0.001	0.123±0.02**	6.97±0.96	93.06±0.91	0.133±0.02*
CI	0.007-0.008	0.11-0.13	6.51-7.43	92.60-93.52	0.12-0.14
* p<0.001 experimental vs. control					
**p<0.001 experimental vs. control					

Volume of the gland.
Stereological estimation of the suprarenal gland volume showed ~41% (p<0.001) and ~37% (p<0.001) increase in one day old and 21day-old PS pups, respectively (Table 3 & 4, Figure 1&2).

Absolute volume of the cortex and medulla.
All densities were converted to absolute quantities for prevention of the "reference trap" These data showed that the volume of the cortex in one day old PS rats increased ~46% (p<0.001). The volume of ZG, ZF, ZR in 21day-old PS rats increased ~24%, ~48%, ~23% (p<0.001), respectively. The volume of the medulla in one day old PS rats did not show significant alteration but the volume decreased ~9% (p<0.05) in 21day-old PS animals (Table 3 & 4, Figure 1&2).

Table 4. Mean ± standard deviation and 95% confidence interval (CI) of the total volume (TV) (mm^3) of the adrenal gland, volume density (Vv) (%) and absolute volume (AV) (mm^3) of the zona glomeruloza (zg), zona fasciculata (zf) , zona reticularis (zr), medulla (m) in 21 day-old control (Con.) and experimental (Exp.) rats

Group	AVm	AVzr	AVzf	AVzg	Vvm	Vvzr	Vvzf	Vvzg	TV
Con.	0.068 ± 0.008	0.119 ± 0.01	0.577 ± 0.04	0.181 ± 0.02	7.47 ± 0.91	12.57 ± 0.79	61.03 ± 1.35	18.91 ± 1.15	0.943 ± 0.007
CI	0.060 - 0.070	0.11 - 0.12	0.556 - 0.598	0.17 - 0.19	7.01 - 7.93	12.17 - 12.97	60.34 - 61.72	18.33 - 19.50	0.90 - 0.98
Exp.	0.062 ± 0.009♦♦	0.147 ± 0.02♦	0.856 ± 0.07■■	0.224 ± 0.02**	4.93 ± 0.28	11.21 ± 0.66	66.38 ± 1.07	17.39 ± 0.67	1.291 ± 0.125*
CI	0.059- 0.065	0.13- 0.15	0.81- 0.89	0.21- 0.23	4.78- 5.07	10.84- 11.54	65.83- 66.92	17.05- 17.73	1.22- 1.35

*   **p<0.001 experimental vs. control**   ♦   **p<0.001 experimental vs. control**
** **p<0.001 experimental vs. control**   ♦♦ **p<0.05 experimental vs. control**
■■ **p<0.001 experimental vs. control**

Discussion

The literature offers data concerning the effects of prenatal immobilization stress on the structure of the developing adrenal gland. The decrease in body weight of PS rats was the first finding in our study. There are conceivably a number of ways by which stress related regulation of the autonomic nervous system and the HPA axis during pregnancy could contribute to poor birth outcomes. For instance, elevated levels of pituitary hormones such as oxytocin and prostaglandins may result in promotion of uterine contractions and contribute to vasoconstriction. This factor may reduce uteroplacental perfusion and exchange and contribute to intrauterine growth retardation (*Wadhwa, 1993*).

Other parameters in our study were estimated using stereological methods. When comparing our result with those of other reports, one should bear in mind that the different methodologies, namely biased versus unbiased stereological methods, have been used over the years. Some researchers have relied on the weight of the gland to report hypertrophy of the adult gland (*Pellegrini et al., 1988; Bauer,*

2001). However, the Cavalieri principle and volume determination should be considered more appropriate for defining the hypertrophy. This method was used in our study and showed that hypertrophy occurred in the cortex of one day and 21 day-old PS rats, especially in the zona fasciculate (ZF). This finding is in agreement with reports in adults (*Bauer, 2001*). But Manjolivic (1998) reported that dexamethasone treatment of pregnant rats led to a decrease in adrenal weight of their offspring.

Some researchers have measured the thickness of the cortical zone (*Pellegrini, 1998*) in histological sections to report the hypertrophy of each zone but it is clear that this might be affected by different factors such as orientation of the gland. In the present study the problems associated with sampling strategies are solved by the use of design-based methods for estimating the reference volume using the Cavalieri principle. Furthermore, because a biological conclusion based on density measurements is difficult to interpret and changes in density due to an alteration in the reference volume can bias the results in unpredictable ways, we estimated the total
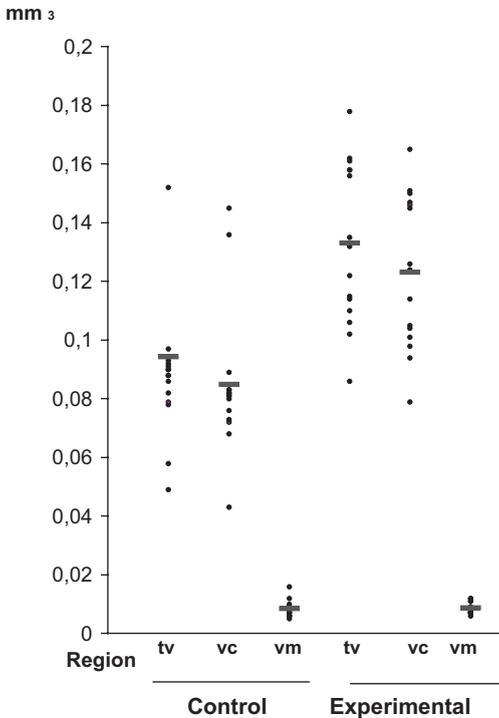
Fig. 1: Plot of the total volume of the adrenal gland (tv), cortex (vc) and medulla (vm) of one day old rats.
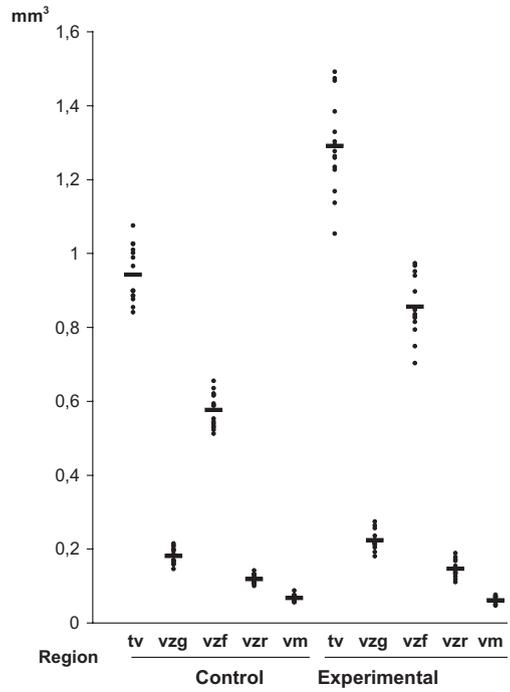
Fig. 2: Plot of the total volume of the adrenal gland (tv), zona glomerulosa (vzg), zona fasciculata (vzf), zona reticularis (vzr) and medulla (vm) of the adrenal gland of 21 days old rats.

volume of the layers by multiplying the density by the gland volume.

In some studies reported changes in blood glucocorticoids, by dexamethasone treatment of pregnant rats, led to a decrease in medullary volume of their pups during fetal and neonatal periods (*Manjolivic, 1998*). We have not found volume reduction of the medulla in one day old PS rats but it was seen in 21day-old animals, and this might be due to the different methodologies that were used.

As discussed by some authors, neurochemical/neurohormonal and neural stimulatory factors might be responsible for adult adrenal hypertrophy (*Rubin, 1996*). These suggested factors include: ACTH, the NH2 terminal fragment of propiomelanocortin, epidermal and fibroblast growth factors, insulin-like growth factor I (somatomedin-C), neurotensin and angiotensin II. With regard to neural influences, there is autonomic (splanchnic noradrenergic) innervations of the adrenal cortex in man as well as in other species. Post-ganglionic unmyelinated fibers with vesicle-containing terminal buttons have been demonstrated in the proximity of endocrine cells in the ZF (*Dorovini-Zis, 1991*) and this may describe the more pronounced hypertrophy of ZF.

From this study it can be concluded that prenatal stress leads to a significant increase in the volume of the adrenal gland and layers of the cortex in one day and 21 day-old offspring. Additionally, the volume of the medulla of the one day old rats remained unchanged although the volume of the medulla of 21 day-old rats was decreased.

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