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ORIGINAL ARTICLE

Maternal treatment with dexamethasone during gestation alters sexual development markers in the F_1 and F_2 male offspring of Wistar rats

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Maternal treatment with dexamethasone (Dex) in threatening preterm delivery alters activities at the hypothalamic–pituitary–adrenal axis in the offspring. This alteration may interfere with reproductive function. The impact of gestational Dex exposure on male reproductive function of the offspring was investigated. A total of 25 pregnant rats randomly assigned to five groups ($n = 5$) were treated with normal saline (control), Dex (100 µg/kg/day sc) during gestation days (GD) 1–7, 8–14, 15–21 and 1–21, respectively. Birth weight, anogenital distance (AGD), pubertal age, sperm parameters, hormonal profile and histopathology of testis and epididymis were determined in the F_1 and F_2 offspring. Results showed a significant increase ($P < 0.05$) in pubertal age, serum corticosterone and gonadotropin-releasing hormone (GnRH) levels in the male offspring of DexGD 15–21 and 1–21 groups and a significant decrease ($P < 0.05$) in serum testosterone, luteinizing hormone, birth weight and AGD at birth in the male F_1 offspring. In the F_2 offspring, there was a significant reduction ($P < 0.05$) in serum corticosterone, testosterone, follicle-stimulating hormone and GnRH when compared with the control. Dex treatment at GD 15–21 and 1–21 significantly reduced ($P < 0.05$) sperm motility and normal morphology in the F_1 and F_2 offspring. Maternal Dex treatment in rats during late gestation may disrupt sexual development markers in the F_1 and F_2 male offspring.

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Key words: dexamethasone, gestation, pubertal age

Introduction

Hazards to reproductive health have become prominent public health issues. Factors associated with reproductive system disorders include nutrition, environment, socio-economic status, lifestyle and stress.[1] Disorders of reproduction in humans include reduce fertility, impotence, menstrual disorders, spontaneous abortions, premature reproductive senescence and various genetic disease affecting the reproductive system and offspring.[1] The reproductive system begins to form early in gestation, but structural and functional maturation are not completed until puberty. Exposure to toxicants early in development can lead to alterations that may affect reproductive function or performance well after the time of initial exposure. Dexamethasone (Dex) is a fluorinated synthetic glucocorticoids with 25-fold more glucocorticoid activity than cortisol.[2] It acts predominantly via genomic effects mediated by glucocorticoid receptor.[2]

Fetal exposure to stress and its glucocorticoid hormone mediators exert influences on organ growth, development, and subsequent offspring physiology and pathophysiology.[3] Sources of maternal exposure to glucocorticoids includes the following: maternal stress, treatment with synthetic glucocorticoid in threatening preterm delivery and treatment of medical conditions such as asthma.[4] Pregnant women who are at risk of delivering a child with congenital adrenal hyperplasia are also likely to receive doses of Dex that are 60-fold higher than mid-gestation glucocorticoids values[5] in order to reduce genital virilization of the female fetus.[6] In rodents and other mammals including non-human primates, prenatal glucocorticoid overexposure as a consequence of maternal stress or treatment with Dex reduced birth weight and permanently altered offspring physiology.[7–9] Reduced birth weight is an established risk factor for testicular dysgenesis syndrome disorder in humans.[10,11]

The observed programming effects of prenatal synthetic glucocorticoids exposure is said to be centrally mediated through the programming of events at the hypothalamic–pituitary–adrenal (HPA) axis.[12] The HPA programming has been reported to lead to the development of hypertension, insulin resistance, type-2 diabetes[3] and alteration in the hematological indices.[13] Evidence is accumulating that the programming effects are not limited to the F_1 offspring and that programming effects may persist in the subsequent generations.[14,15]

Chronic exposure to glucocorticoid is known to affect gonadotropin activity in both males and females. In males it inhibits gonadotropin secretion as evidenced by decreasing their responsiveness to administered gonadotropin-releasing hormone (GnRH) thereby leading to subnormal plasma testosterone concentration. In the females, it also suppresses luteinizing hormone (LH) responsiveness to GnRH, resulting in suppression of estrogen and progestins with inhibition of

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ovulation and amenorrhea.[16] Environmental compounds that possess steroidogenic[17] or anti-steroidogenic[18] activity affect onset of puberty and reproductive function in adulthood. Study by Drake *et al.*[19] showed that exposure of pregnant rats at embryonic days (ED) 13.5–21.5 to combination of Dex and dibutyl phthalate (endocrine-disrupting agent) induced disruption of testosterone and male reproductive development. Moreover, exposure to Dex during the prenatal life caused delayed puberty in female rats.[20]

Despite the reported programming of HPA axis and associated increase in basal corticosterone level, which may alter hormonal activities at the hypothalamic–pituitary–gonadal axis (HPG axis), the multigenerational reproductive effects following maternal treatment with Dex during gestation have not been explored. Reproductive toxicity in rat offspring can occur as a result of exposure to agents from gestation day 8 (GD 8) before the onset of fetal gonadal differentiations or on GD 14 near the onset of fetal testis steroid hormone syntheses. This exposure should continue through at least GD 18 to cover the primary period of reproductive tract development.[21]

It was hypothesized that *in utero* exposure of developing fetus to excess glucocorticoid during development of reproductive organs may have long-term consequences on the phenotypic outcome of the reproductive structures. Therefore, this study was designed to investigate the effects of maternal Dex treatment during gestation on male sexual development in the F_1 and F_2 offspring.

Materials and Methods

Animal

Adult male and female Wistar rats (10 weeks old) that were not from the same litter obtained from the Central Animal House, University of Ibadan, Ibadan, Nigeria were used. These animals were housed singly in cages in the Department of Physiology Animal House and had free access to food (Ladokun Feeds Limited, Ibadan, Nigeria) and water for the entire duration of the study. The female were nulliparous and the males used for the mating were proven male breeder (fertility certified by isolated mating technique). A 12-h dark–light period was maintained throughout the study. At the commencement of mating, the weights of the male and female rats were 150–180 and 180–200 g, respectively. Animals were allowed to acclimatize for 2 weeks to the laboratory conditions. The study was conducted in accordance with the International Ethical Norms on Animal Care and Use as contained in NIH publication/80-23, revised in I985.

Drug

Dex 21-phosphate disodium salt was purchased from Sigma Aldrich Chemical (UK). A dose of 100 µg Dex/kg/day was administered in the drug-treated groups.[3,15]

Experimental protocol

In total, 25 female Wistar rats (12 weeks; 150–180 g) with normal estrous cycle were used. The estrous cycle of the rats were monitored daily according to the method described by Marcondes *et al.*[22] Rats in proestrous were mated with certified fertile male at a ratio of 1:1 overnight and the presence of sperm in their vaginal or copulatory plug in the next morning mark GD 1. After pregnancy has been confirmed, animals were randomly assigned into five groups of five animals each and treated accordingly during gestation. Administration was carried out subcutaneously between 9 am and 10 am daily. Group 1 was administered 0.02 ml/100 g/day normal saline (control). Groups 2–5 were administered 100 µg/kg/day Dex at different GD 1–7, 8–14, 15–21 and 1–21, respectively. The litter size was standardized to seven pups per litter.

After delivery the following parameters were measured: gestational length, birth weight, litter size, anogenital distance (AGD), weight at weaning [postnatal day 21 (PND 21)], weight at puberty, pubertal age, sperm parameters (at PND 12 weeks), hormonal profile [testosterone, follicle-stimulating hormone (FSH), LH, GnRH and corticosterone; PND 12 weeks], histopathology of testis and epididymis (PND 12 weeks). Live birth index (LBI), viability index (VI; 4-day survival index), lactational/weaning index (LI). LBI, VI and LI were determined as described by the United States Environmental Protection Agency (Appendix 1).[1]

Second filia generation offspring

Randomly selected F_1 male from the Dex-treated group were paired with randomly selected F_1 female (both 12 weeks) from the control group at a ratio of 1:1 for 2 weeks. The female animals were checked daily. The day after which sperm was found in their vaginal (positive vaginal smear) was taken as GD 1. The animals were allowed to deliver without any further intervention. Gestational length, birth weight, litter size, AGD, weight at weaning (PND 21), weight at puberty and pubertal age, sperm parameters (PND 12 weeks), hormonal profile (testosterone, FSH, LH, GnRH and corticosterone; PND 12 weeks), and histopathology of testis and epididymis. LBI, VI (4-day survival index), LI were determined as in F_1 offspring.[1]

Evaluation of AGD and pubertal timing

AGD at birth was determined by using a digital Vernier caliper to measure the distance between the posterior base of the sex papilla and the anterior anus at PND 1. At necropsy, AGD was measured by placing the animal with the base of tail on the edge of a table. Then Vernier caliper was used to measure the distance between the posterior base of the phallus and the anterior rim of the anus.[21]

To detect the preputial separation (PPS) (a measure of pubertal timing), male rats were checked daily beginning at 35 days of age to ensure no rats have PPS. Gentle pressure was applied to the prepuce to retract the prepuce and expose the glans penis. PPS is

complete when the entire perimeter of the prepuce can be retracted evenly around the base of the glans penis.[21]

Serum collection and hormonal assay

Blood samples were collected from the orbital sinus (PND 12 weeks) into polythene tubes and allowed to clot for 1 h. The blood samples were then centrifuged at 3000 rpm for 10 min. Serum was then aspirated and stored at 4°C. The collection of the blood sample was done between 8 am and 9 am in the morning. Serum GnRH, FSH, LH, testosterone and corticosterone were measured using enzyme-linked immunosorbent assay. Serum testosterone was measured using rapid lab testosterone kit (UK). Serum FSH and LH were estimated using Dialab FSH and LH kits (UK), respectively. Serum GnRH and corticosterone were measured using cloud clone GnRH and corticosterone kit (UK), respectively.

Histopathology and histomorphometric analysis

Histology of testis and epididymis were done using hematoxylin–eosin stains (H and E stains) as previously described.[23] The animals were killed by cervical dislocation (PND 12 weeks) and dissected to collect the testis and epididymis. The organs were cleared of adherent fats and weighed immediately using OHAUS electronic weighing balance (model no. 031-02-00.0D34). Samples from the organs were fixed in 10% formalin. A thin section (0.05-mm thick) of the tissue was made. The section was stained with H and E stain. Each slide was blotted and mounted with mixture of Distyrene, Polystyrene and Xylene (DPX) mountant. Photomicrographs of the slide preparation were taken after examination under light microscope.

Histomorphometric indices were observed as previously described by Akinloye *et al.*[24] The seminiferous tubular diameter, seminiferous number, epididymal luminal diameter and epididymal ductal diameter of 50 cross-sections/animal were measured using calibrated eyepiece micrometer (Graticules Ltd, Tonbridge Kent) at lower magnification of 100×.

Evaluation of sperm indices

Sperm count and sperm motility were evaluated by microscopy method.[23,25] Epididymal spermatozoa were obtained by mincing the epididymis with anatomical scissors in 5 ml of physiological saline and incubated at 32°C for 2 min. An aliquot of this solution was placed in improved Neubauer counting hemocytometer and motile sperm were counted by using microscope at 400× magnification. Non-motile sperm numbers were first determined, followed by counting of total sperm. Sperm motility was expressed as a percentage of motile sperm of the total sperm counted. Percentage of morphologically abnormal spermatozoa was determined by preparing two slides with H and E stains for morphological examination of live–dead ratio.

A total of 400 sperm cells were counted on each slide under light microscope at 400× magnifications. Sperms with abnormal head and/or tail were considered abnormal.

Statistical analysis

Data were expressed as means ± S.E.M. Statistical comparisons were performed using one-way analysis of variance followed by Tukey's *post-hoc* test to compare the means of the different treatment groups. Differences between the treatment groups with a P-value < 0.05 were considered significant. Data were analyzed with the use of Graphpad Prism version 5.0 for Windows (GraphPad® Software, San Diego, CA, USA).

Results

Effects of maternal Dex treatment during gestation on gestational length, litter size at birth, LBI, VI and LI in the F_1 male offspring

The gestational length was between 20 and 22 days. There was no significant difference in the mean litter size at birth and gestational length in all the treatment groups when compared with the control (Table 1). Table 1 also showed that LBI was significantly reduced ($P < 0.001$) in the DexGD 1–21 when compared with the control. Both VI and LI were also significantly reduced ($P < 0.001$) in the DexGD 15–21 when compared with the control.

Effects of maternal Dex treatment during gestation on body weights, AGD and pubertal age in the F_1 male offspring

The mean birth weights in the DexGD 15–21 and DexGD 1–21 were significantly reduced ($P < 0.001$) when compared with the control (Table 2). The mean weight at weaning was also significantly reduced ($P < 0.05$) in the DexGD 15–21 and DexGD 1–21 when compared with the control (Table 2). It was also observed that the mean weights at puberty was significantly higher ($P < 0.01$) in the DexGD 15–21 and DexGD 1–21 when compared with the control. However, the mean weight at necropsy was not significantly different in all the treatment groups (Table 2).

Maternal Dex treatment at GD 15–21 and 1–21 significantly reduced ($P < 0.001$) AGD when compared with the control (Table 3). The mean AGD at adulthood was, however, not significantly different in all the treatment groups (Table 3). The mean days of PPS was significantly increased in the DexGD 15–21 ($P < 0.01$) and DexGD 1–21 ($P < 0.05$) when compared with the control (Table 3).

Effects of maternal Dex treatment during gestation on sperm indices and serum hormonal profile in the F_1 male offspring at 12 weeks of age

There was no significant difference in the mean sperm count in all the treatment groups when compared with the control. The mean percentage of abnormal sperm was significantly increased in the DexGD 15–21 ($P < 0.01$) and DexGD 1–21 ($P < 0.001$) treatment groups when compared with control (Fig. 1). Meanwhile, there was a significant reduction ($P < 0.01$) in mean

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Table 1. *Effects of maternal dexamethasone treatment during gestation on some indices offspring*

	Live birth index (%)	Viability index (%)	Lactational index (%)	Mean litter size
Control	97.50 ± 0.50	98.30 ± 1.25	98.50 ± 2.25	8.33 ± 1.33
DexGD 1–7	95.00 ± 1.25	86.30 ± 4.50	85.90 ± 3.50	7.66 ± 1.70
DexGD 8–14	98.30 ± 0.67	87.00 ± 5.25	89.20 ± 2.50	7.66 ± 2.3
DexGD 15–21	98.30 ± 0.50	53.30 ± 5.62**	46.50 ± 6.50**	8.00 ± 2.00
DexGD 1–21	75.00 ± 5.50*	52.00 ± 6.71**	42.80 ± 7.25**	7.1 ± 1.34

DexGD, dexamethasone exposure at gestation days.
Values are presented as means ± S.E.M. ($n = 5$).
**$P < 0.01$, *$P < 0.05$ were significant compared with the control.

Table 2. *Effects of maternal dexamethasone treatment during gestation on body weight of F_1 offspring*

	Birth weight (g)	Weight at weaning (PND 21) (g)	Weight at puberty (g)	Weight at PND 12 weeks (g)
Control	4.75 ± 0.14	28.34 ± 2.32	75.46 ± 0.31	223.42 ± 4.31
DexGD 1–7	4.47 ± 0.18	24.98 ± 1.13	74.32 ± 0.24	218.29 ± 3.94
DexGD 8–14	4.52 ± 0.05	23.32 ± 2.52	75.29 ± 0.19	218.47 ± 2.92
DexGD 15–21	3.61 ± 0.01***	21.01 ± 1.02*	84.00 ± 0.21**	208.32 ± 3.17
DexGD 1–21	3.58 ± 0.02***	21.01 ± 0.29*	93.19 ± 1.21**	209.14 ± 2.76

PND 21, postnatal day 21; DexGD, dexamethasone exposure at gestation days.
Values are presented as means ± S.E.M. ($n = 5$).
*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ were significant compared with the control.

Table 3. *Effects of maternal dexamethasone treatment during gestation on anogenital distance (AGD) at birth, AGD at 12 weeks of age and preputial separation in the F_1 offspring*

	AGD birth (cm)	AGD at 12 weeks of age (cm)	Preputial separation (days)
Control	0.41 ± 0.010	2.73 ± 0.24	46.00 ± 2.50
DexGD 1–7	0.4 ± 0.010	2.64 ± 0.43	48.00 ± 2.00
DexGD 8–14	0.41 ± 0.012	2.83 ± 0.62	47.00 ± 1.50
DexGD 15–21	0.30 ± 0.003***	2.60 ± 0.52	57.00 ± 1.50**
DexGD 1–21	0.29 ± 0.003***	2.63 ± 0.36	56.00 ± 2.00*

DexGD, dexamethasone exposure at gestation days.
Values are presented as means ± S.E.M. ($n = 5$).
***$P < 0.001$, **$P < 0.01$, *$P < 0.05$ were significant compared with the control.

sperm motility in DexGD 15–21 and DexGD 1–21 treatment groups when compared with control (Fig. 1).

The mean serum corticosterone levels in the DexGD 15–21 and DexGD 1–21 were significantly increased ($P < 0.001$) when compared with control. Moreover, the mean serum testosterone levels in the DexGD 15–21 and DexGD 1–21 were also significantly reduced ($P < 0.01$) when compared with control (Table 4).

The mean serum LH levels in the DexGD 15–21 ($P < 0.05$) and DexGD 1–21 ($P < 0.01$) were significantly reduced when compared with control (Table 4). As shown in Table 3, the mean serum FSH levels in the DexGD 1–21 was significantly increased ($P < 0.05$) when compared with control (Table 4). In addition, the mean serum GnRH levels in the DexGD 15–21 and DexGD 1–21 were significantly increased ($P < 0.01$) when compared with control (Table 4).

Effects of maternal Dex treatment during gestation on tissue histomorphometry in the F_1 male offspring at 12 weeks of age

Histomorphometric analysis revealed no significant difference in the number of seminiferous tubules, seminiferous tubular diameter, epididymal luminal diameter and epididymal ductal diameter in the male offspring (Table 5).

Effects of maternal Dex treatment during gestation on body weights, AGD and PPS in the F_2 offspring

The birth weight was significantly reduced in the Dex2GD 15–21 ($P < 0.01$) and Dex2GD 1–21 ($P < 0.05$) when

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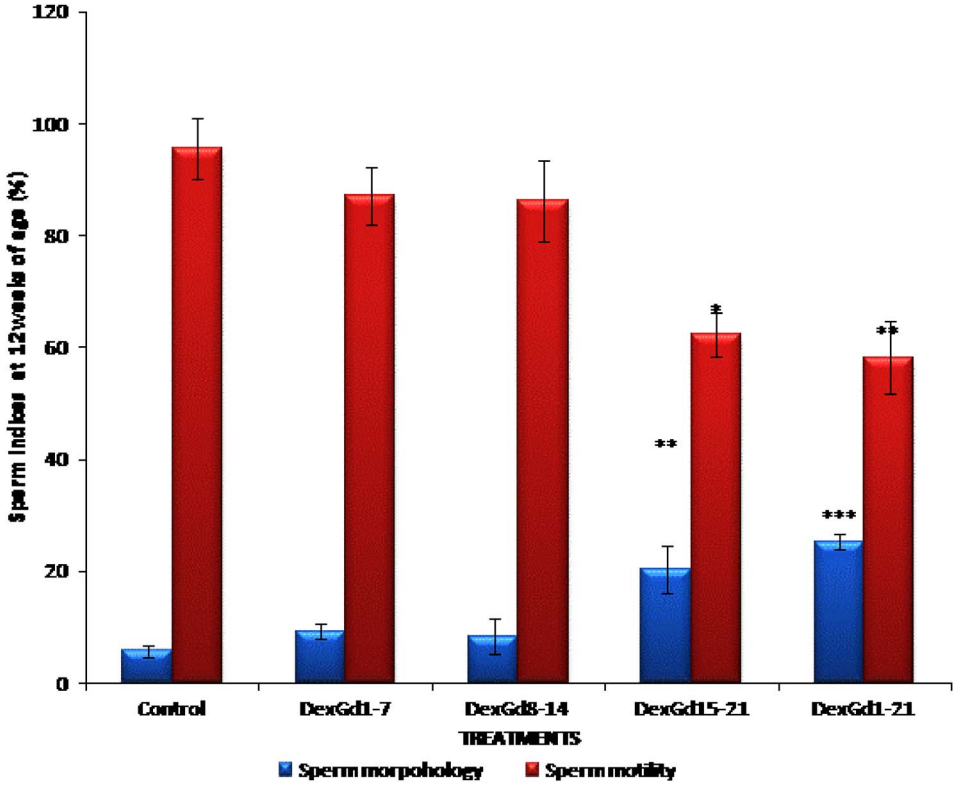

Fig. 1. Effects of maternal dexamethasone treatment during gestation on % abnormal sperm and sperm motility in the F_1 offspring at 12 weeks of age. Values are presented as mean ± S.E.M. ($n = 5$). $^{**}P < 0.001$, $^{*}P < 0.01$, $P < 0.05$ were significant compared with control. DexGd = dexamethasone exposure at gestation days.

Table 4. *Effects of maternal treatment with dexamethasone during gestation on serum hormone concentration in the F_1 offspring at 12 weeks of age*

	Testosterone (nmol/l)	LH (mlU/ml)	FSH (mlU/ml)	GnRH (pg/ml)	Corticosterone (ng/ml)
Control	10.01 ± 1.09	5.70 ± 0.43	5.94 ± 0.44	2.98 ± 0.23	5.2 ± 1.23
DexGD 1–7	8.68 ± 0.53	5.29 ± 0.54	5.24 ± 0.65	2.77 ± 0.34	8.83 ± 2.12
DexGD 8–14	8.95 ± 1.02	4.85 ± 0.49	5.50 ± 0.39	2.74 ± 0.19	5.35 ± 1.01
DexGD 15–21	4.11 ± 1.12**	3.93 ± 0.11*	6.50 ± 0.22	10.21 ± 2.12**	33.7 ± 4.21***
DexGD 1–21	4.32 ± 0.98**	3.50 ± 0.10**	7.90 ± 0.29*	10.87 ± 1.93***	38.57 ± 5.70***

LH, luteinizing hormone; FSH, follicle-stimulating hormone; GnRH, gonadotropin-releasing hormone; DexGD, dexamethasone exposure at gestation days.
Values are presented as means ± S.E.M. ($n = 5$).
$^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ were significant compared with the control.

compared with control2 (Table 6). The mean weight at weaning was also significantly reduced ($P < 0.05$) in the Dex2GD 15–21 and Dex2GD 1–21 when compared with control2 (Table 6). However, the mean weight at puberty and adulthood were not significantly different in all the treatment groups when compared with control2 (Table 6).

The mean AGD at birth were significantly reduced ($P < 0.001$) in the Dex2GD 15–21 and Dex2GD 1–21 when compared with control2 (Table 7). The mean AGD at adulthood was not significantly different in all the treatment groups when compared with control2 (Table 7). The mean days of PPS were significantly increased ($P < 0.05$) in the Dex2GD 15–21 and Dex2GD 1–21 when compared with control2 (Table 7).

Effects of maternal Dex treatment during gestation on sperm parameters and serum hormonal profile in the F_2 offspring at 12 weeks of age

The mean sperm count was significantly reduced ($P < 0.001$) in the Dex2GD 15–21 and Dex2GD 1–21 when compared with control2 (Fig. 2). The mean abnormal sperm was also

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Table 5. *Effects of maternal dexamethasone treatment during gestation on histomorphometric indices in the F_1 offspring at 12 weeks of age*

	Number of seminiferous tubules per section	Seminiferous tubular diameter (μm)	Epididymal luminal diameter (μm)	Epididymal ductal diameter (μm)
Control2	23.00 ± 2.00	0.28 ± 0.01	0.30 ± 0.01	0.49 ± 0.04
Dex2GD 1–7	24.00 ± 1.50	0.29 ± 0.02	0.30 ± 0.03	0.47 ± 0.03
Dex2GD 8–14	26.00 ± 2.50	0.28 ± 0.01	0.29 ± 0.02	0.49 ± 0.02
Dex2GD 15–21	20.00 ± 3.00	0.26 ± 0.03	0.27 ± 0.03	0.46 ± 0.04
Dex2GD 1–21	21.00 ± 2.50	0.27 ± 0.04	0.28 ± 0.02	0.46 ± 0.02

DexGD, dexamethasone exposure at gestation days in the F_1 offspring.
Values are presented as means ± S.E.M. ($n = 5$).

Table 6. *Effects of maternal dexamethasone treatment during gestation on body weight in the F_2 offspring*

	Birth weight (g)	Weight at weaning (PND 21) (g)	Weight at puberty (g)	Weight at PND 12 weeks (g)
Control2	5.32 ± 0.18	35.43 ± 1.93	80.41 ± 1.31	190.3 ± 5.31
Dex2GD 1–7	5.08 ± 0.21	33.67 ± 1.31	78.21 ± 2.49	196.46 ± 2.71
Dex2GD 8–14	4.98 ± 0.31	36.67 ± 1.13	76.29 ± 3.21	202.19 ± 6.31
Dex2GD 15–21	3.31 ± 0.51*	29.34 ± 1.01*	79.21 ± 1.36	197.46 ± 3.21
Dex2GD 1–21	3.59 ± 0.49**	28.49 ± 1.42*	77.32 ± 2.19	199.64 ± 4.91

PND 21, postnatal day 21; Dex2GD, dexamethasone exposure at gestation days in the F_2 offspring.
Values are presented as means ± S.E.M. ($n = 5$).
$^{**}P < 0.01$, $^{*}P < 0.05$ were significant compared with the control.

Table 7. *Effects of maternal dexamethasone treatment during gestation on anogenital distance (AGD) at birth, at 12 weeks of age and preputial separation in the F_2 offspring*

	AGD at birth (cm)	AGD at 12 weeks of age (cm)	Preputial separation (days)
Control2	0.46 ± 0.004	3.09 ± 0.19	48.00 ± 2.00
Dex2GD 1–7	0.43 ± 0.020	2.98 ± 0.24	48.00 ± 2.50
Dex2GD 8–14	0.41 ± 0.003	3.01 ± 0.23	47.00 ± 1.50
Dex2GD 15–21	0.31 ± 0.003**	2.51 ± 0.24	58.00 ± 2.00*
Dex2GD 1–21	0.29 ± 0.003**	2.49 ± 0.19	54.00 ± 1.50*

Dex2GD, dexamethasone exposure at gestation days in the F_2 offspring.
Values are presented as means ± S.E.M. ($n = 5$).
$^{*}P < 0.05$, $^{**}P < 0.001$ were significant compared with the control.

significantly increased ($P < 0.001$) in the Dex2GD 15–21 and Dex2GD 1–21 when compared with control2 (Fig. 3). The mean sperm motility was significantly reduced ($P < 0.001$) in the Dex2GD 15–21 and Dex2GD 1–21 when compared with control2 (Fig. 3).

The mean serum corticosterone levels in the Dex2GD 15–21 ($P < 0.001$) and Dex2GD 1–21 ($P < 0.01$) were significantly reduced when compared with the control2 (Table 8). The mean serum testosterone level in the Dex2GD 15–21 and Dex2GD 1–21 was significantly reduced ($P < 0.01$) when compared with the control2. In addition, the mean serum testosterone was significantly reduced ($P < 0.05$) in DexGD 8–14 when compared with control2 (Table 8). The mean serum LH levels in all the treatment groups were not significantly different in all the treatment groups when compared with control2 (Table 8).

The mean serum FSH level in the Dex2GD 1–21 and Dex2GD 1–15 was significantly reduced when compared with control2 ($P < 0.05$) (Table 8). Similarly, the mean serum GnRH level in the Dex2GD 15–21 and Dex2GD 1–21 was significantly reduced ($P < 0.001$) when compared with control (Table 8).

Effects of prenatal Dex treatment on tissue histomorphometry in the F_2 male offspring at 12 weeks of age

Histomorphometric analysis revealed a significant reduction in the number of seminiferous tubule, seminiferous tubular

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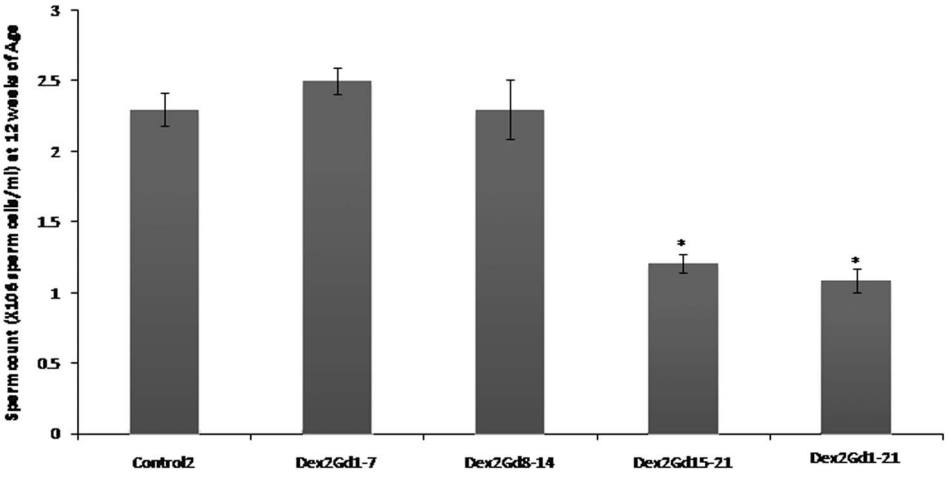

Fig. 2. Effects of maternal dexamethasone treatment during gestation on sperm count in F_2 offspring at 12 weeks of age. Values are presented as mean ± S.E.M. ($n = 5$). *$P < 0.001$ was significant compared with control. Dex2Gd = dexamethasone exposure at gestation days in F_2 offspring.

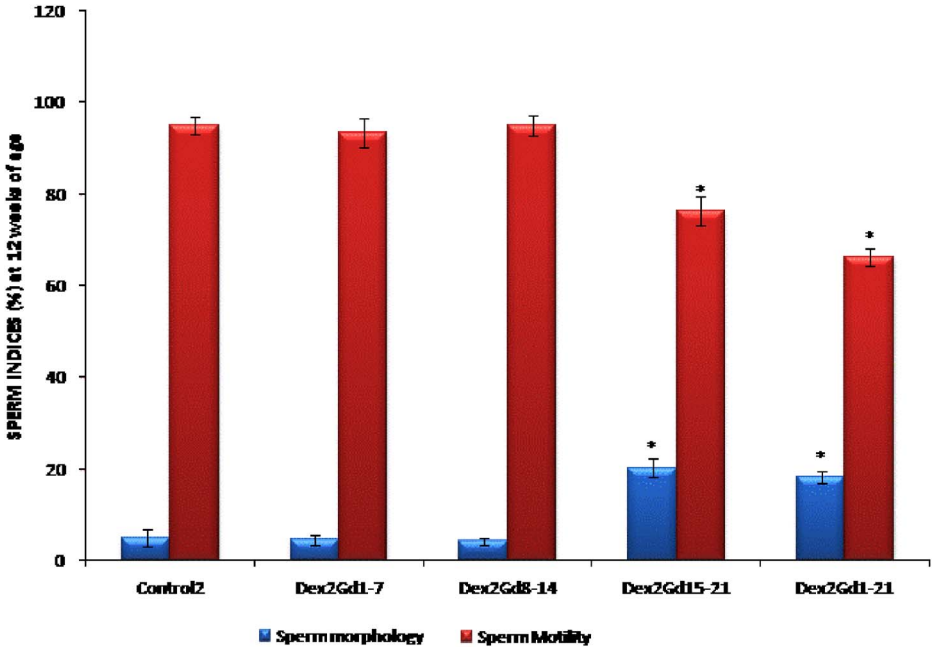

Fig. 3. Effects of maternal dexamethasone treatment during gestation on % abnormal sperm and sperm motility in F_2 offspring at 12 weeks of age. Values are presented as mean ± S.E.M. ($n = 5$). *$P < 0.001$ was significant compared with control. Dex2Gd = dexamethasone exposure at gestation days in F_2 offspring.

diameter, epididymal luminal diameter and epididymal ductal diameter in the Dex2 15–21 and Dex2 1–21 when compared with the control (Table 9).

Discussion

Numerous studies have shown that prenatal administration of synthetic glucocorticoids alters fetal growth, and this phenotypic outcome is strongly dependent on the dosing and timing of exposure during gestation.[14,15,19,26] In agreement with this observation, results from this study indicated that exposure to Dex during the last week of gestation or throughout gestation period reduced birth weight of the offspring. Treatments with Dex limited to the 1st week or 2nd week of gestation does not affect the birth weight (Table 2). Wood and Weeks[27] had earlier reported that prolonged maternal Dex administration (100 µg/kg body weight/day) in rats in late gestation (15–21 days) induced a reduction in birth weight,

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Table 8. *Effects of maternal dexamethasone treatment during gestation on serum hormone concentration in the F_2 offspring at 12 weeks of age*

	Testosterone (nmol/l)	LH (mlU/ml)	FSH (mlU/ml)	GnRH (pg/ml)	Corticosterone (ng/ml)
Control	9.80 ± 0.62	5.90 ± 0.67	9.00 ± 1.36	13.52 ± 2.17	14.90 ± 2.09
Dex2GD 1–7	12.6 ± 1.74	6.00 ± 0.76	7.48 ± 1.44	8.55 ± 1.09	13.78 ± 1.96
Dex2GD 8–14	8.50 ± 0.72	5.25 ± 0.91	9.80 ± 1.91	8.23 ± 0.99	12.00 ± 2.01
Dex2GD 15–21	4.50 ± 0.48*	6.00 ± 0.25	3.90 ± 0.54*	5.09 ± 0.55**	3.45 ± 0.86**
Dex2GD 1–21	4.38 ± 0.78*	7.25 ± 0.34	2.40 ± 045*	2.75 ± 0.62**	5.90 ± 0.78*

LH, luteinizing hormone; FSH, follicle-stimulating hormone; GnRH, gonadotropin-releasing hormone; Dex2GD, dexamethasone exposure at gestation days in the F_2 offspring.
Values are presented as means ± S.E.M. ($n = 5$).
$^{**}P < 0.001$, $^{*}P < 0.01$ were significant compared with the control.

Table 9. *Effects of maternal dexamethasone treatment during gestation on histomorphometric indices in the F_2 offspring at 12 weeks of age*

	Number of seminiferous tubules per section	Seminiferous tubular diameter (μm)	Epididymal luminal diameter (μm)	Epididymal ductal diameter (μm)
Control2	25 ± 2.50	0.3 ± 0.04	0.32 ± 0.01	0.52 ± 0.03
Dex2GD 1–7	22 ± 1.50	0.29 ± 0.02	0.30 ± 0.02	0.53 ± 0.02
Dex2GD 8–14	24 ± 1.50	0.30 ± 0.01	0.28 ± 0.02	0.49 ± 0.05
Dex2GD 15–21	16 ± 1.00**	0.2 ± 0.01*	0.22 ± 0.01***	0.39 ± 0.02*
Dex2GD 1–21	15 ± 1.50**	0.19 ± 0.10*	0.23 ± 0.01**	0.39 ± 0.01*

Dex2GD, dexamethasone exposure at gestation days in the F_2 offspring.
Values are presented as means ± S.E.M. ($n = 5$).
$^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ were significant compared with the control.

whereas administration early in gestation had no effect on birth weight in rats. The reduced birth weight was extended to the second filia generation of male offspring (F_2) without any further intervention in this study (Table 6). Drake and Walker[14] had earlier demonstrated such intergenerational transfer in birth weight due to prenatal Dex treatment in late gestation in rats. This suggests that the programming of birth weight by prenatal Dex exposure may be restricted to the last week of pregnancy in rats.

The reduced body weight observed in DexGD 15–21 and DexGD 1–21 at birth extends to weaning (PND 21). However, immediately after weaning the animals showed a catch-up growth such that at puberty they exhibit increased body weight compared with control and other treatment groups (Table 1). Similar observation was made in the F_2 offspring (Table 6). It has been reported by Somm *et al.*[28] that F_1 pups with intrauterine growth retardation as a result of prenatal Dex treatment or undernourished intrauterine environments failed to recover a normal weight at weaning. However, their study did not evaluate weight at puberty and adulthood. The fact that the offspring from undernourished intrauterine environment and intrauterine Dex exposure experienced reduction in growth rate both at birth and at weaning may suggest a common mechanism. Maternal Dex exposure during gestation may alter nutrient availability for fetal growth and development.

To evaluate the fetal survival as a result of Dex treatment at different stages of gestation LBI, VI and LI were determined. It was observed that LBI was reduced to about 75% in the DexGD 1–21. Both VI and LI were also reduced by prenatal treatment with Dex in the last week of gestation or throughout gestation (Table 1). Pratt *et al.*[29] had earlier reported that repeated administration of betamethasone (100 μg/kg body weight) (fluorinated synthetic glucocorticoids) to the pregnant rabbit either in early gestation (19–20) or late in pregnancy (25–26) significantly reduced fetal survival. They therefore suggested that fetal viability was worse with repeated administration early in pregnancy.[4] This study showed that exposure to Dex during the 1st week or 2nd week of gestation did not affect the viability of the fetus/pups but rather chronic administration throughout pregnancy or in the 3rd week of pregnancy could reduce the viability and therefore the survival of the pups.

The reduction in survival of the offspring of mother exposed to Dex throughout gestation and in the last week of gestation period may be secondary to suppression in the fetal adrenal activities. Fetal adrenal insufficiency has been reported to develop within 2 weeks after maternal use of synthetic

glucocorticoid that crosses the placental in human and the neonate may be born with adrenocorticotrophic hormone (ACTH) suppression.[30] Adrenal insufficiency may become prominent in the neonate and this may be presented with hyponatremia, hypoglycemia and hypotension.[31] These factors may act in concert to reduce the viability and survival of the fetus and neonate.

Disorder in sex steroid balance during fetal development may interfere with male reproductive success and health later in life.[32] Reduction in androgen dominance to estrogens and interference with androgen action are apparent mechanism that could cause demasculinization.[32,33] Agents with androgen action program masculinization during a common early programming window, that is, before ED 19.5 in rats.[34] When pregnant Wistar rats were treated with agent with anti-androgenic actions during the early programming window, a period just after the onset of testicular testosterone production (GD 15.5–17.5) or during the middle window, that is, the period immediately before morphological differentiation of reproductive tract tissues (GD 17.5–19.5), the pups express reduced AGD in postnatal males.[34] In the present study, *in utero* exposure to Dex during late gestation or throughout pregnancy significantly reduced the AGD at birth in the male rats. The reduced AGD resolved before adulthood such that at 3 months of age there was no significant difference in the AGD in all the treated groups when compared with the control (Table 3).

This study also showed that prenatal Dex treatment at GD 15–21 and 1–21 induce delayed puberty in postnatal life (Table 3). This report corroborate the findings of Smith and Waddell[20] that Dex exposure as well as carbenoxolone (an inhibitor of 11β-hydrosteroid dehydrogenase enzyme, which inactivates maternal glucocorticoids during their passage to the fetus and thereby constitute placental barrier) treatments during gestation causes delayed puberty in the offspring. This study indicates that the delay in puberty is transfered to the F_2 offspring.

It has been reported in the literature that reduced AGD[19,21] and delayed puberty[21] are measures of degree of demasculinization, and their presence may be an indicator of increased anti-androgenic activity.[21] This suggests that prenatal Dex exposure at late gestation or throughout pregnancy may be anti-androgenic.

Fetal glucocorticoids exposure could also influence the timing of puberty via direct actions on the HPG axis possibly by acting at all the three levels.[20] Such reprogramming of hormonal axis has been observed in relation to HPA axis.[12,35] In rats, GnRH neurons develop and migrate during fetal period[36] so that altered glucocorticoids exposure at this time may program their number and/or normal function[20] pituitary gonadotrophs[37] and testis are both glucocorticoids targets in the adult and so may also be programmed by fetal glucocorticoids exposure.[20]

It was also observed in this study that serum testosterone level was reduced by prenatal exposure in the last week of pregnancy and throughout gestation. This was accompanied by significant reduction in the LH. However, GnRH was significantly raised by exposure to Dex at GD 15–21 and 1–21, and FSH level was raised only in the group that was treated with Dex throughout gestation (Table 4).

Normally, the system by which gonadal steroid hormone is regulated involves a negative feedback mechanisms from the hypothalamus through GnRH secretion, pituitary through LH and FSH secretion and gonads that produce testosterone in male. Alteration in this feedback mechanism could lead to disruption in reproductive function. It has been previously reported that chronic exposure to glucocorticoid can inhibit gonadotropin (LH and FSH) secretion as evidence by decrease responsiveness to administered GnRH and subnormal plasma testosterone.[16] LH is the hormone involved in the regulation of the steroidogenic acute regulatory (StAR) protein that deliver cholesterol to inner mitochondria for steroidogenesis in the Leydig cell.[38] Therefore, decreased serum level of LH could lead to reduction in testosterone synthesis and release.

As part of negative feedback mechanism in the regulation of gonadal activities, it is expected that reduction in gonadal steroid stimulate an increase in GnRH from the hypothalamus and gonadotropin from the pituitary. Although the GnRH and FSH response follow this pattern, LH level was rather reduced. This may suggest that the programming effect was on the part of HPG axis involved in steroid synthesis in the Leydig cell. It is possible that there was a decreased responsiveness of LH to GnRH secretion. Such kinds of response have been observed in female exposed chronically to glucocorticoid.[16] Moreover, testicular steroidogenic activities in the fetus start at earlier part of the last week of gestation in rats.[21]

To evaluate if corticosterone plays a role in the alterations of the activities at HPG axis as observed in this study, the basal serum corticosterone level in the male offspring was investigated. Treatment with Dex during the 3rd week or throughout pregnancy significantly increased the serum basal circulating corticosterone level in adulthood (Table 4). In agreement with this findings, the report that daily treatment in rat with synthetic glucocorticoids in the 3rd week of gestation or throughout gestation results in elevated basal plasma corticosterone levels in the adult male offspring.[39,40] This may be secondary to the reduction in the maternal HPA axis activity, which programmed and increased adrenal release of corticosterone in the offspring in rats.

It was also observed in this study that there was a decrease in serum corticosterone level in the F_2 offspring. This was accompanied by similar decrease in serum testosterone, FSH and GnRH, but LH levels in the serum remained the same as the control (Table 8). The reduced serum corticosterone level in the F_2 offspring is in line with the findings reported by Dunn *et al.*[41] through maternal transmission. They observed that the female offspring (F_1) that are born to mother that had been exposed to synthetic glucocorticoids during F_0 pregnancy, but had themselves gone through an undisturbed pregnancy, exhibited significant reductions in basal and stress-stimulated salivary cortisol level.[41] This study, however, shows that multigenerational transfer in programming of basal serum

corticosterone is also possible through paternal lineage in rats. Another study had also identified transgenerational influence on liver enzyme and birth weight, which can be transferred through both maternal and paternal lineage in rats.[15] Reduction in serum basal corticosterone level in the F_2 offspring was, however, contrary to what was observed in the F_1 offspring. This observation indicate the complexity of developmental programming effects that depend on several factors that may regulate the entire phenotypic outcome in multigenerational study.

Reduction in both testosterone, GnRH and FSH without any change in LH, suggest a possible disruption in the HPG-feedback mechanism. This may possibly alter the reproductive functions.

Evaluation of sperm indices also showed that treatment with Dex during late gestation or throughout pregnancy reduced sperm motility and increased the percentage of abnormal sperm in the caudal epididymis. Although sperm count is not affected by the treatment. The abnormality in sperm morphology includes abnormal head and/or tail. In addition, in the F_2 offspring, the three sperm indices (sperm count, motility and percentage of abnormal morphology) were significantly affected by the treatment in the late or throughout gestation Dex administered group's offspring (Figs 1–3).

The reproductive system is extremely susceptible to insult from exposure to exogenous steroids during development.[42] When insults occur at a gestation age critical for target organ differentiation, it leads to alterations in the development trajectory of the organ culminating in disruption in the organ function.[43] It has been previously reported that the male programming window is between GD 14.5 and 19.5 in rats.[44,45] Sperm indices might therefore be programmed during this period. Another explanation for this observation is that, reduced androgen level in the DexGD 15–21 and DexGD 1–21 as observed in this study might have altered spermatogenic activity in the seminiferous tubules. Gametogenesis in male is normally androgen dependent.[16,45] The Sertoli cells that surround developing germ cells are known to secret androgen-binding protein under the influences of testosterone and FSH. This protein ensures normal spermatogenic activity by binding testosterone.[46] In addition, there were significant reductions in the histomorphometric indices of the testis and epididymis (Table 9).

The development and functionality of testis and epididymis are androgen dependent and alteration in the androgen activity in the F_1 offspring might have epigenetically programmed these tissues in the F_2 offspring. Although, there was no previous report on the histopathology of these tissues in the F_2 offspring but multigenerational transfer in organ functions and structures have been reported in the literature.[12,14,15] Rodents models examining paternal transmission have identified epigenetic signature in mature sperm as possible substrate of multigenerational programming.[45] In particular, the role of sperm RNA as a mechanistic link between paternal experience and its consequence on offspring behavior and physiology has been emphasized in the recent study.[47]

In conclusion, maternal Dex treatment in rats during late gestation may disrupt reproductive functions by altering the activity at HPG axis. The gestational treatment may be transferred to the F_2 offspring.

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Conflicts of Interest

None.

Ethical Standards

The study was conducted in accordance with International Ethical Norms on Animal Care and Use as contained in NIH publication/ 80-23, revised in I985.

Supplementary material

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