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Morphological alterations in the seminiferous tubules of adult Wistar rats: the effects of prenatal ethanol exposure

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This study presents the effects of prenatal ethanol exposure on the morphology of the seminiferous tubules of the testes in the adult male rat. Timed-pregnant adult female Wistar rats (average weight 200 g) were given daily intragastric intubation of 5.8 g/kg ethanol between gestation days 9 and 12. Pair-fed and ad *lib-fed animals served as controls. The pups were weighed at birth and weaned at 30 days. At 42 days of age the male offspring (n = 10) from each group were anaesthetised and the testes removed and weighed. Another set of male rats from each group (n = 6), were anaesthetised, whole body perfused and the testes removed and processed for paraffin embedding. Sections were subjected to morphological analysis and morphometric measurements based on computerised techniques following haematoxylin and eosin, PAS and reticulin staining.*

The results demonstrated that prenatal ethanol exposure induced persistent growth retardation and a 66% reduction in testicular weight and severely altered the morphology of the seminiferous tubules of adult male rats, causing a reduction in the cross-sectional area of the tubules by 18%, germinal epithelium thickness by 21% ($p < 0.001$) and an inhibition of spermatogenesis. The study showed the absence of reticulin fibres in the peritubular tissue of seminiferous tubules of prenatal ethanol-exposed adult male rats. The results imply that damage following prenatal ethanol exposure occurs irreversibly in utero *and persists into adulthood in the exposed animals, which may have implications for male fertility.*

Key words: germinal epithelium, reticulin, foetal alcohol syndrome, ethanol, male fertility, testes

INTRODUCTION

Ethanol consumption during pregnancy has been proven to be teratogenic to the foetus and leads to a cluster of symptoms termed foetal alcohol syndrome (FAS) [3, 19]. The effects on the development of the central nervous system are the most significant [8, 11, 22, 35] and the most extensively studied [2, 11, 22, 39, 41, 44, 45] and are associated with growth retardation [9, 26, 31, 33] and mental retardation [2, 8, 19, 22].

Prenatal ethanol exposure in the rat is known to interfere with the neurobehavioural sexual differentiation of the male [1, 4, 15, 28], attenuating the postnatal testosterone surge required by the male brain for normal sexual differentiation [10, 27, 28, 29, 32] and lowering serum levels of testosterone

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[40] and luteinising hormone [1, 9, 40]. Udani et al. [40] reported a significant reduction in testicular weight. It has been suggested that the suppression of the perinatal testosterone surge in male rats exposed to alcohol *in utero* and the associated long term demasculinising effects of prenatal exposure to ethanol might be the result of reduced testicular steroidogenic enzyme activity in the perinatal animal [20, 30] and the elevation of regional brain aromatase activity in the males [30].

However, the effects of prenatal exposure to ethanol on the morphology of the seminiferous tubules have received little attention. Earlier reports [29] showed that the testes at birth contained a reduced number of Leydig cells in the prenatal ethanol exposed group. A large number of vacuoles are present in the seminiferous tubules. This study presents the effects of prenatal exposure to ethanol on the morphology of the seminiferous tubules of the testes in the adult male rat.

MATERIAL AND METHODS

Adult female Wistar rats (average weight 200 g) were used for the study. The rats were maintained in the animal holding of the Department of Anatomy and Cell Biology on standard mouse chow (Ladokun Feeds, Ibadan, Nigeria) and water *ad libitum.* They were exposed to 12 hours of light and 12 hours of darkness to stabilise their oestrus cycle and mating was carried out by placing a virile male rat into a cage with two females at 1600 h and examining the females at 1000 h the following morning for the presence of vaginal plugs or spermatozoa [37]. The presence of a vaginal plug was taken as day 0 [37]. Following confirmation of pregnancy the female rats were divided randomly into two groups, A and B (n = 6). Group A received a daily ethanol dose of 5.8 g/kg body weight/day on the 9^{th}, 10^{th}, 11^{th} and 12^{th} days of gestation by intragastric intubation, at 16.00 h daily [37, 42] (PEE) and group B received an isocaloric solution of sucrose for the same duration to substitute for the ethanol in the experimental group. To control the possible effects of under-nutrition, rats in group B were pair-fed with ethanol-treated dams (PF). An additional control group C (n = 4), which was not intubated or pair-fed but received standard chow (C) and water *ad libitum*, was also included [42].

At birth the litters were weighed and weaned at 30 days of age. At 42 days of age the male rats (n = 10 per group) were anaesthetised by an overdose of penthotal intraperitoneally and the testes quickly removed and weighed. Whole body perfusion with Bouin's fluid was carried out on another set of male rats (n = 6). The testes were removed and further fixed by whole immersion in Bouin's fixative for 8 hours and then processed for paraffin embedding. Sections of 6 μ thickness were cut on a rotary microtome and stained with haematoxylin and eosin [43], PAS and DPAS [34] and silver metallic impregnation for reticulin [7]. The sections were examined with the Axioplan-2 Zeiss research microscope attached to a computer with a Sony 3CCD colour video camera. Morphometric measurements (15 per preparation) were performed on the H & E sections, using the computer assisted image analySIS programme (Soft Imaging software GmbH). The intratubular diameters of the seminiferous tubules and the thickness of the germinal epithelium were measured on preparations originating from 6 rats in each group. In each preparation 15 tubules of oval or circular cross-section were measured at a magnification of × 125. The thickness of the germinal epithelium was measured from its base to its free surface [23], again at the same magnification. The data obtained for body weights, weight of the testes and morphometric parameters were subjected to statistical analysis using the paired two-sample t-test and one-way analysis of variance (ANOVA).

RESULTS

Prenatal ethanol exposure during gestational days 9–12 resulted in significant morphological alterations in the seminiferous tubules of adult male rat testes. The seminiferous tubules of the prenatal ethanol-exposed (PEE) adult rats showed marked reduction in size and germinal epithelial thickness, large empty lumens and inhibition of spermatogenesis (Fig. 1b, d), compared with the pair-fed (PF) and chow (C) controls (Fig. 1a, c). There were no differences in morphology between the PF and C groups. Significant sloughing off of the tubular limiting membrane and of the epithelium "desquamation" (Fig. 1b), as well as wide dispersion of the germinal cells (Fig. 1d) were found in the PEE rat tubules. Spermatogenesis was arrested at the spermiogenic phase (stage IX) (Fig. 1b, d) [16], showing multiple spermatids with the absence of residual bodies of Regaud and spermatozoa. In comparison, the seminiferous tubules of the control showed well formed stratified epithelial layers of germinal cells amongst sertoli cells (Fig. 1a, c) at various stages of spermatogenesis with the production of nu-

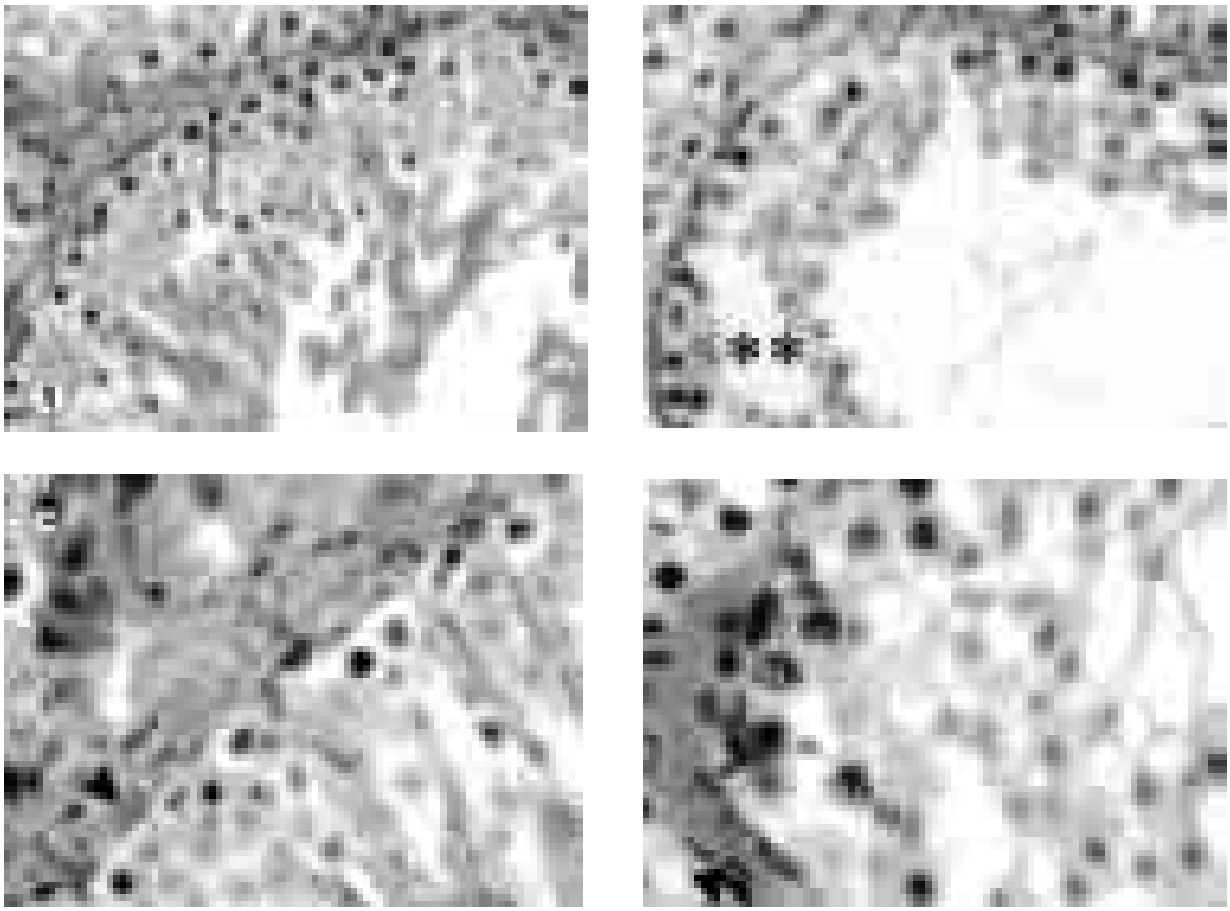

Figure 1. Seminiferous tubules of adult male rats; (**a** and **c**) showing stratified germinal epithelium with numerous 'hook-shaped headed' spermatozoa (Sz) and prominent Leydig cells (Lc) in the interstitium; (**b** and **d**) PEE showing a large empty lumen, desquamation (asterisks) of germinal epithelium (**b**), dispersion of germinal cells, absence of spermatozoa and residual bodies of Regaud (**d**). Haematoxylin and eosin staining. Magnification, **a** and **b**, × 630 and **c** and **d**, × 1000; S° — spermatogonia; S1 — primary spermatocytes; S2 — secondary spermatocytes; Sz — spermatozoa; Rb — residual bodies of Regaud; St — sertoli cells; Lc — leydig cells; Sd — spermatids; arrow head — myoid cells.

merous 'hook shape headed' spermatozoa. The lumens were filled with strands of eosinophilic residual bodies of Regaud (Fig. 1a, c). The interstitial spaces revealed Leydig cells (Fig. 1c, d), which contained granulation in the control group (Fig. 1c) compared to the treatment (Fig. 1d).

The seminiferous peritubular walls and *tunica albuginea* (TA) of the PEE rats did not stain for reticulin fibres (Fig. 2b, d). However, the peritubular wall nuclei of the myoid cell were still present and were also unstained. The control group, in conrast, showed black-stained continuous fibres surrounding the seminiferous tubules (Fig. 2a). The peritubular myoid cells were also black-stained (Fig. 2a). The reticulin fibres appear to extend intraluminally from the wall in between the germinal cells (Fig. 2a). The *tunica albuginea* showed a dense wavy arrangement of stained fibres, which also appeared to be continuous with those of the peritubular fibres (Fig. 2c). The PAS staining only highlighted the granulation of the Leydig cells found in the control group (as in Fig. 1c; otherwise it was not significant).

Data on the body weights, testicular weights and morphometric parameters are shown in Tables 1 and 2 and Figures 3–6. The values are mean ± s.e.m. Prenatal ethanol exposure significantly reduced the litter birth weight by 18%, compared with the control ($p < 0.001$). This reduction in whole body weight was not only sustained, but was further decreased by 10% at 42 days of age (Table 1, $p < 0.001$). The testicular weight was markedly reduced by 66%, compared with the control (Table 2, Fig. 3; $p < 0.001$). The relative testicular weight only improved slightly, by 13%, compared with the percentage reduction in the actual weights (Table 2). The intratubular diameters and germinal epithelium

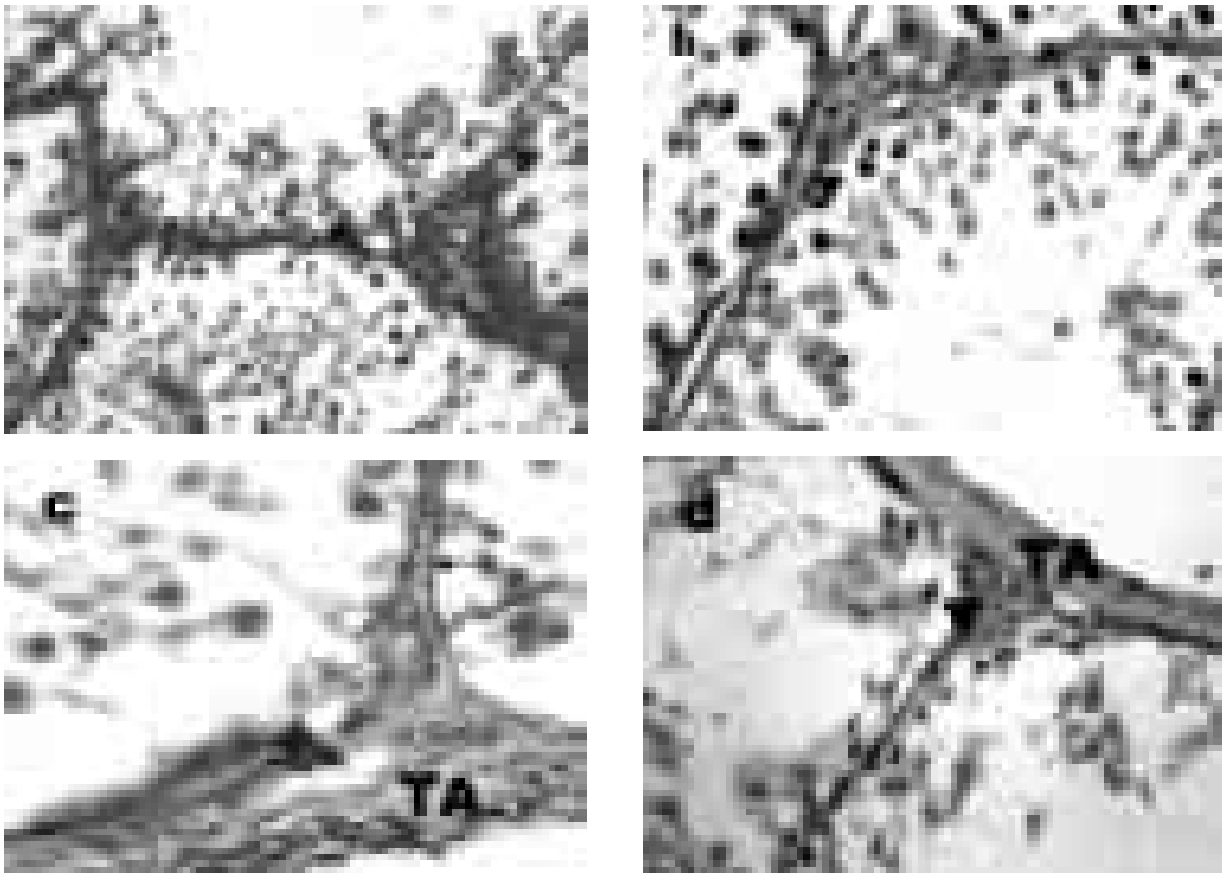

Figure 2. Peritubular wall of seminiferous tubules of PEE rats, unstained for reticulin fibres but showing elongated myoid cell nuclei (arrow head) (**b**) in a single layer. The *tunica albuginea* (TA) is also unstained (**d**). The control tubules shows black-stained reticulin fibres (thick arrows) surrounding the perimeter of each tubule within the peritubular layers. There was also intraluminal extension of the dark stained reticulin fibres (thin arrow) (**a**). The peritubular myoid cell nuclei were also darkly stained (arrow head). The *tunica albuginea* shows a dense wavy arrangement of the black-stained fibres (**c**). Reticulin staining by silver impregnation. Magnification, a, b, c, and d, × 630.

Table 1. Body weight of rats at birth and 42 days; results presented as mean ± s.e.m.

Group of rats	Body weight at birth [g] (n = 20)	Body weight at 42 days [g] (n = 15)
PEE	4.17 ± 0.03*	158.47 ± 2.16*
PF	5.10 ± 0.06	221.33 ± 2.14
C	5.24 ± 0.08	223.60 ± 2.61

Difference compared with PF and C at *$p < 0.001$(t — test and ANOVA); PEE — prenatal ethanol-exposed; PF — pair-fed; C — chow control

Table 2. Actual and Relative Testicular weights at 42 days; results presented as mean ± s.e.m. (n = 10)

Group of rats	Actual Testicular weight [mg]	Relative Testicular weight [mg/100 g]
PEE	691.40 ± 11.7*	436.30 ± 7.38*
PF	2036.90 ± 24.0	920.30 ± 10.84
C	1967.80 ± 24.3	844.55 ± 10.42

Difference compared with PF and C at *$p < 0.001$(t — test and ANOVA); PEE — prenatal ethanol-exposed; PF — pair-fed; C — chow control

thickness were all significantly reduced, by 18% and 21% respectively (Fig. 4, 5; $p < 0.001$ and $p < 0.01$ respectively), following prenatal exposure to ethanol. The tubular cross-sectional area was also reduced by 18% in the prenatally exposed rats compared with the control (Fig. 6; $p < 0.001$). There were no differences between the values of the PF and C groups ($p > 0.05$).

DISCUSSION

General growth retardation due to prenatal exposure to ethanol has been previously reported [9, 31, 33, 40]. This retardation persists into adulthood [31]. All dams successfully littered with an overall average of 6 pups per dam. Prenatal ethanol exposure did not significantly affect the duration of gestation, nor the litter size or sex ratio. Pups were reared

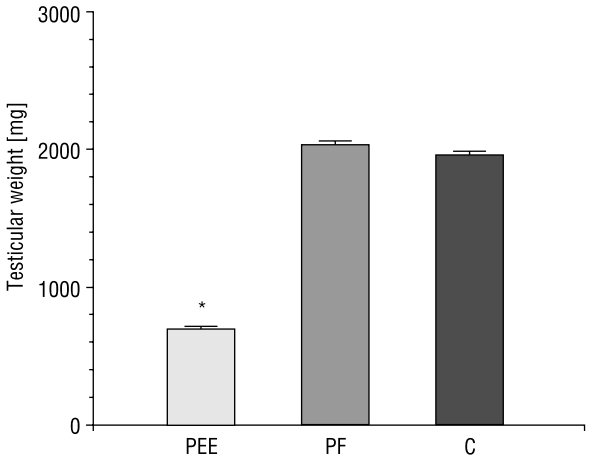

Figure 3. Testicular weight of rats. Values are mean ± s.e.m. [one-way ANOVA, *t*-test (*p < 0.001)]; PEE — prenatal ethanol exposed; PF — pair-fed; C — chow control.

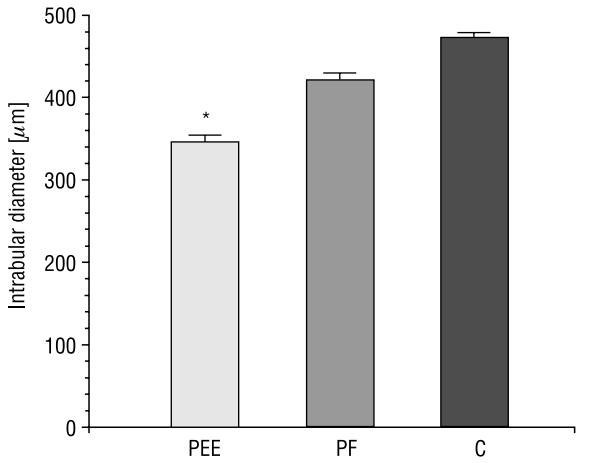

Figure 4. Intratubular diameter of seminiferous tubules. Values are mean ± s.e.m. [one-way ANOVA, *t*-test (*p < 0.001)]; PEE — prenatal ethanol exposed; PF — pair-fed; C — chow control.

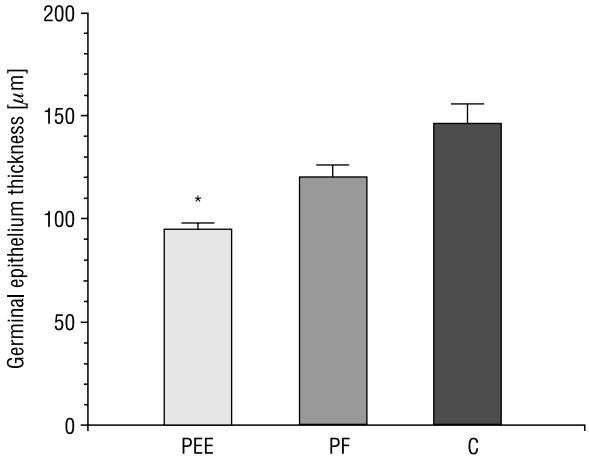

Figure 5. Germinal epithelium thickness. Values are mean ± ± s.e.m. [one-way ANOVA, *t*-test (*p < 0.001)]; PEE — prenatal ethanol-exposed; PF — pair-fed; C — chow control.

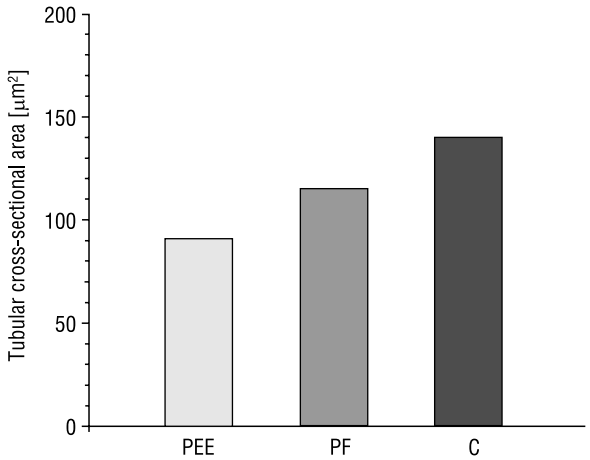

Figure 6. Seminiferous tubules cross-sectional area. Values are mean ± s.e.m. [one-way ANOVA, *t*-test (p < 0.001)]; PEE — prenatal ethanol exposed; PF — pair-fed; C — chow control.

by their mothers and fed on standard mouse chow and weighed regularly. Prenatal ethanol exposure caused an 18% reduction in body weight at birth, which worsened at 42 days of age to 28%, suggesting a persistent retardation of growth and development in the postnatal period. The testicular weight was markedly reduced by 66% following prenatal exposure to ethanol, with the relative weight improving slightly, by 13% only. This finding supports an earlier report [40], that *in utero* exposure to alcohol decreased the testicular weight in the alcohol-exposed animals at postnatal days 55 and 110. The mechanism for this drastic effect on the testes is yet unknown.

The results demonstrate clearly that prenatal ethanol exposure severely alters the morphology of the seminiferous tubules of adult male rats, causing an 18% reduction in the cross-sectional area of the tubules and a 21% reduction in the thickness of the germinal epithelium accompanied by inhibition of spermatogenesis. PEE tubules present mostly germinal epithelia at stage IX of the seminiferous epithelial cycle [16]. These suggest an interference with the spermiogenesis and spermiation processes of the tubules in the treatment rats. Seminiferous tubules are composed of somatic cells (myoid and sertoli cells) and germ cells (spermatogonia, spermatocytes and spermatids). The activities of these germ cells divide spermatogenesis into (a) spermatocytogenesis (b) meiosis and (c) spermiogenesis [17, 18]. The arrest of spermatogenesis at the spermiogenic stage by prenatal ethanol exposure could possibly be due

to the derangement of the germ cell — sertoli cell configuration of the germinal epithelium in the PEE animals (dispersion and desquamation — Fig. 1b, 1d). Kerr [21] suggested that germ cell and sertoli cell functions are intimately related to each other via local intratesticular or paracrine signals, which are suppressed or triggered at certain defined steps in the spermatogenic process. Ethanol *in utero* may interfere with the cellular interaction between these cells, causing the observed alterations.

Testicular peritubular tissue, also known as the *tunica propria*, surrounds the seminiferous tubules and is responsible for contractile, paracrine and transportation functions [14]. The peritubular *laminar propria* of the rats' testes have been shown to be composed of a single layer of myoid cells which secrete collagen of types I and IV, proteoglycans, laminins and fibronectin [6, 13, 24, 36]. In a recent report Fakoya [12] demonstrated the presence of reticulin fibres in the *tunica albuginea* and peritubular tissue of the seminiferous tubules of normal adult rats. The absence of these reticulin fibres in the PEE testes possibly indicates a deleterious effect of prenatal ethanol exposure on the integrity of the testes. The myoid cells stained intensely for reticulin (Fig. 2a) and it has been suggested that they are responsible for the secretion of the stained reticulin fibres in the control group [12]. The absence of the fibres in the PEE rats may mean either that the myoid cells were inhibited by alcohol or its metabolite (since they were still present, but unstained) or that the fibres produced were damaged after production or both. However, since alcohol was given during gestation, it is most probable that the effect is on the secretion of the reticulin fibres by these cells. This may explain the relative thinness of the interstitial spaces in these animals.

Alcohol was administered during days 9–12 of gestation in this experiment, earlier than the onset of gonadal morphogenesis [6]. Differentiated sexual cords, probably, beginning in the inner part of the gonadal ridge, are found in 13-day-old foetuses. The seminiferous cords surrounded by a continuous basement membrane are separated from the coelomic epithelium by the differentiating *tunica albuginea* in 14-day-old foetuses [6]. This implies that *in utero* administration of alcohol does not completely inhibit the morphogenesis of the male gonads but does affect the rate of growth and development, as well as the level of differentiation. Similarly, it also indicates that the primary germ cells were protected against the damaging effects of *in utero* alcohol, or that they had migrated prior to the period of administration of ethanol during gestation. The precise mechanism is yet unknown. Chronic alcohol abuse in adults has been shown to cause testicular atrophy and infertility in alcoholic men and animals [5, 25, 45, 46]. It is known that ethanol exposure disrupts the hypothalamic-pituitary-gonadal axis, adversely affects the secretory functions of sertoli cells and produces oxidative stress within the testes [45]. The histological lesions show a significant decrease in the diameter of the seminiferous tubules, the number of different germ cells at all stages of the seminiferous tubule cycle and the presence of degenerative germ cells [5, 38]. It is still not clear what cellular mechanisms are responsible for the morphological alterations of these testes that result in a reduction of testicular mass as a consequence of ethanol exposure. This damage affects the testicular interstitial cells and seminiferous tubules, particularly the sertoli cells and the peritubular wall of the latter [38]. However, Zhu et al. [45] suggested that ethanol enhances testicular germ cell apoptosis. The morphological findings in prenatal ethanol-exposed animals are somewhat similar to those found in adult animals that ingest alcohol chronically. The mechanism of action may not be similar, considering that alcohol would not be present in any significant amount (if at all) in the adult PEE animals at 42 days of age. It therefore implies that the damage inflicted by prenatal ethanol exposure occurs irreversibly *in utero* and persists into adulthood in the exposed animals. This may have implications for male fertility in this group of animals. It may be important to evaluate fertility in male children born to alcoholic mothers, who may or may not develop the full-blown foetal alcohol syndrome.

In conclusion, this study confirms that prenatal ethanol exposure causes irreversible growth retardation, severe reduction in testicular weight and significant damaging alterations in the seminiferous tubular morphology of adult male rats. There is inhibition of spermatogenesis and damage to the peritubular and testicular integrity.

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