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Low-intensity ultrasound energy applied to the testes of aged rats

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Summary. Previous studies from our laboratory have shown that low-intensity ultrasound applied to the scrotum of prepubertal rats causes a 62% increase in plasma testosterone, suggesting a possible stimulation of LH receptors and/or the enzymes controlling the steroidogenic process. The purpose of the present study was to investigate whether low-intensity ultrasound has a stimulatory effect on the androgenic activity of aged testes. In addition to plasma testosterone, LH and FSH, the testicular spermatogenic status was also analysed.

Ultrasound applied to the scrotum of aged rats did not stimulate sperm production, which was significantly reduced compared to sexually mature animals, and failed to re-establish the steroidogenic testicular function, which was decreased by 74%, suggesting an inherent loss of gonadal steroidogenic competence.

Key words: Ageing, Testis, Testosterone, Ultrasound, Gonadotropins

Introduction

A progressive decline in male reproductive function, observed in many mammalian species with increasing age, includes testicular and epididymal degeneration, reduction in semen quality and loss of fertility (Bishop, 1970; Huber et al., 1980; Gosden et al., 1982; Horn et al., 1996), accompanied by reduction in plasma FSH, LH and testosterone (Ghanadian et al., 1975; Gray, 1978; Miller and Riegle, 1978; Riegle and Miller, 1978; Kaler and Neaves, 1981; Lamano Carvalho et al., 1988; Gruenewald and Matsumoto, 1991; Tsitouras and Bulat, 1995). There is evidence that the impaired endocrine testicular function of aged rats is due to reduced hypothalamic stimulation of pituitary gonadotropin secretion, since the senescent testis retains the capacity to respond to exogenous hCG (Miller and Riegle, 1978; Riegle and Miller, 1978; Jarjour et al., 1986; Lamano Carvalho et al., 1988; Gruenewald and Matsumoto, 1991).

Plasma testosterone concentration is low in prepubertal rats, increase to a maximum during puberty and falls to a lower plateau during sexual maturity (Odell, 1990; Zanato et al., 1994). Previous studies from our laboratory have shown that low-intensity ultrasound energy applied to the scrotum of prepubertal rats caused a 62% increase in plasma testosterone, suggesting a possible stimulation of LH receptors and/or the enzymes controlling the steroidogenic process; from puberty onwards the testicular androgen activity was unaffected by ultrasound (Haddad et al., 1994).

Low-intensity ultrasound has been used in medicine to accelerate the healing of hard and soft tissues (Paul et al., 1960; Dyson et al., 1976; Xavier and Duarte, 1983). The biological effects of ultrasound are mainly attributed to non-thermal mechanisms such as accoustic microstreaming (eddying movement of fluids induced by radiation forces) and stable cavitation (formation and vibration of gas-filled bubbles in fluids as a result of ultrasonically induced pressure changes), that may act as a signal facilitating diffusion of ions and metabolites across cell membranes. As a consequence, stimulation of both cell metabolism and proliferation takes place (Stewart et al., 1985; Dyson, 1987).

The purpose of the present study was to investigate whether a low-intensity ultrasound has a stimulatory effect on the androgenic activity of aged testis, as previously observed in prepubertal rats. In addition to plasma concentrations of testosterone, luteinizing (LH) and follicle stimulating (FSH) hormones, the testicular spermatogenic status was also analysed by morphometric techniques.

Materials and methods

Sexually mature (3 months old, n=10) and aged (2 years old, n=23) male Wistar rats were kept on a standard 12 h light/12 h dark schedule and had free access to food and water. Part of the aged animals (n=10) was stimulated in the scrotum with pulsed ultrasound for 20 min a day during a period of three weeks, followed by 5 weeks lacking stimulation and one

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last week of ultrasound application. The ultrasound generator was calibrated to produce therapeutic doses recommended for tissue repair, as previously described (Haddad et al., 1994, 1996): 1.5 Mhz frequency, 1 Khz repetition pulse rate, 200 μs pulse width, 30 V peak-to-peak amplitude and 20 mW/cm^2 intensity. The transducer was applied to the skin using a contact gel as coupling medium. The remaining aged rats (n=13) were treated in a similar fashion but no ultrasound was applied.

The animals were killed by decapitation, blood was collected and plasma concentrations of testosterone, LH and FSH were determined by double-antibody radioimmunoassay, as previously described (Haddad et al., 1994). The concentration of spermatozoa was estimated in the sperm suspension stored in the right cauda epididymis, according to Kempinas et al. (1988). Right testes were removed, weighed and stored at -20 °C until DNA content determination (Burton, 1956). Left testes were removed, weighed and immersed in Alfac fixing solution (85% ethanol 80°, 10% formaldehyde and 5% glacial acetic acid) for 24 h. Seven μm-thick equatorial paraffin sections were stained with hematoxylin and eosin, for histological and histometric analysis.

Histometric analysis

Sperm production was estimated by the ratio between the number of spermatids in stage 19 of spermiogenesis and the amount of Sertoli cells, counted in 20 seminiferous tubule cross-sections per animal. As the Sertoli cells do not divide in adult rats, presenting a constant number and homogeneous distribution along the seminiferous tubules (Bustos-Obregón, 1970), they have been used as a correction factor for variations in tubular diameter and length (Steinberger and Steinberger, 1975).

Statistical analysis

Results were analysed for statistical significance by a non-parametric test (Kruskall-Wallis, ANOVA).

Results

Adult rats continued to grow up to senility, showing a 70% body weight gain from 3 months to 2 years of age. The absolute testicular weight increased about 40% during the same period, leading to a 30% decrease in the relative gonadal weight. A small (15%) but significant

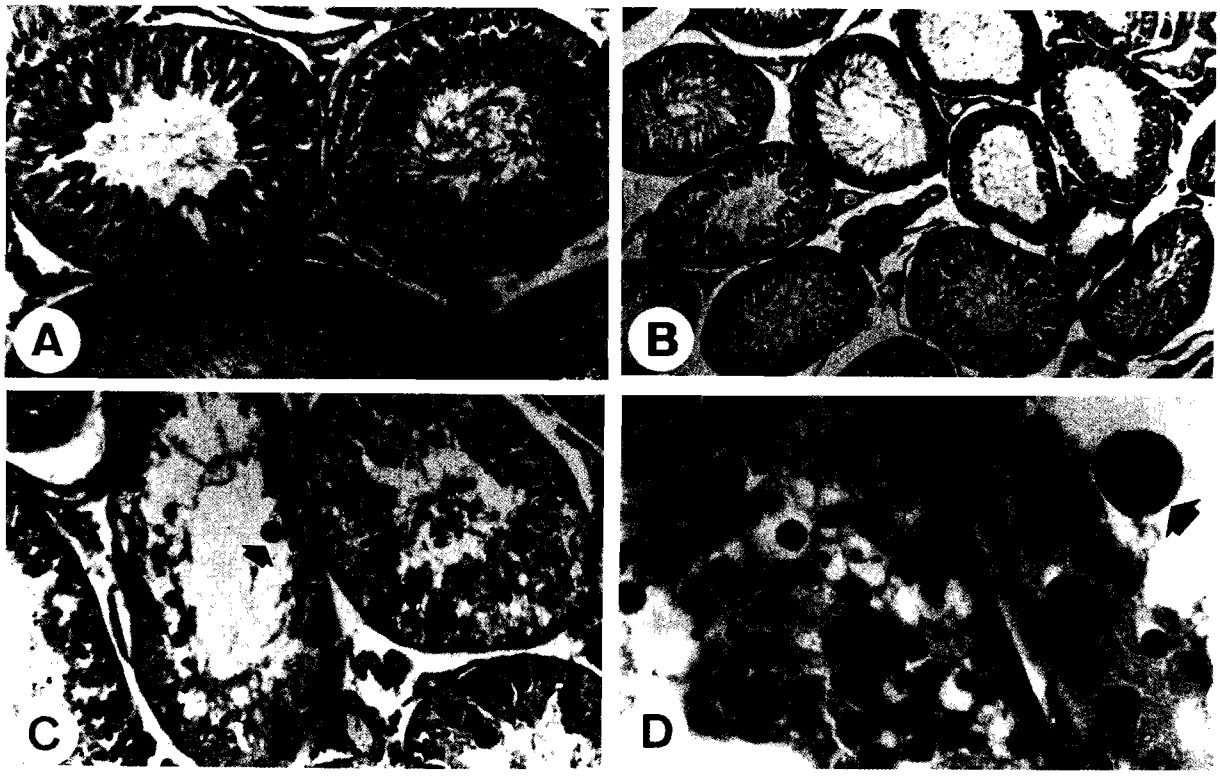

Fig. 1. Histological testicular sections of adult and aged rats (Hematoxylin and eosin). **A.** Seminiferous tubule cross-sections of adult rat showing regular outline and active spermatogenic process culminating with mature spermatids. **B.** Focal degenerative changes in the testis of aged rat exhibiting normal tubular sections side by side with atrophic sections with thin germinal epithelium. **C.** Tubular sections of aged rat showing desquamation of immature germ cells, thinning of the seminiferous epithelium and multinucleated spermatid (arrow). **D.** Depletion of the germinal epithelium and multinucleated spermatid (arrow) in two contiguous tubular sections of aged rat. A, C, x 56; B, x 22.5; D, x 225

increase was observed in the total but not in the relative DNA content in the gonad (Table 1).

Histological examination revealed focal degenerative changes in the testis of aged rats, characterised by atrophic tubular sections lacking spermatids, thinning of the germinal epithelium by precocious loss of immature germ cells and presence of multinucleated spermatids (Fig. 1).

Table 1. Reproductive parameters from adult, aged and aged rats stimulated with low-intensity ultrasound (Aged + US) (Mean±SEM). Different letters mean significant differences between the means ($A \neq B$; $\alpha=0.05$).

PARAMETERS	EXPERIMENTAL GROUP		
	Adult	Aged	Aged + US
Body weight (g)	318.0±4.4^A	560.5±21.8^B	529.2±26.2^B
Testis			
Absolute weight (g)	1.28±0.04^A	1.80±0.03^B	1.77±0.06^B
Relative weight (g/100g)	0.48±0.01^A	0.33±0.01^B	0.35±0.02^B
DNA (mg)	3.98±0.15^A	4.66±0.17^B	4.43±0.21^B
DNA (mg/g)	2.75±0.07^A	2.72±0.10^A	2.54±0.06^A
Sperm production Spermatides/Sertoli)	12.6±0.3^A	10.5±0.4^B	10.3±0.5^B
Sperm concentration (no./mlx10^7)	187,8±12.6^A	162.3±6.6^B	156.5±4.3^B
Plasma testosterone (ng/ml)	1.86±0.45^A	0.47±0.07^B	0.49±0.06^B
Plasma LH (ng/ml)	3.20±0.25^A	1.86±0.15^B	2.13±0.23^B
Plasma FSH (ng/ml)	7.91±0.32^A	6.12±0.50^B	6.42±0.50^B

Ageing resulted in a 17% decrease in sperm production and a 15% decrease in sperm concentration in the cauda epididymidis (Table 1).

A marked decrease (74%) was observed in plasma testosterone of old rats which also exhibited a 42% reduction in plasma LH and 20% reduction in plasma FSH concentrations (Table 1).

Low-intensity ultrasound energy applied to the scrotum of aged rats was inefficient in promoting a recovery of spermatogenic or androgenic testicular function (Table 1). The focal degenerative changes characteristic of senescent gonad persisted in the testis of aged rats submitted to ultrasound stimulation (Fig. 2).

Discussion

Gonadotropin-releasing hormone (GnRH) from the hypothalamus stimulates pituitary gonadotropin secretion, which in turn stimulates spermatozoa and

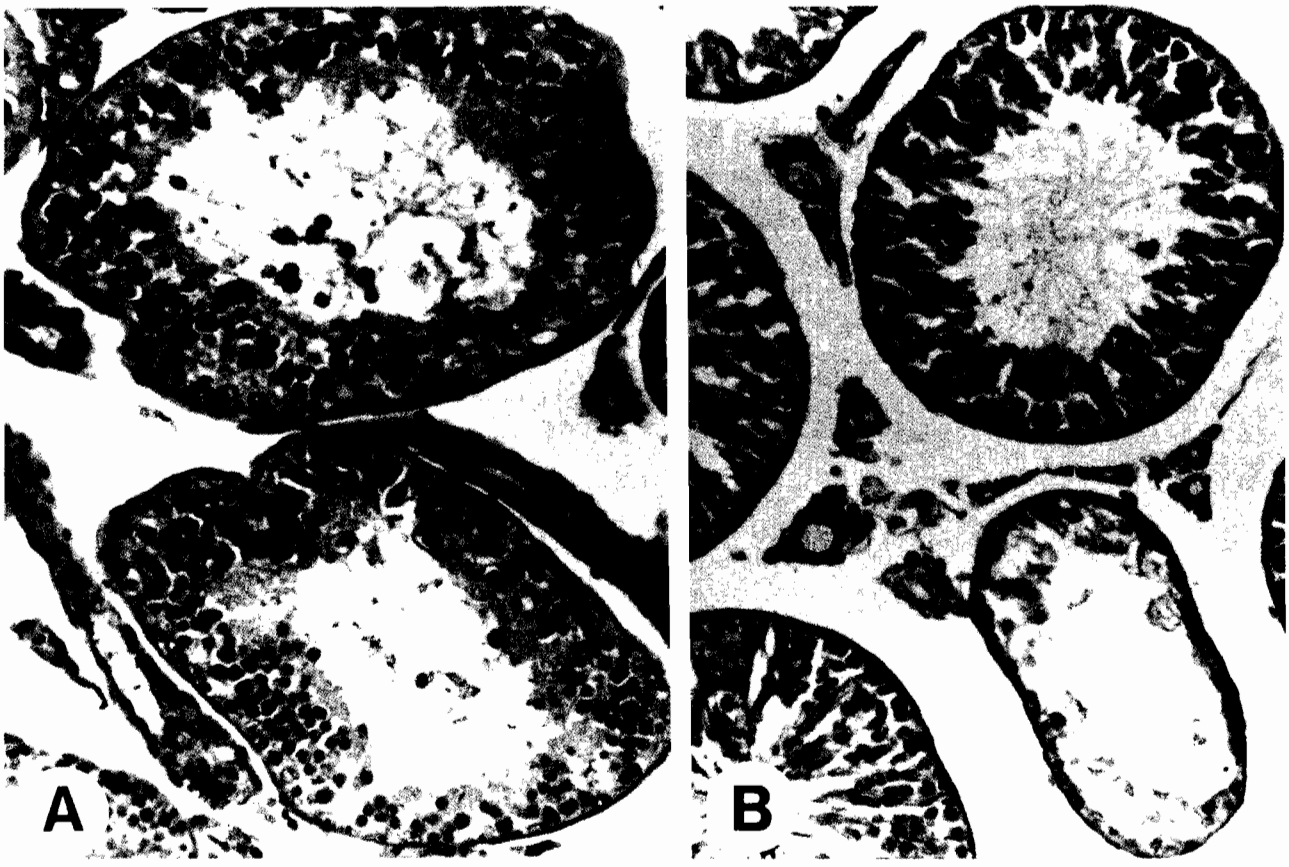

Fig. 2. Histological testicular sections of aged rats stimulated by ultrasound. **A.** Seminiferous tubule cross-sections showing desquamation of immature germ cells and thinning of the seminiferous epithelium. **B.** Focal degenerative change with a normal tubular section side by side with an atrophic section with severe depletion of the germinal epithelium. H-E. x 56

androgen production by the testis. Both LH and FSH are required for the maintenance of the spermatogenic process, the effects of LH being mediated by testosterone from Leydig cells (Steinberger and Steinberger, 1975). In the present study, a reduction was observed in plasma gonadotropins of aged rats in addition to a marked decline in plasma testosterone.

Literature data from the past two decades have shown that the hypothalamic content of GnRH seems to be similar in young and old male rats in spite of a decrease in GnRH release in the latter, probably due to diminished catecholamine content and turnover in the hypothalamus (Miller and Riegle, 1978; Riegle and Miller, 1978). Although pituitary function decreases with age, it is possible to increase plasma LH by GnRH treatment, indicating that the pituitary of aged male rats is capable of sustaining greater gonadotropin secretion. In addition, it has been suggested that there may be no significant decline in the testicular steroidogenic capacity of aged rats, which seems to be capable of sustaining greater testosterone secretion in response to chronic hCG treatment. Thus, the testicular deficiency observed in old animals may result, at least in part, from a prolonged decrease in gonadotropin stimulation (Miller and Riegle, 1978; Riegle and Miller, 1978; Jarjour et al., 1986; Lamano Carvalho et al., 1988).

From middle-age on rats develop an increased sensitivity to the feedback effects of androgen, as they require a much lower level of circulating testosterone to maintain suppression of LH (Pirke et al., 1978; Gray et al., 1980). More recent studies dealing with the mechanisms responsible for this age-related increase in feedback sensitivity showed that there is a reduction in the hypothalamic GnRH synthetic capacity in intact but not in castrated old male rats, suggesting that the hypothalamic secretory decline is dependent upon testicular steroid feedback factors (Gruenewald and Matsumoto, 1991).

Taken together, these data suggest that the primary effect of ageing on the gonadal control mechanisms of male rats concerns a decrease in hypothalamic GnRH synthesis and/or release.

A morphometric study from Kaler and Neaves (1981) added evidence that the diminished androgen status observed in old rats probably cannot be attributed to functional or numerical deficits in the Leydig cells which seem to maintain a normal volume and population, and that low testosterone levels may result from failure of LH levels to rise sufficiently to stimulate additional testosterone secretion.

Conversely, there are literature data suggesting an intrinsic deficiency in the testes of old rats which always show a considerably lower percentage of LH reduction compared to testosterone (Gray, 1978 for references). A recent electron microscopy study by Zirkin et al. (1992) confirmed atrophic Leydig cells in the testis of 24-month-old rats, consistent with the steroidogenic deficit in response to LH challenge.

Application of low-intensity ultrasound to the testis of prepubertal rats proved to have a significant stimulatory effect on androgenic activity, possibly by stimulation of LH receptors and/or the enzymes controlling steroidogenesis (Haddad et al., 1994). In contrast, in the present study the ultrasound energy did not stimulate testosterone synthesis and/or release by the testis of aged rats, attesting to an inherent loss of its steroidogenic competence and corroborating the hypothesis of an intrinsic testicular deficiency.

Histological, histometric and biochemical analysis of the senescent testis in the present study showed focal degeneration of the germinal epithelium, decrease in absolute DNA content and sperm production, in addition to a significant reduction in sperm concentration in the cauda epididymidis, adding evidence to literature data showing impairment of the spermatogenic process in many mammalian species (Bishop, 1970; Gosden et al., 1982; Lamano Carvalho et al., 1988; Zirkin et al., 1992; Horn et al., 1996). The decline in plasma testosterone, LH and FSH levels may account, at least in part, for the marked impairment of seminiferous epithelium of aged rats. The progressive ageing-related loss of germ cells seems to be also associated with specific changes in Sertoli cell gene expression in rats (Wright and Fiore, 1992). Multinucleated spermatids, observed in the testis of old rats, are also seen in other testicular pathologies caused by ischemia, irradiation, ductuli efferent ligation, gonadotoxic agents and vasectomy and are related to arrest in the maturation process causing the spermatids to lose their alignment and fuse their cytoplasm with each other without fusing their nuclei (Anton, 1979; Sarrat et al., 1996).

Ultrasound applied to aged testes did not reverse the degenerative changes observed in the seminiferous epithelium. The therapeutic use of low-intensity ultrasound concerns its capacity to increase cell metabolism and multiplication, increasing the rate of tissue repair (Paul et al., 1960; Dyson et al., 1976; Xavier and Duate, 1983). When applied to healthy male reproductive organs, ultrasound energy was able to increase testosterone secretion and seminal vesicle secretory activity, the germinal epithelium of prepubertal, to adult normal rats, on the other hand, did not respond to ultrasound stimulation (Haddad et al., 1994, 1996). Our results, taken as a whole, lead us to conclude that the germinal epithelium, in contrast to many other mammalian tissues, is refractory to ultrasound stimulation.

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References

Anton E. (1979). Early ultrastructural changes in the rat testis after

ductuli efferentes ligation. Fertil. Steril. 31, 187-194.

Bishop M.H.W. (1970). Ageing and reproduction in the male. J. Reprod. Fertil. 12 (Suppl.), 65-87.

Burton K. (1956). Study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J. 62, 315-232.

Bustos-Obregon E. (1970). On Sertoli cell number and distribution in rat testis. Arch. Biol. 81, 99-108.

Dyson M. (1987). Mechanisms involved in therapeutic ultrasound. Physiotherapy 73, 116-120.

Dyson M., Franks C. and Suckling J. (1976). Stimulation of healing of varicose ulcers by ultrasound. Ultrasonics 14, 232-236.

Ghanadian R., Lewis J.G. and Chisholm G.D. (1975). Serum testosterone and dihydrotestosterone changes with age in rat. Steorids 25, 753-762.

Gosden R.G., Richardson D.W., Brown N. and Davidson D.W. (1982). Structure and gametogenic potential of seminiferous tubules in ageing mice. J. Reprod. Fertil. 64, 127-133.

Gray G.D. (1978). Changes in the level of luteinizing hormone and testosterone in the circulation of ageing male rats. J. Endocirnol. 76, 551-552.

Gray G.D., Smith E.R. and Davidson J.M. (1980). Gonadotropin regulation in middle-aged male rats. Endocrinology 107, 2021-2026.

Gruenewald D.A. and Matsumoto A.M. (1991). Age-related decreased in serum gonadotropin levels and gonadotropin-releasing hormone gene expression in the medial preoptic area of the male rat are dependent upon testicular feedback. Endocrinology 129, 2442-2450.

Haddad S., Petenusci S.O., Anselmo-Franci J.A. and Lamano-Carvalho TL. (1994). Stimulation of prepubertal, pubertal and adult rat testis with low-intensity pulsed ultrasound. Arch. Int. Physiol. Biochem. Biophys. 102, 13-16.

Haddad S., Petenusci S.O. and Lamano-Carvalho T.L. (1996). Direct effect of low-intensity pulsed ultrasound on pubertal rat seminal vesicle. Arch. Physiol. Biochem. 104, 357-359.

Horn R., Pastor L.M., Moreno E., Calvo A., Canteras M. and Pallares J. (1996). Morphological and morphometric study of ealry changes in the ageing golden hamster testis. J. Anat. 188, 109-117.

Huber M.H., Bronson F.H. and Desjardins C. (1980). Sexual activity of the aged male mice: correlation with level of arousal, physical endurance, pathological status and ejaculatory capacity. Biol. Reprod. 23, 305-316.

Jarjour L.T., Handelsman D.J. and Swerdloff R.S. (1986). Effects of aging on the *in vitro* release of gonadotropin-releaisng hormone. Endocrinology 119, 1113-1117.

Kaler L.W. and Neaves W.B. (1981). The androgen status of aging male rats. Endocrinology 108, 712-719.

Kempinas W.G., Lamano Carvalho T.L., Petenusci S.O., Favaretto A.L.V., Lopes R.A. and Azoubel R. (1988). Functional disturbance of rat sexual accessory glands in an early phase of lead intoxication. Gergenb. Morphol. Jahrb. 134, 791-798.

Lamano Carvalho T.L., Favaretto A.L.V., Komesu M.C., Lopes R.A., Petenusci S.O. and Silva-Neto C.R. (1988). Influence of age on the production of rat spermatozoa, on their concentration in the cauda epididymidis and on FSH, LH and testosterone plasma levels. Histol. Histopathol. 3, 413-417.

Miller A.E. and Riegle G.D. (1978). Serum testosterone and testicular response to HCG in young and aged male rats. J. Gerontol. 33, 197-203.

Odell W.D. (1990). Sexual maturation in the rat. In: Control of the onset of puberty. Grumbach M.M., Sizonaenko P.C. and Aubert M.L. (eds). Williams & Wiilkins. London. pp 183-210.

Paul B.J., La Fratta C.W., Dawson A.R., Baab E. and Bullock F. (1960). Use of ultrasound in the treatment of pressure scores in patients with spinal cord injury. Arch. Phys. Med. Rehabilit. 41, 438-440.

Pirke K.M., Vogt H.J. and Geiss M. (1978). *In vitro* and *in vivo* studies of Leydig cell function in old rats. Acta Endocrinol. (Copenh) 89, 393.

Riegle G.D. and Miller A.E. (1978). Aging effects on the hypothalamic-hypophyseal-gonadal control system in the rat. In: The aging reproductive systems. Vol. 4. Schneider E.I. (ed). Raven Pres. New York. pp 159-192.

Sarrat R., Whyte J., Torres A., Lostalé F. and Díaz P. (1996). Experimental vasectomy and testicular structure. Histol. Histopathol. 11, 1-6.

Steinberger E. and Steinberger A. (1975). Spermatogenic function of the testis. In: Handbook of physiology. Vol. 5. Greep R.O. and Astwood E.B. (eds). Baltimore. Williams & Williams. Cap. 1.

Stewart H.D., Stewart H.F., Moore Jr. R.M. and Garry J. (1985). Compilation of reported biological effects data and ultra-sound exposure levels. J. Clin. Ultrasound 13, 167-186.

Tsitouras O. and Bulat T. (1995). The aging male reproductive system. Endocrine aspects of aging. Endocrinol. Metabol. Clin. North. Am. 24, 297-315.

Wright W.W. and Fiore C. (1992). Effects of aging on gene expression by Sertoli cells in the Brown Norway rat. Biol. Reprod. 46 (Suppl. 1), 89.

Xavier C.A.M. and Duarte L.R. (1983). Estimulaçao ultra-sônica do calo ósseo. Rev. Bras. Ortop. 18, 73-80.

Zanato V.F., Martins M.P., Anselmo Franci J.A., Petenusci S.O. and Lamano Carvalho T.L. (1994). Sexual development of male Wistar rats. Braz. J. Med. Biol. Res. 27, 1273-1280.

Zirkin B.R., Santulli R., Strandberg J.D. and Ewing L.L. (1992). Effects of age on male reproductive tract structure and function in the Brown Norway rat. Biol. Reprod. 46 (Suppl. 1), 51.

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