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GnRH agonist induces apoptosis in seminiferous tubules of immature rats: direct gonadal action

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Summary

To investigate direct gonadal effect of buserelin as an agonist of gonadotrophin releasing hormone, the incidence of apoptotic cell death was measured. Thirty 25-day-old immature Wistar male rats were divided into two groups: treated and control rats. Treated rats were given 1.25 mg buserelin acetate/g body weight control rats received vehicle subcutaneously for 5 days. Formalin-fixed paraffin embedded testicles were then investigated for the morphology of seminiferous tubules and occurrence of apoptosis using haematoxylin-eosin staining and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling respectively. Immaturity of rats was proved by the morphological characteristics of testis. In contrast to the control rats, significant increase of apoptotic cell death was found in buserelin-treated rats. Apart from the well-known pituitary-testicular function of buserelin as an agonist of gonadotrophin releasing hormone, our findings suggest that it induces apoptotic cell death via direct gonadal action.

Introduction

The discovery of the structure of hypothalamic gonadotrophin releasing hormone (GnRH) offered the opportunity of designing and synthesising GnRH analogues with agonistic or antagonistic properties, even much more potent than GnRH itself (Cirkel *et al.*, 1989). A paradoxical inhibitory effect of GnRH agonists on the testis has been denoted over 30 years ago, when the expected stimulating effect of buserelin (Superfact®; Sanofi-Aventis Inc, Sanofi-Aventis GmbH, Frankfurt, Germany) as a well-known synthetic analogue of natural GnRH on male genital organs of the treated rats after a few days of treatment was followed by the most unexpected opposite regressive changes in the prostate, the seminal vesicles and the testicles (Auclair *et al.*, 1977). It affects release of follicle stimulating hormone (FSH) and luteinising hormone (LH) 20–170 times stronger than that of luteinising hormone releasing hormone (LHRH) (Sweetman, 2007). Chronic administration of buserelin results in sustained inhibition of gonadotrophin production, suppression of ovarian and testicular steroidogenesis and reduced circulating levels of gonadotrophin and gonadal steroids (Sweetman, 2007).

While the GnRH-related agonists and antagonists acquired considerable attention in the management of infertility in females, the inhibitory effect of GnRH agonist also opened new horizons to the clinical application of GnRH agonists in males, particularly in patients with prostate cancer (Labrie *et al.*, 2005). Due to the molecular castration effect of prolonged chronic treatment with GnRH agonists in men, it was nominated as therapeutic agent in neoadjuvant therapy of prostate cancer. It has been found that the biological activity of LH progressively vanished during long-term treatment of prostate cancer patients with GnRH agonists, thus explaining the castration effect (St-Arnaud *et al.*, 1986).

It was reported earlier that specific binding sites for LHRH are present in breast, ovarian, endometrial, pancreatic and prostatic cancers (Grundker, 2000). Whereas molecular masses of non-pituitary LHRH binding sites are comparable with pituitary receptors, their binding characteristics were of the low-affinity/high-capacity type (Grundker, 2000). Beside the known

hypothalamic-pituitary pathway of the GnRH action, it has been reported that expression of the GnRH and GnRH-R genes is regulated in testicle and ovary during foetal development (Botte *et al.*, 1998) The ontogeny of GnRH and GnRH-R gene expression was investigated in rat gonads from 14.5 to 21.5 days post-coitum and showed that GnRH mRNA could be detected in the ovary at 18.5 days post-coitum, 4 days later than in the testis, and similar levels were found in both sexes at birth. GnRH and GnRH-R mRNA levels increased in both sexes in late foetal development, but expression of the GnRH and GnRH-R genes is regulated in a sex-dependent manner during foetal development. In all cases, expression of GnRH and GnRH-R preceded gonadotrophin receptors in the gonads and initiation of gonadotrophin secretion by the pituitary (Botte *et al.*, 1998). Several studies showed that apoptosis plays a central role in gonads before and after puberty (Tapanainen *et al.*, 1993a; Billig *et al.*, 1995), but it seems that a different mechanism and various factors drive the apoptosis in immature and adult rats. It has been reported that increased DNA fragmentation was found in spermatocytes in selected tubules of rats at 16–24 days of age, but neither Leydig cells nor Sertoli cells were affected. In 32-day-old and adult animals, increased DNA fragmentation was seen in early primary spermatocytes of some tubules (Billig *et al.*, 1995). This study sought to evaluate direct effect of a well-known GnRH agonist, buserelin, on the level of cell death in the spermatocytes of immature rats.

Materials and methods

Animals

All procedures on rats were performed according to the 'Principles of Laboratory Animal Care' (NIH publication no. 85–23, revised 1985), as well as the specific rules of the 'Animal Care and Use Committee', National Medical and Health Service. Twenty 25-day-old immature male rats were included in this study. No significant weight difference was found among them ($P > 0.5$). All rats were allowed to feed *ad libitum* and were kept in a light (12 h light/12 h dark) and temperature (22–24 °C) controlled room.

Chemicals

Buserelin acetate (Injection 1 mg ml^{-1}, Suprefact®) was purchased from the local distributor. All chemicals were procured from Merck (Darmstadt, Germany) or Sigma-Alderich (Sigma-Alderich GmbH, Munich, Germany). *In Situ* Cell Death Detection Kit™ was purchased from Roche (Roche Diagnostics [Schweiz] AG, Rotkreuz, Switzerland).

Treatments

Twenty-five-day-old Wistar rats were randomly divided into two groups, i.e. buserelin-treated ($n = 20$) and control rats ($n = 10$) and subcutaneously received 1.25 μg buserelin acetate/g body weight or vehicle for 5 days respectively.

Terminal deoxynucleotide tranferase-mediated dUTP nick end labelling (TUNEL) detection of apoptosis

The rate of apoptosis in testicular tissues was evaluated in 5 μm thick formalin fixed paraffin embedded tissues testes using the TUNEL assay. Sections were deparaffinised by immersing in xylene, rehydrated and washed in PBS. Subsequently, the sections were permeabilised using proteinase K (30 μg ml^{-1}, 30 min, 37 °C) and washed in PBS. The TUNEL reaction was performed using *In Situ* Cell Death Detection Kits (Roche) which use an optimised terminal transferase (TdT) to label free 3′OH ends in genomic DNA with fluorescein-dUTP, followed by detection of incorporated fluorescein by an anti-fluorescein antibody POD conjugate and visualisation of the antibody by the immunocomplexed POD with diaminobenzine (DAB) as a substrate reaction. Briefly, slides were incubated with TUNEL reaction mixture for 60 min (humid 37 °C), and then washed in PBS twice. Sections were covered by anti-fluorescein-POD and incubated for 30 min at 37 °C. After multiple washing steps, visualisation was performed by adding DAB for 10 min at room temperature, washing in PBS and mounting for light microscopic observations. As positive control, DNase I was used to induce DNA strand breaks. The numbers of TUNEL-positive cells were determined by counting them in the same number of seminiferous tubules. All morphometric measurements were carried out by at least two independent expert examiners in a blinded manner and results of treated rats were expressed in comparison with control rats.

Comparative testis morphometry

Weight, volume and the longest diameter of testes were measured in both treated and control rats by means of analytical balance and digital calliper.

Statistical analysis

Tabularisation of the results and statistical analyses were performed using GraphPad Prism version 3.00 for Windows, GraphPad Software, San Diego, CA, USA. The data are expressed as the mean ± SD and $P < 0.05$ is accepted as statistically significant. Deviation from Gaussian distribution was checked using the Kolmogorov–Smirnov test and normal distribution was accepted.

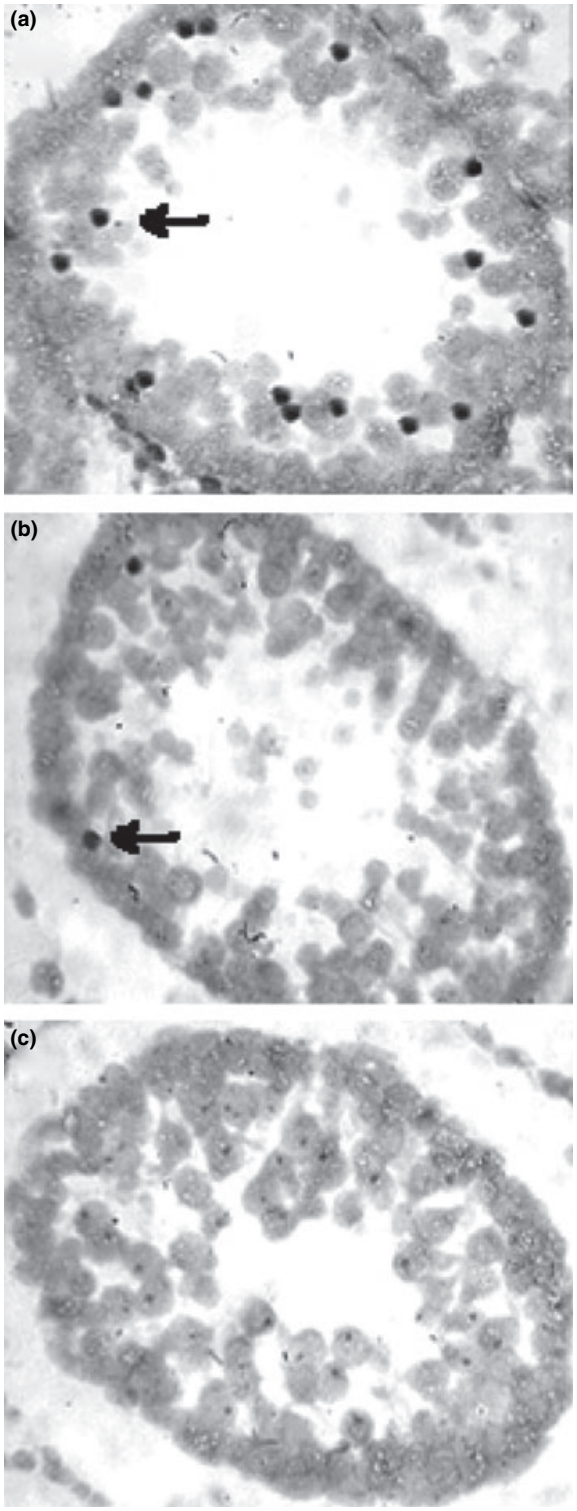

Fig. 1 Evaluation of apoptotic cells in seminiferous tubules using TUNEL staining. In contrast to control rats (b), dense dark TUNEL-positive cells were prevalently found in buserelin-treated rats (a). Negative control was included to rule out possible false positive staining (c). Apoptotic cells are indicated by arrows.

Results

Immaturity of rats was proved by the morphological characteristics of testis. Apoptotic cell death was evaluated using *In Situ* Cell Death Detection Kit™ and TUNEL-positive cells (Fig. 1) in testicular tissues of buserelin-treated rats were compared with control rats.

The seminiferous tubules were more vulnerable than Leydig or Sertoli cells and the percentage of apoptotic cells in control and treated rats was 2.7 ± 2.16 and 20.40 ± 3.24 respectively. Therefore, treated rats had significantly higher apoptotic cells than control rats ($P < 0.001$) (Fig. 2).

The mean of the longest diameter of the testes in the control rats and treated rats was 2.89 ± 0.8 and 2.38 ± 0.57 mm respectively. It confirms that the longest diameter in treated rats was significantly lower than that in control rats ($P = 0.025$) (Fig. 3a).

The mean of testicular volume and weight for treated rats and control rats was 16 ± 3 mm^3, 14.4 ± 2.7 mg and 8.20 ± 1.30 mm^3, 7.38 ± 1.15 mg, respectively, showing similar significant differences ($P = 0.028$ and $P = 0.029$ respectively) (Fig. 3b, c). All vehicle-treated rats were also evaluated for the mentioned parameters and no kind of significant changes was found.

Discussion

All research groups working in this field, including ours, were attempting to better understand the possible clinical advantages or disadvantages of GnRH agonists or antagonists, which would permit the use of GnRH agonists to stimulate fertility in experimental animals and man. As immature male rats were included in this study, the

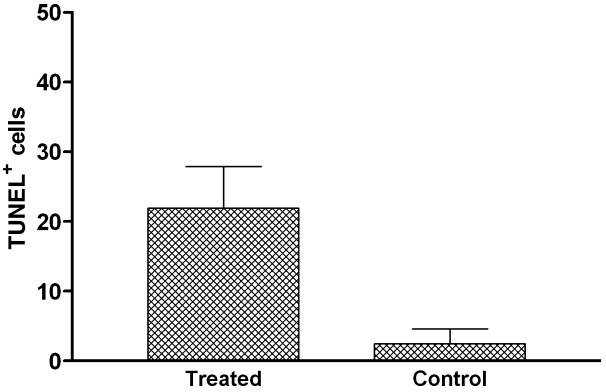

Fig. 2 Frequency of TUNEL-positive apoptotic cells in testicular tissues of buserelin-treated and control rats. Significantly higher numbers of apoptotic cells were found in buserelin-treated rats than in control rats. Data are expressed as mean ± SD and $P < 0.05$ is accepted as statistically significant.

results reveal that buserelin as GnRH agonist is able to elicit direct effect on testicular tissues in absence of physiological hypothalamus–pituitary–testis axis. Direct effects of GnRH or its agonists on the ovary were reported previously (Billig *et al.*, 1994; Sridaran *et al.*, 1998; Kogo *et al.*, 1999) the responses to changes in the hormonal environment differed greatly between infant and adult testes (Huhtaniemi *et al.*, 1985). Apoptosis plays a key role in the development of testicular germ cells which show different gonadotrophin dependence (Billig *et al.*, 1995, 1996). Apoptosis and its augmentation by androgen withdrawal is an important event in the testis of adults (Woolveridge *et al.*, 1998) and treatment of immature rats with FSH and LH/hCG in addition to stimulation of testicular cell differentiation and growth essentially prevent testicular cell death in both seminiferous tubules and interstitial cells (Tapanainen *et al.*, 1993b). The ontogeny of GnRH and GnRH-R gene expression was previously studied in rat gonads at different post-coitum ages and revealed that expression of GnRH and GnRH-R preceded gonadotrophin receptors in the gonads and initiation of gonadotrophin secretion by the pituitary (Botte *et al.*, 1998). Approved therapeutic effect of chronic administration of GnRH agonists in the treatment of prostate cancer (Labrie *et al.*, 2005) shows that the hormonal milieu of testicular tissue controls the rate of cell proliferation or death, which provides multiple sites for therapeutic manipulations. This study showed that the reduced size and weight of testes in treated rats are in keeping with a significant increase of apoptotic cells. This supports that GnRH and its agonists directly induce cell death via apoptosis and could serve as a mechanism of anti-tumour activity of these compounds apart from their suppressing effects at hypothalamus-pituitary axis. The presence of GnRH receptors in nontesticular tissues like the prostate and its connection with pathological processes and apoptosis were recently reported (Kraus *et al.*, 2006; Marelli *et al.*, 2006; Kim *et al.*, 2007; Rothman & Wierman, 2007). Further investigations are needed to find out the signalling pathway by which GnRH receptor-related apoptosis in testicular cells proceeds.

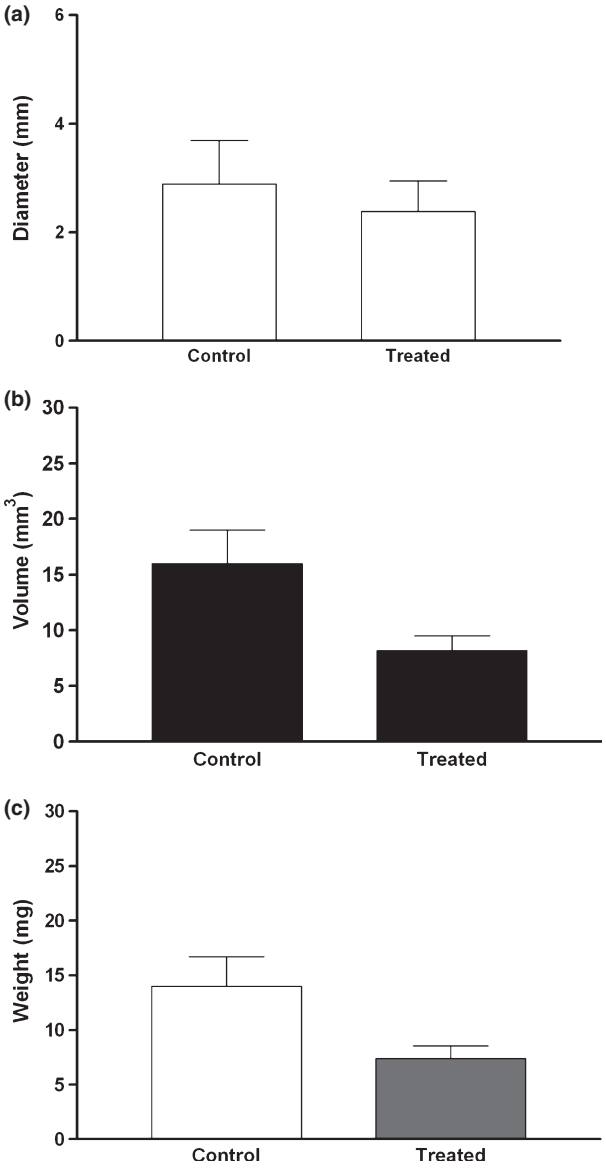

Fig. 3 Morphometry of testes in treated and control rats. Significant reduction of the longest diameter (a), weight (b) and volume (c) was found in testes of treated rats. Data are expressed as mean ± SE and $P < 0.05$ is accepted as statistically significant.

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