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Original Article

Protecting spermatogonia from apoptosis induced by doxorubicin using the luteinizing hormone-releasing hormone analog leuprorelin

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Abstract ***Purpose***: The present study was performed to investigate the protective effect of leuprorelin (LH-RH analog), on spermatogonia apoptosis induced by doxorubicin (DXR) in the Sprague–Dawley rat model.
Methods: Twenty-four adult male rats were divided into the following four groups: (i) control group; (ii) group given doxorubicin (intravenous injection, 8 mg/kg); (iii) group given leuprorelin (subcutaneous injection, 3 mg/kg); and (iv) group given both doxorubicin (intravenous injection, 8 mg/kg) and leuprorelin (subcutaneous injection, 3 mg/kg). Evaluation for quantification of apoptotic spermatogonia was made by the ratio of TUNEL-labeled spermatogonia *versus* 100 Sertoli cells in each seminiferous tubule. Two hundred seminiferous tubules of each rat were assessed.
Results: The ratio of apoptotic spermatogonia *versus* 100 Sertoli cells at stages II–IV of the groups given DXR (groups 2 and 4) were significantly higher than those of the other groups. However, the value at stages II–IV of the group given both DXR and leuprorelin (group 4) was significantly lower than that of the group given DXR (group 2).
Conclusion: The significant prophylactic effect ($P < 0.05$) of LH-RH analog against doxorubicin-induced spermatogonial apoptosis was observed in a stage specific manner by microscopic evaluation with TUNEL.

Key words apoptosis, doxorubicin, leuprorelin, spermatogonia, TUNEL.

Introduction

Chemotherapeutic agents have greatly contributed to improving the survival rate of patients with malignancies. Because some cancers, such as testicular cancer and Hodgkin's disease are likely to occur in young patients before and during their reproductive years, sterility caused by treatments with anticancer drugs is a very significant concern.

The suppression of spermatogenesis induced by a low level of serum testosterone before treatment with cytotoxic agent has been thought to be crucial as a protective measure. In order to protect spermatogenesis from toxicant exposure, many clinical and experimental trials of sex-steroid and gnadotrophic hormones have been attempted. Some experiments using the rat model have demonstrated that hormonal treatments suppressed the serum level of luteinizing hormone and intratesticular testosterone production, and protected spermatogenesis or enhanced the recovery of spermatogenesis.[1–3]

We previously reported that in the Sprague–Dawley rat model, leuprorelin (LH-RH analog) administration before doxorubicin (DXR) treatment could protect the testes of rats from DXR-caused histological damage.[4] DXR was chosen because the toxicity of DXR (the most widely used treatment in the clinical field and effective anthracycline anticancer agent) has been heavily investigated and well documented.[5] Leuprorelin induces

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down-regulation of LH-RH receptors, desensitization of pituitary gonadotrophin, and suppresses serum testosterone and spermatogenesis.[6] Since the inhibition of spermatogenesis in humans and rats is reversible after treatment with leuprorelin,[6] we believed that this drug was eligible in the attempt to protect spermatogenesis from damage by cytotoxic agents.

Recently, however, the conflicting reports of this hypothesis are increasing. One of those reports is by Crawford *et al.* in which an hpg mouse was used.[7] The hpg mouse has complete gonadotrophin deficiency but can undergo the induction of full spermatogenesis by testosterone treatment. Thus, if complete gonadotrophin deficiency was an advantage during cytotoxin exposure, then that mouse should exhibit some degree of germinal protection against cytotoxin-induced damage. In the results, however, they could not detect any evidence of cytoprotection.

Another report advocated that hormonal treatments did not protect any spermatogenesis from anticancer drugs but stimulated the recovery from maturation arrest caused by toxic agents.[3] In accordance with these reports, prolonged azoospermia occurs even though the stem spermatogonia survive the toxic insult[8] because the differentiation of these spermatogonia to produce sperm fails.[3,9] They speculate that the stimulating mechanisms are the hormonal treatments with testosterone or LH-RH analogs, which suppress intratesticular testosterone levels, and relieve the block of maturation arrest.[8]

On the other hand, our preliminary experimental data indicate that 3 mg/kg administration of leuprorelin, which inhibits testosterone to the castration level for more than 4 weeks,[6] reduced cytotoxicity of DXR on germ cells, especially stem spermatogonia, in histological evaluation with stem cell survival rate (data not shown). Stem cell survival rate, which was first advocated by Withers *et al.* roughly represents stem spermatogonia survival after cytotoxic treatment.[10] Based on this result, we speculate leuprorelin pretreatment might prevent spermatogonia damage from the toxic effect of DXR and preserve spermatogenesis.

To date, however, it has not been clearly investigated whether hormonal treatment with LH-RH analog could affect stem spermatogonia survival from the toxic assault of anticancer agents. If LH-RH analog does have this effect, it could be considered that LH-RH analog saves spermatogenesis by protecting stem spermatogonia from anticancer agents.

A recent experimental report of the Sprague–Dawley rat model showed that the DXR-induced damage was the apoptosis of spermatogenic cells and the phenomenon was stage-specific and observed mainly in spermatogonia (both undifferentiated and differentiated types).[11] DXR exerts multiple effects on DNA, with DNA cross-linking thought to be mainly responsible for its cytotoxic activity, causing G2 arrest of the cell cycle.[12,13] It is considered that the DNA repair might occur in arrested cells, but when damage is beyond a certain threshold, apoptosis is initiated.[11] We tried to evaluate if the DXR-induced damage of the stem cells (spermatogonia) was reduced by LH-RH analog and investigated if it was possible that hormonal treatment could protect the stem cells and save spermatogenesis.

Methods

Animals and treatments

Twenty-four adult male Sprague–Dawley rats at 10 weeks of age were obtained from Clea Japan (Tokyo, Japan). The rats were housed in polycarbonate cages with a 12:12 h light/dark cycle, and given MF diet and water *ad libitum*. The rats were divided into four groups (6 rats per group) and treated as mentioned below. This experiment was conducted at the Laboratory Animal Research Center of the University of Tsukuba.

This study and the handling, maintenance, anesthetizing and euthanizing of the rats was reviewed and approved by our institutional review board of animal experiments.

Group 1 (control)

The rats received 0.3 mL of normal saline at week 0 subcutaneously, and 0.5 mL of normal saline at week 4 intravenously.

Group 2 (doxorubicin)

The rats received 0.3 mL of normal saline at week 0 subcutaneously, and 8 mg/kg DXR at a concentration of 0.2% (w/v) at week 4 intravenously.

Group 3 (leuprorelin)

The rats received 3 mg/kg leuprorelin at week 0 subcutaneously, and 0.5 mL of normal saline at week 4 intravenously.

Group 4 (leuprorelin and doxorubicin)

The animals received of 3 mg/kg leuprorelin at week 0 subcutaneously, and 8 mg/kg DXR at a concentration of 0.2% (w/v) at week 4 intravenously.

All animals were euthanized with over-anesthesia by pentobarbital at 24 h after DXR or normal saline administrated intravenously. The incubation time and dose of DXR in this experiment were determined according to the previous report that found TUNEL-labeled spermatogonia was the highest in number at 24 h after the 8 mg/kg of DXR administration.[11]

Immediately after sacrifice, both testes were removed from each rat and blood was collected by puncturing the heart. The testes were weighed, immersed in FSA solution (37% formalin solution, 5% sucrose solution, acetic acid = 5:15:0.8, by volume) for histological and TUNEL evaluations.

Hormone measurement

Serum testosterone concentration was measured using the DPC-Total Testosterone Kit (Diagnostic Product Corporation, Los Angeles, CA, USA).

Histological evaluation

Sertoli cell number

For microscopic examination, testes fixed in FSA solution for 4 days were embedded in paraffin followed by sectioning and staining with PAS and H&E. To clarify the effect of leuprorelin and/or DXR on Sertoli cells, we counted the number of Sertoli cells in each seminiferous tubule and compared these values among all groups. We chose 20 seminiferous tubules with round cross-sections from each rat and assessed them.

TUNEL

Histological detection of DNA fragmentation in testes was performed by the TUNEL method using TACS *in situ* Apoptosis Detection Kit (Trevigen, Gaithersburg, MD, USA). In brief, 5 μm thick sections from the testes were fixed in FSA solution, embedded in paraffin and mounted on glass slides. The sections were deparaffinized by clearing with xylene, and hydrated through a graded series of ethanols to deionized water. They were then treated with proteinase K (20 μg/mL) for 10 min for the digestion of nuclear proteins, and hydrogen peroxide (2%) for 5 min for the inactivation of endogenous peroxidase. Subsequently, the sections were incubated in a solution of terminal deoxynucleotidyl transferase (TdT) and digoxigenin-dUTP in a humidified chamber at 37°C for 1 h and then treated with antidegoxigenin-peroxidase for 30 min at room temperature. Digoxigenin-dUTP end-labeled DNA was visualized by peroxidase detection with diaminobenzidine (0.05%) and hydrogen peroxidase (0.02%) for 5 min. The sections were then counterstained with hematoxylin. For the quantification of TUNEL-labeled germ cells, the seminiferous tubules were divided into seven groups (stage I, II–IV, V–VI, VII–VIII, IX–XI, XII–XIII and XIV) based on the cell types of spermatogonia and spermatocytes according to the description by Hess.[14] The TUNEL-labeled germ cells were identified as spermatogonia, spermatocytes, and spermatid by their morphological features and location within the seminiferous epithelium.

The quantification of TUNEL-labeled spermatogonia was assessed on 200 cross-sectioned seminiferous tubules from each rat of all the groups ($n = 6$) and expressed as numbers of TUNEL-labeled spermatogonia per 100 Sertoli cells in each group of stages.

Statistical analysis

All values are expressed mean ± standard deviation.

Statistical analyzes were performed with one-way analysis of variance and the Mann–Whitney *U*-test. The level of significance was taken as $P < 0.05$, compared with the respective control.

Results

No significant difference was observed in the body weight of sacrificed animals among all groups ($P = 0.245$) (Table 1). Testicular weights of groups treated with leuprorelin (groups 3 and 4) were significantly lower than those of untreated groups (group 1 and 4) (Table 1). However, exposure to DXR had no significant impact on testicular weight in these experimental conditions. Similar tendencies were seen in serum testosterone concentration, i.e. leuprorelin reduced the values of testosterone significantly compared to those of non-treatment groups, however, DXR did not alter it significantly (Table 1). These results show that 3 mg/kg of leuprorelin administration could inhibit testicular functions such as testosterone secretion or testicular weight as the previous report indicated.[6]

Next, we evaluated how DXR treatment alters the number of Sertoli cells in a seminiferous tubule, because there was no previous data available on it. Since there is no statistical difference (group 1, 19.5 ± 2.54; group 2, 19.6 ± 3.64; group 3, 19.0 ± 3.60; group 4, 18.7 ± 4.19), it was concluded that the dose of leuprorelin used in this study did not change the number of Sertoli cells. Based on these results, the ratio between Sertoli cell and spermatogonia could be applied to evaluate the quantitative damage of spermatogonia in this animal model pretreated with leuprorelin.

Table 1 Body weight, testicular weight and serum testosterone level of each group

	Group 1 (Control)	Group 2 (DXR)	Group 3 (leuprorelin)	Group 4 (DXR + leuprorelin)
Body weight (g)	494.8 ± 29.3	495.2 ± 33.9	459.0 ± 28.7	487.7 ± 40.0
lt. testicular weight (g)	1.66 ± 0.17	1.69 ± 0.05	0.99 ± 0.23*	1.14 ± 0.22*
Serum testosterone (IU/L)	1.45 ± 1.26	0.82 ± 0.75	0.18 ± 0.44*	0.35 ± 0.72*

*$P < 0.05$ (compared to control). DXR, doxorubicin; lt, left.

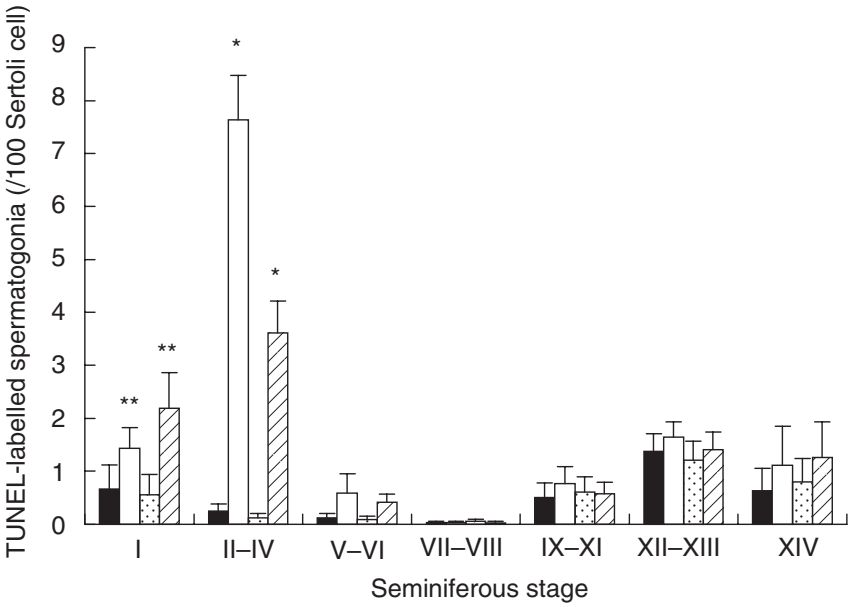

Fig. 1 Stage-specific TUNEL-labeled spermatogonia/100 Sertoli cells in each group. Data indicate means ± SD ($n = 6$). Stage I: groups 1–2; 1–4; 2–3; 2–4;** 3–4 ($P < 0.05$). Stage II–IV: groups 1–2; 1–4; 2–3; 2–4;* 3–4 ($P < 0.01$). Stage V–VI: groups 1–2; 1–4; 2–3; 3–4 ($P < 0.05$). Stages VII–VIII, IX–XI, XII–XIII, XIV showed no significant difference. (■) Control (group 1); (□) doxorubicin (group 2); (⊡) leuprorelin (group 3); (▨) doxorubicin and leuprorelin (group 4).

Quantitative data for TUNEL-labeled germ cells are shown in Fig. 1. In the DXR treatment groups (groups 2 and 4), significant increases of the TUNEL-labeled degenerated spermatogonia–Sertoli cell ratios were observed compared to non-DXR treatment groups (groups 1 and 3) at stage II–IV. However, the ratio of group 4 (both leuprorelin and DXR treatment group) was significantly lower than that of group 2 (DXR monotreatment group) ($P < 0.01$). At stage V–VI, also, the ratio of DXR treatment groups (groups 2 and 4) was significantly higher ($P < 0.05$) than those of non-DXR treatment groups (groups 1 and 3). No significant effect of leuprorelin treatment on the ratio was seen at this stage. Additionally, at stage I of DXR treated groups (groups 2 and 4) significantly higher ratios were also observed compared to others. At stage II–IV, the ratio of group 4 was higher than that of group 2 ($P < 0.05$). However, since the number of degenerated spermatogonia at stage I were low, finding the difference between groups 2 and 4 might be difficult. From these results, especially the result of stage II–IV, leuprorelin treatment was thought to inhibit spermatogonia from degeneration, probably apoptosis, in the whole testis.

No significant difference was found at the other stages: VII–VIII ($P = 0.904$); IX–XI ($P = 0.955$); XII–XIII ($P = 0.210$); and XIV ($P = 0.825$).

Figure 2 shows light micrographs of seminiferous tubules at stage II–IV from DXR treated rats (group 2). Figure 2a shows TUNEL-labeled spermatogonia (arrows). Figure 2b indicates histological features of seminiferous tubules at stage II–IV from both DXR and leuprorelin treated rats (group 4). Figure 2b also shows certain number of TUNEL-labeled degenerating spermatogonia (arrows), but these degenerating cells are less frequently seen than in group 2.

Discussion

Spermatogenetic cells are likely to be targeted by cytotoxic agents such as cancer-chemotherapeutic agents because of their high dividing activity. Damaged

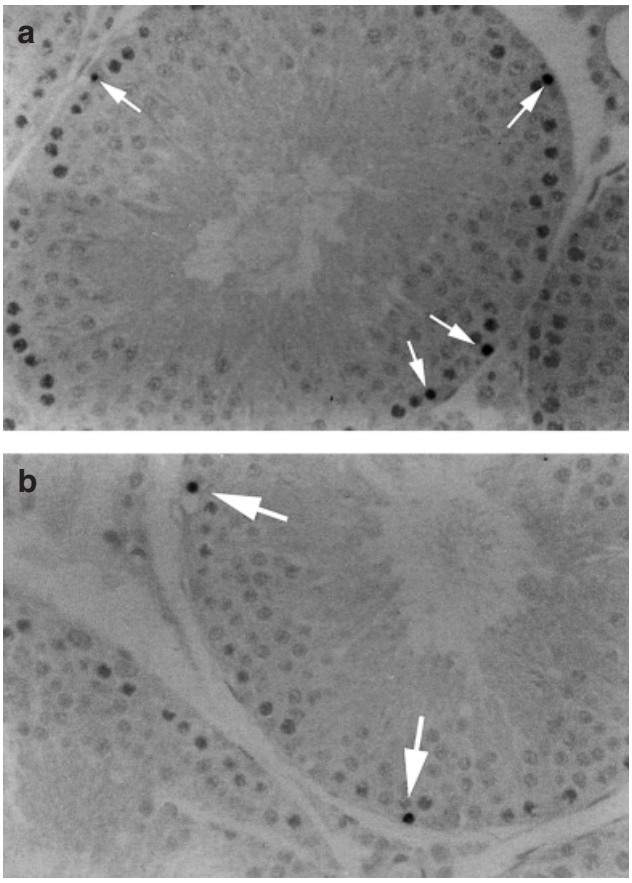

Fig. 2 Light micrographs of seminiferous tubules from doxorubicin (DXR) administered rat group (group 2) and both DXR and leuprorelin administered group (group 4). (a) Stage II–IV tubules from a DXR treated rat. TUNEL-labeled spermatogonia are shown by TUNEL staining (arrows). (b) Stage II–IV tubules from a DXR and leuprorelin treated rat. TUNEL-labeled spermatogonia are shown by TUNEL staining (arrows).

spermatogonia, which are thought to be stem cells for sperm, results in prolonged sterility or oligozoospermia.

Many investigations of DXR-induced testicular toxicity in rodent models have been reported in the past.[15–17] Some experiments have indicated that upon microscopic evaluation, type A1-4, $A_{isolated}$, A_{paird} and $A_{aligned}$ spermatogonia were the most vulnerable cells to DXR.[16,18,19] Recent studies have shown that the acute toxic effect of DXR on spermatogonia was apoptosis in the testis revealed by electron microscope and TUNEL.[11,20,21]

Our result indicated that ratio of TUNEL-labeled degenerated spermatogonia and Sertoli cells increased significantly at stage II–IV in DXR treated groups (groups 2 and 4), which is in agreement with the report of Shinoda *et al*.[11] In these two group (groups 2 and 4), interestingly, the ratio of leuprorelin treated group (group 4) was lower than that of DXR monotreated group. Taken together, we thought that 3 mg/kg of leuprorelin could protect spermatogonia from DXR induced apoptosis, even though the effect was not complete.

However, the precise mechanism for this effect is not understood. There are speculations on the possibility of the 'indirect' and 'direct' effects of leuprorelin on spermatogenesis. The 'indirect', traditional theory is that hormonal treatment suppresses intratesticular testosterone via LH-RH receptor on pituitary cell and also suppresses the germ cell cycle. However, this theory is now very controversial as mentioned before.

The 'direct' theory is that leuprorelin could affect the paracrinological circumstances which are thought to be crucial for spermatogenesis, through LH-RH receptors that are expressed on both Sertoli cells,[22] and Leydig cells.[23]

Recently, stem cell factor (c-kit ligand or SCF), cytokine secreted by Sertoli cells, is of great interest in the research field. Blanchard *et al*. reported that administration of 2,5-hexanedione, which has a specific toxicity to Sertoli cells, induced testicular atrophy in rats, and the following infertility was associated with a decrease in the expression of membrane-bound SCF or SCFm.[24] Interestingly, this atrophy was partly corrected by leuprorelin therapy.[24] The authors insisted that leuprorelin increased the expression of SCFm, which was believed to have a strong positive effect on spermatogenesis.[24–27] Many reports revealed SCFm could exert stronger effects on differentiation, cell–cell contact and antiapoptic effect than those of soluble SCF (SCFs).[25,28–30] The results from this study might support the speculation that SCFm induced by leuprorelin could affect the survival of spermatogonia against DXR treatment.

In conclusion, the present report concerned the possibility that pretreatment with leuprorelin could protect spermatogonial from the DXR-induced apoptosis in rats. Since we observed only the acute phase DXR dosage, further detailed study is required to determine if this effect can is long-term and can contribute to recovering spermatogenesis.

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