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Original Article

GnRH analog, leuprorelin acetate, promotes regeneration of rat spermatogenesis after severe chemical damage

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Abstract

Background: Future fertility is a major concern for cancer patients who undergo intensive chemotherapy. There has been controversy about whether hormonal treatments may have protective effects against the severe spermatogenic damage caused by chemotherapy or irradiation. Recently, it has been proposed that gonadotrophin-releasing hormone (GnRH) analogs administered after testicular damage stimulate the recovery of spermatogenesis. In this study, we have investigated the effects of GnRH agonist, leuprorelin, on the damage to spermatogenesis induced by busulfan.

Methods: Fisher rats were treated with busulfan, 25 mg/kg, intraperitoneally. The effects of subcutaneous injections of leuprorelin before or after treatment were evaluated histologically 18 weeks later.

Results: The percentage of 'recovered' seminiferous tubules was 27.7 ± 12.6% in control rats without leuprorelin and 26.9 ± 10.2% in rats with leuprorelin injected 4 weeks before busulfan. Rats in both groups showed poor recovery of spermatogenesis with an increase of intratesticular fluid. However, rats treated with leuprorelin three times (4 weeks apart) after busulfan showed an improvement of up to 56.5 ± 12.0% ($P < 0.05$). A focal but massive necrotic lesion in the testis was observed only in this group of rats.

Conclusions: The results demonstrated that leuprorelin administered after chemical testicular damage enhanced the recovery of spermatogenesis. At the same time, a possible significant side-effect of leuprorelin was noted.

Key words busulfan, chemotherapy, gonadotrophin-releasing hormone analog, leuprorelin, spermatogenesis, testicular damage.

Introduction

Fertility has been an issue of concern for cancer patients who undergo intensive chemotherapy or regional radiation therapy. Successful chemotherapeutic regimens have significantly increased the survival rate of patients suffering from conditions like testicular cancer, Hodgkin's and non-Hodgkin's lymphomas, or leukemia. In some cases, a complete cure can be achieved. For some of these patients, recovery of fertility after gonadal damage subsequent to the treatments has become an important subject of consideration. Semen cryopreservation has been the most reliable method for this purpose and has been done when requested by patients. However, the quality of cryopreserved sperm after thawing does not always allow fertilization with artificial insemination. In many cases, modern assisted reproductive techniques, such as, intracytoplasmic sperm injection are required to achieve fertilization and childbirth.

In 1981, Glode *et al*. hypothesized that inhibition of spermatogenic cell division during cancer therapy would protect germ cells and hence preserve the fertility of the patient. They used gonadotrophin-releasing hormone (GnRH) agonist to interrupt the pituitary-gonadal axis

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that was thought to reduce the rate of spermatogenesis. It was shown that treatment of mice with GnRH agonist before and during cyclophosphamide administration attenuated damage to spermatogenesis.[1] This initial report of a possible protective effect of GnRH analog raised wide interest and promoted subsequent studies. Animal studies mostly in rats and mice were carried out in an attempt to confirm the protective effect of GnRH analog administered before cytotoxic insults on spermatogenesis. Several researchers successfully demonstrated the protection of spermatogenesis from procarbazine or cyclophosphamide in the rat model.[2–4] However, other researchers found no substantial protective effects in the mouse or in the rat to the same or other drugs.[5–7] Clinical trials have also failed to demonstrate any significant protective effect on spermatogenesis in patients treated with different kinds of anticancer drugs.[8–11]

These results from experimental studies and clinical trials did not provide definite evidence that GnRH agonist can protect spermatogenesis from toxic insults, such as, irradiation or anticancer chemotherapy.

In 1997, 16 years after Glode's report, Meistrich *et al*. demonstrated that treatment with GnRH agonist, after irradiation damage, stimulated the recovery of rat spermatogenesis from surviving spermatogonia.[12] It was an unexpected finding that post instead of pretreatment with GnRH analog reduced toxic damage of irradiation to spermatogenesis. This effect of GnRH analog was thought to be stimulating regeneration of spermatogenesis rather than protecting spermatogenesis from radiation damage. A similar effect of GnRH analog on spermatogenesis has been confirmed in several other studies.[13–16] We designed the present experiment to study whether GnRH agonist treatment also stimulates the recovery of spermatogenesis after exposure to busulfan, an anticancer drug that is selectively toxic to spermatogonia in the testis.[17]

Methods

Animals and drugs

Male Fischer rats (F344), 7-weeks-old (Clea, Tokyo, Japan), were maintained at 24 ± 1°C, 55 ± 1% humidity with a 14 h light/10 h dark cycle. Food and water were provided *ad libitum*.

Busulfan (Sigma, Tokyo, Japan) was first dissolved with dimethyl sulfoxide (DMSO; 8 mg/mL). The same volume of distilled water (final 4 mg/mL) was added just before use. This busulfan solution was given intraperitoneally to all rats at a dose of 25 mg/kg.

Leuprorelin acetate (Leuplin Depot) was provided from Takeda Pharmaceutical (Tokyo, Japan). Leuprorelin injections (0.625 mg/0.300 mL) were prepared by suspending a vial of 3.75 mg leuprorelin acetate into 1.80 mL of its solvent. The leuprorelin was injected subcutaneously in the back.

Bone marrow transplantation

As busulfan of 25 mg/kg dosage is often lethal in rats due to its myelotoxicity, every rat was treated with bone marrow cell transplantation. The bone marrow cells were harvested by flushing the femur, tibia and humerus of non-treated 11-week-old F344 rats with Hank's balanced salt solution. The collected cell suspension was then filtered through a 70 μm pore size membrane to eliminate tissue debris. Five days after the busulfan treatment, rats were injected in the jugular vein with 8×10^7 nucleated bone marrow cells in 0.5 mL of Hank's solution.

Experimental design

The experimental design is illustrated in Fig. 1. The rats were divided into three groups of six males each. All rats received busulfan injection in experimental week 0, at 11 weeks old, followed by bone marrow transplantation 5 days later. Group A (control) rats were injected with solvent in experimental weeks −4, 0, 4 and 8. Group B rats were treated similarly except that leuprorelin instead of solvent was injected in experimental week −4. Group C rats received leuprorelin in experimental weeks 0, 4 and 8.

In the 18th experimental week (29-weeks-old), all animals were killed by CO_2 asphyxiation. Weights of

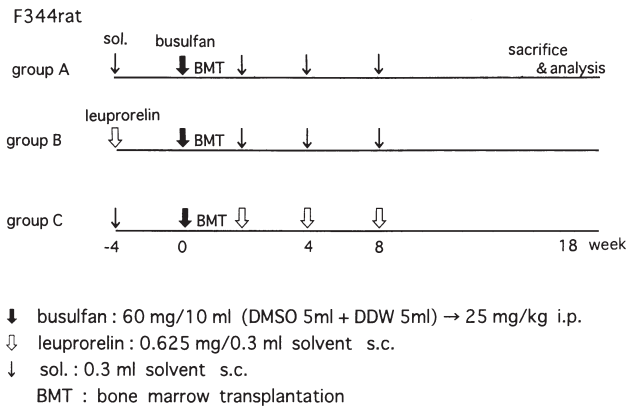


Fig. 1 Experimental design.

body, testis (gross weight) and epididymis were measured. Then the tunica of each left testis was cut open and partially peeled off to remove intratesticular fluid with a paper towel. The left testis was weighed again (net weight).

Evaluation of spermatogenesis

For histological analysis, the right testes were fixed in Bouin's fixative. Every testis was cut in half longitudinally. The fixed testes were then subjected to routine processing for paraffin embedding and thin-sectioning and stained with hematoxylin and eosin (H&E). A single slice from each testis was examined microscopically. The condition of spermatogenesis in each seminiferous tubule was scored according to Johnsen's scoring method.[18] Tubules scored 10–6, 5–4 and 3–1 were classified as 'recovered', 'partially recovered' and 'atrophic', respectively. Proportions of 'recovered' to total seminiferous tubules in number and 'recovered' plus 'partially recovered' to total were calculated in each group of rats.

Approval of the animal study

All animal housing and surgical procedures were in accordance with the guidelines of the institutional animal care and use committee of the Animal Research Center, Yokohama City University School of Medicine, Yokohama, Japan.

Statistical analysis

Statistical analyses of comparisons between groups were first done by using a one-way ANOVA to determine whether there were differences between all groups and then a non-parametric Mann–Whitney *U*-test to determine the significance of the difference between pairs of groups.

Results

General observations

One rat in group A and another in group B died in the course of the experiment, probably due to the toxic effects of busulfan. At necropsy of the remaining 16 rats (29-weeks-old), weights of body, testis and epididymis were measured (Table 1). In group C, the weight of the epididymis was significantly lighter than that of other groups ($P < 0.01$). Because the epididymis is one of the target organs of androgen, this was an expected result. It confirmed that leuprorelin treatment worked to suppress testosterone production. Although there was no statistically significant difference, mean body weight of group C rats tended to be lighter than that of other groups. This may be due to the decreased level of testosterone that works as an anabolic steroid in the male.

Testicular edema, which was due to the increase in intratesticular interstitial fluid, was observed in most of the testes. The amount of fluid was remarkable especially in group A and B. Nearly half of the gross testis weight in group A was intratesticular fluid. Meanwhile, testes of group C rats contained a smaller amount of fluid, about 20% of the gross testis weight. Although gross testicular weights in group A and B were heavier than those in group C, no significant difference in net testicular weight was observed among the groups. This means that the increased gross testis weight in group A and B rats was due to the accumulation of intratesticular fluid.

Every testis in group C animals contained a white, firm lesion in the ventro-caudal region varying in size from 3 to 5 mm in diameter, which was about 30% of the whole testis. They were microscopically confirmed to be massive necrosis of the seminiferous tubules with partial or complete calcification. No such lesion was observed in rats in group A and B.

Table 1 Body and reproductive organ weights

	Body weight (g)	Testicular weight (mg)			Epididymis weight (mg)	
		Right (gross)	Left (gross)	Left (net)	Right	Left
Group A ($n=5$)	314.2 ± 8.03	695 ± 111	774 ± 50	392 ± 85	228 ± 22	238 ± 26
Group B ($n=5$)	301.8 ± 9.02	602 ± 116	697 ± 95	410 ± 108	249 ± 41	237 ± 46
Group C ($n=6$)	290.7 ± 20.2	505 ± 58	519 ± 91	411 ± 78	140 ± 43	150 ± 33

One-way ANOVA, Mann–Whitney *U*-test. * $P < 0.01$.

Histological evaluation of spermatogenesis

For each rat, approximately 300 seminiferous tubule cross-sections were observed and categorized as 'recovered', 'partially recovered', or 'atrophic'. In the testes of group A and B rats (Fig. 2a,b), about half of the tubules were classified as 'atrophic'. Those tubules contained almost no germ cells. The remaining Sertoli cells had lost their original morphological character, becoming flattened and irregularly located along basement membrane (Fig. 2e). The nuclear size was smaller and texture of nuclear context appeared to have become dense losing the original rather grainy characteristics. Even in 'partially recovered' tubules, there were occasionally giant multinucleated cells that seemed to be degenerated germ cells (Fig. 2d). There was also retention of interstitial fluid to different degrees among rats that was observed as eosin-stained homogeneous substance between tubules. On the other hand, in the testes of group C rats, more than half of the seminiferous tubules had complete recovery of spermatogenesis (Fig. 2c). Even in the tubules classified as 'atrophic', Sertoli cells remained aligned regularly on the basement membrane (Fig. 2f). Giant multinucleated germ cells were very rarely observed. The calcified lesion found in the ventro-caudal area of group C rat testes was sharply separated from surrounding intact tubules. The area consisted of necrotic seminiferous tubules with spotted calcification (Fig. 2g). No viable cells were recognized in these seminiferous tubules. A similar lesion was not observed in rats of groups A and B. These degenerated tubules were omitted from categorization.

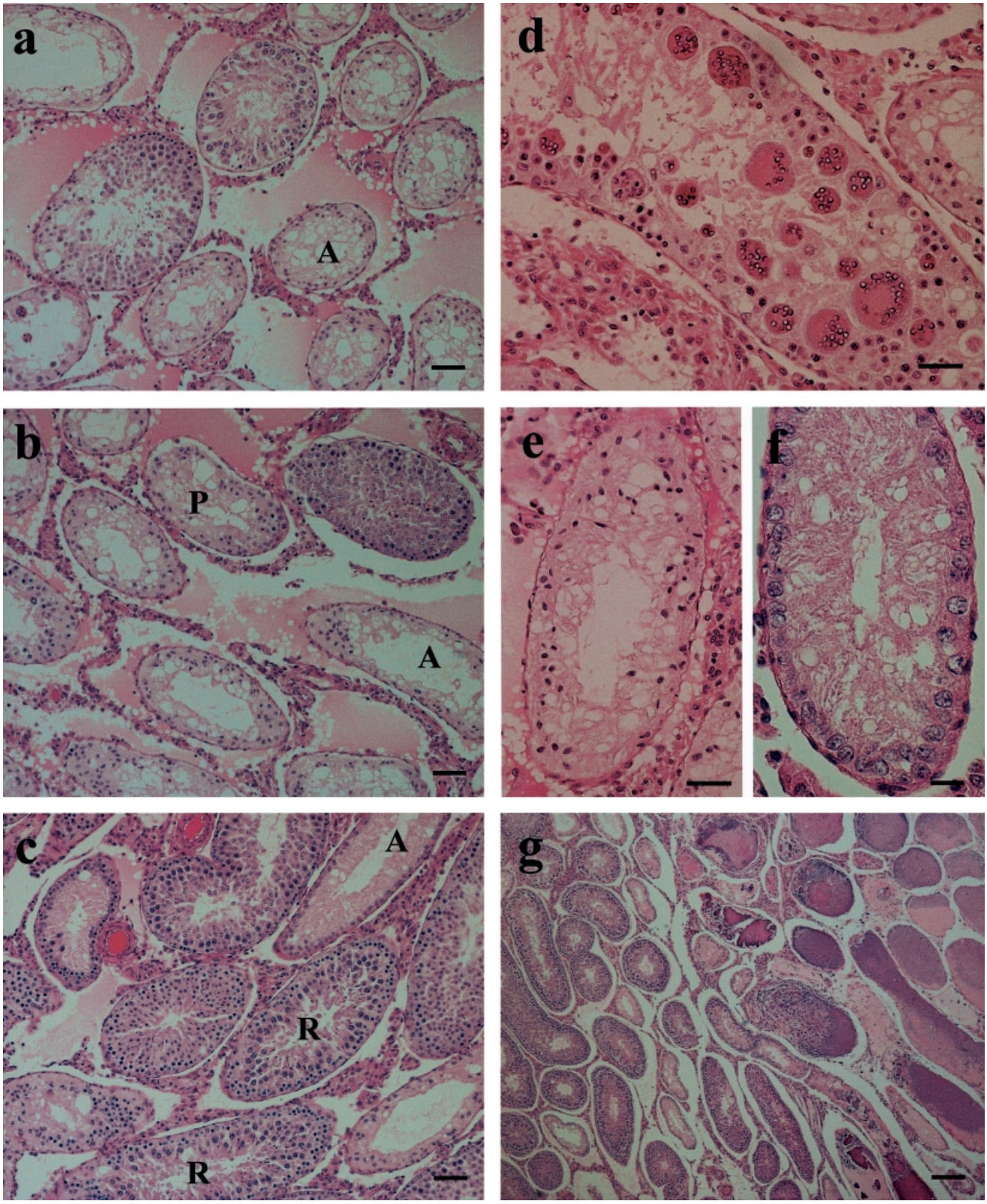
The percentage of 'recovered' seminiferous tubules was significantly different ($P < 0.05$) between the groups: in group C rats $56.5 \pm 12.0\%$ and in group A and B rats $27.7 \pm 12.6\%$ and $26.9 \pm 10.2\%$, respectively (Fig. 3). The percentage of 'recovered' and 'partially recovered' seminiferous tubules was also highest in group C. However, the difference between the groups was not statistically significant (data not shown).

Discussion

In this study, excellent recovery of spermatogenesis was demonstrated in rats that received leuprorelin after busulfan damage. This result agrees with several previous reports that proposed a stimulatory effect of GnRH analogs after spermatogenic impairment.[12,19] We could not find a protective effect of leuprorelin administered before busulfan. Recently, it was demonstrated that complete deficiency of gonadotrophin exhibits no evidence of protection against several different kinds of cytotoxic testicular damage in experiments using *hpg* mice, which is a spontaneous mutant strain with congenital complete gonadotrophin deficiency.[20] The result of this experiment disputed the original hypothesis proposed by Glode.[1] In addition, Meistrich *et al*. showed that numbers of spermatogonia and their proliferative activity were not suppressed during hormone treatment with testosterone plus 17β-estradiol, even though the treatment showed protective effects on the testes from procarbazine-induced damage.[21] This result compromised the theoretical foundation of the hypothesis that suppression of GnRH reduces the rate of spermatogenesis, thus making germ cells more resistant to cytotoxic damage. Taking these recent data into consideration, it seems to be unlikely that GnRH analogs have a protective effect on spermatogenesis when administered before insults. Instead, the stimulatory effect of GnRH analogs on the recovery of spermatogenesis after spermatogenic damage is gaining increasing support.[12–16,19] The results of this study certainly favor this new proposition.

Busulfan is an anticancer drug that is used for several malignant diseases, especially for chronic myelogenous leukemia and polycythemia vera.[22] In many animal experiments, other drugs such as cyclophosphamide, doxorubicin, cisplatin, or procarbazine, have occasionally been used to damage spermatogenesis.[2–7,23] We have chosen busulfan in the present study, because it damages early stage germ cells more severely than other drugs.[17] Therefore, it was expected that busulfan exposure would induce damage to spermatogenesis that might be difficult to

Fig. 2 Evaluation of the recovery of spermatogenesis. Each tubule was classified as atrophic (A), partially recovered (P), or recovered (R). (a) Testis of group A rat. Most seminiferous tubules were classified as 'atrophic'. (b) Testis of group B rat. Most tubules are devoid of germ cells. Note interstitial edema. (c) Testis of group C rat. Regenerated spermatogenesis was observed in most tubules. Interstitial edema was minimal. Scale bar, 30 μm (a, b and c). (d) Some 'partially recovered' tubules in group A and B have giant multinucleated cells. (e) The Sertoli cells in 'atrophic' tubules in group A and B lost their original morphological character. They were irregularly located along the basement membrane. Some of them became flattened with denser chromatin figures. Scale bar, 45 μm (d and e). (f) In 'atrophic' tubules in group C, Sertoli cells kept their original morphology and were aligned along the basement membrane. Scale bar, 20 μm. (g) Calcified or necrotic lesion, right half of the picture, in the ventro-caudal region in group C. Scale bar, 150 μm (H&E).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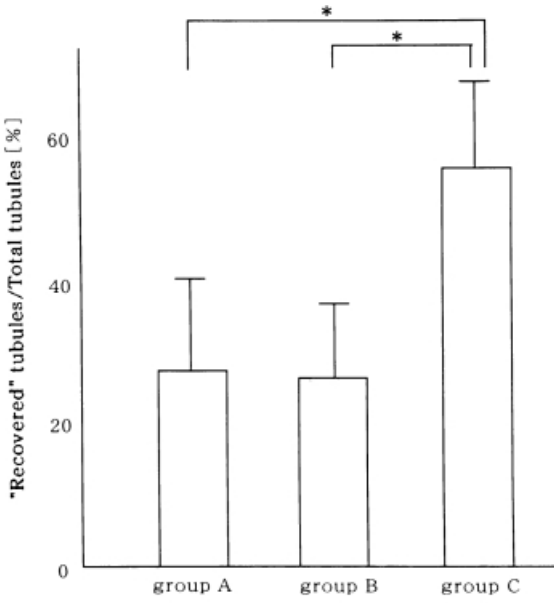

Fig. 3 Evaluation of spermatogenesis (mean ± SD; one-way ANOVA, Mann–Whitney *U*-test). **P* < 0.05.

recover. In a preliminary study, we tried different doses of busulfan in F344 rats. Based on that study, we selected a dose of 25 mg/kg of busulfan that appeared to induce significant damage to spermatogenesis. Because that dose was too toxic especially to bone marrow, bone marrow cell transplantation was necessary to rescue the rats from the myelotoxicity.

The amount of intratesticular fluid was remarkable in group A and B rats. Their testes showed edematous changes confirmed both macroscopically and histologically. The extent of edema corresponded well with the degree of impaired spermatogenesis. When there was less intratesticular fluid, spermatogenesis recovered better. Luteinizing hormone (LH) has been reported to be elevated when spermatogenesis is damaged by irradiation or toxic chemicals.[12] It has also been noted that high LH levels increase vascular permeability which would induce edematous changes in the testis.[24] We speculate that the edematous condition of the testis is unfavorable for spermatogenesis. The reduction of the edema with leuprorelin by way of LH suppression may create more favorable conditions for spermatogenesis.

It was reported that intratesticular testosterone (ITT) levels were elevated after irradiation or chemical treatments.[12] Based on that information, it was suggested that excess of ITT is detrimental to spermatogenesis possibly by its metabolites such as estradiol. Therefore, the reduction of ITT levels may be favorable for maintenance and/or resumption of spermatogenesis. Gonadotrophin-releasing hormone analogs suppress ITT by interrupting the pituitary-gonadal axis which in turn is supposed to stimulate the recovery of spermatogenesis.[12] It is also possible that the intratesticular fluid may contain substances other than testosterone that could be harmful to spermatogenesis.

One possible mechanism that high ITT is detrimental to spermatogenesis could be a changing pattern of stem cell factor (SCF) expression. Blanchard *et al*. have reported that leuprolide treatment recovered spermatogenesis in rats in which testes were irreversibly damaged by 2,5-hexanedione administration.[25] They further showed that the ratio of expression of membrane-bound SCF to soluble SCF was increased by leuprolide. Because membrane-bound SCF is essential for spermatogenesis, the increase of that form of SCF would promote spermatogenesis. Therefore it was suggested that reduction of ITT would induce Sertoli cells to express more membrane-bound type SCF. In our study, the Sertoli cells showed drastic morphological differences between group C and the other groups. These findings may relate to those molecular dynamics.

Every testis in group C rats showed a calcified lesion in the ventro-caudal region. Histologically, these lesions were a partial or complete calcification of necrotic seminiferous tubules. Blanchard *et al*. also reported the presence of atrophic tubular calcification in the lower pole of testes from leuprolide-treated Fischer rats.[25] To the best of our knowledge, this report and our study are the only examples that have shown calcified lesion of the testes after cytotoxic insults. The testicular artery penetrates the capsule and encircles the testis before entering the parenchyma. Then it runs toward the rete and mediastinum followed by the additional branching.[24] The ventro-caudal area is the most peripheral site in terms of arterial distribution. In addition, the testicular artery is an end artery. Therefore it seems that anatomically the ventro-caudal area of the rat testis is the poorest in blood supply. We speculate that poor blood supply, or ischemic changes induced by leuprorelin might have caused necrosis of seminiferous tubules followed by some calcification. In fact, several lines of evidence have shown that LH and luteinizing hormone–releasing hormone (LHRH) affect testicular blood flow. It was also reported that administration of supraphysiological dose of LHRH resulted in severe degenerative changes of seminiferous tubules in rats, possibly because of a disturbance in blood supply.[26]

The result of this study demonstrated that GnRH analog treatment after the cytotoxic damage stimulates

spermatogenesis. However, it was also noticed that leuprorelin induced significant necrotic damage with calcification in a restricted area of the testis. We are not yet aware of the exact mechanism of both phenomena and further studies are needed to elucidate the underlying mechanisms. Through such studies, it is expected that practical hormonal treatment regimens will be developed that could be useful for patients to recover fertility after chemotherapy.

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