Protecting Spermatogenesis from Damage Induced by Doxorubicin Using the Luteinizing Hormone-Releasing Hormone Agonist Leuprorelin

An Image Analysis Study of a Rat Experimental Model

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BACKGROUND. This study was performed to investigate the protective effect of a luteinizing hormone-releasing hormone (LHRH) agonist, leuprorelin, against spermatogenetic damage caused by doxorubicin in rats.

METHODS. Sprague-Dawley rats were divided into 4 groups: (1) a control group, (2) a group given LHRH agonist (subcutaneous injections, total dose 9 mg/kg), (3) a group given doxorubicin (intraperitoneal injections, total dose 7.5 mg/kg), and (4) a group given both LHRH agonist (subcutaneous injections, total dose 9 mg/kg) and doxorubicin (intraperitoneal injections, total dose 7.5 mg/kg). Evaluations were made by measuring body and testicular weights, determining Johnsen's score, and conducting DNA image analysis consisting of DNA content measurement (%1C, %2C, and %4C) by image cytometry.

RESULTS. In the group given doxorubicin, the testicular weight was 1.47 ± 0.24 mg, Johnsen's score was 4.4 ± 1.2, and image analysis revealed %1C: 33.8 ± 9.2, %2C: 43.9 ± 16.3, and %4C: 5.0 ± 4.4. In the group given both LHRH agonist and doxorubicin, the testicular weight was 1.32 ± 0.23, Johnsen's score was 5.90 ± 1.6, and image analysis revealed %1C: 46.9 ± 15.0, %2C: 28.4 ± 13.3, and %4C: 8.8 ± 3.5.

CONCLUSIONS. The significant prophylactic effect ($P < 0.05$) of the LHRH agonist against doxorubicin-induced spermatogenetic damage was demonstrated by Johnsen's score and image analysis (%1C, %2C, and %4C). ***Cancer* 1997;79:1014–21.** © *1997 American Cancer Society.*

KEYWORDS: spermatogenesis, spermatogenetic impairment, anticancer drug, doxorubicin, leuprorelin, Johnsen's score, image cytometry.

Due to advances in anticancer chemotherapy, the survival rates of cancer patients have improved remarkably, but the quality of life (QOL) after successful treatment is still of great concern. Spermatogenetic disorders in male patients are among the major problems caused by chemotherapy.[1–3] Several clinical trials designed to protect spermatogenesis from the damage induced by chemotherapy have been reported, without definite conclusions.[4–8] Animal experiments have produced various results[9–16]; some have been effective[9–13] and others have been ineffective.[14–16] None of these previous experiments have used the appropriate anticancer agents that cause long term and irreversible damage to spermatogenesis, and this might be the reason why they have given uncertain results.

Anticancer drugs damage spermatogenesis to varying degrees, depending on the nature of the drugs. Few drugs have been studied

TABLE 1
Johnsen's Score for the Rat

10	Fully mature spermatogenesis is observed (mature hooked spermatozoa with dense nuclear chromatin lying within tubular lumen). Pyknotic bodies are present.
9	Same criteria as for 10, but the germinal epithelium shows marked sloughing or obliteration of the lumen.
8	More than 10 immature spermatozoa, with dense nuclear staining and hooked heads, are observed; the majority are peripherally placed within the tubule. Pyknotic bodies are sometimes present.
7	Less than 10 spermatozoa are observed. The majority of cells are mature spermatids and are peripherally placed with less dense nuclear staining. No pyknotic bodies are present.
6	Mid-phase spermatids, with pale chromatin and narrow oval heads, are observed; they are radial arranged. No pyknotic bodies are present.
5	Immature spermatids, randomly arranged throughout the tubule, are observed; each has a rounded nucleus with pale chromatin. No pyknotic bodies are present.
4	Only a few spermatocytes (less than 5) are observed and no spermatids or spermatozoa.
3	Spermatogonia are the only germ cells present.
2	No germ cells are present, but Sertoli cells are.
1	No cells are present in the tubular section.

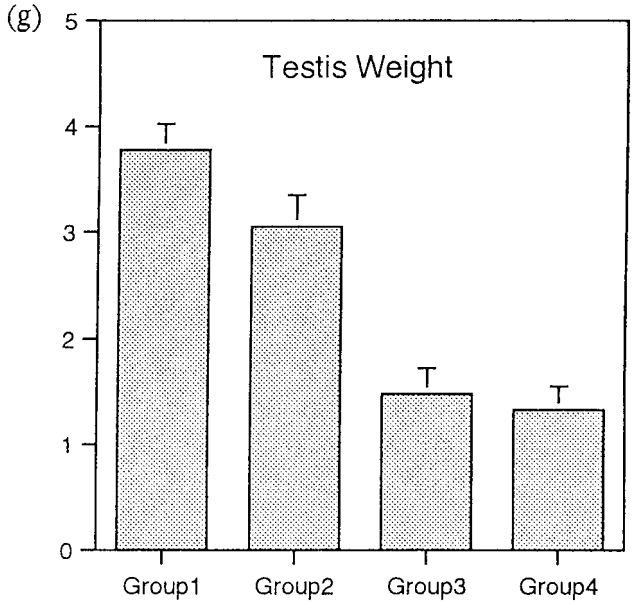

FIGURE 1. Testis weight during Week 25 is shown for groups of Sprague-Dawley rats. Groups 1–2, 1–3, 1–4, 2–3, and 2–4: $P < 0.001$. Group 1: control group; Group 2: administered leuprorelin; Group 3: administered doxorubicin; Group 4: administered leuprorelin and doxorubicin.

in animals in connection with long term, irreversible damage to spermatogenesis. Doxorubicin, the only drug that has been studied in detail, has had its dose effect on spermatogenesis evaluated.[17] We tried to determine the appropriate dose of doxorubicin and administer it to rats to make an experimental model of irreversible spermatogenetic impairment.

Endocrinologic suppression of germ cell division can protect spermatogenesis from anticancer drugs, luteinizing hormone–releasing hormone (LHRH) agonists are thought to have that potential.[5,6] Leuprorelin is an LHRH agonist that suppresses the pituitary-gonadal axis. The testosterone level of rats reaches its maximum 4 days after the administration of leuprorelin. It decreases below the standard value after 2 weeks and falls below the castration level after 3–4 weeks. This effect lasts for over 7 weeks after the administration of a dose of 3 mg/kg of leuprorelin.[18] Germ cell division is also markedly inhibited during that period, and spermatogenesis is reversibly inhibited.[18] Thus, suppressing the pituitary-gonadal axis with leuprorelin might protect spermatogenesis from the damage induced by anticancer drugs.

Evaluation was made from the weight of the testes, histologic examination, and Johnsen's score, as well as measurement of the testicular DNA content by image cytometry. The latter two were applied to evaluate testicular maturity. Image cytometry is thought to be a more accurate method of evaluating testicular maturity than conventional flow cytometry.[19,20]

MATERIALS AND METHODS

Male Sprague-Dawley rats, age 8 weeks, were obtained from Clea Japan Inc (Tokyo, Japan). They were maintained on a 12-hour light and 12-hour dark cycle, and they were allowed free access to food and water throughout these studies. They were also allowed to get accustomed to these conditions for 2 weeks. This experiment was conducted at the Laboratory Animal Research Center of the University of Tsukuba.

Ten-week-old Sprague-Dawley rats (mean weight, 400 g) were divided into 4 groups, with 10 rats in each group.

Group 1 (Control Group)

Ten rats received 0.3 mL of an isotonic sodium chloride solution at Weeks 0, 4, and 8 subcutaneously and 0.5 mL of normal saline 3 times a week from Week 6 to Week 10 (a total of 15 times) intraperitoneally.

Group 2 (Administered Leuprorelin)

Ten rats received 3 mg/kg leuprorelin at Weeks 0, 4, and 8 subcutaneously (total dose 9 mg/kg) and 0.5 mL of normal saline 3 times a week from Week 0 to Week 10 (a total of 15 times) intraperitoneally.

Group 3 (Administered Doxorubicin)

Ten rats received 0.3 mL of normal saline at Weeks 0, 4, and 8 subcutaneously and 0.5 mg/kg doxorubicin 3

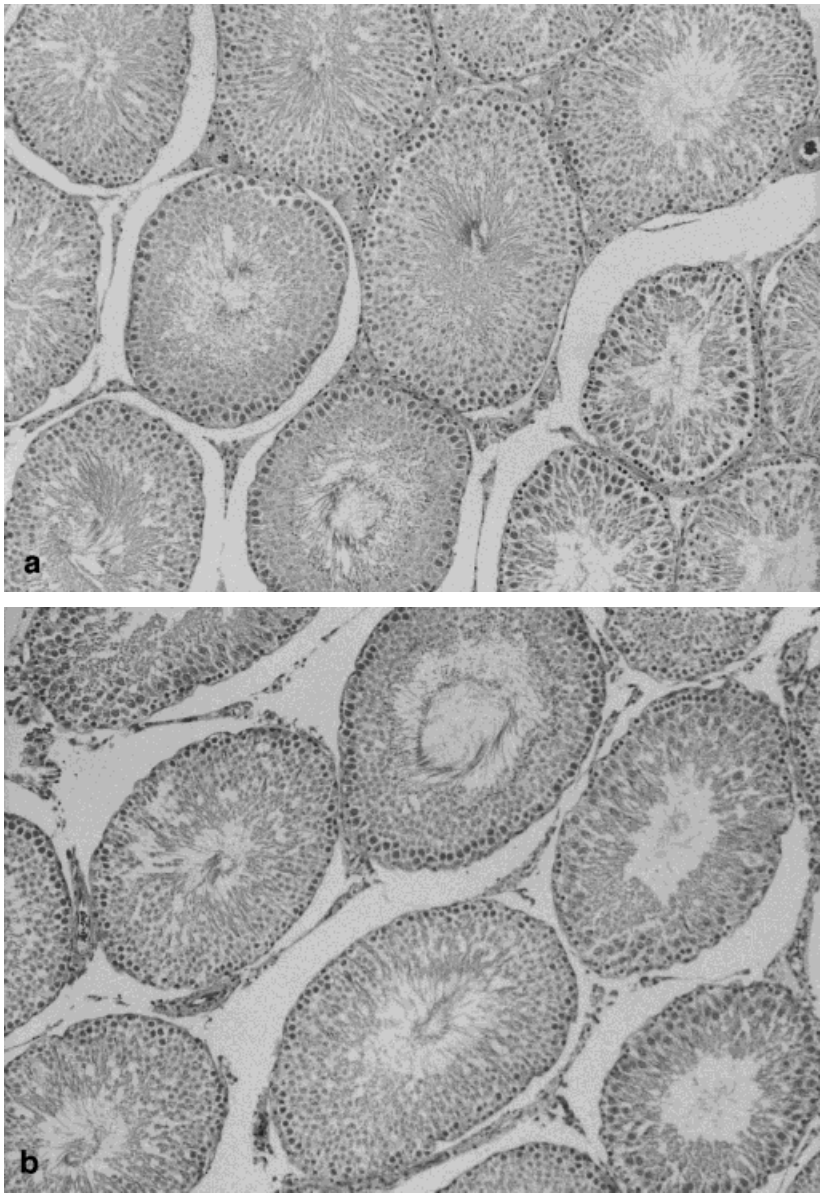

FIGURE 2. Histologic findings in testes of rats during Week 25 are shown. (H&E, original magnification ×100). (a) Control group (Group 1). The seminiferous tubules show normal spermatogenesis. (b) Group administered leuprorelin (Group 2). The seminiferous tubules are almost mature. (c) Group administered doxorubicin (Group 3). Doxorubicin caused severe damage to the seminiferous tubules and reduced the number of germ cells. Conversely, the interstitial cells proliferated. (d) Group administered leuprorelin and doxorubicin (Group 4). The seminiferous tubules show excellent maturation. All germ cell types are present. Interstitial cells proliferated, but to a lesser degree than in Group 3.

times a week from Week 6 to Week 10 intraperitoneally (total dose, 7.5 mg/kg).

Group 4 (Administered Leuprorelin and Doxorubicin)
Ten rats received 3.0 mg/kg leuprorelin at Weeks 0, 4, and 8 subcutaneously and received 0.5 mg/kg doxorubicin 3 times a week from Week 6 to Week 10 intraperitoneally (total dose, 7.5 mg/kg).

During Week 25, all animals were sacrificed with pentobarbital sodium (50 mg/kg). After the body and testes of each rat were weighed, the testes were fixed with 10% formalin and embedded in paraffin. We eval-

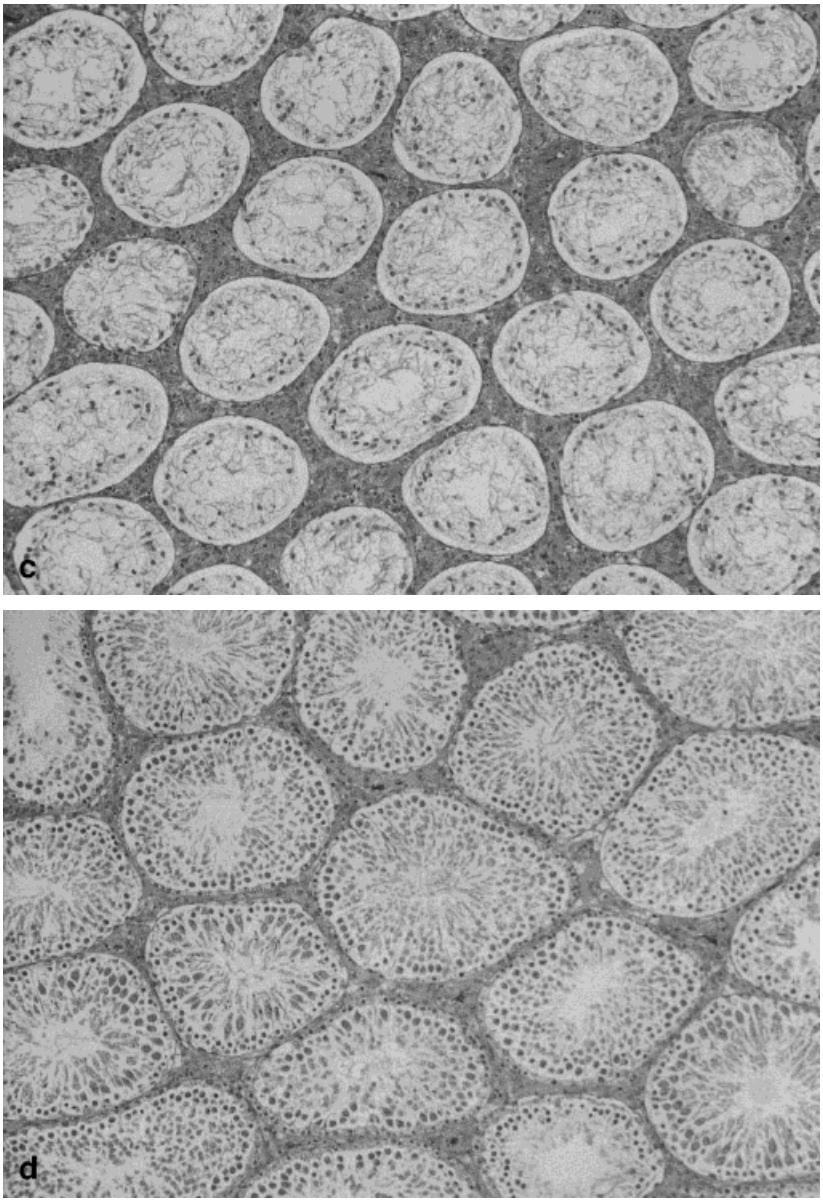

FIGURE 2. *(continued)*

uated the maturation of the seminiferous tubules using Johnsen's score. This scoring system was advocated by Johnsen[21] in 1970 for evaluating the maturation of human seminiferous tubules. It is based on the histologic findings of the testis and specifically focuses on the maturity of germ cells in the seminiferous tubules. Johnsen's score for the rat was devised by Lewis-Johnes and Kerrigan.[22] The detail of the scoring system is shown in Table 1. We calculated the mean Johnsen's score in 200 or more seminiferous tubules in each of the haematoxylin and eosin (H&E) stained specimens, and comparisons were made among the groups. At the same time, the DNA content of germ cells was measured by image cytometry. Specimens sliced into sections 7 μm thick were stained using a Quantitative DNA Staining Kit (Cell Analysis System, Inc., Lombard, IL), and quantitative DNA analysis was measured with a CAS 200 Image Analyzer (Cell Analysis Systems. Lombard, IL). With DNA image cytometry, we were able to distinguish the three ploidy compartments. The percentages of haploid cells (%1C), diploid cells (%2C), and tetraploid cells (%4C) were calculated and compared among all the groups. As a rule, the DNA content of 20 seminiferous tubules was measured for each specimen.

Statistical significance of the Johnsen's scores was

determined by the Mann-Whitney *U* test among all the groups and by the Kruskal–Wallis test for each group. Data from DNA image analysis were analyzed by ANOVA.

RESULTS

At the beginning of Week 25, all experimental rats were alive.

Body Weight

Figure 1 shows the means ± standard deviation (SD) of the body weights at Week 25. Group 1 weighed 660.4 ± 46.5 g; Group 2, 570.3 ± 59.3 g; Group 3, 600.3 ± 49.6 g; and Group 4, 589.4 ± 36.8 g. Body weights of Groups 2, 3, and 4 were significantly smaller than the body weight of Group 1. However, there was no significant difference between the body weights of Groups 3 and 4.

Testicular Weight

Figure 2 shows the means ± SD of the sum bilateral testicular weights at Week 25. Group 1 weighed 3.78 ± 0.24 mg; Group 2, 3.04 ± 0.31 mg; Group 3, 1.47 ± 0.24 mg; and Group 4, 1.32 ± 0.23 mg. There were significant differences between the weights of Group 1 and those of the other groups ($P < 0.0001$). The testicular weights of the groups administered doxorubicin (Groups 3 and 4) were significantly smaller than those of Groups 1 and 2 ($P < 0.001$). However, there were no significant differences between the weights of Groups 3 and 4.

Histologic Examination

Figure 2a shows the histologic findings of the H&E stained specimens of the control group (Group 1) at Week 25. The structure of the seminiferous tubules shows normal spermatogenic maturation. In the group administered leuprorelin (Group 2), the diameters of the testes were smaller than in Group 1. However, the seminiferous tubules of this group showed almost normal spermatogenic maturation (Fig. 2b). In the group administered doxorubicin (Group 3), the seminiferous tubules were atrophic, with little evidence of the existence of germ cells (Fig. 2c). Figure 2d shows the seminiferous tubules of the group administered leuprorelin and doxorubicin (Group 4). The seminiferous tubules of this group had a greater number of germ cells and more mature structures than those in Group 3. However, spermatogenesis in this group was still mildly immature as compared with Group 1, and the interstitial cells proliferated.

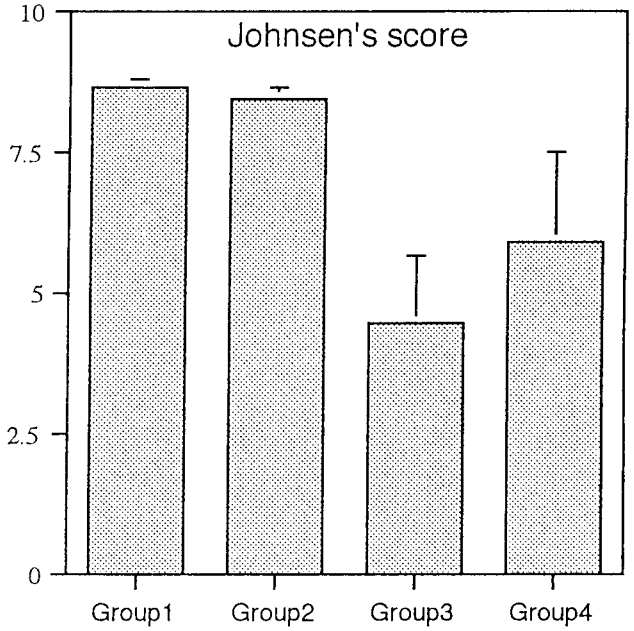

FIGURE 3. Johnsen's scores from Week 25 are shown. Groups 1–2: $P < 0.05$; Groups 1–3, 1–4, 2–3, and 2–4: $P < 0.0005$; Groups 3–4: $P < 0.05$.

Johnsen's Score

Figure 3 shows the means ± SD of Johnsen's scores in the H&E stained specimens. Group 1 was 8.6 ± 0.2; Group 2, 8.5 ± 0.2; Group 3, 4.4 ± 1.2; and Group 4, 5.9 ± 1.6. The Group 3 score was significantly smaller than the scores for the other groups. The Group 4 score was significantly larger than the score for Group 3 ($P < 0.05$) but smaller than those for Groups 1 ($P < 0.0005$) and 2 ($P < 0.0005$).

Image Cytometry

Figures 4a, 4b, and 4c show the means ± SD of the percentages of 3 ploidy compartments (%1C, %2C, and %4C) as determined by image cytometry.

The %1C of Group 1 was 64.7 ± 6.1%; Group 2, 68.0 ± 5.1%; Group 3, 33.8 ± 9.2%; and Group 4, 46.9 ± 15.0%. The %1C of Group 3 was significantly smaller than that of Group 1 ($P < 0.001$), Group 2 ($P < 0.0001$), and Group 4 ($P < 0.005$). The %1C of Group 4 was significantly larger than that of Group 3 ($P < 0.005$) but was smaller than that of Groups 1 ($P < 0.0005$) and 2 ($P < 0.0001$).

The %2C of Group 1 was 17.9 ± 5.6%; Group 2, 15.9 ± 4.1%; Group 3, 43.9 ± 16.3%; and Group 4, 28.4 ± 13.3%. The %2C of Group 3 was significantly larger than that of groups 1 ($P < 0.0001$), 2 ($P < 0.001$), and 4 ($P < 0.005$). The %2C of Group 4 was significantly smaller than that of Group 3 ($P < 0.005$) but larger than that of Groups 1 ($P < 0.05$) and 2 ($P < 0.05$).

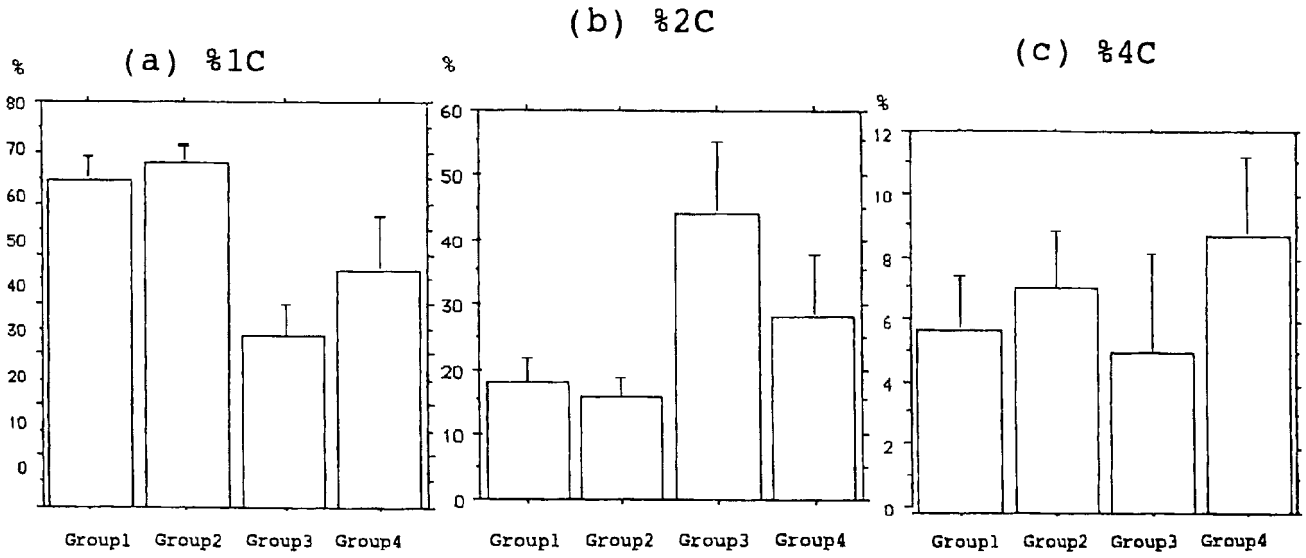

FIGURE 4. Image analysis is shown. (a) Percent of haploid cells (%1C). Groups 1–3: $P < 0.001$; Groups 1–4: $P < 0.0005$; Groups 2–3 and 2–4: $P < 0.0001$; Groups 3–4: $P < 0.005$. (b) Percent of diploid cells (%2C). Groups 1–3: $P < 0.0001$; Groups 1–4: $P < 0.05$; Groups 2–3: $P < 0.001$; Groups 2–4: $P < 0.05$; Groups 3–4: $P < 0.005$. (c) Percent of tetraploid cells (%4C). Groups 3–4: $P < 0.05$.

The %4C of Group 1 was 5.8 ± 2.5%; Group 2, 7.1 ± 2.6%; Group 3, 5.0 ± 4.4%; and Group 4, 8.8 ± 3.5%. The %4C of Group 4 was significantly larger than that of Group 3 ($P < 0.05$).

DISCUSSION

LHRH agonists induce down-regulation of receptors, desensitization of pituitary gonadotrophs, and suppression of spermatogenesis.[18] The inhibition of spermatogenesis in human beings and rats is reversible after treatment with LHRH agonists.[18] Therefore, it is thought that suppression of spermatogenesis by LHRH agonists could protect spermatogenesis from the damage caused by anticancer drugs during systemic chemotherapy. In accordance with these assumptions, LHRH agonists were tested experimentally[9–16] and clinically[4–8] for their ability to inhibit the testicular damage caused by anticancer drugs. Reviewing the results of these studies reveals that the efficacy of this method is still not clear. We thought that the periods of observation might not be long enough to evaluate whether the anticancer drugs used in these studies could be prevented from inducing the irreversible suppression of spermatogenesis. In this study, we designed an experimental model to preclude these problems.

It takes 3–4 weeks for the testosterone levels to reach the castration level after administration of leuprorelin, and that level begins to recover in the 8th week.[18] We administered leuprorelin every 4 weeks, 3 times, to ensure that germ cell division was thoroughly inhibited. The dose of leuprorelin used was 3 mg/kg, in accordance with previously reported data,[18] because leuprorelin at a dose of 0.03–3 mg/kg inhibits spermatogenesis dose-dependently, but inhibition does not differ with a dose of 3 mg/kg or more.

Doxorubicin is known to damage spermatogenesis in rats dose-dependently,[17] and it impairs it at a relatively early stage of spermatogonia (Stages A1, A2, and A3).[23] With the administration of 0.25 mg/kg 3 times a week for 5 weeks, strong but temporary spermatogenetic reduction occurs, and then spermatogenesis is recovered in most of the seminiferous tubules.[17] With 0.5 mg/kg 3 times a week for 5 weeks, the damage is irreversible (unpublished data). Therefore, we selected a dose of doxorubicin of 0.5 mg/kg, administered 3 times a week for 5 successive weeks (for a total dose of 75 mg/kg), in order to create a damaged spermatogenesis model with an irreversible impairment but with germ cells still remaining.

The schedule of leuprorelin and doxorubicin administration was determined on the basis of the following presumption: The castration level of serum testosterone (T) induced by leuprorelin continues for over 7 weeks; thus, the T level is thought to begin to recover 8 weeks after the third administration of leuprorelin in this model. We thought that inhibition of spermatogenesis with an LHRH agonist would continue from Week 4 to Week 16, and doxorubicin was administered from Week 6 to Week 10. Using this time table, we thought that during the administration of doxorubicin, spermatogen-

esis would be protected by leuprorelin. The testicular cycle of Sprague-Dawley rats (the period from spermatogonia to spermatid) was 51.6 days (about 7 weeks), and that period is not influenced by hormonal circumstances.[24] Therefore, we evaluated the efficacy of leuprorelin in Week 25, and that was long enough for the recovery of spermatogenesis.

To evaluate the efficacy, we measured testicular weight and analyzed testicular tissue histologically with Johnsen's score and DNA image analysis. There have been many studies in which DNA flow cytometry has been used to distinguish DNA ploidy compartments (haploid, diploid, and tetraploid cells) of the testicular tissue.[19,20] To the best of our knowledge, however, only Gottschalk-Sabag et al. reported the usefulness of DNA image cytometry in evaluating the maturation of spermatogenesis.[25] We applied DNA image analysis to measure DNA ploidy compartments of the testis in this study. There are several advantages of image analysis over flow cytometry. Flow cytometry is a special technique that requires skill, and it must be performed in a specially designed and equipped laboratory. In contrast, image cytometry involves no special techniques and can be performed easily. Moreover, image cytometry can measure the DNA content of germ cells exclusively (Sertoli cells, Leydig cells, and peritubular cells). However, the main advantage of image cytometry over flow cytometry is its ability to reevaluate a specimen. Using the same specimen, we could evaluate histologic changes in the testis, count the Johnsen's score, and measure the DNA contents.

In our study, the testicular weight of Group 2 (administered leuprorelin) was significantly smaller than that of Group 1 (control group, $P < 0.001$). Histologic examination of the testis revealed that the seminiferous tubules of Group 2 were smaller in diameter than those of Group 1, but the maturity of those in Group 2 did not differ much from the maturity of those in group 1. The Johnsen's score of Group 2 was smaller ($P < 0.05$) than that of Group 1, but there was no significant difference in the result measured by image cytometry between Groups 1 and 2. It was suggested that leuprorelin reduced the quantity of germ cells but had not affected the quality of spermatogenesis by Week 25.

The testicular weight of Group 3 (the group administered doxorubicin) was significantly smaller than that of Group 1 (control group, $P < 0.001$). Histologic examination of Group 3 revealed severe damage to the seminiferous tubules with little evidence of the existence of germ cells. There were significant differences in Johnsen's score ($P < 0.0005$) and image cytometry (%1C:$P < 0.001$, %2C:$P < 0.0001$) between Group 3 and Group 1. These results indicated that the irreversible damage caused by doxorubicin occurred during Week 25. In Groups 3 and 4, proliferation of interstitial cells in the testis was observed. It was reported that testosterone and luteinizing hormone values remained within the normal limits in rats treated with doxorubicin, indicating the resistance of Leydig cells to the drug.[26] In this study, we think that doxorubicin caused severe damage to the seminiferous tubules but not so much to the Leydig cells; and during restoration, luteinizing hormone levels increased via feedback, and the Leydig cells proliferated.

There was no significant difference in the body weight and testicular weight between Group 3 (the group administered doxorubicin) and group 4 (the group administered leuprorelin and doxorubicin). Johnsen's score was significantly higher for Group 4 than for Group 3 ($P < 0.05$).

In the DNA image analysis, Group 4 had significantly higher %1C ($P < 0.005$) and significantly lower %2C ($P < 0.005$) than Group 3. The %4C of Group 4 was significantly higher ($P < 0.05$) than that of Group 3. According to previous flow cytometric studies, %1C indicates the percentage of spermatid and %2C chiefly indicates the percentage of spermatogonia.[19] Thus, high %1C and low %2C is thought to indicate the presence of active spermatogenesis. The %4C indicates the percentage of primary spermatocytes, and, accordingly, a higher %4C indicates the recovery of spermatogenesis.[19] Based on these reported results, Group 4 was thought to have more active spermatogenesis than Group 3, but less than Groups 1 and 2. Therefore, we concluded that leuprorelin has the potential to inhibit the spermatogenetic impairment caused by doxorubicin.

However, a few problems must be solved for its clinical application. One is that a LHRH preparation induces testosterone flare-up immediately after administration. It takes 3–4 weeks to reach the castration level, and during this period spermatogenesis cannot be suppressed. A hormonal preparation may be needed to suppress the flare-up induced by testosterone. Interestingly, Cespedes et al. reported that this initial increase in testosterone could be effectively blocked by flutamide (antiandrogen) and that a combination of an LHRH analog and flutamide provided excellent protection against anticancer drug–induced spermatogenetic damage.[12] Perhaps an antiandrogen alone could be utilized to protect against chemotherapy-induced damage to spermatogenesis; this possibility should be investigated. Another problem is that spermatogenesis in the group administered LHRH agonist and doxorubicin could not reach the level in the control group in this study, and adjuvant agents (hormone, etc.) may be needed to facilitate recovery.

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