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Comparison of the protective roles of L-carnitine and amifostine against radiation-induced acute ovarian damage by histopathological and biochemical methods

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> Abstract

Purpose: The aim of this study was to compare the radioprotective efficacies of L-carnitine (LC) and amifostine against radiation-induced acute ovarian damage.
Materials and Methods: Forty-five, 3-month-old Wistar albino rats were randomly assigned to six groups. Control (CONT, n = 7); irradiation alone RT: radiation therapy (RT, n = 8); amifostine plus irradiation (AMI + RT, n = 8); LC plus irradiation (LC + RT, n = 8); LC and sham irradiation (LC, n = 7); and amifostine and sham irradiation (AMI, n = 7). The rats in the AMI + RT, LC + RT and RT groups were irradiated with a single dose of 20 Gy to the whole abdomen. LC (300 mg/kg) and amifostine (200 mg/kg) was given intraperitoneally 30 min before irradiation. Five days after irradiation, both antral follicles and corpus luteum in the right ovaries were counted, and tissue levels of malondialdehyde (MDA) and advanced

oxidation protein product (AOPP) were measured.
Results: Irradiation significantly decreased antral follicles and corpus luteum (P: 0.005 and P < 0.0001). LC increased the median number of antral follicles and corpus luteum (P: 0.009 and P < 0.0001, respectively). Amifostine improved median corpus luteum numbers but not antral follicle (P < 0.000, P > 0.05). The level of MDA and AOPP significantly increased after irradiation (P = 0.001 and P < 0.0001, respectively). MDA and AOPP levels were significantly reduced by LC (P: 0.003, P < 0.0001) and amifostine (P < 0.0001, P: 0.018). When comparing CONT group with AMI + RT and LC + RT groups, MDA and AOPP levels were similar (P > 0.005). The levels of both MDA and AOPP were also similar when LC + RT is compared with AMI - RT group (P > 0.005).
Conclusions: L-carnitine and amifostine have a noteworthy and similar radioprotective effect against radiation-induced acute ovarian toxicity.

Keywords: Amifostine, kidney, irradiation, L-carnitine, radioprotection

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Introduction

As one of the current cancer treatment strategies, radiotherapy is an important treatment modality in pelvic malignancies. The survival rate of young female cancer patients has steadily increased. Consequently, early and late effects at treatment are gaining greater importance for survivors, their families, and providers. Pelvic irradiation causing premature ovarian failure can often render the patient infertile. [1]

For this reason, ovarian tissues are a dose-limiting organ that restricts application of irradiation on the pelvic area in children and young people. Fortunately, there are several therapeutic interventions, such as gamete collection and freezing for subsequent artificial insemination, freezing of ovarian tissue, and surgical transposition of ovaries out of radiation treatment fields, to preserve fertility for this group of patients. [2] However, the benefit of these techniques remains unproven. [3]

Radiation damages DNA and other cellular targets mainly by its indirect effects through free oxygen radicals. [4] The ionization of cellular water results in the forming of reactive oxygen species (ROS), notably hydroxyl radicals, and increasing oxidative stress. In ovarian tissue, ionizing radiation and the increased ROS cause rapid primordial follicle loss. [5],[6] Since the effect of ionizing radiation is primarily mediated through the action of free radicals, which can cause damage to DNA, proteins, and lipids, some drugs that are acting as a free radical scavenger are getting popular for radioprotection.

Amifostine (S-2{3-aminopropylamino-ethylphosphorothioic acid}; Ethyol; WR-2721) is a prodrug that is converted in vivo by alkaline phosphatase to the active cytoprotective sulfhydryl compound, WR-1065. [7] Amifostine protects normal cells from irradiation damage by scavenging free radicals, by donating hydrogen ions to free radicals, and by depleting oxygen. The selective protection of nonmalignant tissues is believed to be due to higher alkaline phosphatase activity, higher pH, and vascular permeation of nontumoral tissues. It was shown that amifostine has substantial radioprotective effects on several tissues and organs such as oral mucosa, lung, bone, and kidney. [8]

L-carnitine (LC) (i-hydroxy-gammatrimethylammonium butylate) - $(CH_3)_3N^+-CH_2-CH(OH)-CH_2-COO^-$ - (LC) is required for the transfer of long-chain fatty acids from the cytosol into the mitochondria of skeletal muscle and cardiomyocytes during the beta-oxidation of lipids for the generation of energy. [9] LC prevents the formation of ROS produced by the xanthine/xanthine oxidase system, and it has a scavenger effect on ROS, resulting in a stabilizing effect on damaged cell membranes. The radioprotective effect of LC in different organs has been demonstrated in earlier studies. [10],[11],[12] However, to the best our knowledge, no study has yet investigated the efficacy of either LC or amifostine in prevention of radiation-induced acute ovarian damage.

Based on the above-mentioned studies, we hypothesized that amifostine, as well as LC, may have protective effects against radiation-induced ovarian damage. The aim of the present study is to compare the efficacy of these treatments using histopathological and biochemical methods.

Materials and Methods

Animals and experimental design

All animal experiments adhered to the guidelines of the Institutional Animal Ethics Committee. The rats were housed in rat cages with ad libitum access to a standard rodent diet and tap water, with a 12:12-h artificial light cycle, mean temperature 21° C ± 2° C, and mean humidity 55% ±2%. Forty-five 3-month-old animals were randomly assigned into six groups, for the following treatments:

Group 1: Control (CONT, n = 7), 1 mL/kg normal saline by intraperitoneal (i.p.) injection 30 min prior to sham irradiation; Group 2: Irradiation alone (RT, n = 8), 1 mL/kg, i.p., normal saline 30 min prior to irradiation (a single dose of 20 Gy); Group 3: Amifostine and irradiation (AMI + RT, n = 8), 200 mg/kg, i.p., amifostine 30 min prior to irradiation; Group 4: LC and irradiation (LC + RT, n = 8), 300 mg/kg, i.p., 30 min prior to irradiation; Group 5: LC and sham irradiation (LC), n = 7, 300 mg/kg, i.p., 30 min prior to sham irradiation; Group 6: Amifostine and sham irradiation (AMI, n = 7), 200 mg/kg, i.p., amifostine 30 min prior to sham irradiation.

The selection of the 30 min interval between LC administration and exposure to radiation was based on our previous study on animals. [13]

All experimental procedures were performed on anesthetized rats. Anesthesia was maintained with ketamine and xylazine (50 mg/kg body weight [BW] and 5 mg/kg BW, i.m.) during irradiation. The follow-up period was 5 days. During the follow-up, all rats were monitored by veterinary care staff.

Irradiation

The rats in AMI + RT, LC + RT and RT groups were irradiated individually with a single dose of 20 Gy. Doses of irradiation were given with 1.25 MV photon at a depth of 1 cm through an anterior 3 cm × 4 cm single portal to whole abdomen, using 60 co-treatment unit (Cirus, Cis-Bio Int., Gif Sur Yvette, France) at a source skin distance of 80 cm. The rats were anesthetized and then fixed onto a 20 cm × 30 cm blue Styrofoam treatment couch (Med-Tec, Orange City, IA) in a prone position. Correct positioning of the fields was controlled for each individual rat using a therapy simulator (Mecaserto-Simics, Paris, France). Special dosimetry was done for the irregular fields. The dose homogeneity across the field was ± 5%. After irradiation, the animals were closely observed until recovery from anesthesia. The CONT group received an equal field sham irradiation.

Euthanasia

The rats were euthanized 5 days after the radiation therapy. Prior to euthanasia, the rats received anesthesia using ketamine and xylazine combination. Euthanasia was performed by decapitation. Right ovaries of all rats were collected for histopathological analysis.

Histopathological analysis

Formalin fixed ovaries were dehydrated, embedded in paraffin blocks, sectioned serially (5 μm) and stained with hematoxylin and eosin. The stained sections were then observed under an Olympus BX51 Microscope (Olympus BX51, Tokyo, Japan). The number of antral follicles (excluding preantral and primordial) and corpus luteum in the ovaries were counted by examining every fifth serial section of each ovary and then counting the follicles whose plane of section passed through the nucleolus of the oocytes. Antral follicles were classified according to the number of granulosa cell layers and antrum formation.

Biochemical analysis

Tissue specimens were washed with cold 0.9% NaCl solution and stored at −20° C until used for biochemical studies. The frozen tissues were separately weighed and then homogenized in 10 volume of cold potassium chloride in a potter-type homogenizer. Samples were centrifuged at 8,000 xg for 10 min at 4° C.

Tissue levels of malondialdehyde (MDA), a marker of lipid peroxidation, were measured as thiobarbituric acid reactive substances by the method of Ohkawa et al. [13] Spectrophotometric determination of advanced oxidation protein product (AOPP) levels were performed according to Witko's method. [14] The protein content of the tissues was determined by the method of Lowry et al. [15] All results were expressed as nmol/mg protein.

Statistical analysis

The data were analyzed using standard statistical methods Statistica version 7 software (Statsoft, Inc., Tulsa, OK, USA). One-way analysis of variance (ANOVA) was used for statistical comparisons between the groups. The statistical analysis was performed using one-way ANOVA, followed by a post-hoc Bonferroni honestly significant difference test. $P < 0.05$ was considered to indicate significance.

> Results

Histopathologic analyses were made on 45 rats. There were no deaths during the follow-up period. Antral follicles of one rat were not counted in LC + RT group due to tissue damage. Histopathological and biochemical results, such as the count of antral follicles and corpus luteum, the level of MDA and AOPP, were significantly different between the groups ($P < 0.0001$). The median antral follicles and corpus luteum number is summarized for each group in [Table 1].

Table 1: The number of antral follicles and corpus luteum in each group

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Comparing to each group with CONT revealed that AMI or LC injection has not any significantly negative impact on antral follicle and corpus luteum [Figure 1]. (P > 0.05 for each comparison), whereas irradiation significantly decreased numbers of antral follicle and corpus luteum [P: 0.005 and P < 0.0001; [Table 2]] [Figure 2]. The median number of antral follicles and corpus luteum was 35 and 11 in the CONT group compared with 19 and 28 in the RT group, respectively. Pretreatment with LC before RT increased the median number of antral follicles, and corpus luteum (26 and 51, respectively), and the difference was significant when compared to RT, (p: 0.009 and P < 0.0001, respectively) [[Figure 3], panel B]. The protective effect of LC was more distinctive on corpus luteum when comparing CONT (P > 0.05). The median corpus luteum numbers were similar in both groups (LC + RT and CONT). Similarly, pretreatment with amifostine before RT improved median corpus luteum numbers but not antral follicle [[Figure 3], panel A]. The median corpus luteum number significantly improved with the amifostine pretreatment before irradiation (28 vs. 48, P < 0.0001), whereas amifostine did not have a significantly positive impact on antral follicles (19 vs. 31, P > 0.05).

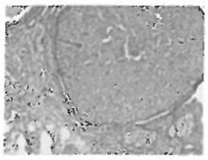

Figure 1: Section of an ovary from control group showing many normal corpus luteums including granulosa luteum cells (arrow) (H and E, ×100)

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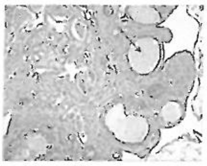

Figure 2: Section of an ovary from irradiation alone group showing immature corpus luteums and some antral follicles (arrow) (H and E, ×12, 5)

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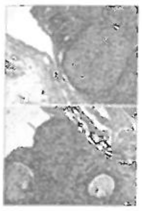

Figure 3: Sections of ovaries from amifostine and irradiation group (panel A), and L-carnitine and irradiation group (panel B), showing many mature corpus luteums (arrow) (H and E, ×50)

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Table 2: Comparisons of groups according to histopathological and biochemical paramaters

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The mean levels of MDA and AOPP are summarized in [Table 3]. The mean MDA and AOPP levels were 1.39 nmol/mg and 46.27 nmol/mg in the CONT group compared with 3.82 nmol/mg and 283.08 nmol/mg in the RT group. The level of MDA and AOPP significantly increased after irradiation (P = 0.001 and P < 0.0001, respectively). MDA and AOPP levels were significantly reduced by LC and amifostine pretreatment before irradiation. The mean MDA and AOPP value was 1.73 nmol/mg (P: 0.003) and 77.36 nmol/mg (P < 0.0001) in LC + RT group, and was 1.45 nmol/mg (P < 0.0001) and 131.19 nmol/mg (p: 0.018) in AMI + RT group. MDA and AOPP levels were similar when comparing CONT group with AMI + RT and LC + RT groups (P > 0.005). The level of both MDA and AOPP were also similar when LC + RT is compared with AMI + RT group (P > 0.005) [Table 2].

Table 3: Biochemical results in each group

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Discussion

Infertility is one of the important chronic adverse effects of radiotherapy reported by childhood of female cancer survivors. [16] Studies to protect their reproductive capacity from radiation-induced damaging may offer potential methods for females of all ages. [17] Using protective agents against radiation-induced ovarian toxicity might be a feasible option to preserve fertility in this group of patients. For this reason, we assessed the radioprotective efficacy of two ROS scavenging agents on ovarian tissue. The main findings of the present study are as follows: (1) irradiation decreased antral follicle and corpus luteum count and increased MDA and AOPP levels. (2) Pretreatment with LC before irradiation increased antral follicle and corpus luteum count and decreased MDA and AOPP levels. (3)

Pretreatment with amifostine before irradiation increased antral follicle but not corpus luteum count and decreased MDA and AOPP levels.

Acute ovarian failure may be transient or permanent, and can occur during or shortly after completion of irradiation or chemotherapy. In contrast, premature ovarian failure or premature menopause typically manifests during a late period. The quantity of ovarian damage is related to patient age and the dose of irradiation applied to the ovarian tissues. [18] Irradiation invariably results in premature ovarian failure and the threshold dose is around 300 cGy. Ovarian failure occurred in 11-13% of patients if exposed to below 300 cGy, compared with 60-63% if above that value. [19] The ovarian follicle damage occurred after irradiation, which results in ovarian atrophy and reduced follicle stores. The oocytes show a rapid onset of pyknosis, chromosomal condensation, disruption of the nuclear envelope, and cytoplasmic vacuolization. [20] Since this radiation-induced acute morphological changes might be visible as soon as the 4 th day after irradiation, according to Lee et al., in the present study we sacrificed the rats and harvested ovaries on the 5 th day after irradiation. [21] In a pioneer study in 1927, Brambell and Parks showed that ovarian follicles disappeared after irradiation in mouse ovaries. [22] They stated that the follicular apparatus shows the different radio sensitivity probability correlated with oocyte size. Baker irradiated rhesus monkeys and assessed the ovaries histopathologically. [23] That author observed that the irradiation induced cytoplasmic eosinophilia in oocytes, pyknosis in many granulosa cells, and the destruction of most of the multi-layered follicles. He concluded that the primordial oocyte of the rhesus monkey is markedly resistant to radiation-induced cell death, and the number of germ cells surviving exposure varies according to the dose administered and the postirradiation interval. In the present study, we used the antral follicle and corpus luteum count to assess the acute radiation toxicity on ovarian tissue and observed that, similar to previous studies, irradiation had a significant detrimental impact on ovaries: Antral follicle and corpus luteum counts significantly decreased after irradiation (median 35 antral follicles and 41 corpus luteum in the CONT group vs. median 19 and 28 in the RT group, respectively).

Amifostine is dephosphorylated by alkaline phosphatase in normal tissues to an active free thiol metabolite. The thiol metabolite can also scavenge ROS generated by exposure to radiation. [24] Amifostine is believed to be responsible for the reduction of the cumulative renal toxicity of cisplatin and for the reduction of the toxic effects of radiation on normal oral tissues. The radioprotective effect of amifostine on kidney, bone, and rectal tissue was shown in earlier studies. [8],[11],[25] However, it was reported that amifostine has undesirable side-effects, including nausea, vomiting, sneezing, hot flashes, mild somnolence, hypocalcaemia, and hypotension. [26] Amifostine 200 mg/kg before irradiation decreased radiation-induced acute histopathologic as well as biochemical ovarian damage in this study.

There are a few studies in which the radioprotective effect of amifostine on ovary cells was assessed. However, these are both in vitro cell culture studies and the authors evaluated the radioprotective efficacy of amifostine on ovary tumor cells. [27],[28] On the contrary, Yoon et al. studied mice ovaries in vivo for the radioprotective effects of amifostine. [29] 3-week-old female mice were irradiated with 6.42 Gy of gamma-ray with or without pretreatment with amifostine. The proliferation of granulosa cells reduced and the incidences of follicular degeneration increased in the irradiated mice, compared to amifostine-treated group. Our results corroborate and extend the observation that pretreatment with amifostine before irradiation protects ovary-increasing antral follicle numbers and decreasing MDA and AOPP levels.

Another important radioprotective agent is LC. It is available from the diet or synthesized endogenously by skeletal muscle, heart, liver, kidney, and brain, or can be given as a nutritional supplement. It is also a relatively well tolerated and safe compound. [8],[30] LC has the capacity to control carbohydrate metabolism and to maintain cell membrane structure and cell viability, and it is an essential cofactor in the oxidation of long-chain fatty acids. [30] It also affects several key enzymes involved in protein and lipid metabolism. [31] In addition, LC is a substance that can act as an antioxidant and free radical scavenger. [32] Moreover, it increased endogenous antioxidant defence mechanisms, which might have protected the animals from radiation-induced organ toxicity. Altas et al. showed that LC could improve radiation-induced cochlear damage in guinea pigs. [12] LC also was shown to serve as a protective agent against irradiation-induced lens damage, in a rat study by Kocer et al. [33] The radioprotective properties of LC in delaying the onset and reducing the severity of radiation-induced oral mucositis, kidney, and bone damage have also been reported in other animal studies. [11],[25]

In addition, since there is a relationship between ovarian tissue and fat methabolism, LC may have a protective role for ovaries against irradiation. Rhodes et al., [34] stated that greater dietary fat ingestion has a direct effect on ovarian structures, and Hawkins et al. declared that fat may result in higher progesterone production. [35] The importance of LC on oocyte metabolism was shown in earlier studies. Oocyte metabolism is closely linked with oocyte quality. Dunning et al. had shown that beta-oxidation of lipids is essential for developing of oocyte competence. [36] They then investigated the treatment with LC, the fatty acid transport cofactor of beta-oxidation, and whether it could improve folliculogenesis and developmental competence of mouse follicles. [37] LC did not alter survival, growth, or differentiation of follicles. However, LC supplementation during in vitro follicle culture increased lipid metabolism and improved oocyte developmental competence.

To our knowledge, so far there has been no study that assessed the effectiveness of LC on radiation-induced ovarian damage. Our results revealed that pretreatment with LC before RT significantly increased the median number of both antral follicles and corpus luteum, when compared to RT (P: 0.009 and P < 0.0001, respectively), whereas amifostine just improved median corpus luteum numbers but not antral follicle (P < 0.0001, P > 0.05, respectively). However, both agents presented a similar radioprotective efficacy by biochemical methods. The increased mean MDA and AOPP levels after irradiation were decreased to control levels with both amifostine and LC (P < 0.0001, and P: 0.018 for amifostine; and P: 0.003, and P < 0.0001 for LC, respectively). Somfai et al. reported that using LC enhanced the mitochondrial functions, improved the oocyte maturation and cleavage underlining the importance of lipid metabolism for nuclear and cytoplasmic maturation of porcine oocytes. [38] Furthermore, the ROS levels in LC-treated oocytes were significantly lower compared to control level. Usta et al. evaluated histopathological changes and MDA levels in rat ovaries, which were subjected to torsion and detorsion and treated with LC. [39] Ovarian total tissue damage scores, and tissue MDA levels were found significantly lower in those treated with LC, compared with the control group.

There have been several studies in which the radioprotective effectiveness of amifostine and LC on several tissues and organs such as kidney, bone and intestine was assessed comparatively. [8],[11],[25] However, to the best of our knowledge there has been no study to evaluate the protective effect of LC on radiation-induced ovarian toxicity, so far. LC was used as a possible modulator of radiation-induced toxicity, based on the previous reports. Caloglu et al. compared the protective effects of LC and amifostine against radiation-induced late nephrotoxicity. [11] They found that the tubular damage was less common in the LC and amifostine group than in the irradiation group. Yurut-Caloglu et al. showed that amifostine, as well as LC, decreased the radiation-induced growing bone damage at the same level. [25] Similar protective results were noted for radiation-induced acute intestinal damage by Caloglu et al. [8]

The presented study has some limitations. First, we did not assess the endocrinological results of ovarian irradiation. Functional changes in irradiated rats have been investigated in several studies. However, it has long been recognized that cellular function of the ovary is more sensitive than the endocrinological function. [22] Thus, we aimed to assess histopathological changes as a substitute for functional changes in irradiated ovaries and did not take into consideration the estrous cycles of rats. Second, we did not investigate the levels of serum antioxidants. In conclusion, based on the results of the present study, it may be stated that LC protects the ovaries against the single fraction irradiation-induced acute toxicity as much as amifostine. Since recent advances in cancer therapy have resulted in increasing numbers of long-term survivors, it would also be worthwhile to study the effects of in vivo administration LC and amifostine in radiation-treated cancer patients, with the hope of reducing radiation-induced ovarian damage.

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Figures

[Figure 1], [Figure 2], [Figure 3]

Tables

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