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ORIGINAL PAPER

Antiapoptotic effect of L-carnitine on testicular irradiation in rats

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Abstract We evaluated the effects of L-carnitine on apoptosis of germ cells in the rat testis following irradiation. Male Wistar rats were divided into three groups. Control group received sham irradiation plus physiological saline. Radiotherapy group received scrotal gamma-irradiation of 10 Gy as a single dose plus physiological saline. Radiotherapy + L-carnitine group received scrotal irradiation plus 200 mg/kg intraperitoneally L-carnitine. Twenty-four hours post-irradiation, the rats were sacrificed and testes were harvested. Testicular damage was examined by light and electron microscopy, and germ cell apoptosis was determined by terminal deoxynucleotidyltransferase-mediated deoxyuridine triphosphate in situ nick end-labeling (TUNEL) technique. Morphologically, examination of irradiated testis revealed presence of disorganization and desquamation of germinal cells and the reduction in sperm count in seminiferous tubule lumen. Under electron microscopy, the morphological signs of apoptosis were frequently detected in spermatogonia. Apoptotic spermatogonia showed the marginal condensation of chromatin onto the nuclear lamina, nucleus and cytoplasm shrinkage and still functioning cell organelles. TUNEL-positive cells were significantly more numerous in irradiated rats than in control rats. L-carnitine treatment significantly attenuated the radiation-induced morphological changes and germ cell apoptosis in the irradiated rat testis. In conclusion, these results suggested that L-carnitine supplementation during the radiotherapy may be beneficial for spermatogenesis following testicular irradiation by decreasing germ cell apoptosis.

Keywords Irradiation · L-Carnitine · Testicular toxicity · Germ cell apoptosis · Rat

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Introduction

Radiotherapy is known to be one of the most common and important methods for cancer treatment (Agrawal et al. 2001a). The killing action of ionizing radiation is mainly mediated through the free radicals generated from the radiolytic decomposition of cellular water (Agrawal et al. 2001b), including superoxide radical, hydroxyl radical, and hydrogen peroxide (Fang et al. 2002). These free radicals can stimulate chain reactions by interacting with proteins, lipids and nucleic acids, causing cellular dysfunction and even death (Filho et al. 2004). Previous studies have indicated that radiation-mediated oxidative stress can induce apoptosis (Lee et al. 2002).

Germ cell apoptosis has been reported by several investigators to play an important role in normal testicular physiology (Modi et al. 2003). It is required for normal spermatogenesis and is believed to ensure cellular homeostasis and maintain the fine balance between germ cells and Sertoli cells (Said et al. 2004). Besides its role in normal testicular physiology, germ cell apoptosis can also be induced by pathological conditions (Sukhotnik et al. 2007; Otala et al. 2004) and toxic chemicals (Shoda et al. 2001; Yu et al. 2001). Apoptosis of germ cells has been reported recently as a mechanism responsible for infertility in irradiated testis (Dewey et al. 1995; Hasegawa et al. 1997). Exposure of the testis to ionizing radiation induces

apoptosis of germ cells, primarily affecting actively dividing spermatogonia and preleptotene spermatocytes. On the other hand, testicular somatic cells that support the germ cells are relatively resistant to radiation. For example, Sertoli cells and Leydig cells were shown to be resistant to radiation-induced apoptosis (Pinon-Lataillade et al. 1988; Vergouwen et al. 1995).

L-carnitine is a small water-soluble molecule important in mammalian fat metabolism (Bremer 1983). It is known that L-carnitine and its derivatives have antioxidant and anti-inflammatory effects on various pathophysiological conditions (Izgut-Uysal et al. 2003; Onem et al. 2006; Ferrari et al. 2004). L-carnitine has been recently shown to act as an important antiapoptotic mediator (Moretti et al. 2002; Cifone et al. 1997). In addition, L-carnitine enhances the activity of DNA repairing enzyme poly(ADP-ribosyl) polymerase and also other related repair mechanisms (Boerrigter et al. 1993). The use of L-carnitine and its derivatives in therapy has been proposed in recent years for treatment of male infertility, and a number of human and animal studies have been published that indicate a possible role for application of carnitine (Lenzi et al. 2004; Stradaioli et al. 2004). However, the mechanisms by which carnitines control male fertility are not yet understood. In this study, antiapoptotic effects of L-carnitine in rat testes exposed to gamma (γ)-radiation were examined by light microscopy, electron microscopy and terminal deoxynucleotidyltransferase-mediated deoxyuridine triphosphate in situ nick end-labeling (TUNEL) technique.

Materials and methods

Animals

Wistar albino male rats were obtained from the Experimental Animal Center of Trakya University Medical Faculty. The animals were housed at 21°C under a 12-h (h) light–dark cycle and were allowed tap water and standard pellet diet for rats (Gebze Food Factory, Kocaeli, Turkey). The study was approved by the local animal ethics committee.

Experimental design

Eighteen rats were randomly divided into three groups of six rats each. Group 1 (control group) received sham irradiation plus 0.2 ml intraperitoneally (i.p.) physiological saline, as placebo. Group 2 (radiotherapy group) received scrotal γ-irradiation of 10 Gy as a single dose plus 0.2 ml i.p. physiological saline. Group 3 (radiotherapy + L-carnitine group) received scrotal γ-irradiation plus 200 mg/kg i.p. L-carnitine (CARNITINE ampule, Sigma-tau, Pomezia, Italy) starting 1 day before irradiation. The dose of L-carnitine used in the present study (200 mg/kg) was based on previous reports (Ramadan et al. 2002; Ucüncü et al. 2006). Because of L-carnitine has been reported to be well tolerated without any toxic effects over the dose range 100–300 mg/kg (Ucüncü et al. 2006), its middle dose was chosen in our study protocol.

Scrotal γ-irradiation

Prior to radiotherapy, the rats were anesthetized with xylazine/ketamine (10/90 mg/kg, i.p.) and immobilized from their 4 extremities on a tray. Irradiation was delivered by a cobalt-60 (^{60}Co) teletherapy unit (Cirus, *cis*-Bio Int., Gif Sur Yvette, France) at a source-surface distance of 65 cm. A single dose of 10 Gy radiation was given at a depth of 1.5 cm (half thicknees) with a dose rate of 1.05 Gy/min to an area of 5 × 5 cm of the scrotum in a supine position. Control group received equal-field sham irradiation.

Sample collection

Twenty-four hours following irradiation, the rats were sacrificed by cervical dislocation. Testes from each animal were dissected out and weighed with tunica albuginea intact. The specimens of the all groups were processed for histopathological examination, and apoptosis was detected using the TUNEL technique.

Light microscopic examination

Testis tissues were fixed in Bouin's solution, conventionally dehydrated and embedded in paraffin blocks. Five micrometer (μm) sections were obtained by a manual microtome and stained with hematoxylin-eosin and periodic acid-Schiff.

The morphometric parameters were measured on paraffin sections that were stained with hematoxylin-eosin. The tubular diameter (minor axis) and the height of seminiferous tubule epithelium were measured at 200× magnification under a light microscope (Olympus Cx 31) using a micrometric ocular lens. At least 30 tubular profiles that were round or nearly round were chosen randomly and measured for each animal. The height of the epithelium was obtained from the same tubules utilized to determine tubular diameter.

Evaluation of germ cell apoptosis

Germ cell apoptosis was evaluated by the TUNEL assay. The TUNEL method, which detects fragmentation of DNA in the nucleus during apoptotic cell death in situ, was

employed using an apoptosis detection kit (TdT-Fragel™ DNA Fragmentation Detection Kit, Cat. No. QIA33, Calbiochem, USA). All reagents listed below are from the kit and were prepared following the manufacturer's instructions. Deparafinised and rehydrated 5-μm-thick testis sections were incubated with 20 mg/ml proteinase K for 20 min and rinsed in Tris Buffered Saline (TBS). Endogenous peroxidase activity was inhibited by incubation with 3% hydrogen peroxide. Sections were then incubated with equilibration buffer for 10–30 s and then TdT-enzyme, in a humidified atmosphere at 37°C, for 90 min. They were subsequently put into pre-warmed working strength stop/wash buffer at room temperature for 10 min. Sections were incubated with anti-digoxigenin-peroxidase antibody for 30 min at room temparature and the signals were visualized with diaminobenzidine. Sections were counterstained with methyl green, and sections were dehydrated, cleared and mounted.

The number of apoptotic cells was evaluated by counting the positively (dark-brown) stained nuclei in 30 cross sections of seminiferous tubules per testis section.

Transmission electron microscopic examination

For electron microscopical observation, testis specimens of 1 × 2 mm size were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer and after primary fixation tissues were washed in 0.1 M phosphate buffer overnight. The tissues were postfixed with 1% osmium tetraoxide in phosphate buffer for 1 h at 4°C. Then the postfixed tissues were washed in 0.1 M phosphate buffer and dehydrated by graded ethyl alcohol and finally with propyleneoxide. Dehydrated tissues were processed for making araldite blocks. Ultrathin sections were obtained by ultramicrotome (RMC-MTX Ultramicrotome-USA) and collected on copper grids for double staining (uranyl acetate and Reynold's lead citrate). Stained sections were finally observed under a Jeol-JEM 1010 transmission electron microscope.

Statistical analysis

Statistical analyses were performed with Statistica 7.0 software. A P value <0.05 was considered statistically significant. The data obtained from morphometric measurements were expressed as mean ± standard deviation (SD). Normality distribution of the variables were tested using one sample Kolmogrov-Simirnov test. Differences in measured parameters among the groups were analyzed by one-way analysis of variance test due to normal distribution. For TUNEL, the data were expressed as median (min–max). Normality distribution of the variables were tested using one sample Kolmogrov-Simirnov test. Differences in measured parameters among the groups were analyzed with a nonparametric Kruskal–Wallis test due to non-normal distribution. When a significant difference was found, Mann–Whitney U tests with correction were performed to compare the control with samples of radiotherapy and radiotherapy + L-carnitine groups (P: α/n).

Results

The mean testis weight, seminiferous tubule diameter and epithelium height

Ionizing radiation caused no statistically significant alterations in testis weights in relation to the individual body weights, the tubular diameter and the height of seminiferous tubule epithelium at 24 h post-irradiation. Overall, no statistically significant differences were determined between the L-carnitine treated group and the radiotherapy group (Data not shown).

Light microscopic findings

On histopathological examination, control rat testes showed normal morphology and spermatogenesis, containing abundant amounts of spermatids and sperm in the lumen (Fig. 1a, b). In contrast to control, the arrangement of the cells was disturbed in the seminiferous tubules of γ -irradiated rats. Germinal epithelial cells were separated from each other and the tubular basement membrane. There was desquamation of germinal cells and consequent appearance of irregular spaces in the epithelium and spermatogenic cells were also decreased. The numbers of spermatozoa in the lumen were significantly low (Fig. 1c, d). However, seminiferous tubule basement membranes in this group of animals were observed to be normal (Fig. 1d).

L-carnitine treatment improved the radiation-induced histopathological changes in rat testes. The number of spermatogenic cells in the L-carnitine-treated rats was higher compared to the radiotherapy group and the disturbance in the arrangement of the cells was slight in this group (Fig. 1e, f).

Electron microscopic findings

Under electron microscopy, the seminiferous tubule sections of the control group showed a normal histology (Data not shown). After irradiation, most of the anormalities were found in spermatogonia. Using electron microscopic analysis, the most common type of cell death caused by irradiation was found to be apoptosis. The morphological signs of apoptosis were frequently detected in spermatogonia, whereas most of the spermatocytes and spermatids had

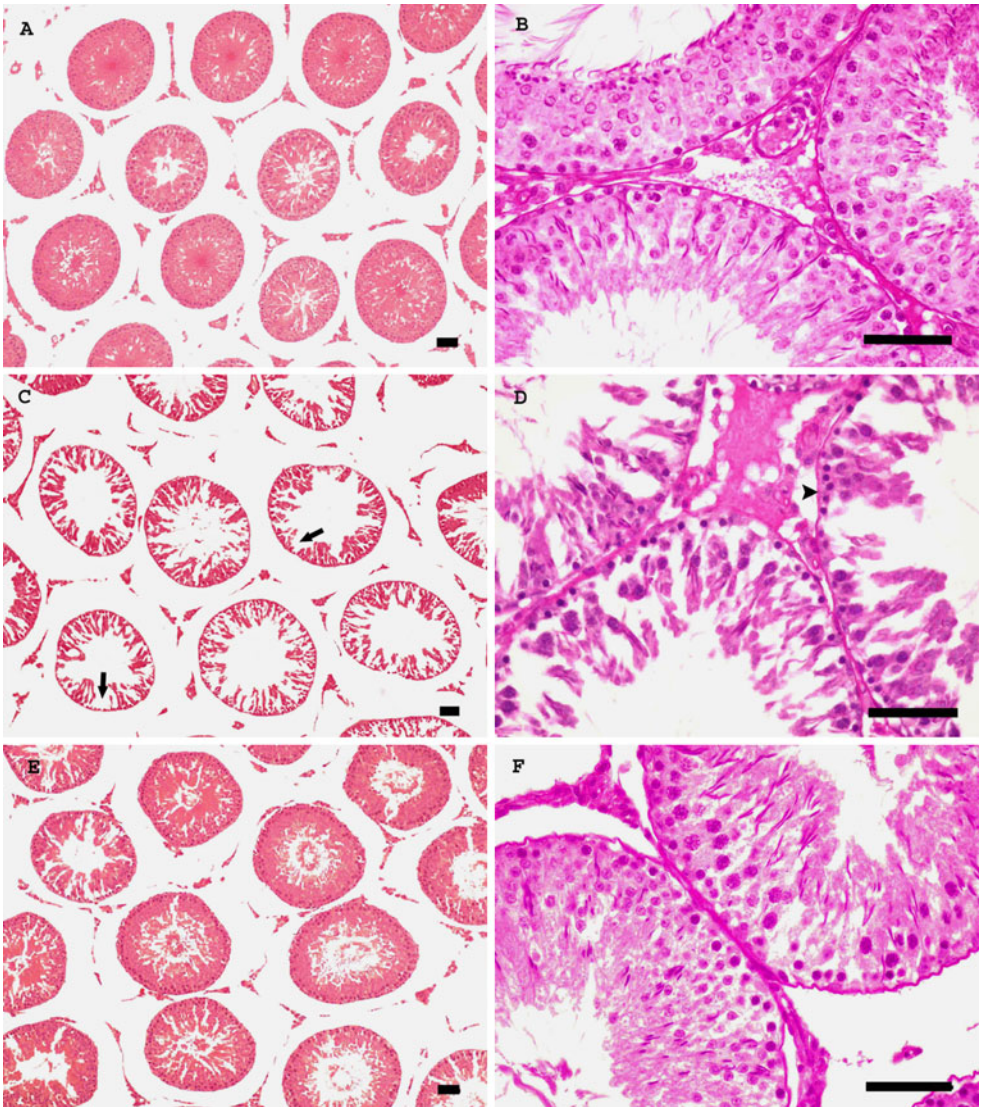

Fig. 1 Light micrographs of testicular tissue. **a, b** Control group. Testis shows normal seminiferous tubule morphology. Note the orderly arrangement of germinal cells. **c, d** Radiotherapy group. Disorder of the germinal cells arrangement and irregular spaces (*arrow*) in the epithelium due to the desquamation of cells are seen in the seminiferous tubules of irradiated rats, but tubular basal membrane (*arrowhead*) is relatively normal. The numbers of spermatozoa in the lumen are seen to be low. **e, f** Radiotherapy + L-carnitine group. Treatment with L-carnitine resulted in almost normal seminiferous tubule morphology. The germinal cells appear well preserved. (**a**, **c**, **e** Hematoxylin-eosin, **b**, **d**, **f** Periodic acid-Schiff). *Scale bars*: 50 μm

retained their normal appearance. Apoptotic spermatogonia showed the marginal condensation of chromatin onto the nuclear lamina, nucleus and cytoplasm shrinkage and still functioning cell organelles. In addition, dilatation of smooth endoplasmic reticulum cysternas were detected in the cytoplasm of Sertoli cells (Fig. 2a, b).

An ultrastructural examination of seminiferous epithelium in the L-carnitine treated group revealed little evidence of damaged cellular structure. The treatment of L-carnitine partially reduced radiation-induced apoptotic changes in spermatogonia (Fig. 2c, d).

Evaluation of germ cell apoptosis

TUNEL-positive cells for each group are represented in Table 1. The number of TUNEL-positive cells in the control group was negligible (Fig. 3a). Testicular irradiation resulted in a marked increase in germ cell apoptosis compared to control testis ($P < 0.005$) (Fig. 3b). TUNEL-positive cells were commonly spermatogonia (Fig. 3c). Treatment of L-carnitine resulted in a significant decrease in programmed germ cell death in the irradiated testis ($P < 0.005$), compared to irradiated-untreated animals (Fig. 3d).

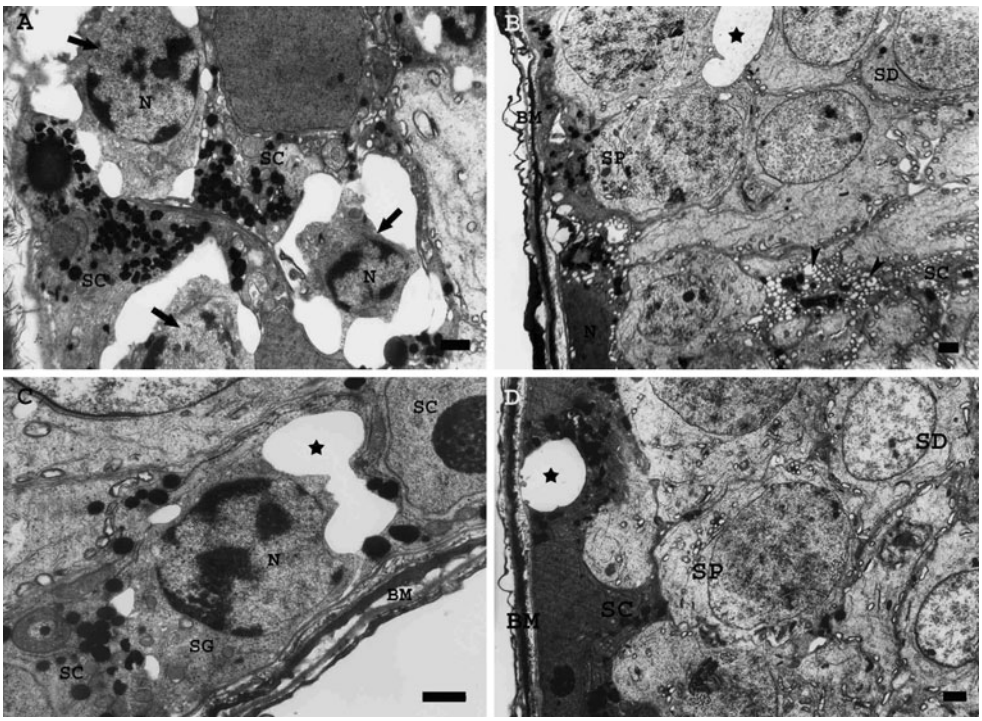

Fig. 2 Electron micrographs of seminiferous tubules from irradiated-rats and L-carnitine-treated rats. **a, b** Radiotherapy group. Note the apoptotic spermatogonia (*arrow*) with nuclear chromatin condensation at the nuclear periphery and shrinkage of cytoplasm and nucleus (N). Slightly dilated smooth endoplasmic reticulum cisternae (*arrowhead*) can be seen in the cytoplasm of Sertoli cells. **c, d** Radiotherapy + L-carnitine group. L-carnitine treatment partially prevented the dilated cyternae of smooth endoplasmic reticulum and shrinkage of cytoplasm. Enlarged intercellular spaces (*asteriks*), basal membrane (BM), Sertoli cell (SC), spermatogonia (SG), primary spermatocyte (SP), spermatid (SD). *Scale bars*: 1 μm

Table 1 Effect of testicular irradiation and treatment with L-carnitine on germ cell apoptosis in rat testis

	Control	Radiotherapy	Radiotherapy + L-carnitine	*P*
TUNEL-positive cells	0 (0–2)	3 (0–30)**	1 (0–20)***	<0. 000*

Values are median (min–max), ($n = 6$)

* Kruskal–Wallis variance analysis, statistically significant; ** $P < 0.005$ compared to control group; *** $P < 0.005$ compared to radiotherapy group

Discussion

The present study demonstrated that scrotal γ irradiation of 10 Gy as a single dose caused histopathological changes in rat testis and L-carnitine pretreatment improved these changes. Besides, the number of TUNEL-positive cells in the germinal epithelium was significantly increased in the irradiated rats, an indication of apoptosis induced by γ radiation in male rats. However, the number of TUNEL-positive cells was reduced after L-carnitine treatment in irradiated rats.

Apoptosis described as programmed cell death, is a physiological phenomenon characterized by cellular morphological and biochemical alterations that cause a cell to commit suicide (Vaux and Flavell 2000). Animal studies have suggested that apoptosis is a key regulator of spermatogenesis in normal and pathological states (Kerr 1992). Relatively high rates of apoptosis have been reported in testicular biopsies from infertile men with various degree of testicular insufficiency (Juriscova et al. 1999). An altered apoptotic process has been observed to be closely associated with male infertility (Lin et al. 1997).

The cell- and stage-specific mode of apoptosis induction in the seminiferous epithelium depends on the chemical type used, probably due to the difference in its activity and mechanisms of action and in the susceptibility of male germ cells at different stages of spermatogenesis (Kim et al. 2003; Nandi et al. 1999; Shin et al. 1999; Krishnamurthy et al. 1998). It is well known that ionizing radiation exerted oxidative stress on the testis and induced apoptosis primarily in the germ cells (Lee et al. 2002). Sensitivity to radiation-induced apoptosis was highest in the spermatogonia and

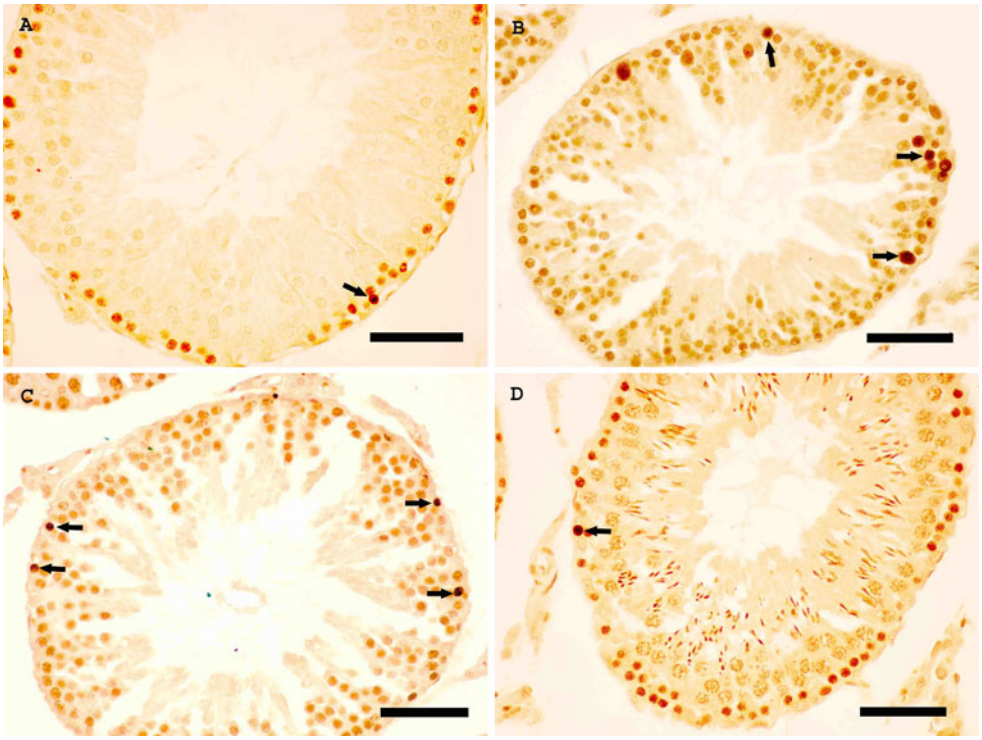

Fig. 3 In-situ end labelling of DNA fragmentation on testis sections. **a** Testis from control rat. Few TUNEL-positive germ cells are observed in the seminiferous epithelium. **b** Testis from irradiated rat. Labelled cells are frequently found in the seminiferous epithelium. **c** Positivity is mainly observed in spermatogonia of testis from irradiated rats. **d** Testis from L-carnitine-treated rat. Labelled germ cells are rarely observed in the seminiferous epithelium. TUNEL-positive germ cells (*arrow*). *Scale bar*: 50 μm

spermatocytes, while the Sertoli and Leydig cells were comparatively resistant (Pinon-Lataillade et al. 1988; Vergouwen et al. 1995). In the present study, apoptosis occurred predominantly in spermatogonia the most radiosensitive cell type, as reported by other previous studies (Hasegawa et al. 1997; Henriksen et al. 1996). Mauduit et al. (2001) have shown an increase in premeiotic apoptotic germ cell number was detected after 24 h post-irradiation and reached a maximum after 48 h post-irradiation. Previous studies reported that the maximal number of apoptotic premeiotic germ cells (evaluated by TUNEL approach) was reached after 12–18 h post-irradiation (Hasegawa et al. 1997) or 42 h post-irradiation (Henriksen et al. 1996).

The mode of cell death of the spermatogonia was further characterized by microscopy and the TUNEL assay. In this study, TUNEL-positive cells were commonly spermatogonia and the typical morphological features of apoptosis were frequently detected in spermatogonia by electron microscopy. Apoptotic spermatogonia showed the marginal condensation of chromatin into the nuclear lamina, nucleus and cytoplasm shrinkage and still functioning cell organelles, as reported by other previous studies (Andriana et al. 2004; Saraste 1999; Liepins and Bustamante 1994).

L-carnitine exhibits a wide range of biological activities including anti-inflammatory (Izgut-Uysal et al. 2003), neuroprotective (Onem et al. 2006), cardioprotective (Ferrari et al. 2004) and gastroprotective (Dokmeci et al. 2005), properties. In the previous studies, it was reported that L-carnitine was effective in preventing radiation-induced oral mucositis (Ucüncü et al. 2006), cataract (Kocer et al. 2007), bone marrow (Dokmeci et al. 2006) and testicular (Ramadan et al. 2002; Amendola et al. 1989) toxicity. Furthermore, these effects are attributed to its antioxidative and free radical scavenging activity and it also acts on cellular DNA and membranes, protecting them against damage induced by free oxygen radicals. L-carnitine has been recently shown to act as an important anti-apoptotic mediator (Ishii et al. 2000; Moretti et al. 2002). Inhibition of apoptosis by L-carnitine has been reported in primary cultured neuronal cells (Ishii et al. 2000), non-neuronal cell lines like P 19 teratoma cells (Galli and Fratelli, 1993) and C2.8 hepatocyte (Revoltella et al. 1994). L-carnitine enhances the activity of the DNA repairing enzyme poly(ADP-ribosyl) polymerase and other related repair mechanisms (Boerrigter et al. 1993). In additional to these reports, Sundaram and Panneerselvam (2006) reported that L-carnitine reduced DNA single strand breaks in skeletal muscle of aged rats.

Carnitine's protective role is further sustained by reducing toxicity and accelerating repair processes following

physical (Amendola et al. 1991; Ramadan et al. 2002) and chemical (Palmero et al. 1990) damages to the testicular parenchyma. However the molecular mechanism of action by which carnitine system affects male fertility has not been completely clarified. Our results illustrate that pretreatment of rats with L-carnitine 1 day before exposure to γ radiation significantly attenuated the radiation-induced morphological changes and germ cell apoptosis in the irradiated testis. To our knowledge, this is the first report of any relationship between L-carnitine and testicular germ cell apoptosis following irradiation.

Infertility after irradiation is caused by the spermatogonia undergoing apoptosis instead of differentiation (Boekelheide et al. 2005). The ability of the testis to recover spermatogenesis depends on the survival of some stem spermatogonia and their ability to repopulate the testis with differentiating cells (Shetty and Meistrich 2005). Amendola et al. reported that the administration of exogenous L-acetylcarnitine influences the early stages of the spermatogenic process by affecting mainly spermatogonial cells, thus allowing a faster recovery after irradiation with 10 Gy *x*-rays (1989) and after hyperthermi (1991) in mice. L-acetylcarnitine represents a free available source of acetyl groups substaining the energy needs of these cells engaged in processes requiring high metabolic activity (Amendola et al. 1989, 1991). In a previous work (Topcu-Tarladacalisir et al. 2009), we have demonstrated that the administration of L-carnitine enhanced the spermatogenic recovery after irradiation in rats. This may be attributed to the protective effect of L-carnitine against radiation-induced germ cell apoptosis.

L-carnitine is an endogenous substrate that is widely distributed among tissues including the male reproductive organs (Hinton et al. 1979). This compound readily penetrates across the cell membrane and acts as an essential co-factor for the transport of long-chain Acyl CoA through the inner mitochondrial transport functions such as energy metabolism (Rosenthal et al. 1992) and pyruvate dehydrogenase activity (Bogaert et al. 1994). It is well known that one of the initial events of apoptosis is cytochrome c release from mitochondria to the cytoplasm (Kluck et al. 1997) due to mitochondrial dysfunction. The antiapoptotic action of L-carnitine, therefore, is likely to be releated to recovery from mitochondrial dysfunction (Ishii et al. 2000).

In the present study, we demonstrated that the number of apoptotic germ cells significantly increased in the irradiated rat testis compared with the control testis. Treatment with L-carnitine prior to irradiation decreases the germ cell apoptosis, suggesting that L-carnitine can protect testes from radiation injury. Consequently, the present results suggest that L-carnitine may be beneficial to spermatogenesis and infertility following testicular irradiation by decreasing germ cell apoptosis. However, further investigations and clinical studies are required to elucidate the exact mechanism of antiapoptotic effects of L-carnitine in testis.

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