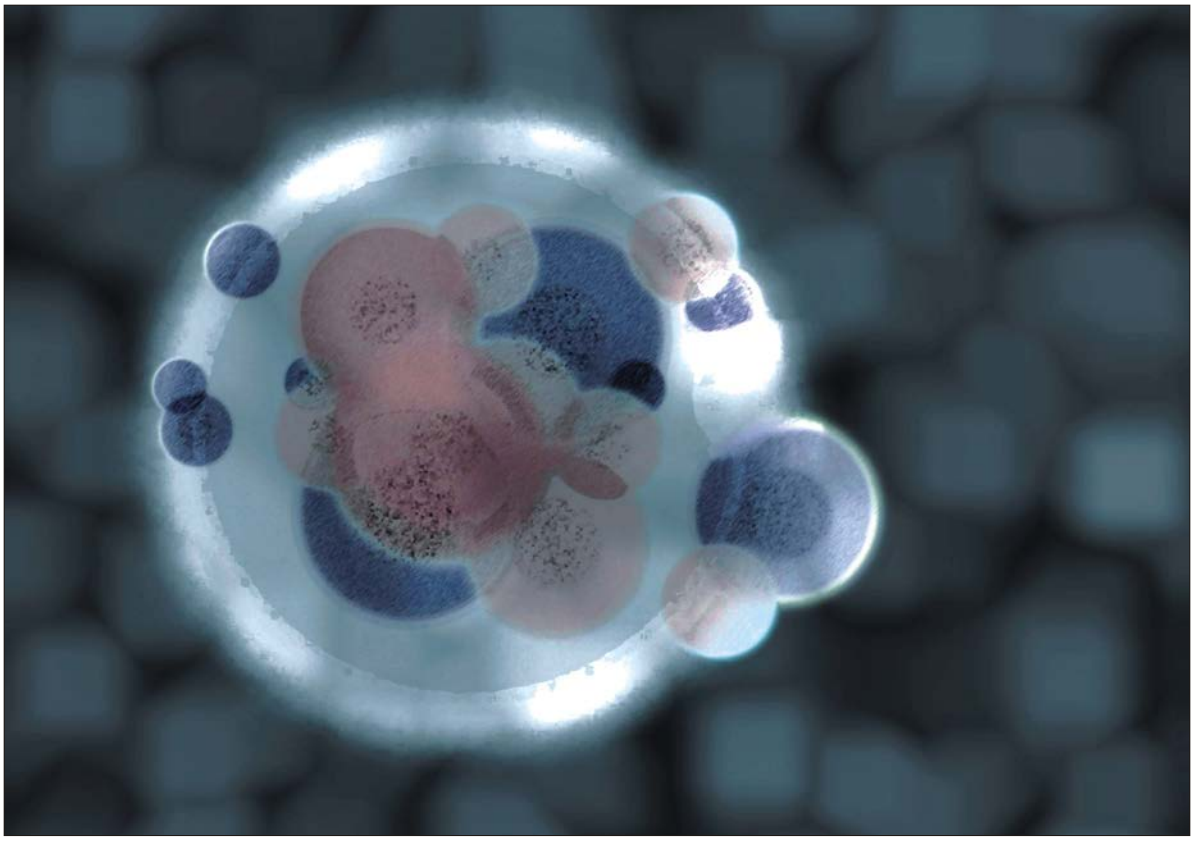

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Low Doses of Gamma Radiation may Impair Testicular Tissue in a Rat Treated CCl_4 Model: Role of BM Transplantation

N. Hanafi
Department of Radiation Biology, National Centre for Radiation Research and Technology (NCRRT), P.O. Box 29 Nasr City, Egypt

Abstract: Treatment of carbon tetrachloride (CCl_4) 1 mL kg^{-1} in olive oil (1:1) twice a week for 8 weeks to albino male rat caused a significant increase in serum level of alamine transaminase (ALT) and aspartate transaminase (AST). Injury to liver, resulting in loss of its normal physiological/biochemical functions, may adversely affect a secondary organ like testis. In the current study young adult rats were treated by Carbon tetrachloride (CCl_4) two times per week and/or continuously whole body γ-irradiated (R) at a dose level 0.5 Gy, two times per week for 8 weeks. The previous groups were treated by bone marrow transplantation (BMT). Plasma estradiol and testosterone concentrations in animals sera were analyzed. Histopathological, apoptosis and necrosis examinations were done in testicular tissues. Either CCl_4 or R exposure alone or combined reflect testicular seminiferous tubules atrophy, peritubular fibrosis and apoptotic cells in seminiferous epithelium and Leydig cells. BMT reflect some recurrence of normal structure in testis tissue of CCl_4 group. Meanwhile R and CCl_4 R groups showed atrophied testicular seminiferous tubules, great interstitial hyperplasia, deposition of collagen fibres around blood vessels and presence of interstitial apoptotic and necrotic tissue cells. CCl_4 treatment recorded anon significant change in plasma testosterone and a significant decrease in estradiol concentration. γ-irradiation either alone or combined with CCl_4 treatments recorded a significant reduction in testosterone level and significant increase in estradiol concentration. BMT recorded a significant increase in testosterone level and anon significant change in estradiol level following CCl_4 or irradiation either alone or combined. In conclusion low doses of γ- radiation impair testicular tissue in a rat treated CCl_4 model. BM transplantation recorded increase in this testicular damage.

Key words: Liver, testis, carbon tetrachloride, γ-irradiation

INTRODUCTION

Carbon tetrachloride is used extensively in experimental models to induce oxidative stress in rats (Onori *et al*., 2000; Nabeshima *et al*., 2006; Noori *et al*., 2009). A single dose of CCl_4 can rapidly lead to both oxidative stress and acute liver injuries such as centrilobular necrosis and steatosis in rats (Weber *et al*., 2003; Lin *et al*., 2008; Khan and Alzohairy, 2011). Previous data demonstrated that rats with advanced liver cirrhosis showed reduced testicular size and weight and severe histopathological testicular abnormalities, including reduced tubular diameters, loss of the germinal line and diminutions in cellular proliferation and spermatogenesis (Castilla-Cortazar *et al*., 2000). Also previous data demonstrated that rats treated by CCl_4 showed histopathological testicular abnormalities and loss of the germinal line (Khan and Ahmed, 2009).

The biological effects of low-level radiation have attracted the attention of investigators for more than 20 years. The biological effects of low-level radiation on cellular metabolisms and defence systems sometimes called hormesis (Feinendegen, 2005) by increased immune responses and antioxidant capacity (Lee *et al*., 2000; Gong *et al*., 2000; Joksic and Petrovic, 2004). However, Liu *et al*. (2006) reported that the exposure of the experimental animals to low-level radiation induces increased apoptosis in male germ cells. Bone marrow transplantation (BMT) is increasingly used in the therapy of solid malignancies, as well as in non-malignant disorders such as thalassemia and immunodeficiency (O'Reilly, 1983; Champlin and Gale, 1987). Also the preclinical and clinical studies have demonstrated that bone marrow stromal cells (MSCs) can be used for tissue repair (Yoon *et al*., 2005). The successful application of Bone Marrow Transplantation BMT to the treatment of several potentially fatal disorders (Storb *et al*., 1976; Powles *et al*., 1980; O'leary *et al*., 1983) has resulted in a variety of late complications on gonadal function and future fertility.

The present study was done to recognize the effect of exposure to low dose of γ-radiation on testis tissue in rat treated CCl_4 model and the role of BM transplantation.

MATERIALS AND METHODS

Experimental animals: Male Swiss albino rats (100-120 g) purchased from the Egyptian Organization for Biological Products and Vaccines were used for the different investigations carried out in this work. Animal maintenance and treatments were conducted in accordance with the National Institute of Health Guide for Animal, as approved by Institutional Animal Care and Use Committee (IACUC). Animals were housed in specially designed cages and maintained in conditions of good ventilation, normal temperatures and humidity ranges and kept under observation for one week prior to experimentation. The rats were fed on standard pellets, containing all nutritive elements (proteins, fats, carbohydrates, vitamins, salts and minerals). Drinking water and food were provided *ad libitum* throughout the study.

Radiation facility: Whole-body γ-irradiation was performed at the National Centre for Radiation Research and Technology (NCRRT), Cairo, Egypt, using an AECL Gamma Cell-40 biological irradiator. Animals were irradiated at dose level of 0.5 Gy 2 times/week for 8 weeks. The γ-irradiation delivered at a dose rate of 0.46 Gy/min.

Rat bone marrow preparation: Donors and recipients rats were chosen of the same inbred strain, brother to brother (isologues or synergic or allogeneic transplantation). Rats sacrificed by exposure to ether in a dessicator kept in a well-functioning hood. Femur bones were dissected out and cleaned. The ends of the bones were chipped by a bone nibbling forceps. Then the marrow was blown out of the femur into isotonic solution under sterilized conditions inside a laminar flow cabinet. The marrow was collected into a sterile container surrounded by ice cubes and mixed by drawing and expelling it several times from the syringe without needle in order to avoid mechanical damage to the cells. Total viable cells of about 75×106±5% were injected intravenously (IV) through the caudal vein. All Rats treated with bone marrow cells transplantation were killed after four weeks of bone marrow cells transplantation.

Carbon tetrachloride administration: Rats were intraperitoneally injected with 1 mL kg^{-1} of carbon tetrachloride (CCl_4) dissolved in olive oil (1:1) twice a week for 8 weeks.

Experimental design: A total of 48 rats were divided into the following sub groups.

Non Bone marrow administrated groups including:

- **Control group (C):** untreated normal rats
- **Irradiated group (R):** group of animals exposed to 0.5 Gy of γ- radiation two times/week for 8 weeks
- **Carbon tetrachloride administrated group (CCl_4):** group of animals treated by CCl_4 twice a week for 8 weeks
- **Irradiated and carbon tetrachloride treated group (CCl_4 R):** group of animals exposed to 0.5 Gy of γ-radiation 2 times/week and treated by CCl_4 twice a week for 8 weeks

Bone marrow administrated groups including:

- **Bone marrow treated group (BM):** group of control animals treated with bone marrow cells transplantation and killed after four weeks of bone marrow cells transplantation
- **Irradiated bone marrow transplantation group (RBM):** group of animals irradiated for 4 weeks then treated with bone marrow cells transplantation followed by exposure to γ-radiation for another 4 weeks
- **CCl_4 bone marrow transplantation group (CCl_4 BM):** group of animals treated by CCl_4 for four weeks and then treated with bone marrow cells transplantation followed by the same dose of CCl_4 treatment for another four weeks
- **CCl_4 R bone marrow transplantation group (CCl_4 RBM):** group of animals treated by CCl_4, exposed to γ-radiation for 4 weeks then treated with bone marrow cells transplantation followed by the same dose of CCL_4 and γ-radiation exposure treatments for another four weeks

Histopathological examination: Excised liver and testis tissues from each rat were fixed in 100 mL L^{-1} neutral formalin, embedded in paraffin and stained with hematoxylin-eosin (HE) and the fibrous lesion areas were determined via Masson's trichrome method which is used to stain collagen fibers.

Apoptosis and necrosis examination: For apoptosis and necrosis examination according to Bank (1988) fluorescence microscopy was used. Deparaffinization was done by immersing tissue sample slides in 3 changes of xylene for 5 min each followed by washing in graded alcohol as follows: 100, 95, 80 and 50% for re-hydration. Two changes for 3 min in each alcohol concentration were done. Then, they were rinsed in 3 changes of PBS. Afterwards, slides were directly incubated in (5 μg mL^{-1}

of propidium iodide and 50 μg mL^{-1} of acridine orange in phosphate-buffered saline) in dark for 20 min at room temperature.

Analysis of biochemical assay: Serum obtained from the blood samples were analyzed for aspartate aminotransferase (AST) (Bergmeyer *et al*., 1985), alanine aminotrasnferase (ALT) (Klauke *et al*., 1993) and total protein (Keller, 1984) by using kit purchased from Stanbio (USA). However, serum testosterone and estradiol ware quantitatively determined in the sera by enzyme immunoassay kit (Meddix Bioech Inc, 420 Lincoln Centre Drive, Foster City, CA 94404, USA, Catalog Number: KEF4057).

Statistical analysis: Statistical analysis for obtained results was carried out with the aid of the SPSS computer software program.

OBSERVATIONS AND RESULTS

At baseline, in Table 1 compared to control level ($p<0.05$) CCl_4 treated group showed a significant increase in serum ALT and AST levels but total proteins represented, no significant change. Exposure of control rats to fractionated low dose of γ-radiation recorded a no significant change in serum ALT and AST levels but total proteins represented, a significant increase compared to control level ($p<0.05$). On the other hand exposure of CCl_4 treated group to fractionated low dose of γ-radiation recorded a decrease in serum levels of ALT, AST but total proteins level recorded a significant increase in compression to CCl_4 group level ($p<0.05$). Bone marrow transplantation in control animals represented a significant decrease in serum ALT, a significant increase in serum AST and no significant change in total proteins. However, BM transplantation in CCL_4 or CCl_4 R groups showed ameliorating effect in AST, ALT and total proteins levels compared to CCl_4 treated group.

Histopathologically liver tissue sections in rats suffered from CCl_4 treatment (two times per week) for eight weeks showed hepatocytes degeneration, necrosis, mononuclear cells and neutrophil infiltration. Also, collagen fibers extend within the hepatic plate was observed. Bone marrow cells transplantation showed normal hepatic tissue section. Exposure of the CCl_4 treated group to 0.5 Gy (two times per week) for eight weeks showed some sort of regeneration in a considerable number of hepatocytes, inhibition of inflammatory cellular infiltration in many areas and less prominent of cytoplasmic vacuolation. On the other hand liver section in CCl_4 (two times per week for eight weeks) rats irradiated with 0.5 Gy and treated with bone marrow cells (one time at the fourth week) transplantation recorded that bone marrow cells helped very much in regain of most hepatocytes cellular structure. There are no signs of cell weakness as many hepatocytes appeared with well defined membranes, homogenous cytoplasm and healthy normal nuclei. Many mitotic figures were noted, blood sinusoids were normal in size and the blood vessel was well organized.

Table 1: Effect of CCl_4, radiation and BM either alone or combined on liver functions

	Parameters		
Groups	ALT (IU L^{-1})	AST (IU L^{-1})	TP (g dL^{-1})
C	44.42±3.33	132.10±5.64	5.73±0.51
CCl_4	55.01±2.87*	365.83±28.91#	5.56±0.20
R	43.82±2.08#	137.86±9.47#	7.10±0.56*#
CCl_4 R	51.64±4.88*	143.1±1.50*#	7.38±0.44*#
BM	30.78±2.26*#	190.89±17.69*#	5.60±0.32
CCl_4 BM	41.71±3.68*#	144.19±5.64*#	5.98±0.13#
RBM	36.69±4.04	206.38±17.32*	6.71±0.31#
CCl_4 RBM	39.32±2.23*#	253.1±20.52*#	6.57±0.65*#

Values are given as mean ±SD (n = 6), *Significantly different from control at 0.05, #Significantly different from CCl_4 treatment at 0.05

TESTICULAR TISSUE OBSERVATIONS

H and E stain: In Fig. 1 the light microscopic examination of the testis of control rats showed normal structure and completely enveloped by a thick capsule, tunica albuginea which is composed mainly of dense collagenous fibrous connective tissue. The structural components of the testis are the seminiferous tubules and interstitial tissues. The seminiferous tubules are two types of cells, the Sertoli cells, resting on the thin basal lamina (basement membrane) and the spermatogenic cells. These cells are many layers, namely, the spermatogonia, primary and secondary spermatocytes; spermatoids and finally mature spermatozoa. Treatment of the experimental animals by CCl_4 (two times per week) for eight weeks noted wide interstitial space, seminiferous tubuli in testis appear seriously damaged and animals show a decrease of tubular diameter, vacuolization on germinal epithelium, loss of germinal line, total or partial reduction of spermatogenesis and presence of abnormal spermatids (multinucleated cells and cells with an intense stained nuclei). Exposure of control rats to 0.5 Gy (two times per week) for eight week showed atrophy and decrease in size of seminiferous tubule, tubular profiles completely depleted of germ cells and some hyperplasia in the Leydig cell was observed. Increase in testicular disorganization was detected in seminiferous tubules when CCl_4 (two times per week for eight weeks) treated animals exposed to 0.5 Gy γ-radiations (two times per week for eight weeks). Seminiferous tubules in testis appear seriously

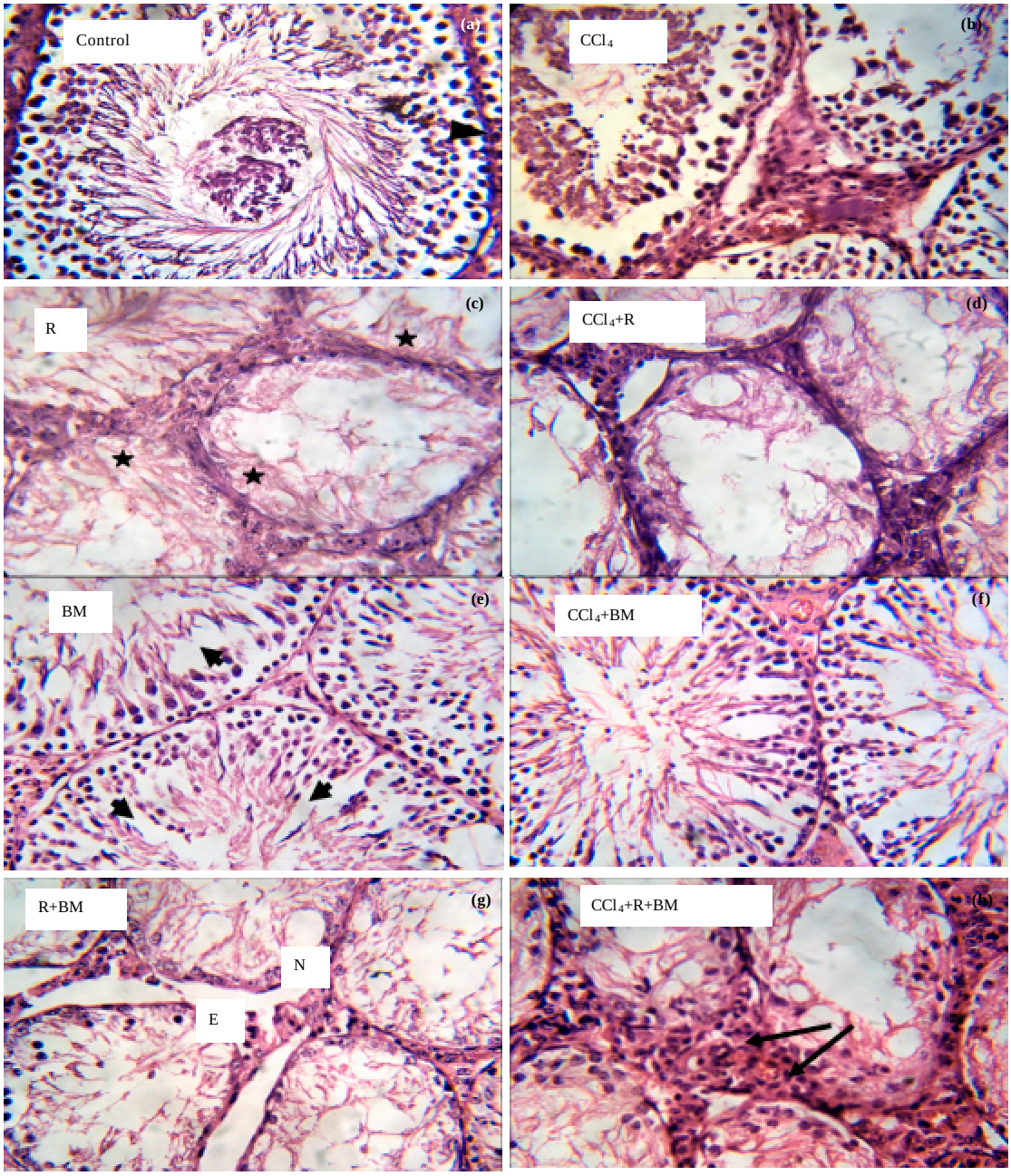

Fig. 1(a-h): Microscopy of testes (×400 magnifications, H&E stain). Testicular histological sections of rat demonstrated active spermatogenesis in normal-size seminiferous tubuli with thin basement membranes (▲). Leydig cells were scarce, being widely separated by seminiferous tubuli. BM represented discontinuous seminiferous epithelium (short arrow). Either CCl_4 or R exposure alone or combined reflect testicular seminiferous tubules atrophy and completely depleted of germ cells (✶). BM treatment of CCl_4 group reflects great recurrence of seminiferous tubules normal structure in testes. Exudation (E) into the interstitial space and degeneration/necrosis (N) of spermatogenic and great interstitial hyperplasia (long arrow) in R+BM and CCl_4+R+BM groups

Table 2: Effect of CCl_4, radiation and BM either alone or combined on testosterone and estradiol testicular hormones

	Groups							
Parameters	C	CCl_4	R	CCl_4 R	BM	CCl_4 BM	RBM	CCl_4 RBM
Testosterone (ng mL^{-1})	63.61±1.69	66.55±2.72	28.45±2.66*	47.502±2.26*#	42.88±2.63*	53.02±2.70*#	33.57±0.21*	38.43±2.01*!
Estradiol (pg mL^{-1})	5.11±0.37	3.03±0.25*	10.16±0.22*	6.598±0.51*#	6.08±0.45	4.10±0.33#	5.67±0.23	4.63±0.44!

Each value represents the mean of 6 records±SE. Means with different superscripts are significantly different at the 0.05 level.* significatly different from control, #significantly different from CCl_4. ! significantly different from CCl_4+BM and R+BM

damaged, decrease in tubular diameter, vacuolization on germinal epithelium, loss of germinal line, total reduction of spermatogenesis and increase in hyperplasia in the Leydig cell were observed.

Treatment of control group with BM cells represented discontinuous seminiferous epithelium. Great recurrence of seminiferous tubules normal structure in testis when experimental animals treated by CCl_4 (two times per week) for eight weeks and treated by bone marrow cells transplantation (one time at the fourth week) was occurred. Exudation into the interstitial space and degeneration/necrosis of spermatogenic cells were observed when rats irradiated for four weeks and then bone marrow cells transplantation occurred followed by irradiation again for another four weeks. Atrophied testicular seminiferous tubules and great interstitial hyperplasia were shown in testis of CCl_4 irradiated rats for four weeks, followed by bone marrow cells transplantation one time and continue the process of irradiation and CCl_4 treatments for another four weeks.

Masson's trichrome stain: In Fig. 2 treatment of the experimental animals with CCl_4 or low dose of γ- radiation exposure either alone or combined represented peritubular fibrosis or increase in collagen deposition. Treatment of the previous groups with BM represents a remarkable depletion in peritubular collagen deposition with its great deposition on blood vessels sides.

Apoptosis and necrosis observations: In Fig. 3 testicular sections of normal rat (Control) demonstrated anon apoptotic observations in seminiferous epithelium. CCl_4 treatment (two times per week for eight weeks) recorded the presence of apoptotic cells in seminiferous epithelium. Both 0.5 Gy of γ-radiation exposure (two times per week for eight weeks) or CCl_4 and 0.5 Gy irradiation (two times per week for eight weeks) treatments represented apoptosis in the Leydig cells. BM cells transplantation showed many apoptotic observations in the spermatogonia, sertoli cells and primary spermatocytes. Interstitial tissue represented necrotic observations. Treatment of CCl_4 group by BM cells transplantation recorded apoptotic and necrotic cells. Also treatment of irradiated animals either alone or combined with CCl_4 treatments by BM cells transplantation represented many interstitial apoptotic and necrotic tissue cells.

Analysis of testosterone and estradiol testicular hormones: In Table 2 testicular hormonal analysis of testosterone concentration was investigated in this study. A non-significant reductions in testosterone concentration mean values when compared with the control concentration ($p<0.05$) following CCl_4 treatment (two times per week for eight weeks). However, exposure of control rats to fractionated dose of γ-radiation (0.5 Gy two times per week for eight weeks) recorded a significant reduction in testosterone concentration level when compared with the control level ($p<0.05$). Combined treatment of the experimental animals by CCl_4 and γ-irradiation recorded a significant decrease in testosterone level compared to control or irradiated group. BM cells transplantation in rat's empire their testosterone level either alone or following CCl_4 or γ-radiation exposure registered a significant decrease comparing to control level.

Also Table 2 represented Estradiol levels in different studied groups. Estradiol levels showed a significant decrease for the treatment of the experimental rats by CCl_4. Meanwhile radiation exposure represented a significant increase in compression to control level. Combined treatment of CCl_4 and fractionated dose of γ-irradiation (0.5 Gy two times per week for eight weeks) recorded a significant increase in estradiol level compared to control or irradiated group levels. On the other hand BM cells transplantation in experimental rats recorded a non-significant change in estradiol level when compared with the control level ($p<0.05$) following CCl_4 treatment or radiation exposure.

DISCUSSION

The present study was designed in order to gain more insights into the effect of exposure to consecutive low dose of γ-radiation and bone marrow transplantation were evaluated in CCl_4 treated rats and the altered changes in testis tissue associated with liver disease.

Our study demonstrates that rats treated by CCl_4-induced many histopathological and biochemical altered changes in liver tissue show a severe testicular atrophy and gonadal insufficiency. Both testicular histopathological abnormalities and low levels of sex hormones have been described in previous years in patients with alcoholic and nonalcoholic cirrhosis (Pajarinen and Karhunden, 1994; Van Steenbergen, 1993).

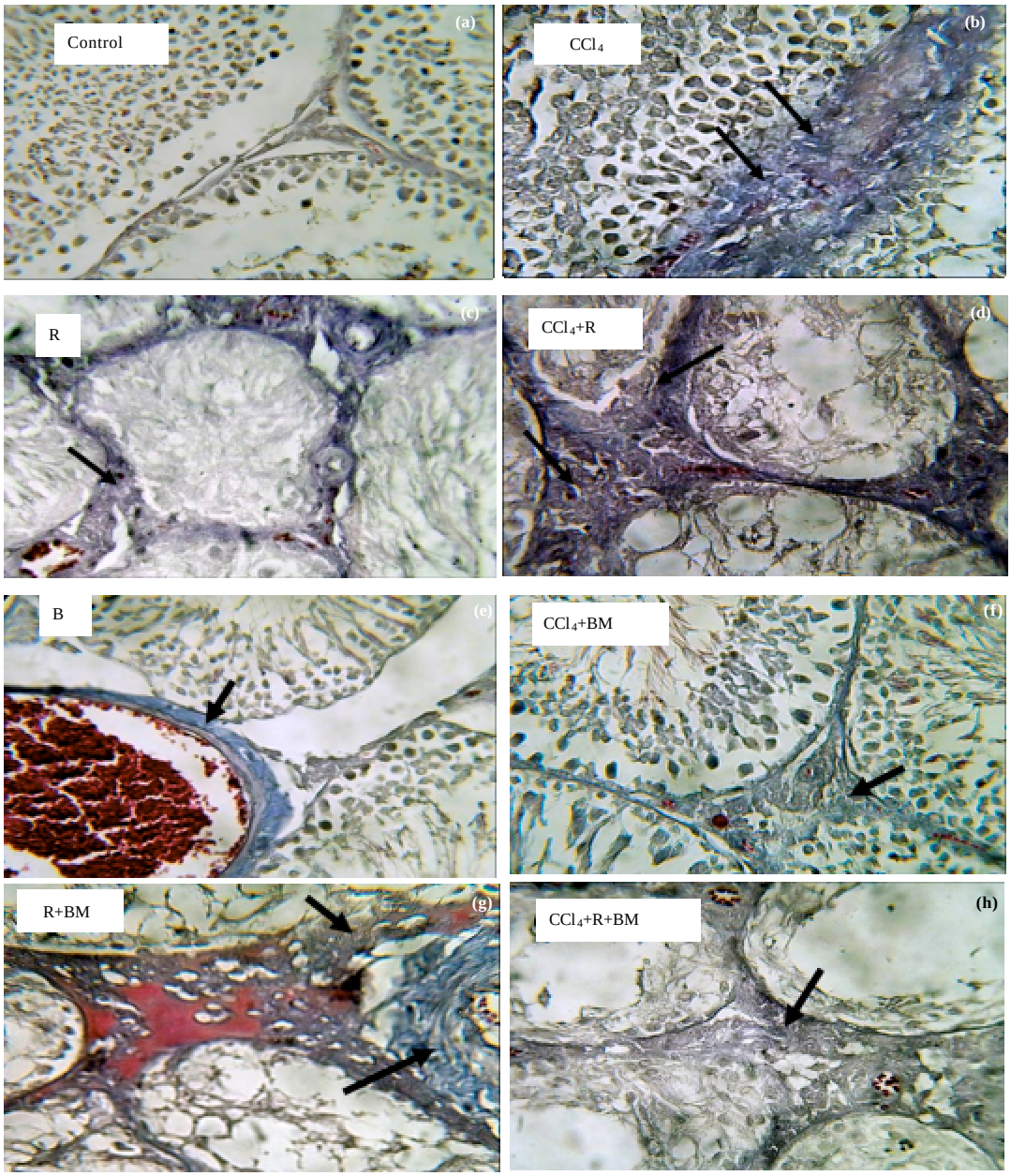

Fig. 2(a-h): Microscopy of testes (x400 magnifications, Masson's stain). Testicular histological sections of normal rat (Control) demonstrated minimal peritubular fibrosis. Evidence of peritubular fibrosis and other alterations were found in testes from R, CCl_4 and CCl_4+R treated animals. Treatment with BM recorded great deposition of collagen fibres (↑) around blood vessels.

Our data show a severe testicular damage as manifested by a variety of histopathological abnormalities that include alterations in tubular diameters, presence of aberrant cells in tubular lumen, peritubular fibrosis, loss of the germinal line and the presence of apoptotic cells in seminiferous epithelium. These alterations resemble those reported in experimental models of testicular damage, such as chronic testicular ischemia (Santamaria *et al*., 1995; Al-Jahdali and Bisher, 2007). The occurrence of testicular atrophy and gonadal dysfunction in advanced cirrhosis

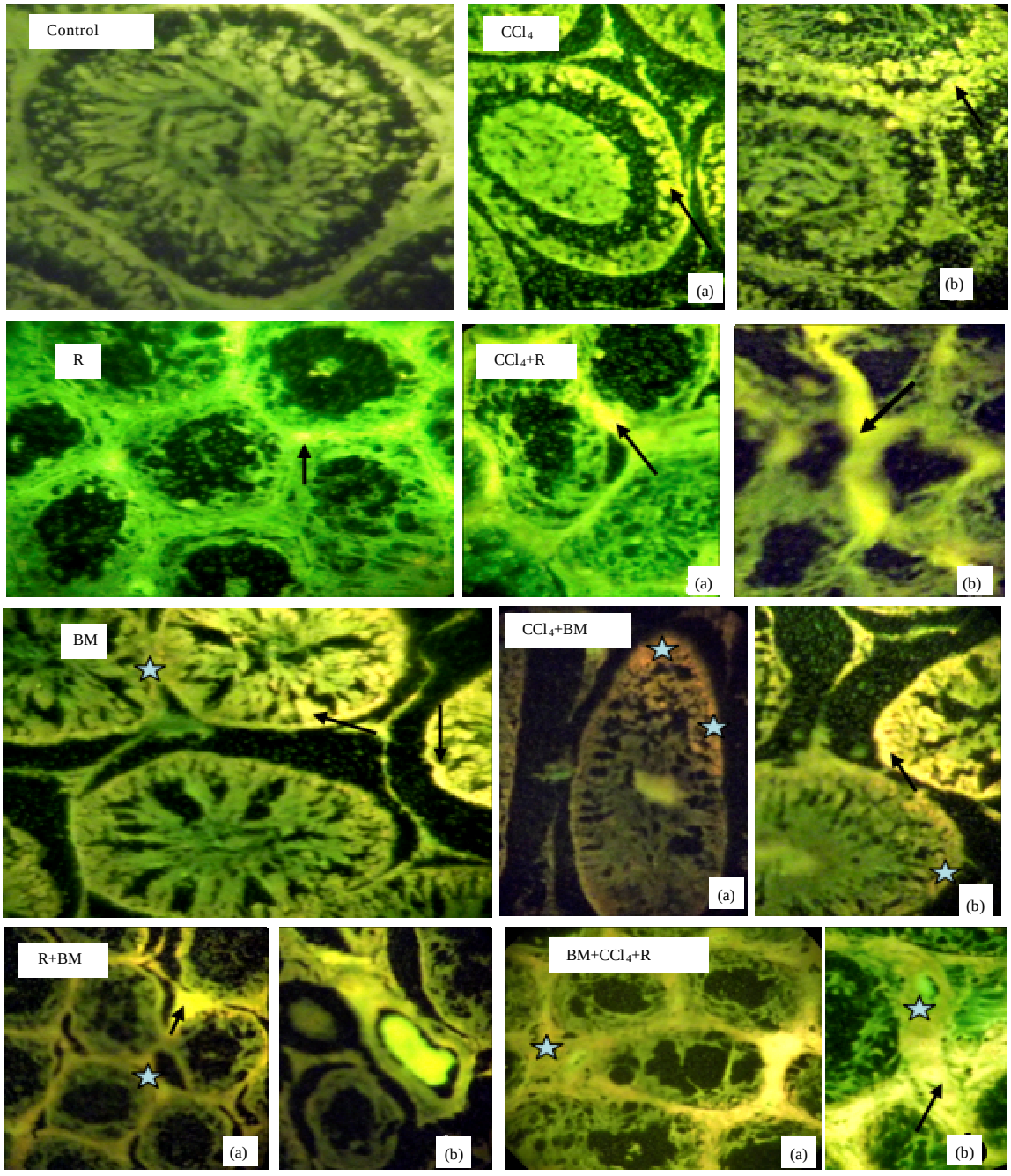

Fig. 3: Fluorescent Microscopy of testes (x400 magnifications, propidium iodide/acridine orange stain). Testicular sections of normal rat (Control) demonstrated anon apoptotic observations in seminiferous epithelium. CCl_4 treatment recorded the presence of apoptotic cells in seminiferous epithelium (↑). Either Radiation exposure or CCl_4 and irradiation treatments represented apoptosis in the Leydig cells (↑). BM transplantation showed many apoptotic observations in the spermatogonia, sortoli cells and primary spermatocytes. Interstitial tissue represented necrotic observations. CCl_4BM treatment recorded apoptotic (↑) and necrotic cells (✶). Treatment of irradiated animals by BM represented many interstitial apoptotic and necrotic tissue cells. Treatment of CCl_4 and irradiated group by BM also represented interstitial apoptotic (↑) and necrotic tissue cells (✶)

is a well-known clinical event (Van Thiel *et al*., 1980; Bannister *et al*., 1986; Pajarinen and Karhunden, 1994).

Ranl *et al*. (2007) proposed that the cause of testicular histopathology may be attributed to the malfunctioning of liver (Lox, 1984) which causes general systemic toxicity due to some toxic factors in peripheral circulation which influence testicular functions.

The non significant change in testosterone level and the significant decrease in estradiol level of CCl_4 treated animals in our study was context with finding of Frezza *et al*. (1993).

According to Withers *et al*. (1974) changes in weight and size of the irradiated testis is related more to depletion and regeneration of much more numerous cells in various stages of differentiation and are not a direct indication of stem cell depletion.

Nomura and Yamaoka (1999) proposed that Low-dose gamma-ray irradiation reduces oxidative damage induced by CCl_4 in mouse liver and suggest that low-dose radiation relieved functional disorder at least in the liver of mice with active oxygen diseases.

In the present study γ-irradiation treatments represented great atrophy in testis tissue, pretubular fibrosis and apoptosis in the Leydig cells, context with the finding of Kangasniemi *et al*. (1996) and Meistrich *et al*. (1996) who proposed after exposure to low doses of gamma rays differentiating spermatogonia are killed. The depletion in spermatogonia resulted in a reduction in subsequent spermatozoa. Also, Konoplia *et al*. (1996) examined the microscopy morphological characteristics of Sertoli cells. Leydig's cells and other populations of testicular cells after prolonged of low dose whole-body gamma-irradiation and suggests that the existence of gamma-sensor in brain of mammals that involved on hypothalamic-pituitary-testicular levels in realisation of radiation stress suppression of Sertoli cell functions at a relatively "low" (0.1-0.5 Gy) doses by means of hypothalamic releasing factors.

Isao *et al*. (2004) proposed that mice with BMC transplants with continuous CCl_4 injection had reduced liver fibrosis and a significantly improved survival rate after BMC transplantation compared with mice treated with CCl_4 alone. This finding introduces a new concept for the therapy of liver fibrosis.

In the present study BM transplantation empire testicular hormones, many apoptotic and necrotic observations in the spermatogonia, sertoli cells and primary spermatocytes and discontinuous seminiferous epithelium context with the finding of Yong-Hoon *et al*. (2009). Also, the same observations were recorded when CCl_4 or CCl_4 irradiated group treated by bone marrow transplantation. Autoimmune-like complication after BMT leads morphological and functional changes of target tissues (Levy *et al*., 2000) and also associated with gonadotoxicity (Wagner *et al*., 2005).

In conclusion exposure to consecutive low dose of γ-radiation and bone marrow transplantation impair testicular tissue in a rat treated CCl_4 model.

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