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Curcumin protects against testis-specific side effects of irinotecan

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Abstract. – OBJECTIVE: Irinotecan (IR/CPT-11) is a semisynthetic, water-soluble derivative of the alkaloid camptothecin. It is a topoisomerase I group antineoplastic drug commonly used for the treatment of many cancer types, although it has side effects in tissues such as the testis. Curcumin (CRC) is a polyphenol compound produced from the Indian saffron root; it is used as food colouring and food flavouring. This study examined the testis-specific side effects of IR and the ability of CRC to protect against these side effects.

MATERIALS AND METHODS: Forty male Sprague-Dawley rats were used in our study (n = 10). The rats were randomly divided into the following four groups: control, IR, IR + CRC, and CRC. IR 10 mg/kg/day was administered intraperitoneally and CRC 100 mg/kg was administered orally. Blood and testicular samples were collected from rats in all four groups on day 30 after drug administration. Histological, biochemical, and spermatological analyses were conducted.

RESULTS: Testis tissue and blood samples were collected from the four groups. Tissue samples from the control and CRC groups demonstrated normal histological appearance on light microscopy. The IR group exhibited the following findings: vascular congestion in the tunica albuginea layer; tubular degeneration and vascular congestion in the interstitial area; oedema, vacuolisation, and luminised cells in the seminiferous tubule; and cells that temporarily stopped dividing at any stage of division in the seminiferous tubule epithelium. In the IR+CRC group, histopathological damage was significantly reduced by CRC treatment. Biochemical analysis showed that the level of thiobarbituric acid reactive substance (TBARS) was significantly increased in the IR group, compared with the other groups. CRC treatment significantly decreased this IR-mediated increase in TBARS level, and the TBARS level in the IR + CRC group approached the level observed in the control group. IR treatment caused significant decreases in glutathione (GSH), superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) levels. However, CRC administration tended to ameliorate the decreases in GSH, SOD, CAT, and GPx levels.

CONCLUSIONS: In this study, IR had some toxic effects in rat testis tissue; these effects were ameliorated by CRC treatment. Further studies are warranted to confirm our results.

Key Words:
Irinotecan, Testis, Curcumin, Rat, SN-38.

Introduction

Irinotecan (IR/CPT-11) is a semisynthetic and water-soluble derivative of topoisomerase I group antineoplastic drugs, which is produced from the tree Camptotheca acuminata[1]. IR comprises a prodrug that is converted by the enzyme carboxylesterase into the active metabolite SN-38[2,3]. Although SN-38 is approximately 250-1000-fold more potent than IR, the plasma concentration of IR is higher than the plasma concentration of SN-38[4-6]. Common side effects of IR treatment are nausea, vomiting, hair loss, diarrhoea, and bone marrow suppression[7]. In addition, some scholars[8] have shown that SN38 has cytotoxic effects on male germ cells in the prepubertal mouse testis; however, few studies[7,8] have investigated the testis-specific side effects of IR.

Curcumin (CRC) is a polyphenol compound produced from the Indian saffron root "Curcuma Longa," especially in southeast Asia. It is used as food colouring and food flavouring[9]. CRC reduces lipid peroxidation by activating enzymes such

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as catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx). Thus, it protects cells from the destructive effects of free oxygen radicals[10]. Furthermore, CRC exerts anti-inflammatory effects by suppressing the effects of pro-inflammatory cytokines including interleukin 1 beta (IL-1β), IL-6, IL-12, tumour necrosis factor alpha, and interferon gamma[11]. It also contributes to wound healing through enhancement of granulation tissue formation, wound contraction, and epithelialisation[12,13], as well as antimicrobial effects[14]. Finally, CRC reduces the production of B-cell lymphoma 2, cyclin D1, cyclooxygenase 2, and matrix metalloproteinase 9 in carcinogenic cells; reduces the dose-dependent phosphorylation of Janus kinase/signal transducer and activator of transcription 3; activates caspase enzymes; and induces apoptosis through these pathways[15,16].

In this study, we investigated the testis-specific side effects of IR, which is used in the treatment of many cancers. We hypothesised that these effects could be eliminated by treatment with CRC because of its antioxidant activity. In addition, we examined whether CRC could be used to prevent infertility, a possible side effect of IR.

Materials and Methods

IR was obtained from a local pharmacy in Malatya, Turkey. The other chemicals were purchased from Merck (Darmstadt, Germany). Forty male Sprague-Dawley rats (age, 3-4 months; weight, 290-310 g) were obtained from the Experimental Animals Unit of Inönü University (Malatya, Turkey).

Rats were placed in sterilised polypropylene cages with a 12-h:12-h light/dark cycle at an ambient temperature of 21°C. Food and water were provided ad libitum. The study protocol was approved by the Inonu University Experimental Animals Ethics Committee (Protocol No. 2016/A-06).

The rats were randomly divided into the following four groups (n = 10 each): control, IR, IR + CRC, and CRC.

Group 1: Control group (n=10)

Group 2: Irinotecan (CPT-11) group (n=10)

Group 3: Irinotecan (CPT-11) + Curcumin (CRC) group (n=10)

Group 4: Curcumin (CRC) group (n=10)

The control group was administered 0.01% carboxymethyl cellulose by oral gavage every other day and 0.5 mL saline intraperitoneally once weekly (four total doses) for 1 month. The IR group received IR dissolved in saline (10 mg/kg/day intraperitoneally) for 1 month. The CRC group received CRC suspended in 0.01% carboxymethyl cellulose (100 mg/kg/day by oral gavage) for 1 month. The doses of IR and CRC were determined in accordance with published methods[16-18]. The IR + CRC group was administered both substances concurrently at the above doses.

After 30 days of drug administration, rats were sacrificed and testicular samples were removed. Rat blood samples were collected from the left ventricle of the heart.

The collected samples were used for histological, biochemical, immunohistochemical, and spermatological examinations.

Histological Methods

Tissue samples obtained for histopathological examination were fixed in 10% formaldehyde. Routine tissue follow-up procedures were performed on the detected tissues, and tissue samples were embedded in paraffin blocks. Subsequently, 5-µm-thick sections were prepared. Haematoxylin and eosin staining was conducted for light microscopy evaluation. Caspase-3 staining was conducted for immunohistochemical evaluation, using the following protocol. Testis tissue samples were sectioned on polylysine-coated slides. The sections were placed in citrate buffer (pH 7.6) and heated in a microwave for 20 min. They were then cooled at room temperature for 20 min, washed with phosphate-buffered saline, and incubated in 0.3% hydrogen peroxide for 7 min to block endogenous peroxidase activity. After sections had been washed again with phosphate-buffered saline, they were incubated with polyclonal anti-caspase-3 antibody (Ab4051; Abcam, Cambridge, MA, USA) for 2 h at room temperature, then washed with phosphate-buffered saline, followed by incubation with secondary antibody for 10 min at room temperature and streptavidin peroxidase for 10 min at room temperature. The AEC chromogen/substrate was applied for 15 min, then sections were stained in Mayer's haematoxylin solution for 1 min and examined by light microscopy using the Leica DFC 280 light microscope (DMLB 2/11888110; Leica Microsystems, Wetzlar, Germany).

Biochemical Methods

After collection, rat testicular tissue samples were stored at -45°C until analysis. The tissues

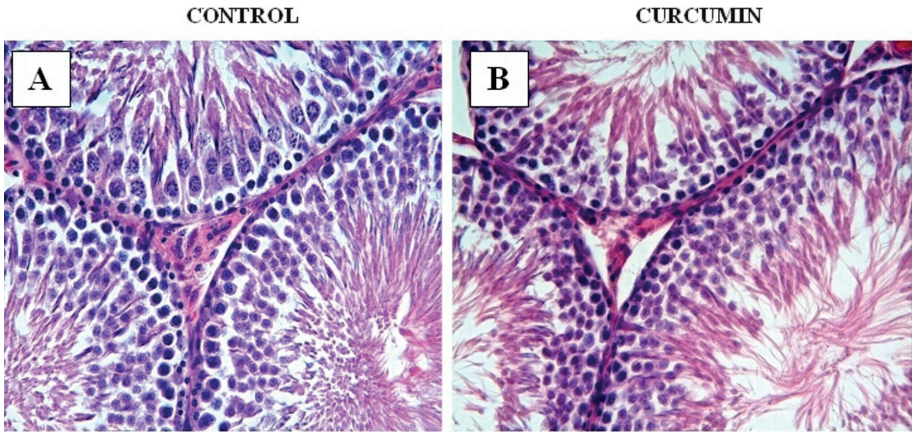

Figure 1. Control and Curcumin group (**A, B:** H-E; X40).

were then removed from the freezer, weighed, and placed in glass tubes. Potassium chloride (1.15%; diluted 1:10 [g/h]) was added, and the sections were homogenised in a glass Teflon homogeniser at a speed of 16,000 revolutions per min for approximately 3 min. The prepared homogenate was centrifuged at 4°C for 45 min at 3500 rpm to obtain supernatant, and the enzyme activities of GSH-Px and CAT were measured in the supernatant. The chloroform/ethanol (3:5, v/v) mixture was added to the supernatant at a ratio of 1:1 (v/v) and vortexed, followed by centrifugation at 3500 rpm for 45 min. SOD enzyme activity and protein levels were measured again in the chloroform/ethanol phase.

In separate analyses, the sperm parameters were evaluated in testes, epididymis, seminal vesicles, and prostate.

Statistical Analysis

SPSS 13.0 (SPSS Inc. Chicago, IL, USA) and MedCalc 11.0 (Belgium) program were used for statistical evaluations. One-way ANOVA variance analysis was used in comparison of sperm characteristics and biochemical findings. The results were shown as mean ± standard error. $p < 0.01$ for biochemical parameters and $p < 0.05$ for sperm analysis were considered statistically significant.

Results

Light Microscopic Findings

The control (Figure 1A) and CRC-treated (Figure 1B) specimens had a normal histological appearance, as assessed by light microscopy. The seminiferous tubules, basement membrane, and interstitial areas demonstrated normal histological structure. Spermatogenic serial cells, Sertoli cells, and interstitial connective tissue forming the seminiferous tubule epithelium also had a normal histological appearance. The IR group exhibited the following findings in the testis: vascular congestion in the tunica albuginea layer (Figure 2A); tubular degeneration and vascular congestion in the interstitial space (Figure 2B, 2C); oedema, vacuolisation (Figure 2C, 2D), and luminised cells in the seminiferous tubule (Figure 2E); and cells that temporarily stopped dividing at any stage of division in the seminiferous tubule epithelium (Figure 2F). Light microscopy analysis of the IR + CRC group showed that histopathological damage was significantly decreased and the numbers of spermatogenic serial cells were increased (Figure 3A, 3B).

Immunohistochemical Findings

Comparison of spermatogenic serial cells among the control (Figure 4A), IR (Figure 4C), IR + CRC (Figure 4D), and CRC (Figure 4B) groups revealed substantially greater caspase-3-positive cell staining in the IR group. In the IR + CRC group, there were significant reductions in the number of caspase 3-positive cells and the staining intensity. Caspase-3 immunoreactivity was not observed in the control and CRC groups. There were significant decreases in mean seminiferous tubular diameter in the IR and IR + CRC groups. Furthermore, germinal epithelial cell thickness was reduced in the IR and IR + CRC groups (Table I).

Biochemical Findings

TBARS, SOD, CAT, GSH, and GPx levels in all groups are shown in Table II. The TBARS le-

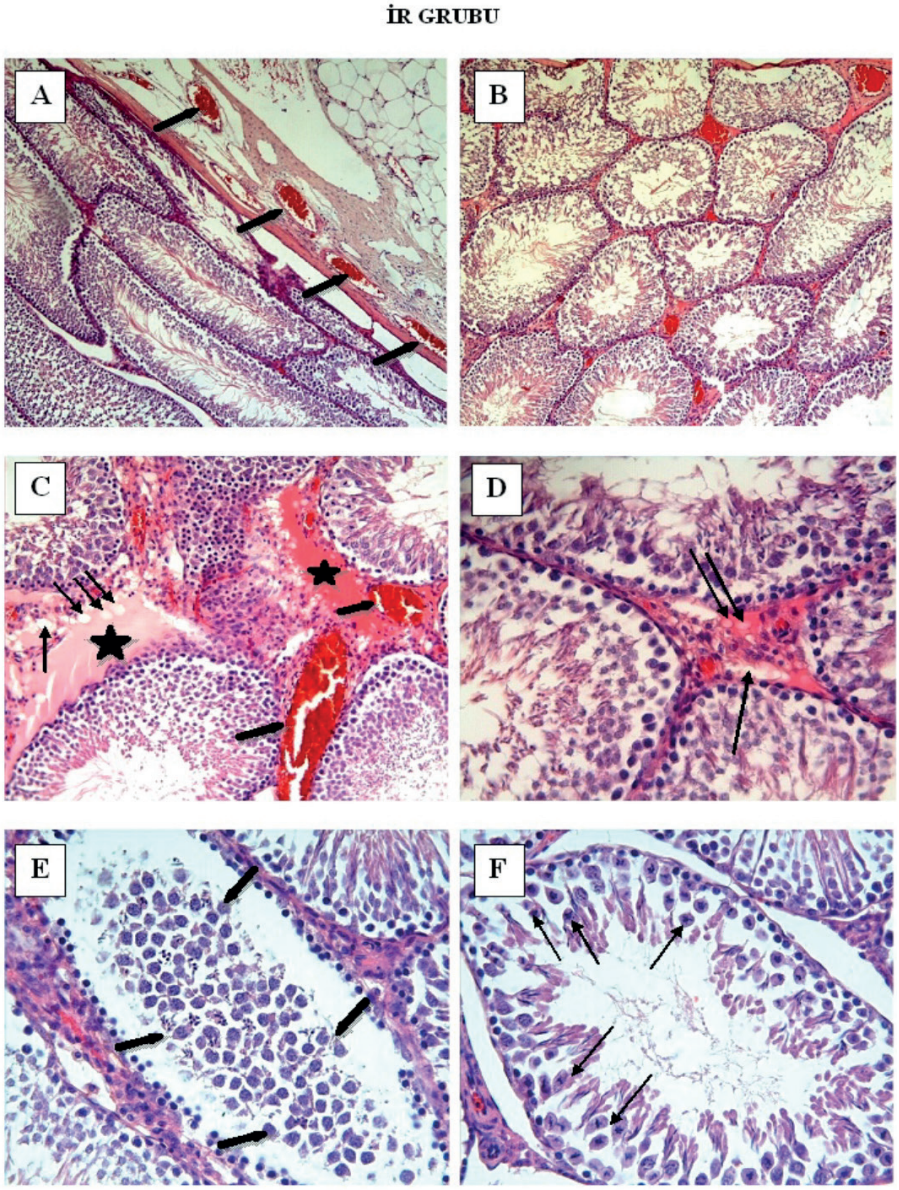

Figure 2. Irinotecan group (A, B: H-E; X10, C: H-E; X20, D, E, F: H-E; X40).

vel, which is indicative of oxidative damage, was significantly increased in rats that received IR, compared with the other groups. However, CRC treatment significantly ameliorated the IR-mediated increase in TBARS level in the IR + CRC group. Treatment with IR caused significant decreases in GSH, SOD, GPx, and CAT levels, which are the elements of the antioxidant defence system. However, CRC treatment ameliorated the IR-related decreases in GSH, SOD, CAT, and GPx levels in the IR + CRC group. There were no differences in any parameters between the control and CRC groups.

Spermatological Findings

CRC and IR treatments did not cause statistically significant differences in reproductive system organ weights, compared with the control group (Table III). IR treatment resulted in a decrease in sperm motility and concentration, compared with the control and CRC groups. In the IR + CRC group, CRC ameliorated this IR-mediated decrease and led to significantly greater sperm motility and concentration, compared with the IR group. There were no statistically significant differences between the control and IR + CRC groups. However, IR administration caused an increase in the

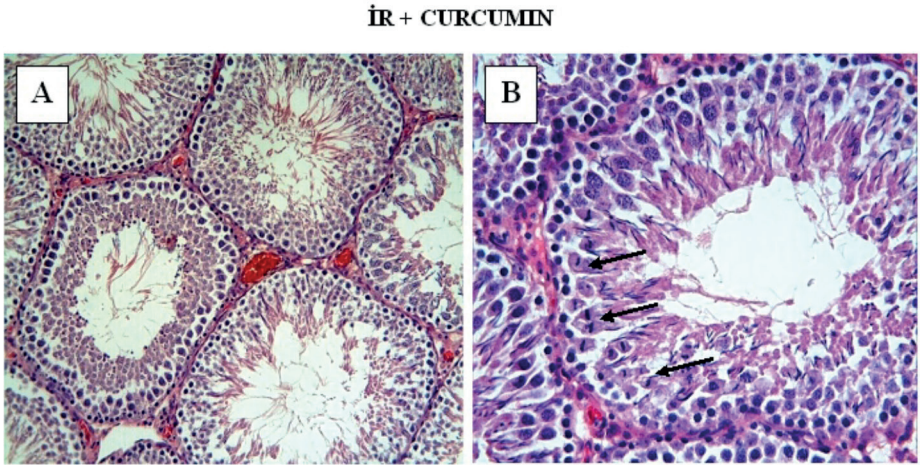

Figure 3. Irinotecan + Curcumin group (**A:** H-E; X20; **B:** H-E; X40).

amounts of abnormal sperm (head, tail, and total); these effects were ameliorated by CRC treatment (Table IV).

Discussion

Chemotherapy drugs may have adverse effects on reproductive cells by inducing spermatogenesis disorders, oligospermia, azoospermia, asthenozoospermia, teratozoospermia, hormonal changes, sperm DNA, and chromosomal structural damage[19]. In particular, chemotherapy used for the treatment of childhood cancers can result in varying degrees of gonadotoxicity and adversely affect future fertility[20]. To prevent the gonadotoxic effects of chemotherapeutic drugs, methods such as antioxidant and hormonal treatment are used, along with germ cell or sperm freezing approaches[19].

The chemotherapy agent IR used in our study is a prodrug that is converted by the enzyme carboxylesterase into the active metabolite SN-38[1]. It is often used for the primary and secondary treatment of colorectal cancers, as well as some other malignancies[21]. IR has various side effects on specific tissues, but its effects on fertility have not yet been fully identified[8]. Utsunomiya et al[22] showed that IR treatment triggered apoptosis in granulosa cells. Lopes et al[8] showed a marked increase in Sertoli cell-only tubules in histological sections acquired from testicular tissue, which was exposed to high concentrations of SN38 *in vitro*. The seminiferous tubule density was not affected by this treatment, while the diameter of the seminiferous tubules was significantly reduced. In our study, rats that received IR exhibited the following findings: vascular congestion, tubular degeneration in the tunica albuginea layer and vascular congestion in the interstitial space, and oedema and vacuolisation in the luminal cells of the seminiferous tubule. In addition, we observed significant reductions in the germinal epithelial cell thickness and mean seminiferous tubular diameter in the IR and IR + CRC groups. In the study by Lopes et al[8], the number of germ cells and percentage of immunostained area were approximately 2-20-fold smaller in SN38-exposed tissue. There was also a marked reduction in the percentage of proliferating germ cells. In our study, IR

Table I. Effects of curcumin on changes in irinotecan-induced tubule diameter and tubule epithelial thickness (mean ± SEM n = 7). The lower case letters a, b, c in the same column indicate differences between the groups. (p<0.0001).

Groups	Tubule Diameter (µm)	Tubular Epithelial Thickness (µm)
Control	297.15 ± 2.29[a]	88.12 ± 1.04[a]
CPT-11	255.60 ± 2.24[b]	42.49 ± 0.78[b]
CRC	283.77 ± 3.36[a]	74.57 ± 1.06[a]
CPT-11+ CRC	265.46 ± 3.14[c]	62.64 ± 1.41[c]

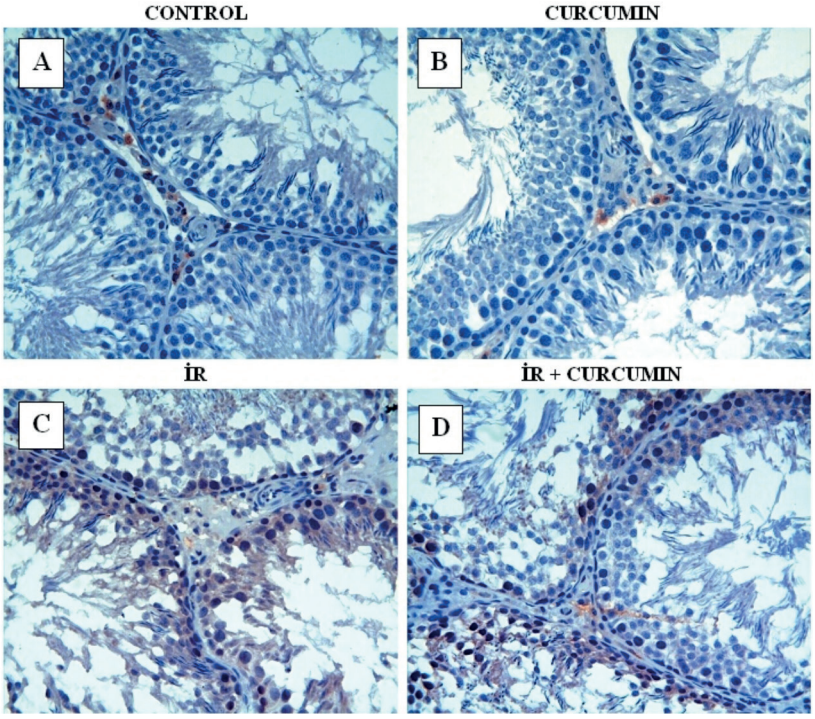

Figure 4. Caspase-3 activities in experimental groups (X40).

treatment caused a significant decrease in sperm concentration, compared with the control and CRC groups. In addition, cells that temporarily stopped dividing at any stage of division were observed in the seminiferous tubule epithelium.

In our study, caspase-3 expression was used as a marker of apoptosis. Caspases are serine protease-derived enzymes that cleave aspartic peptide bonds, inactivate the enzymes required for DNA repair and replication, and cause apoptosis. Immunohistochemical staining to detect caspase-3 expression is commonly performed to assess apoptotic activity[23]. In the study by Lopes et al[8], there were no statistically significant differences among groups in caspase-3 expression in testicular tissue that had been exposed to high concentrations of SN38 in vitro. In the study by Du et al[24], the effects of panaxadiol and IR combination treatment on apoptosis were evaluated by measuring the expression levels of caspase-3 and caspase-9. Combined treatment led to increased expression levels of caspase-3 and caspase-9[24]. In our study, the IR group exhibited substantially greater caspase-3-positive cell staining.

While many researchers define CRC as a pro-apoptotic molecule[15,25,26], some have described it as an anti-apoptotic molecule[27]. Khadrawy et al[28] reported a significant increase in the level of caspase-3 in brain cells from rats that had received cisplatin. This apoptotic effect was completely eliminated in rats that had also received CRC, such that caspase-3 levels were not significantly different from levels in the control group. In our study, caspase-3 immunoreactivity, a marker of

Table II. SOD, CAT, GPx, GSH and TBARS levels in testicular tissue of rats (Mean ± SD). The letters a, b and c in the same column indicate statistical differences between the groups (p <0.01).

	TBARS nmol/g	GSH nmol/ml	CAT k/mg protein	GPx U/mg protein	SOD mg/protein
Control	8.18±1.25[a]	126.1±14.3[a]	0.093±0.0019[a]	154.3±18.1[a]	24.8±2.76[a]
CPT-11	13.23±1.95[b]	79.8±12.1[b]	0.056±0.0018[b]	96.9±21.6[b]	15.9±2.15[b]
CRC	7.16±1.49[a]	119.5±17.5[a]	0.089±0.0023[a]	160.4±19.4[a]	25.3±2.84[a]
CPT-11+CRC	10.78±1.18[c]	113.8±15.7[a]	0.078±0.0025[c]	139.7±16.8[c]	21.1±2.92[c]

Table III. Testicular weights, Epididymis weights, Seminal vesicle weights, Prostate weights (mean ± SEM) in curcumin and irinotecan-treated rats.

Groups	Testicular Weight (gr)		Epididymis Weight (gr)		Seminal Vesicle Weight (gr)	Prostate Weight (gr)
	Right	Left	Right	Left		
Control	1.477±0.05	1.310±0.04	0.639±0.03	0.645±0.02	1.376±0.02	0.421±0.02
CPT-11	1.283±0.04	1.333±0.04	0.645±0.01	0.581±0.03	1.333±0.04	0.426±0.02
CPT11+CRC	1.255±0.03	1.371±0.02	0.677±0.01	0.589±0.02	1.353±0.05	0.405±0.03
CRC	1.375±0.05	1.396±0.03	0.672±0.01	0.581±0.01	1.181±0.09	0.394±0.03

apoptosis, was not observed in the control or CRC groups. In addition, we observed significant decreases in the number of caspase-3-positive stained cells and the staining intensity in the IR + CRC group, indicating that CRC reduced the apoptotic effect of IR.

In the study by Rtibi et al[29], increased levels of malondialdehyde and myeloperoxidase, indicators of oxidative stress, were observed in the intestinal mucosa of rats that received intraperitoneal IR, compared with the control group. There were significant decreases in the levels of antioxidant enzymes (e.g., SOD, CAT, and GPx) in the IR group. In the present study, we also measured the levels of SOD, CAT, GPx, GSH, and TBARS. We found that the level of TBARS, an indicator of oxidative damage, was increased in IR-treated rats, compared with the other groups. IR treatment also caused statistically significant decreases in GSH, SOD, GPx, and CAT levels.

Edrees et al[30] showed that malondialdehyde and nitric oxide levels were increased in brain and kidney cells from rats treated with colistin. CRC treatment partially reduced this oxidative effect. In our study, CRC treatment also ameliorated IR-related decreases in GSH, SOD, CAT, and GPx levels.

In our study, CRC and IR treatments did not cause significant changes in the testicular, epididymis, seminal vesicle, and prostate weights, compared with the control group. However, IR treatment caused statistically significant decreases in sperm motility and concentration, compared with the control and CRC groups. In the IR + CRC group, CRC reversed the IR-mediated decreases in sperm motility and concentration. IR administration also caused statistically significant increases in the amounts of abnormal sperm (head, tail, and total); these effects were ameliorated with CRC treatment. Although the IR + CRC group exhibited significant decreases compared with the control group, the magnitudes of these differences were small.

Conclusions

To our knowledge, there have been few studies[19,20] regarding the side effects of IR, which is used in chemotherapy for testis tumours in both children and adults. Our study showed that IR had some toxic effects in rat testis tissue, including histopathological changes, induction of apoptosis, reduction of antioxidant effects, enhancement

Table IV. Sperm motility, epididymal sperm concentration, abnormal sperm ratio (mean ± SEM) in curcumin and irinotecan-treated rats. The mean difference between the bearing and the superscript bearing in the same column (a,b and c; $p < 0.05$).

Groups	Sperm Motility (%)	Epididymal Concentration (million/gr tissue)	Abnormal Sperm Ratio (%)		
			Head	Tail	Total
Control	92.90±1.02[a]	340.85±7.45[a]	5.14±0.34[a]	4.57±0.36[a]	10.00±0.69
CPT-11	57.85±3.24[b]	237.57±8.38[b]	8.57±0.42[b]	8.28±0.52[b]	16.85±0.26
CPT-11+CRC	77.14±2.64[ab]	272.28±8.39[a]	5.57±0.57[ab]	5.14±0.26[ab]	10.71±0.80
CRC	93.808±0.70[a]	361.00±2.24[a]	5.14±0.26[a]	4.14±0.45[a]	8.85±0.85

of oxidative stress, reduction of sperm quality, and enhancement of abnormal sperm production. These effects were significantly ameliorated with CRC treatment. The results of this study are important because there have been some published studies[8,24,29,30] regarding the ability of CRC to protect against IR-mediated side effects. Larger studies are needed to confirm the results of this investigation.

Conflicts of Interest

The authors declare no conflicts of interest.

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