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The Therapeutic Effects of Epigallocatechin-3-gallate and Rutin, either Alone or in Combination with Low-dose Radiation, against Testicular Injury Evoked by Nicotine in Rats

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GROWING evidence suggests that nicotine, the addictive component of cigarettes, plays a direct role in testicular injury and infertility. The present study was intended to investigate the therapeutic effect of epigallocatechin-3-gallate (EGCG) and rutin (RUT), either alone or in combination with a low-dose radiation (LDR), against testicular injury evoked by nicotine in rats. For the induction of testicular injury, nicotine (1mg/kg) was administered orally for 30 days. Following that, rats were administered EGCG (100 mg/kg), RUT (30 mg/kg) orally, either alone or in combination with LDR (2 x 0.25 Gy) for an additional 14 days. Rats were sacrificed on day 45, the testes were then dissected for histopathological analysis, and several biochemical parameters in serum and testicular tissue were also evaluated. The results showed that nicotine administration significantly increased the testicular thiobarbituric acid reactive substances and decreased the reduced glutathione contents. Besides, the activities of testicular androgenic enzymes (3 beta-hydroxysteroid dehydrogenases and 17 beta-hydroxysteroid dehydrogenases) were reduced, whereas serum lactate dehydrogenase activity was considerably raised. In addition, the follicle-stimulating hormone, luteinizing hormone, and testosterone serum levels were reduced, indicating hormonal alterations. The testicular seminiferous tubules structure was also deformed after histological examination. On the other hand, treatment with LDR combined with either EGCG or RUT dramatically reduced the deleterious effects of nicotine compared to their individual effects, as evidenced by biochemical and histological findings. Accordingly, exposure to LDR combined with natural antioxidants, either EGCG or RUT, may be a promising candidate for treating testicular injury caused by nicotine.

Keywords: Epigallocatechin-3-gallate, Low-dose radiation, Nicotine, Rutin, Testicular injury.

Introduction

Testicular injury and male infertility are the most common long-term consequences of abusing chemicals and drugs that have a detrimental effect on the male reproductive system. For instance, nicotine accounts for 90% of the total alkaloid content of cigarette smoke and is one of the most likely causes of male infertility (Borgerding & Klus, 2005). Heavy smokers' seminal fluids have been shown to contain nicotine, indicating a high level of transfer from the circulation to testicular cells, which may have an adverse effect on spermatogenesis, epididymal sperm count, motility, and sperm fertilization potential (Reddy et al., 1995; Oyeyipo et al., 2014).

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Several investigations were conducted to induce testicular damage with daily nicotine administration at doses ranging from 0.5 to 1.0 mg/kg body weight. These findings revealed a dose-dependent drop in sperm count and motility, seminiferous tubule and spermatogenic derangement in the testes, and an overall decrease in testicular weight (Oyeyipo et al., 2010; Budin et al., 2017). In addition, Jana et al. (2010) showed that nicotine decreases gametogenesis and prevents the secretion of gonadotropic hormones like follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Koskenniemi et al. (2017) reviewed that FSH and LH are essential for testicular function and spermatogenesis. LH is the main tropic hormone secreted by the Leydig interstitial cells to produce testosterone, whereas FSH regulates spermatogenesis through its action on the Sertoli cells. Moreover, nicotine causes a decrease in the level of testosterone (Oyeyipo et al., 2013) via inhibition of multiple steps of testosterone biosynthesis, decreases the testicular androgenic enzymes, namely 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-hydroxysteroid dehydrogenase (17β-HSD) (Nair & Rajamohan, 2014). Other causative factors may include DNA damage, elevation in reactive oxygen species (ROS), increased testicular lipid peroxidation, hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\bullet}OH$) generation. At the same time, it reduces the level of glutathione (GSH), the activities of antioxidant enzymes, and the mitochondrial membrane potential of the testis (Jana et al., 2010).

Recently, several studies have attempted to develop effective techniques and medicines to decrease or prevent testicular damage. Antioxidants, particularly natural ones, have recently sparked much attention due to their ability to defend against free radical damage. For instance, epigallocatechin-3-gallate (EGCG) is the major catechin found in green tea. It is among natural products that have recently attracted the attention of researchers due to its antioxidant (Dewaantari et al., 2021), anti-inflammatory (Braegelmann et al., 2021), anti-apoptotic (Zong et al., 2021), and anticancer (Zhu et al., 2021) properties. Moreover, in several models, ECCG has shown to be renoprotective (Kanlaya & Thongboonkerd 2019), neuroprotective (Xu et al., 2021), and anti-diabetic (Li et al., 2020). Furthermore, according to Guvvala et al. (2017), EGCG protects the reproductive system by reducing the testicular oxidative stress induced by arsenic exposure. In addition, previous studies showed that EGCG enhanced testosterone production in rat Leydig cells (Yu et al., 2010), protect testicular seminiferous tubules, and spermatogenesis during testicular torsion/detorsion (Al-Ajmi et al., 2013).

Rutin (RUT, named vitamin P) is another widely utilized antioxidant. RUT is composed of flavonol quercetin and disaccharide rutinose (Ganeshpurkar & Saluja, 2017a). It is present mainly in buckwheat, onions, apples, tea, and red wine (Hosseinzadeh & Nassiri-Asl, 2014). RUT exhibits multiple pharmacological activities, including antioxidant (Iova et al., 2021) and anti-inflammatory (Yao et al., 2021), anticancer (Zhao et al., 2003), antiulcer (Liu et al., 2013), anti-apoptotic (Wang et al., 2021) properties. RUT has also been shown to act as a vasodilator (Li et al., 2012), immunomodulator (Ganeshpurkar & Saluja, 2017b), hepatoprotective, and renoprotective effects (Abarikwu et al., 2017a). Moreover, previous studies reported that RUT detoxifies the oxidative stress caused by various drugs and chemicals, as it has shown a protective impact against reproductive toxicity induced by cyclophosphamide (Abarikwu et al., 2012), cadmium (Abarikwu et al., 2017b), and cisplatin (Jahan, 2018) in rats. It also showed a promising effect against oxidative insult associated with male infertility in mice (Mehfooz et al., 2018).

Radiation-induced hormesis hypothesizes that low doses of radiation (LDR, <1.0 Gy) can induce protective, radio adaptive, and/or reparative mechanisms in a biological system (Betlazar et al., 2016), where hormesis, in general, is any biphasic dose-response when low doses are useful while higher doses are detrimental (Calabrese, 2018). A series of studies were conducted to investigate the therapeutic potential of LDR in cancers (Takahashi & Nagasawa, 2020), arthritis (El-Ghazaly et al., 2020), diabetes (Rashed et al., 2016), cardiac inflammation (Zhang et al., 2011), and neurodegenerative diseases such as Parkinson's disease (El-Ghazaly et al., 2015).

Seldom researches demonstrated the therapeutic benefits of LDR in conjunction with natural products against nicotine-induced testicular damage. Our research group Ashoub et al. (2021) is concerned with studying the effect of LDR in combination with ellagic acid

against nicotine-induced testicular toxicity. This prompted us to continue in the present study to investigate the effect of more natural antioxidants, for instance, EGCG and RUT as an adjunctive therapy on the therapeutic activity of LDR on the nicotine-induced testicular injury rat model.

Materials and Methods

Experimental animals

Male Wistar rats weighing 120-180 g were used in the experiments, obtained from the National Research Center's animal breeding section (Dokki, Giza, Egypt). One week before the experiment, the rats were allowed to acclimate at the laboratory of the National Center for Radiation Research and Technology (NCRRT)- Egyptian Atomic Energy Authority (Nasr City, Cairo, Egypt). The rats were fed a standard pellet diet purchased from the National Research Center (Dokki, Cairo, Egypt) and given free access to water ad libitum. All experiments were carried out in accordance with the rules established by the European Commission (EEC) (updated directive 86/609/EEC), and the Ethics Committee for Animal Experimentation, Faculty of Pharmacy, Cairo University granted ethical permission (Permit no: PT 1175).

Drugs and chemicals

Nicotine hydrogen tartrate (95% nicotine) and RUT were purchased from Sigma Aldrich (Saint Louis, Missouri, USA), while EGCG was supplied by Alfa Aesar (Karlsruhe, Germany). All chemicals and reagents were obtained from Sigma-Aldrich Co.or were of the highest analytical quality available.

Irradiation procedures

Rats were exposed to whole-body irradiation at the NCRRT using a Gammacell®-40 biological irradiator with a Caesium-137 source (Atomic Energy of Canada Limited; Sheridan Science and Technology Park, Mississauga, Ontario, Canada). Non-anesthetized rats were placed in the plastic sample tray and irradiated at 0.5 Gray (Gy) applied as a fractionated exposure (2 x 0.25 Gy /1week interval) delivered at a 0.46 Gy/min rate. The selection of radiation dose and exposure period was based on previous studies by our research groups Ashoub et al. (2021) and El-Ghazaly et al. (2020).

Experimental design

The rats were randomly divided into seven groups; 8 rats/group.

- Normal control group: Rats received normal saline solution orally.

- Nicotine group: Nicotine dissolved in normal saline was administered orally to rats at a dose of 1 mg/kg for 30 consecutive days. The selection of nicotine dose and duration of administration was according to Ashoub et al. (2021). In addition to numerous studies, for instance, Oyeyipo et al. (2010), Kolawole et al. (2019), and Ukwenya et al. (2020).

- Nicotine + LDR treated group: After 30 days of nicotine administration, rats received the first and second radiation fraction of 0.25 Gy on days 31 and 37, respectively.

- Nicotine + EGCG treated group: After 30 days of nicotine administration, the EGCG was dissolved in normal saline and given to rats orally at a dose of 100 mg/kg for an additional 14 days, according to Wu et al. (2017).

- Nicotine + LDR + EGCG treated group: Rats received nicotine for 30 days, followed by treatment with LDR combined with EGCG for an additional 14 days.

- Nicotine + RUT treated group: After 30 days of nicotine administration, the RUT was dissolved in normal saline and given to rats orally at a dose of 30 mg/kg for an additional 14 days, according to Alhoshani et al. (2017).

- Nicotine + LDR + RUT treated group: Rats received nicotine for 30 days, followed by treatment with LDR combined with RUT for an additional 14 days.

Rats were sacrificed on day 45, blood samples were taken from the heart for serum separation, and testes were removed under mild ether anesthesia. The right testis was used for histopathological investigations, whereas the left testis was utilized to create 10% homogenate in a different medium according to the parameters to be evaluated using a Glass-Col homogenizer (Terre Haute, IN, USA). The homogenate was centrifuged using Mikro 22R centrifuge (Hettich GmbH, Tuttlingen, Germany).

Biochemical assessment

Determination of testicular lipid peroxides

Lipid peroxides formation was determined in testicular tissue homogenate by estimation of thiobarbituric acid reactive substances (TBARS) colorimetrically using a Unicam 8625 UV/V spectrophotometer (Cambridge, UK) at 535 nm, according to the method of Uchiyama & Mihara (1978).

Determination of testicular reduced glutathione (GSH)

The GSH was spectrophotometrically measured at 412 nm in testicular tissue homogenate, according to Beutler et al. (1963).

Determination of androgenic enzymes activities

The testes were homogenized in 15% glycerol containing 5 mmol potassium phosphate and 1 mmol ethylenediaminetetraacetic acid to measure the activity of 3 beta-hydroxysteroid dehydrogenases (3β-HSD) according to Talalay (1962), and 17 beta-hydroxysteroid dehydrogenases (17β-HSD) according to Jarabak et al. (1962). One unit of enzyme activity corresponds to a 0.001/min change in absorbance at 340 nm.

Determination of lactate dehydrogenase activity

Lactate dehydrogenase (LDH) activity in serum was determined according to the method of Gay et al. (1968) using a kinetic kit purchased from BioSystems (Barcelona, Spain).

Hormonal study

According to the manufacturer's instructions, an enzyme-linked immunosorbent assay (ELISA) kit was used to measure serum levels of follicle-stimulating hormone (FSH) obtained from LifeSpan BioSciences, Inc. (Seattle, Washington, USA), luteinizing hormone (LH) obtained from Elabscience Biotechnology Inc. (Houston, Texas, USA). Testosterone was obtained from DRG® International, Inc. (Mountain Ave, Springfield Township, USA). The optical density of each sample was determined using an ELISA plate reader (Dynatech® MR5000, Guernsey, Channel Islands, UK) set at 450 nm.

Histopathological examination

The rats' testes were fixed in Bouin's solution overnight at 4°C (Bilinska et al., 2018), embedded in paraffin blocks, and cut to a thickness of 4-6 µm. The specimens were deparaffinized and stained using hematoxylin and eosin (H&E). The morphology of the testes was evaluated under a light microscope (Bancroft & Stevens, 1996). According to Tuglu et al. (2015), the degree of testicular injury was assessed. In brief, several parameters were calculated for each testicular region, and the microscopic score was rated on a scale of mild (+), moderate (++), and severe (+++). Desquamation in germinal cells, disarray in germinal cells, interstitial edema, germinal cell degeneration, and a decrease in germinal cell numbers were among these characteristics.

Data analysis

For data management, IBM SPSS Statistics for Windows, version 23.0 (IBM Corp., Armonk, NY, USA) was utilized. The Shapiro-Wilk test was used to ensure that the data were normal and the mean ± standard error of the mean (s.e.m) was given. A one-way analysis of variance technique (ANOVA) was used to compare the means. The Games-Howell post hoc test was used when the test of homogeneity of variances was significant, and Duncan's Multiple Range Tests were used when the variances were not significant because ANOVA is resistant for small deviations from normality but not for heterogeneity of variances. Differences were considered statistically significant at a probability value less than 0.05 ($P < 0.05$).

Results

Effect of EGCG and RUT either alone or in combination with LDR on oxidative stress biomarkers in nicotine-induced testicular injury in rats

Thiobarbituric acid reactive substances (TBARS)

Data given in Table 1, and Fig. 1A elucidate that administration of nicotine (1 mg/kg) induced a significant elevation in the testicular TBARS content from 2.81 ± 0.09 nmol/g to 4.55 ± 0.27 nmol/g ($P < 0.05$), which is the hallmarks of lipid peroxidation. Exposure to LDR (2 × 0.25 Gy) after 30 days of nicotine administration led to a slight reduction of the TBARS testicular content from 4.55 ± 0.27 nmol/g to 3.91 ± 0.09 nmol/g ($P < 0.05$). On the same line, treatment with either EGCG (100 mg /kg) or RUT (30 mg/kg) led to a non-significant reduction in TBARS testicular content. On the other hand, the combination therapy

of LDR with EGCG resulted in the normalization of testicular TBARS ($P < 0.05$). However, when LDR and RUT were given together, the TBARS level only decreased from 4.55 ± 0.27 nmol/g to 3.46 ± 0.054 nmol/g, which was not significantly different from when given separately.

Reduced glutathione (GSH)

The nicotine administration led to a severe drop in GSH testicular content from 111.82 ± 2.00 µg/g to13.65 ± 1.64 µg/g ($P < 0.05$). Exposure to LDR tended to prevent the reduction in GSH content by 74%, compared to the normal control ($P < 0.05$). On the same line, either EGCG or RUT protected GSH testicular content from further reduction, reaching 65%-67%, respectively, compared to the normal control. However, the combined treatment (LDR + EGCG) or (LDR + RUT) showed a more pronounced decrease in GSH content by 39% or 54%, respectively, compared to the normal control at $P < 0.05$ (Table 1 and Fig. 1B).

Effect of EGCG and RUT either alone or in combination with LDR on androgenic key enzymes activities in nicotine-induced testicular injury in rats

3β-hydroxysteroid dehydrogenase (3β-HSD)

The activity of 3β-hydroxysteroid dehydrogenase (3β-HSD) in the nicotine group was significantly reduced from 8.16 ± 0.38 U/g to 2.45 ± 0.09 U/g ($P < 0.05$). In comparison, treatment with LDR (2 × 0.25 Gy) has amended the inhibitory responses to this testicular enzyme activity. The reduction in 3β-HSD activity after treatment with EGCG (100mg/kg) was 49% from the normal control group ($P < 0.05$). The combined treatment with LDR and EGCG revealed significant protection to this enzyme activity as the decrease in its activity from the normal group was 33% only ($P < 0.05$). Administration of RUT (30 mg/kg) to nicotine treated group has shown a reduction in 3β-HSD activity by 53% at $P < 0.05$ linked to the normal. On the other hand, the combination of LDR and RUT displayed a significant protector against a further decrease in androgenic key enzymes activities at $P < 0.05$ (Table 2 and Fig. 2A)

17β-hydroxysteroid dehydrogenase (17β-HSD)

The activity of 17β-HSD in nicotine administered group decreased significantly by 46% of normal control (from 6.39 ± 0.05 U/g to 3.47 ± 0.08 U/g), while this decrease in activity after treatment with either LDR (2 × 0.25 Gy) or EGCG (100 mg/kg) was found to be 26% and 32%. The combined treatment with EGCG and LDR resulted in a more profound reservation of 17β-HSD activity than the individual treatment reaching 20% from the normal control ($P < 0.05$). Furthermore, RUT treatment at a dose of (30mg/kg) showed a decrease in 17β-HSD activity by only 34% from the normal. Whereas RUT combination with LDR resulted in a reduction equal to 20% only from the normal ($P < 0.05$) (Table 2 and Fig. 2B)

TABLE 1. Effects of epigallocatechin gallate (EGCG) and rutin (RUT) either alone or in combination with low-dose radiation (LDR) on thiobarbituric acid reactive substances (TBARS) and reduced glutathione (GSH) content in rats testicular injury evoked by nicotine

Parameters / Groups	TBARS (nmol/g wet tissue)	GSH (µg/g wet tissue)
Normal control	2.81^{e} ± 0.09	111.82^{a} ± 2.00
Nicotine (1 mg/kg)	4.55^{abcd} ± 0.27	13.65^{f} ± 1.64
Nicotine + LDR (2x0.25 Gy)	3.91^{b} ± 0.09	28.70^{e} ± 1.27
Nicotine + EGCG (100 mg/kg)	3.47^{d} ± 0.145	39.76^{d} ± 1.20
Nicotine + LDR + EGCG	2.78^{e} ± 0.094	68.53^{b} ± 2.64
Nicotine + RUT (30 mg/kg)	3.97^{b} ± 0.081	36.40^{d} ± 0.50
Nicotine + LDR + RUT	3.46^{cd} ± 0.054	51.02^{c} ± 0.66

Data are expressed as mean ± s.e.m (n= 8).

Groups with different superscript letters are significantly different ($P< 0.05$).

Groups sharing the same superscript letters are non-significantly different ($P< 0.05$)

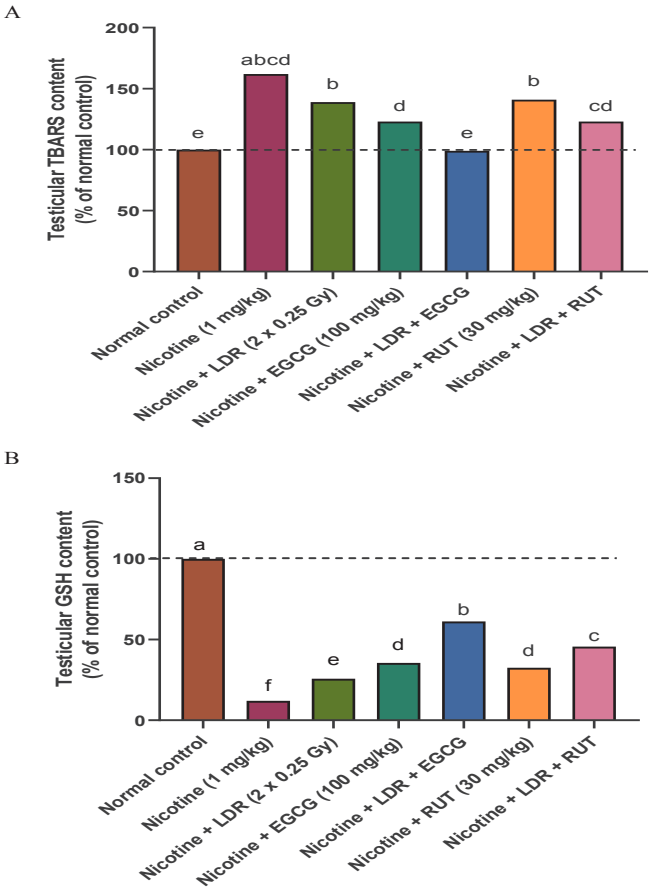

Fig. 1. Effects of epigallocatechin gallate (EGCG) and rutin (RUT) either alone or in combination with low-dose radiation (LDR) on (A) thiobarbituric acid reactive substances (TBARS) and (B) reduced glutathione (GSH) content in rats testicular injury evoked by nicotine [Nicotine was administered at 1 mg/kg once daily for 30 days. Twenty-four-hours later, rats were treated either by exposure to LDR (2x 0.25 Gy/1 week interval), oral administration of EGCG (100 mg/kg/day; for 14 days), RUT (30 mg/kg/day; for 14 days), or a combination of LDR with EGCG or RUT. Rats have been sacrificed 24 hrs after the last dose of treatment. All values are expressed as mean %. Groups with different superscript letters above the column are significantly different (P < 0.05). Groups sharing the same superscript letters above the column are non-significantly different (P<0.05)]

TABLE 2 Effects of epigallocatechin gallate (EGCG) and rutin (RUT) either alone or in combination with low-dose radiation (LDR) on 3 beta-hydroxysteroid dehydrogenase (3β-HSD), and 17 beta-hydroxysteroid dehydrogenase (17β-HSD) testicular activities, in addition to serum lactate dehydrogenase (LDH) activity in rats testicular injury evoked by nicotine

Parameters / Groups	3β-HSD (U/g wet tissue)	17β-HSD (U/g wet tissue)	LDH (IU/L)
Normal control	$8.16^{a} \pm 0.38$	$6.39^{a} \pm 0.05$	$92.51^{a} \pm 6.35$
Nicotine (1 mg/kg)	$2.45^{d} \pm 0.09$	$3.47^{d} \pm 0.08$	$151.3^{b} \pm 12.36$
Nicotine + LDR (2x0.25 Gy)	$3.69^{c} \pm 0.15$	$4.73^{bc} \pm 0.18$	$88.35^{ac} \pm 5.59$
Nicotine + EGCG (100 mg/kg)	$4.17^{c} \pm 0.28$	$4.33^{c} \pm 0.13$	$74.01^{c} \pm 3.03$
Nicotine + LDR + EGCG	$5.45^{b} \pm 0.07$	$5.10^{b} \pm 0.04$	$73.09^{ac} \pm 4.93$
Nicotine + RUT (30 mg/kg)	$3.80^{c} \pm 0.09$	$4.20^{c} \pm 0.08$	$92.09^{a} \pm 1.28$
Nicotine + LDR + RUT	$5.01^{b} \pm 0.05$	$5.13^{b} \pm 0.07$	$84.25^{ac} \pm 3.43$

Data are expressed as mean ± s.e.m (n=8).

Groups with different superscript letters are significantly different (P < 0.05).

Groups sharing the same superscript letters are non-significantly different (P < 0.05).

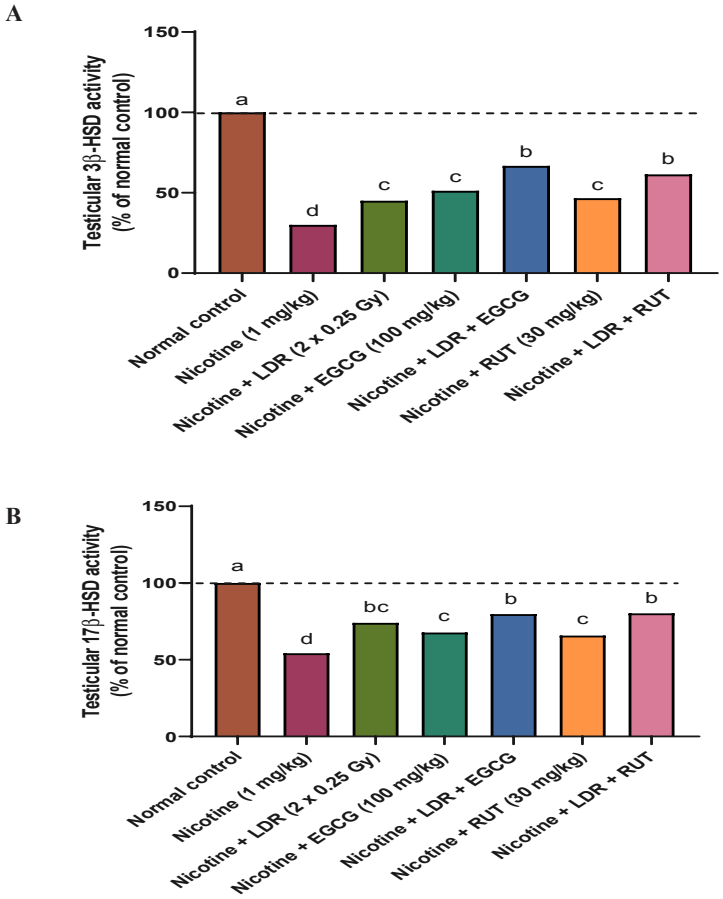

Fig. 2. Effects of epigallocatechin gallate (EGCG) and rutin (RUT) either alone or in combination with low-dose radiation (LDR) on (A) 3 beta-hydroxysteroid dehydrogenase (3β-HSD) and (B) 17 beta-hydroxysteroid dehydrogenase (17β-HSD) activities in rats testicular injury evoked by nicotine [Nicotine was administered at 1 mg/kg once daily for 30 days. Twenty-four-hours later, rats were treated either by exposure to LDR (2 x 0.25 Gy/1 week interval), oral administration of EGCG (100 mg/kg/day; for 14 days), RUT (30 mg/kg/day; for 14 days), or a combination of LDR with EGCG or RUT. Rats have been sacrificed 24 hrs after the last dose of treatment. All values are expressed as mean %. Groups with different superscript letters above the column are significantly different (P < 0.05). Groups sharing the same superscript letters above the column are non-significantly different (P<0.05)]

Effect of EGCG and RUT either alone or in combination with LDR on lactate dehydrogenase (LDH) enzyme activity in nicotine-induced testicular injury in rats

As illustrated in Table 2 and Fig. 3, nicotine-induced a significant rise ($P < 0.05$) in serum LDH activity, from 92.51 ± 6.35 IU/L to 151.3 ± 12.36 IU/L. After nicotine administration, exposure to LDR (2 × 0.25 Gy) tended to normalize the LDH activity. Regarding the treatment with EGCG (100 mg/kg), either alone or in combination with LDR, the activity of LDH in serum leans to normal value. Similarly, treatment with RUT (30 mg/kg) alone or combined with LDR tended to normalize the activity of the LDH enzyme.

Effect of EGCG and RUT either alone or in combination with LDR on the reproductive hormone levels in nicotine-induced testicular injury in rats

Follicle-stimulating hormone (FSH)

As demonstrated in Table 3 and Fig. 4A, nicotine caused a significant reduction in FSH serum levels, reaching 55% of the normal control (from 11.22 ± 0.28 ng/ml to 5.05 ± 0.23 ng/ml). Exposure to LDR (2 × 0.25 Gy) after nicotine administration reversed the decline in FSH hormone compared to the normal control as it reached 6.96 ± 0.43 ng/ml. On the same line, the administration of EGCG (100mg/kg) prevented a decrease in FSH level as this decline reached 32% only from the normal level ($P < 0.05$). Likewise, combined therapy (LDR + EGCG) displayed a further protection of this hormone. Moreover, administered RUT

(30 mg/kg) offered a restoration of FSH level as the reduction from normal was 39% ($P < 0.05$). Moreover, the combination of (LDR + RUT) has shown a more pronounced effect in preventing the decrease in FSH serum level than the individual treatment.

Luteinizing hormone (LH)

As presented in Table 3 and Fig. 4B, nicotine resulted in a marked drop in LH serum levels relative to normal control, from 14.39 ± 1.02 mU/ml to 5.88 ± 0.34 mU/ml. Treatment with LDR (2 × 0.25 Gy) failed to prevent LH serum level from decreasing as it reached only 5.12 ± 0.21 mU/ml. While the LH level reduced by 47% when rats were treated with EGCG (100 mg/kg) and by 36% in the case of combined therapy with (LDR+ EGCG) when compared to the normal control ($P < 0.05$). Administration of RUT (30 mg /kg) alone or combined with LDR was shown to prevent the decline in the LH level, which turned out to be 43% and 34% of the normal control level ($P < 0.05$).

Testosterone hormone

As indicated in Table 3 and Fig. 4C, testosterone levels dropped significantly after nicotine administration, from 9.60 ± 0.30 ng/ml to 3.36 ± 0.12 ng/ml. LDR (2 × 0.25 Gy) caused a significant elevation in testosterone level reaching 5.45 ± 0.11 ng/ml, compared to the nicotine group. On the same line, treatment with EGCG (100mg/kg) exhibited a significant elevation in the testosterone level, which reached 75% of the normal control level. Whereas the combined treatment with EGCG and LDR revealed a substantial increase in testosterone level as it reached 84% of normal control ($P < 0.05$). Furthermore, administration of RUT at a dose of 30mg/kg alone or in combination with LDR has shown a significant increase in the testosterone level in rat serum as it was 68% and 87%, respectively, of the normal control group at $P < 0.05$.

Histopathological findings

Microscopic examination of the normal control rats' testicular tissue showed closely packed seminiferous tubules lined by stratified germinal epithelium. Spermatogonia and Sertoli cells rested on intact basement membranes (Fig. 5A1). The spermatogenic cells were well-ordered with a regular structure. Furthermore, clusters of a few Leydig cells with acidophilic cytoplasm were distributed between the seminiferous tubules and blood vessels in the thin interstitium in-between the tubules (Fig. 5A2). On the other hand, after nicotine administration, the spermatogenic cells were disorganized, the seminiferous tubules were shrunken with wide interstitium in-between.

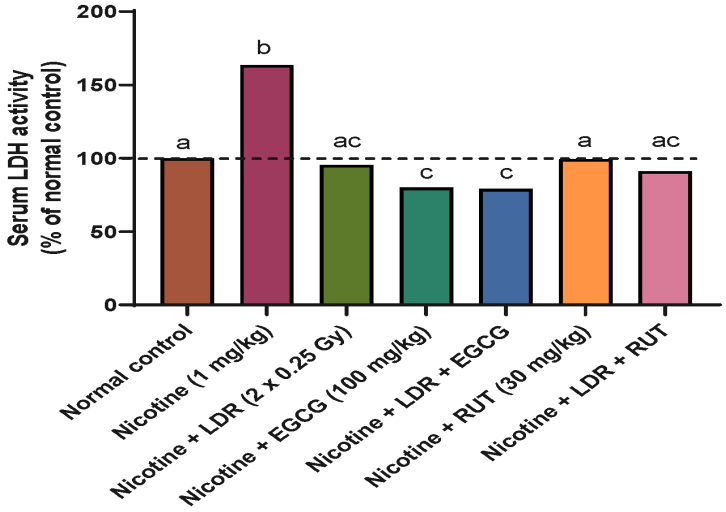

Fig. 3. Effects of epigallocatechin gallate (EGCG) and rutin (RUT) either alone or in combination with low-dose radiation (LDR) on serum lactate dehydrogenase (LDH) activity in rats testicular injury evoked by nicotine [Nicotine was administered at 1 mg/kg once daily for 30 days. Twenty-four-hours later, rats were treated either by exposure to LDR (2 x 0.25 Gy/1 week interval), oral administration of EGCG (100 mg/kg/day; for 14 days), RUT (30 mg/kg/day; for 14 days), or a combination of LDR with EGCG or RUT. Rats have been sacrificed 24 hrs after the last dose of treatment. All values are expressed as mean %. Groups with different superscript letters above the column are significantly different ($P < 0.05$). Groups sharing the same superscript letters above the column are non-significantly different ($P<0.05$)]

TABLE 3. Effects of epigallocatechin gallate (EGCG) and rutin (RUT) either alone or in combination with low-dose radiation (LDR) on follicle-stimulating hormone (FSH), luteinizing hormone (LH), and testosterone serum levels in rats testicular injury evoked by nicotine.

Parameters / Groups	FSH (ng/ml)	LH (mU/ml)	Testosterone (ng/ml)
Normal control	$11.22^{a} \pm 0.28$	$14.39^{a} \pm 1.02$	$9.60^{a} \pm 0.30$
Nicotine (1 mg/kg)	$5.05^{e} \pm 0.23$	$5.88^{d} \pm 0.34$	$3.36^{f} \pm 0.12$
Nicotine + LDR (2x0.25 Gy)	$6.96^{bcd} \pm 0.43$	$5.12^{d} \pm 0.21$	$5.45^{e} \pm 0.11$
Nicotine + EGCG (100 mg/kg)	$7.57^{c} \pm 0.12$	$7.60^{c} \pm 0.21$	$7.21^{c} \pm 0.15$
Nicotine + LDR + EGCG	$8.32^{b} \pm 0.15$	$9.28^{b} \pm 0.19$	$8.08^{b} \pm 0.17$
Nicotine + RUT (30 mg/kg)	$6.89^{d} \pm 0.13$	$8.26^{bc} \pm 0.07$	$6.56^{d} \pm 0.06$
Nicotine + LDR + RUT	$7.77^{bc} \pm 0.04$	$9.54^{b} \pm 0.06$	$8.31^{b} \pm 0.17$

Data are expressed as mean ± s.e.m (n=8).

Groups with different superscript letters are significantly different ($P < 0.05$).

Groups sharing the same superscript letters are non-significantly different ($P < 0.05$).

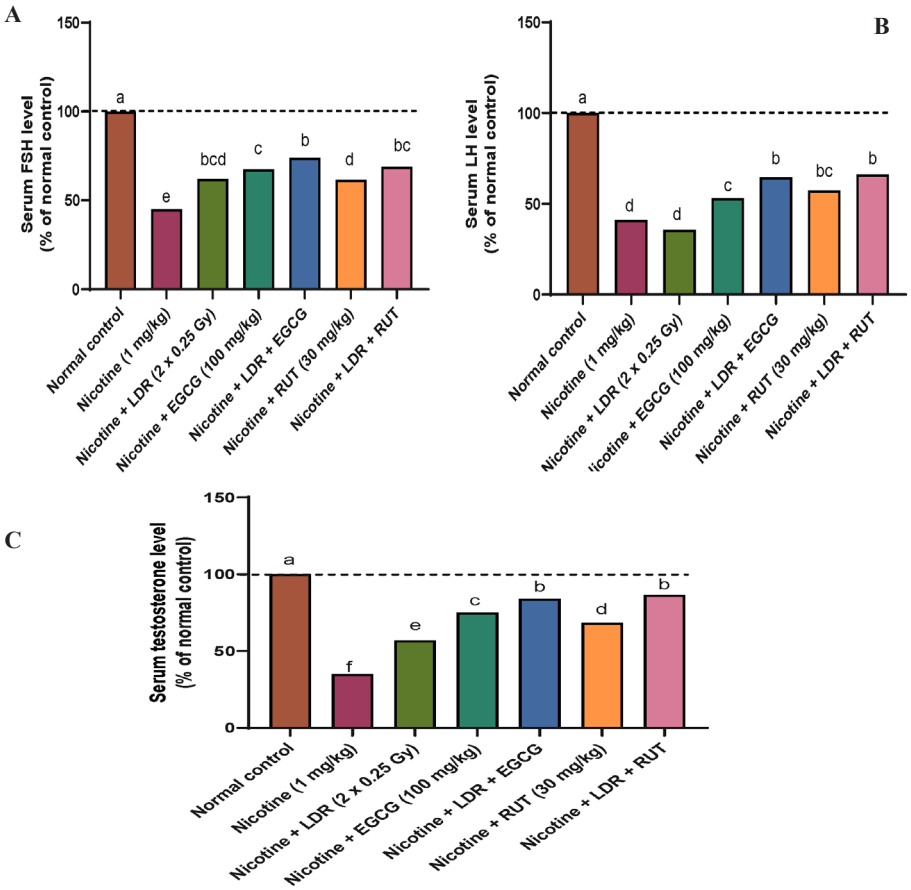

Fig. 4. Effects of epigallocatechin gallate (EGCG) and rutin (RUT) either alone or in combination with low-dose radiation (LDR) on (A) follicle-stimulating hormone (FSH), (B) luteinizing hormone (LH), and (C) testosterone serum levels in rats testicular injury evoked by nicotine [Nicotine was administered at 1 mg/kg once daily for 30 days. Twenty-four-hours later, rats were treated either by exposure to LDR (2 x 0.25 Gy/1 week interval), oral administration of EGCG (100 mg/kg/day; for 14 days), RUT (30 mg/kg/day; for 14 days), or a combination of LDR with EGCG or RUT. Rats have been sacrificed 24 hrs after the last dose of treatment. All values are expressed as mean %. Groups with different superscript letters above the column are significantly different ($P < 0.05$). Groups sharing the same superscript letters above the column are non-significantly different ($P<0.05$)]

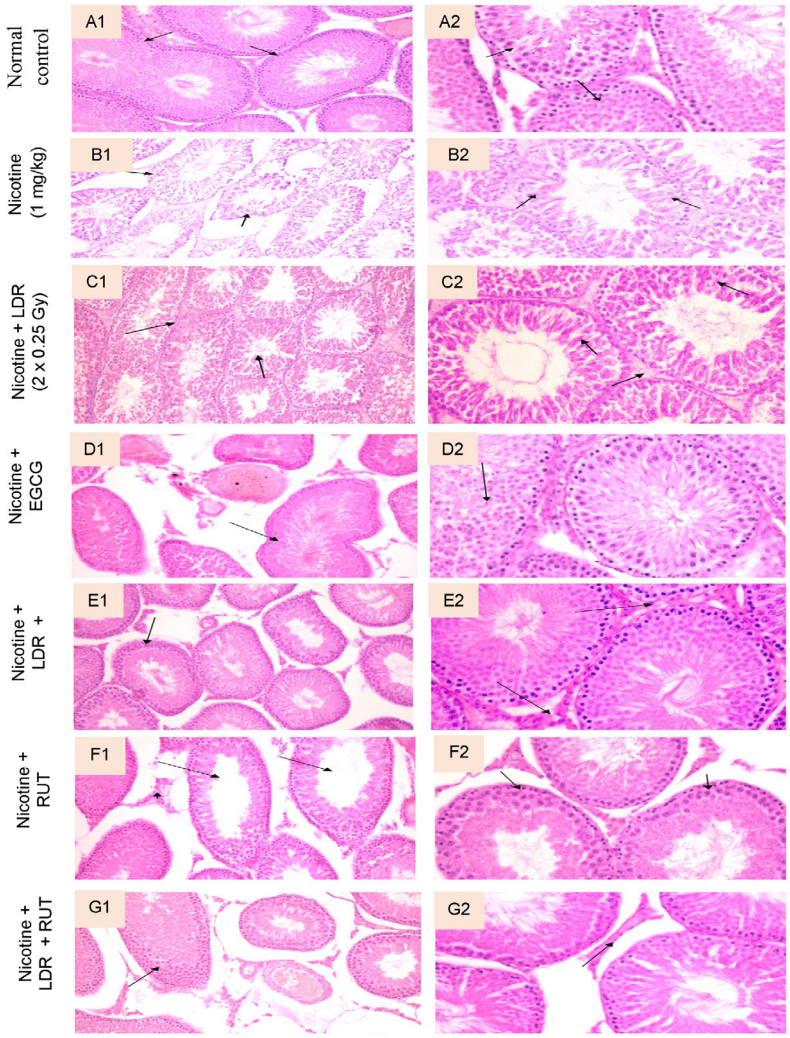

Fig. 5. At different magnifications, representative photomicrographs of testes sections stained with hematoxylin and eosin (HE) [x 200 (A1-G1) and x 400 (A2-G2)] [A: Histological slices of normal rats' testis indicated active spermatogenesis in normal-size seminiferous tubules with thin basement membranes (A1, arrow), as well as multiple layers of spermatogenic cells, including primary and secondary spermatocytes and spermatids (A2, arrow). B: The nicotine group's testicular tissue had deformed, shrunken seminiferous tubules with broad interstitium (B1, arrow), and the spermatogenic cells had pyknotic nuclei, necrotic cells, and vacuolated cytoplasm (B2, arrow). C: Exposure to low-dose radiation (LDR, 2 x 0.25 Gy/1week interval) developed sloughed germinal cells with reduced pyknotic nuclei, less defined seminiferous tubule boundaries (C1, arrow), spermatogenic cell disarray, and interstitial edema (C2, arrow) in rat's testicular tissue. D: Treatment with epigallocatechin gallate (EGCG, 100 mg/kg/day; for 14 days) after nicotine administration showed reductions of seminiferous tubules size, interstitial edema, and congestion of blood vessels (D1, arrow), disorganization, and reduction of spermatogenic cells (D2, arrow). E: Exposure to LDR combined with EGCG showed non-cohesive germinal cells, closely packed seminiferous tubules (E1, arrow), edema, and reduced interstitial cells (E2, arrow). F: Treatment with rutin (RUT, 30 mg/kg/day; for 14 days) after nicotine administration showed empty tubules from spermatids (H1, arrow), widening of interstitium spaces with edema (F1, arrowhead), pyknotic nuclei, and vacuolated cytoplasm of spermatogenic cells (F2, arrow). G: Exposure to LDR combined with RUT showed disorganization of spermatogenic cells within the seminiferous tubules (G1, arrow), reduction of interstitial cell number, and edema (G2, arrow)]

Additionally, the germinal epithelium thickness in the seminiferous tubules was reduced, and the lumina were wide (Fig. 5B1). Besides, as shown in Fig. 5B2, histological changes in the spermatogenic cells included pyknotic nuclei, necrotic cells, and vacuolated cytoplasm. The number of cells in the spermatogenic series was considerably reduced, and Sertoli cells with

vacuolated cytoplasm were seen. As a result, the pathological grade in the nicotine group was remarkably higher than that of the normal group as the damage severity score reached 3.

As shown in Fig. 5C, the exposure of rats to LDR after nicotine administration resulted in the elimination of the majority of histological lesions caused by nicotine taking score = 2. Moreover, the histopathological findings of the testicular parenchyma after nicotine administration and treatment with EGCG showed a reduction of seminiferous tubules size, interstitial edema, and congestion of blood vessels. In the spermatogenic series, the seminiferous tubules revealed disorganization and decrease in the spermatogenic cells and sloughed germinal cells with shrunken pyknotic nuclei. Some seminiferous tubules contained well-developed spermatids, and others were empty (Fig. 5D1). After being exposed to LDR combined with EGCG, the testicular parenchyma revealed non-cohesive germinal cells and tightly packed seminiferous tubules. The spermatogenic series all seen Spermatogonia, primary and secondary spermatocytes, and spermatids. Wide interstitium in-between the tubules was observed compared with nicotine group. Edema and reduction of interstitial cells were seen (Score 2) (Fig. 5D2).

Regarding the treatment with RUT after nicotine administration, the seminiferous tubules significantly reduced size with widening interstitium space. Spermatogenic cells also showed different histological alterations included pyknotic nuclei and vacuolated cytoplasm. The number of the cells in the spermatogenic series showed a significant decrease and empty tubules from spermatids. In addition, the interstitial spaces showed edema, which appeared as deeply eosinophilic homogenous areas in-between seminiferous tubules with a reduction of interstitial cells number (Fig. 5E1). As illustrated in Fig. 5E2, the testicular parenchyma of the rats administered the combined treatments (LDR + RUT) showed shrunken seminiferous tubules. Disorganization of spermatogenic cells within the seminiferous tubules was seen. The seminiferous tubules showed a significant reduction in the number of spermatogonia, primary spermatocytes, secondary spermatocytes, and spermatids. Reduction of interstitial cell number and edema with a widening interstitium space was seen (Score 2).

Discussion

Nicotine is well known for promoting ROS generation and causing oxidative stress in all tissues, particularly the testis, causing lipid peroxidation in spermatozoa and other testicular cells and altering DNA, RNA, and protein activities, as well as decreasing antioxidant enzyme activity (Erat et al., 2007). In the present study, rats that received 1mg/kg of nicotine for 30 continuous days had substantial changes in the testicular TBARS, a marker of lipid peroxidation, and reduced GSH contents, indicating nicotine-induced oxidative stress. These results align with previous researches (Oyeyipo et al., 2014; Ray & Majumder, 2018; Ashoub et al., 2021).

Hormones are important for spermatogenesis, and nicotine-induced ROS can reduce male reproductive hormone levels and disrupt hormonal balance directly or indirectly by causing oxidative stress or acting on hormone release from the hypothalamic axis, leading to infertility. (Jana et al., 2010; Spiers et al., 2015; Ashoub et al., 2021). In parallel with the previously mentioned studies, this current investigation has confirmed that following nicotine administration, the activities of 3β-HSD and 17β-HSD, which are the major enzymes responsible for the synthesis of the male reproductive hormones, were reduced. Accordingly, the FSH and LH, anterior pituitary gonadotropic hormones, were subsequently reduced due to oxidative stress activation and nicotine's adverse effect on the central nervous system by decreasing neuronal stimulation necessary for pituitary gonadotropin release. (Reddy et al. 1995). Moreover, testosterone levels were reduced following nicotine administration, consistent with prior findings (Jana et al., 2010; Kolawole et al., 2019; Ashoub et al., 2021). Many factors contributed to the decline in testosterone levels, including a decrease in the FSH and LH levels, which maintains the testosterone levels via the hypothalamic-pituitary-testicular axis (Tweed et al., 2012; Oyeyipo et al., 2013), disruption of testicular cytoarchitecture, which negatively affects the number and function of Leydig cells (Oyeyipo et al., 2010) and cholinergic nicotine agonist activity, that inhibits testosterone secretion (Ni et al., 2020).

Furthermore, nicotine increased LDH serum activity in the current study, which was interpreted as a sign of testicular degeneration. These results

agree with those of Salahipour et al. (2017) and Ashoub et al. (2021). Lactate produced by Sertoli cells by the action of LDH and the disruption of this pathway negatively affects the normal reproductive physiology (Mullaney et al., 1994). Evidence of testicular injury was also reflected histologically by the changes in the spermatogenic cells, atrophy, and degenerative alteration in seminiferous tubules. Taking into consideration all the histological findings, it could be suggested that nicotine produces significant histological alterations in the testes, which might be the cause of the increase of LDH in serum. This finding is also corroborated with the previous findings related to the effects of nicotine on testicular injury (Castro et al., 2018; Ray & Majumder, 2018; Ashoub et al., 2021).

During the past years, a remarkable progress has been made towards understanding the mechanism of action of LDR in biological systems. Several studies have been conducted to examine the benefits of LDR in various animal models, such as those of El-Ghazaly et al. (1985, 2015, 2020) and Nowosielska et al. (2009). The present study intended to investigate the beneficial effects of LDR alone or in combination with natural antioxidants (EGCG or RUT) in testicular injury induced by nicotine.

The results revealed that LDR decreased the TBARS testicular content. This suppression could arguably be due to the free radicals within the cells as carriers/transcription factors to stimulate the body›s defense system. Furthermore, the present findings concerning the GSH system have also clearly demonstrated that LDR exposure affords an increase in the reduced GSH testicular content. This improved action could be attributed to the scavenging effect of LDR towards free radicals responsible for the depletion of GSH (Kojima et al., 2002; Yukawa et al., 2005). To further assess the impact of LDR on the testis, the present study results showed that LDR attenuates the androgenic enzymes (3β-HSD, 17β-HSD) and LDH activities, in addition to serum sex hormones (FSH, LH, and testosterone) levels as well as the histopathological changes induced by nicotine. The finding of the current study is in harmony with the observations reported in the study of Zhao et al. (2010) and Ashoub et al. (2021), all of which suggest that LDR›s improved action is most likely due to its antioxidant properties.

According to previous studies, natural antioxidants can prevent and repair cell damage, impacting the semen quality parameters and fertility potential (Maneesh & Jayalekshmi, 2006; Kopa et al., 2021). In terms of EGCG, it exerts direct antioxidant actions by scavenging free radicals or chelating iron and other metals via EGCG phenolic groups (Legeay et al., 2015; Zwolak, 2021). Besides, EGCG can exert indirect antioxidant effects by stimulating the synthesis of endogenous antioxidant enzymes, such as superoxide dismutase, glutathione reductase, glutathione-S-reductase, and catalase (Bernatoniene & Kopustinskiene, 2018; Zhang et al., 2020). Owing to these effects, EGCG inhibited testicular oxidative stress after nicotine administration by restoring the TBARS and the reduced GSH testicular content. These results agree with those of Guvvala et al. (2017), who suggested that EGCG reduces the testicular oxidative stress induced by arsenic poisoning and protects the reproductive system. Furthermore, treating rats with EGCG can ameliorate various parameters related to androgenic enzymes, LDH, and sex hormones, as well as preserve the histological architecture of the testis. This protective effect may be related to its antioxidant activity emphasized by different publications (Sugiyama et al., 2012; Bagherpour et al., 2019).

In the same line, other flavonoids with antioxidant properties as RUT have also been reported to prevent testicular toxicity in different models. For instance, Abarikwu et al. (2012) showed RUT's antioxidant potential against cyclophosphamide-induced reproductive toxicity. In addition, Jahan et al. (2018) reported that RUT protected the testicular tissues against cisplatin's detrimental effects and reduced oxidative stress. RUT antioxidant effect was proven in the present study as RUT ameliorated the testicular tissue of TBARS and reduced the GSH content induced by nicotine administration. Furthermore, the obtained data revealed that RUT reverted the deleterious effects of nicotine on testicular steroidogenesis as manifested by restoring the androgenic enzymes, LDH activities, and sex hormones serum levels. This amendment effect may be attributed at least partially to improving the oxidative stress and antioxidant defense system. The antioxidant activities of RUT are due to its chemical structure, which can directly scavenge ROS, and its ability to increase the production of GSH and the cellular defense system (Abarikwu et al., 2012;

Pivec et al., 2019). As a result, RUT showed some tendency to improve the architecture of the histological deterioration of the testis induced by nicotine administration. The present finding is in harmony with the observations reported by Abarikwu et al. (2020), which showed that RUT administration tended to improve the architecture of the seminiferous epithelium of the rat's testes after busulfan treatment.

Conclusion

Employing natural antioxidants alongside LDR as part of a dual treatment strategy appears to be safe for achieving the study's aim. The present research showed that combining either EGCG or RUT with LDR effectively reduces and repairs nicotine-induced testicular injury by regulating all of the nicotine's detrimental consequences, as evidenced by biochemical and histological findings, which may explain the presence of the potent antioxidant effect. As a result, the present findings urge further research in a more clinically relevant context to see whether this is a viable approach for preserving fertility among heavy smokers in the future.

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