Quercetin Guards Against Nicotine-Induced Testicular Changes in Albino Rats Via LC3, P62 and Nrf2 Regulation

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Research Article

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ABSTRACT

Introduction: Cigarette smoking is hazardous to cells. Nicotine disturbs cell division and destroys the testes. It decreases testicular antioxidants and raises testicular lipid peroxidation. Quercetin has a powerful antioxidant property by scavenging reactive oxygen species.

Aim of the Work: To investigate the possible ameliorating effect of quercetin on the nicotine-induced testicular changes in rats.

Study Design: Twenty-four adult male rats were divided randomly into 3 groups of 8 rats each; Negative control: Were injected subcutaneously (SC) by distilled water, nicotine treated group: Were injected SC by nicotine (6mg/kg/d) for 4 weeks, and nicotine + quercetin group: Were injected SC by nicotine (6mg/kg/d) and received quercetin (302 mg/kg/day) orally for 4 weeks.

Materials and Methods: Testes were stained by H & E to check the alterations in the testicular architecture. Immunohistochemical detection of oxidative stress marker (Nrf2) and autophagy markers (P62 and LC3) were performed. Also, immunohistochemical quantitation of Nrf2, P62 and LC3 was done.

Results: Distorted seminiferous tubules with degenerated and vacuolated germ cells were found in the nicotine group. The testicular architecture was restored after quercetin administration. The area % of positive P62 and LC3 immune reaction was statistically larger in nicotine treated group and it reduced after coadministration of quercetin. On contrary, Nrf2 area % of positive reaction statistically reduced in nicotine treated group and it increased in nicotine + quercetin group.

Conclusion: Quercetin has the ability to reverse nicotine-induced testicular damage via modulation of Nrf2 as well as autophagic markers (P62 and LC3). Also, coadministration of quercetin markedly normalized the testicular histological architecture.

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INTRODUCTION

Cigarette smoking is a widespread addictive habit, particularly among teenagers and young adults. Tobacco smoke contains over 4,000 harmful compounds, including oxidants and carcinogens, which contribute to cellular damage and inflammation[1]. Epidemiological studies have demonstrated a strong association between smoking and several diseases, including cardiovascular disorders, hypertension, infertility, and cancer[2].

Nicotine, a toxic alkaloid derived from the tobacco plant, is rapidly absorbed by the body, disrupting cellular function by interacting with intracellular components such as tubulin proteins. This leads to impaired cell division, particularly in germ cells, ultimately affecting spermatogenesis and testicular function[3]. Nicotine exposure in laboratory animals has been shown to induce testicular degeneration, sperm abnormalities, and oxidative stress, increasing lipid peroxidation while depleting testicular antioxidants[4].

Quercetin (QCT), a naturally occurring flavonoid, is found in various fruits and vegetables, including apples, onions, and red wine. Due to its unique chemical structure, QCT exhibits potent antioxidant and cytoprotective properties, reducing oxidative damage by scavenging reactive oxygen species[5]. Additionally, QCT inhibits lipid peroxidation and enhances cellular defense mechanisms[6].

Autophagy, a conserved catabolic process, plays a crucial role in cellular homeostasis by degrading damaged cytoplasmic components. It is particularly important in spermatogenesis, facilitating sperm maturation, acrosome

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formation, and mitochondrial restructuring[7]. Autophagy-related proteins such as LC3 and P62 are key regulators of this process, with LC3 involved in autophagosome formation and P62 acting as a substrate for autophagic degradation[8].

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a critical transcription factor that regulates antioxidant responses. Under oxidative stress, Nrf2 translocates to the nucleus, activating cytoprotective genes. Studies have shown that Nrf2 overexpression protects against oxidative damage in various tissues[9].

This study investigates quercetin's protective role against nicotine-induced testicular toxicity, focusing on its immunohistochemical effects on Nrf2 and autophagic markers (P62 and LC3).

MATERIALS AND METHODS

Ethics approval

The study was approved by the Animal Care and Use Committee – Mansoura University (approval no.: VM.R.23.08.121). The rats were kept under veterinary care at Veterinary Medicine, Mansoura University, Egypt, where the experiment was carried out, and all effort was made to reduce both their number and degree of discomfort.

Chemicals

Nicotine and quercetin were obtained from Sigma Chemical Company (St. Louis, MO, USA) in powdered form and dissolved in distilled water prior to administration.

Animals

Twenty-four adult male albino rats (3 months old, weighing 200-250g) were housed under a 12-hour light/dark cycle with ad libitum access to food and water.

Experimental design

The rats were split into three groups (n=8) after two weeks of habituation; Negative control: Were injected subcutaneously (SC) by distilled water, nicotine group: Were injected SC by nicotine (6mg/kg/d) for 4 weeks, and nicotine + quercetin group: Were injected SC by nicotine (6mg/kg/d) and received quercetin (302 mg/kg/day) orally for 4 weeks[10] (Figure 1).

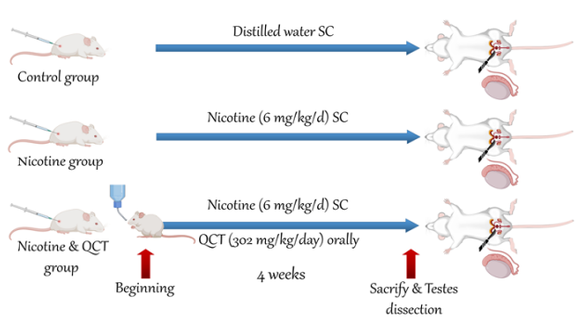

Fig. 1: The study design. Three groups were used. Nicotine and QCT were given subcutaneously for 4 weeks.

Sample preparation

Under anaesthesia, cervical dislocation was used to kill the rats. Testicles were removed. After fixing all of the specimens in 10% formalin, they were sectioned, processed to create paraffin blocks, and stained with hematoxylin and eosin (H & E). Testicular tissues were also immunostained for the Nrf2, P62, and LC3 markers.

Immunostaining of LC3, P62, and Nrf2 in testicular tissue

After dewaxing and rehydrating, 4 µm slices were heated to 60° for an hour in citrate buffer (PH = 6.0) to retrieve antigens. They were then coated with 3% hydrogen peroxide for 10–30 minutes at 37°C to inhibit endogenous peroxidase, and finally washed in phosphate-buffered saline (PBS). After 30 minutes of blocking nonspecific binding sites with 10% normal goat serum (100 mL/L), the sites were three times cleaned in PBS. Polyclonal antibodies against NRF2, P62, and LC3 (1:300 dilution, 1:500 dilution), were diluted and incubated on sections for an entire night at 4°.

After three PBS washes, the sections were incubated for 30 minutes at 37°C with the secondary antibody. To colorize the slides, 3, 3-diaminobenzidine was incubated. Positive cells with a brown or brownish-yellow hue were easily seen[11].

Measurement of % area of positive LC3, P62, and Nrf2 immunoreaction in testicular tissue

Every group of rats had five randomly selected regions examined. The area fraction of immunological expression was computed with a 20 objective. A computerized image analysis system called Image-j (version 1.48) was used to analyze the immune-positive reaction. Each section's strata almost entirely exhibited a unique, immune-expressing brownish color. The color deconvolution plugin was used to separate each image's color content. To improve precision, the cutoff point was adjusted[11].

Statistical analysis

Version 26.0 of IBM SPSS for Windows was used to analyze the data. The data were found to be normally distributed according to the results of the Shapiro-Wright test for normality. The significance level was set at the (0.05) level, and the data were reported as mean ±SD. The three study groups' means were compared using the one-way ANOVA test, and pairwise comparisons were performed using the Post Hoc Games-Howell test.

RESULTS

The effect of QCT administration on testicular histological architecture

The testicular sections of control rats stained with hematoxylin and eosin showed a variety of sizes of seminiferous tubules that appeared nearly circular in cross-section. These tubules were separated by a small

amount of Leydig cell-containing interstitial tissue, lined by several layers of spermatocytes, and filled with sperm (Figure 2A). Seminiferous tubules of nicotine-impaired rats showed deformities, including some germ cells that had deteriorated and vacuolated, no sperm, and acidophilic substances in the lumen. A small number of Leydig cells and dilated blood vessels were present in the interstitial tissue where inflammatory cells had spread throughout (Figure 2B). Rats given nicotine and quercetin, on the other hand, displayed testicles that were full of sperm and had seminiferous tubules that were essentially normal. Interstitial tissue revealed an increased number of Leydig cells (Figure 2c).

In the control group, the spermatocytes showed a negative immune response (Figure 3A). On the other hand, the immunoreactivity in the nicotine group was strongly positive in the spermatocytes (Figure 3B). Furthermore, spermatocytes of quercetin-treated group exhibited minimally favourable immune response (Figure 3C). Nicotine group showed significantly higher area % of positive reactivity for P62 compared to control group (p=0.001). Additionally, rats treated with quercetin demonstrated considerably lower area % of positive reactivity for P62 compared to the nicotine group (p= 0.04) (Figure 3D).

Effect of quercetin on the immunohistochemical expression& area percentage of positive LC3 in the testis tissue

The spermatocytes in the control group revealed a negative immune response (Figure 4A). while in the nicotine group, the spermatocytes displayed moderate immunoreactivity (Figure 4B). On the other hand, there was mild immune response in the spermatocytes of quercetin treated group (Figure 4C). The Nicotine group showed a significantly higher area % of positive reactivity for LC3 compared to control group (p=0.001). Additionally, rats treated with quercetin demonstrated considerably lower area % of positive reactivity for LC3 compared to the nicotine group (p= 0.001) (Figure 4D).

Effect of quercetin on the immunohistochemical expression & area percentage of positive Nrf2 in the testis tissue

Spermatocytes of the control group exhibited moderate immune response (Figure 5A). However, nicotine-intoxicated rats showed mild positive immunoreactivity in the spermatocytes (Figure 5B). Furthermore, quercetin-received rats displayed strongly positive immunoreactivity in the spermatocytes (Figure 5C). Area % of positive Nrf2 of nicotine group was significantly lower compared to control group (p=0.001). however, quercetin group revealed considerably higher area % of positive reactivity for Nrf2 compared to nicotine group (p= 0.001) (Figure 5D).

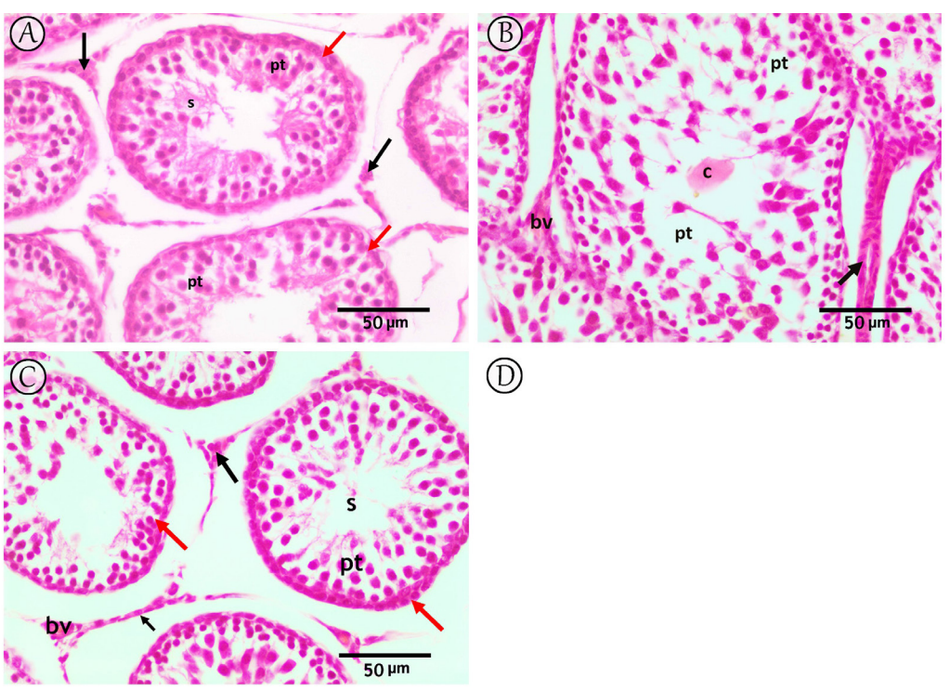

Fig. 2: Effect of quercetin on the testis structure in the study groups:
(A) sections of the testis of control group, displayed variable sized seminiferous tubules which appeared nearly circular in cross section (red arrow) separated by small amount of interstitial tissue containing Leydig cells (black arrow), lined by multiple layers of spermatocytes (pt) and filled with sperms (s). (B) nicotine group showed distorted seminiferous tubules with degenerated and vacuolated some germ cells (pt), acidophilic material was found in the lumen of some tubules(c). Interstitial tissue was diffusely infiltrated with inflammatory cells (I), few number of Leydig cells (black arrow) and congested blood vessel (bv) were seen. (C) seminiferous tubules of quercetin treated group appeared more or less normal (red arrow), lined by multiple layers of spermatocytes (pt) and filled with sperms (s). Interstitial tissue showed increased number of Leydig cells (black arrow) and congested blood vessel (bv) was seen.

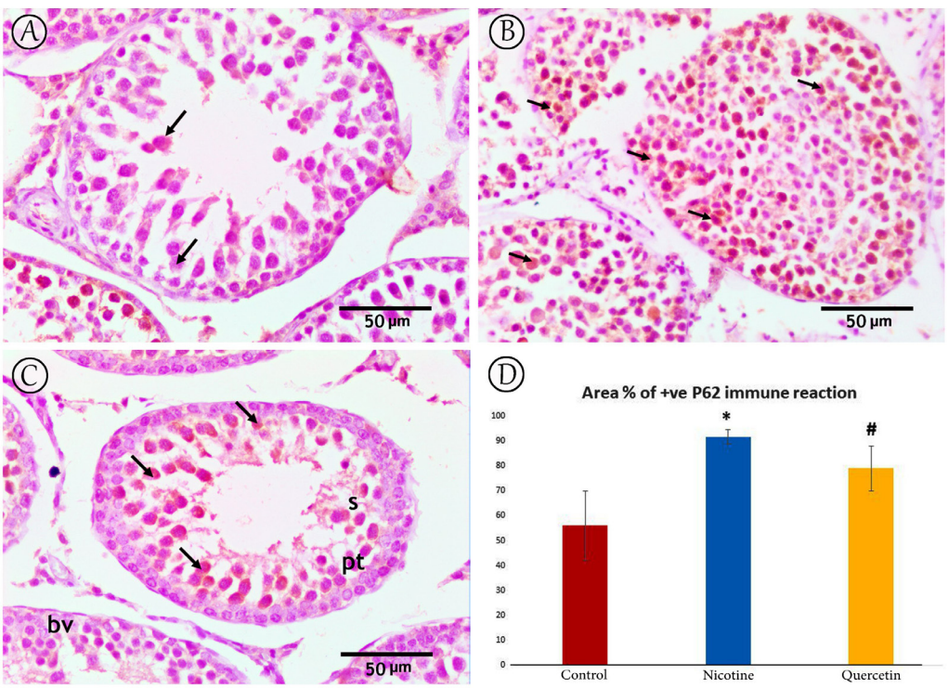

Fig. 3: Effects of quercetin on immunohistochemical expression and area % of positive P62 in testis-stained sections.
(A)The control group showed negative immune reaction in the spermatocytes (arrows). (B) strong immunoreactivity was present in nicotine group (arrows). (C) the spermatocytes of the quercetin group revealed minimally favorable immune response (arrows). (D) A one-way ANOVA was utilized, followed by Games Howell post- hoc analysis*significance versus the control group: p≤0.001 and # significance versus nicotine group: $p \leq 0.04$.

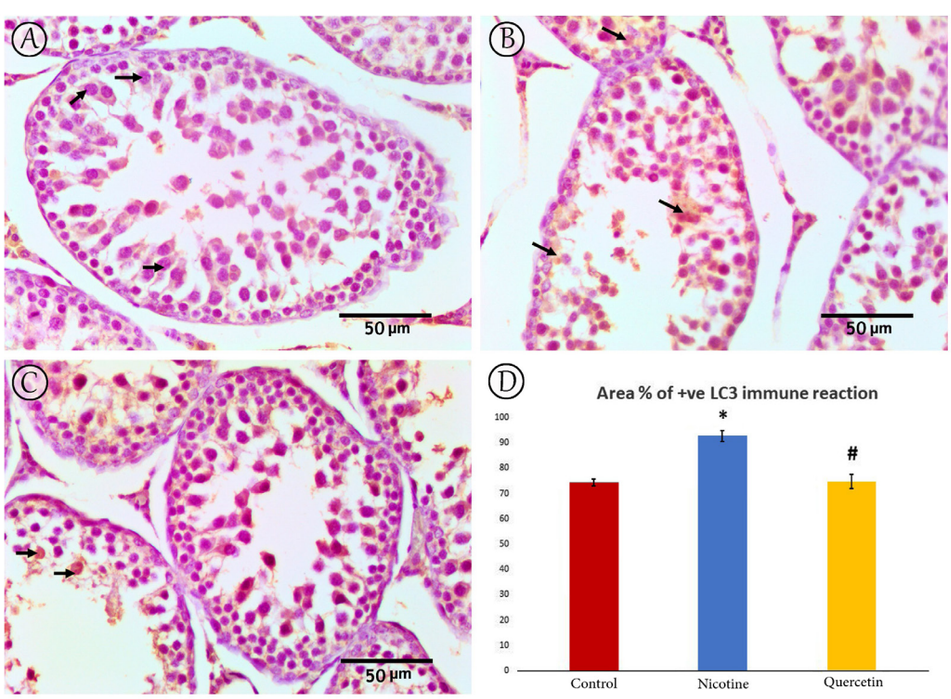

Fig. 4: Effects of quercetin on immunohistochemical expression and area % of positive LC3 in testis-stained sections.
(A)The spermatocytes in the control group revealed negative immune response (arrows). (B) moderate immunoreactivity was present in nicotine group (arrows). (C) while, quercetin treated group exhibited minimal immune response in the spermatocytes (arrows). (D) A one-way ANOVA was utilized, followed by Games Howell post- hoc analysis*significance versus the control group and # significance versus nicotine group: $p \leq 0.001$.

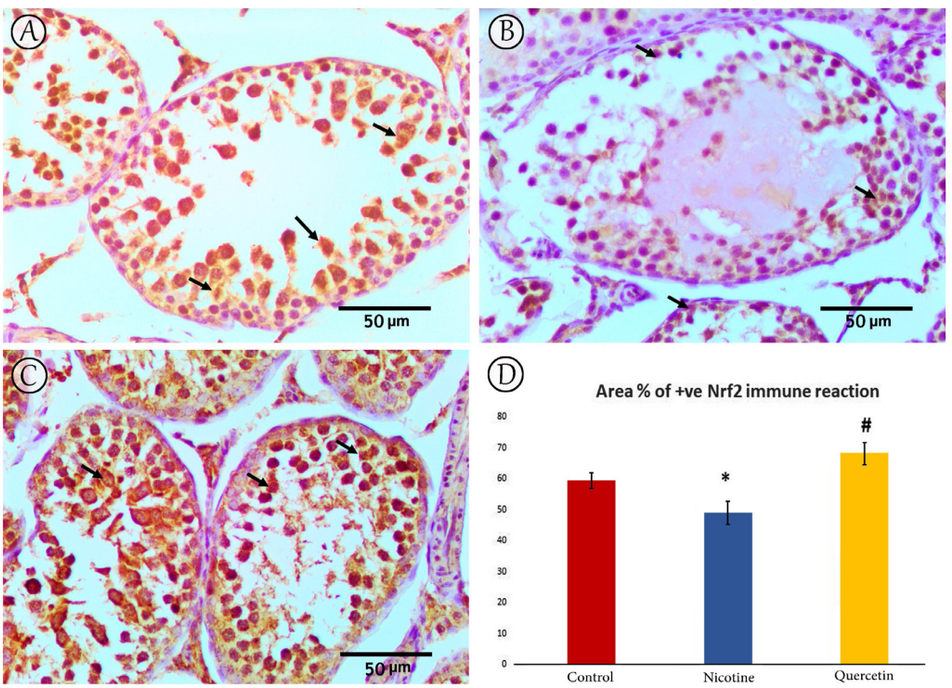

Fig. 5: Effects of quercetin on immunohistochemical expression and area % of positive Nrf2 in testis-stained sections.
(A) The control group showed moderated immune reaction in the spermatocytes (arrows). (B) the spermatocytes of nicotine group displayed mild immunoreactivity (arrows). (C) however, significant positive immune response was present in the quercetin treated rats (arrows). (D) A one-way ANOVA was utilized, followed by Games Howell post- hoc analysis*significance versus the control group and # significance versus nicotine group: $p \leq 0.001$.

DISCUSSION

The current study declared that nicotine hampered P62 and LC3 autophagic degradation and reduced the oxidative defense component Nrf2. Notably, the testicular histological architecture was significantly normalized when quercetin and nicotine were administered together. Quercetin caused the autophagic degradation of P62 and LC3 and significantly raised Nrf2. These findings were consistent with earlier research that documented the toxicity of nicotine on the testicles.

Growing research in both human and animal models has linked nicotine to male reproductive damage[12]. Research is still ongoing to determine the precise underlying mechanisms of nicotine-induced testicular damage and the most effective preventive medicines against it[13]. The current work sought to determine how nicotine affects testicular antioxidant defense factor (Nrf2) and autophagic indicators (LC3 and P62), and how quercetin protects against these effects by modifying these factors.

In accordance with[14-17], the current results showed that rats exposed to nicotine had reduced Leydig cell counts and impaired spermatogenesis when compared to controls. It is possible that decreased testicular testosterone resulted from defective spermatogenesis and the absence of mature sperm in rats exposed to nicotine[18]. Both directly and indirectly through its effects on Leydig cells and the hypothalamus-pituitary-testicular axis[19], nicotine reduces testicular testosterone[20]. Apoptosis and/or autophagy after nicotine exposure cause Leydig cell death[15,21]. Furthermore, according to[17], nicotine dramatically reduced the expression of the steroidogenic acute regulatory protein (StAR) gene. According to[21], nicotine impedes the delivery of cholesterol to mitochondria, which is thought to be the first stage of testosterone production in Leydig cells. Contrary to the current findings,[22] discovered that nicotine exposure did not affect Leydig cells. The variation in the experimental design concerning the length of exposure and the species used could account for the discrepancy.

Normal spermatogenesis requires a careful balance between reactive oxygen species (ROS) and antioxidant defense components. Testicular oxidative stress is exacerbated by nicotine and its active metabolite cotinine, as seen by elevated malondialdehyde (MDA) levels, which are consistent with low levels of antioxidants such as superoxide dismutase (SOD)[23].

A crucial upstream component in the transcription of several antioxidants is Nrf2. The Nrf2-Keap1 (Kech-like ECH-associated protein 1) signalling pathway regulates Nrf2's activity[22]. After cleaving Keap1, Nrf2 moved to the nucleus and activated multiple antioxidant genes, including heme oxygenase 1, glutathione peroxidase, SOD, and catalase[24]. In comparison to controls, the current study demonstrated a significant lack of Nrf2 protein expression in the testes of rats exposed to nicotine. In a similar vein, 22 observed that oral nicotine delivery resulted in dose- and

time-dependent decreases in testicular Nrf2 expression. Consequently, oxidative stress, inflammation, and reduced StAR as a result of insufficient Nrf2 were used by[25] to explain the poor spermatogenesis associated with nicotine. According to[24], nicotine significantly suppressed the Nrf2 encoding gene.

Regarding autophagy, P62 and LC3 are well-known indicators of macroautophagy, a process in which lysosomes and an autophagosome, a double-membrane vesicle, merge to facilitate the breakdown of macromolecules[26]. One of the macroautophagy substrates, P62, increases with inefficient autophagy and decreases with efficient autophagy[27]. By cleaving LC3-I into LC3-II, microtubule-associated protein 1 light chain (LC3) is activated. Beclin-1 and LC3-II cooperate to generate and lengthen the autophagosome membrane. Elevated LC3 may result from elevated gene expression or the build-up of unprocessed autophagosomes in cases of poor autophagosome membrane fusion with lysosomes, which are indicative of improper autophagy[28].

Thus, in both the in *vitro* Leydig cell line and the in *vivo* model of C57BL/6 J mice, nicotine enhanced the expression of both LC3 and Beclin-1, resulting in an increase in autophagosomes and a decrease in autophagic degradation in Leydig cells[21]. Furthermore, nicotine's modifying influence on the AKT/mTOR pathway via protein kinase B was linked to decreased autophagic breakdown. Rats exposed to nicotine had defective autophagy in their testes, which resulted in lower testosterone synthesis as seen by a decrease in StAR detection[21]. Autophagy plays a favourable role in the physiological process of cell remodeling during spermatogenesis and in the functions of Sertoli cells. Cell death is a result of both insufficient and excessive autophagy[29].

In contrast to the findings of the previous investigations and our own, nicotine was linked to lower levels of Beclin-1 and LC3-II gene expression in the germinal cells of Wistar male rats. It was believed that the evidence of testicular damage and blockage of the autophagosome formation stage would result in the activation of inflammation and apoptosis[13]. The variations in nicotine exposure dosage and route duration may be expected to result from various study designs. Autophagy and apoptosis are both involved in Leydig and germinal cell death[21]. According to certain theories, moderate to early stress promotes autophagy, whereas severe, ongoing stress causes necrosis, inflammation, and apoptosis[30].

Remarkably, earlier research focused on the reciprocal association between the Nrf2-Keap1 antioxidant pathway and the autophagic marker P62[31]. Phosphorylated P62 binds to the Keap1 domain of the Nrf2-Keap1 complex in one direction to create the LC3-P62-Keap1 complex, which is then selectively autophagically destroyed. To promote the production of antioxidant genes, active free Nrf2 is translocated to the nucleus[32]. On the other hand, Nrf2 suppresses the inhibitory mTOR pathway and enhances the expression of autophagic genes, such as LC3 and P62. Frias *et al*.[33] have reported that Nrf2 is a recognized inducer of efficient autophagy in response to stress. In the present study, nicotine reduced Nrf2 expression in testicular cells in correlation with noticeably elevated P62 and LC3 expression. One possibility is that a deficit in Nrf2 inhibits effective autophagy. It is highly advised to investigate the precise pathway that is impacted, particularly about mTOR. Since nicotine has been shown to lower Nrf2 gene expression, enhanced P62 in the current study was unable to effectively activate Nrf2[24].

As compared to the nicotine group, the co-administration of quercetin and nicotine in the current study significantly enhanced spermatogenesis and increased the number of Leydig cells, which is consistent with lower interstitial inflammation. Similarly, in rats exposed to nicotine, cotreatment with quercetin alone[34] or with additional antioxidants[35] preserved the testes' normal histological architecture. Thus, by reducing testicular oxidative stress and inflammatory alterations, quercetin co-administration counteracted the harmful effects of lead acetate and aluminium trioxide nanoparticles[36]. Consistent with what we found, quercetin prevented cotinine, an active metabolite of nicotine, from negatively affecting the motility and viability of sperm[23]. Additionally, it has been observed that quercetin improves spermatogenesis through its anti-inflammatory, anti-apoptotic, and antioxidant properties[37].

In the present investigation, testicular Nrf2 expression was significantly elevated in the quercetin cotreatment group when compared to the nicotine group. According to research, quercetin counteracts nicotine dependence by boosting Nrf2 antioxidant activity in the mice's brain reward system when they are exposed to nicotine[12]. Consistent with our findings, quercetin increased Nrf2 expression in a streptozotocin-induced type 1 diabetic rat model, thereby reversing the oxidative and apoptotic testicular damage[38]. Furthermore, by raising Nrf2 activity, quercetin reduced testicular oxidative stress, inflammation, and mitochondrial-induced apoptosis caused by the antiepileptic levetiracetam[19]. Quercetin's effect on Nrf2 may be explained by increasing nuclear translocation of the protein and gene forms of Nrf2, which enhances Nrf2 activity[39].

In the current data, quercetin reduced the testicular expression of both P62 and LC3 as compared to the nicotine group. This could be explained by quercetin-induced autophagic breakdown of P62 and LC3. In a similar vein, quercetin prevented mice that had been inebriated with cadmium from having an increase in P62 and LC3 expression in their testes[26]. Reduced P62 expression with quercetin was explained by[40], who enhanced P62 degradation by inducing the autophagic flux phase, which resulted in the fusion of an autophagosome with a lysosome. Additionally, it has been observed that quercetin stimulates the production of autophagosomes and activates genes linked to autophagy in gastric cancer cells[41] and Sertoli cell lines exposed to toxic zinc oxide nanoparticles[42].

Quercetin nanoparticles have been shown to decrease P62 in human neuroglioma cells while increasing LC3 in the form of an elevated LC3-II/LC3-I ratio[43], which is in partial agreement with our findings. They linked that to quercetin's capacity to disrupt the signalling pathway of phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT), hence decreasing the autophagic inhibitory pathway mTOR. The discrepancy between this study and others on the impact of quercetin on LC3 may be attributed to variations in study design concerning the experimental model employed and the LC3 detection technique.

To the best of our knowledge, this was the first-time quercetin's ability to modulate Nrf2 and autophagic markers (P62 and LC3) was demonstrated to repair nicotine-induced testicular injury. Therefore, quercetin may be viewed as a helpful protective factor against the toxicity of nicotine on male reproduction. To determine the precise mechanisms (such as PI3K -AKT-mTOR and MAPK-mTOR) by which quercetin mediated its autophagic protective action against nicotine, more research is strongly advised.

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CONFLICT OF INTERESTS

There are no conflicts of interest.

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الملخص العربى

يقى الكيرسيتين من التغيرات التي يسببها النيكوتين في الخصيتين لدى الجرذان البيضاء من خلال تنظيم LC3 وP62 وNrf2

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مقدمة: يُعد تدخين السجائر ضارًا بالخلايا إذ يُعيق النيكوتين انقسامها ويُدمر الخصيتين. كما يُقلل من مضادات الأكسدة ويزيد من أكسدة الدهون في الخصيتين.
يتميز الكيرسيتين بخاصية قوية مضادة للأكسدة عن طريق إزالة مواد الأكسجين التفاعلية.
الهدف من البحث: دراسة التأثير المُحسِّن المُحتمل للكيرسيتين على التغيرات الخصوية المُحفزة بالنيكوتين لدى الجرذان.
مواد و طرق البحث: قُسِّم أربعة وعشرون جرذًا بالغًا ذكرًا عشوائيًا إلى ثلاث مجموعات، تضم كل مجموعة ثمانية جرذان؛ مجموعة التحكم السلبية: حُقنت تحت الجلد بالماء المقطر، المجموعة المعالجة بالنيكوتين: حُقنت تحت الجلد بالنيكوتين (٦ ملغ/كغ/يوم) لمدة ٤ أسابيع، ومجموعة النيكوتين + كيرسيتين: حُقنت تحت الجلد بالنيكوتين (٦ ملغ/كغ/يوم) وتلقت كيرسيتين (٣٠٢ ملغ/كغ/يوم) عن طريق الفم لمدة ٤ أسابيع.
ثم صُبغت الخصيتان بصبغة الهيماتوكسيلين والإيوسين للتحقق من التغيرات في بنية الخصية كما أُجري الكشف المناعي الكيميائي عن مؤشر الإجهاد التأكسدي (Nrf٢) ومؤشرات الالتهام الذاتي .(P٦٢, LC٣) كما أُجري تحديد كمي مناعي كيميائي لـكل من Nrf٢ وP٦٢ وLC٣.
النتائج: وُجدت أنابيب منوية مشوهة مع خلايا جرثومية متحللة ومتفرعة في مجموعة النيكوتين. استُعيدت بنية الخصية بعد إعطاء الكيرسيتين. كانت نسبة مساحة التفاعل المناعي الإيجابي لـ P٦٢ وLC٣ أكبر إحصائيًا في المجموعة المعالجة بالنيكوتين، وانخفضت بعد الإعطاء المشترك للكيرسيتين. و على العكس من ذلك، انخفضت نسبة مساحة التفاعل المناعي الإيجابي لـ Nrf٢ إحصائيًا في المجموعة المعالجة بالنيكوتين، وزادت في المجموعة التي تناولت النيكوتين مع الكيرسيتين.
خلاصة البحث: يمتلك الكيرسيتين القدرة على معالجة تلف الخصية الناتج عن النيكوتين من خلال تعديل Nrf٢، بالإضافة إلى علامات الالتهام الذاتي .(P٦٢, LC٣) كما أدى الإعطاء المشترك للكيرسيتين إلى استعادة البنية النسيجية للخصية بشكل ملحوظ.