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ORIGINAL ARTICLE

Male Reproduction

Comparison of quercetin and resveratrol in the prevention of injury due to testicular torsion/detorsion in rats

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Quercetin (QE) and resveratrol (RSV) are powerful antioxidants with the potential to protect the testes against ischemia/reperfusion (I/R) injury. We compared their effects in testicular torsion/detorsion (T/D) in adult rats. Twenty-four male Wistar rats were divided into four groups: sham (group A), T/D (group B), T/D treated with QE (group C), and T/D treated with RSV (group D). QE (20 mg kg^{-1}) and RSV (20 mg kg^{-1}) were injected intra-peritoneally at 60 min of torsion. After 90 min of surgically induced torsion, the testicular cord was restored to its anatomical position. Twenty-four hour after torsion, blood and tissue samples were obtained for further examination. Testicular tissue malondialdehyde (MDA) and nitric oxide (NO) levels and serum total oxidant status (TOS) were higher in group B than in group A ($P < 0.05$). Group A had higher serum total antioxidant status (TAS) than group B. ($P < 0.05$) QE and RSV significantly lowered MDA, NO, and TOS levels and TAS consumption ($P < 0.05$). QE reduced the MDA and TOS levels more than RSV ($P < 0.05$), but their effects on NO reduction and TAS consumption were similar ($P > 0.05$). Group A had normal testicular architecture (grade 1). Groups C (mean grade 2.60) and D (mean grade 3.00) had lower testicular injury grades than group B (mean grade 3.45) ($P < 0.05$). Group C had lower testicular injury grade than group D ($P < 0.05$). Treatment with QE and RSV protects against I/R injury after testicular T/D. QE may exhibit better function than RSV at the doses tested in this study.

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INTRODUCTION

Testicular torsion, a serious urologic emergency, occurs primarily in newborns, children, and young male adults.[1] It causes testicular ischemia injury due to rotation of the spermatic cord, initially leading to obstruction of venous return and subsequently leading to obstruction of arterial flow; this ultimately leads to either potential serious infertility or subfertility. Immediate diagnosis and surgical intervention are essential in managing this emergent condition. Reperfusion injury is inevitable when blood flow is reestablished, after which reactive oxygen species (ROS)[2,3] and cytokines are overproduced and released, resulting in cellular and tissue damage. Testicular atrophy and apoptosis of germ cells and spermatogenesis can be observed in the laboratory during the ischemia/reperfusion (I/R) process.[1,4–6]

Antioxidants extracted from natural plants have been used in recent studies to protect the testes against I/R injury.[7] According to previous studies, quercetin (QE) is a powerful antioxidant agent with the ability to reduce tissue damage after I/R processes.[8–11] Resveratrol (RSV) is found in many plants, particularly grapes and peanuts. RSV has protective effects in preventing lipid peroxidation in the cell membrane and DNA damage caused by excessive ROS production.[12,13] The aims of this study were to evaluate the potential protective effect of QE and RSV against testicular I/R injury in an experimental model of rats, and compare the results of their administration.

MATERIALS AND METHODS

Animals and reagents

The experimental protocol was reviewed by the Ethics Committee of The First Affiliated Hospital of Henan University of Traditional Chinese Medicine, and the research was conducted within the national animal welfare guidelines. In this study, 24 adult male Wistar albino rats (12–14 weeks old, weighing 250–300 g) were divided in four groups, with six rats per group. The animals were obtained from the Zhengzhou University Animal Research Center and were acclimatized for 1 week prior to the beginning of the experiment. They were housed under standard laboratory conditions with a temperature of 22 ± 2°C, 60% relative humidity, and 12 h light and dark cycles. They were anesthetized with an intra-muscular injection of chloral hydrate (100 mg kg^{-1}) and xylazine (5 mg kg^{-1}), and they breathed spontaneously throughout the procedures.

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Animal models

Animals in group A underwent a sham operation to determine the effect of surgical stress on the testes. First, the right testes were exposed through an incision in the scrotum. The right testes from group B was exposed in the same way as in group A. Second, torsion of each testis was performed by twisting the testicular cord 720° clockwise. The testis was then fixed to the scrotum, and the scrotal incision was closed. Third, after 90 min of testicular torsion, the scrotum was reopened, and the right testicular cord was restored to its anatomical position with the testis replaced back to its normal position. Rats in group C underwent the same procedure as group B, and QE of 20 mg kg^{-1} (Sigma, St. Louis, MO, USA) was injected intra-peritoneally at 60 min of torsion. Rats in group D underwent the same procedure as groups B and C, and RSV of 20 mg kg^{-1} (Sigma) was injected intra-peritoneally at 60 min of torsion.

All rats were eventually sacrificed by exsanguination at 24 h after the surgical procedure under 100 mg kg^{-1} of chloral hydrate anesthesia; the rats' blood and right testes were removed. Testicular tissue and blood samples were obtained for further biochemical and histopathological investigation.

Biochemical parameters

Tissue malondialdehyde

Testicular tissues were weighed and homogenized in ice-cold 1.15% KCl (2 and 10% w/v^{-1}). The homogenate was centrifuged at 2000 ×*g* for 10 min. Malondialdehyde (MDA) levels in the tissue samples were determined by the method of Uchiyama and Mihara.[14] Tetramethoxypropane was used as a standard, and tissue MDA levels were calculated as nmol g^{-1} wet tissue.

Tissue nitric oxide determination

Tissue nitrite (NO_2^-) and nitrate (NO_3^-) levels were estimated as an index of nitric oxide (NO) production. The quantification of NO_2^- and NO_3^- was based on the Griess reaction, in which a chromophore with a strong absorbance at 540 nm is formed by the reaction of nitrite with a mixture of naphthylethylenediamine and sulfanilamide. The results are expressed as μmol g^{-1} wet tissue.

Total oxidant status

The total oxidant status (TOS) was determined using the method previously described by Erel.[15] Serum TOS levels were calculated in μmol H_2O_2 equivalent/l^{-1}.

Total antioxidant status

The total antioxidant status (TAS) level was determined using the method developed by Erel.[16] Serum TAS levels were calculated in mmol Trolox equivalent/l^{-1}.

Histopathological examination

The testicular tissue samples were fixed in Bouin solution and embedded in paraffin. Five micrometer-thick sections were cut, stained with hematoxylin and eosin, and examined with a light microscope (Olympus, Tokyo, Japan). Histological changes in the testes caused by I/R were scored in **Table 1**, according to the grading system proposed by Cosentino *et al.*[17] The I/R injury caused tissue damage with severity ranging among areas in each testis. Therefore, according to Nick *et al.*[18] each area was graded separately, and the final result for each testis was calculated by multiplying the grade for each area by the percentage of the total surface that it occupied. The presence of foci with pyknotic nuclei surrounded by apoptotic bodies was evaluated as apoptosis. Necrosis was defined as the presence of disrupted cell membranes. A pathologist blinded to the study graded the histological changes in the testes.

Statistical analysis

The Shapiro–Wilk test was used to assess the normal distribution of data, and the Levene test was used to assess the homogeneity of variance. Analysis of variance with Bonferroni correction was performed for comparisons among groups. Statistical analysis was performed using SPSS, version 17.0 software (SPSS Inc., Chicago, IL, USA). The results were considered statistically significant if $P < 0.05$.

RESULTS

Biochemical parameters

The results of biochemical oxidant and antioxidant parameters of all groups are presented in **Table 2**. Our results demonstrated significant testicular damage in group B. Testicular tissue MDA and NO levels and serum TOS were higher in group B than in group A ($P < 0.05$). Serum TAS was higher in group A than in group B. ($P < 0.05$) Both the QE and RSV treatment groups showed significantly less I/R injury, with decreased MDA, NO, and TOS levels and less TAS consumption compared with group B ($P < 0.05$). In addition, treatment with QE led to a greater reduction in both MDA and TOS levels than treatment with RSV, and these differences were statistically significant ($P < 0.05$); however, there was no significant difference in the NO level or TAS consumption between the two treatment groups ($P > 0.05$).

Histopathological findings

The results for each group according to the percentage of the total testicular surface corresponding to each grade of testicular injury are shown in **Table 3**, and histological images from each group are demonstrated in **Figures 1–4**. All rats in group A showed a normal testicular structure with an orderly arrangement of germinal cells, corresponding to grade 1 testicular injury findings, which have a total score of 1. Rats in group B (mean grade 3.45, range 3.05–3.90) had severe tissue lesions characterized by disorganized seminiferous tubules packed with irregularly arranged necrosis of the germinal cells, severe vacuolization, and fewer spermatozoa. Rats in group D (mean grade 3.00; range 2.85–3.25) treated with RSV had moderate tissue damage consisting of disordered, sloughed germinal cells with shrunken pyknotic nuclei. There was also a considerable portion with

Table 1: Histological grading system of testicular damage proposed by Cosentino *et al.*

Grade 1 - Showed normal testicular architecture with an orderly arrangement of germinal cells
Grade 2 - Injury showed less orderly, noncohesive germinal cells and closely packed seminiferous tubules
Grade 3 - Injury exhibited disordered sloughed germinal cells with shrunken pyknotic nuclei and less distinct seminiferous tubule borders
Grade 4 - Injury defined seminiferous tubules that were closely packed with coagulative necrosis of the germinal cells

Table 2: Levels of biochemical oxidant and antioxidant parameters and statistical comparisons between the groups

	MDA (nmol g^{-1} wet tissue)	*NO (μmol g^{-1} wet tissue)*	*TOS (μmol H_2O_2 equivalent l^{-1})*	*TAS (mmol trolox equivalent l^{-1})*
Group A	492.07±30.14	3.17±0.19	7.08±0.31	4.97±0.19
Group B	915.33±17.35[a]	7.19±0.16[a]	20.22±1.09a	1.64±0.16[a]
Group C	626.55±21.42[a,b]	4.13±0.18[a,b]	11.16±1.19[a,b]	2.99±0.27[a,b]
Group D	716.32±13.94[a,b,c]	4.14±0.11[a,b]	14.56±0.62[a,b,c]	2.95±0.20[a,b]

Values are expressed as mean±s.d. [a]Significantly different from the group A ($P<0.05$); [b]Significantly different from the group B ($P<0.05$); [c]Significantly different from the group C ($P<0.05$). MDA: malondialdehyde; NO: nitric oxide; TOS: total oxidant status; TAS: total antioxidant status; s.d.: standard deviation

Table 3: Results of each rat according to Cosentino *et al*. grading system

Rat	*Grade 1 (%)*	*Grade 2 (%)*	*Grade 3 (%)*	*Grade 4 (%)*	*Total grades*
A1	100	0	0	0	1
A2	100	0	0	0	1
A3	100	0	0	0	1
A4	100	0	0	0	1
A5	100	0	0	0	1
A6	100	0	0	0	1
B1	0	5	15	80	3.75
B2	0	0	10	90	3.90
B3	0	35	25	40	3.05
B4	0	20	20	60	3.4
B5	0	20	35	45	3.25
B6	0	15	35	50	3.35
C1	15	30	30	25	2.65
C2	0	35	40	25	2.9
C3	0	50	30	20	2.7
C4	10	40	25	25	2.65
C5	10	50	20	20	2.5
C6	0	50	40	10	2.2
D1	5	25	30	40	3.05
D2	0	15	45	40	3.25
D3	0	35	35	30	2.95
D4	5	30	40	25	2.85
D5	0	35	30	35	3
D6	0	40	30	30	2.9

severe changes consisting of closely packed seminiferous tubules with necrotic germinal cells and vacuolization. Rats in group C (mean grade 2.60; range 2.20–2.90) treated with QE showed milder lesions than those in groups B and D. Images from group C show ill-defined seminiferous tubules with disordered, sloughed germinal cells; closely packed but better defined seminiferous tubule borders; and less severe vacuolization. We found statistically significant differences among groups B, C, and D. The treated groups C and D had significantly lower testicular injury grades (better results) than group B ($P < 0.05$ and $P < 0.05$, respectively). Furthermore, rats in group C had significantly lower testicular injury grades (better results) than those in group D ($P < 0.05$) (**Figure 5**).

DISCUSSION

I/R injury involves neutrophil recruitment;[4] the generation of ROS, proinflammatory cytokines, and adhesion molecules; lipid peroxidation; apoptosis; anoxia; and alteration to the microvascular blood flow, which can result in infertility.[19] The main pathophysiological consequence of testicular torsion is I/R injury of the testis, generated by the twisting of the spermatic cord that renders the tissue ischemic, followed by reperfusion on the release of the twisted cord.[5,20] The two most important factors that exacerbate the degree of testicular damage are the duration of torsion and the degree of twisting of the spermatic cord.[4,21]

Effective treatment after testicular torsion is essential to protect the testis against dangerous I/R damage. Several plant extracts have been studied in animal models for their potential as secondary treatments to surgical repair of testicular torsion. Aktoz *et al*.[11] demonstrated that I/R of 5 h causes obvious injury to the testis, and the administration of QE improved the histopathological parameters, increased the expression of testicular eNOS, and increased germ cell apoptosis in the affected testis. In an ethanol-induced testicular injury model in rats, RSV protected against DNA damage and lipid peroxidation in the cell membrane caused by ROS.[22] RSV may work by reducing oxidative stress in the seminiferous tubules and increasing sperm development.[23]

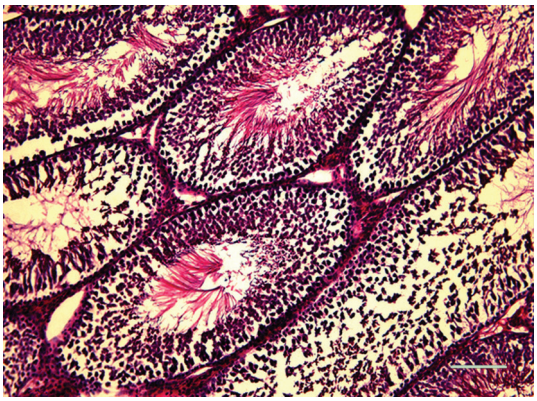

Figure 1: Testis from group A showed normal seminiferous tubules and spermatozoon with rounded figure, and germinal cells were regularly arranged. Scale bar = 20 μm.

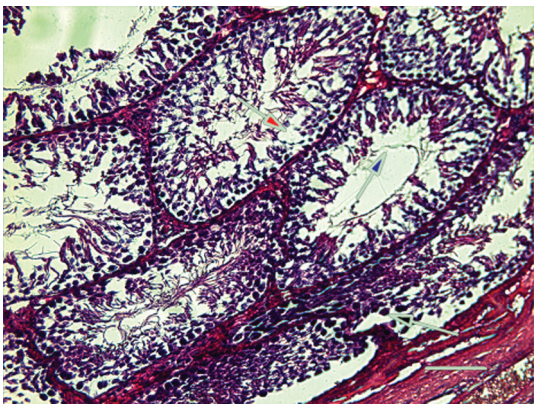

Figure 2: Testis from group B showed disorganized seminiferous tubules packed with irregularly arranged necrosis of the germinal cells (black arrow), severe vacuolization (red arrow) between epithelial cells and lessened spermatozoa (blue arrow). Scale bar = 20 μm.

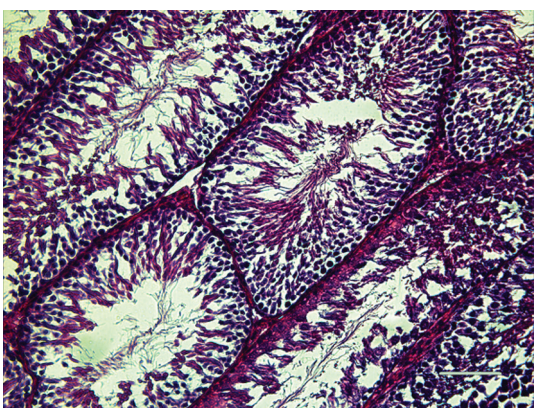

Figure 3: Group C showed the pathological lesion was less severe than group B. Most of the seminiferous tubules were closely packed with both normal germinal epithelial cells and immature germinal cells, with preservation of tubular structure. The maturation is almost up to the level of spermatozoa. Scale bar = 20 μm.

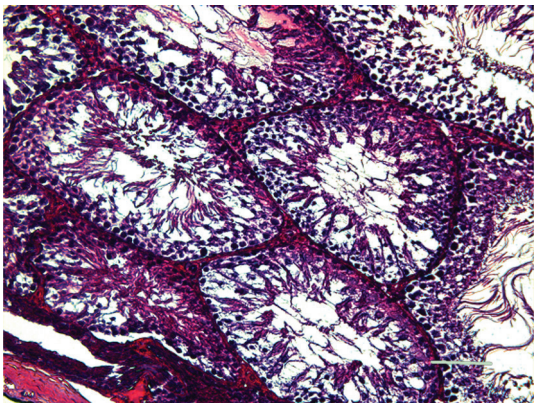

Figure 4: Testis from group D: Most seminiferous tubules showed degenerated, anomalistic germinal cells with necrosis and vacuolization. The lesion was also less severe than group B, but more severe than group C. Scale bar = 20 μm.

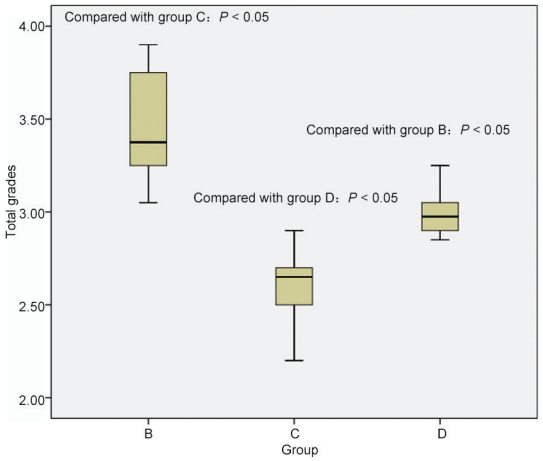

Figure 5: Total histological grades of the rats from groups B, C and D.

In this study, for the first time, we compared the effect of QE and RSV on protecting the testis against I/R injury in rats.

The most important indicator of tissue injury due to I/R injury is the MDA level. MDA is an indirect indicator of lipid peroxidation in cells due to ROS effects. ROS cause chain reactions of lipid peroxidation in the cell membranes, which eventually leads to the generation of the major lipid peroxidation product, MDA.[5,20,24–26] As an indicator of ROS injury, MDA levels in rats with testicular torsion were elevated compared with those in group A in the present study. As free radical scavenger agents and powerful antioxidant hormones in the human body, both QE and RSV decreased the levels of MDA, but QE had a more powerful effect on reducing MDA levels.

As a water- and lipid-soluble free radical, NO plays an essential role in modulating blood flow in normal and pathological states, and its levels seem to affect I/R injury.[5,27,28] NO synthase activity increases and NO levels increase during ischemia.[29] Although blood flow to the testis is re-established, reperfusion results in the generation of excess superoxide radical (O_2^-). In addition, the interaction between NO and O_2^- further promotes cell damage.[30] The increase in NO levels in the I/R group and the partial restoration of NO levels in the drug-treated groups in our study support the hypothesis that QE and RSV play a beneficial role in protecting the testis from I/R injury by inhibiting NO production. However, their protective effect in decreasing NO levels did not differ statistically in our study. Therefore, we consider that another physiological mechanism is involved in the I/R course of events that influence the NO levels and this requires further investigation.

Reactive oxygen surfaces and oxidative defense capacity are balanced in healthy cells under normal circumstances. TOS and TAS parameters are a combination of oxidant and antioxidant parameters such as MDA, glutathione peroxidase, and catalase. TAS and TOS have been evaluated in several studies in a testicular T/D model. Koksal *et al*.[31] found that at 24 h after 1 h of testicular torsion, there was no change in the TOS or TAS level in any of the studied groups, yet testicular injury based on the Johnsen score was evident. It has been reported that the TOS level, MDA level, and oxidative stress index were significantly increased in the torsion group compared to those in the control group in a 2-h torsion and 4-h detorsion model,[32] but no biochemical antioxidant parameters were evaluated in the previous study. We introduced TAS and TOS parameters to determine oxidant and antioxidant status as described by Erel.[15,16] In our study, oxidative stress during the I/R process in group B was manifested as an increase in the TOS level and a decrease in the TAS level in the I/R group compared with those in group A. The results of our study also indicated that QE and RSV injected intra-peritoneally resulted in significantly decreased biochemical damage associated with an I/R injury. Although the results showed no significant differences in the TAS levels between the QE and RSV treatment groups, a remarkable difference was found in the TOS levels.

When administered intra-peritoneally 30 min before detorsion, both QE and RSV significantly ameliorated the deleterious effects of torsion and detorsion on the affected testis, which was verified by the lower grades of histological damage, fewer abnormal germinal cells, less severe vacuolization, and better defined seminiferous tubules in the treated groups. This suggests that QE and RSV can protect the testes from an I/R injury. In addition, we compared the actions of these two drugs, and histological results showed that QE had superior protective effects compared with RSV.

CONCLUSION

Both QE and RSV were able to prevent I/R injury of the testes after testicular torsion and detorsion. The results suggest that a testis is less affected by an I/R injury when these two drugs are injected intra-peritoneally before detorsion, and QE may exhibit better function than RSV at the doses tested in this study. The limitation of our study was that the exact administration timing and dose for achieving the maximum effects of QE and RSV have not yet been clarified. Therefore, different doses and administration timings should be tested in future studies to determine an optimal treatment for clinical application.

AUTHOR CONTRIBUTIONS

KKC designed the study, drafted the manuscript, and coordinated and participated in every part of the experiments. WHZ and ZC participated in the design of the study and helped draft the manuscript. YC participated in the biochemical assays and statistical analysis of the study. WH performed the histopathological assays. SGW participated in the experiments and histopathological assays. CZ coordinated among the authors and helped draft the manuscript. JC performed the biochemical analysis. GCW participated in the experiments of the study. All authors read and approved the final manuscript.

COMPETING INTERESTS

All authors declare no competing interests.

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