Iran Red Crescent Med J. 2017 April; 19(4):e44765. doi: 10.5812/ircmj.44765.

Published online 2017 February 28. **Brief Report**

The Effects of Rutin on the Gene Expression of *Dazl*, *Bcl2*, and *Caspase3* in Idarubicin-induced Testicular Damages in Mice

Mohammad Deihimi,[1] Sahar Moghbelinejad,[2] Reza Najafipour,[2] Kazem Parivar,[1] and Marjan Nassiri-Asl[2,*]

[1]Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, IR Iran
[2]Cellular and Molecular Research Center, Qazvin University of Medical Sciences, Qazvin, IR Iran

[*]*Corresponding author*: Marjan Nassiri-Asl, Cellular and Molecular Research Center, Department of Pharmacology, Qazvin University of Medical Sciences, P.O. Box: 341197-5981, Qazvin, Iran. Tel: +98-2833336001, Fax: +98-2833324970, E-mail: mnassiriasl@qums.ac.ir

Received 2016 December 13; **Revised** 2017 January 13; **Accepted** 2017 February 01.

Abstract

Background: Idarubicin (IDA), as a chemotherapeutic drug, has side effects on testicular tissue; different studies have shown the protective and antioxidant effects of rutin.
Objectives: The current study aimed at investigating the protective effects of rutin on damages induced by IDA in the testes of mice.
Methods: In the current experimental study, Balb/c mice were divided into 8 groups, including saline (10 mL/kg), rutin (single doses of 50 and 100 mg/kg via intraperitoneal (i.p) as the control groups, saline-IDA group (saline for 7 days, IDA, 10 mg/kg, i.p), rutin 50 and 100 mg/kg for 7 days before IDA, and rutin (single doses of 50 and 100 mg/kg) before IDA. The expression of *Bcl2*, *Caspase3* and *Dazl* at the mRNA level was assessed.
Results: Administration of rutin 100 mg/kg for 7 days before IDA could significantly downregulate *Caspase3* expression by 45% compared with saline-7d-IDA ($P < 0.001$). Also, in the R100-7d-IDA group, the expression level of *Dazl* (12.4 ± 3.50), *Bcl2* (2.5 ± 0.5) significantly increased compared to those of the saline-7d-IDA group (0.84 ± 0.5, 0.12 ± 0.001, respectively) ($P < 0.001$).
Conclusions: It seems that the preventive effects of rutin against damages caused by IDA can be attributed to its ability to reduce apoptosis, which may be mediated by underexpression of *Caspase3* and overexpression of *Bcl2* genes. Also, it could increase the expression of *Dazl* that may be important in spermatogenesis.

Keywords: Idarubicin, Rutin, Apoptosis, Spermatogenesis

1. Background

Fertility disorder is one of the main complications in patients with cancer, and infertility can be an important factor in the psychological morbidity of survivors (1). It is well known that chemotherapy induces azoospermia or reduces spermatogenesis (2). Idarubicin (IDA) is an effective drug for the early management of adult acute myeloid leukemia (AML), particularly in patients expressing high level of MDR (multiple drug resistant). IDA is a DNA intercalating agent, which reacts with topoisomerase II and has an inhibitory effect on nucleic acid synthesis (3). Doxorubicin and its derivative IDA, as anthracyclines, could prevent chromosomal segregation, and induce significant increases in the frequencies of disomic and diploid sperm (4). Epirubicin, another analogue of anthracyclines, could affect the abnormal reproductive outcomes by both the clastogenic and aneugenic potential in cancer survivors and medical personnel exposed to it (5). IDA could also induce oxidative stress, caspase activation, and apoptotic death in leukemic cell line (6). Therefore, reproductive toxicity induced by anthracyclines is a great concern for the toxicities associated with chemotherapy and it is necessary to find a protective agent against it for patients. In this regard, the use of antioxidant agents can reduce the toxic effect of such drugs.

Rutin (3 , 3' , 4' , 7 - pentahydroxyflavone - 3 - rhamnoglucoside) is a flavonoid of the flavonol-type found in many foodstuff and vegetables (7). Rutin has several pharmacological effects such as antioxidant properties (8, 9). It was shown that rutin (30 μM) decreased lipid peroxidation induced by tert-butyl hydroperoxide in human sperm (10). Moreover, rutin as a flavonoid could restore motility of metal ($AlCl_3$, $CdCl_2$, and $PbCl_4$)-exposed sperm and protect it against lipid peroxidation (11). On the other hand, doxorubicin in some studies increased the expression of *Caspase3* (12, 13) and decreased *Bcl2* (13) in the testicular tissues of animals.

Furthermore, the DAZ (deleted in azoospermia) family refers to germ cell-specific transcription factors bound to RNA and play role in the regulation of several transcripts (14). It is expressed in primordial germ cells and/or premeiotic and meiotic germ cells of both genders, and the

most common cause of infertility is the deletion of DAZ gene in humans (15). Therefore, the current study aimed at assessing the protective effect of rutin by evaluating the expression of *Caspase3* as a proapoptotic and *Bcl2* as anti-apoptotic genes, and *Dazl* as a key gene involved in spermatogenesis in IDA-induced testicular damage in mice.

2. Methods

2.1. Animals

A total of 64 male Balb/c mice with weight range of 20 to 22 g were provided by Razi institute (Karaj, Iran) and maintained at a constant room temperature (21 ± 2°C) under a 12:12 light: dark cycle. All animals had free access to food and water. All experiments of the current study were conducted in accordance with the European communities council directive of 24 November 1986 (86/609/EEC).

2.2. Chemicals

IDA (4-demethoxydaunorubicin) was purchased from Pharmacia (Italia, S.P.A), rutin from Sigma (Sigma-Aldrich Co, Saint Louis, MO, USA), ketamine from Rotexmedica (GmbH, Germany), xylazine from Loughrea Co. (Galway, Ireland), RNeasy Mini Kit from Qiagene (Germany), and cDNA Synthesis Kit from Thermo Scientific, Fermentas (Waltham, MA, USA).

2.3. Experimental Design

In the current experimental study, mice were divided into 8 groups. The control group was given an intraperitoneal (i.p) injection of saline (10 mL/kg) daily for 7 days (n = 8). Two other groups, R50-7d and R100-7d, rutin (50 and 100 mg/kg, i.p) were given daily for 7 days (each group n = 8). In the saline-IDA group, saline (10 mL/kg, i.p) was given daily for 7 days, and on the last day, IDA (10 mg/kg, i.p) was injected 15 minutes after the administration of saline (n = 8). In the other 2 groups (R50-7d-IDA and R100-7d-IDA) rutin (50 and 100 mg/kg, i.p) was administered daily for 7 days, and on the last day, IDA (10 mg/kg, i.p) was injected 15 minutes after the administration of rutin (each group n = 8). In the 2 groups of R50-IDA and R100-IDA, rutin (50 and 100 mg/kg, i.p) was administered as a single dose, and on the same day, IDA (10 mg/kg, i.p) was injected 15 minutes after the administration of rutin (each group n = 8). Then, all of the mice in each group, 24 hours after treatment, were anaesthetized with i.p injection of ketamine (60 mg/kg)/xylazine (6 mg/kg) and were subsequently sacrificed. The testes of the animals were immediately removed, and cleaned with chilled 0.9% saline.

2.4. Expression Study of Candidate Genes

In the current study, testes tissues were homogenized using an ultrasonic processor UP100H (Hielsher, Germany), and then, RNAs were extracted from tissue samples using RNeasy Mini Kit (Qiagene, Germany). The quality and quantity of the isolated total RNA was measured using Nano Drop 2000c (Thermo, USA); thereafter, the RNA samples with A260/A280 ratios of > 2 were selected for the quantitative analysis. The extracted RNAs were, then, frozen at -80°C. First strand complementary DNA (cDNA) synthesis was also performed using Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, Fermentas, Waltham, MA, USA).The *beta-actin* gene was used as an internal control to quantify target genes expression. Three target genes, such as *Dazl*, *Bcl2*, and *Caspase3*, and beta-actin, as internal control), were amplified with appropriate primers as follows:

Dazl F: GATGGACATGAGATCATTGGA, R: ATACCAGGGAGCAATCCTGA

Bcl2 F: GAGTACCTGAACCGGCATCT, R: TTTGACCCAGAATCCACTCA

Caspase3 F: TGTCATCTCGCTCTGGTACG, R: AAATGACCCCTTCATCACCA

Beta-actin F: TTACTGAGCTGCGTTTTACAC, R: ACAAAGCCATGCCAATGTTG.

Real-time polymerase chain reaction (PCR) was carried out in a total volume of 20 μL containing 10 μL Taq man master mix (Takara, Shiga, Japan), 0.2 μM forward and reverse primers, and 2 μL cDNA. Thermal cycling was performed on calibrated ABI-7500 (Applied Biosystems, Foster, CA, USA) sequence detection system using the following cycling condition: 30 seconds at 95°C as the first denaturation step, followed by 40 cycles at 95°C for 5 seconds and 60°C for 34 seconds. The $2^{-\Delta CT}$ method of relative quantification was used to determine the fold change in expression. It was performed by normalizing the resulting threshold cycle (CT) values of the target mRNAs to the CT values of the internal control (beta-actin) in the treated and untreated samples ($\Delta CT = CT_{target} - CT_{Beta}$) (16).

2.5. Statistical Analysis

Statistical analysis, including mean and standard deviation (SD), was conducted using Prism (version 5) software. Additionally, one-way analysis of variance (ANOVA) and post hoc Tukey test were used to determine the significant differences between the studied groups. P value < 0.05 was considered as level of significance.

3. Results

According to the results of the current study, in saline-7d-IDA group, the expression of *Caspase3* as a proapoptotic

gene elevated significantly ($P < 0.001$). The administration of rutin 100 mg/kg for 7 days significantly downregulated *Caspase3* expression, compared with the saline-7d-IDA group ($P < 0.001$). However, administration of rutin 50 mg/kg for 7 days could not downregulate *Caspase3* expression and the difference between this group and saline was significant ($P < 0.05$). No significant difference was observed in the expression of *Caspase3* gene in R50 and R100-IDA groups, compared with the saline group (Table 1).

The expression of *Bcl2* gene, as an anti-apoptotic gene, between the saline-7d-IDA and saline groups was significant ($P < 0.01$). Overexpression of *Bcl2* gene in the mice receiving rutin 100 mg/kg for 7 days prior to IDA was shown compared with those of the saline-7d-IDA and saline groups ($P < 0.001$). However, similar to the saline-7d-IDA group, the expression of *Bcl2* gene in the treated group with rutin 50 mg/kg for 7 days before IDA was significantly downregulated, compared with the saline group ($P < 0.01$). Furthermore, the *Bcl2* expression significantly downregulated in the R50 and R100-IDA groups, compared with the saline group ($P < 0.01$) (Table 1).

The *Dazl* gene was significantly downregulated in the saline-7d-IDA group, compared with the saline group ($P < 0.05$). *Dazl* showed significant overexpression in the groups of R50 and 100-7d-IDA, compared with the saline-7d-IDA group ($P < 0.05$ and $P < 0.01$, respectively). There was no significant difference between R50 and R100-IDA groups, compared with the saline group (Table 1).

4. Discussion

In the current study, the expression of *Caspase3*, and *Bcl2* genes were over- and under- expressed, respectively in the saline-7d-IDA group. Therefore, IDA-induced oxidative stress in testicular cells leads to the induction of apoptosis in germ cells by causing the activation of *Caspase3* and downregulation of *Bcl2* gene. Similarly, the mRNA level of *Bcl2* decreased in spermatogonia, spermatocytes, and spermatids from testis tissues treated with 0.5 and 1 mM of doxorubicin (17). Underexpression and overexpression of *Caspase3*, and *Bcl2* genes were observed with rutin 100 mg/kg for 7 days in mice. Similarly, pretreatment of rutin could suppress the increment of Bax and *Caspase3*, and decrement of *Bcl2* expression induced by H_2O_2 at mRNA and protein levels (9). The protective effect of rutin on reproductive toxicity may be caused by scavenging reactive oxygen species (ROS), increasing superoxide dismutase, and catalase activities previously found in other studies (18-20). Generally, ROS produced as a result of oxidative stress, generate single strand break and double strand break in DNA by intrinsic mechanisms and causes adverse biological effects in cells. The biological consequences of exposure to ROS include gene mutation, chromosome aberrations, cellular transformation, and cell death (21). Several studies showed that doxorubicin could induce apoptosis due to increase in the expression of various genes such as p38, p53, *Caspase3* and 9, and Bax/Bcl-xL, and downregulation of *Bcl2* (22, 23). Moreover, chronic administration of rutin (50 mg/kg, i.p, 3 w) protected testicular toxicity induced by adriamycin (doxorubicin) in rats. Rutin could increase testosterone and antioxidant enzyme levels, decrease testicular enzymes levels, and prevent expression of inflammatory markers (24).

In the current study, *Dazl* expression was evaluated in different studied groups. Rutin significantly upregulated *Dazl* expression dose dependently. Underexpression of *Dazl* gene in the saline-7d-IDA group was observed in the current study. Similarly, the microdeletion and sensitivity of DAZ gene (human homologue of *Dazl*) to the 4-Gy radiation were shown in other studies (25). The loss of *Dazl* expression results in apoptosis of the postmigratory germ cells (26).

In the current study, for the first time, the effect of rutin on the expression of *Caspase3*, *Bcl2*, and *Dazl* were studied in IDA-induced testicular damage in mice. Administration of rutin 100 mg/kg for 7 days increased the expression of *Bcl2* and *Dazl*, and decreased *Caspase3*. Furthermore, it seems that pretreatment and protection with 100 mg/kg of rutin for 7 days before IDA plays a more effective role when compared with a single dose of rutin. However, further studies are necessary on the sperm parameters, stress oxidative damage, and in the field of histopathology.

Acknowledgments

The protocol was approved by the local ethics committee of science and research branch, Islamic Azad University, Tehran, Iran (No IR.IAU.SRB.REC.1395.11). Authors thank cellular and molecular research center of Qazvin University of Medical Sciences for providing laboratory facilities. The results reported in the article were parts of a PhD thesis.

Footnotes

Authors' Contribution: Mohammad Deihimi: performing the experiments and drafting the manuscript; Sahar Moghbelinejad: designing the study, performing molecular genetic assays, preparing and drafting the manuscript; Reza Najafipour: drafting the manuscript; Kazem Parivar: designing the study; Marjan Nassiri-Asl: designing the study, performing the statistical analysis, drafting and revising the manuscript. All authors read and approved the final manuscript. Mohammad Deihimi and Sahar Moghbelinejad contributed equally.

Table 1. Effect of Rutin on the Expression of *Caspase3*, *Bcl2*, and *Dazl* Genes in the Study Groups[a]

Treatment	*Caspase3*	P Value	*Bcl2*	P Value	*Dazl*	P Value	Statistical Test
Saline	0.5 ± 0.15	-	0.95 ± 0.12	-	3.72 ± 0.99	-	Tukey-Kramer
R50-7d	0.45 ± 0.1	> 0.05	0.8 ± 0.35	> 0.05	2.07 ± 1.20	> 0.05	
R100-7d	0.4 ± 0.1	> 0.05	0.9 ± 0.3	> 0.05	3 ± 1.30	> 0.05	
Saline-7d-IDA	1.1 ± 0.25	< 0.001[b]	0.12 ± 0.001	< 0.01[c]	0.84 ± 0.5	< 0.05[d]	
R50-7d-IDA	0.9 ± 0.3	< 0.05[d]	0.45 ± 0.14	< 0.01[c]	5.20 ± 1.20	> 0.01c	
R100-7d-IDA	0.5 ± 0.23	< 0.001[e]	2.5 ± 0.5	< 0.001[b,e]	12.4 ± 3.50	< 0.001[b,e]	
R50-IDA	0.55 ± 0.11	> 0.05	0.15 ± 0.08	< 0.01[c]	1.05 ±.45	> 0.05	
R100-IDA	0.8 ± 0.12	> 0.05	0.23 ± 0.1	< 0.01[c]	1.50 ± 0.63	> 0.05	

[a]Values are expressed as mean ± SD.
[b]P < 0.001, compared to the saline group.
[c]P< 0.01, compared to the saline group
[d]P < 0.05, compared to the saline group
[e]P< 0.001, compared to the salins-7d-IDA group (n = 8), using Tukey-Kramer test.

Conflict of Interest: The authors declared no conflict of interest exists.

References

1. Tschudin S, Bitzer J. Psychological aspects of fertility preservation in men and women affected by cancer and other life-threatening diseases. *Hum Reprod Update.* 2009;**15**(5):587–97. doi: 10.1093/humupd/dmp015. [PubMed: 19433413].
2. Ku JY, Park NC, Jeon TG, Park HJ. Semen Analysis in Cancer Patients Referred for Sperm Cryopreservation before Chemotherapy over a 15-Year Period in Korea. *World J Mens Health.* 2015;**33**(1):8–13. doi: 10.5534/wjmh.2015.33.1.8. [PubMed: 25927057].
3. de Figueiredo-Pontes LL, Pintao MC, Oliveira LC, Dalmazzo LF, Jacomo RH, Garcia AB, et al. Determination of P-glycoprotein, MDR-related protein 1, breast cancer resistance protein, and lung-resistance protein expression in leukemic stem cells of acute myeloid leukemia. *Cytometry B Clin Cytom.* 2008;**74**(3):163–8. doi: 10.1002/cyto.b.20403. [PubMed: 18200595].
4. Attia SM. Comparative aneugenicity of doxorubicin and its derivative idarubicin using fluorescence in situ hybridization techniques. *Mutat Res.* 2011;**715**(1-2):79–87. doi: 10.1016/j.mrfmmm.2011.07.012. [PubMed: 21856314].
5. Attia SM, Ahmad SF, Okash RM, Bakheet SA. Aneugenic effects of epirubicin in somatic and germinal cells of male mice. *PLoS One.* 2014;**9**(10):ee109942. doi: 10.1371/journal.pone.0109942. [PubMed: 25303090].
6. Ristic B, Bosnjak M, Arsikin K, Mircic A, Suzin-Zivkovic V, Bogdanovic A, et al. Idarubicin induces mTOR-dependent cytotoxic autophagy in leukemic cells. *Exp Cell Res.* 2014;**326**(1):90–102. doi: 10.1016/j.yexcr.2014.05.021. [PubMed: 24907655].
7. Hosseinzadeh H, Nassiri-Asl M. Review of the protective effects of rutin on the metabolic function as an important dietary flavonoid. *J Endocrinol Invest.* 2014;**37**(9):783–8. doi: 10.1007/s40618-014-0096-3. [PubMed: 24879037].
8. Sharma S, Ali A, Ali J, Sahni JK, Baboota S. Rutin : therapeutic potential and recent advances in drug delivery. *Expert Opin Investig Drugs.* 2013;**22**(8):1063–79. doi: 10.1517/13543784.2013.805744. [PubMed: 23795677].
9. Zhou YF, Guo B, Ye MJ, Liao RF, Li SL. Protective Effect of Rutin Against H2O2-Induced Oxidative Stress and Apoptosis in Human Lens Epithelial Cells. *Curr Eye Res.* 2016;**41**(7):933–42. doi: 10.3109/02713683.2015.1082186. [PubMed: 26576853].
10. Moretti E, Mazzi L, Terzuoli G, Bonechi C, Iacoponi F, Martini S, et al. Effect of quercetin, rutin, naringenin and epicatechin on lipid peroxidation induced in human sperm. *Reprod Toxicol.* 2012;**34**(4):651–7. doi: 10.1016/j.reprotox.2012.10.002. [PubMed: 23064111].
11. Jamalan M, Ghaffari MA, Hoseinzadeh P, Hashemitabar M, Zeinali M. Human Sperm Quality and Metal Toxicants: Protective Effects of some Flavonoids on Male Reproductive Function. *Int J Fertil Steril.* 2016;**10**(2):215–23. [PubMed: 27441055].
12. Olusoji MJ, Oyeyemi OM, Asenuga ER, Omobowale TO, Ajayi OL, Oyagbemi AA. Protective effect of Gallic acid on doxorubicin-induced testicular and epididymal toxicity. *Andrologia.* 2016 doi: 10.1111/and.12635. [PubMed: 27421682].
13. Yeh YC, Liu TJ, Wang LC, Lee HW, Ting CT, Lee WL, et al. A standardized extract of Ginkgo biloba suppresses doxorubicin-induced oxidative stress and p53-mediated mitochondrial apoptosis in rat testes. *Br J Pharmacol.* 2009;**156**(1):48–61. doi: 10.1111/j.1476-5381.2008.00042.x. [PubMed: 19133991].
14. Smorag L, Xu X, Engel W, Pantakani DV. The roles of DAZL in RNA biology and development. *Wiley Interdiscip Rev RNA.* 2014;**5**(4):527–35. doi: 10.1002/wrna.1228. [PubMed: 24715697].
15. Fu XF, Cheng SF, Wang LQ, Yin S, De Felici M, Shen W. DAZ Family Proteins, Key Players for Germ Cell Development. *Int J Biol Sci.* 2015;**11**(10):1226–35. doi: 10.7150/ijbs.11536. [PubMed: 26327816].
16. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc.* 2008;**3**(6):1101–8. [PubMed: 18546601].
17. Habas K, Anderson D, Brinkworth MH. Germ cell responses to doxorubicin exposure in vitro. *Toxicol Lett.* 2017;**265**:70–6. doi: 10.1016/j.toxlet.2016.11.016. [PubMed: 27890809].
18. Wei SM, Yan ZZ, Zhou J. Protective effect of rutin on testicular ischemia-reperfusion injury. *J Pediatr Surg.* 2011;**46**(7):1419–24. doi: 10.1016/j.jpedsurg.2010.09.044. [PubMed: 21763845].
19. Akondi BR, Challa SR, Akula A. Protective effects of rutin and naringin in testicular ischemia-reperfusion induced oxidative stress in rats. *J Reprod Infertil.* 2011;**12**(3):209–14. [PubMed: 23926504].
20. Abarikwu SO, Otuechere CA, Ekor M, Monwuba K, Osobu D. Rutin Ameliorates Cyclophosphamide-induced Reproductive Toxicity in Male Rats. *Toxicol Int.* 2012;**19**(2):207–14. doi: 10.4103/0971-6580.97224. [PubMed: 22778522].
21. Wyman C, Kanaar R. DNA double-strand break repair: all's well that ends well. *Annu Rev Genet.* 2006;**40**:363–83.

22. Zhang S, Liu X, Bawa-Khalfe T, Lu LS, Lyu YL, Liu LF, et al. Identification of the molecular basis of doxorubicin-induced cardiotoxicity. *Nat Med.* 2012;**18**(11):1639–42. doi: 10.1038/nm.2919. [PubMed: 23104132].
23. Sharifi S, Barar J, Hejazi MS, Samadi N. Doxorubicin Changes Bax /Bcl-xL Ratio, Caspase-8 and 9 in Breast Cancer Cells. *Adv Pharm Bull.* 2015;**5**(3):351–9. doi: 10.15171/apb.2015.049. [PubMed: 26504757].
24. Salem EA, Salem NA, Hellstrom WJ. Therapeutic effect of ozone and rutin on adriamycin-induced testicular toxicity in an experimental rat model. *Andrologia.* 2017;**49**(1) doi: 10.1111/and.12603. [PubMed: 27136444].
25. Moghbeli-Nejad S, Mozdarani H, Behmanesh M, Rezaiean Z, Fallahi P. Genome instability in AZFc region on Y chromosome in leukocytes of fertile and infertile individuals following exposure to gamma radiation. *J Assist Reprod Genet.* 2012;**29**(1):53–61. doi: 10.1007/s10815-011-9626-5. [PubMed: 21898105].
26. Lin Y, Page DC. Dazl deficiency leads to embryonic arrest of germ cell development in XY C57BL/6 mice. *Dev Biol.* 2005;**288**(2):309–16. doi: 10.1016/j.ydbio.2005.06.032. [PubMed: 16310179].