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GENERAL GYNECOLOGY

Evaluation of preventive effect of shilajit on radiation-induced apoptosis on ovaries

Mete Kececi[1] · Meryem Akpolat[1] · Kanat Gulle[1] · Ercan Gencer[2] · Ahmet Sahbaz[3]

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Abstract
Purpose Cancer is the second leading cause of death in children in developed countries and most of childhood malignancies can be treated with chemo-radiotherapy. Although radiation therapy is a successful treatment modality in cancer patients, it has various adverse effects. Especially the gonads are very sensitive and prone to radiation-related damage. Radiation impairs the ovaries by triggering apoptosis of follicular cells and chromosomal damage and oxidative stress. Shilajit, a traditional medicinal agent in India, Russia, and other parts of the world, contains various antioxidant agents and has ovogenic effects. To evaluate the ability of shilajit to prevent radiation-induced ovarian damage.
Methods Forty Wistar albino female rats were divided into four groups as: Control group, shilajit group, radiation only group, and radiation + shilajit group. Four days after radiation exposure, the rats were sacrificed and the ovaries were removed and evaluated immuno-histopathologically.
Results There was a statistically significant difference in follicle counts (primordial, primary, preantral, antral, and atretic follicles) between the groups ($p < 0.001$). Almost all follicles at all stages were atretic in the radiation only group whereas normal-looking primordial follicles were detected in the radiation + shilajit group. In radiation + shilajit group, p53, Bax and caspase 3 expression was less intense than that in the radiation only group follicles.
Conclusion This is the first reported study evaluating the effects of shilajit on radiation-related ovarian damage prevention. Shilajit decreased the expression of p53, Bax, and caspase 3, thereby blocking the apoptotic pathways. Shilajit was found to be especially protective of primordial follicles.

Keywords Apoptosis · Folliculogenesis · Ionizing radiation · Oxidative stress · Rat ovary · Shilajit

Introduction

Cancer is the second leading cause of death in children in developed countries [1]. Leukemia, lymphoma, Wilms' tumor, rhabdomyosarcoma, central nervous system tumors, and germ cell tumors constitute the majority of childhood malignancies, and most can be treated with chemo-radiotherapy [2, 3]. Radiotherapy is one of the most common treatment modalities in cancer management, and more than 50 % of patients with cancer require radiotherapy during their management [4]. Although radiation therapy is a successful treatment modality in cancer patients, it has also several long-term side effects on healthy tissues, such as the pulmonary, hepatic, and cardiac and reproductive systems (testes and ovaries) [5–7]. The ovaries are very sensitive and prone to radiation-related damage, which may adversely affect the patient's life by causing premature menopause, osteoporosis, infertility, psychological problems, and impaired quality of life.

Radiation causes damage via ionization and formation of reactive oxygen derivatives. Radiation not only damages

✉ Ahmet Sahbaz
drsahbazahmet@yahoo.com

[1] Department of Histology and Embriology, Bulent Ecevit University School of Medicine, Zonguldak, Turkey

[2] Department of Physiology, Bulent Ecevit University School of Medicine, Zonguldak, Turkey

[3] Department of Obstetrics and Gynecology and Department of Histology and Embriology, Bulent Ecevit University School of Medicine, Zonguldak, Turkey

tissues with oxidative stress, but also causes damage by induction of apoptosis through activation of the cytochrome *c* and caspase pathway [8–10]. It has been reported that 2 Gy radiation depletes half of the ovarian reserve, and radiation doses of 10–20 Gy in children and 4–6 Gy in adults can cause permanent cessation of ovarian function [11, 12]. It has been reported in several studies that radiation impairs the ovaries by triggering apoptosis of follicular cells and chromosomal damage [13–15]. Several studies have evaluated the effects of various anti-oxidant agents, such as melatonin, selenium, sphingosine, and resveratrol, on the prevention of radiation injury in the ovaries [16–19].

Shilajit (also known as silaras adrija, girija, asphalt, and mineral pitch) has been used as a medicinal agent in traditional medicine in India, Russia, and other parts of the world. Shilajit consists of a mixture of organic substances, herbal and microbial metabolites, leaked from rock roots [20]. Shilajit includes benzoic acid, fatty acids, and vitamins, such as B1 and B12, and various other anti-oxidant agents [21–23]. For thousands of years, shilajit has been used to treat a wide range of diseases, such as hypertension, anemia, emesis, and various neurological diseases, and as an immune-modulator and also has anti-oxidative, spermatogenic and ovogenic effects [24–26]. The safety of the shilajit is well studied and is generally regarded as a safe substance [27, 28]. Because shilajit contains various anti-oxidant agents and has reported ovogenic effects, we evaluated the ability of shilajit to ameliorate radiation-induced ovarian damage.

Materials and methods

Animals

Wistar albino rats (pre-pubertal, female, 90 ± 10 g) were obtained from the Bulent Ecevit University Animal Care and Research Unit (Zonguldak, Turkey). The animals were housed under standard conditions (20 ± 1 °C room temperature, 60 ± 10 % humidity, and a 12/12-h light/dark cycle) in regular cages and allowed free access to food (Gebze Food Factory, Kocaeli, Turkey) and water. All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals, published by the US Public Health Service. The study was approved by the Institutional Animal Ethical Committee of Bulent Ecevit University (Zonguldak, Turkey; 2012-20-00-33).

Experimental design

Forty Wistar albino female rats were divided into four groups, as follows:

Control group ($n = 10$): no treatment was applied throughout the study.
Shilajit group ($n = 10$): 100 mg/kg/day shilajit was applied orally throughout the study.
Radiation only group ($n = 10$): a single dose of 8.3-Gy whole body radiation was applied.
Radiation + shilajit group ($n = 10$): 100 mg/kg/day shilajit was applied for 10 days (every other day) prior to radiation (8.3 Gy whole body radiation) and continued for 4 days (every other day) after radiation.

Total body irradiation

The radiation procedure was performed at Bulent Ecevit University, Radiation Oncology Department. A rat simulation was made using a 1-mm slice computed tomography (CT) scan, and the dose calculation was performed using the Eclipse treatment planning system (ver. 8.9; Varian Medical Systems, Palo Alto, CA, USA). On day 10 of the study, for whole body radiation, animals were anesthetized with 10 mg/kg xylazine and 90 mg/kg ketamine. Animals were fixed in a supine position and exposed to total body irradiation of 8.3 Gy using a linear accelerator (Clinac, Varian Medical Systems).

Four days after radiation exposure, the rats were killed and the ovaries were dissected and removed from the surrounding tissues. The ovaries were weighed and recorded in mg/100 g for each animal. Right ovaries were stored at −80 °C for biochemical evaluation, and left ovaries were processed for histopathological evaluation.

Light microscopic procedures

For light microscopic analysis, ovarian specimens were fixed overnight in 10 % formalin and embedded in paraffin wax blocks, from which 4–5-μm sections were cut and stained with hematoxylin and eosin (H–E) and periodic acid-Schiff (PAS). The preparations were evaluated using a light microscope and photographed (Carl Zeiss Axio Lab A1, Germany).

Follicle counting

Ovarian follicle classification was performed according to Devine et al. [29]. Serial sections were cut from the paraffin wax blocks. Every fifth section throughout the entire ovary was stained with H–E, and follicles with a nucleus present in the oocyte were counted in a blinded fashion. All sections were prepared by one author (MK) and were blinded to the reader (MA). The number of follicles per ovary was estimated by multiplying by a correction factor of five and dividing by the total section number for each ovary, as described previously [30].

Immunohistochemical evaluation

To investigate p53, Bax, and anti-caspase 3 (active) activities, avidin–biotin complex was applied to the sections. Paraffin sections were incubated at 55 °C overnight, deparaffinized in xylene, and rehydrated through a series of graded alcohols. Antigen retrieval was performed by boiling the sections in 0.1 M sodium citrate. The sections were immersed in 3 % H_2O_2 to inhibit endogenous peroxidase activity. The sections were then incubated with 10 % normal goat serum for 1 h at room temperature to block the non-specific binding of antibodies, after which they were incubated with primary antibodies targeting p53 (Abcam, rabbit polyclonal IgG, Cat. #ab8105), active caspase 3 (Novus, rabbit polyclonal IgG, Cat. #NB100-56113), or Bax (Chemicon, rabbit polyclonal IgG Cat. #AB2915) for 1 h. Biotinylated anti-rabbit antibody (Vector Laboratories, Burlingame, CA, USA) was added to the sections for 30 min at room temperature. Negative controls comprised tissue sections stained without the primary antibody. The antigen–antibody complex was detected using a streptavidin–biotin–peroxidase kit (Vector Laboratories). Finally, the sections were developed using a 3,3-diaminobenzidine substrate kit (DAB, Vector Laboratories) to visualize immunolabelling and were counterstained with Mayer's hematoxylin. The preparations were evaluated using a light microscope and photographed (Carl Zeiss Axio Lab A1).

Statistical analysis

Statistical analyses were performed using SPSS software (ver. 18.0; SPSS Inc., Chicago, IL, USA). Variables are expressed as medians (minimum–maximum). Differences among the groups were analyzed using the Kruskal–Wallis test. Dual comparisons among groups with significant values were evaluated using the Bonferroni-corrected Mann–Whitney U test. P values <0.05 were considered to indicate statistical significance in all tests.

Results

Throughout the experiment, no animal was lost due to shilajit administration. There was no statistically significant difference in body weights among the groups ($p > 0.05$).

Histopathological findings

The control and shilajit groups showed normal ovarian morphological findings (Fig. 1a, b). However, in the radiation group, almost all of the follicles were atretic (Fig. 1c). The granulosa layer in the atretic follicles was found to be loosened generally, and many picnotic nuclei were detected in the granulosa layer. Apoptotic bodies were found both in the antral and granulosa layers. Most of the primordial and primary follicles were found to lack

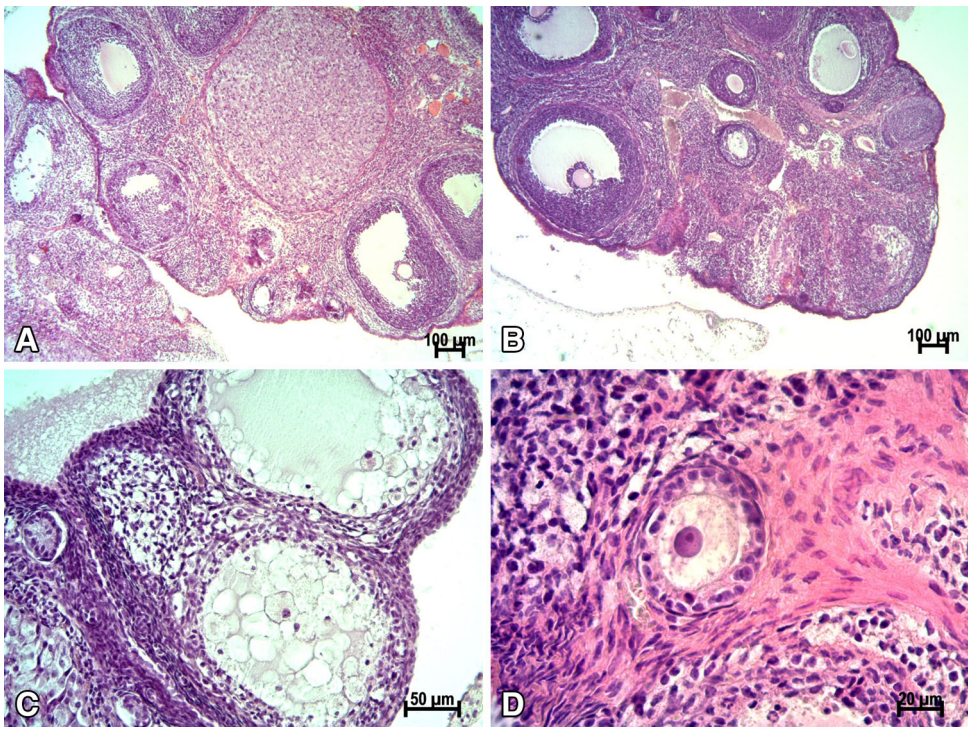

Fig. 1 Hematoxylin and eosin staining. **a** A In the control group, different stages of follicle development could be seen with normal-looking follicles, corpus luteum, and several atretic follicles. **b** As in the control group, normal-looking follicles can be seen in the shilajit group. **c** Many atretic follicles with apoptotic bodies can be seen in the radiation-only group. **d** Although early apoptotic changes can be seen in the radiation + shilajit group, the preantral follicles with advanced-stage follicles, especially, showed normal morphology

Table 1 Follicle counts in each group

	Control ($n = 10$) Median (min–max)	Shilajit ($n = 10$) Median (min–max)	Radiation only ($n = 10$) Median (min–max)	Radiation+Shilajit ($n = 10$) Median (min–max)
Primordial follicles	158.50 (113–213)[a]	195.50 (153–218)[b]	6.50 (3–21)[c]	19.50 (7–26)[d]
Primary follicles	34.50 (26–63)[a]	41.00 (30–57)[a]	2.50 (0–5)[b]	2.00 (0–5)[b]
Preantral	10.50 (6–18)[a]	12.50 (7–19)[a]	3.00 (1–5)[b]	3.00 (0–7)[b]
Antral	5.00 (1–10)[a]	6.00 (2–19)[a]	0.00 (0–3)[b]	0.00 (0–1)[b]
Atretic	29.00 (21–35)[a]	28.00 (18–32)[a]	86.50 (56–95)[b]	92.50 (78–105)[b]

Same letters denote similar groups. Min (minimum), max (maximum)

oocytes. Almost all follicles at all stages were atretic in the radiation only group, and no primordial follicles were observed. In contrast to the radiation group, the preantral-stage follicles were in the early phases of atresia, and normal-looking primordial follicles were also detected in the radiation + shilajit group (Fig. 1d).

Follicle counts of each group are given in Table 1, and primordial follicle counts are demonstrated in Fig. 2. There was a statistically significant difference in follicle counts (primordial, primary, preantral, antral, and atretic follicles) between the groups ($p < 0.001$). The primordial follicle count was statistically different between the radiation only and radiation + shilajit groups ($p < 0.001$). There was no statistically significant difference in the primary or antral follicles between the radiation only and radiation + shilajit groups ($p > 0.05$).

Using PAS staining, in the control and shilajit only group, the integrity of the zona pellucida was intact, whereas in the radiation group, the integrity of the zona pellucida was lost. In the radiation + shilajit group, the integrity of the zona pellucida was maintained significantly compared with the radiation only group. The PAS staining of the specimens is shown in Fig. 3a–d.

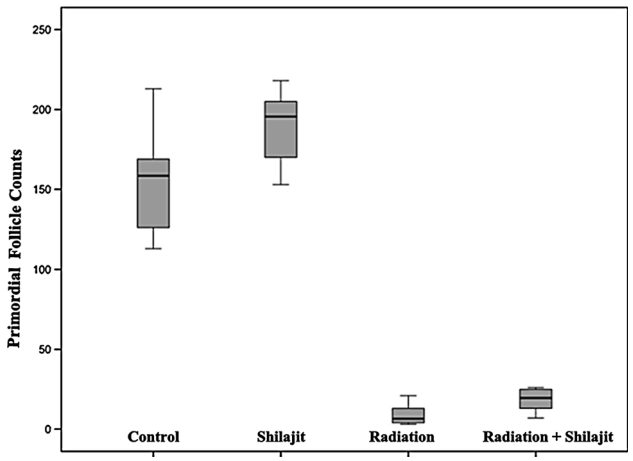

Fig. 2 Primordial follicle counts in each group

Immunohistochemical findings

p53 staining of the specimens is shown in Fig. 4a–d. p53 expression was not detected in primordial or primary follicles in the control or shilajit groups. In the radiation only group, strong p53, Bax, and caspase-3 expression was present in the primary follicles. Intense p53, Bax, and caspase-3 expression was present in the antral follicles, especially in the granulosa cells along the antral area. In the thecal layer, only Bax expression was observed. Bax and caspase-3 stainings of the specimens are shown in Figs. 5 and 6a–d.

In the radiation + shilajit group, expression was weaker than that in the radiation group, and it was striking that partially normal-looking follicles were present. In the thecal layer, no expression was observed. Although Bax and caspase 3 expression was evident in the follicles, it was less intense than that in the radiation only group follicles.

Discussion

Radiation triggers the apoptotic pathway by causing oxidative stress directly or via ionization. Free radicals formed by ionizing radiation disrupt mitochondrial membrane integrity, liberating cytochrome *c* from the inner mitochondrial membrane into the cytosol, subsequently triggering apoptosis by caspase activation.

In addition, oxidants adversely affect steroidogenesis by disrupting mitochondrial integrity. The apoptotic process is further increased in preantral and antral follicles, because their development shows a higher dependency on steroidogenesis. In primordial follicles, in which development is less dependent on steroidogenesis, the reduction in radiation-related oxidative stress by shilajit caused significantly higher primordial follicle count preservation after radiation exposure in the present study. Partially preserved follicular morphology and weaker apoptotic marker expression in preantral and antral follicles in the radiation + shilajit group versus the radiation only group suggests

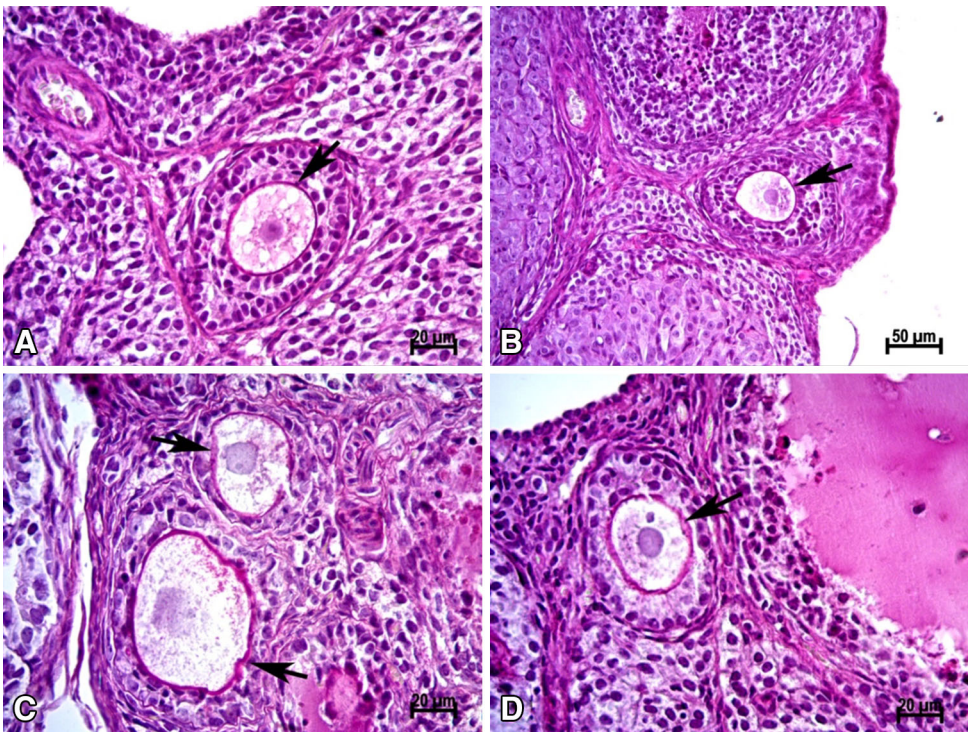

Fig. 3 PAS staining. **a** In the control group, the normal shape of the zona pellucida (*arrow*) is clear. **b** In the shilajit group, the shape of the zona pellucida is normal, as in the control group. **c** In the radiation-only group, the destroyed appearance of the zona pellucida is prominent. **d** Although the shape of the zona pellucida is damaged in the radiation + shilajit group, it is more normal than that in the radiation-only group

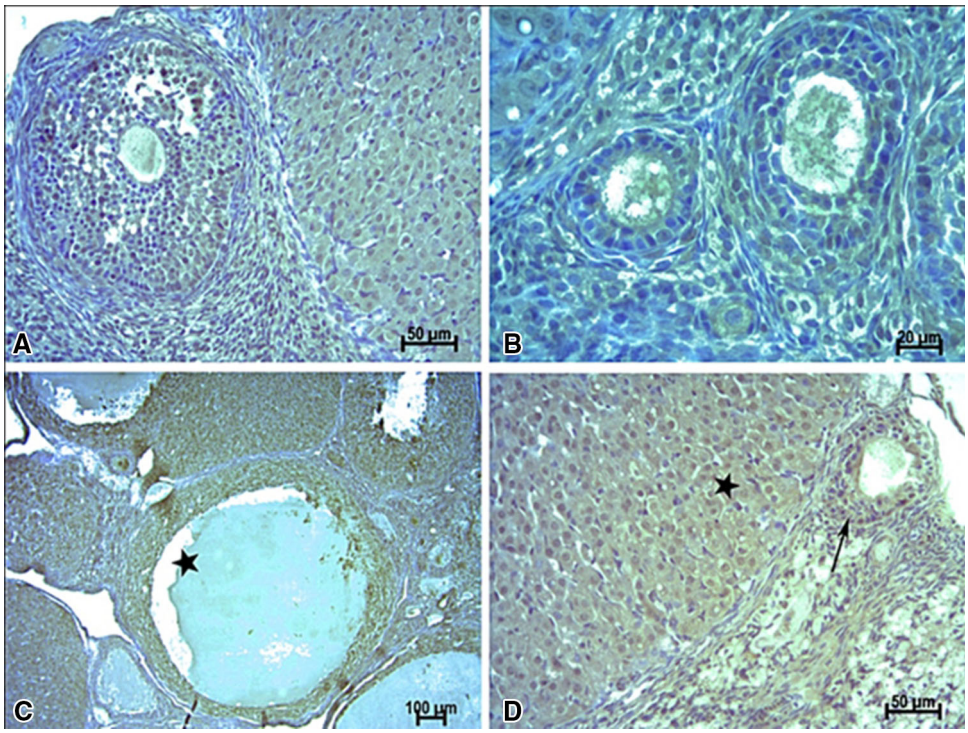

Fig. 4 Immunohistochemical demonstration of p53 expression in all groups. **a** Control group. **b** Shilajit-only group. **c** In the radiation only group, especially in the granulosa cells (*Asterisk*) close to the antral border, intense p53 expression was striking. **d** In contrast to the radiation only group, granulosa cells in the radiation + shilajit group showed less intense p53 expression (*arrow*)

that shilajit was also effective in ameliorating radiation-related damage in the ovarian follicles at this stage.

Radiation can accelerate follicular atresia [15]. Radiation induces apoptosis and impairs follicular function [13]. The early follicular phases are more sensitive to radiation-induced damage [31]. Follicular degeneration has been associated with granulosa cell apoptosis [29]. Follicular apoptosis is regulated both by pro-apoptotic factors (e.g., p53, Bax, androgens, oxidative stress end products, tumor necrosis factor-α) and anti-apoptotic factors (e.g.,

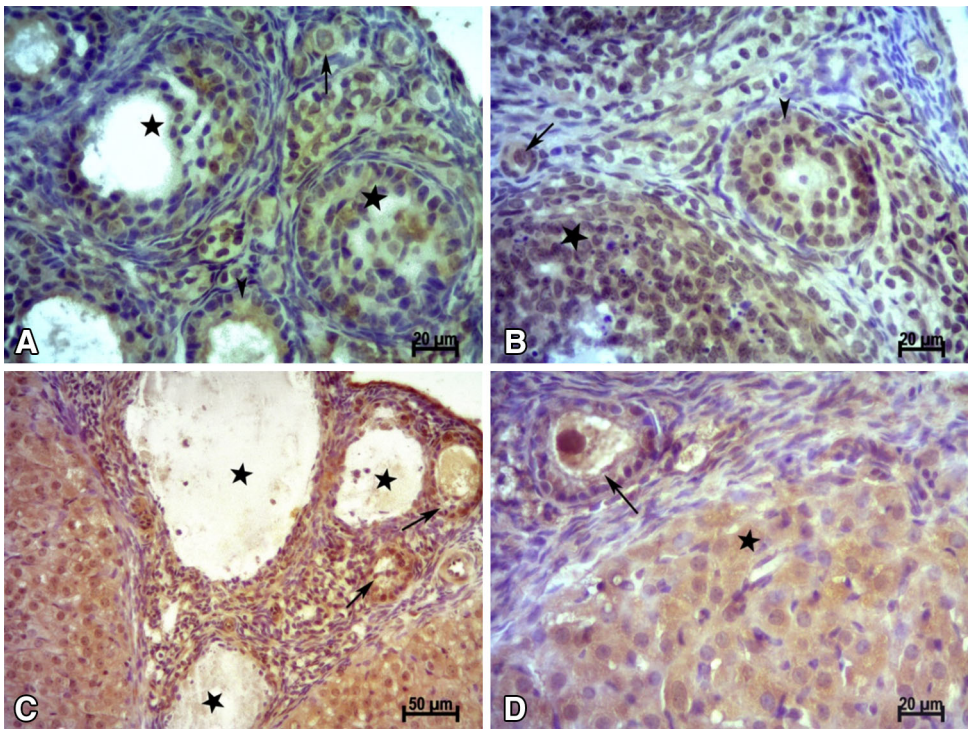

Fig. 5 Immunohistochemical demonstration of Bax expression in all groups. **a** No expression was detected in the control group granulosa cells in the primordial follicles (*arrow*). In advanced-stage follicles, slight expression was detected in granulosa cells (*asterisk*). No expression was detected in the thecal layer. **b** The shilajit group was similar to the control group. **c** In the radiation only group, dense expression was detected in the granulosa layer and corpus luteum and in the thecal layer (atretic follicles in different stages (*asterisk*) and preantral follicles (*arrows*)). **d** In the radiation + shilajit group, morphologically near-normal preantral follicles and early thecal layer were seen; weaker expression was detected in contrast to the radiation only group (*arrow*). In this group, the corpus luteum shows slight expression in contrast to the radiation only group

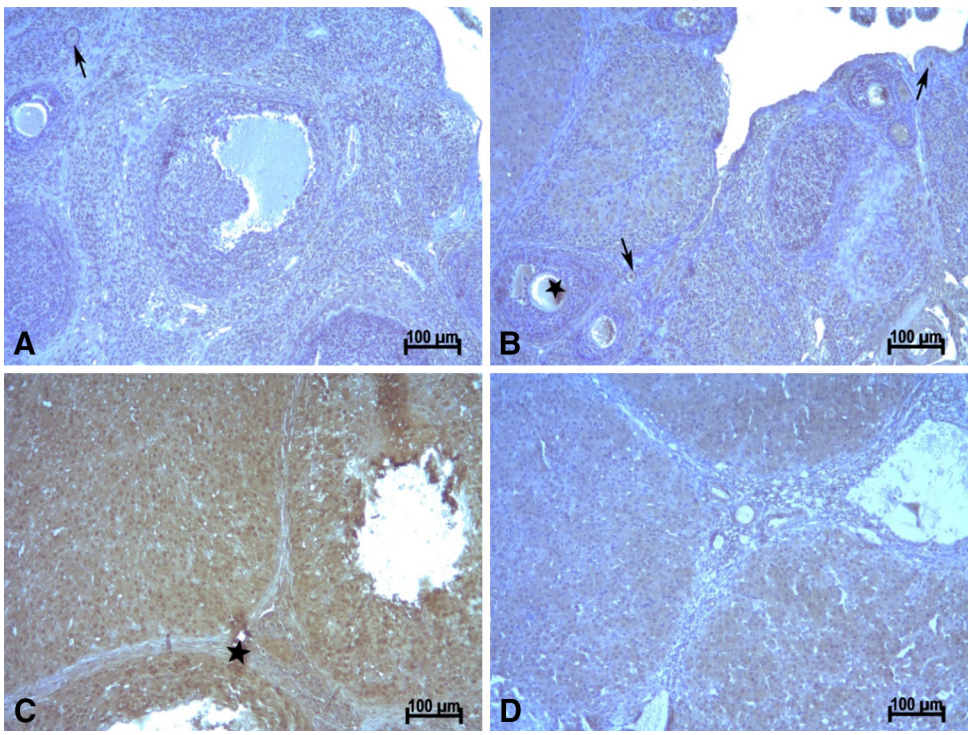

Fig. 6 Immunohistochemical demonstration of caspase-3 expression in all groups. **a** Control group, normal-looking primary follicle (*arrow*) and granulosa cells in advanced-stage follicles; no caspase-3 expression was detected. **b** Shilajit only group. Similar to the control group, no caspase-3 expression was detected in the granulosa cells of the shilajit group. Antral follicle (*asterisk*), primordial follicle (*arrow*). **c** In the radiation only group, dense expression was detected in the granulosa layer and corpus luteum. In the thecal layer (*asterisk*), expression was weak, but stronger than that in the control and shilajit groups. **d** Caspase-3 expression in the radiation + shilajit group granulosa cells was weaker than that in the radiation only group

estrogens, gonodatropins, insulin-like growth factor, Bcl-2, anti-oxidative agents) [32].

Several agents and techniques have been used to prevent radiation-related damage in the ovaries. In Simsek et al., resveratrol (a well-known anti-oxidant agent) was found to be effective in preventing the toxic effects of radiation in the ovaries, possibly through decreasing oxidative stress [19]. Other anti-oxidant agents that have been evaluated in radiation-exposed animals include melatonin and selenium [16, 17]. The researchers concluded that reducing oxidative stress-related substances may be beneficial in reducing radiation-related damage.

Shilajit, an ancient remedy for various diseases, recently gained attention because of its anti-oxidant, immune-modulating, and anti-aging effects. It contains humus (60–80 %), benzoic acid, hippuric acid, fatty acids, ichthyol, albuminoids, dibenzo-α-pyrones, essential oils, and various vitamins and minerals, such as B1 and B2 [20–24]. It is believed that the main physiological effects of shilajit depend on the presence of fulvic acid, humic acid, and dibenzo-α-pyrones, which are carrier molecules for active components [21]. Recently it was reported that it has both spermatogenic and ovogenic effects in animals [25, 26]. It was shown to trigger follicular development and differentiation and increase the frequency of ovulation [25].

In terms of primordial follicle numbers, a statistically significant difference was found between the radiation + shilajit and radiation only groups. Although the serum MDA values were lower and TAC levels were higher in the radiation + shilajit group than the radiation only group, the differences were not statistically significant, which might be due to sample collection and storage techniques.

In the histopathological evaluation of the groups, ovarian damage was highest in the radiation group. Almost all of the follicles were atretic in the radiation group. At least some normal-looking primordial follicles were encountered in the radiation + shilajit group. Although most of the advanced-stage follicles were undergoing apoptosis, some were in the early stages of apoptosis. In the radiation + shilajit group, ovarian tissue damage was reduced compared with the radiation only group.

In the present study, we assessed the apoptotic process in ovarian follicles by evaluating the expression of p53, Bax, and caspase-3 in tissues. The highest expression was found in the radiation group, whereas in the radiation + shilajit group, some primordial follicles showed no expression, and a few advanced-stage follicles showed slight expression.

Ovarian radio-protection by shilajit may be explained by the anti-oxidative characteristics of its components and the anti-apoptotic effects, shown immunohistochemically in the present study by the decreased expression of p53, Bax, and caspase 3.

The present study has several limitations. First, we did not evaluate different doses of shilajit. Second, although radiation apparently known to cause oxidative stress, in the present study we did not evaluate serum oxidative stress and anti-oxidant capacity, and we could not evaluate tissue oxidative stress and anti-oxidant capacity due to technical difficulties. Such evaluation of tissues might provide more precise values for oxidative damage. Third, although the anti-apoptotic effect of the shilajit is essential in protection and amelioriation of the radiation induced damage on ovaries, its effect on cancer cells could not be evaluated. So, anti-apoptotic effects of the shilajit should also be evaluated in cancer models in the prospective studies.

To our knowledge, this is the first reported study evaluating the effects of shilajit on radiation-related ovarian damage amelioration. Shilajit was found to be especially effective on amelioration of damage on primordial follicles. Further studies are needed to evaluate whether higher protection can be achieved with higher doses.

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Compliance with ethical standards

Conflict of interest None.

References

1. Kaatsch P (2010) Epidemiology of childhood cancer. Cancer Treat Rev 36(4):277–285
2. Bath LE, Hamish W, Wallace B, Critchley HOD (2002) Late effects of the treatment of childhood cancer on the female reproductive system and the potential for fertility preservation. BJOG 109(2):107–114
3. Miller RW, Young JL, Novakovic B (1995) Childhood cancer. Cancer 75(1):395–405
4. Delaney G, Jacob S, Featherstone C, Barton M (2005) The role of radiotherapy in cancer treatment: estimating optimal utilization from a review of evidence-based clinical guidelines. Cancer 104(6):1129–1137
5. Hudson MM (2010) Reproductive outcomes for survivors of childhood cancer. Obstet Gynecol 116(5):1171–1183
6. Geenan M, Cardous-Ubbink M, Kremer L et al (2007) Medical assessment of adverse health outcomes in long-term survivors of childhood cancer. JAMA 297(24):2705–2715
7. Oeffinger KC, Mertens AC, Sklar CA et al (2006) Chronic health conditions in adult survivors of childhood cancer. N Engl J Med 355(15):1572–1582
8. Shinomiya N (2001) New concepts in radiation-induced apoptosis: 'premitotic apoptosis' and 'postmitotic apoptosis'. J Cell Mol Med 5(3):240–253
9. Feinendegen LE (2002) Reactive oxygen species in cell responses to toxic agents. Hum Exp Toxicol 21(2):85–90
10. England K, Cotter TG (2005) Direct oxidative modifications of signalling proteins in mammalian cells and their effects on apoptosis. Redox Rep 10(5):237–245

11. Chemaitilly W, Mertens AC, Mitby P et al (2006) Acute ovarian failure in the childhood cancer survivor study. J Clin Endocrinol Metab 91(5):1723–1728
12. Pouilles JM, Tremollieres F, Bonneu M, Ribot C (1994) Infl uence of early age at menopause on vertebral bone mass. J Bone Miner Res 9(3):311–315
13. Hendry JH, West CM (1997) Apoptosis and mitotic cell death: their relative contributions to normal-tissue and tumour radiation response. Int J Radiat Biol 71(6):709–719
14. Chapman RM (1982) Effect of cytotoxic therapy on sexuality and gonadal function. Semin Oncol 9(1):84–94
15. Kim JK, Lee CJ, Song KW, Do BR, Yoon YD (1999) γ-Radiation accelerates follicular atresia in immautre mice. Vivo 13(1):21–24
16. Kaya H, Delibas N, Serteser M, Ulukaya E, Ozkaya O (1999) The effect of melatonin on lipid peroxidation during radiotherapy in female rats. Strahlenther Onkol 175(6):285–288
17. Abedelahi A, Salehnia M, Allameh AA (2008) The effects of different concentrations of sodium selenite on the in vitro maturation of preantral follicles in serum-free and serum supplemented media. J Assist Reprod Genet 25(9–10):483–488
18. Morita Y et al (2000) Oocyte apoptosis is suppressed by disruption of the acid sphingomyelinase gene or by sphingosine-1-phosphate therapy. Nat Med 6(10):1109–1114
19. Simsek Y et al (2012) Ameliorative effects of resveratrol on acute ovarian toxicity induced by total body irradiation in young adult rats. J Pediatr Adolesc Gynecol 25(4):262–266
20. Rajesh K, Witt M, Anwer MK, Agarwal SP, Koch BP (2008) Spectroscopic characterization of fulvic acids extracted from the rock exudate shilajit. Org Geochem 39(12):1719–1724
21. Ghosal S, Lal J, Singh SK, Goel RK, Jaiswal AK, Bhattacharya SK (1991) The need for formulation of shilajit by its isolated active constituents. Phytother Res 5(5):211–216
22. Frolova LN, Kiseleva TL (1996) Chemical composition of mumijo and methods for determining its authenticity and quality (a review). Pharm Chem J 30(8):543–547
23. Al-Himaidi AR, Mohammed U (2003) Safe use of salajeet during the pregnancy of Female mice. Online J Biol Sci 3(8):681–684
24. Agarwal SP, Khanna R, Karmarkar R, Anwer MK, Khar RK (2007) Shilajit: a review. Phytother Res 21(5):401–405 **(review)**
25. Park Jeong-Soog, Kim Gee-Young, Han Kun (2006) The spermatogenic and ovogenic effects of chronically administered shilajit to rats. J Ethnopharmacol 107(3):349–353
26. Biswas TK, Pandit S, Mondal S, Biswas SK, Jana U, Ghosh T, Tripathi PC, Debnath PK, Auddy RG, Auddy B (2009) Clinical evaluation of spermatogenic activity of processed shilajit in oligospermia. Andrologia 42(1):48–56
27. Velmurugan C, Vivek B, Wilson E, Bharathi T, Sundaram T (2012) Evaluation of safety profile of black shilajit after 91 days repeated administration in rats. Asian Pac J Trop Biomed 2(3):210–214
28. Stohs SJ (2014) Safety and efficacy of shilajit (mumie, moomiyo). Phytother Res 28(4):475–479
29. Devine PJ, Payne CM, McCuskey MK, Hoyer PB (2000) Ultrastructural evaluation of oocytes during atresia in rat ovarian follicles. Biol Reprod 63(5):1245–1252
30. Qian H, Xu J, Lalioti MD, Gulle K, Sakkas D (2010) Oocyte numbers in the mouse increase after treatment with 5-aminoisoquinolinone: a potent inhibitor of poly(ADP-ribosyl)ation. Biol Reprod 82:1000–1007
31. Baker TG (1971) Comparative aspects of the effects of radiation during oogenesis. Mutat Res 11(1):9–22
32. Gosden Roger (1997) Norah spears. Programmed cell death in the reproductive system. Br Med Bull 52(3):644–661