http://dx.doi.org/10.1590/1678-4162-12608

Arq. Bras. Med. Vet. Zootec., v.74, n.5, p.778-784, 2022

Protective effect of kaempferol against cisplatin-induced acute ovarian damage in a mouse model

[*Efeito protetor do kaempferol contra o dano ovariano agudo induzido pela cisplatina em um modelo murino*]

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ABSTRACT

The flavonoid kaempferol has attracted research attention as a potential adjuvant during chemotherapy. This study aimed to evaluate the protective effects of kaempferol against ovarian damage in cisplatin-treated mice. Two groups of mice received saline solution (intraperitoneal injection [i.p.]; control) or a single dose of cisplatin (5 mg/kg body weight, i.p.). Moreover, two other mice groups were pretreated with kaempferol (1 or 10 mg/kg body weight, i.p.) 30 min before of the cisplatin administration. Thereafter, their ovaries were harvested and subjected to histological (follicular morphology and activation) and fluorescence (reactive oxygen species [ROS] production, glutathione [GSH] concentration, and mitochondrial activity) analyses. Compared with cisplatin treatment alone, pretreatment with 1 mg/kg kaempferol maintained normal follicular morphology, reduced ROS production and mitochondrial damage, and enhanced GSH concentration. However, pretreatment with 10 mg/kg kaempferol did not prevent cisplatin-induced damage. The rate of primordial follicle activation was greater in mice pretreated with 1 mg/kg kaempferol than in the other treatment groups. In conclusion, pretreatment with 1 mg/kg kaempferol prevents cisplatin-induced ovarian damage and stimulates primordial follicle activation in mice.

Keywords: antioxidant, chemotherapy, ovary, oxidative stress, primordial follicle

RESUMO

O flavonoide kaempferol tem atraído a atenção como um potencial adjuvante durante a quimioterapia. O presente estudo objetivou avaliar os efeitos do kaempferol contra os danos ovarianos em camundongos tratados com cisplatina. Fêmeas de camundongos receberam solução salina (injeção intraperitoneal [ip]; controle) ou uma dose única de cisplatina (5 mg/kg, ip) ou foram pré-tratadas com kaempferol (1 ou 10 mg/kg, ip) 30 min antes da administração de cisplatina. Os ovários foram recuperados e destinados para as análises histológicas (morfologia e ativação folicular) e de fluorescência (produção de espécies reativas de oxigênio [ERO], concentração de glutationa [GSH] e atividade mitocondrial). Em comparação ao tratamento apenas com cisplatina, o pré-tratamento com 1 mg/kg de kaempferol manteve a morfologia folicular normal, reduziu a produção de ERO, bem como os danos mitocondriais, e aumentou a concentração de GSH. Entretanto, o pré-tratamento com 10 mg/kg de kaempferol não preveniu os danos induzidos pela cisplatina. A taxa de ativação do folículo primordial foi maior em camundongos pré-tratados com 1 mg/kg de kaempferol do que nos outros grupos experimentais. Em conclusão, o pré-tratamento com 1 mg/kg de kaempferol previne o dano ovariano induzido pela cisplatina e estimula a ativação do folículo primordial em camundongos.

Palavras-chave: antioxidante, quimioterapia, ovário, estresse oxidativo, folículo primordial

INTRODUCTION

Cisplatin (*cis*-diamminedichloroplatinum-II) is one of the most effective antineoplastic drugs used to treat various cancers (Deavall *et al.*, 2012). However, its use is associated with serious side effects, including ovarian toxicity (Motwani and Curhan, 2020; Rybak *et al*., 2019). This toxicity is due to an imbalance between antioxidant capacity and reactive oxygen species (ROS) production, which leads to mitochondrial

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Submitted: November 11, 2021. Accepted: April 27, 2022.

and DNA damage and consequent follicle death (Barberino *et al.*, 2017; Lins *et al.*, 2020). The administration of natural compounds with antioxidant properties may substantially prevent the toxic effects of cisplatin on ovaries.

In the last decade, kaempferol has attracted attention from the medicinal chemistry community and the pharmaceutical industry because of its pharmacological properties such as anti-inflammatory, anticancer, and antioxidant effects (Oh, 2016; Wang *et al.*, 2018). Kaempferol [3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4-H-1-benzopyran-4-one] is a flavonoid found in tea, beans, broccoli, tomatoes, strawberries, and grapes (Chen and Chen, 2013; Imran *et al.*, 2019), as well as in plants used in traditional medicine, such as *Aloe vera* (Keyhanian and Stahl-Biskup, 2007) and *Amburana cearensis* (Gouveia *et al.*, 2015).

In vivo studies using a mouse model have demonstrated that kaempferol pretreatment ameliorates cisplatin-induced toxicity in the cardiac muscle (Qi *et al.*, 2020) and kidney (Wang *et al.*, 2020). Overall, kaempferol exerts its protective effects by removing ROS and/or increasing the activity of endogenous antioxidants such as catalase, superoxide dismutase, and glutathione (GSH), thus preventing an inflammatory response, mitochondrial damage, and the incidence of cell death (Shakya *et al.*, 2014; Feng *et al.*, 2017; Wang *et al.*, 2020). However, to the best of our knowledge, the effects of kaempferol on cisplatin-induced ovarian damage have not yet been investigated. Thus, the present study was conducted to evaluate whether pretreatment with kaempferol prior to cisplatin administration could preserve normal ovarian follicle morphology and influence the activation of primordial follicles, oxidative stress, and mitochondrial activity in a mouse model.

MATERIAL E METHODS

This study was conducted in accordance with the ethical guidelines of the Ethics Committee on Animal Use of the Federal University of São Francisco Valley, Brazil (protocol number 0012/080716). Adult female Swiss mice (n = 16; age: 8 weeks; weight: 30–40 g) were housed in an air-conditioned atmosphere at 25ºC and kept under a 12-h/12-h light/dark cycle. Animals were provided *ad libitum* access to standard chow and water.

The mice were randomly divided into four experimental groups (four animals per group) and treated as follows: the first group (control) received a single intraperitoneal injection (i.p.) of 0.9% saline solution (0.15 M, 0.15 mL/mouse; Sigma Aldrich Chemical Co., St. Louis, MO, USA). The second group received 0.9% saline solution (0.15 M, 0.15 mL/mouse, i.p.; Sigma Aldrich Chemical Co.) and 30 min later, a single dose of cisplatin (5 mg/kg body weight, i.p.; Libbs Farmacêutica Ltda, São Paulo, Brazil). For the third and fourth groups, mice were pretreated with 1 or 10 mg/kg body weight (i.p.) kaempferol (Sigma Aldrich Chemical Co.), respectively, and 30 min later, received a single dose of cisplatin (5 mg/kg body weight, i.p.). The administered dose of cisplatin was based on a previous study that showed a loss of ovarian reserve 1 day after a single injection of the drug (Nguyen *et al.*, 2019). Kaempferol doses were based on a previous study showing its cytoprotective effects in cardiac cells (Xiao *et al.*, 2012). The mice were euthanized 24 h after the experimental treatment, ovarian tissues were dissected, washed in 0.9% saline solution (Sigma Aldrich Chemical Co.), and used for histological and fluorescence analyses.

For light microscopic evaluation, ovaries (one ovary per animal) were fixed in 10% buffered formalin (Dinâmica, São Paulo, Brazil) and embedded in paraffin wax (Dinâmica). Paraffin-embedded tissue blocks were serially sectioned at a thickness of 5 μm, mounted on glass slides, and stained with hematoxylin and eosin (HE; Vetec, São Paulo, Brazil). The tissue samples were examined blindly by an experienced investigator using a light microscope (Nikon, Tokyo, Japan), and follicles were classified based on the Pedersen and Peters (1968) morphological criteria as primordial (one layer of flattened or flattened and cuboidal granulosa cells), primary (a complete layer of cuboidal granulosa cells surrounding the oocyte), secondary (two or more layers of cuboidal granulosa cells with no sign of antrum formation), and antral (multiple granulosa cell layers with some antral space). Follicles were further classified as morphologically normal if no overt signs of degeneration were noted, including shrunken oocytes, disorganization of

the granulosa cell layer, condensed nuclear chromatin, and/or cell swelling. Overall, 160 follicles with visible oocyte nuclei were evaluated for each treatment (40 follicles per treatment × four replicates = 160 follicles). To evaluate follicular activation, only morphologically normal follicles were recorded, and the ratio of primordial to growing (primary, secondary, and antral) follicles was calculated for different treatments.

The intracellular ROS production, GSH concentration, and mitochondrial activity were evaluated using previously described methods (Barberino *et al.*, 2017). Briefly, 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA; Invitrogen Corporation, Carlsbad, CA, USA), CellTracker® Blue (Invitrogen Corporation), and Mitotracker® Red (Molecular Probes, Melbourne, Australia) were used to determine ROS production, GSH concentration, and mitochondrial activity as green, blue, and red fluorescence, respectively. Preantral and antral follicles (n = 40 in each treatment) were mechanically isolated from the ovaries (one ovary per animal) using 26-gauge needles and incubated in the dark at 37ºC for 30 min with 10 µM H2DCFDA, 10 µM CellTracker Blue, and 100 nM Mitotracker Red. After incubation, the follicles were washed with phosphate-buffered saline (Sigma Aldrich Chemical Co.) for 30 min, and fluorescence was observed under an epifluorescence microscope (Nikon) with UV filters (460 nm for ROS, 370 nm for GSH, and 579 nm for active mitochondria). The fluorescence intensities were analyzed using the ImageJ software (Version 1.41; National Institute of Health, Bethesda, MD, USA) and normalized to control group.

For statistical analysis, data of morphologically normal, primordial, and growing (activation) follicles were compared using the chi-square test and expressed as percentages. Data of ROS production, GSH concentration, and mitochondrial activity were evaluated using the Shapiro–Wilk test to verify the normal distribution of residuals and homogeneity of variances. Thereafter, the Kruskal–Wallis nonparametric test was used for comparisons. When the main effects or interactions were significant, the means were compared using the Student–Newman–Keuls test. The results were expressed as the mean ± standard error of the mean, and differences were considered significant when $P<0.05$.

RESULTS

Morphological analysis indicated that ovaries from mice in the control group showed normal follicles with centrally located oocytes surrounded by organized granulosa cells (Fig. 1A). Cisplatin treatment damaged the ovarian structure, resulting in follicles with swollen and disorganized granulosa cells and vacuolated oocytes (Fig. 1B). Pretreatment with 1 mg/kg kaempferol decreased the follicular damage caused by cisplatin, and the morphological features of follicles were similar to those of the follicles of the control group (Fig. 1C). Follicular morphology in mice pretreated with 10 mg/kg kaempferol was not different from that in the cisplatin-treated mice (Fig. 1D).

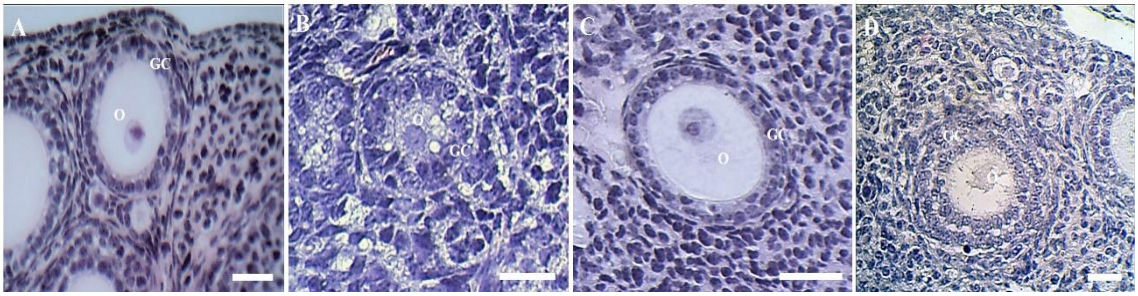

Figure 1. Histological sections of mouse ovarian fragments after HE staining: follicles from the (A) control group, (B) exposed to cisplatin alone, and pretreated with (C) 1 or (D) 10 mg/kg kaempferol before cisplatin administration. A and C: note the normal centrally located oocytes surrounded by well-organized granulosa cells; B and D: note the shrunken or vacuolated oocyte surrounded by swollen and disorganized granulosa cells; O: oocyte; GC: granulosa cells; Scale bars: 25 µm (400x).

Cisplatin treatment decreased (P<0.05) the percentage of total normal follicles compared with that in the control group. Ovaries from mice pretreated with 1 mg/kg kaempferol showed similar (P>0.05) percentages of normal follicles as those observed in the control group and greater (P<0.05) percentages than those in the cisplatin and 10 mg/kg kaempferol groups (Fig. 2A). To verify whether any follicular category was more susceptible to the experimental treatment, the effects of cisplatin and/or kaempferol were evaluated at different developmental stages (Fig. 2B). After treatment with cisplatin, there was a decrease (P<0.05) in the percentage of primordial and primary follicles compared to that in the control group. Only pretreatment with 1 mg/kg kaempferol prevented (P<0.05) primordial and primary follicles loss caused by cisplatin treatment, with percentages similar (P>0.05) to those observed in the control group. Interestingly, mice pretreated with 1 mg/kg kaempferol showed a higher (P<0.05) percentage of normal secondary follicles than the mice in both control and cisplatin groups. There was no significant difference (P>0.05) in the percentage of normal antral follicles among the groups (Fig. 2B).

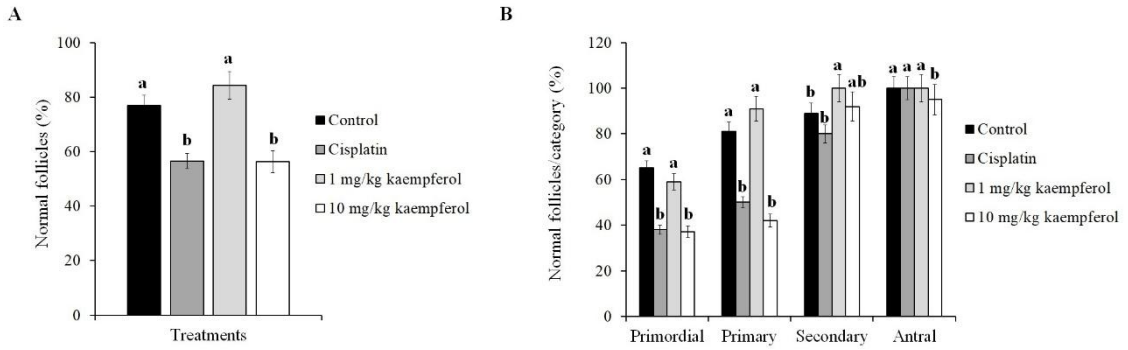

Figure 2. Percentages of (A) total morphologically normal follicles, and of (B) morphologically normal follicles in different stages (primordial, primary, secondary, and antral follicles) from mouse ovaries of the control group, exposed to cisplatin alone or pretreated with kaempferol (1 or 10 mg/kg) before cisplatin administration. (^{a,b}) Bars with different letters are significantly different (P<0.05).

Compared with the saline (control), cisplatin, and 10 mg/kg kaempferol treatments, pretreatment with 1 mg/kg kaempferol increased (P<0.05) the ratio of growing versus primordial follicles, indicating an increase in primordial follicle activation (Fig. 3).

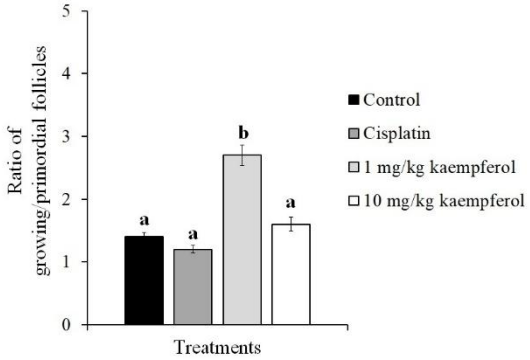

Figure 3. Ratio of growing (primary, secondary and antral) versus primordial follicles (follicular activation) from mouse ovaries of the control group, exposed to cisplatin alone or pretreated with kaempferol (1 or 10 mg/kg) before cisplatin administration. (^{a,b}) Bars with different letters are significantly different (P<0.05).

Cisplatin treatment increased (P<0.05) ROS production and decreased (P<0.05) GSH concentration as well as mitochondrial activity compared with the control treatment (Fig. 4). These changes were reversed (P<0.05) by pretreatment with 1 mg/kg kaempferol. Furthermore, mice pretreated with 10 mg/kg kaempferol showed lower (P<0.05) mitochondrial activity than mice from other groups.

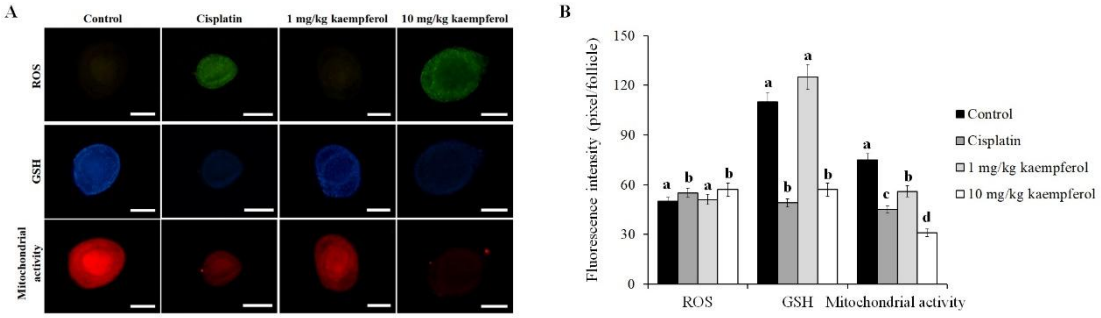

Figure 4. (A) Representative fluorescence images showing ROS, GSH and mitochondrial activity staining in follicles from mouse ovaries of the control group, exposed to cisplatin alone, and pretreated with 1 or 10 mg/kg kaempferol before cisplatin administration. Scale bars: 50 μm (100x). (B) ROS production, GSH concentration and mitochondrial activity in follicles from mouse ovaries of experimental groups exposed to cisplatin alone or in combination with kaempferol (1 or 10 mg/kg). (^{a,b,c,d}) Bars with different letters are significantly different (P<0.05).

DISCUSSION

In the present study, we used an *in vivo* mouse model in which a single-dose administration of cisplatin (5 mg/kg) decreased the percentage of normal follicles, with the highest negative effects on primordial and primary follicles. A previous study also demonstrated that a short-term treatment (24 h) was sufficient to assess the damage induced by 5 mg/kg cisplatin in the mouse ovary, characterized by the depletion of primordial and primary follicles (Nguyen *et al.*, 2019). These direct effects of cisplatin on the ovarian reserve can be related to increased ROS production and decreased cellular antioxidant capacity. To reduce cisplatin-induced damage, we tested kaempferol as an ovarian protective adjuvant during chemotherapy for the first time.

In recent years, kaempferol has been considered as a potential therapeutic compound because of its remarkable beneficial effects on human and animal health (Chen and Chen, 2013; Imram *et al.*, 2019). In the present study, pretreatment with 1 mg/kg kaempferol before cisplatin administration showed more morphologically normal follicles (total, primordial, primary, and secondary follicles), decreased ROS production and mitochondrial damage, and increased GSH concentration compared with cisplatin treatment alone. In previous studies using rodent models, kaempferol attenuated cisplatin-induced nephrotoxicity (Wang *et al.*, 2020) and cardiotoxicity (Qi *et al.*, 2020) by preventing inflammation and/or oxidative stress. Because of its lipophilicity, kaempferol can be absorbed by passive diffusion (Alam *et al.*, 2020) and act directly on cell components, especially in the mitochondria where it activates the thioredoxin reductase system, which is essential for the maintenance of oxidative balance and cell survival (Choi, 2011). Therefore, we believe that pretreatment with 1 mg/kg kaempferol prevented cisplatin-induced follicle loss through its direct antioxidant action, inhibiting mitochondrial damage and oxidative stress (by reducing ROS production and increasing GSH concentrations). Kaempferol enhances the anticancer effects of cisplatin on human ovarian cancer cells (line OVCAR-3: Luo *et al.*, 2010), and has no toxic effects on normal ovarian epithelial cells (line IOSE39: Luo *et al.*, 2012). Therefore, kaempferol may be suitable for use as a therapeutic agent to preserve ovarian morphology and function during anticancer therapy.

In our study, after treatment with 10 mg/kg kaempferol, the percentage of normal follicles, ROS production, and GSH concentration were

similar to those found in the cisplatin group, whereas mitochondrial activity was lower. Although the antioxidant action of kaempferol is clear, it is known to have toxic or pro-oxidant effects, depending on its concentration or dose (Santos *et al.*, 2019a; Wang *et al.*, 2019). Thus, we believe that treatment with 10 mg/kg kaempferol plus cisplatin can exert a pro-oxidant activity in the ovarian tissue in this experimental design.

Depending on the dose and/or treatment period, cisplatin may cause atresia of growing follicles, followed by increased activation of primordial follicles (Chang *et al.*, 2015). Although cisplatin did not induce activation in the present study, pretreatment with 1 mg/kg kaempferol before cisplatin administration increased primordial follicle activation without affecting the survival of growing follicles, as evidenced by the greater percentage of normal secondary follicles in the mice of this group. Kaempferol has been shown to enhance primordial follicle activation by stimulating granulosa cell proliferation after *in vitro* culture of ovine ovarian cortex (Santos *et al.*, 2019b). Moreover, after *in vitro* culture of ovarian tissue, secondary follicles have been isolated and cultured to generate mature human oocytes for embryo production (McLaughlin *et al.*, 2018). Therefore, one of the clinical applications of our findings is the preservation of fertility in individuals with a reduced ovarian reserve. Secondary follicles from mice treated with 1 mg/kg kaempferol plus cisplatin can be retrieved from the ovarian cortex, cryopreserved (before or after isolation), and used for *in vitro* culture and oocyte maturation. This process may facilitate the restoration of ovarian function as an alternative to autologous transplantation, particularly in cases with a possibility of tumor reintroduction due to grafting. Further studies are necessary to evaluate the efficiency and safety of these approaches.

CONCLUSION

A single dose of kaempferol (1 mg/kg) maintained follicle survival during acute experimental treatment with cisplatin in mice by preventing mitochondrial damage and oxidative stress (ROS production reduction and GSH expression stimulation) and induced primordial follicle activation. However, the mechanisms underlying the effects of kaempferol on mammalian ovaries need to be elucidated. Additionally, future studies evaluating the effects of long-term kaempferol treatment are necessary to support its potential role as an adjuvant in anticancer therapy.

ACKNOWLEDGMENTS

L.M.R. Barbosa received a scholarship from National Council for Scientific and Technological Development (CNPq, Brazil). M.H.T. Matos is supported by a grant from CNPq, Brazil.

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