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·Complementary Medicine ·

# Herbal extracts counteract cisplatin-mediated cell death in rat testis

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# Abstract

**Aim:** To evaluate the protective effects of ginger (Gin) and roselle (Ros) against testicular damage and oxidative stress in a cisplatin (CIS)-induced rodent model. Their protective effects against CIS-induced apoptosis in testicular and epididymal sperms is also investigated. **Methods:** Ethanol extracts of Gin or Ros (1 g/kg·day) were given orally to male albino rats for 26 days. This period began 21 days before a single CIS intraperitoneal injection (10 mg/kg body weight). **Results:** Gin or Ros given orally significantly restored reproductive function. Both tested extracts notably reduced the CIS-induced reproductive toxicity, as evidenced by restoring the testis normal morphology. In Gin and Ros, the attenuation of CIS-induced alterations of testicular lipid peroxidation were markedly improved by these plant extracts. **Conclusion:** The present results provide further insights into the mechanisms of protection against CIS-induced reproductive toxicity and confirm the essential anti-oxidant potential of both examined extracts. *(Asian J Androl 2008 Mar; 10: 291–297)* 

Keywords: cisplatin; cell death; toxicity; flow cytometry; ginger; roselle

# 1 Introduction

Cis-diamminedichloroplatinum (II) or cisplatin (CIS) is a highly effective antineoplastic DNA alkylating agent used to treat many types of solid tumors including testicular, ovarian, breast, lung, bladder, and head and neck. However, adverse side-effects, including testicular toxicity, limit its application [1]. Both short-term and long-term effects of CIS treatment on testicular func-

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tion have been previously documented in human [1] and in other animal models [2, 3]. Within days of CIS injection, animals develop severe testicular damage characterized by germ cell apoptosis, Leydig cell dysfunction and testicular steroidogenic disorder [2, 4]. Germ cell apoptosis has been reported to play an important role in CIS-induced testicular damage [2, 4]. CISinduced DNA adduct formation in rat's spermatozoa was observed after treatment with CIS at a dose of 10 mg/kg body weight [5].

Free radicals have been reported to mediate reactions responsible for a wide range of CIS-induced sideeffects. Consequently, anti-oxidants have been shown to protect non-malignant cells and organs against damage by CIS [6]. CIS has previously been shown to induce lipid peroxidation (LP) with a concomitant decrease in the

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level of testicular anti-oxidants [3].

Ginger rhizome (Gin, *Zingiber officinale* R., family Zingiberaceae) is a spice commonly used as a digestive aid and an anti-nausea remedy [7]. Gin extract has recently been shown to have a variety of biological activities, including anticancer, anti-oxidation, anti-inflammation and antimicrobial properties [8–10]. All Gin's major active ingredients, such as zingerone, gingerdiol, zingibrene, gingerols and shogaols, are known to possess anti-oxidation anti-activities [10, 11].

Roselle (ROS, *Hibiscus sabdariffa* L., family Malvaceae) is an annual shrub commonly used to make jellies, jams and beverages. In folk medicine, Ros has commonly been used for its antihypertension properties [12]. The anthocyanin pigments that confer Ros's color [13] make it a valuable food product. Many biological activities, such as anti-atherosclerosis, anticarcinogenic [14], hepatoprotective [15] and anti-oxidative properties [16], have been reported in Ros and its anthocyanin.

This investigation was set to evaluate the protective effects of Gin and Ros against CIS-induced testicular toxicity in male rats and to investigate whether apoptosis mediates this protection.

# 2 Materials and methods

# 2.1 Chemicals

The dried plants, Ros flowers and Gin roots, were purchased from a local herbal store (Al-Ain, United Arab Emirates). CIS was purchased from Bristol-Myers Squibb (Hopewell, NJ, USA). Thiobarbituric acid, Folin's reagent and bovine serum albumin were obtained from Sigma-Aldrich Chemicals (St. Louis, MO, USA). All other chemicals were purchased from local commercial suppliers.

## 2.2 Animals

Albino rats (150–200 g) of the Wistar strain were obtained from the Animal House, United Arab Emirates University (Al-Ain, United Arab Emirates). They were maintained on a standard pellet diet and tap water *ad libitum* and were kept in polycarbonate cages with woodchip bedding under a 12 : 12 h light : dark cycle and room temperature 22–24°C. Rats were acclimatized to the environment for 2 weeks prior to experimental use. This study was conducted following the guidelines of the Animal Ethics Committee, United Arab Emirates University.

# 2.3 Plant extraction

To increase the yield of extraction in a shorter time and a lower temperature, the liquid-phase microwaveassisted process was used for extraction of Gin and Ros according to the methods described by Alfaro *et al.* [17]. These microwave-assisted extraction applications are based on the selective heating of the matrix containing the target extract when the matrix is immersed in a transparent solvent (ethanol and water). This solvent allows for selective heating of particular components within the matrix without using excessive heating. One-hundred grams of dried plants, Ros flowers and Gin roots, were mixed in 1 000 mL of 70% ethanol. Mixtures were then irradiated with microwaves for 2 min and extracts were finally filtered through gauze and evaporated under vacuum at 40°C using a rotary evaporator.

#### 2.4 Experimental protocol

CIS solution was freshly prepared, protected from light in a saline solution, and was given in a volume of 1 mL/100 g body weight. Control animals received an equivalent volume of saline based on body weight. Plant extracts were given orally by gavage at volumes of 1 g/kg body weight. Rats were randomly divided into four groups (n = 5) and were subjected to the following treatment: The control group was treated daily with distilled water; In the CIS-treated group, animals were given a single intraperitoneal dose of CIS (10 mg/kg body weight), used previously to induce testicular toxicity in various animal species [2, 4]; Animals of the third group were given Gin extract for 21 days prior to CIS treatment and for 5 days after CIS injection; The fourth group was fed Ros extract daily for 21 days prior to CIS treatment and for 5 days after CIS treatment. After 5 days of CIS treatment and 26 days of extracts and vehicle solution treatment, blood and testes were collected from all groups.

#### 2.5 Sample collection and preparation

Following diethyl ether anesthesia, blood was collected from the retro-orbital plexus. After the animals were killed the testes were removed. For histopathological examination, one testis was immediately fixed in 10% buffered formalin. For biochemical determination, the other testis was homogenized in ice-cold KCl<sub>4</sub> (150 mmol/L). The ratio of tissue weight to homogenization buffer was 1:10. From the latter, suitable dilutions were prepared in different buffers to determine levels of malondialdehyde (MDA) and total proteins. To obtain serum, blood was collected in centrifuge tubes and centrifuged at  $1 300 \times g$  for 20 min at 4°C.

# 2.6 Biochemical assays

MDA is the most abundant individual aldehyde resulting from LP breakdown in biological systems and is used as an indirect index of LP. Determination of MDA in biological materials, as described in Uchiyama and Mihara [18], is based on its reaction with thiobarbituric acid to form a pink complex with the absorption maximum at 535 nm.

The total protein content of testis was determined according to a modified Lowry's method [19]. Absorbance was recorded using a Shimadzu recording spectrophotometer UV-160A (Tokyo, Japan) in all measurements.

#### 2.7 Histology

For the histological examinations, small pieces of testis were fixed in 10% neutral phosphate-buffered formalin and the hydrated 5  $\mu$ m-thick sections were stained with hematoxylin and eosin. Sections were examined under a Leica DMRB/E light microscope (Heerbrugg, Switzerland).

#### 2.8 Immunohistochemistry

Apoptosis was assessed in deparaffinized sections using the terminal deoxynucleotidyl transferase-mediated triphoshate nick-end labeling (TUNEL) technique. In this technique, the manufacturer's protocol for the ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (Chemicon International, Temecula, CA, USA) was followed. This method detects the DNA fragmentation associated with apoptosis by labeling 3-OH DNA termini with digoxigenin nucleotides, a process facilitated by terminal deoxynucleotidyl transferase. The labeled fragments are then allowed to bind to anti-digoxigenin antibody conjugated with peroxidase. Color was developed by adding sufficient peroxidase substrate to specimens. p53 protein expression was assessed on sections by immunohistochemistry. Briefly, after deparaffinization and rehydration; tissue sections were treated with 3% hydrogen peroxide for 20 min to diminish non-specific staining. Sections were immersed in 10 mmol/L citrate buffer solution (pH 6.0) in a microwave oven twice for 5 min then incubated with normal goat serum for 20 min. Sections were incubated overnight at 4°C with the rabbit p53 primary antibody, then washed with phosphate-buffered saline (PBS). Sections were exposed to the avidin-biotin peroxidase complex (1/400; Dako, Glostrup, Denmark) for 1 h at room temperature. The chromogenic substrate of peroxidase was developed using a 0.05% solution of 3,3 diaminobenzidine tetrahydrochloride, 0.03% hydrogen peroxide, and imidazole in Tris-HCl buffer (pH 7.6). Sections were counterstained with hematoxylin. The number of apoptotic and p53-positive cells in each section was calculated by counting the number of positive cells in 10 fields per slide at × 400 magnification. This was repeated for all five animals in each group and the average was plotted.

## 2.9 Flow cytometry

The number of apoptotic sperms was analyzed by flow cytometry. Sperms were washed twice and resuspended in PBS (Nissui Pharmaceutical, Tokyo, Japan), and fixed with 70% ethanol. The suspension was treated with 10 mL PBS containing 2.5 mg RNase A (USB, Cleveland, OH, USA) and 10  $\mu$ L Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) at 37°C for 30 min. The treated nuclei were stained with 50  $\mu$ g/mL propidium iodide (Sigma-Aldrich) at room temperature (22 ± 2°C) for 30 min with gentle agitation. The dye fluorescence to reflect the relative DNA ploidies was measured using FACSCalibur (Becton Dickinson, San Jose, CA, USA) equipped with an argon ion laser tuned at 488 nm at a low flow rate. Data were analyzed by Cell Quest software (Becton Dickinson, San Jose, CA, USA).

# 2.10 Statistical analysis

Data are expressed as group mean  $\pm$  SE. The statistical analysis was carried out using ANOVA, with SPSS version 10.0 (SPSS, Chicago, IL, USA). ANOVA was carried out to detect differences between all various groups. When significant differences were detected, analysis of a difference between the means of the treated and control groups was carried out using Dunnett's *t*test.

# 3 Results

# 3.1 Histological effects of Gin and Ros

Control rats showed normal testicular architecture with an orderly arrangement of germinal and Sertoli cells. CIS treatment induced moderate to severe testicular atrophy with degeneration of germ cells in seminiferous tubules (Figure 1). The tubules were shrunken and

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greatly depleted of germ cells. There were depleted numbers of Leydig cells between the tubules. Sertoli cells with few germ cells were observed in the lumen. Animals pretreated with Gin or Ros showed normal testicular morphology with irregular arrangement of germ cells and slight degeneration of seminiferous epithelium and shedding of germ cells in some tubules.

# 3.2 Apoptotic cell death

TUNEL assay was used to identify apoptotic cells of seminiferous tubules. Brown staining, indicating TUNEL-positive nuclei, was visible in seminiferous tubules of control and CIS-treated animals (Figure 2). However, TUNEL-positive cells were significantly (P < 0.001) increased in the CIS-treated group compared to the control group (Figure 2B, E). Pretreatment with Gin or Ros



Figure 1. Photomicrograph of the seminiferous tubules of control rats (A) showing the normal arrangement of germinal cells and Sertoli cells. Testis of cisplatin-treated rats (B–D) showing tubular atrophy (asterisks) with extensive degeneration of germinal epithelium. The atrophic tubules contained degenerated Sertoli cells with few germ cells. However, ginger pretreated (E) or roselle pretreated (F) rats had less degeneration in some tubules and irregular arrangement of germ cells with shedding of cellular materials from seminiferous epithelium in some tubules. Cells stained with hematoxylin and eosin. Bars: (A)–(C), (E), (F) = 100  $\mu$ m; (D) = 10  $\mu$ m.

prior to CIS treatment significantly attenuated the increase in the number of TUNEL-positive cells in the CIS-treated group (Figure 2C, D, E).

# 3.3 Effects on apoptosis in sperms

The effect of CIS on the percentage of apoptosis in sperm was determined by flow cytometry. As shown in Figure 3, CIS induced significant increases in the per-



Figure 2. Terminal deoxynucleotidyl transferase-mediated triphoshate nick-end labeling (TUNEL)-positive cells in seminiferous tubules of rats treated with vehicle (control) (A), cisplatin (CIS) (B), ginger (Gin) + CIS (C), and roselle (Ros) + CIS (D). Photomicrographs (A–D) and the semiquantitative analysis (E) show variable levels of apoptosis in different experimental groups. The number of apoptotic cells in each section was calculated by counting the number of TUNEL-positive cells in 10 fields per slide at × 400 magnification. This was repeated for all five animals in each group and the average was plotted. Brown staining indicates TUNEL-positive cells. TUNEL-positive cells are denoted by arrows (counterstained with hematoxylin, Bar = 10 µm). \*P < 0.05, \*\*\*P < 0.001, compared with control; +++P < 0.001, compared with CIS-treated group.

centage of apoptosis in sperm. When rats were pretreated with either Gin or Ros, this percentage of apoptosis was significantly decreased as compared with the CIS group.

## 3.4 Effects on p53 protein expression

p53 protein expression was detected in seminiferous tubules of both control and CIS-treated animals (Figure 4A, B). Brown staining, indicating positive immuno-



Figure 3. Flow cytometry DNA histogram of epididymal sperm cells in rats. The histogram represents the counts for one representative rat from each treatment group: control (A), cisplatin (CIS) (B), ginger (Gin) + CIS (C) and roselle (Ros) + CIS (D). When excited with blue light, fluorescence stain associated with double-stranded DNA emits pink fluorescence, whereas single-stranded DNA emits blue fluorescence. (E): Effect of ginger (Gin) and roselle (Ros) on the percentage of apoptotic sperm cells in cisplatin (CIS)-treated rats. Each column represents the mean  $\pm$  SE for five rats in each treatment group. \*\*P < 0.01, \*\*\*P < 0.001, compared with control; +P < 0.05, +++P < 0.001, compared with CIS-treated group.

stained cells, was significantly more (P < 0.001) in the CIS-treated group compared to the control group (Figure 4B, E). The concomitant treatment with Gin or Ros before CIS treatment significantly prevented the increase in the number of p53-positive cells in the CIS-treated group (Figure 4C, D, E).

3.5 Effects on testicular MDA

A significant increase (P < 0.001) of testicular MDA



Figure 4. Protein expression of p53 in the seminiferous tubules of rats treated with vehicle (Control) (A), cisplatin (CIS) (B), ginger (Gin) + CIS (C) and roselle (Ros) + CIS (D). Photomicrographs (A)–(D) and semiquantitative analysis (E) show the degree of immunostained cells in different experimental groups. The number of p53-positive cells in each section was calculated by counting the number of immunostained cells in 10 fields per slide at × 400 magnification. This was repeated for all five animals in each group and the average was plotted. Brown staining indicates positive-stained cells. p53-positive cells are denoted by arrows (counterstained with hematoxylin, Bar = 10 µm). \*\*\*P < 0.001, compared with CIS-treated group.



Figure 5. Effect of ginger (Gin) and roselle (Ros) on testicular malondialdehyde (MDA) levels in cisplatin (CIS)-treated rats. Each column represents the mean  $\pm$  SE, for five rats in each group. \*\*\*P < 0.001, compared with control; \*\*\*P < 0.001, compared with CIS-treated group.

was recorded after CIS treatment (Figure 5B). Although MDA levels of pretreated animals (given Gin or Ros before CIS treatment) did not return to the control level, there was no significant difference compared to the control.

## 4 Discussion

Recent studies have shown the important role of apoptosis in the pathogenesis of CIS testicular damage [1]. In the present study, the protective effect of Gin and Ros against testicular damage induced by CIS was shown in rats. In addition to its role in normal testicular physiology [1], apoptosis of germ cells has been recently reported as a mechanism responsible for the toxic damage to spermatogenesis. CIS was reported to cause apoptosis to testicular germ cells and Sertoli cells [2, 4]. In this study, apoptotic DNA fragmentation was determined in testicular tissue using the TUNEL technique and in epididymal sperm using cytometric assessment of DNA damage. A single dose of CIS caused apoptosis in testes (germ cells and Sertoli cells) and in epididymal sperms. Consistent with the results of apoptosis, histological changes were observed in the CIS-treated animal group. The high proportion of apoptosis in the present study suggests that apoptosis is an important mechanism that might account for the marked loss of spermatogenic cells in the CIS-intoxicated testes. The CIS-induced testicular damage was also associated with upregulation of p53

expression. Elevation of p53 protein expression in response to DNA damage triggers either a transient cell cycle arrest or apoptosis [20, 21]. Sperms respond to exposure to a DNA-damaging agent by elevating p53 protein levels [22]. It is therefore suggested here that p53 is a necessary component in the CIS-mediated apoptotic pathway of testicular epithelia.

Consistent with results reported elsewhere [3], the current study shows that histological damage in testis is associated with increase in testicular LP. CIS-treated animals have shown an elevation in testicular MDA levels compared with the control group. The decreased formation of anti-oxidants and the augmented activity of free radicals might account for the increase of MDA production in CIS-induced tissues. In our previous work, the levels of hepatic reduced glutathione as well as the enzyme activities of catalase and superoxide dismutase in the testis were lower in CIS-treated animals compared to control animals. Several studies have shown that CIS toxicity in kidney is mediated by depletions of anti-oxidants and elevations of LP [23, 24]. CIS has also been suggested to generate free radicals by interaction with DNA [25]. Therefore, overproduction of free radicals and hence oxidative stress might account, at least in part, for testicular injury associated with CIS treatment.

Recently, much attention has been focused on the protective effects of anti-oxidants and naturally occurring substances against oxidative stress damage. Gin or Ros extracts given before CIS treatment clearly attenuated the testicular damage and decreased apoptotic damage both in testes and sperms. It also retained the control value of p53 protein expression in the testicular tissue. The protective effect of plant extracts is accompanied by normalizing the increase of MDA. Gin crude extract and its individual constituents, such as zingerone, gingerdiol, zingibrene, gingerols, and shogaols, have been shown to protect against LP in various established models [10, 11]. Accumulating evidence suggests that the protective effects of Ros against oxidative damage could be attributed to its anti-oxidative properties [26-28]. The antioxidant activity of Ros could be attributed to its phenolic contents, namely protocatechuic acid [28] and anthocyanins [13, 16]. Ros has also been reported to prevent or attenuate decrease in tissue anti-oxidant enzymes in different animal models and to provide cellular protection against oxidative stress [3, 15]. In conclusion, this study showed that apoptotic cell death might play an important role in the development of CIS-induced testicular damage. Both Gin and Ros are reported here to have a potent protective effect on CIS-induced testicular damage and apoptotic cell death in rats. The protective effect of Gin and Ros might be due to their anti-oxidant properties.

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