Experimental research

Assessment of the protective effect of green tea extract against ciprofloxacin-induced chondrotoxicity in albino rats (histochemical, and immunohistochemical study)

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Abstract

Introduction: Ciprofloxacin is a broad-spectrum antibiotic which can cause a chondrotoxic effect in the growing condyle. Researchers found that green tea showed significant health benefits for a large number of disorders. The objective of the present research was to investigate the preventive potential of green tea extract against mandibular chondrotoxicity induced by ciprofloxacin in juvenile Wistar rats.
Material and methods: Twenty juvenile male rats were used and divided into four equal groups, the saline/water (S/W), saline/green tea (S/G), ciprofloxacin/water (C/W) and ciprofloxacin/green tea (C/G) treated groups. On day 32 of age, all the animals in C/W and C/G treated groups were subcutaneously injected with ciprofloxacin as two subcutaneous injections of 600 mg/kg of body weight, eight hours apart, while the S/W and S/G groups were subcutaneously injected with saline. The S/G and C/G groups were intragastrically gavaged by green tea extract in an oral dose of 300 mg/kg/day, 8 days before the subcutaneous injection of saline or ciprofloxacin. On day 34, all the animals were anaesthetized, and the mandibular condyle samples were taken immediately and processed.
Results: In comparison with the C/W treated group, the C/G treated group showed a significant increase in the thickness of the mandibular condylar cartilage, cartilage cell number, glycosaminoglycan content, and Bcl-2 immune expression ($p < 0.05$).
Conclusions: This study provides the first evidence that green tea extract can significantly decrease the chondrotoxic effects of ciprofloxacin in a rat mandibular condyle cartilage model.

Key words: antibiotics, mandibular condylar cartilage, Bcl-2, safranin O.

Introduction

Ciprofloxacin (CIP) hydrochloride is a yellow substance which consists of 1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-1-piperazinyl-3-quinolinecarboxylic acid with a molecular weight of 331.4 and a formula of C17H-18FN3O3 [1]. The susceptibility of multi-resistant pathogens to CIP, greater bioavailability and tissue penetration, and higher plasma concentrations were the reasons for its use [2, 3]. It is indicated for the treatment of joint infections [4], gastrointestinal infections [5], anthrax [6], respira-

tory infections [7], and urinary infection [8]. It can interrupt the DNA replication in bacterial cells and prevent cell division [9].

Previously reported cases indicated that CIP can induce chondrotoxicity and tendinopathy [10]. It must not be used as a first line agent in children due to its risk of injury to the joints [11]. It causes necrosis of the chondrocytes, matrix degeneration and cleft formation in the center of articular cartilage [12]. Supplementation with magnesium [13], calcium [14], zinc [15] or vitamin E [16] can diminish the induced chondrotoxicity by preventing DNA oxidation and oxidative stress and inhibiting overexpression of COX-2, which is a key enzyme in chondrocyte apoptosis.

Chondrocytes are specialized cells which synthesize extracellular matrix for condylar cartilage. Several studies have found that Bcl-2 plays a role in controlling apoptosis of cartilage cells [17–19]. Bcl-2 can be activated by proinflammatory cytokines, and its overexpression can suppress apoptosis as a response to different stimuli [20, 21].

(-)-Epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG), and (-)-epigallocatechin gallate (EGCG) are polyphenolic flavonoids found in green tea (GT). The most active component of GT is EGCG [22]. The other constituents present in GT are: minerals (e.g. magnesium, calcium and zinc), vitamins (e.g. vitamin E), proteins, amino acids, carbohydrates and others [23]. GT has shown significant health benefits for various disorders [24]. Previous research has revealed the importance of dietary sources. Dietary therapy, if effective, might be a safer method for the prevention of the chondrotoxic effect of CIP. The prevention of chondrotoxicity by GT extract (GTE) can facilitate the use of CIP in children and during pregnancy and lactation periods.

The objective of the present research was to study the preventive potential of GTE in the mandibular chondrotoxicity induced by CIP in juvenile Wistar albino rats.

Material and methods

The total number of animals used in the study was 20 male Wister Albino rats. They were aged 24 days, weighing 35–45 g. All rats were kept in standard conditions, and maintained on a 12-hour light and dark cycle, at 25 ±6°C, fed with standard rat chow, and allowed to drink water ad libitum. The research project was approved by the USM Institutional Animal Care and Use Committee (USM IACUC).

Experimental design

The 20 male rats were divided into four equal groups (Table I). All rats of the C/W and C/G groups were administered CIP hydrochloride (Bactiflox, Switzerland, 750 mg) as two subcutaneous injections of 600 mg/kg of body weight, 8 h apart [16]. The GTE was prepared by adding 2.5 g of green tea (Alwazah, Sri Lanka) to 50 ml of boiling water in a porcelain tea pot and steeped for 20 min. The extract was cooled to room temperature then filtered. The GTE was prepared daily to prevent degradation of important constituents [25] and used in an oral dose of 300 mg/kg/day [26, 27].

Rats' anesthesia, dissection, tissue processing, and staining

Rats were first anaesthetized with intramuscular injection of xylazine and ketamine. After separation of the mandible, the right and left condylar heads were collected, fixed in 4% neutral buffered formalin with pH 7.4 for 1 day at 4°C, and then decalcified with 10% ethylene diamine tetra acetic acid containing dimethyl sulfoxide (pH 7.2) for 2 weeks, processed, sectioned and stained.

Hematoxylin and eosin stain (H&E)

Two sagittal sections per animal were stained with H&E. The histomorphometric measurements were made in a blinded and nonbiased manner using an objective micrometer (EW10X/20/Japan).

Table I. Experimental design of the study

Group	Description
Group 1 (S/W)	On day 32 of age, all the animals in this group were subcutaneously injected with physiological saline (0.9% NaCl) in the same manner as CIP, and treated by intragastric gavage of distilled water 8 days before the subcutaneous injection of physiological saline and continued for 10 days (day 24 to day 34)
Group 2 (S/G)	On day 32 of age, all the animals in this group were subcutaneously injected with physiological saline (0.9% NaCl) in the same manner as CIP, and treated by intragastric gavage of GTE 8 days before the subcutaneous injection of physiological saline and continued for 10 days (day 24 to day 34)
Group 3 (C/W)	On day 32 of age, all the animals in this group were subcutaneously injected with CIP, and treated by intragastric gavage of distilled water 8 days before the subcutaneous injection of CIP and continued for 10 days (day 24 to day 34)
Group 4 (C/G)	On day 32 of age, all the animals in this group were subcutaneously injected with CIP, and treated by intragastric gavage of GTE 8 days before the subcutaneous injection of CIP and continued for ten days (day 24 to day 34)

The total thickness of the MCC was measured, which is represented by the distance from the external articular surface to the external boundary of the subchondral bone. Three different measurements for the MCC thickness were made, one in the middle of the thickest portion of the middle third, and one 200 µm anterior and one 200 µm posterior to this line. The number of cartilage cells was measured by counting the cells that intersected these three lines [28]. The result of three measurements per section of condyle 100× magnification resulted in a total of 6 measurements per animal, and the mean value was then calculated.

Safranin O stain

The safranin O uptake is a measure of the glycosaminoglycans loss and distribution. The pathologic condition in MCC was assessed using the modified system of Mankin scoring [29, 30]. Background staining intensity of the pericellular matrix: normal staining = 0, slight reduction = 1, severe reduction = 2. Staining intensity of the territorial and interterritorial matrix: normal staining = 0, slight reduction = 1, moderate reduction = 2, severe reduction or no stain = 3. Spatial arrangement of cartilage cells: normal = 0, cluster = 1, hypocellularity = 2. Structure of cartilage: normal and smooth noneroded cartilage surface = 0, irregular surface = 1, vacuoles = 2, blister, cleft, erosion, or separation of calcified from uncalcified cartilage = 3. The maximum score for the degenerative articular cartilage is 10.

Immunohistochemistry for Bcl-2

Sections were deparaffinized by two changes of xylene 10 min each, hydrated in a series of alcohols (100%, 70%, 50%) and then distilled water, three minutes each. Tris-EDTA was used as antigen retrieval solution. Sections were retrieved in Thermo Scientific for 15 min using the PathnSitu Multi-Epitope Retrieval System, allowed to cool, washed in distilled water and then in PBS for 3 min, two changes each, blocked for 10 min with PolyExcel H_2O_2, and washed in PBS. Tissue sections were then incubated with primary antibody for 45 min (PathnSitu PolyExcel Detection System, ready to use, London, a monoclonal rabbit oncoprotein, Isotope Rabbit IgG, Clone EP36, and dilution 1 : 50), washed in PBS, incubated with the PolyExcel Target Binder for 10 min, washed in PBS, followed by a PolyExcelPoly HRP labeled polymer for 10 min of incubation at room temperature, and washed in PBS. The staining procedure was completed by incubation with 3,3′-diaminobenzidine (PolyExcel Stunn DAB) substrate-chromogen for 5 min, which results in a brown color at the antigen site [31]. Tissue sections were then covered with Mayer's hematoxylin solution for 30 s as a nuclear counterstain, dehydrated through graded alcohol, cleared and then coverslipped.

With each batch of stain, a negative control tissue specimen was used. In the middle third of the MCC, which is the thickest portion, the total numbers of immune-positive and immune-negative cells were counted. The positive cells were expressed as the percentage of the total cells in a high-power field 400× in five randomly selected areas per section. The terminal hypertrophic chondrocyte was not quantified because of the extremely low degree of its immunoreactivity [32].

Statistical analysis

Results are given as mean ± standard deviation. Using the ANOVA test, the potential differences among groups were evaluated. Statistical calculations were done using SPSS computer program (Statistical Package for the Social Science; SPSS Inc., version 24). The non-parametric Kruskal-Wallis test was used to analyze the significant differences in histochemical and immunohistochemical data. A p-value less than or equal to 0.05 was considered statistically significant.

Results

Histochemical results

Histological structures of the MCC in the S/W and S/G groups were compose of different layers which can be obviously distinguished (Figure 1). In comparison with the C/G group, all cases of the C/W group were associated with a decrease in the thickness of the MCC, irregularly distributed chondrocytes causing difficulty in differentiating the different cartilaginous layers, and severe reduction in the safranin O stain (Figure 2). Two cases of the C/W group were associated with a cleft in the intermediate zone of the cartilage.

A nonsignificant difference was found between the S/W and S/G groups regarding the MCC thickness, cell numbers, and safranin O stain uptake ($p > 0.05$). The C/W group showed a significant decrease in MCC thickness and cell numbers, with a severe reduction in the safranin O stain in comparison with the other three groups ($p < 0.05$), as seen in Table II.

Immunohistochemical results

In the S/W, S/G, and C/G groups, the Bcl-2 immunoreactivity displayed marked cytoplasmic immune activity of Bcl-2 (Figure 3). Statistical analysis did not show a significant difference between the S/W and S/G groups regarding the percentages of Bcl-2 positive cells ($p > 0.05$), but a significant decrease was seen in the C/W and

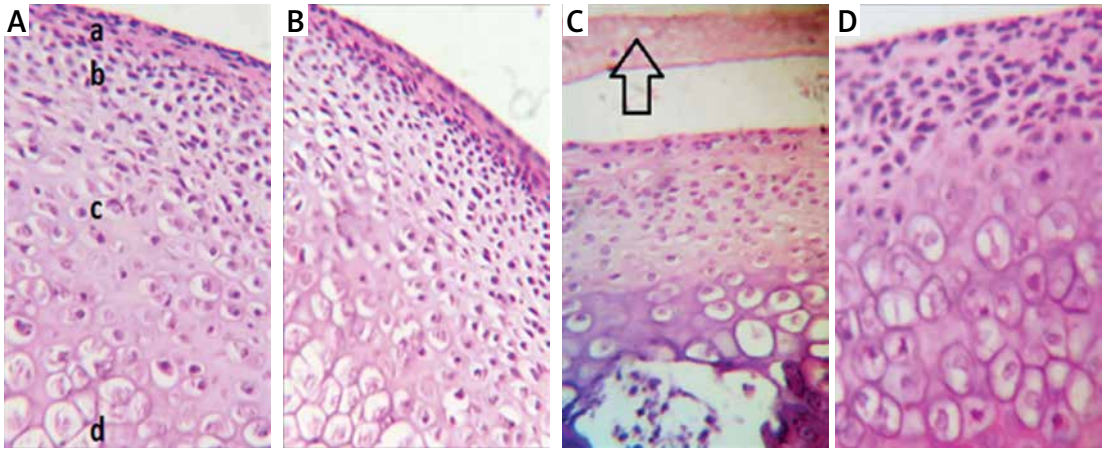

Figure 1. Photomicrograph of the rat's mandibular condylar cartilage section in the S/W (**A**) and S/G (**B**) groups showing the different cell layers: a – articular; b – proliferative; c – mature; and d – hypertrophic cell layers. **C** – Photomicrograph of a section in the middle third of the rat's mandibular condyle cartilage near the thinnest area of the condylar disc (arrow) in the C/W group shows decreased thickness and irregularity of cellular arrangement. **D** – The C/G group shows greater thickness in comparison with the C/W group (H&E 400×)

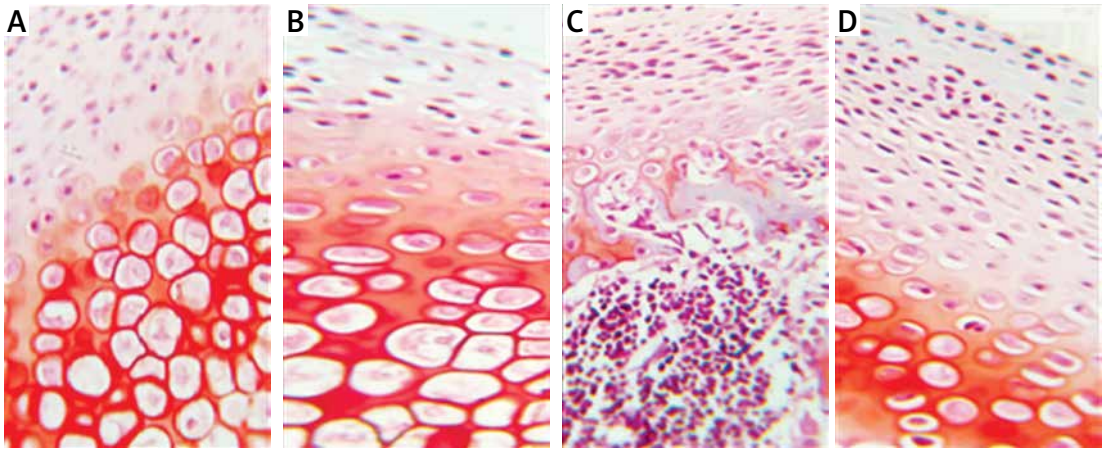

Figure 2. Photomicrographs of sections of rat's mandibular condylar cartilages. In the S/W (**A**) and S/G (**B**) groups, the safranin O staining of the intercellular matrix appears normal. **C** – The C/W group shows a severe reduction in the staining. **D** – A moderate reduction is seen in the mandibular condylar cartilage of the C/G group (safranin O 400×)

C/G groups in relation with them. The C/G group showed significantly higher Bcl-2 immuno-expression in comparison with the C/W group ($p < 0.05$), as seen in Table II.

Discussion

The present study showed that the MCC layer mean thickness and number of cartilage cells in the S/W group were 373 ±0.160 µm and 20.8 ±2.039, respectively. Oksayan *et al.* [33] found that the anterior and posterior total thickness of MCC were 342.198 ±241.77 and 314.69 ±194.15 µm, respectively. Cavalli *et al.* [34] found that the mean number of cartilage cells in MCC in 60-day old adult rats was 416.4 ±79.9. The differences in the results may be due to the method used for measurement. Glycosaminoglycans are polysaccharides that can play an important role in determining the biomechanical properties of the MCC [35]. In the S/W group, the Mankin score for glycosaminoglycan distribution was 0.4 ±0.489. This result is in agreement with that of the study by Yuan *et al.* [36].

Statistical analysis showed no significant difference between the S/W and the S/G groups regarding the MCC layer mean thickness, number of cartilage cells, and glycosaminoglycan score. The Huang *et al.* [37] study also found a non-toxic effect of catechins in chondrocyte proliferation and later on condylar cartilage matrix components.

The present study showed that the MCC layer mean thickness, cell number, and the glycosaminoglycan score in the C/W group were significantly lower than in the other groups. In addition, the study revealed two cases with cleft formation within the intermediate zone. Yabe *et al.* [38] found that vesicles were observed on the articular surfaces of all juvenile dogs after two doses of ofloxacin. Halawa's [10] study also found that the

Table II. Histochemical and immunohistochemical analysis for rats' mandibular condylar cartilage in different groups of the study

Group	Thickness [µm]	*P*-value	Number of cartilage cells	*P*-value	Mankin score	*P*-value	Bcl-2%	*P*-value
S/W S/G	373 ±0.160 356 ±10.198	0.143	20.8 ±2.039 18.4 ±1.496	0.094	0.4 ±0.489 0.6 ±0.489	0.601	74.166 ±5.367 71.5 ±2.432	0.3366
S/W C/W	373 ±0.160 162 ±7.483	0.009*	20.8 ±2.039 10.4 ±2.059	0.009*	0.4 ±0.489 6.166 ±1.067	0.006*	74.166 ±5.367 8.166 ±2.544	0.0039*
S/W C/G	373 ±0.160 210 ±14.142	0.009*	20.8 ±2.039 15.8 ±1.166	0.009*	0.4 ±0.489 2.2 ±0.748	0.016*	74.166 ±5.367 45.333 ±5.185	0.0039*
S/G C/W	356 ±10.198 162 ±7.483	0.009*	18.4 ±1.496 10.4 ±2.059	0.009*	0.6 ±0.489 6.166 ±1.067	0.006*	71.5 ±2.432% 8.166 ±2.544	0.0039*
S/G C/G	356 ±10.198 210 ±14.142	0.009*	18.4 ±1.496 15.8 ±1.166	0.028*	0.6 ±0.489 2.2 ±0.748	0.021*	71.5 ±2.432% 45.333 ±5.185	0.0039*
C/W C/G	162 ±7.483 210 ±14.142	0.009*	10.4 ±2.059 15.8 ±1.166	0.012*	6.166 ±1.067 2.2 ±0.748	0.006*	8.166 ±2.544 45.333 ±5.185	0.0039*

**Significant p < 0.05.*

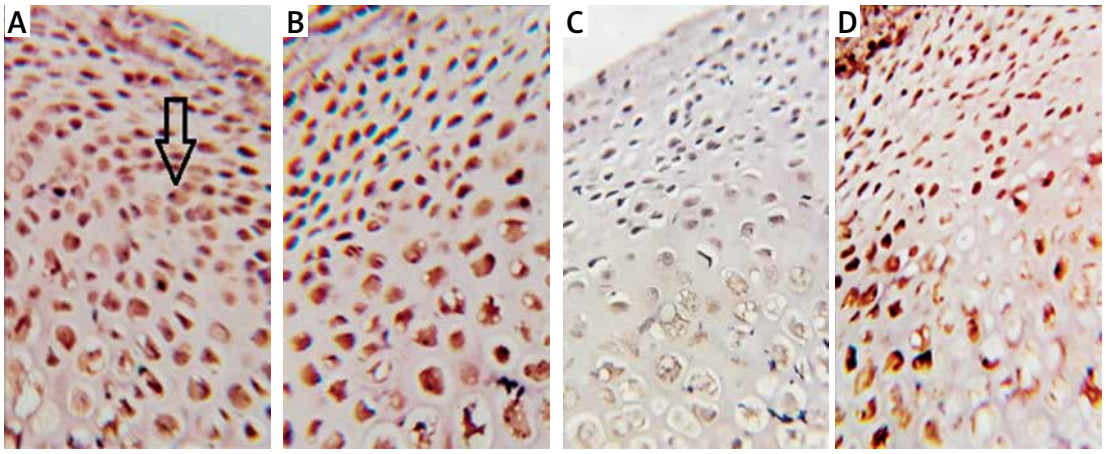

Figure 3. Photomicrographs of sections of rat's mandibular condylar cartilages, immune stained with Bcl-2, in S/W (**A**), S/G (**B**), C/W (**C**) and C/G (**D**) groups. The positive cells show brown staining in the cytoplasm, arrow (immunohistochemistry 400×)

CIP treated animals showed a significant decrease in the condylar cartilage thickness, the number of chondrocytes, and the glycosaminoglycan content of the femoral articular matrix with areas of decreased staining affinity. The articular cartilage damage in young animals can be caused by DNA oxidative damage of the condylar cartilage chondrocytes and collagen with loss of the integrity of extracellular proteins [39]. The formation of CIP-chelate complexes can induce a deficiency of the functionally available Mg, which resulted in articular cartilage damage [40].

The present study showed that the MCC layer mean thickness, number of cells, and the glycosaminoglycan score in the C/G group were significantly higher than in the C/W group. The GT polyphenols can increase levels of serum glutathione peroxidase, reduce serum malondialdehyde, increase the anti-oxidation ability and decrease the damage to tissues [41]. In human articular chondrocytes, EGCG can also enhance aggrecan core protein synthesis [42]. Aggrecan is a large aggregating proteoglycan and its degradation can be inhibited by consumption of GTE or EGCG, which can selectively inhibit the activities of aggrecanases [43]. It was also found that the production of MMP-1 and MMP-13 was inhibited in human chondrocytes which were pre-treated with EGCG [44]. The GT polyphenols can also inhibit the gelatinolytic activity of MMP-2 [45].

Guay's study [46] revealed that multivalent cations such as Mg, Ca, Zn and other minerals can interact with fluoroquinolone and substantially reduce its bioavailability, leading to subtherapeutic drug concentrations at the infection site. Minerals present in GT may be responsible for this action. GT also contains various vitamins such as vitamins E and C which are associated with different activities including antioxidant, anti-apoptosis [47, 48], and radical scavenging antioxidant activity [49]. It was found that 100 lM of vitamin E or 10 mM of ascorbic acid can inhibit the generation of CIP-induced hydrogen peroxide and significantly inhibit CIP cytotoxicity due to the possible antagonistic

properties of vitamins E and C when they are used with CIP [50]. These constituents of GTE may have anti-chondrotoxic effects against CIP activity.

In the present study, a significant decrease in Bcl-2 immuno-expression as an antiapoptotic marker in MCC of C/W group was observed. Condylar cartilage damage in growing animals was mainly due to DNA oxidative damage of the chondrocytes [51], deficiency of functionally available Mg [52], inhibition of mitochondrial dehydrogenase [53], and altered metabolism of DNA [54]. The beneficial effect of GTE by the significant increase in the Bcl-2 immune expression in the C/G group in comparison with the C/W group can be attributed to the high content of catechins. Studies by Schroeder *et al.* [55] and Cong *et al.* [56] revealed that GT can restore the function of Bcl-2. In contrast to these studies, Singh *et al.* [57] reported that EGCG could effectively lower pro-apoptotic gene activities with no effect on the anti-apoptotic genes. The differences in the results may be due to the different types and concentrations of catechins or GTE used in these studies. In conclusion, this study provides the first evidence that GT extract can significantly decrease the chondrotoxic effects of CIP in a rat model by increasing the MCC thickness, cartilage cell number, glycosaminoglycan distribution, and Bcl-2 immune expression.

Conflict of interest

The authors declare no conflict of interest.

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