Effect of Remote Ischemic Preconditioning on Systemic Toxicity and Ototoxicity Induced by Cisplatin in Rats: Role of TNF-α and Nitric Oxide

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Abstract
Background/Aims: Cisplatin is a chemotherapeutic agent. The use of remote ischemic preconditioning (RIPC) was proposed after the observation that ischemic preconditioning of a cardiac vascular area could protect another completely distinctly. Methods: This is an experimental study. Male Wistar rats were anesthetized, and they underwent a hearing evaluation via measurement of the brainstem auditory evoked potential (BSAEP). Then, cisplatin was administered intraperitoneally (IP) at a dose of 8 mg/kg/day for 4 consecutive days to group 1, whereas saline solution was administered IP to group 2. In groups 3 and 4, ischemia of the right hind paw was performed for 10 min, followed by reperfusion for 30 min, after which cisplatin or saline was administered IP to group 3 or group 4, respectively. Afterwards, all animals were evaluated via the BSAEP. The right cochlea was dissected for immunohistochemistry. Results: RIPC lowered the increase in BSAEP of the animals treated with cisplatin ($p = 0.0146$). Weight loss decreased in the animals subjected to RIPC ($p < 0.005$). In group 3, RIPC reversed immunostaining for tumor necrosis factor-α and inducible nitric oxide synthase in the stria vascularis injured by cisplatin ($p < 0.05$). Conclusion: RIPC protects against systemic toxicity and ototoxicity induced by cisplatin in rats.
Introduction

Cisplatin (cis-diamminedichloroplatinum, CDDP) is a chemotherapeutic agent commonly used to treat a variety of cancers, including those of the head and neck, lung, bladder, ovary, tests, and gastrointestinal system [1]. Its side effects include ototoxicity, nephrotoxicity, neurotoxicity, bone marrow suppression, and gastrointestinal disorders. This kind of toxicity can interfere with drug treatment in many patients by necessitating decreases in the dosage, frequency, and duration of chemotherapy [2]. There is no cure or preventive treatment of ototoxicity in these patients [3]. This effect was first described by Rossof and has been widely studied since then [4]. It is estimated that between 11 and 97% of treated patients present with hearing loss [5]. The risk factors include the use of cisplatin combined with other ototoxic drugs such as aminoglycosides and furosemide, dosage and cumulative dosage, craniofacial irradiation, advanced age, and male gender [6–8].

The molecular mechanism of action of cisplatin involves dependent and independent DNA damage pathways. The independent pathways are important in cochlear injury [9] and occur via formation of reactive oxygen species (ROS), depletion of the glutathione system, increase in lipid peroxidation, oxidative modification of proteins, and nitrosylation of cochlear proteins [10]. The cytokines involved in the formation of lesions induced by cisplatin are proinflammatory and include tumor necrosis factor-α (TNF-α) [11], interleukin (IL)-1β, and IL-6 [12]. An increase in the levels of inducible nitric oxide synthase (iNOS) and nuclear factor-κB has also been observed [13, 14]. Some compounds protect against the ototoxicity induced by cisplatin, including melatonin [15], vitamin E [16], N-acetylcysteine [17], allopurinol-ebselen [18], D-methionine [19], Ginkgo biloba [20, 21], trolox [22], metformin [3], silymarin [23], and amifostine [24].

Ischemic preconditioning (IPC) was first described in 1986. The concept of IPC has been demonstrated in the myocardium of dogs, with a reduction of 25% in the infarcted area in the IPC group after sustained ischemia for 40 min and reevaluation of the condition on the fourth day [25]. IPC is a protection mechanism against ischemia-reperfusion injury (IRI). The pathophysiology of IRI involves the formation of ROS [26] during short periods of ischemia, followed by reperfusion and a prolonged ischemic insult [27]. A reduction in liver damage was observed in an IPC model of rabbits subjected to IRI [28]. An analysis of the blood parameters in rats indicated an anti-inflammatory, protective effect of IPC in the intestinal response to IRI [29]. The pathophysiology of IPC remains unclear but appears to be associated with the modulation of the organic response to the insult from IRI [30]. IPC is characterized by early intermediate protection, later protection observed at up to 3 h, delayed protection observed between 18 and 24 h, and a remote effect, that is, remote ischemic preconditioning (RIPC). RIPC is characterized by the protection (at the same intensity) of organs or tissues distinct from those directly subjected to IPC [31, 32]. RIPC was first proposed in 1993, after the observation that IPC of a cardiac vascular area could protect a completely distinct area [27]. RIPC techniques that involve ischemia and reperfusion of anterior and posterior members in animals are widely used owing to their easy application and low cost [33]. A study on skeletal muscle revealed that the early stage of RIPC is the most effective for protecting against IRI [34].

A study [35] was carried out in rats for the evaluation of IPC and RIPC for protection against right paw edema induced by carrageenan or dextran, with reduction of edema with IPC in the ipsilateral paw, and also in another group with RIPC in the left paw protecting the contralateral paw. Dextran edema occurs by degranulation of mast cells with a low protein concentration in the fluid and few neutrophils. The mechanism of action of carrageenan consists of an exudate with a high protein concentration and many neutrophils. Therefore, IPC and RIPC act both in polymorphonuclear and in mast cell-dependent edema. This action is related to a reduction in chemotactic TNF-α and IL-1. Also in this study, there was an eval-
The objective of the present study was to evaluate the effect of RIPC on systemic toxicity and ototoxicity induced by cisplatin in rats.

**Materials and Methods**

All the experiments were approved by the Ethics Committee for Animal Research of the Federal University of Ceará under protocol No. 101/2014.

For this study, we used male Wistar rats weighing between 200 and 275 g, maintained in cages with free access to food and water, subjected to natural cycles of sleep and wakefulness, and reared according to the standards established by the Brazilian College of Animal Experimentation (Colégio Brasileiro de Experimentação Animal – COBEA) (available at www.cobea.org.br). Animals with signs of external or middle ear disease on otoscopy and those with an electrophysiological threshold higher than 20 dB HL before drug administration, as assessed by evaluation of the brainstem auditory evoked potential (BSAEP), were excluded.

The rats were divided into four groups: group 1, CDDP (*n* = 8) – rats treated with cisplatin at a single daily dose of 8 mg/kg/day, corresponding to a total dose of 32 mg/kg divided into 4 doses; group 2, control (C) (*n* = 8) – rats treated with saline at a daily dose of 8 mL/kg/day for 4 days; group 3, RIPC + CDDP (*n* = 9) – rats subjected to RIPC in the right hind paw on the first and second days and treated with cisplatin at a single daily dose of 8 mg/kg/day, corresponding to a total dose of 32 mg/kg divided into 4 doses; and group 4, RIPC + C (*n* = 7) – rats subjected to RIPC in the right hind paw on the first and second days and then treated with saline solution at a single daily dose of 8 mL/kg/day for 4 days. All animals were evaluated before treatment (D0) and 4 days after treatment (D4) via evaluation of the BSAEP and were evaluated before treatment (D0) and 4 days after treatment (D4) via evaluation of percent weight variation. At last, after euthanasia, all right cochleae were removed for immunohistochemistry for iNOS and TNF-α (Fig. 1).
The rats were subjected to deep anesthesia with ketamine at 80 mg/kg and xylazine at 10 mg/kg on the first, second, and last day. An otoscopy was performed before treatment, and the animals with external and middle ear changes were excluded from the study (exclusion criteria). Those with normal otoscopy results were subjected to a hearing evaluation via measurement of the BSAEP immediately before drug administration. Subsequently, cisplatin and saline were administered intraperitoneally (IP) to group 1 and group 2, respectively. In groups 3 and 4, right hind paw ischemia was performed using an elastic band, followed by intravenous injection of Evans blue at a dose of 2.5 mg/kg into the dorsal penile vein and confirmation of ischemia by the absence of blue staining in the ischemized paw. After 10 min, the elastic band was loosened, and reperfusion was performed for 30 min and confirmed by the presence of Evans blue. Subsequently, cisplatin and saline were administered IP to group 3 and group 4, respectively. The same procedure was conducted on the first and second days (D0 and D1); however, only the IP administrations were performed on the third and fourth days (D2 and D3), i.e., ischemic induction and reperfusion was performed before IP application of cisplatin on the first 2 days, followed by the absence of ischemia-reperfusion on the last 2 days. At D4, all animals were subjected to hearing assessment via the BSAEP. Immediately after the last hearing evaluation, euthanasia was performed by decapitation using a self-manufactured guillotine, the right temporal bone was removed, and the cochlea was dissected for immunohistochemistry. After the experiment, the rat carcasses were incinerated.

For evaluation of the BSAEP, an ICS CHARTR EP 200 (Otometrics, Taastrup, Denmark) was used in a quiet environment. After the animals were anesthetized, subdermal platinum electrodes were placed on the vertex (positive electrode), in the right retroauricular region (negative electrode), and on the right foreleg (ground). ER-3A earphones coupled to a probe used for hearing assessment of infants were introduced into the right external auditory canal of the rats. The stimuli used were rarefaction clicks, released at a rate of 15 clicks per second, with a maximum of 1,000 promediations in a 15-ms evaluation period. The bandwidth used was 0–2,000 Hz. Auditory stimuli were started at 80 dB HL and progressively decreased until they disappeared completely. To determine the electrophysiological threshold, the lowest stimulus intensity with an evident wave II was considered.

Immunohistochemistry for iNOS and TNF-α was performed using streptavidin-biotin-peroxidase [36]. On D5, the animals were sacrificed, and their cochleae were removed and fixed in 10% formalin for 24 h. These samples were subjected to demineralization in 10% EDTA for 14 days, washed, processed, and embedded in paraffin. After this procedure, 4-μm-thick serial sections were prepared using an appropriate microtome and placed on 1-lysine-coated slides used specifically for immunohistochemistry. For antigen recovery, the sections were deparaffinized and hydrated in xylene and ethanol and immersed in 0.1 M citrate buffer (pH 6.0) under heating in a water bath at 65 °C for 20 min. After cooling at room temperature for 20 min, the samples were washed in phosphate-buffered saline (PBS) solution, and endogenous peroxidase was blocked by incubation in a 3% H2O2 solution for 20 min. The sections were incubated for 2 h at 4 °C with anti-iNOS rabbit primary antibody diluted 1:100 and with anti-TNF-α primary rabbit polyclonal antibody diluted 1:200 in antibody diluent (DAKO). After washing in PBS, the sections were incubated with the HRP polymer (DAKO) for 30 min. After another washing step in PBS, the sections were stained with the chromogen 3,3′-diaminobenzidine peroxide and counterstained with Mayer's hematoxylin. The samples were then dehydrated and mounted on slides. Negative controls were processed simultaneously as described above, wherein the primary antibody was replaced with antibody diluent. All images were acquired using a Nikon microscope at a magnification of x400 coupled to a camera and using Motic image software version 2.0. Immunohistochemistry was quantified using the open-source processing software ImageJ.

For graph preparation and statistical analysis, GraphPad Prism version 5.00.288 was used. The normality of the data was evaluated using the Kolmogorov-Smirnov test. The results are expressed as the mean ± standard error of the mean (SEM). The minimum significance accepted was 5%. The various experimental procedures were compared using the following tests: (1) analysis of variance (ANOVA), wherein the significance between the groups was determined using Tukey's test to compare the variation in weight of the animals of each group; (2) the log-rank test, used for pairwise comparisons of the survival curves between the study groups; (3) ANOVA, wherein the significance between the groups was determined using the Bonferroni test to compare the mean electrophysiological threshold values of the study groups via measurement of the BSAEP between the first (D0) and the fifth (D4) day of evaluation; and (4) ANOVA, wherein the significance between the groups was determined using Bonferroni's test to compare, using ImageJ, the mean percentages of the areas immunohistochemically stained.
Results

Changes in the Middle or External Ear

One animal in group 3 (RIPC + CDDP) presented middle ear alterations, and 1 animal in group 4 (RIPC + C) presented a change in the external ear upon otoscopy during the experiment. These animals were eliminated from the study.

Systemic Toxicity of Cisplatin

Weight Curve

The Kolmogorov-Smirnov test was used to evaluate data distribution, and it indicated that the data followed a normal distribution \((p > 0.10\) in groups C, CDDP, and RIPC + CDDP; \(p = 0.595\) in group RIPC + C). There was a significant difference in weight variation between the groups (weight at D4 – weight at D0), with \(p < 0.05\) according to one-way ANOVA and Tukey’s post hoc test. The weight decreased in all groups except in group RIPC + C, which presented a weight gain (Fig. 2).

Fig. 2. Weight variation in the study groups as the difference between the final weight (on day 4) and the initial weight (on day 0). The weight increased significantly in group RIPC + C according to one-way ANOVA (Tukey’s test). Asterisks represent significant differences between the groups: * \(p < 0.05\), ** \(p < 0.05\), *** \(p < 0.05\), **** \(p < 0.05\). C, control; CDDP, cisplatin; RIPC + CDDP, remote ischemic preconditioning + cisplatin; RIPC + C, remote ischemic preconditioning + control.

Fig. 3. Mean threshold values in groups 1, 2, 3, and 4. * Significant difference between D0 and D4 in the CDDP group according to one-way ANOVA (Bonferroni’s test). C, control; CDDP, cisplatin; RIPC + CDDP, remote ischemic preconditioning + cisplatin; RIPC + C, remote ischemic preconditioning + control; BSAEP, brainstem auditory evoked potential.
Survival Curve

The survival curve indicated the loss of 1 animal in group RIP C + CDDP. There was no significant difference in survival between the groups according to the log-rank test.

Evaluation of Functional Hearing

The Kolmogorov-Smirnov test was used to evaluate the distribution of the hearing threshold data obtained by the BSAEP and indicated that the data did not follow a normal distribution ($p = 0.0002$). The Bonferroni test was then applied, and the evaluation of the BSAEP indicated a significant difference between the beginning (D0) and the end of the experiment (D4) only in the cisplatin group ($p = 0.0164$). No significant difference was observed between the remaining groups ($p > 0.05$) (Fig. 3).

Immunohistochemistry of TNF-α and iNOS

The immunohistochemical analysis indicated that RIPC significantly reversed immunostaining for TNF-α in the stria vascularis ($p < 0.05$) according to Bonferroni’s test (Fig. 4, 5). Immunostaining for iNOS was also reversed in group RIPC + CDDP compared with group CDDP ($p < 0.05$) according to Bonferroni’s test (Fig. 6, 7). The control group showed no significant change ($p > 0.05$).

Discussion

Cisplatin is a chemotherapeutic agent widely used in current medical practice. It is used in many antineoplastic protocols, including those for malignant neoplasms of the head and neck, lung, bladder, ovary, testis, and gastrointestinal system [1]. Some of its side effects, including ototoxicity and neurotoxicity, are irreversible. An ideal otoprotective agent for preventing ototoxic lesions caused by cisplatin would have the following characteristics: it would be nontoxic, i.e., have few side effects; it would reach sufficiently high concentrations in the inner ear; and it would not interfere with the systemic action of CDDP [2].

A literature review indicated that protection against the ototoxicity caused by cisplatin using RIPC has not yet been studied. IPC is a mechanism of protection against injuries caused by ischemia-reperfusion with experimental and clinical applicability. Therefore, a study of the protection against cisplatin-induced injury by IPC seemed to be feasible.
Fig. 5. Photomicrographs of the immunostaining for TNF-α in the stria vascularis in the study groups. ×400. Scale bar division, 1 μm. 

- **a** Group C with discrete TNF-α immunostaining.
- **b** Group CDDP with increased TNF-α immunostaining.
- **c** Group RIPC + C with discrete TNF-α immunostaining.
- **d** Group RIPC + CDDP with discrete (reversed) TNF-α immunostaining.

C, control; CDDP, cisplatin; RIPC + CDDP, remote ischemic preconditioning + cisplatin; RIPC + C, remote ischemic preconditioning + control; TNF-α, tumor necrosis factor-α.

Fig. 6. Percent area immunostained for iNOS compared with the total area. iNOS immunostaining was reversed by RIPC. *p < 0.05 for the comparison between the CDDP and RIPC + CDDP groups according to Bonferroni’s test. C, control; CDDP, cisplatin; RIPC + CDDP, remote ischemic preconditioning + cisplatin; RIPC + C, remote ischemic preconditioning + control; iNOS, inducible nitric oxide synthase.
RIPC is a model of protection against IRI used successfully in various organs [35, 37–39]. Understanding the role of mediators in the mechanism of RIPC is the aim of various research groups. Inflammatory mediators such as TNF-α, iNOS, and IL-1 are involved in the action of RIPC [35] and in the lesions induced by cisplatin [11, 12]. One way RIPC protects against IRI-induced lesions is by reducing or eliminating oxidative stress in various organs, including the liver, kidney, lung, skeletal muscle, and heart.

The analysis of the systemic toxicity of cisplatin via measurement of the weight change in the study groups revealed two important results: (1) a significant reduction in weight loss in the RIPC + CDDP group compared to the CDDP group and (2) a significant increase in weight in the RIPC + C group compared with the other groups. A plausible explanation for this finding is the anti-inflammatory effect – as demonstrated in other studies involving RIPC [30, 40] – which would provide a better response to stress, as shown in the RIPC + C group. The significant weight loss in the C group may be explained by the stress of the animals during the experiment, reinforcing the observation of weight gain in the preconditioning control group (RIPC + C). The significant reduction in weight loss during exposure to cisplatin observed in the group subjected to RIPC may represent a possible effect of RIPC on the systemic (antineo-

Fig. 7. Photomicrographs of iNOS immunostaining in the stria vascularis in the study groups. ×400. Scale bar division, 1 μm. a Group C with discrete iNOS immunostaining. b Group CDDP with increased iNOS immunostaining. c Group RIPC + C with discrete iNOS immunostaining. d Group RIPC + CDDP with discrete (reversed) iNOS immunostaining. C, control; CDDP, cisplatin; RIPC + CDDP, remote ischemic preconditioning + cisplatin; RIPC + C, remote ischemic preconditioning + control; iNOS, inducible nitric oxide synthase.
plastic) action of cisplatin. However, further studies on the systemic action of cisplatin should be performed to elucidate the exact role of preconditioning in this action.

Other studies that evaluated the ototoxicity of cisplatin indicate that some protective agents interfere with both its systemic action and ototoxicity. Two of these agents, the antioxidants sodium thiosulfate and 4-methyl-thiobenzoic acid, are applied systemically [41]. Other agents interfere only with the ototoxicity of cisplatin, including *Ginkgo biloba* [42], ebselen (a synthetic compound with an activity similar to that of glutathione peroxidase) [43], and amifostine [24]. Other protective compounds, whose antineoplastic effects have not been evaluated, include metformin [3] and silymarin, a lipophilic extract derived from the plant *Silybum marianum* [23]. A suggestion made by some studies is the application of protective agents such as b-methionine and N-acetylcysteine directly to the eardrum or via the round window to abolish the interference with the systemic toxicity induced by cisplatin [13].

We investigated the otoprotective mechanism of RIPC by measuring the BSAEP. Cisplatin damages the cochlea by activating pathways that are dependent and independent of DNA damage [9]. The cytokines involved in the lesion induced by cisplatin, such as TNF-α, IL-1, and iNOS, are affected by preconditioning, indicating a possible mode of action of RIPC. The decreased generation of ROS by RIPC can explain the decrease in cisplatin-induced cell damage in the DNA damage-independent pathway, and the main mechanism of action may be a decrease in oxidative stress [10]. We evaluated the BSAEP because this evaluation is easy to perform and has less variability and greater reproducibility than assessment of distortion product otoacoustic emissions [44], though many studies still use the latter method [45]. The protection of the BSAEP thresholds by RIPC observed in almost all animals indicates a significant reduction in ototoxicity, and this was supported by the decrease in weight loss. These findings suggest an effect of RIPC on the systemic and ototoxic action of cisplatin.

The effect of RIPC on the ototoxicity induced by cisplatin in rats is based on the interrelation of the mechanisms involved in the modulation of cytokines. The proinflammatory cytokines associated with the lesions caused by cisplatin include TNF-α [11] and IL-1β [12]. The protection against the ototoxic effects of cisplatin by RIPC in mice is related to the modulation of these cytokines, as shown in other organs [35]. Moreover, the production of ROS is essential in cisplatin-induced lesions [10] and is most likely decreased by RIPC, as demonstrated in other organs [46]. The effect of RIPC on cisplatin-induced ototoxicity in rats also depends on the modulation of other cytokines, such as iNOS. Nitric oxide chemically reacts with ROS to form peroxynitrite, which is a highly reactive and toxic compound to cells [47]. Therefore, the decrease in oxidative stress promoted by RIPC in cisplatin-induced lesions may occur via a decrease in the levels of iNOS. RIPC in the hind paw of Wistar rats significantly protects against the increase in electrophysiological thresholds and the weight loss induced by cisplatin. The mechanism of otoprotection is associated with modulation of TNF-α and iNOS.

**Disclosure Statement**

The authors have no conflicts of interest to declare.

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Author Contributions

M.J.B.M. performed all the experiments, analyzed the data, and wrote the manuscript. A.M.A.B. and Y.N.F.B. performed the experiments with the animals. P.M.G.S. and C.d.S.M. performed the immunohistochemistry experiments. R.d.A.R. and G.A.d.C.B. designed the study and analyzed the data. M.R.d.F. designed the study, analyzed the data, and wrote the manuscript.

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