Can daily consumption of a nephroprotective homeopathic product protect the kidneys from gentamicin toxicity?

O consumo diário de um produto homeopático nefroprotetor pode proteger os rins da toxicidade da gentamicina?

¿Puede el consumo diario de un producto homeopático nefroprotector proteger los riñones de la toxicidad de la gentamicina?

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Abstract
The objective of this work was to determine whether preventive consumption of a homeopathic product via drinking water would protect mouse kidneys from a challenge with the nephrotoxic antibiotic gentamicin. We used 40 Swiss mice divided into four groups with ten animals each. The homeopathic product was supplied through water for 30 days in a preventive manner and gentamicin for 10 days to induce an experimental renal failure. The groups were as follows: Negative-CT (homeopathic and gentamicin was not provided), Positive-CT (did not receive homeopathic, but received gentamicin 40 mg/kg), T2 (received 0.002 ml of the product per animal/day, and received gentamicin 40 mg/kg), and T4 (0.004 ml of the product per animal/day and received gentamicin 40 mg/kg). On days 12 and 20, blood and tissue samples were collected from five animals in each group. No histopathological lesions were found in mouse kidneys. However, levels of thiobarbituric acid reactive substances, reactive oxygen species, and nitrite/nitrate ratios in the kidney of the Positive-CT group were higher compared to the other groups. As for glutathione S-transferase, on the 20th day, the groups that used the homeopathic product (T2 and T4) had higher activities than the positive TC. Therefore, the results suggest that prophylactic consumption of the hepatoprotective homeopathic product can decrease lipid
peroxidation, nitrous stress, and oxidative stress at the renal level when consecutive doses of gentamicin induce insufficiency.

**Keywords**: Gentamicin; Homeopathic; Renal failure.

### 1. Introduction

Renal failure is characterized by the loss of the kidneys' ability to perform functions such as filtering toxic substances from the blood. Renal failure leads to gradual nephron loss and ultimate disruption of electrolyte balance (Rufato et al., 2011). This disease affects pet animals regardless of their age or developmental stage (Rufato et al., 2011). Oliveira et al. (2001) reported that one of the most common causes of nephrotoxicity is the continued use of some drugs, especially aminoglycosides such as gentamicin.

Oxidative stress occurs when the antioxidant and oxidant systems are out of balance, this generates an increase in the production of free radicals, such as reactive oxygen species (ROS), thioarbituric acid reactive substances (TBARS) (Gutteridge and Halliwell 2018). In this way, the antioxidant system reduces excessive levels of free radicals (Birben et al., 2012). Antioxidants can be classified as enzymatic (superoxide dismutase [SOD], catalase [CAT], glutathione peroxidase [GPx], glutathione reductase [GR], glutathione S-transferase [GST], and glucose-6-phosphate dehydrogenase [G6PD]) and non-enzymatic (gluthathione, vitamins E and C) (Jyothilakshmi et al., 2014). Renal failure causes oxidative stress, affecting pet health and performance (Jyothilakshmi et al., 2014).

Homeopathy is an alternative therapy characterized by medicines produced with natural substances, from plants, animals, or minerals, and less aggressive to the body, as they do not accumulate in the body (Faria et al., 2014). Homeopathic
drugs have low concentrations, which stimulates defense reactions and decreases the risk of toxicity, unlike allopathic drugs (Faria et al., 2016). Among the constituents of the homeopathic Berberis vulgaris, which is a shrub from South America, stands out, and the bark of its root has been used in research with albino rats to treat diseases of kidney stones and urolithiasis (Jyothilakshmi et al., 2014). Cantharis vesicatoria is obtained from a beetle, which affects the urinary tract, especially in situations of frequent urination and pain when urinating (De Paula Coelho et al., 2017). Causticum is a mineral used to reduce inflammation in mice induced in infection by Trypanosoma cruzi (Lopes et al., 2016). However, studies using homeopathic in experimental conditions and showing their behavior in the animal organism are rare. Therefore, this study aimed to evaluate whether the consumption of the homeopathic product in a preventive way in mice, via drinking water, has a protective effect on the kidneys against the challenge with nephrotoxic antimicrobial when used for a prolonged time (gentamicin).

2. Metodology

This research had an exploratory and quantitative nature (Pereira et al., 2018). The project was approved by the Ethics Committee for Animal Well-Being at the State University of Santa Catarina-UDESC, under protocol n° 5860021218.

2.1 Acquisition of the homeopathic product

In the present study, the commercial product Orgarim® (OrgaPet Line, Organic Homeopathy Veterinary, Chapecó, SC, Brazil) was evaluated, indicated for preventing and treating problems related to the urinary tract. The drug was formulated based on Berberis vulgaris (14 CH), Cantharis vesicatoria (14 CH), Causticum (30 CH), qsp vehicle (30 ml).

2.2 Gentamicin

Gentamicin was used as a vehicle to cause renal failure in animals, using GARAMYCIN, Schering-Plow, USA. This was administered via intraperitoneal injections, at a dose of 40 mg/kg of body weight, in which it followed the methodology and dose proposed by Duarte et al. (2016).

2.3 Animals and experimental design

Forty female mice (swiss) were used, divided into four groups of ten animals in each. They were housed in specific cages for the species, measuring 41 x 34 x 17.8 cm, kept in a controlled environment between 22 to 24 °C. The animals received food and water ad libitum. The water was changed every two days, following the methodology described by Jaguezesk et al. (2020). The homeopathic product was supplied through water, and the dosage was measured using an automatic pipette, with the following dosages: The Negative-CT group (homeopathic and gentamicin was not supplied), Positive-CT (did not receive the homeopathic, but received gentamicin, 40 mg/kg) T2 (received 0.002 ml of homeopathic product per animal/day and received gentamicin, 40 mg/kg) and T4 (0.004 ml of homeopathic product per animal/day and received gentamicin, 40 mg/kg). The product was provided for 30 days in a preventive manner. After that period, the animals were medicated for ten consecutive days with the antibiotic gentamicin; the protocol was carried out to induce renal failure in mice. In this way, it was possible to assess the homeopathic nephroprotective effect, as stated in the product's technical sheet.

2.4 Sample collection

On days 12 and 20, five mice per group were anesthetized with isoflurane in an anesthetic chamber. Subsequently, blood was collected by heart puncture and placed in tubes without anticoagulant. The serum was obtained by centrifuging the blood at 3500 rpm for 10 min. Kidney fragments were used for histological and oxidative analysis. The samples were frozen at –20 °C for subsequent biochemical analysis and oxidative and antioxidant status.
2.5 Serum biochemistry

In the biochemical analyses, the total proteins, albumin, urea, and creatinine levels were evaluated. For this purpose, a commercial kit (ANALISA®) was used, and the reading was performed by the equipment (BioPlus 2000®). Globulin levels were calculated using the mathematical formula: globulin = total protein - albumin.

2.6 Oxidizing and antioxidant status in the kidneys

The collected kidney was homogenized in Tris-HCL solution (1 y 9 v). Then it was centrifuged at 2800 g for ten minutes; the supernatants were collected and frozen at -20 °, for further analysis of oxidant and antioxidant status.

2.6.1 Measurement of reactive oxygen species

The production of ROS (especially peroxides) was determined according to Halliwell and Gutteridge (2007). This experimental method of analysis is based on the deacetylation of the 2,7-dichlorofluorescein diacetate probe (DCFH-DA) by cellular esterases to form 2,7-dichlorodihydrofluorescein (DCFH) and its subsequent oxidation by ROS to 2',7'-dichlorofluorescein (DCF), which is highly fluorescent, with excitation and wavelength emission of 498 and 522 nm, respectively. The results were expressed as U DCF/mg protein.

2.6.2 Determination of thiobarbituric acid reactive substance levels

Lipid peroxidation was estimated in samples measuring the formation of malondialdehyde (MDA). The MDA, when heated, reacts with thiobarbituric acid to form a pink complex. Serum samples were analyzed according to Jentzsch et al. (1996) were incubated in a water bath at 90 °C for 45 min in a medium containing distilled water, 0.2 M orthophosphoric acid, and 0.1 M TBA. The tissue samples were incubated at 95 °C for 60 min in an acid medium containing 8.1% sodium dodecyl sulfate, 0.5 ml of acetic acid buffer (500 mM, pH 3.4), and 0.6% TBA (Ohkawa et al., 1978). Absorbances were measured at 532 nm and compared to the standard malondialdehyde curve. The results were expressed in nmol MDA/ml.

2.6.3 Protein determination

The protein content was determined by the Coomassie blue method, according to Bradford (1976), using bovine serum albumin as a standard. The protein supernatant of the samples was maintained with 0.8–1.0 mg/ml of protein until the analysis of the experiments.

2.6.4 Glutathione S-transferase activity

GST activity was analyzed according to the colorimetric method described by Habig et al. (1974) based on the reaction of GST catalyst between reduced glutathione and substrate, 1-chloro-2,4-dinitrobenzene (CDNB). The mixture contained test samples, 0.1 M potassium phosphate buffer (pH 7.4), 100 mM GSH, and 100 mM CDNB. The absorbance was determined at 340 nm at 37 °C, and the enzyme activity was expressed in μmol CDNB/min/mg of protein.

2.6.5 Nitrate and nitrite detection

The principle of this test is the reduction of nitrate by vanadium (III) combined with detection by Griess reagents, according to Miranda and Espey (2001). The samples were deproteinized after the addition of vanadium (III) chloride (VC1₃) and quickly followed by the Griess reagents [sulfanilamide and N- (1-naphthyl) ethylenediamine dihydrochloride (NEDD)]. The absorbance was measured at 540 nm, and the results expressed as μM/mg of protein.
2.6.6 Catalase enzyme activity

Catalases are antioxidant enzymes that catalyze the conversion of hydrogen peroxide into water and molecular oxygen. The decomposition of hydrogen peroxide analyzed the enzymatic activity by decreasing the absorbance at 240 nm. The absorbance difference per unit of time was used to measure catalase activity. The method was according to Nelson and Kiesow (1972), the solution whose samples diluted 1:10 and 50 mM potassium phosphate buffer (TFK) pH 7.0 was placed in a quartz cuvette, and hydrogen peroxide (H$_2$O$_2$) 30 mM was added. The decomposition of H$_2$O$_2$ was measured, and the results expressed as nM/mg of protein.

2.7 Histopathology

The collected kidney fragments were stored in a 10% formaldehyde solution, and after 48 hours, they were removed from the formaldehyde, sieved, and transferred to a new formaldehyde solution. Slides with histological cuts were made and stained with hematoxylin and eosin.

2.8 Statistical analysis

The data were first subjected to normality testing, and when they were not normally distributed, data were transformed to logarithms. With normally distributed data, a one-way analysis of variance was performed to compare groups at the two different moments of sample collection. The Tukey test was used to assess the accuracy of the data. It was considered significant when P < 0.05. The results were presented as mean and standard deviation.

3. Results

On days 1, 15, and 30, after the start of the supply of the homeopathic product, there was no significant difference in the bodyweight of the mice (P > 0.05; Figure 1). However, on the 50th day after the intraperitoneal application of gentamicin, lower body weight was observed in the animals of the Positive-CT, Trat 2, and Trat 4 groups in relation to the Negative-CT group (P < 0.05; Figure 1).

Figure 1: Average mouse weight of mice on days 15, 30, 40, and 50 of the experiment.

Source: Authors.
The levels of creatinine and urea in the groups treated with the homeopathic product were lower than the Positive-CT and Negative-CT groups (P < 0.05). The total protein levels on day 12 were lower in the serum of Positive-CT animals compared to the other groups (P < 0.05). Globulin levels were higher on day 20 in the groups of animals treated with the homeopathic product than the other groups (P < 0.05; Table 1). The levels of TBARS, ROS, and NOx in the kidney of the rodents of the Positive-CT group were higher than the other groups. As for the GST activity on the 20th, in the groups that used the homeopathic product (Trat 2 and Trat 4) and had the highest concentrations than the Positive-CT (P < 0.05). There was no significant difference between the groups at the two evaluated moments (P > 0.05; Tab 2).

**Table 1:** Biochemical analysis of the serum from the experimental model after 30 days of treatment with the homeopathic product Orgarim on the 12th and 20th days after the intraperitoneal application of gentamicin.

| Variable          | Day | Negative-CT | Positive-CT | TRAT-2  | TRAT-4  | P-value |
|-------------------|-----|-------------|-------------|---------|---------|---------|
| Creatinine (mg/dl)| 12  | 0.34 (0.05) | 0.35 (0.05) | 0.32 (0.05) | 0.32 (0.05) | > 0.05 |
|                   | 20  | 0.90 (0.09) | 0.92 (0.21) | 0.68 (0.19) | 0.8 (0.12) | > 0.05 |
| Urea (mg/dl)      | 12  | 56.5 (5.5)a | 32.7 (2.0)b | 35.2 (9.8)b | 34.6 (8.0)b | <0.05  |
|                   | 20  | 59.5 (11.7) | 52.0 (15.9) | 49.8 (10.6) | 54.6 (11.2) | > 0.05 |
| Protein total (g/dl)| 12  | 4.05 (0.46)a | 3.05 (0.63)b | 3.47 (0.25)ab | 3.12 (0.78)ab | <0.05  |
|                   | 20  | 4.85 (1.12) | 5.06 (1.3)  | 4.73 (1.07) | 4.48 (1.07) | > 0.05 |
| Albumin (g/dl)    | 12  | 1.97 (0.25)a | 1.40 (0.21)b | 1.97 (0.25)a | 1.70 (0.56)ab | <0.05  |
|                   | 20  | 2.63 (0.74) | 1.92 (0.42) | 1.80 (0.7)  | 1.92 (0.57) | > 0.05 |
| Globulin (g/dl)   | 12  | 2.07 (0.18)a | 1.50 (0.26)b | 1.50 (0.43)ab | 1.95 (0.20)a | <0.05  |
|                   | 20  | 2.2 (0.69)b  | 3.14 (1.3)ab | 3.80 (1.03)a | 2.56 (1.3)ab | <0.05  |

Note Negative-CT (neither received the product nor received the injections); Positive-CT (did not receive the product and received the injections); T2, (received 0.002 ml of the product per day/animal, received the injections); and T4, (0.004 ml of the product per day/animal); P <0.05 informs that there is a difference between groups; this difference is illustrated by different letters on the same line. Source: Authors.
Table 2: Concentration of oxidant and antioxidant variables in the kidneys of experimental models treated with homeopathic product Orgarim on days 12 and 20 after undergoing intraperitoneal injection of gentamicin.

| Variable      | Day | Negative-CT | Positive-CT | TRAT-2 | TRAT-4 | P-Value |
|---------------|-----|-------------|-------------|--------|--------|---------|
|               | 12  | 4.0 (1.8)   | 5.61 (0.95) | 4.74 (0.45) | 5.89 (1.8) | <0.05   |
| TBARS (nmol MDA/g) | 20  | 3.10 (0.47) | 3.64 (0.28) | 3.84 (1.0) | 3.27 (1.13) | >0.05   |
| ROS (x10^5 U DCF/mg protein) | 12  | 1.35 (0.26) | 2.14 (0.68) | 1.28 (0.46) | 1.94 (0.46) | <0.05   |
|               | 20  | 2.16 (0.9)  | 1.72 (0.35) | 2.79 (1.44) | 2.58 (0.86) | >0.05   |
| NOx (µM/mg protein) | 12  | 1.93 (0.5)  | 3.09 (0.82) | 2.75 (0.21) | 2.31 (0.8)  | <0.05   |
|               | 20  | 2.30 (1.0)  | 2.01 (0.57) | 2.57 (1.11) | 1.54 (0.42) | >0.05   |
| GST (µmolCDNB/min/mg protein) | 12  | 79.8 (27.0) | 78.6 (14.0) | 72.2 (8.5)  | 89.6 (23)   | >0.05   |
|               | 20  | 84.8 (20.6) | 68.5 (18.4) | 99.7 (13.6) | 105 (30)    | <0.05   |
| CAT (nmol/mg protein) | 12  | 3.15 (0.08) | 2.03 (0.30) | 1.61 (0.24) | 2.52 (0.69) | <0.05   |
|               | 20  | 1.66 (0.29) | 2.36 (0.38) | 1.96 (0.51) | 1.59 (0.41) | <0.05   |

Note: Negative-CT (neither received the product nor received the injections); Positive-CT (did not receive the product and received the injections); T2, (received 0.002 ml of the product per day/animal, received the injections); and T4, (0.004 ml of the product per day/animal); TBARS, Substances reactive to thiobarbituric acid; ROS, reactive oxygen species; NOx, Oxidation Number; GST, Glutathione S-Transferase; CAT, Catalase; P < 0.05 informs that there is a difference between groups; this difference is illustrated by different letters on the same line. Source: Authors.

4. Discussion

Inside the nephrons are located the glomeruli that are considered kidney functional units, responsible for blood filtration, that is, the place where substances such as glucose, bicarbonate, and salts reabsorb, in addition to the excretion of components (Oliveira et al., 2001). Thus, when the glomeruli begin to fail, it generates an accumulation of substances in the kidneys, which leads to anemia (Rufato et al., 2011). Often, the body can keep the disease under control only with the appearance of some signs (Rufato et al., 2011). However, in some more severe cases, when undiagnosed, animals can die (Rufato et al., 2011). Pre-existing diseases in the individual can trigger renal failure due to age, fever, diabetes, or other reasons such as lesions of the renal parenchyma due to infectious processes or by obstruction of the urinary tract (Oliveira et al., 2001).

Thus, renal failure can be diagnosed through tests such as urinalysis and blood count. Treatment can be done through medication administration, dialysis treatments, and renal therapy (Pöppl et al., 2004). Gentamicin is an injectable aminoglycoside antibiotic widely used to treat infections caused by bacteria. Usually, treatments with this drug are indicated for seven to ten days. These treatments can cause toxicity (Mathias et al., 2019). This antibiotic can remain in the body for a longer time due to an accumulation inside the cells, being more recurrent when multiple applications are used compared to single doses. Thus, it can cause nephrotoxicity and ototoxicity (Mathias et al., 2019).

There was no evidence of histopathological lesions in the kidneys of the mice that were treated with gentamicin. This fact, different from what was reported by Duarte et al. (2016), who used the same dose as the present study. These described that gentamicin applications increased the occurrence of renal necrosis and apoptosis by 20% and 25%, respectively, a result not found in our study; however, oxidative renal toxicity was found.

Homeopathy is based on treating diseases with extremely diluted components, so they tend to be safer and have a lower risk of toxicity (Jaguezeski et al. 2019). Jyothilakshmi et al. (2014) reported that homeopathic products could reduce oxidative
stress by reducing ROS formation. These results are consistent with the present study, in which it was observed that in the groups treated with the homeopathic product, lower levels of ROS and TBARS. Therefore, this may suggest that the homeopathic product decreased lipid peroxidation, and therefore, it may have had a protective effect. This reduction in free radicals occurs by stimulating antioxidant enzymes (GST and CAT). These are responsible for eliminating excess radicals in the body (Sakamoto et al. 2018) to decrease cellular damage and tissues in the mouse. CAT is the first enzyme to be used in this mechanism, in which it donates its hydroxyl to the free radical, and therefore is quickly depleted (Gius et al. 1999). This mechanism may explain the lower CAT activity in the groups that received the homeopathic.

GST forms the group of enzymes that catalyze and promote compound detoxification to protect cells from peroxidative damage (Jyothilakshmi et al. 2014). The increase in GST activity is one way that the body finds to decrease the free radicals responsible for oxidative stress and acts as a detoxifying enzyme (Fatemi et al. 2006). Jyothilakshmi et al. (2014) found that B. vulgaris has an intense antioxidant activity against oxidative stress and related disorders, thus protecting renal tissue. They also report that B. vulgaris can prevent damage that may occur in antioxidant enzymes. This could explain its protective role.

C. vesicatoria acts mainly on the urinary tract (Jyothilakshmi et al. 2014). You can modulate the interaction of macrophages, change the dynamics of cell migration at the site of infection, and also perform phagocytic activity. Thus, it can be said that there was possible protection of the kidneys due to this nephroprotective effect (De Paula Coelho et al. 2017). When the Causticum compound was used, reductions in inflammation of the lymphatic organ tissues and a significant decrease in the parasitic load were observed in rats infected with T. cruzi (Lopes et al. 2016), with this inflammatory reduction being related to a lower parasitic load (Nagajyothi et al. 2012). The same researchers found that the highly diluted homeopathic product increased the cellular apoptosis of infected mice and cell renewal, immunity, and the body’s defense responses, an improvement in the animals’ survival was also observed. They also had a smaller number of inflammatory foci due to the activation of immunoregulatory mechanisms that regulate the recruitment of mediators (histamine, serotonin, prostaglandin) and defense cells (Prado Neto 2004).

The groups that used the homeopathic product showed improved renal function, as urea levels were lower (Duarte et al., 2016). This effect demonstrates an improvement in the use of the protein or merely a lower inflammatory response (Scanes 2015). Urea is excreted in the urine, and high levels are related to the lower filtration rate of the glomerulus (Motta 2003). Also, the homeopathic product prevented the decrease in total protein. This can prevent leakage of fluid carried by protein loss, as well as dehydration (Stevens 2005).

5. Conclusion

The homeopathic product administered preventively consumed was able to minimize oxidative stress, nitrous stress, and lipid peroxidation in the kidney tissue of mice that received gentamicin for a long time to induce renal failure. It is still necessary to do research to know the effects caused by gentamicin on the renal system, in the future it will be necessary to carry out further research, as there are few articles that show how homeopathic products act to combat these effects.

Conflict of interest

The authors declare no conflict of interest.

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