TOXICITY STUDY USING RAT (WISTAR) MODEL OF A HYDROGEL DRESSING WITH SILVER NANOPARTICLES CROSSLINKED AND STERILIZED BY GAMMA RADIATION

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ABSTRACT

Silver nanoparticles (NPAg) have a bactericidal and bacteriostatic action in combination with hydrogels to recover the damaged tissue, promoting healing of the wound. The objective of this study was to evaluate the toxicity of the hydrogel dressing with NPAg from the analysis of possible toxic effects on renal and hepatic functions. It is an experimental study with 85 male Wistar rats, young adults. Nanoprate hydrogel and bidrogel dressings were used at concentrations of 22 and 44 ppm, both crosslinked and sterilized by irradiation with gamma rays at the 25 kGy dose at the center of radiation technology (CTR). The animals were distributed according to the treatment received after surgical induction of the wound on the animal's back. They were euthanized with 24 hours, 3, 7, 14, 21 and 30 days and after collecting the blood to determine the biochemical parameters. The project was approved by CEUA FAPAC ITPAC PORTO. Data were analyzed using the Past, Shapiro-Wilk and Kruskal-Wallis programs. For distribution and comparison data ANOVA and Tukey test with a significance level of 95%. No changes were observed in relation to biochemical parameters (TGP, TGO, urea and creatinine), and there were no statistically significant differences between the three groups of animals, independent of time. It was possible to observe that the animals treated with 44 ppm had always lower mean values than the other two groups in all analyzes. The study showed that the dressings of NPAg tested may not induce toxicity, being necessary to complement with other tests, such as histopathological study and atomic absorption spectroscopy.

1. INTRODUCTION

Wound is any lesion that causes discontinuity on the epithelial tissue, occurring in any people, from all ages, they can be classified in many ways: by the type of the causer agent, the contamination level, time and depth of lesions. It is considered a huge health problem, because it involves factors related to the patient and its external environment (IRION, 2005).

The big challenge for health professionals is the ideal treatment that stimulates tissue healing, either chronic or acute wound. Healing is a physiological and dynamic process reaching to restore tissue continuity. In the tissue repairing process three phases are emphasized: inflammatory, proliferative and maturation phase.

With the development of health technology, it has been produced many types of dressings, to intensify the healing process and improve the conditions and treatment of patients. This variety of dressings allows better management and adequation of wounds. The dressing, besides protecting and keeping the wound zone wet, should combat the infection and inflammation, accelerate the healing process, reduce pain and provide comfort to the patient (FRANCO; GONÇALVES, 2008).

Choosing the dressing is important for a well succeed treatment, it is necessary to evaluate the cause, place and size of the lesion, the amount of exudate, the center of the wound and its potential for contamination. Associated with these topics it must be considered the non-adherence to the center of the wound, decreasing the number of changes, exudate absorption, lesion protection, the influence in the absence or decrease of pain, heal tissue stimulus and low cost (IRION, 2005).

The hydrogel is among the most used products in open wounds treatment, because it maintains the place ideally wet for the healing process, they are soft and don't cause damage to the granulation tissue, and can act like delivery systems assets increasing the efficiency of treatment.

Hydrogel is a polymeric network capable of absorbing and retaining a large amount of water and body fluids without melting. It presents in its structure hydrophilic domains which are hydrated and crosslinked in their polymer chains. It has in its composition high water content, low interfacial tension enabling low tendency to adsorb proteins from body fluids and cell adhesion, physical properties similar to human tissue, microporous structure and good oxygen permeability (ALCANTRA, 2013).

The use of radiation in the formation of hydrogels enables crosslinking and simultaneous sterilization in which it provides the synthesis of a product with more purity.

Several compounds are included into wounds covering, among them it can be included the use of Silver or silver nanoparticles, which has bactericidal and bacteriostatic effect to the microorganisms in the center of the wound, guaranteeing the contamination security level, preventing the disarrangement that leads to infections.

Nanoparticles are defined as materials sized between 1 and 100 nanometers (1nm=10-9m), conferring mechanical, optical, electric properties and advanced structures, besides a large superficial area in relation to the original substance (BEER, et al., 2012). Reducing the size of the particle is an efficient way to improve the biocompatibility of materials and that allied to the fact that these materials can be modified, makes their applications in cosmetics, pharmaceutical and electric products easier (KIM et al., 2008).

Silver has been used as an antimicrobial and anti-inflammatory therapeutic agent for over 2000 years. It is effective against a large range of aerobic, anaerobic, gram positive, gram negative bacteria; besides the activities against fungi and viruses. It is biologically active in its soluble form, ionic or not (Ag+ or Ag0). The ionic form has a strong antimicrobial effect through immediate block of respiratory chain, destruction of the cell membrane, bacterial wall. The form Ag0 is in nanocrystalline compounds (WRIGHT, 2002).

It's worth pointing out that not all forms of silver are anti-inflammatory. The anti-inflammatory properties depend of the deliver vehicle, concentration available and the duration of the release (FERNANDES, 2010).

Many studies point to a promising future to the use of NPAg, because its antimicrobial activity is influenced by the size of the particles, with reduced size and without aggregation showing action against Gram-negative and Gram-positive bacteria. Besides, NPAg, can promote proliferation of keratinocytes in the reepitalization process and also lead to the differentiation of fibroblasts into myofibroblasts (NOGUEIRA, PAINO and ZUCOLOTTO, 2013).

However, the same characteristics that make NPAg interesting, also can be potentially risky, may causing health problems to humans and other living beings, resulting in a negative impact on the environment such as public health (LEE, et al., 2007).

Studies indicate that AgNPs induce toxicity via activation of the cascade resulting in apoptosis. During the process of programmed cell death, activation of proteolytic proteins such as caspases occurs. AgNPs appear to induce apoptosis via activation of caspases (SOARES, 2014).

Human exposure to NPAg may occur in different ways: inhalation, ingestion, injection and dermal contact. Once inside the body, the NPAg gets quickly into the circulation and can migrate to the liver, kidney, spleen, lungs and brain, inducing toxicity (PAULA, 2013). AgNPs can enter and penetrate these organs. This penetration depends on the size, shape, and surface characteristics of NPs (VIEGAS, 2018).

The liver is the most affected organ by the toxic action of several substances and for having a big importance maintaining homeostasis, an analysis is needed to have a safe use of them. Too much accumulation of NPAg in the liver can lead to some adverse effects such as pathological changes in the liver morphology, generation of reactive oxygen species (ROS), DNA damage and changes in liver enzymatic activities (COSTA, 2009).

The liver is a target of NPAg exposure leading to changes the hepatic mitochondria functions. Thus, the mitochondrial toxicity may play a crucial part in the toxicity from the silver nanoparticles exposure (FONG e WOOD, 2006).

Subacute tests of toxicity in rats, shown that they tolerate doses of 1000 mg/kg -1 of NPAg without significant alterations in body weight. Increased levels of alkaline phosphatase and cholesterol at doses above 300 mg / kg-1 indicating functional changes in hepatic tissue (PAL; TAK and SONG, 2007).

Several dressings with silver and silver nanoparticles have been introduced in the market, and it is necessary to evaluate the actual effect of this metal on wound healing and anti-inflammatory action, as well as its toxicity. Some studies indicate possible acute toxic effects, demonstrating the need for a better understanding of the effects of these materials before they are used in everyday processes or products. Although the toxicology of silver and its compounds has been studied, there are several gaps for further knowledge about the risks caused by nanoparticles of silver, both for humans and the environment. There are still few studies on silver toxicity in the body that are sometimes contradictory. The study of the hydrogel with silver nanoparticles at concentrations of 22 and 44 ppm produced in IPEN Chemistry and Environment Center (CQMB) laboratory becomes important to understand its toxicity, distribution, bioaccumulation and excretion.

The objective of this study was to evaluate the toxicity of the hydrogel dressing with AgNP from the analysis of possible toxic effects on renal and hepatic functions.

2. MATERIALS AND METHODS

This is an experimental study that seeks to evaluate the toxicity of silver nanoparticle using hydrogel and hydrogel dressings with silver nanoparticles at concentrations of 22 and 44 ppm in the treatment of superficial lesion wounds. The dressings were produced at the Chemistry and Environment Center (CQMA) of IPEN under the coordination of Dr. Ademar Benevolo Lugão . Its preparation consists of PVP (ca. 11%), Glycerol (ca 1.5%), Agar (ca 1.5%), Reverse osmosis water (ca.86%) with concentrations of 22 and 44 ppm of silver nanocrystalline obtained from AgNO3. The crosslinked synthesis of NPAg and simultaneous sterilization was through irradiation with gamma rays at the dose up to 25 kGy carried out the center of radiation technology (CTR) in IPEN.

2.1. Experimental Design

There were 85 Wistar male rats, young adults with mean age of 90 days were obtained from IPEN - CNEN / SP Biotério. The animals involved in the study were received 30 days before the beginning of the experiments for acclimatization and kept in the Vivarium of Faculdade Presidente Antônio Carlos - FAPAC ITPAC Porto Nacional. They were all weighed when arrived to follow the weight gain and stress recovery of the transport and allocated right away in two rooms with controlled temperature of 22 + 2ºC, humidity (50-70%), in individual suspended cages, sterile shawl bed, cardboard cylinders to minimize stress, commercial sterile feed (Nuvilab CR-1) and water ad libitum in a 12-hour dark / dark cycle environment

throughout the experiment period. The animals were monitored for water intake (10 to 20 ml / day) and feed (10 to 20 g / day) and weighed every 03 days for weight monitoring. All procedures are in accordance with the recommendation of the Brazilian College of Animal Experimentation and approved by the Animal Research Ethics Committee of FAPAC ITPAC Porto Nacional under process number 005/2017.

2.2. Group Standardization

The animals were randomly distributed into three groups, according to the treatment received after surgical induction of the wound on the animal's back, each one consisted of four animals with one (n) of 72 rats:

H: control group: 24 animals whose lesions were treated with the hydrogel and monitored for 24 hours, 3, 7, 14, 21 and 30 days (n = 4 rats per treatment time).

HP22: 24 animals whose lesions were treated with hydrogel containing silver nanoparticles at 22 ppm and monitored for 24 hours, 3, 7, 14, 21 and 30 days (n = 4 rats per treatment time).

HP44: 24 animals whose lesions were treated with hydrogel containing silver nanoparticles at 44 ppm and monitored for 24 hours, 3, 7, 14, 21 and 30 days (n = 4 rats per treatment time).
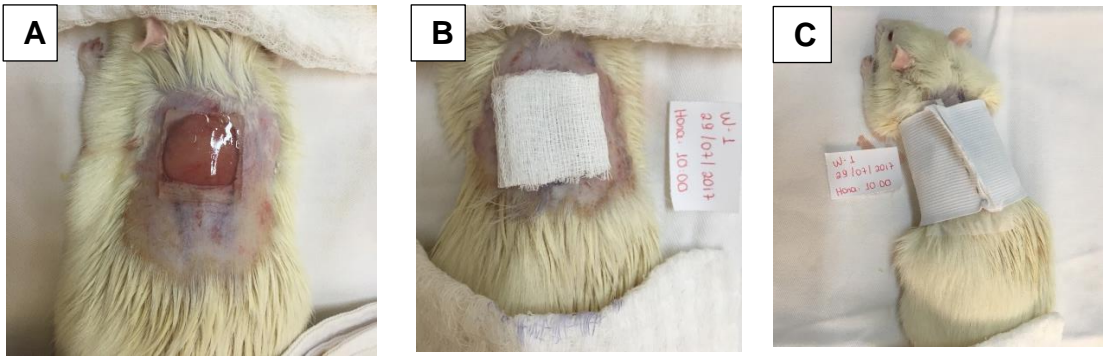
2.3. Surgical Procedure

In surgical procedure days to generate the injury the animals were weighed to adjust the medication dose, thereafter they were analgesic pre -medicate with acepromazine at a dose of 2.5 mg / kg body weight, anesthetized with 1.5 mg / kg xylazine 2% and 50 mg / kg of intraperitoneal ketamine. Afterwards, the animals were tricotomized in the dorsal region, positioned on the operative table in ventral decubitus position, delimited the skin and after antisepsis with cloroxidine, it was made a rectangular cutaneous lesion with tissue removal from the area (2cm x 2cm), of superficial depth and healing by second intention subsequently. (Figure 01)
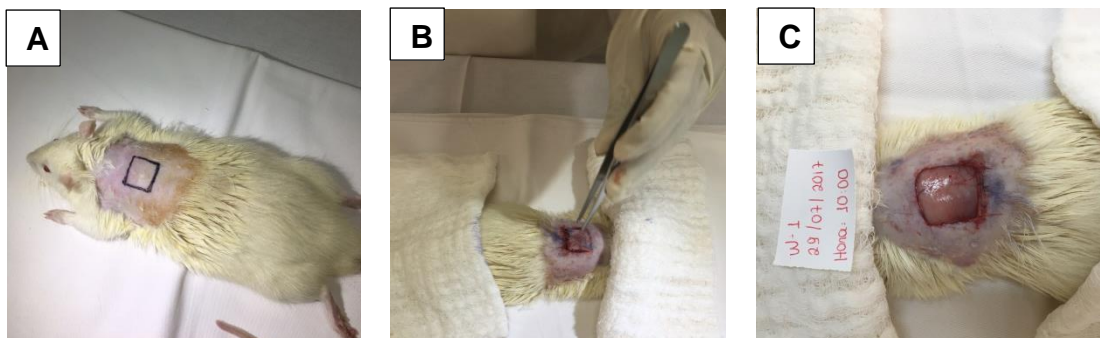
(A) Trichotomized animal on anesthetic effect in ventral position with delimitated area. **(B)** Performing the surgical lesion using a scalpel. **(C)** Animal after superficial surgical injury.

Figure 01: Stages of Surgical Procedure

Each wound was treated immediately after the surgical procedure according to the type of treatment. The hydrogel and hydrogel dressings with silver nanoparticles were humidified with distilled water and covered with a sterile gauze and fixed to the animal using a micropore type tape and elastic mesh to ensure that the animal did not remove the dressing (Figure 02).

(A) Wound that received the hydrogel. (B) Sterile gauze cover (C) Fixation with adhesive tape and elastic mesh.
Source: The author, August 2017

Figura 02: Curativo Oclusivo Após Receberem os Tratamentos

After the surgical procedure, the animals were placed in individualized cages. For the pain relief, it was administered 50-600 mg / kg of dipyrone subcutaneous 12/12 hours when needed. The animals were observed after the surgical procedure for 2 hours in order to be followed up in the recovery and reestablishment of the routine. Some animals had to receive extra anesthetic dosage and 11 animals died due to an overdose of the anesthetic.

2.4. Injury Treatment and Sample Collection

Treatment of lesions was performed every three days, once a day and applied on the cover of the injured area. At the determined time of evaluation of the groups, the animals were euthanized by chemical method with dose of anesthetic, 90 mg / kg of intraperitoneal thiopental, using a 2% lidocaine anesthetic button. Blood sample collection was performed by puncturing the abdominal and cardiac aorta using a 5 ml syringe and a 28x7 needle.

2.5. Clinical Biochemistry Evaluation

Part of the collected blood was packed in heparin-containing tubes, centrifuged (Centribio ® TDL80-2B) at 2500 rpm for 10 minutes, resulting in separation between plasma and figured elements. The obtained plasma was carefully removed and stored in an eppendorf flask and frozen in a freezer (-20 ° C).

The aim of this study is determining the biochemical parameters for the evaluation of liver function through ALT / TGP (Alanine Aminotransferase) and AST / TGO (AspartateAminotransferase) analyzes and the evaluation of renal function through the Creatinine and Urea tests. Labtest ® kits specific for each parameter were used for the analyzes following the instructions determined by the manufacturer in a semi-automatic Bioplus ® BIO-2000 analyzer.

2.6. Statistical Methods

The results of the tests to determine the hematological parameters were used from a table containing all the numerical information and then the program Past v 3.22 (Øyvind Hammer , Natural History Museum , University of Oslo) to make the analyzes. Initially, the Shapiro- Wilk test was performed to verify the normality of the data. For non-parametric data, the Kruskal- Wallis test (paired combination) was used, and the multiple comparisons between the groups were done by Dunn's post- test. For normal distribution data, analysis of variance (ANOVA) and means comparison tests were performed using the Tukey test.

3. RESULTS E DISCUSSION

Analysis of serum urea and creatinine concentrations of the animals showed that they were similar at different experimental times and no significant statistical differences were observed between the three control groups, 22 ppm and 44 ppm, considering p <0.05. Data are presented as mean - MED. (Table 01)

Table 01: Serum Urea and Creatinine Concentrations of Animals at Different Times and Experimental Groups

Euthanasia Time	Urea (mg/dL)						Creatinine (mg/dL)					
	Control	(n)	22 ppm	(n)	44 ppm	(n)	Control	(n)	22 ppm	(n)	44 ppm	(n)
24 hours	51,515	4	52,58	3	56,515	4	0,35	4	0,4	3	0,4	4
03 days	60,12	3	61,065	4	51,92	3	0,4	3	0,35	4	0,4	3
07 days	42,43	3	44,13	2	49,6	3	0,35	3	0,4	2	0,4	3
14 days	50,16	3	49,72	3	54,6	4	0,4	3	0,4	3	0,45	4
21 days	69,66	1	60,045	2	48,4	3	0,4	1	0,4	2	0,4	3
30 days	53,45	4	52,72	4	47,23	4	0,35	4	0,4	4	0,4	4

(n) = number of animals   (22 ppm e 44 ppm) = concentrations of AgNP dressing in the hydrogel

The AST / TGO (Aspartate Aminotransferase) and ALT / TGNP (Alanine Aminotransferase) liver injury indicators were similar and no statistically significant differences were observed between groups (control, hydrogel dressing with silver nanoparticles at concentrations of 22 and 44 ppm) in relation to 24 hours, 03, 07, 14, 21 and 30 days. (Table 02)

Table 02: Serum AST and ALT Concentrations of Animals at Different Times and Experimental Groups

Euthanasia Time	AST (U/L)						ALT (U/L)					
	Control	(n)	22 ppm	(n)	44 ppm	(n)	Control	(n)	22 ppm	(n)	44 ppm	(n)
24 hours	216,2	4	200,4	3	221,2	4	40	4	38,1	3	48,8	4
03 days	237,1	3	231,4	4	220,6	3	40,6	3	47,15	4	39,9	3
07 days	81,4	3	103,85	2	93	3	19,6	3	17,9	2	25,9	3
14 days	175,5	3	194,6	3	175,3	4	34,9	3	34,6	3	36,9	4
21 days	260,8	1	297,85	2	110,8	3	50,5	1	34,3	2	27	3
30 days	232,95	4	179,65	4	237,3	4	39,85	4	35	4	47,45	4

(n) = number of animals   (22 ppm e 44 ppm) = concentration of AgNP of dressing in the hydrogel

The results showed that in 24 hours, the urea levels and ALT in animals used hydrogel dressing with AgNP with a concentration of 44 ppm showed higher compared to the other groups, but this difference was not presented as significant by the test analysis ANOVA.

Regarding the time of 03 days, it was observed that the animals treated with dressings of 44 ppm had urea, AST and ALT values always lower in relation to the control group and the dressing with 22 ppm, but according to the variance analysis there was no meaningful statistical difference.

The data showed that in the biochemical assays in the animals sacrificed at 7, 14, 21 and 30 days of treatment did not present significant statistical differences. However the samples of four animals euthanized at 07 days (1 of the control group, 2 of the 22 ppm group and 1 of the 44 ppm group) and six samples of 21 days (3 control , 2 of 22 ppm and 1 of 44ppm) hemolysis, impairing the statistical analysis in function of the reduced number of samples.

In the study, no toxic effect of the hydrogel dressings with AgNP was observed in the concentrations of 22 and 44 ppm in the kidneys, since the serum concentrations of urea and creatinine were not altered.

No toxic effect of the nanoparticles was observed in the liver, considering that the AST and ALT levels of the animals were not altered and were similar to the control group.

Studies indicate that the main target organ of accumulation of AgNPs has been the liver and toxicity may be related to particle size, the lower the greater the probability of its toxic effect, this possibly is related to the combination of size and its dissolution (VIEGAS, 2018).

In Pourhamzeh, *et al*. (2015), after oral exposure of AgNPs in mice at concentrations of 30, 125, 300, and 700 mg / kg for 28 days observed that the AST and ALT parameters did not show changes, suggesting that at these concentrations did not cause any significant dysfunction in liver of animals.

4. CONCLUSIONS

To date the study has shown that crosslinked AgNP bandages and sterilized by gamma irradiation produced in the IPEN / USP may not induce toxicity and should be complemented with other tests such as histopathological study and atomic absorption spectroscopy.

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