AUTARQUIA ASSOCIADA À UNIVERSIDADE DE SÃO PAULO

SYNTHESIS AND CHARACTERIZATION OF HYDROGELS LOADED NEOMYCIN FOR INFECTION TOPIC TREATMENT AND VETERINARY USE

Angélica Tamião Zafalon

Tese apresentada como parte dos requisitos para obtenção do Grau de Doutor em Ciências na Área de Tecnologia Nuclear - Materiais

Orientadora:
Profa. Dra. Duclerc Fernandes Parra

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INSTITUTO DE PESQUISAS ENERGÉTICAS E NUCLEARES
Autarquia associada à Universidade de São Paulo

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DEDICATION

To my husband, Eric Zafalon for his unconditional support,
love, strength and inspiration to follow my dream
and make it a reality.
To my parents, Marilza Tamião, Watson Maximo
and my brother Guilherme, for their encouragement,
support and love.

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"Your mind has the power to transform reality. What you think, you feel, what you feel, you vibrate, what you vibrate, you attract to yourself. We are vibrational beings that interact with the universe. What you send out is what you get back. Decide what you want. Believe you can have it. Believe that you deserve it and believe that it is possible"

The Secret

"If you want to find the secrets of the universe, think in terms of energy, frequency and vibration"

Nikola Tesla

ABSTRACT

Hydrogels are natural or synthetic polymer systems that have been vastly applied in the pharmaceutical industry due to their high soft tissue biocompatibility. These hydrogels have been used in dressings as a controlled drug release system. In this study, hydrogels were prepared using poly (N-vinyl-2-pyrrolidone) (PVP), poly (ethylene glycol) (PEG), agar and neomycin followed by gamma irradiation to promote crosslinking and sterilization. The influence of the irradiation process at 25 kGy dose was investigated. The gel fraction and maximum swelling were estimated using physicochemical methods and found about 95% gel fraction and 1100% swelling after 8 hours of immersion. Neomycin released from the hydrogel was measured by the Liquid Chromatography-Mass Spectrometry method and the drug concentration remained constant for 48 hours. Hydrogel / neomycin exhibited antibacterial effect against bacteria and biofilm of *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The hydrogel was used in the treatment of pododermatitis in penguins healing process without signs of contamination. With these results, it can be inferred that the hydrogel / neomycin is a suitable candidate for wound dressings.

Keywords - hydrogel, gamma irradiation, drug-controlled release system, antimicrobial, antibiotic-resistant bacteria, penguins, pododermatitis

Síntese e caracterização de hidrogéis com neomicina para tratamento de infecções tópicas e uso veterinário

RESUMO

Hidrogéis são sistemas poliméricos naturais ou sintéticos que ganharam interesse na indústria farmacêutica devido à sua alta biocompatibilidade com tecidos moles. Estes tipos de hidrogéis têm sido usados em curativos como um sistema de liberação controlado de drogas. Neste estudo, os hidrogéis poliméricos foram preparados usando poli (N-vinil-2-pirrolidona) (PVP), poli (etilenoglicol) (PEG), ágar e neomicina seguido de irradiação gama para promover a reticulação e esterilização. A influência do processo de irradiação com a dose de 25kGy foi investigada. A fração gel e o intumescimento máximo foram estimados usando métodos físico-químicos e encontraram cerca de 95% de fração de gel e 1100% de intumescimento após 8 horas de imersão. A neomicina liberada do hidrogel foi mensurada pelo método de Cromatografia Líquida-Espectrometria de Massa e a concentração do fármaco permaneceu constante por 48 h. Hidrogel/ neomicina exibiu efeito antibacteriano contra bactérias e biofilme de *Pseudomonas aeruginosa e Staphylococcus aureus*. O hidrogel foi utilizado no tratamento de pododermatites em pinguins e após 5 dias de tratamento, as lesões apresentaram processo de cicatrização acelerado e sem sinais de contaminação. Com estes resultados, pode-se inferir que o hidrogel/ neomicina é um candidato adequado para curativos.

Palavras chaves: hidrogéis, irradiação gama, sistema de liberação controlada de drogas, bactérias resistentes, antimicrobiano, pinguins, pododermatites.

TABLE OF CONTENTS

1 INTRODUCTION..... 12

2 OBJECTIVE..... 14

3 LITERATURE REVIEW……..... 15

3.1 Hydrogels..... 15

3.2 Production of hydrogels using ionizing irradiation..... 16

3.3 Intelligent Polymers..... 18

3.4 Hydrogels as wound dressings for treatment of wounds..... 20

3.4.1 Sustained drug release from hydrogel..... 20

3.4.2 Poly (N-vinyl-2-pyrrolidone) (PVP)..... 22

3.5 Skin Structure..... 22

3.6 Chronic wounds and infections..... 23

3.7 Biofilms..... 25

3.8 Neomycin..... 27

3.9 "In vivo" test..... 28

3.9.1 Animal model: Magellanic Penguin (*Spheniscus magellanicus*)..... 28

3.10 Ethical protocol..... 32

4 MATERIALS AND METHODS..... 33

4.1 Materials..... 33

4.2 Preparation of hydrogels..... 33

4.3 Swelling behavior..... 33

4.4 Determination of gel fracrion..... 34

4.5 Infrared Spectroscopy FTIR..... 34

4.6 Neomycin analysis by HPLC-MS/MS..... 34

4.7 Neomycin stability assay…….. 35

4.8 Release of neomycin..... 35

4.9 Thermal analysis……..... 36

4.10 Cytotoxicity assay……..…….. 35

4.11 Antimicrobial activity..... 36

4.11.1 Preparation of Bacteria Inoculum..... 36

4.11.2 Disc diffusion test……..……... 37

4.11.3 Antibacterial activity of hydrogel in bacterial suspension…… 37

4.12 Time Kill curve…….…… 37

4.13 Measurement of biofilm growth..... 38

4.14 Efficacy hydrogel against established biofilm..... 39

4.14.1 Treatment of stablished biofilm in 96-well plate..... 39

4.14.2 Treatment of stablished biofilm in slides..... 39

4.15 Microbial penetration……..... 39

4.16 Scanning electron microscopy (SEM)…… 40

4.17 Evaluation of the treatment of pododermatitis in penguins..... 40

5 RESULTS AND DISCUSSION..... 41

5.1 Preparation of hydrogel / neomycin..... 41

5.2 Swelling Behavior..... 42

5.3 Gel fraction test..... 43

5.4 LC – MS/MS method..... 44

5.5 Calibration curve of neomycin..... 45

5.6 Effect of gamma irradiation on the neomycin stability……..…… 46

5.7 TG/ DTG e DSC curves..... 49

5.8 FTIR spectra..... 53

5.9 Drug release assay..... 54

5.10 Cytotoxicity test..... 57

5.11 Antimicrobial activity assay..... 57

5.11.1 Disk diffusion..... 57

5.11.2 Suspension of bacteria assay……..... 60

5.12 Time kill curve..... 61

5.13 Biofilm Formation and quantification..... 62

5.14 Efficacy hydrogel against established biofilm ……..... 67

5.15 SEM images..... 68

5.16 Microbial penetration..... 69

5.17 Evaluation of the treatment of pododermatitis in penguins …… 70

6 CONCLUSIONS..... 78

APPENDIX 80

REFERENCES..... 79

LIST OF FIGURES

FIGURE 1	^{60}Co decays by emitting beta decay	16
FIGURE 2	Subtraction of •H from the PVP polymer chain and formation of the stable macrochains	18
FIGURE 3	Illustration of hydrogel pH and temperature sensitive	19
FIGURE 4	Representation of the skin layers.	23
FIGURE 5	Chemical structure of neomycin; R1 = CH_2 NH_2; R2 = H_2	27
FIGURE 6	Magellanic Penguin (*Spheniscus magellanicus*)	29
FIGURE 7	Bilateral Pododermatitis in Magellanic Penguin	30
FIGURE 8	Hydrogel / neomycin obtained by ionizing irradiation, at 25 kGy dose.	41
FIGURE 9	Swelling curve in seawater of hydrogels obtained by gamma irradiation at different doses.	42
FIGURE 10	Swelling curve in distilled water of the hydrogels obtained by gamma irradiation at different doses.	42
FIGURE 11	LC-MS positive full scan mode with neomycin fragments peak.	45
FIGURE 12	Calibration curve of neomycin	46
FIGURE 13	Neomycin stability under irradiation at 25kGy. Comparison between different radioprotectors.	46
FIGURE 14	TG/ DTG curves	49
FIGURE 15	TG curves for non-irradiated neomycin, hydrogel/neomycin and hydrogel, obtained under N_2 flow	50
FIGURE 16	DSC profiles of neomycin irradiated and non-irradiate	53
FIGURE 17	FTIR spectra of PVP polymer, neomycin and hydrogel/ neomycin synthetized by irradiation at 25 kGy	54
FIGURE 18	Drug released curve	55
FIGURE 19	Cell viability curve	57
FIGURE 20	Antimicrobial activity assay by diffusion disk in Hydrogel/neomycin against *P. aeruginosa.* and *S. aureus*	58
FIGURE 21	Result of disc diffusion against *P. aeruginosa* after successive	59

plate changes.

FIGURE 22 Result of diffusion in agar against *S. aureus*. 60

FIGURE 23 Bacterial growth inhibition in liquid medium 61

FIGURE 24 Time kill curve of hydrogel / neomycin against *P. aeruginosa* bacteria. 62

FIGURE 25 Time kill curve of hydrogel / neomycin against *S. aureus*. 62

FIGURE 26 Three slides were placed into the Coplin jar and filled LB Broth 63

FIGURE 27 Established biofilm by *P. aeuriginosa* 64

FIGURE 28 Established biofilm by *S. aureus* 64

FIGURE 29 Biofilm formation observed under optical microscopy 65

FIGURE 30 SEM pictures of biofilm formation by *P. aeruginosa.* Lamellar formation following the biofilm maturation 66

FIGURE 31 Antimicrobial activity of hydrogel against biofilme of *P. aeruginosa* 68

FIGURE 32 SEM images showing the formation of *P aeruginosa* biofilm before and after hydrogel / neomycin treatment 69

FIGURE 33 Pododermatites in both paws of Penguin 71

FIGURE 34 Pododermatitis in paw of penguin 71

FIGURE 35 Pododermatitis lesion measured with ruler 72

FIGURE 36 Hydrogels applied on podermatitis. 73

FIGURE 37 Sequence for hydrogel application and penguin in its aquatic environment. 74

FIGURE 38 Penguin after surgical procedure and hydrogel application 74

FIGURE 39 Evolution of the treatment of popodermatitis in penguin 75

FIGURE 40 Pictures were taken before and after treatment 75

FIGURE 41 (a) hydrogel before and after treatment; b) hydrogels removed after 5 days 76

LIST OF TABLES

TABLE 1	Gel Fraction versus dose of irradiation	44
TABLE 2	TG / DTG degradation values for PVP, agar, PEG and hydrogel, under nitrogen flow.	50
TABLE 3	TG data of degradation for neomycin, hydrogel/neomycin and hydrogel under nitrogen flow	51
TABLE 4	Comparison of inhibition halos after successive plate changes	59
TABLE 5	DO_{540nm} X incubation time	66
TABLE 6	Biofilm treatment with neomycin released from the hydrogel	67
TABLE 7	Microbial penetration inhibition	70

1. INTRODUCTION

Infections are considered a common problem in chronic wounds. In general, these lesions have low blood perfusion and it is necessary to associate the systemic treatment with the use of topical dressings. (HUB, 2011, THEURETZBACHER, 2013, BOEKEME, 2013, PIMENTA, 2014). *Pseudomonas aeruginosa* and *Staphylococcus aureus* are opportunistic Gran negative and Gran positive bacteria, respectively, responsible for infections in debilitated patients. These bacteria produce virulence factors including biofilms that increase their pathogenicities, make treatment difficult and promote chronicity of wounds (GONÇALVES 2017). With the increasing prevalence of antibiotic-resistant bacteria, new strategies are requested, such as the synergistic use of antibiotics and modified release system, where antibiotics already available on the commerce can be used, but with a different form of action. These new therapeutic approaches have been used in the treatment of biofilms (LUCENA, 2014; BAZZI, 2015; NEWITT, 2015). In this way, hydrogels have been widely studied as a drug delivery system, with the purpose of releasing the drug in the local, by the controlled way, reducing toxicity and overcoming bacterial resistance (KENZEVIC, 2013; XIONG, 2014; WANG, 2014). The hydrogels are three-dimensional insoluble structures, formed by crosslinked, biocompatible, non-toxic and capable of swelling in water (PEPPAS, 2000, THEURETZBACHER, 2013, DRAGAN, 2014, OLIVEIRA, 2014 and NASIN, 2015). The formation of hydrogels by polymer crosslinking can be achieved by various methods, including ^{60}Co source gamma radiation, which induces the modification of the rheological characteristics of the polymers and has the advantage of not using toxic initiators or reagents during the process (ROSIAK, 2003; LUGÃO, 2007; KADLUBOWSKI, 2010). Poly (N-vinyl-2-pyrrolidone) (PVP) is a synthetic polymer used to obtain hydrogels with specific characteristics, mainly for the development of a drug release system (PEPPAS, 2000B; KADLUBOWSKI, 2010; VILLANOVA, 2010; DRAGAN, 2014, HALAKE, 2014). For instance, Neomycin is a broad spectrum antibiotic applied topically for the treatment and prophylaxis of skin infections. This drug was chosen for the present work because it has low cost and was selected by the

Brazilian regulatory agency to be free antibiotic prescription (BRUTON, 2006; KOROLKOVAS, 2010). In the present study, the “in vivo” tests were performed in Magellan Penguins, a wild species susceptible to develop pododermatitis in the paw due to habitat conditions in captivity. Pododermatitis is a multifactorial syndrome that appears in the paws of birds, mainly in captivity. The evolution of the disease can lead to an increase in body temperature, abscesses, osteomyelitis, and septicemia. The current therapeutics available for the treatment of pododermatitis include surgical procedure followed by drug therapy and some authors report the use of phototherapy, however, there are no studies in the literature that involve the treatment of pododermatitis using a controlled drug release system. The hydrogel / neomycin consists of a new treatment approach since it consists of a polymeric matrix that releases the drug continuously on the injury, reducing the side effects of the systemic application facilitating the management of the animal in captivity. In this way, the present work evaluates the chemical-physical parameters, antimicrobial effect and the action of the neomycin released from the hydrogel in the treatment of pododermatitis in Magellan Penguins (*Spheniscus magellanicus*).

2. OBJECTIVE

This research proposes the development and characterization of the neomycin loaded PVP-hydrogel obtained by gamma irradiation for topical treatment of infections and biofilm through its biocide activity and in veterinary application to the treatment of pododermatitis in penguins.

3. LITERATURE REVIEW

3.1 Hydrogels

Biomaterials are defined as substances or materials, synthetic or natural, which may be employed to treat, replace tissues, for organs or physiological functions, for an indeterminate time. One of the most promising categories of biomaterials is the hydrogel (ROSIAK, 1999; KADLUBOWSKI et al., 2010; KADLUBOWSKI, 2014; WANG et al., 2016; VEDADGHAVAMI et al., 2017; WEINSTEIN-OPPENHEIMER et al., 2017; PELLA et al., 2018).

Hydrogels are insoluble three-dimensional structures with soft and elastic consistency, formed by crosslinked polymers and stabilized by covalent crosslinks, hydrogen bonding, ionic strength and hydrophobic interactions present between the polymer chains (PEPPAS et al., 2000; OLIVEIRA, 2013; MAHINROOSTA et al., 2018). The hydrogels have hydrophilic characteristics with high water content, which can vary from 30 to 90% in relation to the total weight. They are known to be capable of absorbing and swelling in the presence of water, physiological fluids and/or saline solution (WANG, 2018, DeMetter et al., 2017, CAPANEMA et al., 2018). The degree of swelling may be influenced by the type of polymer and functional groups present, for example, -OH, -COOH, -CONH2, -SO3H, amines, and R4N + (PEPPAS et al., 2000; DRAGAN, 2014; DEMETER et al. al., 2017). At maximum swelling, the volume of the hydrogel can increase up to 1000% without integrity loss.

Hydrogels may be derived from synthetic or natural polymers and copolymers and are classified according to their physico-chemical and biological characteristics, as well as their methods of manufacturing.

Hydrogels derived from only one polymer may have poor mechanical properties and slow response to swelling. In order to improve these characteristics, polymer blends are used to form the three-dimensional network (PEPPAS et al., 2000, PEPPAS, 2000; KAMOUN, 2017). The combination of these polymers results in hybrid hydrogels with mechanical and physico-chemical characteristics more attractive for industrial and commercial use, as it

increases the elasticity, the degree of swelling and the biodegradability (PEPPAS et al., 2000; DRAGAN, 2014; HUBER et al., 2017; POONGUZHALI, 2017).

3.2 Production of hydrogels using ionizing irradiation

The formation of hydrogels by means of polymer crosslinking may occur by chemical and physical processes which include the use of catalysts, crosslinking agents, freezing and thawing cycles, free radicals from the decomposition of peroxides, by UV light or ionizing radiation (PEPPAS, 2000; NAIR et al., 2007).

The use of ionizing radiation to obtain hydrogels was first proposed by ROZIAK in 1989, and since then the gamma ray sources most used for this purpose are those of ^{60}Co and ^{137}Cs (MOZALEUSKA et al., 2017; YANG et al., 2017). Gamma radiation is a high-energy electromagnetic wave and the ^{60}Co radioisotope is industrially most used because it has a half-life of 5.27 years and energy intensity greater than ^{137}Cs. The decay of ^{60}Co occurs by emission of γ rays giving the ^{60}Ni isotope, as shown in FIG. 1.

$$^{60}_{27}\mathrm{Co} \longrightarrow \beta^{-} + {}^{60}_{28}\mathrm{Ni}^{+} \longrightarrow \gamma + {}^{60}_{28}\mathrm{Ni}$$

FIGURE 1 - ^{60}Co decays by emitting beta decay

The 25 kGy dose has been employed to induce crosslinking of hydrophilic polymers and to obtain the three-dimensional hydrogel structure. This dose is also responsible for the sterilization of the dressing in its final package, concomitantly with the crosslinking process. The use of this technique to obtain hydrogel presents other advantages such as the low cost of operation, the absence of chemical initiators and non-generation of toxic residues in the hydrogel (ROSIAK, et al., 1995; MOZALEUWSKA et al., 2017).

The irradiation process can be controlled by the choice of polymer, the type of irradiation (pulses or continuous), rate and dose (ROZIAK et al., 2003). In addition, the irradiation may influence the cleavage of the molecule and

modification of the rheological characteristics of the polymer chains (CHAPIRO, 2002; LUGÃO et al., 2007; FERRAZ, 2013; OLIVEIRA 2013).

The irradiation of the polymers can be carried out with the polymer in the solid state or aqueous solution (ROSIAK, 1999). When irradiation occurs in solid-state polymers there is a restriction of movement of the polymer chains, which requires a higher dose of radiation for the production of free radicals necessary to initiate the crosslinking process (ROSIAK et al., 1995; ROSIAK, 1999; ROSIAK, 2003). The presence of oxygen during irradiation may lead to polymer chain breakage and polymer molecular weight decrease (ROSIAK et al., 1995). In aqueous media, the polymer chains can move easily, dissipating the oxygen present and decreasing the sheer process (ROSIAK et al., 1995). With the process of irradiation in an aqueous medium, reactive intermediates are formed in the polymer chain due to the direct action of the radiation on the polymer and/or by the indirect action of free radicals from the water (ROSIAK, 1999).

The formation of free radicals occurs when the energy of the radiation is absorbed by water giving free radicals OH-, H+, e^-eq and the molecules H_2 and H_2O_2, represented in equation 1. This process is known as water radiolysis.

$$H_2O + \gamma \text{ radiation} = OH^- + H^+ + H_2O_2 + e^-_{eq} + H_2 + H_3O$$

Among the ions formed, OH- is the most reactive. This radical interacts with the polymer chains by subtracting hydrogen atoms and forming macroradicals. These, in turn, combine with each other by crosslinking, originating macromolecules with higher molar mass and insoluble. This process is also called the crosslinking process (ROSIAK, 1993; ROSIAK, 1999; ROSIAK et al., 2003). FIG. 2 represents the formation of macroradicals derived from the H subtraction of the PVP polymer (ROSIAK, 1999; KADLUBOWSKI, 2014; SEDLACEK et al., 2017).

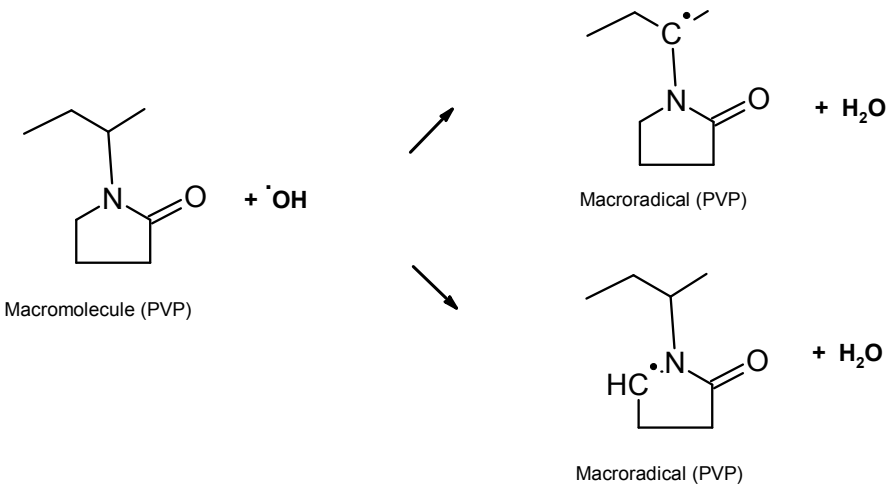

FIGURE 2 - Subtraction of •H atom from the PVP macrochain and formation of the stable macroradicals

Hydrogels have been used in the textile industry, in the manufacturing of paper, solvents, dispersants, and stabilizers, in cosmetics, construction and biomedicine fields. Their use in biomedicine has been investigated in the last decades due to their similarity with living tissues. Such interest has been increasing every year (PEPPAS et al., 2000; KADLUBOWSKI et al., 2010; DRAGAN, 2014; KAMOUN, 2017).

The first use in this area was applied in contact lenses due to good mechanical stability and favorable refractive index of these materials (WICHERLE, 1960). Currently, contact lenses are made with silicone hydrogels that allow the transport of oxygen and the permeability of nutrients and ions to the cornea. Other applications for hydrogels are in the manufacturing of tendons, membranes, articular cartilage, and artificial skin. They are used to expand blood volume, in prosthetic materials for reconstruction of the sexual and maxillofacial organ, cell cultures, encapsulation and coating of tablets, healing bioadhesives and as a controlled drug release system (TAE et al., 2007, KADLUBOWSKI et al., 2010, KAMOUN, 2017, AJOVALASIT et al., 2017).

3.3 Intelligent Polymers

Intelligent or sensitive hydrogels are biomaterials capable of detecting and responding to environmental stimuli such as pH, temperature, analyte

concentration, ionic strength, light, electric and magnetic field, chemicals, enzymes, ultrasonic irradiation, among others, due to the functional groups present in their polymer structure that interact in a non-covalent way (KADLUBOWSKI et al., 2010; KAMOUN, 2017). This particularity has been widely explored in the pharmaceutical industry for the development of controlled drug delivery systems. In general, healthy tissues and organs may have their pH altered due to pathologies. This pH change can be used to alter the structure of the hydrogel and lead to drug release directly on the locus to be treated. This way, the release would be denominated dependent pH (Moore et al., 2008).

pH-sensitive hydrogels are derived from polymer chains composed of weak acids (-COOH) or ionizable bases (amines) present in the side chains, branching and crosslinking of polymer chemical structures (KADLUBOWSKI et al., 2010). The carboxylic groups are deprotonated with raising the pH leading to swelling of the hydrogel while the basic groups deprotonate with the decrease in pH (MOORE, 2009). This electrostatic change alters the osmotic pressure of the hydrogel and influences the final volume of the gel, FIG. 3. The absorption and adsorption of the water can occur simultaneously according to the functional groups present in the polymer chains (KOETTING et al. 2015; MAHINROOTA et al., 2018).

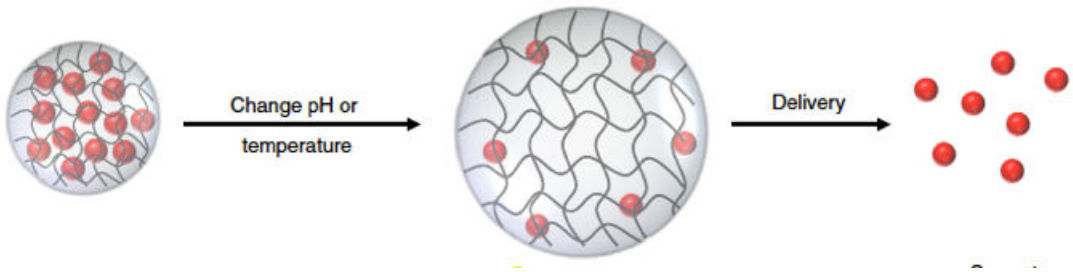

FIGURE 3 - Illustration of hydrogel pH and temperature sensitive (MOORE, 2009).

Temperature-sensitive hydrogels have the ability to change their water volume according to the ambient temperature. Crosslinked polymers exhibit

critical solubility temperatures according to each monomer. Below this temperature, the polymers are highly swollen. Above such temperature, the hydrogels collapse and the polymers precipitate causing water loss, phase separation and gel volume decrease (GOTTLIEB, 2005; MOORE, 2009; MA et al., 2010). Metal particles can be used to alter the sensitivity of the hydrogel to temperature because the metal absorbs light that is converted into heat and transmitted locally to the polymer (Moore et al., 2009)

The hydrogels can be made with polymer blends and thus present double responses, both for pH and temperature changes (Moore, 2009). The most commonly used polymers for obtaining pH-sensitive hydrogels are poly (N-vinyl-2-pyrrolidone), polyacrylic acid, methacrylic acid, diethylaminoethyl methacrylate, dimethylaminoethyl methacrylate (VLASSOULOS, 2012; SINGH, 2012).

3.4 Hydrogels as wound dressings for treatment of wounds

3.4.1 Sustained drug release from hydrogel

The aim of the controlled drug delivery system is to keep the therapeutic drug dose constant at the site of action for a prolonged time through of a system that controls the rate of drug release (SINKO, 2008). Such system uses polymer matrix or coating different from those applied in conventional pharmaceutical forms. And natural or synthetic polymers are the new strategies for the development of matrices for active principle delivery (OLIVEIRA, 2013).

The major synthetic hydrophilic polymers used as support for the drug delivery system are (YU et al., 2005; COIMBRA, 2012).

- Acrylate: poly (methyl methacrylate) (PMMA); poly (acrylic acid) (PA); poly (2-hydroxyethyl methacrylate) (PHEMA)
- Acrylamides: polyacrylamides; poly (N-isopropylacrylamide)
- Poly (dimethylsiloxane) (PDMS)
- Polyethylene glycol (PEG)
- Ethylene vinyl acetate (EVA)
- Poly (N-vinyl-2-pyrrolidone) (PVP)

- Poly (vinyl alcohol) (PVAL)

The mechanisms of drug release from the polymeric matrix include diffusion, swelling and diffusion, degradation and active efflux (SINKO, 2008). During the crosslinking process, macroradicals recombine creating the pores in the hydrogel which in turn have the ability to adsorb solute (ROSIAK et al., 1995; KOETTING et al., 2015). During the synthesis of the hydrogel, the drug may be added into the solution with the polymer which is subsequently deposited into molds to obtain the form of a film, disk or sphere. After the cross-linking process, the drug remains trapped in the pore and immobile within the gel matrix (PLUNGPONGPAN et al., 2013). When the hydrogel comes in contact with water or biological fluids there will be swelling and macromolecular relaxation of the polymer and consequently the release of the drug into the external environment. The diffusion of the drug through the gel will occur until equilibrium, and the release kinetics will be determined by water activity, polymer structure and drug concentration (PLUNGPONGPAN et al., 2013; KAMOUN, 2017). The physicochemical properties of the gel, as well as the characteristics of the drug, also influence the release of the latter from the hydrogel.

In recent years, the increasing amount of development and applications of hydrogels as a drug delivery system can be observed, since hydrogels are inert to biological processes, resistant to degradation and heat sterilization and are not absorbed by the body. Hydrogels can replace traditional dressings, which include cotton gauze, and bandages that may adhere to the wound causing dryness, trauma, and pain during dressing removal (SINGH, 2014; LIU et al., 2018). Hydrogels exhibit characteristics of an ideal dressing, such as mechanical stability, flexibility, resistance to pressure and tension, and barrier against external microorganisms. They allow the exchange of gases, do not adhere to the surface of the lesion, have low cost, promote the absorption of exudate and the acceleration of the healing process (PERSON et al., 2014; ABDEL-MALEK, 2017; ZHAO, 2017; KAMOUN, 2017, AJOVALASIT et al., 2018; KOEHLER et al., 2018). Furthermore, the transparency of the hydrogel allows observing and accompanying the healing process under the dressing without the need to remove it (MOZALEWSKA, 2017).

New methods for controlled release of chemotherapeutic drugs, insulins, hormones and antibiotics, such as ciprofloxacin, gentamicin, tetracycline and silver sulfadiazine, have been reported. However, there have been few reports in the literature of the use of hydrogels with neomycin for the treatment of topical infections (KAVIMANDAN, 2006; SINGH, 2012; SINGH, 2014; SKORUPSKA et al, 2014; LIV et. al. 2018; REFAT el. al. 2018).

Hydrogels can be combined with drugs and growth factors for the treatment of topic diseases. The associations can provide a number of benefits to patient such as avoiding the adverse effect of oral administration, avoiding first-pass metabolism, decreasing pharmacokinetic interactions and treating directly to the injury in the locus (ASHARA et al. 2014).

3.4.2 Poly (N-vinyl-2-pyrrolidone) (PVP)

Poly (N-vinyl-2-pyrrolidone) (PVP) hydrogel is a gel formed from the synthetic polymer known as polyvinylpyrrolidone (PVP). PVP is a white powder, derived from cyclic amide polymerization, non-toxic, highly polar with amphoteric characteristics and electrical properties, hydrophilic, complexing ability, stable, biocompatible, easy to process and high transparency (POONGUZHALI 2017; KREZOVIC et al. al., 2017). This polymer was patented by BASF in 1939 and firstly used as a blood volume expander during the World War II. Since then it has been widely required in the pharmaceutical industry for the manufacture of surfactant polymers, emollients, solubilizers and thickeners (ROSIAK, 1993; VILLANOVA, 2010, HALAKE et al., 2014, Kadlubowski, 2014). PVP can be used alone or in combination with other polymers to obtain hydrogels with specific characteristics, and it can also be used in liquid, tablets and films forms (KADLUBOWSKI, 2014; KREZOVIC et al., 2017).

3.5 Skin Structure

The skin is responsible for the body's first barrier of protection and defense against external agents. In addition, it maintains homeostasis, secrets and excrets water and metabolites, regulates temperature, and anchors

sensitive nerve terminals (WYSOCKI, 2000; FERREIRA, 2010). The skin is formed of 3 layers as shown in FIG. 4.

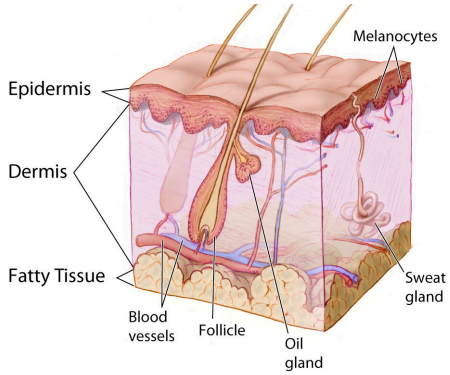

FIGURE 4 - Representation of the skin layers.
https://sv.wikipedia.org/wiki/%C3%96verhud

The outermost layer is known as the epidermis. It is a stratified squamous epithelium with keratinocytes producing keratin, melanocytes and Merckel cells. This layer has little vascularization (WYSOCKI, 2010; ALVARADO-GOMEZ et al., 2018).

The dermis is the second layer, located between the epidermis and subdermal tissue. This layer consists of connective tissue, blood and lymphatic vessels, hair follicle, nerve endings, sweat glands and sebaceous glands. The presence of collagen and elastin fibers in this layer provides skin elasticity (WYSOCKI, 2010; FERREIRA, 2010).

The subdermal tissue is the deepest layer of skin and contains fat cells. This layer is responsible for the mechanical protection against physical trauma, besides being a deposit of energy (WYSOCKI, 2010).

The loss of skin integrity favors the exposure of subcutaneous tissue to the colonization, proliferation and dissemination of opportunistic and pathogenic microorganisms leading to the appearance of lesions, causing pain, exudate and an accompanying fetid smell (TELICHOWSKA et al., 2013; ALVARADO-GOMEZ et al. (Lefebvre et al., 1981).

3.6 Chronic wounds and infections

Chronic wounds are a major public health problem and can be described as skin lesions that fail to heal in the hoped time (POOGOZHALI, 2017, LEFEBVRE, 2018). In general, these lesions are located in regions of the skin with interruption of blood supply (WYSOCKI, 2000; TELICHOWSKA et al., 2013). This type of wound includes venous ulcers, ischemic wounds, decubitus ulcer and is more likely to occur in debilitated patients, such as elderly, diabetic, malnourished, burned people or people with multiple systemic problems (VALAZQUEZ-VELAZQUEZ et al. 2015, and ALVARADO-GOMEZ, 2018). In general, patients who are bedridden or have restricted locomotion are susceptible to developing decubitus ulcers. These lesions are exposed to fecal contamination with a high concentration of anaerobic microorganisms. These bacteria are often opportunistic and take advantage of the patient's low resistance and loss of skin integrity to proliferate (SIDDIQUI, 2010; BRAGA et al., 2013; SINGH, 2013).

It is estimated that chronic wounds affect more than 20 million patients annually, with treatment costs exceeding 20 billion dollars (KOEHLER, 2018).

A common problem encountered in treating chronic wounds is injury infection. This fact is related to the increased morbidity and mortality of patients. Infection may occur by bacteria and fungi from the skin adjacent to the wound, by the professionals responsible for caring for the patient, and even by the local environment (SIDDIQUI, 2010; SIMÕES et al., 2018).

The main bacteria found in wounds are *Staphylococcus aureus, Streptococcus pyogenes, Enterobacteriaceae, Pseudomonas aeruginosa and Acinetobacter baumannii* (SIDDIQUI, 2010; BRAGA et al., 2013; SINGH, 2013).

However, the type of bacteria changes over time, and the longer a wound remains uncured, the greater the probability of acquiring antibiotic-resistant microorganisms (SIDDIQUI, 2010). The presence of antibiotic-resistant pathogens has been associated with the indiscriminate use of antibiotics since their discovery in the 1920s (BASU et al., 2018). This class of microorganisms can contribute to the chronicity of the wounds mainly in debilitated patients (BRAGA et al., 2013).

In general, the treatment involves the discovery of the cause, debridement and removal of the necrotic tissue. Systemic treatment can be performed with the administration of antibiotics such as β-lactams, aminoglycosides, fluoroquinolones and polymyxins. However, these drugs can cause toxicity, teratogenicity and hypersensitivity. In some cases, the low effectiveness of antibiotics may be explained by the low blood perfusion of the affected areas, which reduces the drug's action and increases the risk of developing resistance (LANE et al., 2017). However, the presence of antibiotic-resistant bacteria causes the number of effective drugs to progressively decrease. In general, antimicrobial treatment is long, with high doses and may require prolonged hospitalization of the patient, increasing the cost and contributing even more to the selection of resistant bacteria (KNEZEVIC, 2013; LANE et al., 2017; SIMÕES et al. 2018; TELECHOWSKA et al., 2013).

3.7 Biofilms

Biofilm is defined as an organized growth of bacteria in a three-dimensional community embedded in an extracellular matrix (EM). This matrix is composed mainly of polysaccharides, extracellular DNA, polypeptides and represents up to 85% of the total biofilm biomass (ALVARADO-GOMEZ et al., 2018). The EM allows the maintenance of the biofilm structure, providing physical resistance, stability, protection, as well as water and nutrient retention (LEE et al., 2013; LANE et al., 2017; JUNKA, 2017; THET et al., 2016).

The mechanism of biofilm growth is complex and includes the interaction between three structures: bacteria, surface and also the environment (ALVARADO-GOMEZ et al., 2018). Biofilm formation is initiated by the reversible adhesion of bacteria to a surface, such as a wound or skin lesion followed by microcolony formation. Subsequently, the irreversible binding of the bacteria occurs and begins the production of the extracellular matrix, maturation and growth of the three-dimensional community. From this phase, the biofilm structure can rupture, releasing bacterial cells that may give rise to new colonies and infectious processes (EVEN et al., 2017).

Over the years, increased strains of antibiotic-resistant bacteria have been reported (LEE et al., 2013). This can be explained by the inappropriate

prescription of antibiotics, inefficient drugs, plus many types of bacteria produce virulence factors including toxins and biofilm formation. These virulence factors increase the pathogenicity of the microorganism. The growth in the biofilm form increases the pathogenicity of the microorganism, as it increases the resistance of the bacterium to the treatment with antibiotics in more than 100 times and leading to the treatment inefficiency and the chronicity of the lesions. (LEE et al. 2013; THET et al., 2016; LANE et al. 2017; JUNKA, 2017;)

P. aeruginosa and *S. aureus* are gran negative and positive bacteria, respectively, responsible for infections mainly in debilitated patients. In this context, *P. aeruginosa* is one of the main hospital pathogens and presents resistance to several antibiotics due to its intrinsic and acquired mechanisms of resistance, as for example the capacity of growth in a biofilm. *P. aeruginosa* is capable of producing at least three different polysaccharides known as alginate, Pel and Psl. These polysaccharides are important and determinant for the stability of the biofilm structure. Alginate, for example, provides resistance to biofilms against antibiotic treatment and human antibacterial defense mechanisms (RASAMIRAVAKA, et al., 2015).

Different strategies can be used to combat biofilm formation, which includes: avoiding the binding of bacteria to a surface, preventing the development and preventing maturation of biofilms (GHAFOOR 2011; RASAMIRAVAKA et al. 2017; LEFEBVRE et. al., 2018).

The need to develop new techniques for the treatment of wounds with fewer side effects and that is effective against antibiotic-resistant biofilms and bacteria makes the hydrogel a promising material for this application (KNEZEVIC, 2013; PERSIN et al., 2014; AJOVALASIT, 2018; BASU, 2018). Hydrogels have been widely used as an antibiotic delivery system because it allows the drug to reach the affected area avoiding the drawbacks of systemic use and successive applications of creams and ointments (BOEKEMA et al., 2013). Hydrogel dressings can be impregnated with antimicrobial and antifungal agents, collagen, enzymes, methylene blue, honey, iodine and among others for topical treatment of lesions (KNEZEVIC, 2013; MOGASANU, 2014; WANG 2014; KOEHLER).

3.8 Neomycin

Neomycin is a broad-spectrum aminoglycoside antibiotic produced by *Streptomyces fradiae* and consists of two major components called Neomycin B and Neomycin C. The chemical structure of neomycin is shown in FIG.5.

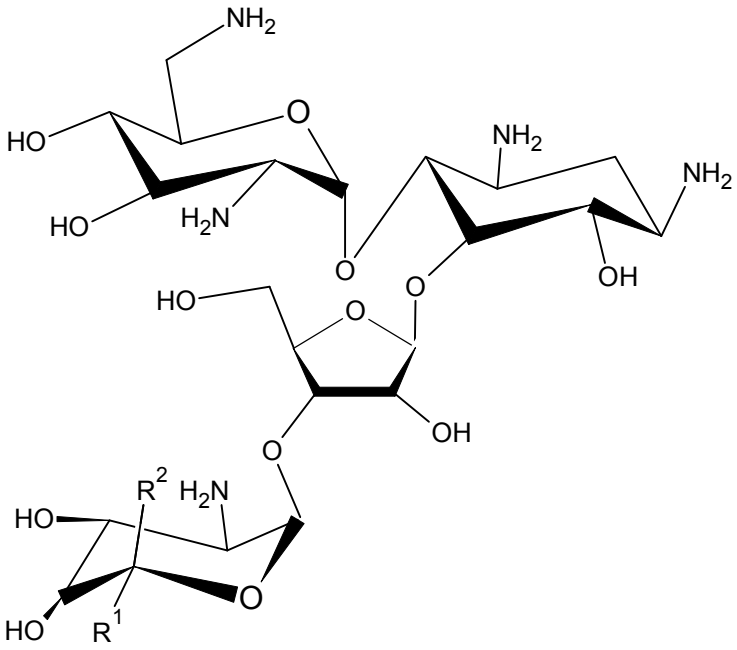

FIGURE 5 - Chemical structure of neomycin B; R1 = CH_2 NH_2; R2 = H_2

This drug has high polarity, low volatility and water solubility. It can be found in topical formulations at concentrations of 5 to 10 mg mL^{-1}. It is available in cream and ointments for the treatment and prophylaxis of skin infections, including traumatic or surgical wounds, boils, abscesses and cutaneous ulcers. There is no absorption by the intact skin, but the injured skin easily absorbs it as the case of large burns or deep wounds. Systemic neomycin is ototoxic and nephrotoxic (KOROLKOVAS, 2010).

The mechanism of action is based on the binding of the drug to the 30s subunit of the ribosome, interference of protein synthesis and disorganization of the cell membrane of the microorganism. Effective against aerobic Gram-negative bacteria and also against some Gram-positive, its use may be associated with other antibiotics such as bacitracin and gramicidin. This synergistic effect increases the spectrum of action of neomycin (KOROLKOVAS, 2010). Other associations can be found with triamcinolone, fluocinolone, dexamethasone, clostebol and nystatin (KOROLKOVAS, 2010).

However, there are no conclusive studies on the use of neomycin in a controlled release system for wound treatment. According to resolution RDC 20/2011 from ANVISA 20/2011 (National Health Surveillance Agency in Brazil – ANVISA), which obligates all drugstores and pharmacies in Brazil to sell antibiotic for only the patients who have two copies of the prescription written by a doctor, a veterinarian or a dentist. In this context, topical neomycin sulfate is a prescription-free drug, therefore hydrogel formulation with neomycin would not require a prescription to be commercialized (BRASIL, 2010).

3.9 "In vivo" test

3.9.1 Animal model: Magellanic Penguin (*Spheniscus magellanicus*)

Magellanic penguins are medium-sized migratory seabirds found in the Atlantic and Pacific Oceans of South America, between the coasts of Argentina, Chile, Uruguay, the Falkland Islands (Malvinas), and Brazil (STOKES et al., 2014; BENEDITTO, 2017).

Penguins live in large colonies with thousands of individuals and do not have apparent sexual dimorphism. Moreover, males are in average 21% larger than females (AKST, 2002). In recent years the reduction of the penguin population has been observed. Such increase of mortality can be attributed to factors such as climate change and human interference in the marine environment due to oil extraction (STOKES et al., 2014).

These animals are birds with a migratory habit. The migration is carried out mainly by young animals that disperse from the colonies in search of the food. The feeding of penguins is based on small fish, cephalopods and crustaceans. After traveling long distances, these animals arrive to the Brazilian coast, weakened and with low immunity due to inanition, exhaustion due to the long trip and also due to the exposure to pollution from the oceans (BRASIL, 2011; STOKES et al., 2014; MWANGI et al., 2016; POLITO, 2016; BENEDITTO, 2017). The animals rescued in the Brazilian coast are taken to rehabilitation centers, zoos and aquariums for treatment and rehabilitation, FIG. 6. With the decrease in the population of Magellanic penguins, the responsibility of the rescue teams and the veterinarians who receive these animals increases,

since it is a wild animal at risk (SILVA FILHO, 2006; NASCIMENTO, 2014; SELLERA, 2018).

FIGURE 6 - Magellanic Penguin (*Spheniscus magellanicus*). Source https://en.wikipedia.org/wiki/Pinguim-de-magalh%C3%A3es and photo personal collection.

In their natural environment, penguins spend most of their time swimming. Their paws are not adapted to remain a long time in contact with the land and rigid surfaces such as of the aquarium. Thus, the challenge encountered by rehabilitation centers during the management of this species is to mimic wild habitat and keep the animals in their routines (SILVA-FILHO, 2006; NASCIMENTO, 2014; SELLERA, et al., 2018).

Among the pathologies found in penguins, pododermatitis or bumblefoot is a multifactorial syndrome that occurs in the paws of birds, especially in captivity. This disease is characterized by an inflammatory and ischemic process in the plantar region with local stiffening, excoriations, swelling up and difficulty of movement. The evolution of the inflammatory process may lead to increased body temperature, abscesses, osteomyelitis, septicemia and necrosis (OSORIO et al., 2017; REISFELD, 2013; BLAIR, 2016; NASCIMENTO, 2014). The evolution of the lesion can compromise the bone structure, tendons and ligaments of the legs, and systemically reach vital organs such as kidneys, liver and pancreas (BLAIR, 2016). The degrees of injury can be classified depending on the severity and clinical stage (OAKS, 1993; BLAIR, 2016) into:

• Initial devitalization of the region without discontinuation of the epithelial barrier, hyperemia and early ischemia;
• Inflammation of adjacent tissues with signs of infection;
• Infection with local swelling, fibrosis and exudate; or
• Infectious vital organ envolvement.

Penguins are animals with a tendency to develop this type of injury, FIG. 7. The change in habitat causes penguins to spend more time out of the water, acquiring sedentary habits and consequently gaining weight. These factors lead to ischemia in the paws and to the appearance of the lesion (Reisfelde, 2013, BLAIR, 2016, OSORIO et al., 2017, SELERRA, 2018).

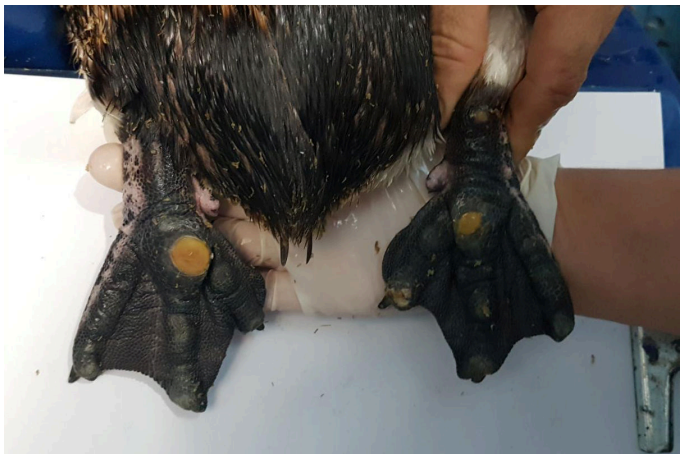

FIGURE 7 - Bilateral Pododermatitis in Magellanic Penguin (Spheniscus magellanicus). Personal collection photo.

A tendency of the penguins to present pododermatitis in both paws can be observed by the follow-up of these animals in rehabilitation centers and aquariums. On the first sign of the lesion in one of the paws, the animal deposits its weight in the adjacent paw, inducing the appearance of the second lesion (COOPER, 2002; OSORIO et al., 2013).

These lesions can be colonized by pathogenic microorganisms, such as *Staphylococcus aureus, Escherichia coli, Klebisiella sp*. and *Pseudomonas aeruginosa* (BLAIR, 2016). Those bacteria can come from the microbiota of the skin, digestive tract and also from human manipulation. With the emergence of

super-resistant bacteria, the treatment of these infections has become increasingly difficult.

Knowledge of the pathogenesis of pododermatitis is well established, but little progress has been obtained in the treatment (COOPER, 2002; NASCIMENTO, 2014). Therefore, prevention is still the best method employed by aquariums.

Preventative actions were proposed by rehabilitation centers to avoid the beginning of these lesions and keeping wild habits of these animals in captivity, such as the practice of exercises in an open environment, access to swimming pools and aquatic activities. This practice allows the control of animal weight and thus decreases the formation of pododermatitis (BLAIR, 2013). However, some penguins may still have these lesions during their lifetime, and treatment is necessary. The treatment of penguins occurs according to the degree of each bumblefoot. Procedures can be performed to reduce pressure and swelling in the affected areas: hygiene, topical and systemic antibiotic therapy, laser application, acupuncture and even more invasive procedure such as debridement of the site. Another option has been the synergism in the use of associated antibiotics and also the alternative use of techniques that prolong the availability of these drugs in the active site (COOPER, 2002; BLAIR, 2013; REISFELD, 2013). Systemic antibiotics are not very effective, due to the low blood perfusion presented by the pathogenesis of the lesions. In this way, drugs are used topically by means of dressings (REMPLE, 2006). In these cases, it is necessary to use materials that ensure an anatomical dressing adapted to the penguin's paw, with mechanical resistance compatible with the weight of the animal and that keeps the site free from recontamination. The recontamination of the area by bacteria can delay healing, cause more pain to the animal and require greater care by the veterinary team.

Traditional dressings with gauzes and bandages have limitations such as frequent replacements, which require more time spent by the veterinary team in handling the animal, as well as penguin stress and pain. The use of a hydrogel has many advantages over cotton dressings, such as flexibility, softness, swelling in the presence of water or liquids without losing its integrity, besides releasing in a controlled and continuous form of the drug. The hydrogel can reduce the impact of the weight of the animal on the paw injured, allowing the

penguin to develop its aquatic activities without prejudice to the treatment. The animal does not need to be isolated during treatment, as the hydrogel does not lose its integrity in humid environments. In addition, the hydrogel is also indicated to prevent recontamination from urine and feces, which allows the dressing to remain longer without the need for replacement. These advantages facilitate the management of the animal in captivity, reducing the trauma and stress due to the animal spending more time in a group.

3.10 Ethical protocol

The project entitled "Preparation, characterization and antimicrobial study of hydrogel impregnated with neomycin for the treatment of topical infections" was analyzed and approved at the 40th Meeting of the Ethics Committee on Animal Use of the Nuclear and Energy Research Institute (CEUA / IPEN) (appendix 1).

The objective of this work is to use the hydrogel with neomycin for the treatment of infected lesions in a group of penguins that already have pododermatitis and have previously been treated with techniques available in their rehabilitation centers. The choice of this animal is justified by the pre-existence of the problem, and it is not necessary to induce an injury or expose the animal to stress of suffering. It is well known to aquarium veterinarians that the incidence of pododermatitis in penguins requires treatment and prevention of injury. In this context the hydrogel appears as a new treatment approach, thus facilitating the management of the animal in captivity.

4. MATERIALS AND METHODS

4.1 Materials

N-vinyl-2-pyrrolidone (PVP) K-90 (Mw 360.000), polyethylene glycol – 300 (PEG), agar and neomycin sulfate were obtained from Exodo Cientifica, Oxiteno, Oxoid and Sigma Aldreich, respectively in pharmaceutical degree. Agar nutrient, LB Broth, Muller Hinton e ISO-SENSITEST agar were purchased Sigma-Aldrich. *Staphylococcus aureus* (ATCC 6835P) and *Pseudomonas aeruginosa* (ATCC 27853 and 481997 A-SPM resistant strain) bacterias.

This work was developed in the laboratories of the Chemistry and Environment Center of the Institute of Energy and Nuclear Research (CQMA-IPEN), the Center for Radiation Technology (CTR-IPEN) and the Biomedical Sciences Institute of the University of São Paulo (ICB-USP), São Paulo Aquarium, Municipal Aquarium of Santos, Aquarium of Ubatuba, Sabina School of Knowledge Park of Santo André, Department of Materials Science and Engineering and the Department of Pathology, Tuskegee University, Alabama - United States

4.2 Preparation of hydrogels

The hydrogels syntheses were performed on the mixture of PVP polymer (6 %wt), PEG (0,45 %wt) and agar (1,5 %wt). The compounds were mixed in water and heated to 85 °C for 5 min, then cooled to 50 °C for the addition of neomycin (0,5 %wt). The solution was placed in polyethylene terephthalate (PET) molded forms in volumes of 10 mL and vacuum-sealed, in the sequence. The sealed samples were irradiated at 25 kGy gamma irradiation dose at 6 kGy h^{-1} rate in order to crosslink and sterilize the polymers gelified solution.

4.3 Swelling behavior

Hydrogels were dried to constant weight. Then they were immersed in distilled water and seawater and weighed each hour for 10, 24, 48 and 72 hours. The swelling was calculated as follows:

$$S\,(\%) = \frac{Wo - Ws}{Wo}\ x\ 100$$

Where *S* is water absorption, *Ws* weight swollen sample, *Wo* is the weight of initial gel sample.

4.4 Determination of gel fraction

Extraction of the sol fraction was carried out by Soxhlet apparatus for 6 h using water as solvent. Then dried at 50 °C to a constant weight. The gel fraction was determined gravimetrically by using the following equation.

$$G\ (\%) = \frac{Wo - Wg}{Wo}\ x\ 100$$

Where *G* is the gel fraction (%), *Wg* the weight of sample after extraction and *Wo*, before.

4.5 Infrared Spectroscopy FTIR

Hydrogels were lyophilized before characterization by Fourier Transform Infrared (FTIR) spectroscopy using KBr pellet prepared by mixing KBr with dried hydrogels samples (10:1 w/w). The TGA analysis was carried out using a Mettler-Toledo TGA/SDTA 851 under an inert atmosphere of N_2 from 25 to 600 °C at the heating rate of 10 °C min^{-1}. The DSC experiments were carried out using Mettler-Toledo 822e under an inert atmosphere of N_2 from 25 to 600 °C at the heating rate of 10 °C min^{-1}

4.6 Neomycin analysis by HPLC-MS/MS

The standard of neomycin sulfate was weighed into volumetric flasks and dissolved with the distilled water to produce a stock solution at concentration 1 mg mL^{-1} (1000 ppm) and successively dilutions were done to obtain five required concentration (20, 50, 100 200 and 400 µg mL^{-1}). Neomycin

concentration was measured by LC-MS/MS (Agilent Technologies 1290 Infinity) coupled with a C18 Aquasil column (100 x 2.1) 5µm and MS–MS system used was AB Sciex 3200 Q Trap equipped with an electrospray interface (ESI). The mobile phase was a mixture of acetonitrile, water and formic acid and a flow rate of 250 µL min^{-1}. The MS-MS analyses were carried out in the positive mode and full scan acquisition 250 – 620 m/z.

4.7 Neomycin stability assay

Neomycin powder, neomycin in aqueous solution and standards of neomycin sulfate with mannitol, PEG and isopropyl alcohol (1:3 w/w) were irradiated at 25 kGy dose. The samples were analyzed by LC-MS/MS. Neomycin pure no irradiated was used as the control.

4.8 Release of neomycin

Hydrogels samples were placed in beakers and immersed in 100 mL distilled water and incubated at 37 °C under agitation of 80 rpm. 2 mL aliquots were retained at times intervals of 2, 5, 8, 10 and 15 min, 1, 1.5, 2, 4, 8, 24, and 48 h, and were replaced by 2 mL of distilled water at aliquot. These samples were further analyzed by LC – MS/MS.

4.9 Thermal analysis

The hydrogels were lyophilized and thermogravimetric analysis (TG) and derived thermogravimetry (DTG) were carried out from Mettler Toledo equipment. A sample mass of 5.0 ± 0.1 mg was weighted. The program consisted in the heating rate of 10 °C min^{-1} in the range of 25 to 600 ºC, under N_2 atmosphere flowing at 10 mL min^{-1}. The DSC curves analyses were carried out using Mettler Toledo equipment. The samples mass of 5.0 ± 0.1 mg were heated at 10 °C min^{-1} from 25 to 350 °C in a nitrogen atmosphere at 10 mL min^{-1}.

4.10 Cytotoxicity assay

According to the method proposed by ROGERO (2003), the cytotoxicity tests of hydrogels loaded neomycin were carried out *"in vitro"* using the incorporation of the neutral red method.

Hydrogels samples measuring 2.0 X 2.0 cm were placed in sterile Becker and dried in an oven at 100 °C until constant weight.

Eagle culture medium, sodium pyruvate, nonessential amino acids and fetal bovine serum were used to obtain extracts at concentrations of 100; 50; 25; 12.5 and 6.25 %. HDPE (high density polyethylene) was used as negative control and natural rubber Latex, as the positive control. Aliquots of 200 µl of each dilution were transferred into 96-well microplate wells containing mouse connective tissue cells (NCTC L929) and incubated at 37 °C for 24 h.

After incubation, the sobrenadantes were discarded and 200 µL of neutral red solution was added. After 3 h of incubation at 37 °C, the supernatants were discarded and the wells were washed with phosphate buffered saline pH 7.4 and 200 µL of wash solution (10 % $CaCl_2$ in 0.5 % formaldehyde solution).

The percentage of cell viability was calculated by means of optical density values obtained at 520 nm in a Tecand Sunrise model spectrophotometer in relation to the control cells in the assay.

4.11 Antimicrobial activity

4.11.1 Preparation of Bacteria Inoculum

P. aeruginosa and *S. aureus* were cultured in LB agar and incubated at 37 °C for 24 h to obtain isolated colonies. One colony was inoculated in LB broth at 37 °C for 24 h. The density optical was measured to turbidity corresponding to a spectrophotometric absorbance at 0.08 at 540 nm, which is equivalent to a bacteria inoculum size of approximately 10^6 CFU/mL.

4.11.2 Disc diffusion test

Antimicrobial effect of the hydrogel / neomycin was carried out by disc diffusion with Gram negative *P. aeruginosa* and *S. aureus* strain. Bacteria were incubated at 37°C in LB broth overnight. The sterile cotton swabs were dipped three times in bacteria suspensions gently spread onto ISO-SENSITEST agar plates. The hydrogels were cut in small circular disks and gently placed on the bacterial lawn. The plate was incubated overnight at 37 °C. Ciprofloxacin was used as positive control and hydrogel without neomycin, as the negative control. The inhibition halos were observed and measured with a ruler. Images of the plates were taken using Aplha Imager HP. The experiments were carried out in triplicate. After 24 h incubation, the hydrogel disk was taken, transferred to freshly seeded plates and incubated for the same time. The diameters of the inhibition halo were measured. This method was carried out until the inhibition halo decreased to zero.

4.11.3 Antibacterial activity of hydrogel in bacterial suspension

Hydrogels samples were cut into small circles of 8 mm in diameter and incubated in tubes with 500 µL of bacterial suspension (10^8 CFU / mL) in 3 mL of LB broth. The tubes were incubated at 37 °C for 24 h at 50 rpm. Tubes containing LB broth and bacteria in suspension were used as negative and positive controls, respectively. After 24 h of incubation, the tubes with the samples were compared with the controls to infer bacterial growth or biocidal effect of the samples.

4.12 Time Kill curve

P. aeruginosa (ATCC 27853 and 481997 A-SPM) and *S. aureus* (ATCC 6835) strains were individually inoculated in Muller Hinton broth and adjusted to 0.5 McFarland scale and subsequently diluted to 10^6 CFU mL^{-1} concentration. Aliquots of 1 mL of liquid medium, 50 µL of the bacterial suspension and the hydrogel sample were added in a 12-well plate. An aliquot of each well was taken at times 0; 2; 4; 8; 12 and 24 h and placed in agar plates. The

MacConkey medium was used for the *P. aeruginosa* and Muller Hinton for the *S. aureus* strain. The result of the time kill curve was plotted as a graph in relation to CFU and time.

4.13 Measurement of biofilm growth

P. aeruginosa ATCC 27853 was used initially to optimize the parameters of biofilm formation. Bacteria were grown in LB broth overnight and optical density (OD_{540nm}) were adjusted by 0.08 using Gen 5 2.0 Powerwave XS spectrometer, considering a concentration of approximately 10^6 CFU/mL. Biofilm quantification was measured in 96 well plates and the assays were tested in triplicate. Aliquots 100µL of bacteria suspension were incubated in 96 well plate for 24 at 37 °C under stationary condition. After incubation, the absorbance was read at 520 nm. For easier interpretation of OD results, strains were classified into three categories according to OD: no biofilm or weak biofilm producer (ODs ≤ OD or ODc < ODs < 2xODc), moderate biofilm producer (2xODc<ODs<4xODc) and strong biofilm producer (ODc <ODs), where ODs is the optical density of the strains and ODc was established an average OD of negative growth control + 3x SD of negative control (SPASOJEVIC et. al., 2016; GONÇALVES et. al., 2017).

Evaluate to establish biofilm of *P. aeruginosa* and *S. aureus* was carried out placed three sterile slides in sterile Coplin jar containing 30 mL LB broth, added 100µL bacterial suspension and incubated at 37 °C to grow the biofilm at the liquid-solid air interface. Slides were taken out aseptically at the different times (24h, 48h, 72h and 7 days) fixed in the flame three times, covered with methylene blue for 30 seconds, washed with water and air dried. The slides were examined microscopically under Olympus Cellsens Dimension Microscope and photographed.

4.14 Efficacy hydrogel against established biofilm

4.14.1 Treatment of stablished biofilm in 96-well plate

For biofilm treatment, aliquots of 100 µL of *P. aeruginosa* and *S. aureus* bacterial suspension with approximately 10^8 CFU/mL were incubated in a 96-well plate at 24 h at 37 °C. The supernatant was discarded and the wells were washed with sterile distilled water. The hydrogel was immersed in LB broth for 24 h for the release of neomycin from the polymeric matrix and 100 µL of medium was added to the wells. The plate was incubated for 24 h at 37 °C and OD was measured at 540 nm. Wells with bacterial suspensions were used as positive controls, while wells with LB broth were considered negative controls of the tests.

4.14.2 Treatment of stablished biofilm in slides

Sterile slides were placed in a Coplin jar containing 30 mL LB broth and 100 µL bacterial suspension. The jar was incubated for 24 h at 37 °C. Slides with biofilm were removed from the jar, rinsed with sterile distilled water to remove no-adherent cells and covered with hydrogel/neomycin. The slides were placed in the conical tube for 24h at 37 °C. Then, 30 mL LB broth was added in tube and vortexed by 30 seconds. In a dilution series, 10 µL each dilution were plated on Isosentitest agar plates and incubated at 37 °C for 24 h. Colonies were counted and the results were given by the reductions of colony forming unit per mL (CFU mL^{-1}). All experiments were evaluated in triplicate and the final results are reported in a graph as the mean with standard deviation.

4.15 Microbial penetration

The hydrogel was tested to measure the efficiency to prevent microbial penetration in the wound. For that, 3 mL LB broth were placed in conical tubes. Hydrogels were cut and placed in the top of the tubes to seal them. The tubes were incubated in the ambient environment and OD540 nm of the medium was read on different days. All tests were carried out in triplicate.

4.16 Scanning electron microscopy (SEM)

The slides with biofilms were treated in 2% glutaraldehyde solution and dehydrated by dipping them in ascending concentration of ethyl alcohol. SEM images were obtained on the FE-SEM (Jeol JSM-7200F) microscope with an increase of 3700, 4500, 5000 and 6000x

4.17 Evaluation of the treatment of pododermatitis in penguins

The management and treatment of the animals were carried out in the aquariums of São Paulo, Ubatuba, Santos cities and Sabina School of Knowledge Park, in Santo Andre city.

The lesions of pododermatitis in penguins were hygienized with 2 % chlorexidine solution and debrided. These two procedures were performed according to the protocols of each aquarium.

The hydrogels were applied on the lesions and wrapped with a bandage for fixation. The penguins were kept in an aquatic environment and the dressings were changed every 5 days. Photos were taken before and after the application of the hydrogel to evaluate the evolution of the treatment.

5 RESULTS AND DISCUSSION

5.1 Preparation of hydrogel / neomycin

After the irradiation process, the hydrogels presented an insoluble three-dimensional structure, transparency, elasticity and softness. These characteristics are required for application as dressings. FIG. 8 illustrates the hydrogel after irradiation.

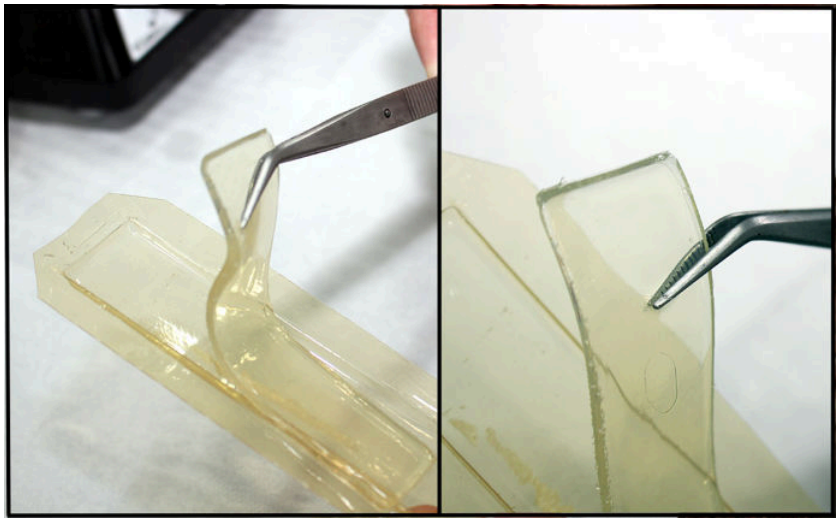

FIGURE 8 - Hydrogel / neomycin obtained by ionizing irradiation, at 25 kGy dose.

The softness and elasticity can be explained by the presence of PEG in the hydrogel formulation. During synthese of hydrogel, PEG is positioned between the polymer chains that decreasing the physicochemical interactions of the polymer chains during irradiation and resulting in greater flexibility. Thus, the concentration of PEG directly influences the rheological characteristics of the hydrogel (LUGÃO, 2002; AJJI et al., 2005).

Synthesis of the hydrogel by gamma irradiation method showed to be easy in process control, low process cost and concomitant membrane sterilization (CHAPIRO, 2002; ROZIAK, 2003; LUGÃO 2007). Thus, it can be inferred that the concentrations of the raw materials, the preparation technique and the dose employed were adequate to obtain the hydrogel with the desirable characteristics.

5.2 Swelling Behavior

The swelling assay was performed with samples of hydrogels obtained with 15, 20, 25 30 and 40 kGy and two different medium: distilled water and seawater (water from the sea). Seawater was applied for the swelling test because the animal model used in the "in vivo" test has a marine aquatic habit.

The results of swelling of hydrogels as a function of time in distilled water and seawater at room temperature can be observed in FIG 9 and 10 respectively.

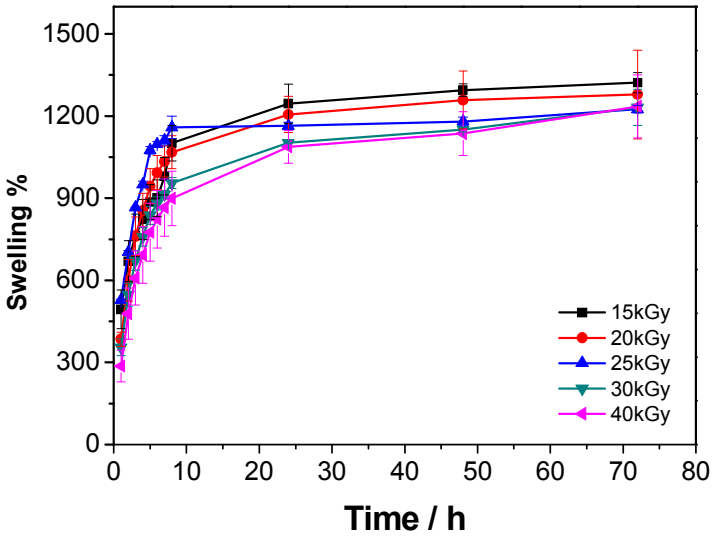

FIGURE 9 - Swelling curve in seawater of hydrogels obtained by gamma irradiation at different doses.

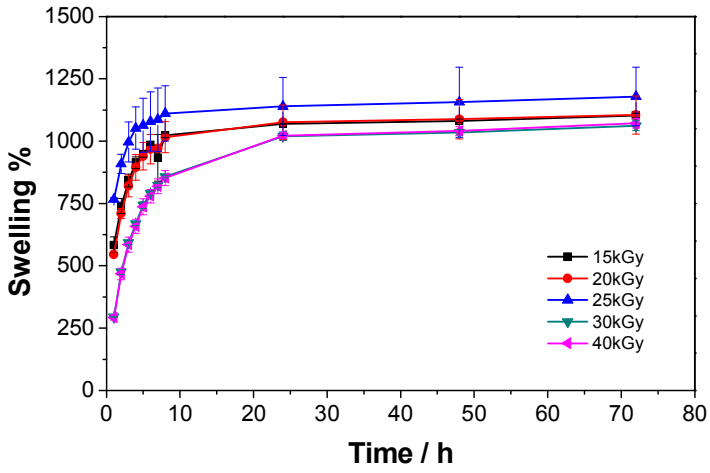

FIGURE 10 - Swelling curve in distilled water of the hydrogels obtained by gamma irradiation at different doses.

The water penetrated and diffused into the hydrogel rapidly in the first few hours and the maximum swelling reached equilibrium after 24 hours of immersion.

After 24 hours of immersion, the hydrogels showed a weak change in swelling. The maximum swelling was achieved at the dose of 15 kGy. The maximum peak reached was 19 % higher in seawater, after 72 h.

One of most important characteristic of a hydrogel is swelling capacity without losing its form and the water cannot be removed from hydrogel under pressure (SOOD, 2017). Several parameters affect the swelling ratio such as hydrophilicity attributed to the presence of hydrophilic groups on the polymer chain, stiffness, polymer concentration, plasticizer, irradiation dose rate. Those factors modify the average molecular weight crosslinks and thus the spaces between them. This influences the structure and water absorption (ROSIAK et al., 1995; PEPPAS et al., 2000; AJJI, 2005). The swelling can be explained in steps. Swelling increased rapidly during the first period in contact with solution due to the porosity of the crosslinked polymer, enabling water to diffuse rapidly. This relaxation of the polymer chains allows diffusion of the water into the hydrogel interface until saturation, in this moment the maximum swelling is achieved (SOOD, 2017). This makes the structure stable for fluid retention and integrity of wound dressing (PEPPAS et al., 2000; AJJI, 2005; ADL-ALL, 2007; SKORUPSKA et al., 2014).

5.3 Gel fraction test

The TAB. 1 shows the results of the gel fraction in a function of the irradiation dose.

TABLE 1 – Gel Fraction versus dose of irradiation

Dose kGy	% Gel Fraction
15	95.4 ± 0.4
20	96.3 ± 0.9
25	97.7 ± 0.8
30	98.4 ± 0.2
40	99.1 ± 0.3

The increase of the gel fraction was proportional with increasing irradiation dose. The gel fraction is directly influenced by the polymer concentration, irradiation dose and presence of plasticizing agent. AJJI et al. (2005) studied the relationship between the concentration of PVP and the gel fraction. The results obtained by this author showed that increasing the concentration of the polymer increases the crosslinking density and the insolubility of the PVP hydrogel; and 6 % (w/v) PVP results in a gel fraction above 94 %. However, the concentration of PEG decreases the crosslinking density due to its plasticizing effect (LINK 2002; LUGÃO 2002; AJJI 2005).

In our work, a concentration of 0.45 % (w / v) PEG was used in relation to the total weight of the membrane and a gel fraction of 97.0 ± 0.5 % was obtained.

5.4 LC – MS/MS method

The reference method described in the European and American pharmacopoeias (USP) for antibiotic determination is the bioassay or turbidimetric assay method, however, this method has given low efficiency and reproducibility. Other analytical methods may be employed, for example, LC-MS/MS (ORTEL, 2004; STYPULKOWSKA, 2013; APYARI, 2013).

Aliquot of neomycin sulfate solution standard of 1000 µg mL^{-1} was introduced into the MS-MS system in positive full scan mode to recognize fragmentation pattern. The peaks obtained in the fragmentation are described in FIG. 11.

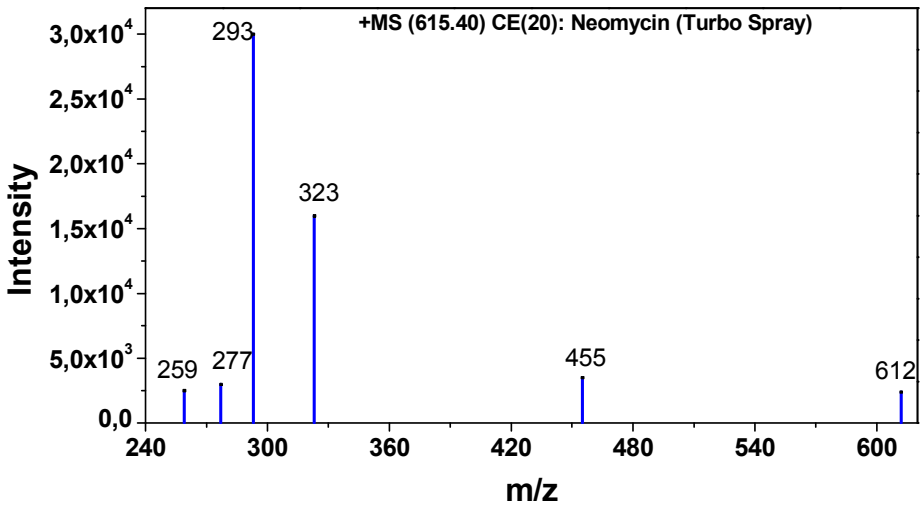

FIGURE 11 - LC-MS positive full scan mode with neomycin fragments peak.

Peak at 612.3 m/z was attributed to the protonated molecular ion of neomycin [M+H]+ (the precursor ion), and five others peaks (259, 277, 293, 323 and 455 m/z) corresponded to the protonated ions resulting from the proposed fragmentation scheme (ORTEL, 2004; STYPULKOWSKA et al., 2013).

5.5 Calibration curve of neomycin

A standard solution at 2500 µg mL -1 was prepared followed by successive dilutions to obtain concentrations of 250, 500, 1000, 1500, 2000 and 2500 µg mL^{-1}. The standards solutions were analyzed by LC-MS / MS and the calibration curve obtained is shown in FIG. 12.

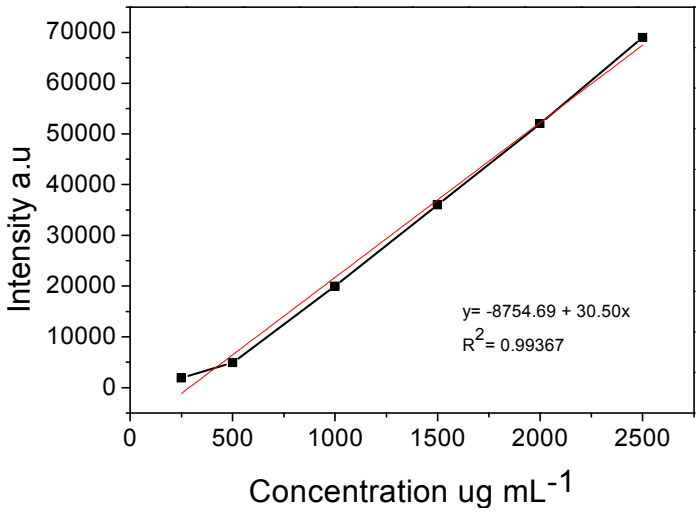

FIGURE 12 – Calibration curve of neomycin

5.6 Effect of gamma irradiation on the neomycin stability

Neomycin solution was irradiated separately with 1.5% (w / w) mannitol, PEG and isopropyl alcohol at 25 kGy and analyzed by the LC-MS / MS method. The results of the calibration curves of each sample were compared with neomycin non irradiated and neomycin powder sample irradiated at 25 kGy and the results are shown in FIG. 13.

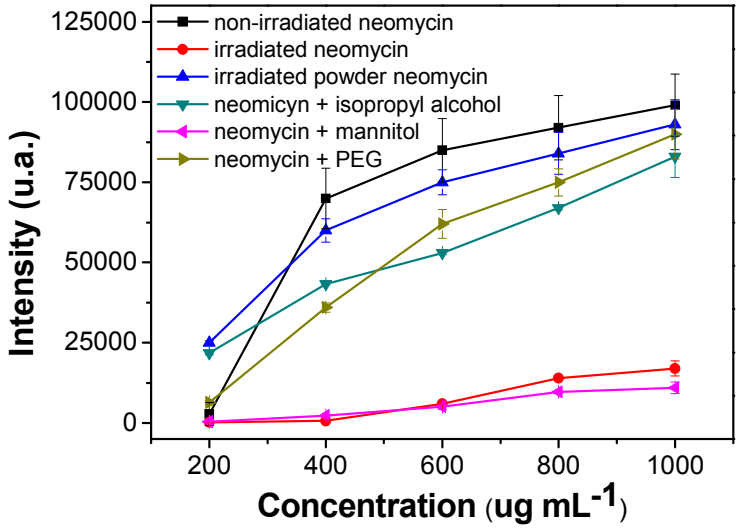

FIGURE 13 - Neomycin stability under irradiation at 25kGy. Comparison between different radioprotectors.

Irradiated Neomycin solution in the presence of PEG and isopropyl alcohol presented peak intensity reduction of 9.01 and 16.17%, respectively. These results indicate that PEG and alcohol showed a protective effect of neomycin against the free radicals produced by the gamma radiation. LC-MS/MS analysis showed that the neomycin aqueous solution decreased 80% of its intensity after irradiated at 25 kGy.

Gamma irradiation has been explored to produce hydroxyl free radical for oxidation pharmaceuticals in treatment wastewater to avoid environmental bioaccumulation. In this way, it is possible to compare the dose used in this method to degrade these compounds in water and to discuss the relation of the impact of the radiation on the drugs during crosslinking. In summary, drug degradation rate increases with increasing radiation dose when drug was irradiated in aqueous solutions. Previous studies have been evaluated and the results showed that amphetamine solution was completely degraded at 2,8 kGy and anti-inflammatory drugs such as ketoprofen, and diclofenac were stable until 5 kGy dose. Antibiotic degradation in aqueous environment is well documented, with many research evidenced: chlorafenicol was destroyed with 7 kGy, while penicillin and amoxicillin were degraded with dose above 12 kGy (CHOI et al., 2016; SINGH, 2017; YASASVINI, et al., 2017; SINGH, 2017).

In this context, gamma radiation has revealed to be an important method to degradate pharmaceuticals in wastewater. However, it remains unclear what impact radiation has on the stability of the drug when it is irradiated with excipients and polymers for obtaining hydrogels. Considering neomycin loaded hydrogel, drug stability was evaluated under radiation in excipients presence such as mannitol, PEG, isopropyl alcohol, and quantitatively analyzed by the HPLC-MS/MS method. It can be observed in FIG. 13 that the irradiation process influenced in a different way the stability of each sample. The non-irradiated neomycin obtained the highest intensity, followed by the irradiated powder sample. Aduhonoglu (2010) observed in their studies a reduction of 5% in neomycin concentration as a result of irradiation at 25 kGy dose. In our work, neomycin was irradiated in powder, no presence water, so it can be affirmed that no free radical from radiolysis was produced and there was not degradation (WICTTERLE, 1960; ADUHONOGLU, 2010).

The results of LC-MS/MS analysis showed that neomycin solution decreased 80% its intensity after irradiated at 25 kGy. Gamma radiation is known as an effective method to degrade pharmaceutical in water presence due mainly hydroxyl radical produced from radiolysis. Therefore, it is important to study the behavior of neomycin under the radiation, as well as its interaction of the other components of the formulation.

In pharmaceutical industry, non-pharmacological active substances, known as excipients, may be incorporated into the production of medicine. When present, excipients improve the properties active pharmaceuticals. It has been reported that some excipients may interact with free radicals from water radiolysis decreasing degradation of pharmacological agents under radiation (KADLUBOWSKI, 2010; HALAKE, et al., 2014; SINGH, 2017).

Mannitol is an alcohol from mannose family vastly used as excipient. Neomycin sample was irradiated with mannitol at 25 kGy dose. Stability curve obtained presented similar result to the irradiated neomycin solution, thus indicating the degradation in both samples. This result proved that mannitol had no protective function. Although, Slegers (2006) studied metoprolol degradation under gamma radiation suggested that radiolysis of metoprolol powder, at the dose of 25 kGy in the solid-state, is not enough to degradate it (SLEGERS 2006; NITANAN et al., 2013). And when metropolol was irradiated in solution in presence of mannitol, the later reacts quite well with hydroxyl radicals and protect the drug against gamma degradation. The appropriate choice of excipients, contributed to radioprotecting pharmaceutical, but depends on the concentration of the radioprotector. In low concentration may be insufficient for the effect.

The influence of PEG as radioprotector is also observed in FIG 13. The results showed radioprotector function for PEG. It can be easily explained once PEG has high solubility. When in aqueous solution, PEG chains remained around the neomycin molecule and this effect can be utilized to control the free radical attack protecting neomycin from degradation during the crosslinking process (LUGÃO et al., 2002; PARK et al., 2012; FRYKBERG, 2015). Different concentrations of PEG have been employed on the hydrogel formulation to modify the crosslink density and swelling degree. Studies have reported that addition of PEG to the hydrogel composition could improve efficiency of the

hydrogel barrier against microrganism from environment and prevent secondary infection (AJJI, 2005; LUGAO, 2001; SOOD, 2017).

These characteristics of PEG provide important information and explain the importance of the presence of the PEG in the hydrogel formulation.

It is well known that drug stability also depends on chemical reactions that can occur during the manufacturing process. These chemical reactions include exposure to heat, moisture, light, or an oxidizing atmosphere for example free radical from radiolysis. Many excipients are employed to increasing shelf life of drugs and protecting them against humidity, atmospheric oxygen and thermal degradation.

5.7 TG/ DTG e DSC curves

TG / DTG assay were carried on polymer PVP, PEG, agar and hydrogel samples to investigate the thermal stability of each component and the hydrogel.

In FIG. 14 and TAB. 2 it can be seen that the first events between 25 and 120 °C are characteristic of water loss. This water may be residual, mainly in hydrophilic compounds such as PVP, PEG and agar (BRANT, 2008).

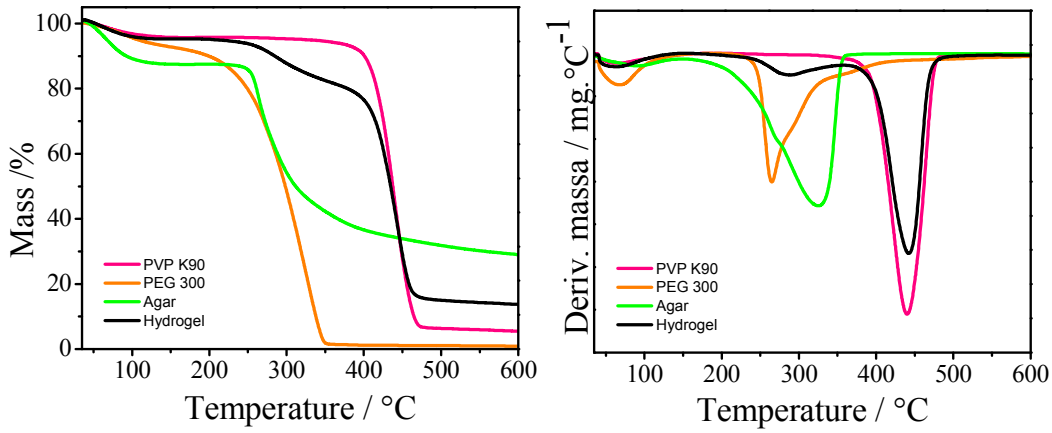

FIGURE 14 - TG/ DTG curves

TABLE 2 - TG / DTG degradation values for PVP, agar, PEG and hydrogel, under nitrogen flow.

Samples	Mass loss / %	T onset (°C)	T endset (°C)	Degradation peaks (°C)
PVP	4.0	35.0	134.2	64.6
	89.3	364.8	478.5	440.0
PEG	6.7	34.2	140.7	90.5
	91.8	140.7	367.1	324.9
Agar	12.5	35.5	171.5	68.0
	58.4	230.2	600.0	265.0
Hidrogel	4.6	36.8	156.9	64.8
	12.7	229.2	357.7	289.4
	66.8	357.7	495.7	442.1

The results illustrate that the decomposition of PEG and agar began at 140.7 and 230.2 °C, respectively. PVP is a thermally stronger polymer and degraded at about 364.8 °C. The hydrogel ixhibited three stages of loss of mass, the first one was attributed to water loss, the second to the decomposition of PEG and agar, and the third to the degradation of PVP, in the range of 364.8 - 478.5 °C. FIG. 15 illustrates the profiles of the TG / DTG curves of the hydrogel, neomycin and hydrogel / neomycin. The mass loss and the corresponding temperature ranges are shown in TAB. 3.

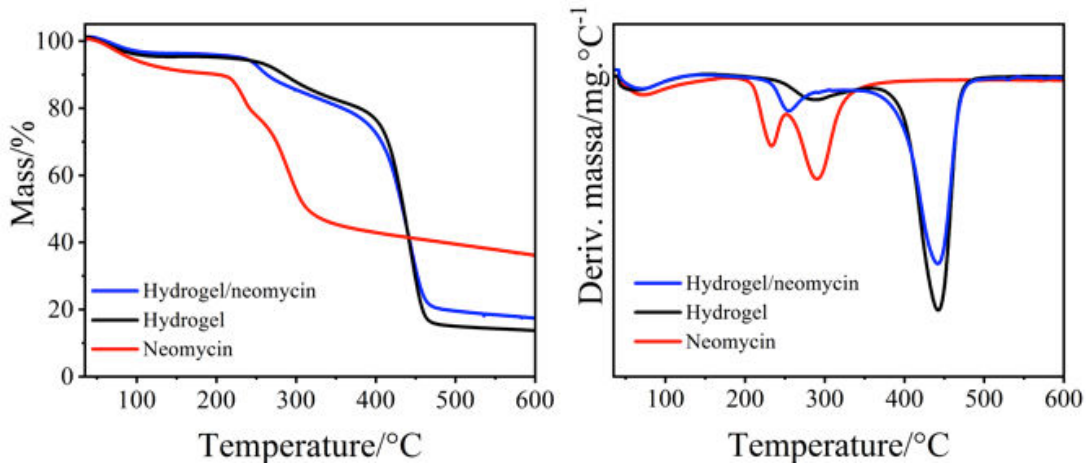

FIGURE 15 - TG curves for non-irradiated neomycin, hydrogel/neomycin and hydrogel, obtained under N_2 flow.

TABLE 3 - TG data of degradation for neomycin, hydrogel/neomycin and hydrogel under nitrogen flow.

Sample	Mass loss (%)	T *onset* (°C)	T *endset* (°C)	DTG max (°C)
Neomycin	9,5	35,9	179,9	71,8
	12,9	185,8	251,5	233,3
	33,8	251,5	384,4	290,5
Hydrogel/neomycin	3,7	40,6	142,2	70,8
	13,0	175,4	325,6	254,7
	61,8	343,1	499,6	441,7
Hydrogel	4,6	36,8	156,9	64,8
	12,7	229,2	347,7	289,4
	66,8	357,7	495,7	442,1

The difference melting point between samples reported in FIG. 15 can be attributed by the effect of gamma irradiation. Irradiated neomycin curve had a shift to the left, with a melting peak of 3 degrees lower than non-irradiated neomycin curve. FIG. 15 shows the TG / DTG profile of the non-irradiated neomycin, hydrogel and hydrogel/neomycin.

No irradiated neomycin exhibited a mass loss of 9.5 % to 179.9 °C, which is mainly due to water loss and it was stable up to 185.8 °C, above which it begins to decompose into two distinct peaks around 233.3 and 290.5 °C, with a residual mass of 43.5 %.

In the TG/DTG curves of hydrogel/neomycin, second step is shifted toward lower temperature compared to the hydrogel, indicating the presence of neomycin degradation. From TAB. 2, it can clearly see that neomycin present in hydrogel exhibit higher thermal stability than pure. The major mass losses are observed in the third decomposition step, in the range of 343.1- 499.6 °C. This peak is characteristic of degradation PVP.

TG profiles clearly showed that neomycin can be heated and gamma radiation sterilized because its melting peak and Tonset decomposition are above sterilization temperature by autoclave (121 °C) as sterilization temperature and hydrogel showed Tonset above 176 °C, resulting high thermal stability.

Therefore, neomycin as a topic antibiotic, it is not necessary to be sterile, but wound dressing should be due to the manipulation process.

It is important to add other event in this discussion. The initial solution concentration could influence in degradation rate. The solute increase could increase concentration of the free radicals from radiolysis, in competitive effect. In view of the fact that hydroxyl radicals and hydrogen atoms interact with polymer to crosslink reaction, few reactive radicals were available to degrade the neomycin.

When an interaction occurs between excipient and drug, the physicochemical properties such as solubility, hydrolysis, oxidation and polymerization, may be modified, and thus the stability can be changed. A DSC is frequently used to provide information of the thermal properties of the solid pharmaceuticals and it has been used to evaluate drug such as angiotensin converting enzyme, lisinopril and enalapril. Thermal stability study of the solid pharmaceutical drugs is important to evaluate losing of the drug activity, degradation rate or also toxins generation that can decrease the effectiveness and increase the toxicity of the drug (LIN, 2012).

For DSC evaluation of neomycin thermal stability, the neomycin was irradiated at 25 kGy dose, and its stability was compared with non irradiated sample. In the results shown in FIG. 16, two melting peaks can be observed with maximum values of 154.42 and 158.63 °C.

Neomycin is composed of two isomers (B and C), and their concentration can change according to the production process. However, the B isomer concentration is always higher than C. In the DSC profile, the first peak corresponds to the crystalline fusion of the C isomer, and the second is associated to B isomer.

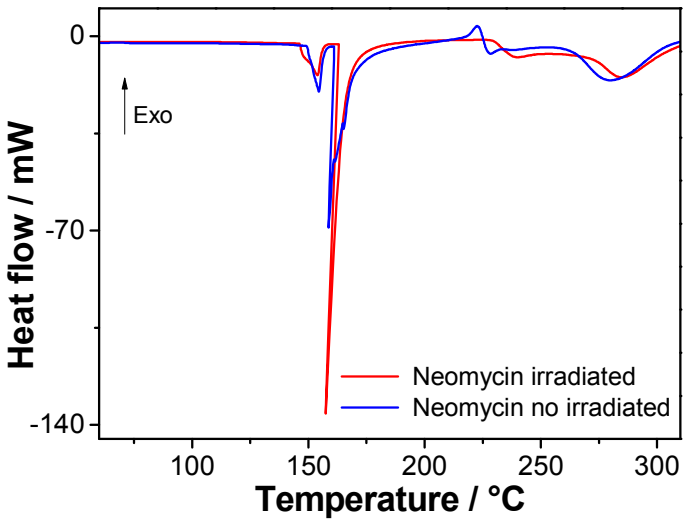

FIGURE 16 - DSC profiles of neomycin irradiated and non-irradiated

Non-irradiated neomycin curve overlapped with date of the irradiated neomycin. Irradiated neomycin powder has shown minimal degradation when undergoing gamma radiation dose of 25 kGy

In irradiated samples curve, the C isomer melt has a T_{onset} 146.32 °C, and the B isomer, 163.10 °C, where 398.86 mJ was required to complete the C isomer melting and 2666.53 mJ, for the B isomer.

In the non-irradiated neomycin curve, the C isomer melting was found to have T_{onset} 149.75 °C, and the B isomer, 160.97 °C, where 402.57 mJ was required for C isomer and 1967.28 mJ for B isomer.

According to these results, it was possible to inquire the ratio between B and C isomers. The ratio was 87/13 (w%) for non-irradiated neomycin and 83/16 (w%) for irradiated neomycin. Similars results were reported by Roetz (1995) in their studies for the determination of neomycin isomers by liquid chromatography.

5.8 FTIR spectra

Functional groups of the hydrogel and composition were characterized by FTIR. The FIG 17 shows some characteristic peaks of PVP at 1286 cm^{-1} for

C-N stretching vibration of amid group (C=N), presence of C=O stretching adsorption peaks in 1652 cm^{-1}.

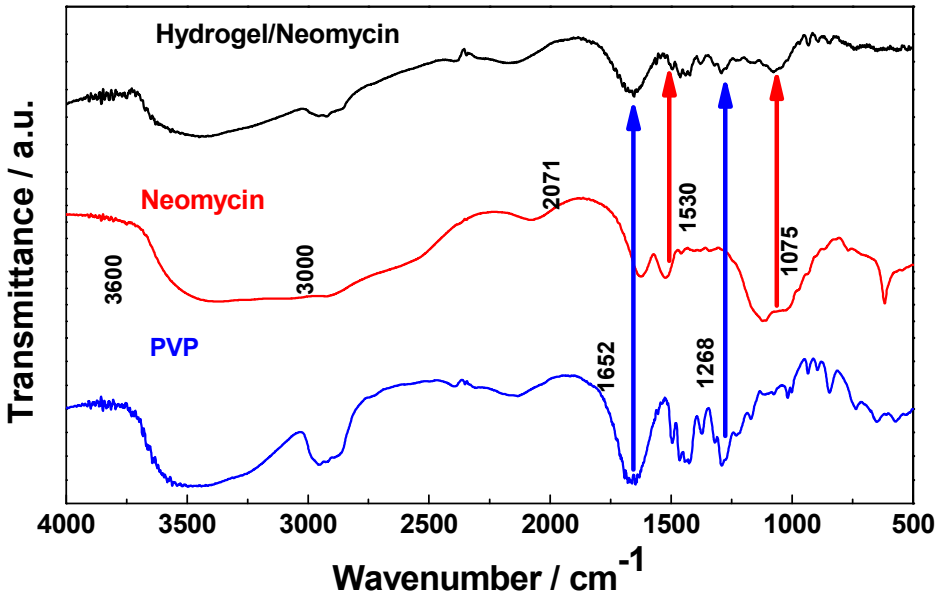

FIGURE 17 - FTIR spectra of PVP polymer, neomycin and hydrogel/ neomycin synthetized by irradiation at 25 kGy

According to the literature, vibration carbonyl stretching around 1650 and 1680 cm^{-1} is assigned to the presence of PVP. Peaks between from 3000 to 3500 cm^{-1} are attributed to water presence. In the neomycin spectrum, the peak at 2071 cm -1 is derived from NH_3^+ (MA, 2009; ABDEL-MOHDY, 2013; LIAN, 2013).

5.9 Drug release assay

The release kinetic of the drug is illustrated in FIG. 18, where neomycin released from hydrogel was evaluated for 48 h.

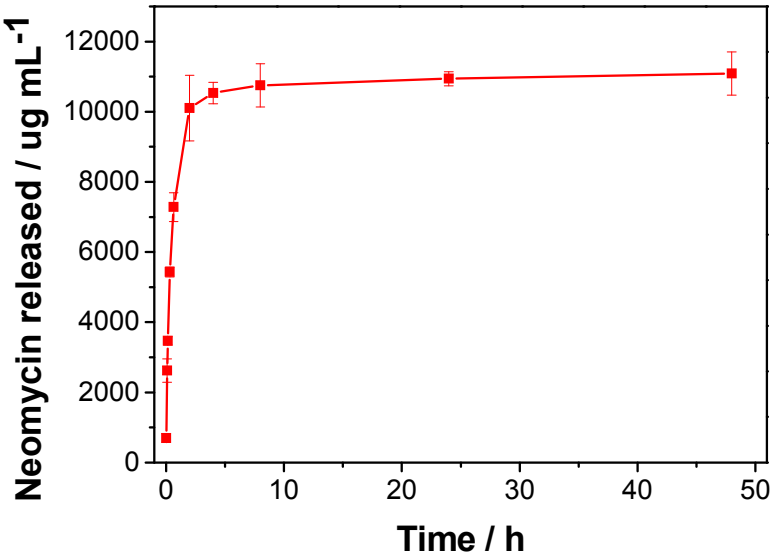

FIGURE 18 – Drug release curve of neomycin.

It is observed that neomycin was released fastly in the early hours of experiment with the maximum release concentration reached at 8 hours and remaining stable up to 48 hours, the testing time.

The drug release method or also known as the dissolution method is used in the development and quality control of pharmaceuticals. The dissolution process can be influenced by the rate of dissolution of the drug, the solubility of the drug in the medium and by the reaction time.

Some mathematical models are employed to describe this process, among them, Fick's law, which relates dissolution to the diffusion process (RITGER, 1987; ABDOU et al., 2000; RODRIGUES, 2008). The diffusion process is the passive movement of solute from a medium in which it is in highest concentration to the medium of lower concentration. The difference of concentration between the two media is defined as the concentration gradient and diffusion process continue until the concentration in both media reach an equilibrium, in that point, the gradient is eliminated. Thus, the rate of drug release is proportional to the difference between concentrations in the membrane and the aqueous medium, that the hydrogel is immersed.

Higuchi's diffusion law is a mathematical model derived from Fick's law and it can be used to explain the release kinetics of neomycin from polymer matrices because the drug release from hydrogel, via diffusion, is dependent on the swelling. The hydrogel with neomycin is a polymeric membrane, in which case, the interaction of the drug with water occurs in two stages. When the hydrogel is immersed in water, the

neomycin that is on the surface of the hydrogel is released rapidly. The second step is related to the swelling of the matrix. When water penetrates in the hydrogel, a relaxation of the three-dimensional structure occurs and neomycin is released from the system. This step is limiting in the dissolution process and the surface area and membrane thickness also affect the process (RODRIGUES, 2008; CHITRATTHA, 2016; MAHINROOSTA et al., 2018).

The immediate release drug products are developed to release the drug rapidly after administration, reach the maximum concentration and valleys associated with absorption of the drug. The release curve is described as a peak (ALLEN, 2014). On the other hand, modified release pharmaceutical forms are developed to modify the release of the drug and prolonging its rate of dissolution. In this way, the drug dissolves in a certain volume of liquid, forming a saturated solution. The release will be constant as long as saturation is maintained inside (BURI, 1997). The polymeric matrix was shown to be very useful as a matrix for controlled releasing formulations, since it was possible to observe this release in increasing form until reach the peak and maintaining its drug percent concentration as a function of time, over an extended period of time.

The results illustrated in FIG. 18 show that the neomycin was released fastly until 8 hours because there was a high gradient between the aqueous medium and the hydrogel. In fact, for the treatment of topical infections, it is indicated that the antibiotic to be rapidly released from the matrix because its pharmacological effect depends on the concentration in the site of action (ANSEL, 2007).

In addition, after 8 h of the experiment, neomycin release rate remained constant until 48h. In the releasing system, when the aliquots were withdrawn for HPLC-MS/MS analysis and replaced with an equally fresh medium volume (for each aliquot), there was gradient formation again, and the neomycin was released by the hydrogel, continuously up to 48h of the test. For that, the neomycin released from the hydrogel in a controlled manner (ANSEL, 2007; JABEEN et. al., 2017). If no neomycin has remained in the hydrogel, the line would have one negative inclination. And if the aliquots were replaced with the same tested aliquot the line would have one positive inclination.

In fact, hydrogel to be used as a controlled release system, the drug concentration must remain constant over time and thus avoid the daily exchange of the dressing. According to the release result of neomycin, the hydrogel succeeded both in releasing neomycin rapidly and maintaining its concentration constant.

5.10 Cytotoxicity test

Cytotoxicity assay was determined by the neutral red incorporation method and the results are given in FIG. 19.

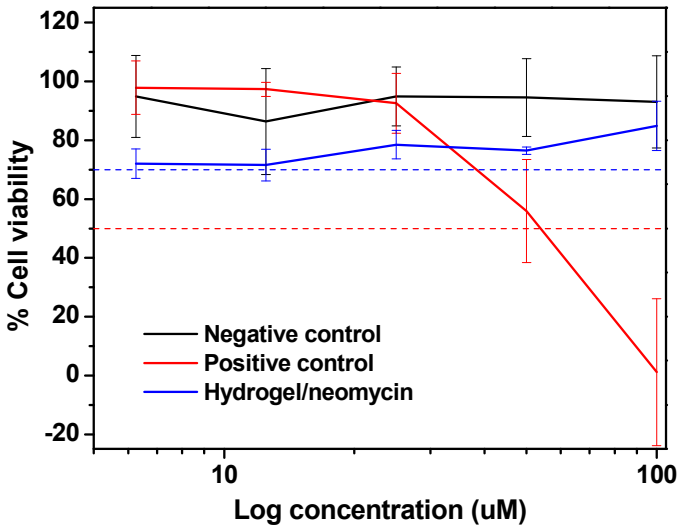

FIGURE 19 – Cell viability curve of hydrogel / neomycin

This method is a quantitative test that determines the cytotoxic potential of the material under analysis. The negative (-) and positive (+) controls were used to verify the performance of the test. According to FIG. 20 the cell viability curve of the hydrogel showed a behavior similar to the control curve (-), indicating that there was no death or damage to the cells, characterizing the hydrogel as nontoxic when in contact with the mousse cell culture.

5.11 Antimicrobial activity assay

5.11.1 Disk diffusion

Diffusion disk test was used to measure the antimicrobial activity of the hydrogel, since it simulates the application of the hydrogel on the infected skin (HUBER, 2017). Hydrogels samples were cut, placed in bacterial lawn and incubated by 24h. The inhibition zones can be observed in FIG. 20.

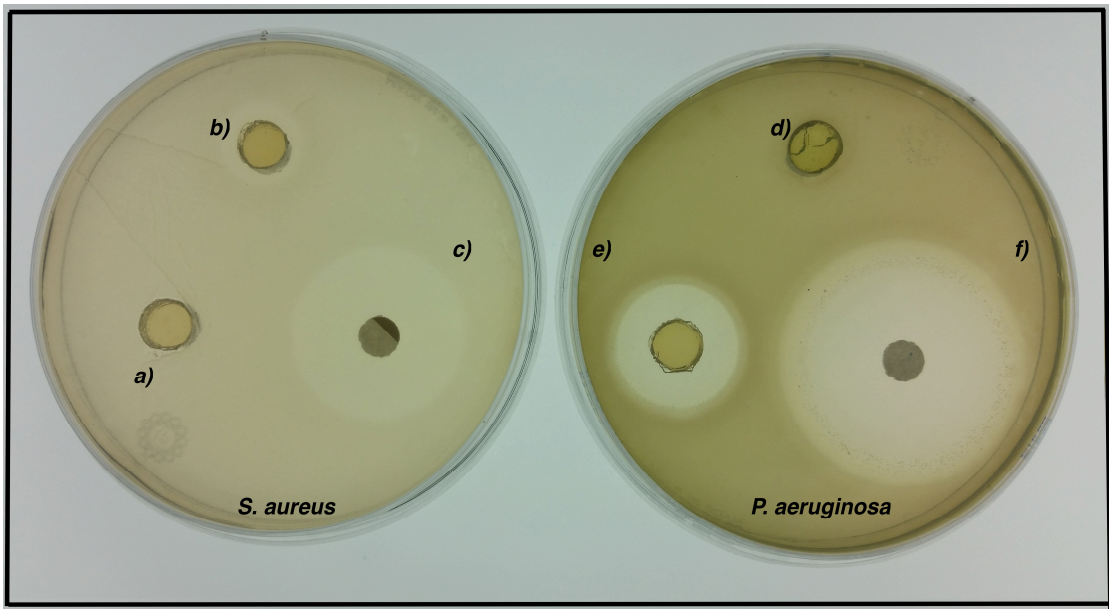

FIGURE 20 - Antimicrobial activity assay by diffusion disk in Hydrogel/neomycin against *P. aeruginosa* (ATCC 27853) and *S. aureus* (6835P).

After 24 hours of incubation, it was observed inhibition halo around the positive controls, FIG. 21c and 21f, and also of the hydrogel / neomycin samples, FIG. 21b and 21e.

The diameters of the inhibition halos are listed in the TAB. 5. The inhibition halo against *S. aureus* was 16.0 ± 2.0 mm, while the diameter against *P. aeruginosa* was 21.0 ± 1.0 mm. In evidence the results indicate that neomycin was released from hydrogel, diffused into the culture medium and exerted antimicrobial effect against both strains. The negative control (hydrogel without drug) did not exhibit inhibition halo, indicating that the hydrogel formulation has no bactericidal effect.

Controlled release of neomycin from hydrogel was tested by the same disk diffusion test, however, the hydrogel disks were removed every 24 h and transferred to fresh Petri dishes containing *P. aeruginosa* or *S. aureus* lawns. The diameters of inhibition halos were measured with a ruler and the results are given in TAB. 4 and FIG. 21 and 22.

TABLE 4 - Comparison of inhibition halos after successive plate changes

Inhibition Halo (mm)		
Plate chance	Bacterias	
	P. aeruginosa	*S. aureus*
1	20.0 ± 1.0	14.0 ± 1.0
2	19.0 ± 3.0	-
3	13.0 ± 2.0	-
4	10.0 ± 1.0	-

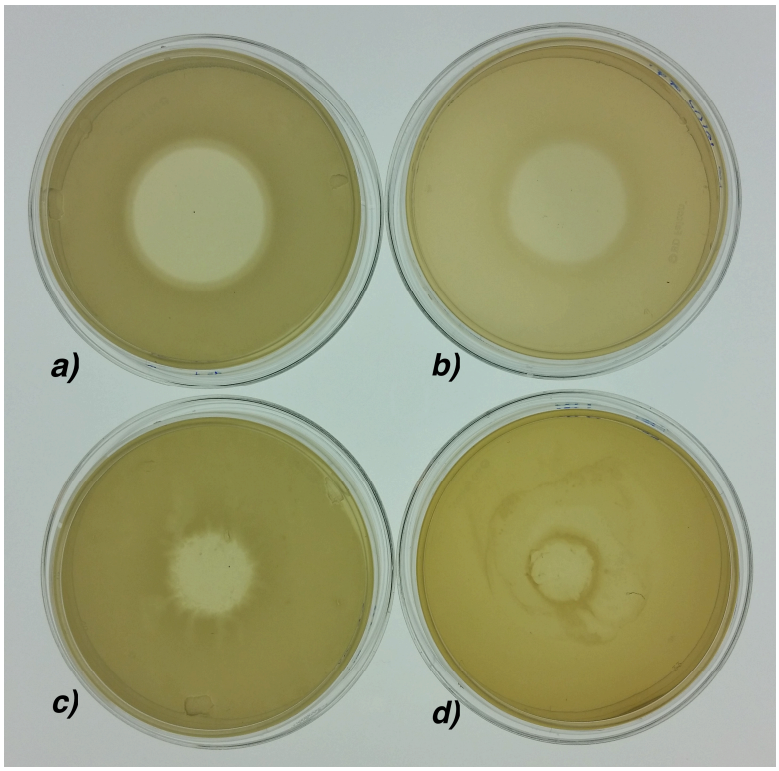

FIGURE 21 - Result of disc diffusion against *P. aeruginosa* after successive plate changes.

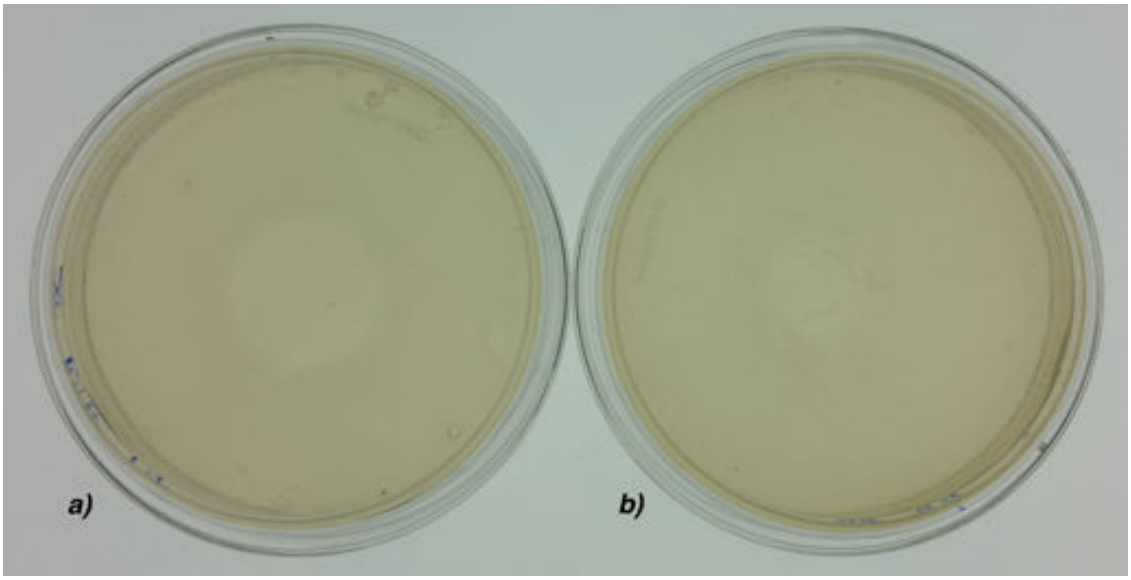

FIGURE 22 - Result of diffusion in agar against *S. aureus*.

The diameter of the inhibition halo on the plate with *P. aeruginosa* decreased 30% after the third change, FIG. 22c, and no halo was observed after the fourth plate, FIG. 22 d. The test performed against *S. aureus* showed halo only on the first plate, FIG. 23.

Results obtained in this assay confirm one more advantage presented by the hydrogel on traditional dressings, creams and pomade. Neomycin was released continuously from hydrogel on the bacterium for 3 consecutive days, no dressing change was necessary. According to LANE (2017), sustained drug release from hydrogel was probably due to its reversible electrostatic interaction with polymer chains, porosity of the hydrogel network and higher concentration of neomycin in hydrogels (HUBER, 2017; LANE et al. , 2017).

5.11.2 Suspension of bacteria assay

Antimicrobial activity was performed by incubating hydrogel disks in suspension of bacteria. In FIG. 23, tubes are shown after 24 h of incubation at 37 °C.

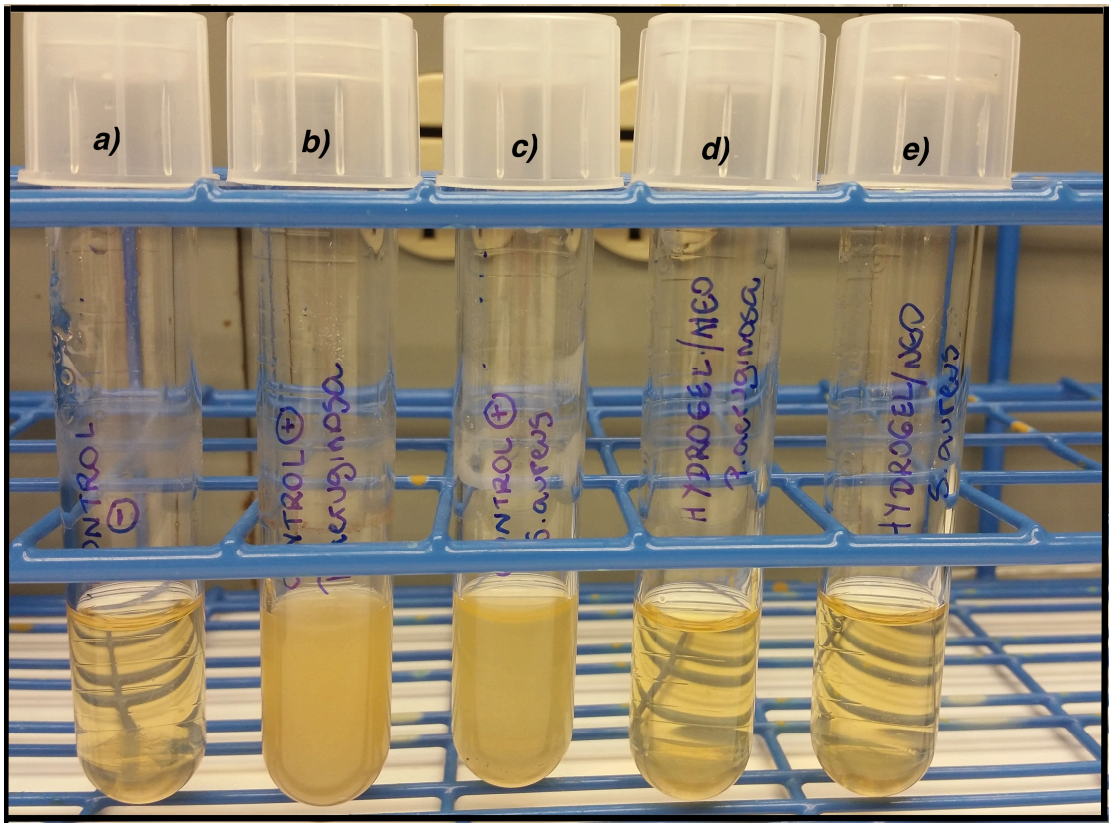

FIGURE 23 - Bacterial growth inhibition in liquid medium

In FIG. 23, tube (a) was called the negative control. No turbidity was observed, indicating that the test was performed asseptically and that there was no contamination.

Tubes identified (b) and (c) were positive controls with *P. aeruginosa* and *S. aureus*, respectively. Bacteria were incubated with hydrogel no neomycin. The turbidity of the medium indicates the microbial growth and absence of biocidal action of the hydrogel matrix.

The tubes labeled with letters (d) and (e) were prepared with the bacteria inoculum and hydrogel with neomycin. The culture medium kept clear after 24 hours of incubation in both tubes. These results indicate that there was no microbiological growth, since neomycin was released from the hydrogel and exhibited biocidal activity.

5.12 Time kill curves

Time kill assays were performed using *P. aeruginosa* (ATCC 27853; 481997 A SPM) and *S. aureus* ATCC 6835 strains. Hydrogel samples were

incubated with a suspension of the bacteria and aliquots were taken in time interval followed by counting viable cells. The results shown in FIG. 24 and 25 indicate the susceptibility of the resistante bacterium (481997 A SPM) and ATCC strains to the hydrogel / neomycin.

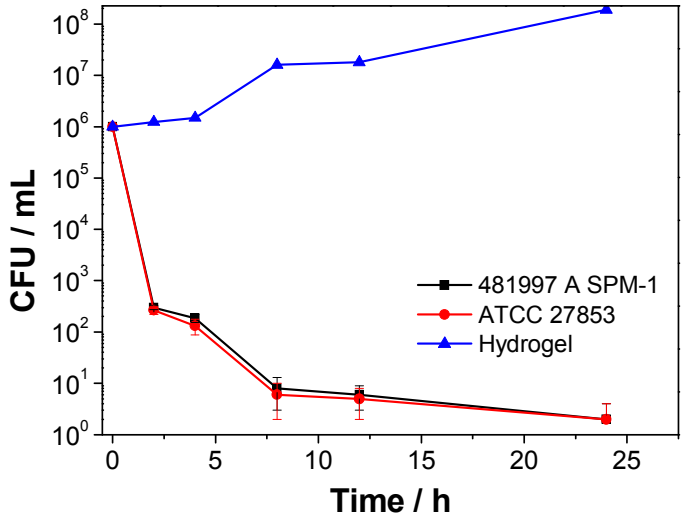

FIGURE 24 - Time kill curve of hydrogel / neomycin against *P. aeruginosa* ATCC and r481997 SPM resisten strain.

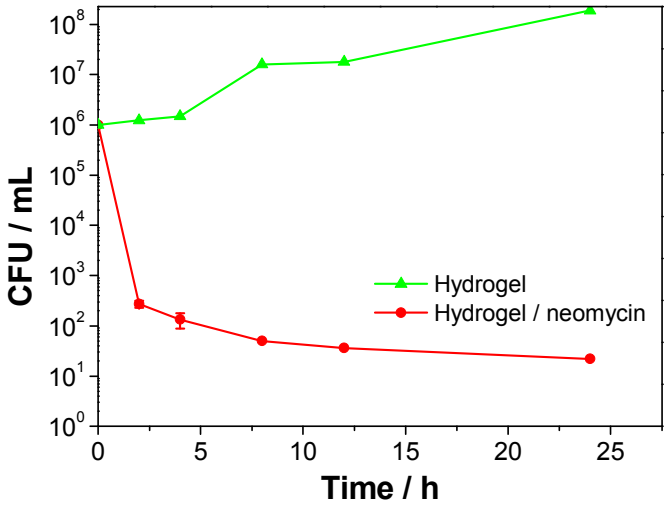

FIGURE 25 - Time kill curve of hydrogel / neomycin against *S. aureus*.

The kinetics of the time kill curves were monitored for 24 hours. The experiment was carried out with two P. aeruginosa strains, ATCC and 481997 A SPM, to evaluate the ability to hydrogel to kill the resistant strain. After two hours of testing, it was observed rapid bactericidal activity with a sustained 3-log killing achieved at 2 hours. The hydrogel without neomycin was used as a positive control, indicating once again that the formulation has no biocidal effect.

5.13 Biofilm Formation and quantification

Biofilm formation test was carried out using Coplin jar. In FIG 26, it is illustrated slides inside Coplin jar. Slides were taken and the established biofilms were washed with sterile distilled water to remove salts from the culture medium and no attached cell. The slides were coated with methylene blue to take pictures. Biofilm formation was observed in different periods of incubation, as illustrated in FIG 27.

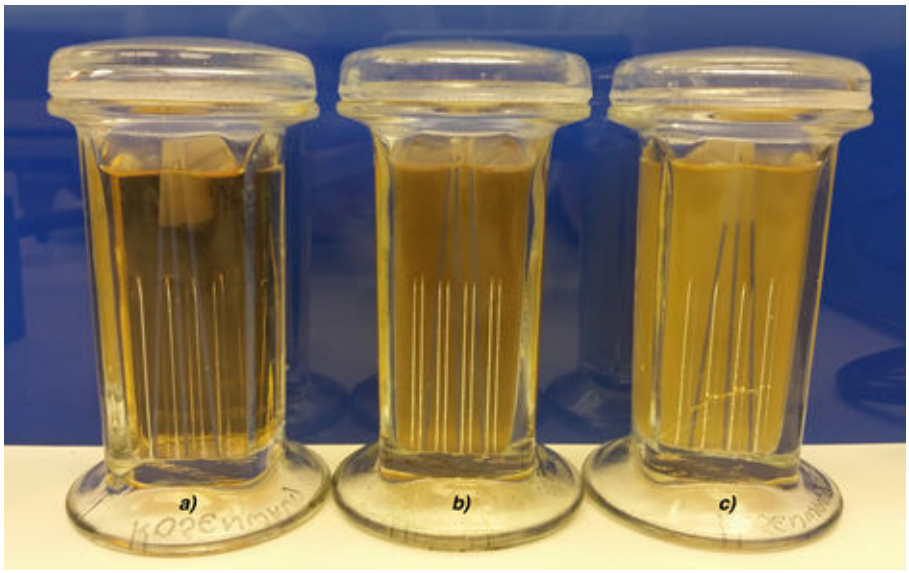

FIGURE 26 - Three slides were placed into the Coplin jar and filled LB Broth; a) negative control, only LB broth; b) slides incubated with *P. aeruginosa*; c) slides incubated with *S. aureus*.

In this test, biofilm formation was considered as bacteria had attached to the slides and was not easily removed during the washing process (HABIMANA et al 2018).

The experiment was observed for 7 successive days and, as expected, biofilm grew at the air-liquid interface slides, in different stages, which includes since initiating cell attachment until biofilm maturation and growth of the three dimensional community. As illustrated in FIG 27 and 28, the process of attachment and biofilm formation was improved with the time.

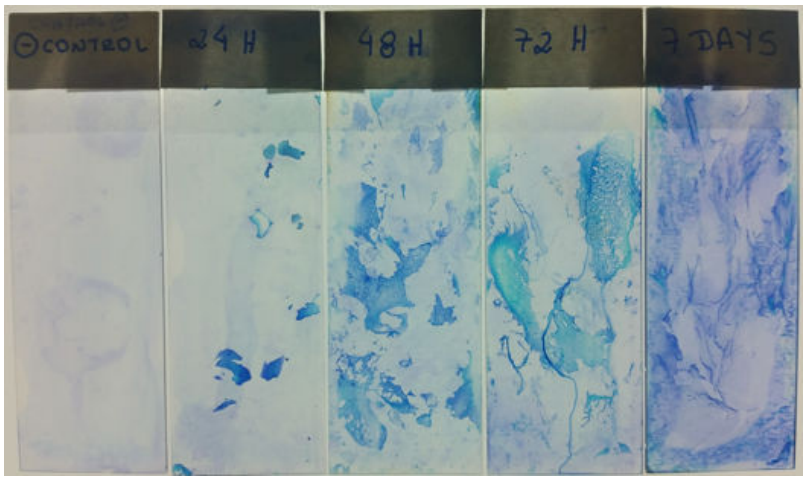

FIGURE 27 - Established biofilm by *P. auriginosa*.

FIGURE 28 - Established biofilm by *S. aureus*

It can observe moderate biomass density after 48 h incubation, followed by the intense biofilm after 72h. *P. aeruginosa* strains can produce different biofilm depending on their phenotypes and mucoid strains are known for

producing more alginate in their biofilm matrix than no-mucoid strains (DINGEMANS et al., 2016). In FIG 29, after 72 h of incubation, it can be observed filamentous structures derived from the higher concentration of alginate present in the extracellular matrix.

Biofilm slides were observed by optical microscopy, FIG. 30. Initially, it can be seen in FIG. 30a and 30b, colonies dispersed in the slides, then the colonies were aggregated into denses structures embedded in an extracellular matrix, characterizing the formation of biofilm (Figure 30 c and 30 d).

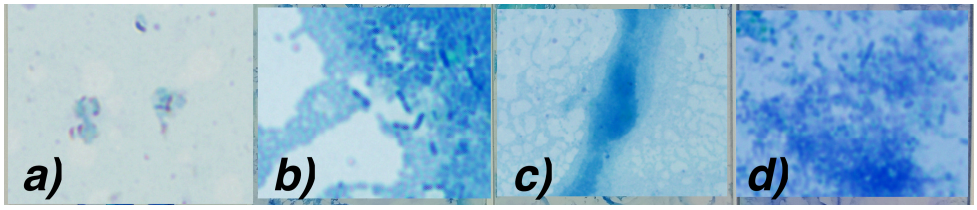

FIGURE 29- Biofilm formation observed under optical microscopy

P. aeruginosa and *S. aureus* bacteria were chosen to perform this test because they are the two most commonly isolated bacterial species of chronic wounds and they have been known for their biofilm formation capacity (FINLEY et al., 2013; SIMÕES et al., 2018).

Biofilm formation occurs at the air-liquid interface of the injury surface and the environment. This formation takes place in different stages, from cell adhesion to maturation and biofilm growth in a tridimensional community.

SEM pictures were taken scanning electron microscopy, FIG. 30.

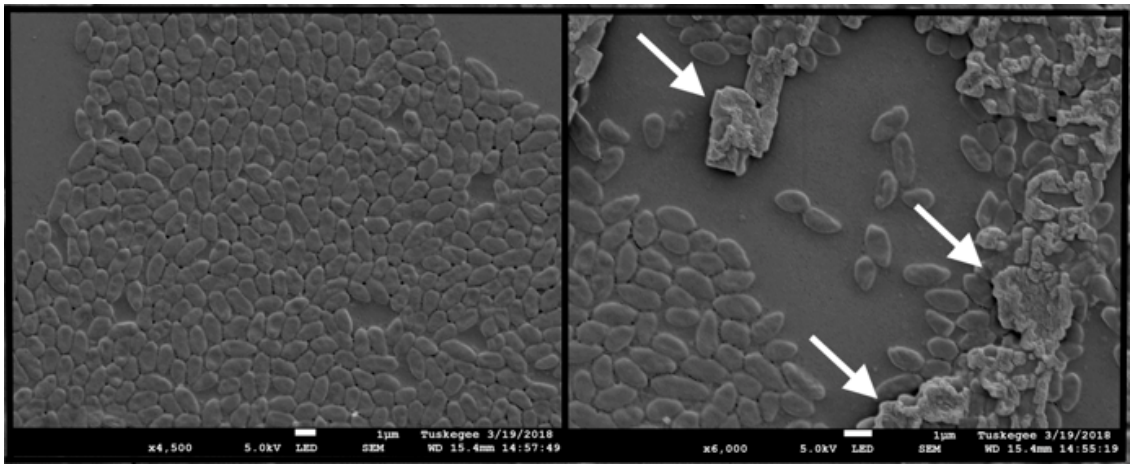

FIGURE 30 - SEM pictures of biofilm formation by *P. aeruginosa.* Lamellar formation following the biofilm maturation

FIG. 30, the SEM pictures show the lamellar formation, maturation and three-dimensional structure characteristic of the biofilm indicated by the white arrows.

Bacteria growth were accompanied by OD_{540nm} measurement. The values are described in the TAB. 5.

TABLE 5 - DO_{540nm} versus the incubation time.

Incubation (days)	Blank (negative control)	LB broth and neomycin released from hydrogel	*P. aeruginosa*	*S. aureus*
0	0.062 ± 0.002	0.062 ± 0.002	0.064 ± 0.004	0.061 ± 0.007
1	0.067 ± 0.001	0.061 ± 0.004	0.529 ± 0.025	0.235 ± 0.016
2	0.067 ± 0.005	0.064 ± 0.001	0.773 ± 0.021	0.225 ± 0.008
3	0.066 ± 0.003	0.067 ± 0.002	1.563 ± 0.031	0.280 ± 0.011
5	0.069 ± 0.003	0.066 ± 0.005	0.881 ± 0.014	0.287 ± 0.033
7	0.068 ± 0.002	0.063 ± 0.005	0.741 ± 0.026	0.242 ± 0.027
10	0.063 ± 0.004	0.068 ± 0.003	0.399 ± 0.040	0.209 ± 0.005
12	0.065 ± 0.003	0.063 ± 0.002	0.442 ± 0.037	0.188 ± 0.010
15	0.066 ± 0.002	0.067 ± 0.005	0.295 ± 0.040	0.182 ± 0.005

The OD_{540nm} increased with time incubation and the peak of microbial growth was observed after 72h of incubation for both strains.

After 24 h of incubation, *P. aeruginosa* ATCC was considered a strong biofilm producer with an average OD_{540nm} 0,529 ± 0,025, since *S. aureus* exhibited OD_{540nm} about 0,235 ± 0,016 and it was therefore considered a moderate biofilme producer.

No biofilm growth was observed when hydrogel / neomycin was placed in Coplin jar. This result is explained by the presence of neomycin in the culture medium from the polymer matrix release. The drug exhibits its biocide effect and prevented the microbiological growth and consequently, the formation of the biofilm. In this way, evaluation of the effect of the neomycin loaded hydrogel on stablished biofilms of *P. aeruginosa* is extremely important and it becomes essential to increase the field of hydrogel performance. It is known that biofilm in the wound inhibits healing process and, hence, can increase the time and costs of patient hospitalization (GONÇALVES 2017, HUBER 2017). Thus, evaluating the biocide activity of the hydrogel on established biofilm becomes essential, since the treatment of biofilm is more difficult than the treatment of single colony because mechanisms of resistance presented in the biofilm. (GONÇALVES 2017, HUBER 2017).

5.14 Efficacy hydrogel against established biofilm

96-well plates method was used to evaluate the efficacy neomycin released from the hydrogel on biofilm. *P. aeruginosa* and *S. aureus* inoculum were placed in the 96-well plate to produce biofilm.

Established biofilms were treated with 100 µL of the neomycin solution. The $OD_{540\ nm}$ were measured and are listed in TAB. 6.

TABLE 6 - Biofilm treatment with neomycin released from the hydrogel

Strain	No treatment	After treatment
P. aeruginosa	1.530 ± 0.015	0.189 ± 0.020
S. aureus	1.342 ± 0.025	0.094 ± 0.018

After treatment, *P. aeruginosa* biofilm decreased 87.6 %, while *S. aureus* reduced 92.0 % when compared with biofilm untreated.

Treatment of the biofilm produced by *P. aeruginosa* was also measured by counting cells in Petri dishes. Established biofilm on the slides was covered with hydrogel loaded neomycin and incubated for 24 h at 37 °C. In sequence, it was added 30 ml of sterile culture medium and vortexed 30s.

After in serial dilution, 10 uL of each dilution was inoculated into agar medium plates and incubated for 24 h. The results obtained were exhibited by log reduction calculated relative no treatment and after treatment. FIG. 31 shows anti-biofilm activity of hydrogel / neomycin with 3 log reduction values when compared biofilm no treatment.

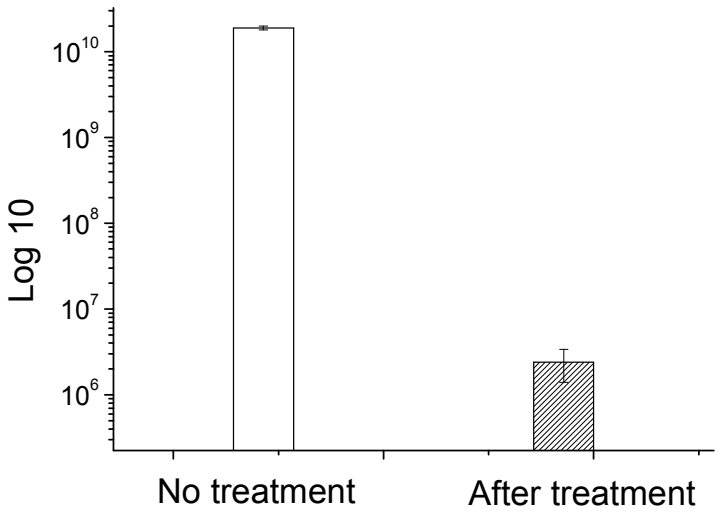

FIGURE 31 - Antimicrobial activity of hydrogel against biofilme of *P. aeruginosa*.

5.15 SEM images

SEM images shown in FIG 32 were obtained from slides with biofilm treated and without treatment (negative control). The images in Fig 6a and 6b were obtained with an increase of 6000x and exhibit a microbial aggregate of three-dimensional formation, characteristic of biofilm formation. In Fig. 6c and

6d, the images obtained after the treatment with hydrogel / neomycin and captured with an increase of 5,000 and 3,500x showed the reduction of the formation of the biofilm structure. Alteration of the bacterial membrane is indicated by the white arrows and refers to the action of the hydrogel / neomycin in the cell.

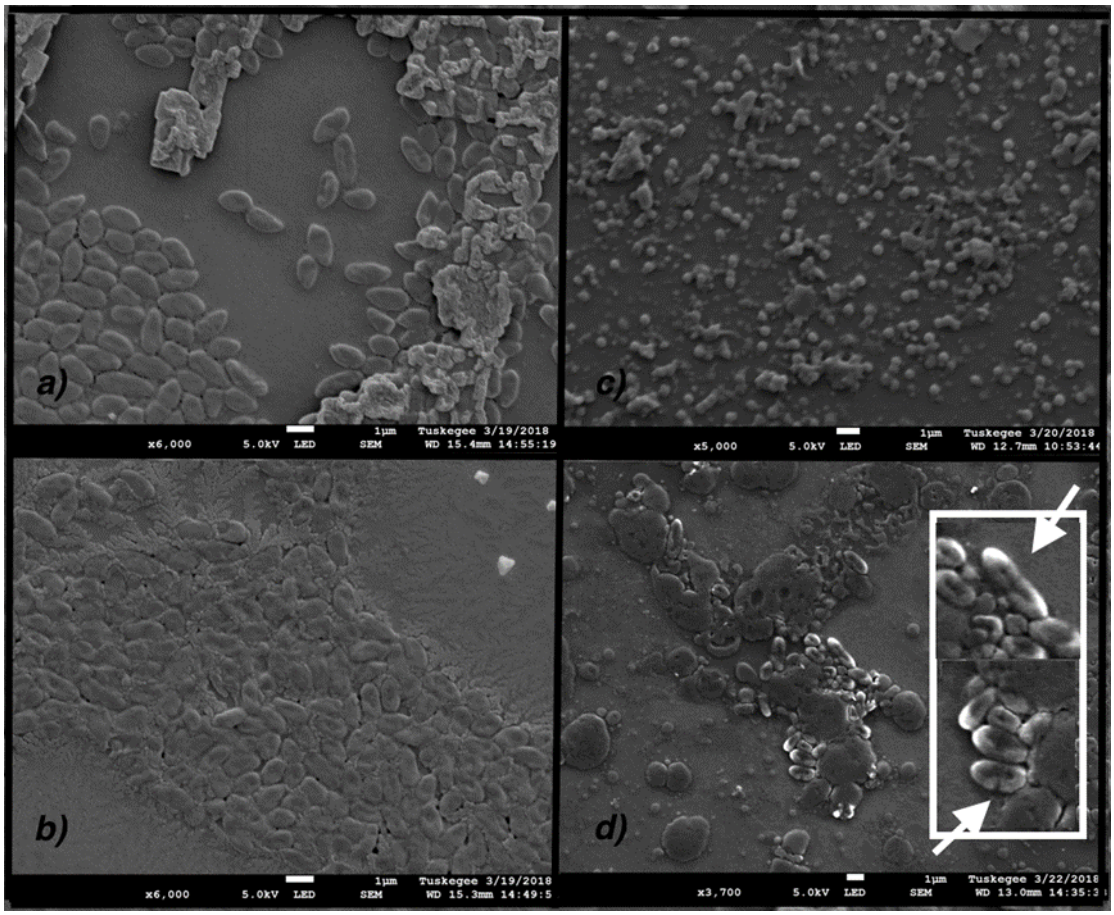

FIGURE 32 - SEM images of *P. aeruginosa* biofilm before and after hydrogel / neomycin treatment

5.16 Microbial penetration

Microbial penetration assay was carried out with a sealed tube with hydrogel. The hydrogel without neomycin was used since that the presence of the drug could modify the test result. The results showed in TAB. 7 indicate that no turbidity was observed in sealed tubes with hydrogel, kept in open environment during 30 days, while high turbidity was observed in the positive control as a result of microbial contamination.

TABLE 7 - Microbial penetration inhibition

Penetração microbiana			
Incubation time (days)	Hydrogel	Positive control	Negative control
1	0.069 ± 0.003	0,066 ± 0,003	No turbidity
2	0.068 ± 0.001	0,123 ± 0,003	No turbidity
3	0.066 ± 0.001	turbidity	No turbidity
7	0.068 ± 0.003	turbidity	No turbidity
10	0.067 ± 0.002	turbidity	No turbidity
20	0.067 ± 0.001	turbidity	No turbidity
30	0.067 ± 0.004	turbidity	No turbidity

Hydrogel prevented contamination in the tube; this result means that hydrogel is able to prevent microbial penetration through the dressing during the wound healing period.

Results above demonstrate another advantage of the use of the hydrogel as a wound dressing; it acts as a barrier against microorganisms and as a consequence, must prevent secondary infections, which can retard the healing process.

5.17 Evaluation of the treatment of pododermatitis in penguins

“In vivo” tests were performed in penguins of the rehabilitation centers and aquariums. Ten animals with lesions of pododermatitis were monitored and treated with the hydrogel / neomycin. Ten animals with lesions with a mean diameter of 12.0 ± 3.6 mm were treated. The lesions diameters were measured with a ruler before and after the treatment. FIG. 33 and 34 illustrate the bilateral and unilateral pododermatitis in penguins.

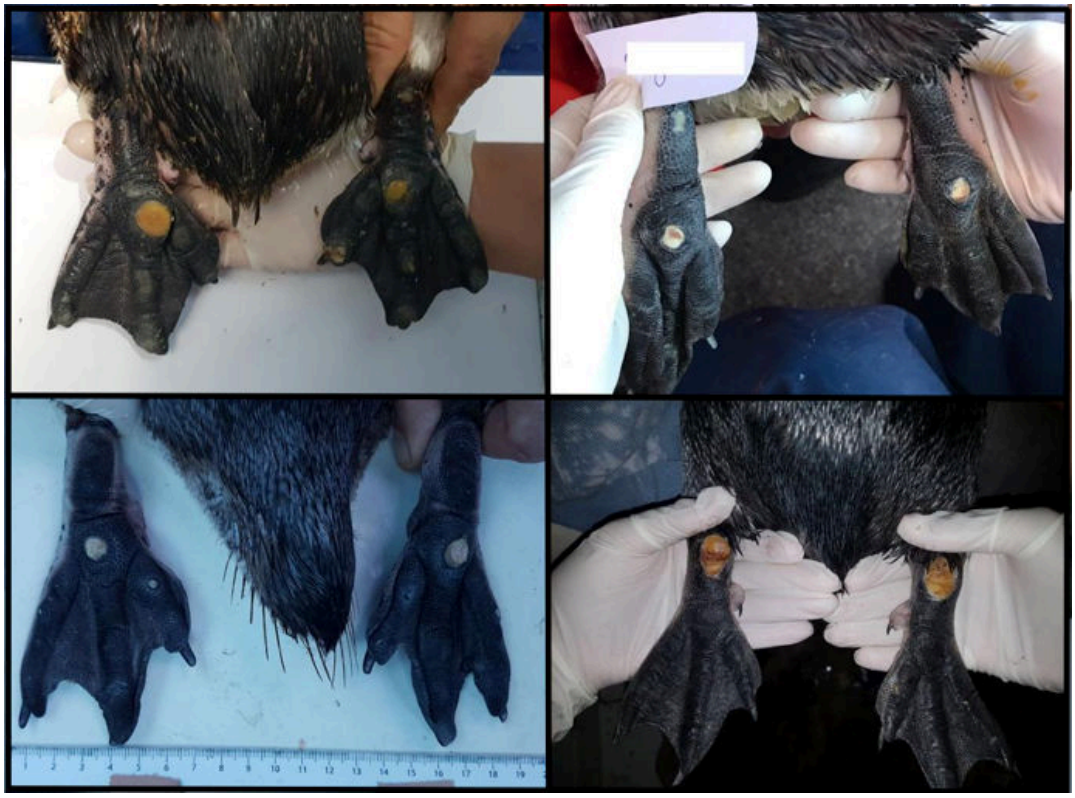

FIGURE 33 – Pododermatitis in both paws of Penguin

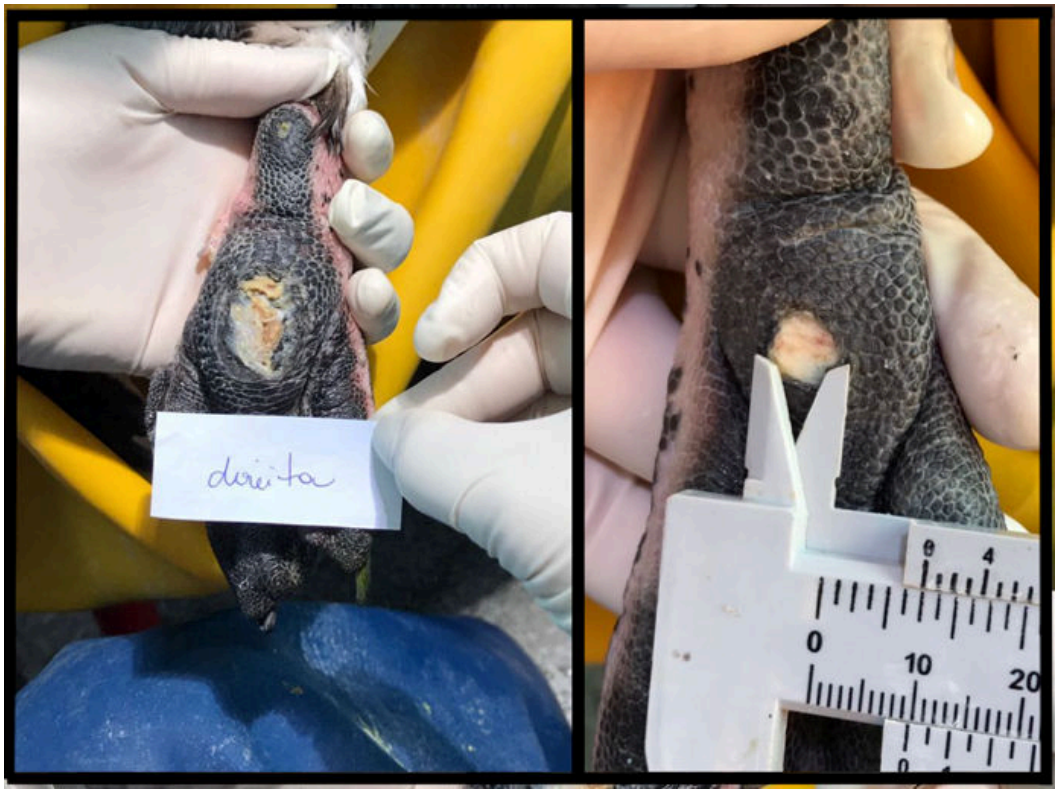

FIGURE 34 – Pododermatitis in paw of penguin

During the experiments, it was observed that 70% of the birds had bilateral pododermatitis. This result is expected because the animals are susceptible to develop lesions on both paws at the same time due to the compensation of body weight.

The animal with lesions of pododermatitis was submitted to treatment with hydrogel / neomycin. The lesions were measured with the ruler according to FIG. 35.

FIGURE 35 – Pododermatitis lesion measured with ruler

The lesions were debrided, cleaned and coated with hydrogels, as can be seen in FIG. 36.

FIGURE 36 - Hydrogels applied on podermatitis.

Cotton bandages were used to keep the hydrogel on the lesions and the animals continued their aquatic routines throughout the treatment. The

sequence of the procedure and the penguin with the dressing in its aquatic environment are shown in FIG. 37 and 38.

FIGURE 37 – Sequence for hydrogel application and penguin in its aquatic environment.

FIGURE 38 – Penguin after surgical procedure and hydrogel application.

The penguins were kept into the humid environment because, according to REIDARSON (1999) and BALLABIO (2008), remaining in water favors

treatment, decreases pressure on the lesions and also stimulates conviviality with other animals of their species, easing their stress.

After 5 days, the hydrogels were removed and the lesions were measured to follow the evolution of the treatment.

In FIG. 49 and 40 are compared to the lesion before and after the treatment. The border of the lesion showed decreased size and no reinfection was observed. FIG. 39 c.

FIGURE 39 – Evolution of the treatment of popodermatitis in penguin

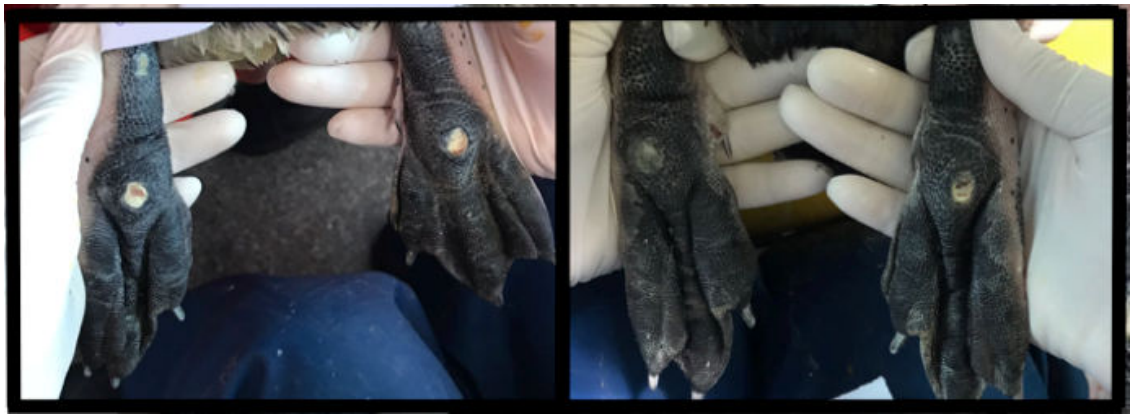

FIGURE 40 – Pictures were taken before and after treatment

After the application of the hydrogel, it was possible to observe the change in its color, probably due to the absorption of the exudate from the lesion and also liquids from the aquatic environment. The hydrogel had kept

intact throughout the time, and it was resistant to water and animal weight, FIG. 41.

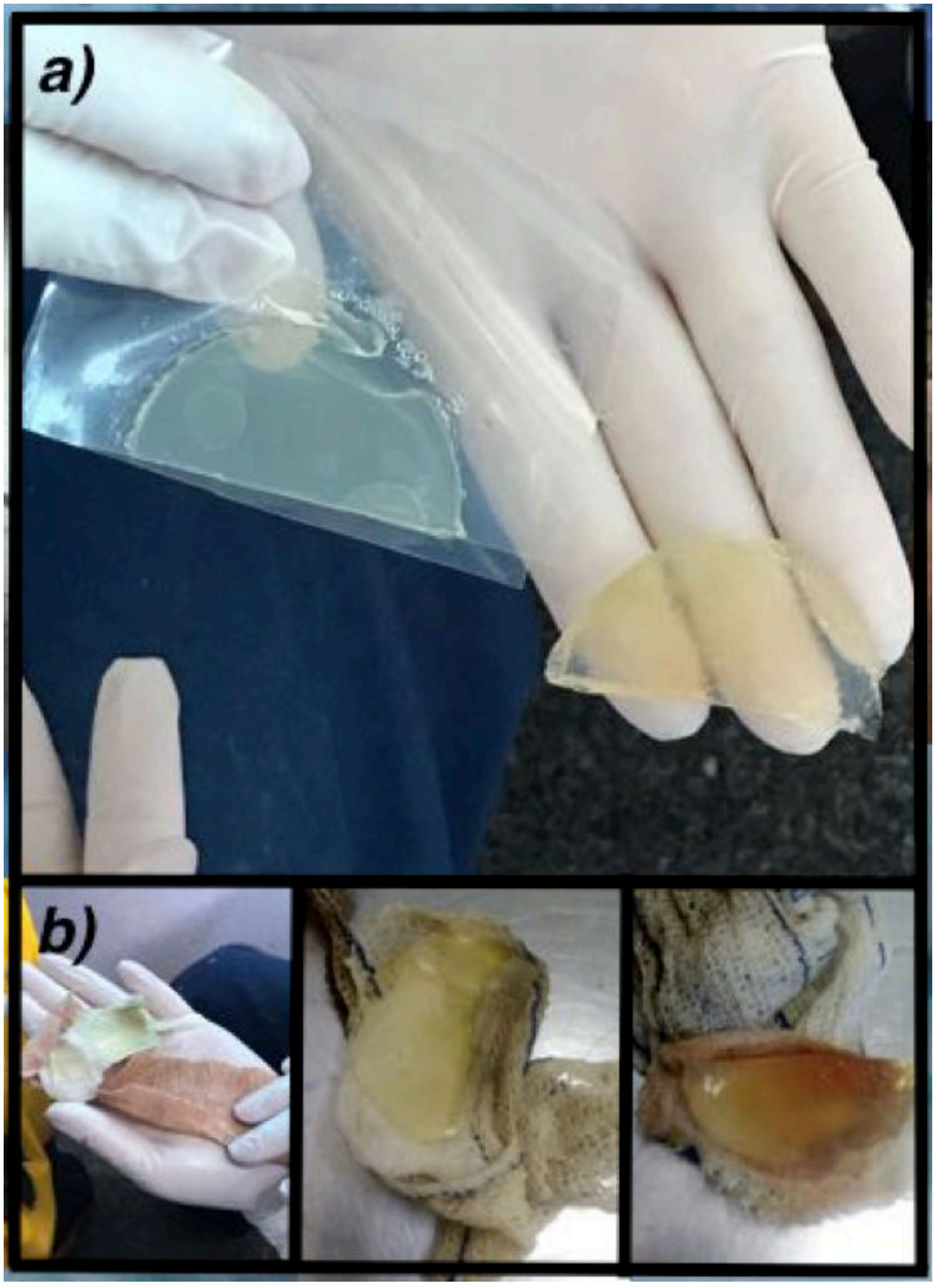

FIGURE 41 – (a) hydrogel before and after treatment; b) hydrogels removed after 5 days

Pododermatitis is a chronic lesion that requires a long time of treatment and the rate of recurrence is higher than 60% of treated animals (REMPLE,

2006; NASCIMENTO, 2014). This number is alarming because the pathogenicity of the disease involves intrinsic factors of the animal, such as sedentarism and weight gain. Also, the handling alternatives available in the literature have the objective of stimulating and increasing the time in which the animal remains in the water, decreasing the time of contact of the feet with the soil and weight control. The treatment includes surgical procedures for debridement of the lesion and drug therapy with anti-inflammatories and antibiotics (OAKS, 1993, AZA AMERICAN ZOO AND AQUARIUM ASSOCIATION, 2005).

Studies carried out by NASCIMENTO (2014) with Magellanic penguins reported the use of topical antibiotic and change of the dressing with gauze every 3 days and proposed the use of dressings that avoid the recontamination of the lesions as a determining factor to reduce the recidivism.

In this context, the hydrogel / neomycin has been synthesized and used in the treatment of topical lesions to offer advantages over cotton bandages routinely used in the treatment of pododermatitis. The hydrogel has a soft consistency and contributes to decreasing the pressure of the animal's weight on the lesions. This pressure is one of the factors that may induce the onset of the injury. Thus, the hydrogel decreases the friction of soil on the lesion when compared to the gauzes. The controlled release of neomycin allowed the dressing to be changed every 5 days. The increase in days between dressing changes implies in reducing the frequency of human intervention in the animal and reducing its stress. Some microorganisms found in penguin lesions may originate from the manipulator and consequently will be, as lower the exposure to humans, as lower the chances of contamination of the animal will occur.

6. CONCLUSION

From the point of view of the characterization of neomycin under ionizing irradiation, the thermal analysis indicated that neomycin has thermal stability when irradiated in powder form and the components of the hydrogel formulation acted as radioprotectors of the drug during the irradiation process. The infrared spectroscopy curves indicated characteristics peaks of PVP and neomycin.

The hydrogel / neomycin has been classified as non-toxic and can be used “in vivo” as a topical drug delivery dressing without compromising patient health.

The kinetics of swelling in seawater was important to elucidate the absorption capacity of the hydrogel in solution with higher concentration of mineral salts. The result of this assay indicates that the hydrogel can be used in marine animals.

The release of the antibiotic occurred rapidly in the first few hours and remained constant for 48 hours. This result is extremely important for the use of the hydrogel as a drug delivery system. The hydrogel can remain topically on the lesion and not be replaced for daily dressing changes, reducing the cost of treatment, patient pain and nursing time.

The microbiological results indicated that the hydrogel released the neomycin and exhibited its biocidal effect against the bacteria *P. aeruginosa* and *S. aureus*. Tests performed on biofilm indicated that the hydrogel / neomycin is able to reduce microbiological growth even when it is expressed embedded in a polysaccharide matrix. The action on the biofilm makes the hydrogel / neomycin an available tool for the treatment of chronic wounds.

Antimicrobial studies were carried out in collaborative efforts among at Tuskegee University and our group at Nuclear and Energy Research Institute (IPEN).

The partnership brought additional knowledge, technological and scientific development to our laboratory and graduate course at IPEN, as well as the improvement of the relationship between the researchers.

The "in vivo" study was carried out in Magellanic Penguins, with the assistance of the aquarium veterinarians. With the results presented, it can be inferred that the hydrogel was effective in the treatment of pododermatitis in

penguins. The lesions regressed, there were no signs of recontamination of the lesions by feces and urine, a fact that contributes to accelerating the cicatrization process. The hydrogel remained intact during the dressing application time, being resistant to water and animal weight.

The results obtained in the present study ensure the efficacy of the hydrogel / neomycin used as a controlled release system of neomycin and may be a therapeutic alternative for the treatment of topical infections even in the presence of biofilm, besides being a therapeutic resource in the veterinary area

The result of the microbial penetration test showed the hydrogel ability in preventing the passage of the microorganism through its polymer matrix. These results can be also observed “in vivo” test since after 5 days of treatment no recontamination of the urine and feces were noticed in the lesion, indicating another advantage of hydrogel over gauzes and bandages dressings.

2.2. AVALIAÇÕES DO PROJETO DE PESQUISA:

2.2.1. Eletrotransferência do gene do hormônio de crescimento de camundongo associada à administração de células-tronco mesenquimais em modelo murino de osteogênese imperfeita

Pesquisador Responsável: Dra. Cibele Nunes Peroni
Pesquisador(es) Executante(s): Alissandra de Moura Gomes;
Gustavo Protasio Pacheco de Jesus;
Enio Aparecido Zacarias;
Paolo Bartolini;
Ana Carolina Pedroso Romeiro Garcia.

Foi feita uma exposição do Projeto pelo Coordenador e após análise de vários itens do projeto pela CEUA, o projeto foi aprovado.
PARECER: PROJETO APROVADO

2.2.2. Preparação, caracterização e estudo antimicrobiano de hidrogel impregnado com neomicina e nanoprata para tratamento de infecções tópicas

Pesquisador Responsável: Dra. Duclerc Fernandes Parra
Pesquisador(es) Executante(s): Angélica Tamião Zafalon;
Franscinne Braint Narita;
Cristiane Lassálvia Nascimento.

Foi feita uma exposição do Projeto pelo Coordenador e após análise dos itens, a comissão considerou que o projeto não necessita de aprovação da CEUA.
PARECER: PROJETO DISPENSA APROVAÇÃO DA CEUA

2.3. REAVALIAÇÃO DO PROJETO DE PESQUISA

2.3.1. Estudo da interferência de radiofármacos na neuroimunomodulação em animais de laboratório

Pesquisador Responsável: Dr. Carlos Roberto Jorge Soares
Pesquisador(es) Executante(s): Maria Helena Bellini Marumo, Elaine Bortoleti de Araújo, Glaucie Jussilane Alves

Em 10/01/2018 foi apresentado novo formulário com os esclarecimentos/correções solicitados anteriormente pela CEUA-IPEN.
PARECER: PROJETO **APROVADO**

2.4. COMUNICADOS DO COORDENADOR:

Não houve ocorrência.

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