Latin American Journal of Pharmacy
(formerly *Acta Farmacéutica Bonaerense)*
Lat. Am. J. Pharm. **30** (6): 1104-8 (2011)

Regular Article
Received: April 6, 2010
Revised version: December 23, 2010
Accepted: January 2, 2011

In Vitro and *In Vivo* Toxicology Evaluation to Determine Suitable Biomedical Polymers for Development of a Papain-Containing Drug Delivery System

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***SUMMARY*. Papain has been known by many decades for wounded tissues repair. However, papain stability is not high enough to be commercialized in a stable pharmaceutical form; therefore its use is limited. The strategy to entrap papain into a polymeric matrix to provide an adequate drug delivery system consists of an alternative to this problem. The purpose of this study was to assess *in vitro* and *in vivo* four polymers cytotoxicity and ability to cause cutaneous irritation to be applied as a suitable papain delivery system. A Monocomponent (MSD) and Bicomponent Silicone Dispersions (BSD) and, Natural Rubber Bicentrifuged Latex (NRBL) and an Acrylic Adhesive (AA) were selected. The cytotoxicity was firstly assessed by the Neutral Red Uptake Method. Non-cytotoxic polymers were then submitted to *in vivo* Cutaneous Irritation Test. Both silicone dispersions were found non-cytotoxic, and NRBL and AA polymers showed cytotoxicity. MSD and BSD polymers did not cause any cutaneous reactions.**

INTRODUCTION

Enzymatic debridement uses proteases to degrade necrotic tissue, thus allowing the healthy tissue to migrate into the wound area. Autolytic debridement is a process that occurs naturally in a moist wound [1].

Another role of debridement is to remove the tissue in excess that surrounds chronic wounds. Neuropathic ulcers are often associated with callus formation. Excision of the callus allows tissue-healing cells to proliferate, migrate, and heal [2,3]. Another benefit of debridement is its effect over the growth factor activity. It is suggested that chronic wounds may lack these proteins or they may be unavailable for wound healing processes due to protein binding present in the chronic wound [4,5]. Debridement releases activated platelets that promulgate various growth factors and cytokines [6].

Papain is an archetypal cysteine proteinase, whose family includes chymopapain, caricain, bromelain, actinidin, and ficin [7]. Papain digests necrotic tissue by liquefying scar [8], facilitating the migration of viable cells from the wound edge into the wound cavity [1].

Debridement helps to remove bacteria. Evidence suggests that significant numbers of bacteria in a wound may slow healing. When bacteria exceeds 100,000 (10^5) bacteria/tissue (g), wound healing processes do not proceed normally [9], even though it is unclear if bacterial burden is a cause or a consequence of impaired healing [3]. However, it is known that necrotic material in a wound promotes growth of anaerobic bacteria that are deleterious [6].

Papain is also useful in reducing the bacterial burden, decreasing exudates, and increasing granulation tissue formation [1]. The use of papain latex for wound healing by American, African and Caribbean Island tribes has been reported by Monetta [10-12]. Its therapeutical value is specially recognized in hard recovery treatments [11-13].

KEY WORDS: Cutaneous irritation test, Cytotoxicity test, Drug delivery system, Papain.

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ISSN 0326-2383

However, papain stability is not high enough to be applied into formulations and as a consequence its products hold short life. Thus its use is limited to extemporaneous or 60 days shelf life formulations.

A 0.4% (w/v) papain containing gel has been standardized by Velasco for the treatment of patients presenting abscess and pressure ulcer in a daily application [14]. This formulation presented a shelf life of 60 days and no protection against external agents.

An alternative to papain usage as a healing agent is to entrap the enzyme into a polymeric matrix sterilized by gamma radiation and obtain a topical drug delivery system with extended shelf-life. Medical devices that hold direct contact to the affected skin must be sterile. Gamma radiation is an interesting option to meet this requirement. Sterilizing agents cause the inactivation of microorganism by an irreversible damage caused to essential molecules such as proteins and DNA [15]. According to Phillips, ionizing radiation sterilization of medical products present advantages over conventional agents: no radioactive residues are formed, the product may be sterilized inside the final package and the process presents an excellent reliability [16]. ISO 13409 is the normative reference for the establishment of the sterilizing dose, which determined 25 kGy as the necessary radiation dose for the inactivation of viable microorganisms [17].

Dermal irritation tests in small animals are often performed to help identify substances which may be irritants to human skin and/or mucosal tissue. A primary irritant is a substance which produces inflammatory changes in the skin as a result of a direct damaging effect characterized by the presence of inflammation, or in the case of severe allergenic reactions, vesiculation and/or necrosis [18].

In vitro tests minimize the use of animals in researches and are usually performed as an initial screening test in the first phase of the biocompatibility evaluation. *In vitro* cytotoxicity assays are techniques that evaluate the cell damage caused by a specific material, which provides low cost and quick results, using cell culture and determine cells lyses (cell death), inhibition of cell growth and other effects caused by devices, materials and/or their extracts [19].

The aim of this study was to assess four polymers by *in vitro* Cytotoxicity Test and by *in vivo* Cutaneous Irritation Test in order to identify the suitable polymers for development of a papain-containing drug delivery system.

MATERIALS AND METHODS

Selection and preparation of polymeric membranes for Cytotoxicity and Cutaneous Irritation Tests

Initially four polymers were selected to prepare the polymeric matrix of the papain-containing membranes. These polymers corresponded to Natural Rubber Bicentrifuged Latex (NRBL), Acrylic Adhesive (AA), a monocomponent silicone dispersion (MSD) and a bicomponent silicone dispersion (BSD). AA, MSD and BSD are medical grade polymers. NRBL is a hypoallergenic polymer that cures by drying at room temperature after approximately 24 h. AA polymer is cured at 110 °C for 1 min. MSD cures by chemical reaction at room temperature after five days. BSD cures by thermal treatment at 75 °C for 45 min.

NRBL was prepared at Instituto de Pesquisas Energéticas e Nucleares (IPEN). AA was purchased from Rohm & Raas Company (USA). MSD and BSD were purchased from NuSil (USA).

Approximately 1.0 g of each polymer was used to prepare its respective membrane (5 cm diameter) to be used as samples for the Cytotoxicity assay and the Cutaneous Irritation Test. Membranes were not irradiated to perform this test.

In vitro Cytotoxicity Test

In order to assess the *in vitro* biological safety of the selected polymers a cytotoxicity test was performed by using the Neutral Red Uptake Method [20].

NCTC clone L929 cell line from ATCC bank which corresponds to mouse connective tissue, was selected as cell line due to its well established and easy culture and its ability to provide reproducible results [21]. Cells were cultivated in Eagle Minimum Essential Medium (MEM) and maintained in 75 cm2 bottles at 36 °C, undergoing subcultures at 72 h in average each.

The cell culture was prepared in a 96 wells microplate containing 200 µL of a 2 x 10^5 cells/mL suspension per well. Microplate was kept at 37 ºC for 24 h for the cell monolayer formation.

The polymeric membranes prepared and not irradiated were extracted with 1 mL/cm^2 Eagle MEM, at 37 °C for 24 h. PVC pellets were used as the negative control and a 0.02% phenol solution as the positive control. Both controls were extracted with Eagle MEM and after that diluted with the same solution to reach 100 %,

50 %, 25 %, 12.5 % and 6.25 % dilutions. Approximately 200 µL of the dilutions were added to each well and each dilution was assessed in triplicate.

The plates were prepared and maintained for 24 h at 37 °C. After that the extracts were removed from the plate and 200 µL of a Neutral Red solution was added to each well. After being kept for 3 h at 37 °C in order to be absorbed by the viable cells and the dye solution was discarded. The plate was washed twice with a (pH 7.4) phosphate buffer solution. After that a washing solution (10 % calcium chloride in formaldehyde 0.5 % solution) was added and then replaced by an extraction solution containing 50 % ethanol and 1 % acetic acid in Milli-Q water for cell lyses and dye release.

Optical density was measured at 540 nm by using a 620 nm standard filter in a microplate reader. The percentage of viable cells was calculated by dividing the optical density average of each dilution by the optical density average of cell control, multiplied by 100. CI_{50} (Cytotoxicity Index at 50 %) was calculated by the graph extract concentration versus percentage of cell viability. For more details please refer to Rogero *et al.* [22].

The polymeric membranes which presented no toxicity were submitted to *in vivo* biocompatibility tests in rabbits, known as "cutaneous irritation test".

In vivo Cutaneous Irritation Test

Non-cytotoxic polymers were submitted to cutaneous irritation test in order to estimate the irritant potential to human skin, through the observation and quantification of cutaneous reactions that may take place after a single product application [23].

For each assessed polymeric membrane three male and three female rabbits of New Zealand breed, which weighted at least 2 kg were used. Twenty four hours prior to the beginning of the test the animals had two areas of 6.25 cm² tricotomized in their back. After that the animals were housed separately in metal cages measuring 80x60x40 cm, equipped with an automatic watering system. All animals received water and food ad libitum and were maintained under controlled temperature at 20 ± 2 °C and 30 to 70 % relative humidity and lighting (12/12 light/dark cycle) conditions.

For each animal one area corresponded to the negative control and the other was used for the application of the 1 cm² not irradiated membrane sample. Both areas were covered with a bandage and a plaster and then protected by another bandage the involving animal body. The samples were put into contact to the animal skin over 24 h and then removed. The measurement of the tested areas was performed at 24 and 72 h after the beginning of the test by an evaluation of erythema and edema formation. The erythema formation is visually evaluated according to Draize method [24,25]. Edema area measurement was performed with a caliper and calculated by the following equations:

$Ed\ (mm) = (L_{24} - L_i) / 2$ and $Ed\ (mm) = (L_{72} - L_i) / 2$

where: Ed is the edema value in millimeters; L_{24} is the measured test area after 24 h; L_i is the measured test area before sample application; L_{72} is the measured test area after 72 h.

The classification of the reactions intensity is based on Draize Method and is showed in Tables 1 and 2 [22].

RESULTS AND DISCUSSION

Considering that papain holds a high efficiency of a healing agent and its low stability, a drug delivery system offers a good alternative for this enzyme usage in medical applications.

Membranes-samples were prepared with these polymers in order to be assessed for their *in vitro* biological safety by the Cytotoxicity Test through the Neutral Red Uptake Method. This test allowed an analysis of the material ability to

Reaction level	Reaction	Characteristics
0	Normal skin	White or light pink skin.
1	Slight erythema	Slightly reddish skin, with a different colour compared to the control area.
2	Well defined erythema	Reddish skin, normally the entire area.
3	Moderate erythema	Intense and diffuse redness in skin.
4	Severe erythema	Dark red skin showing scar formation.

Table 1. Classification of the erythema formed by the cutaneous reaction to products which do not modify skin color.

Reaction level	Reaction	Characteristics
0	No edema	Edema value ranges from 0 to 0,24 mm.
1	Very slight edema (weakly perceptible)	Edema value ranges from 0,25 to 0,49 mm.
2	Slight edema	There are well defined edge areas with a perceptible elevation. Edema should be 0,5 to 0,74 mm.
3	Moderate edema (well perceptible)	Edema value should from 0,75 to 1 mm.
4	Severe edema	Edema value is greater than 1 mm or even bigger than the tested area.

Table 2. Classification of the edema formed by cutaneous reaction to products which do not modify skin color.

induce biological reactivity over the cell culture, using cell viability as a parameter [22].

The cytotoxicity test by the Neutral Red Uptake Method allows the assessment of the ability of a material to kill the cells of a standardized culture. The percentage of culture cell viability determines weather the material is safe to be used as a biomaterial [22]. Cytotoxicity index is the concentration of a sample which causes injury or death to 50 % of cell population assessed. All viability curves under cytotoxicity index line were considered cytotoxic and above CI_{50} non-cytotoxic. In the viability curse intersection and CI50 line, the cytotoxicity index of the sample was obtained.

The cell culture viability after contact to both silicone dispersions was greater than CI_{50}, as represented in Fig. 1. However, NRBL and AA polymers were identified as cytotoxic in the cell culture applied (Fig. 2) and the AA membrane presented higher toxicity. The viability curve is located around zero percent under the cytotoxicity index line, indicating that even at a 6.25 % sample concentration the cell viability was about zero. All cellular population in the test was injured by toxic compounds extracted from the sample. NRBL membrane showed cytotoxic effects with a viability curve crossing CI_{50} line and presenting CI_{50} of about 22.

Both silicone dispersions were found non-cytotoxic, which is an expected result for a biomaterial. However, NRBL and AA polymers showed to be cytotoxic under the test conditions.

The selection of materials to be used in biomedical devices considers the *in vitro* Cytotoxicity test results as well as the primary cutaneous irritation test outcome in order to establish their irritant potential to human beings [23].

As NRBL and AA polymers showed cytotoxi-

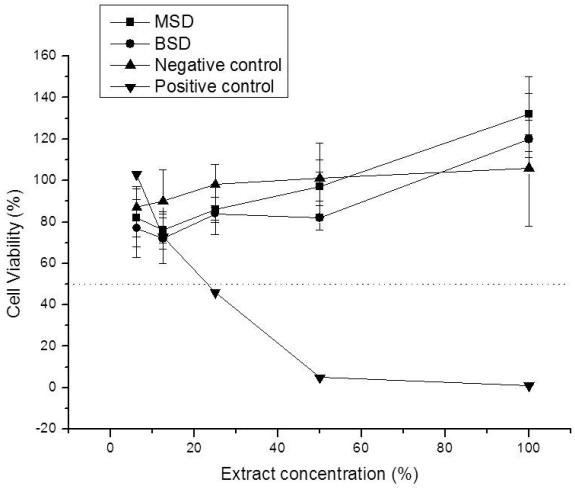

Figure 1. Cell viability curves of MSD (Monocomponent Silicone Dispersion) and BSD (Bicomponent Silicone Dispersion) polymers in the cytotoxicity test by Neutral Red Uptake Method.

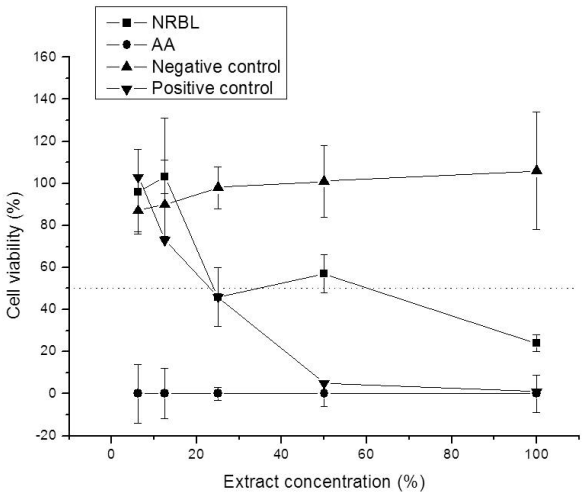

Figure 2. Cell viability curves of NRBL (Natural Rubber Bicentrifuged Latex) and AA (Acrylic Adhesive) polymers in the Cytotoxicity assay by Neutral Red Uptake Method.

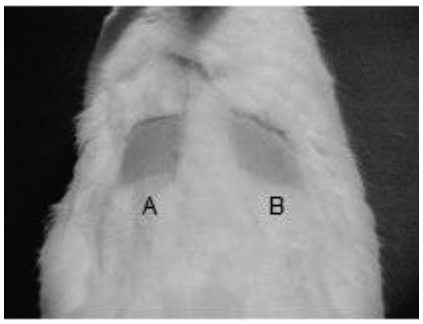

Figure 3. Back region of an animal submitted to the test with MSD polymer. A: control area; B: sample area.

city they were not submitted to the primary cutaneous irritation test.

All twelve tested animals presented zero erythema grade (normal skin) and zero edema grade (no edema), according to classification presented in Tables 1 and 2, as observed in Figure 3. Therefore, cutaneous irritation index for both silicone dispersions was zero and considered non-irritant.

MSD and BSD polymers did not cause any cutaneous reaction onto animal skin. These results indicated that these materials do not represent an irritant potential to human skin and may be used as biomaterials.

CONCLUSIONS

From the toxicological point of view the results obtained indicate that both monocomponent and bicomponent silicone dispersions are suitable for development of a papain-containing drug delivery system, sterilized by gamma radiation.

Acknowledgments. We are thankful to Dr. Olga Zazuco Higa for her great contribution. We also thank to Adolfo Lutz Institute for the special help to carry this study out and to CAPES and IPEN for the financial support.

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