In vivo toxicity of enoxaparin encapsulated in mucoadhesive nanoparticles: Topical application in a wound healing model

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Abstract: Wound healing comprises four distinct phases and involves many cell events and biologic markers. The use of nanoparticles for topical application has gaining attention due to its deeper penetration in the skin and the retention capacity of the drug in the site of application. In this study the effect and toxicity of mucoadhesive polymeric nanoparticles loaded with enoxaparin was evaluated in in vivo model of skin ulcer. Our results showed an interesting formulation based on mucoadhesive nanoparticles with enoxaparin that improved wound healing without cytotoxicity in vitro in all endpoint evaluated. Then, this semi-solid formulation is a promising option for skin ulcer treatment.

1. Introduction

Wound healing is a dynamic and complex process that results in anatomical and functional restoration of the injured area [1]. It comprises four distinct phases: hemostasis, inflammation, proliferation and remodeling. There are some pathological conditions which this efficient process is altered, resulting in not healed ulcers, like in diabetes [2-4]. An interesting drug for treatment of these ulcers is low molecular weight heparin (enoxaparin) due to its anti-inflammatory property, analgesia, neoangiogenesis and epithelization [5, 6]. An area that has been widely investigated is the topical application of nanoparticles, due to its deeper penetration in the skin and the retention capacity of the drug in the site of application [7]. The encapsulation of enoxaparin in polymeric nanoparticles for topical use can help in the healing process, increasing the drug stability and skin penetration and decreasing its toxicity. To increase their adhesiveness and to helps in the wound healing, chitosan is being used. Chitosan is a biocompatible and biodegradable polymer, nontoxic and low cost, and also presents interesting biological properties like antimicrobial activity, bioadhesiveness, potent topical analgesic action and acceleration of the healing process [8,9].

In this study polymeric nanoparticles recovered with chitosan were prepared in one step, with the aim to improve the economical aspect of the process and the properties of these nanostructures (increase of the skin adhesion and antimicrobial effect) with enoxaparin, to promote an efficient wound healing ulcers.
Actually, the interest for the safe use of nanometric materials became valuable. The risk/benefit about the use of nanoparticles must be measured by medical and technological methods [10]. One way to test nanoparticles in vitro is through cytotoxicity evaluation. Fibroblasts from mouse BALB/c 3T3 could be consider a cell model to estimate the toxicity of nanoparticles. The evaluation of toxicity in vivo was made through hepatic and renal enzymes, hemogram and oxidative stress. Wound healing is affected according the quantity of oxygen reactives species (ROS), since the ROS suppression will result in infection and their elevation could result in destruction of healthy stromal tissue [11].

In this study the in vivo effect in wound healing and in vitro toxicity of mucoadhesive nanoparticles loaded with enoxaparin was evaluated. The results showed an interesting nanocarrier system for enoxaparin improving the wound healing and did not exhibit in vitro toxicity.

2. Material and Methods

2.1. Preparation of polymeric nanoparticles recovered with chitosan in the encapsulation of enoxaparin

The nanoparticles were prepared with the biodegradable polymer poly(ε-carbolactona) (PCL) (Aldrich) recovered with the natural polymer chitosan (105 g/mol ~81% acetylation, Polymar). The preparation of nanoparticles loaded enoxaparin was held by the double emulsion modified technique and evaporation of the solvent as describe by Meng et al [12].

2.2. Characterization of the nanoparticles

2.2.1. Size, distribution and Zeta potential

The dispersion of nanoparticles was diluted with water and the average diameter (Z-average), distribution and superficial charge was measured in Zeta Sizer Manlvern®.

2.2.2. Encapsulation efficiency

The encapsulated enoxaparin in nanoparticles was quantified through the modified Azure II colorimetric method as describe by Lam et al [13].

2.3. In vivo evaluation

2.3.1. Experimental animals

This study was performed using 50 male Wistar rats with age of 6-8 weeks. All the experiments were carried out with the standards established by Brazilian law for the animal experiments approved by Ethics Committee on the use of Animals (CEUA – Unicamp) certificate number 2290-1.

After intramuscular anesthesia with ketamine (75 mg/kg) and xylazine (10 mg/kg), a 1 cm² quad format dermatological lesion was performed with scissor and tweezer. Posteriorly, the animals were divided in five groups of daily treatment, with 10 animals each: G1- free ENOXA gel (2mg/g gel), G2- Sham, G3- pure gel, G4- empty nanoparticles gel (2 mg/g gel), G5- ENOXA nanoparticles gel (2m/g gel).

Macroscopically, the evaluation of the wound healing was made by measurement of wound contraction in established days (4th day, 7th day and 10th day) and analyzed through Image J software. After 10 days of the lesion, the animals were anaesthetized and sacrificed. The blood was collected by cardiac puncture.

2.3.2. Enzymatic evaluation, hemogram and oxidative stress

The hemogram was accomplished in the automation system Cell Dyn 1700 ABBOTT.

The blood was washed with the cold buffer to catalise analysis, as describe by Aebi [14]. The plasm was utilized to evaluate lipid peroxidation (TBARS) according Yagi methodology [15] and nitrite dosage was realized through Griess reaction [16]. The plasm was also analyzed to verify the presence of enoxaparin in the bloodstream with the evaluation of the anti-Xa activity (Kit IEL-equipment Top 500).

The serum was used to analyze aspartate aminotransferase (AST) and alanine aminotransferase (ALT) both using the kinetic method (Kit Laborlab). Urea was measured by enzymatic colorimetric
method (Kit Laborclin) and creatinine was measured by kinetic method (Kit Laborlab), as indicators of hepatic and renal function.

2.4. Free-enoxaparin cytotoxicity, empty nanoparticles and encapsulated enoxaparin

The cytotoxicity test of free-enoxaparin, empty nanoparticles and encapsulated enoxaparin was made in mouse embryo fibroblasts (3T3) from BALB/c. The cells were plated in the density of 2 x 10^4/mL in 96 wells plates and incubated for 48 hours until reach the semi-confluency phase. Then, the cultures were exposed to free-enoxaparin, empty and encapsulated nanoparticles, in different concentrations (0-100 µM for free and encapsulated enoxaparin and 0-400 µM for empty nanoparticles). The cells were incubated for 2 hours with the samples. Each concentration was tested in 6 replicates. In the end of incubation, MTT was used to evaluate the cell viability.

3. Results and discussion

3.1. Preparation and characterization of mucoadhesive enoxaparin nanoparticles

The preparation of PCL/chitosan nanoparticles had a relation 6:1, with or without enoxaparin, and presented a average particles size of 496 ± 491 nm, respectively, and positive surface charges with average values of +25 mV (without enoxaparin) e +20 mV (with enoxaparin) (Table 1). The encapsulation efficiency was 98% (Table 1) and the particles were stable during 90 days.

Table 1. Particles sizes, surface charge (Zeta potential), and encapsulation efficiency of mucoadhesive nanoparticles with and without enoxaparin

| PCL/Chitosan ratios | Enoxaparin (mg) | Zeta Potential (mV) | Particles sizes (nm) | PdI | Encapsulation efficiency (%) |
|---------------------|-----------------|---------------------|----------------------|-----|-----------------------------|
| 6:1                 | 0               | +25                 | 491                  | 0.357 | ---                         |
| 6:1                 | 200             | +20                 | 496                  | 0.369 | 98                          |

3.2. Macroscopically analysis, enzymatic evaluation, hemogram and oxidative stress

In the macroscopically analysis the values are expressed in percentage of size lesion through mean ± SD in the different groups according the analyzed days (d4- 4th day, d7- 7th day, d10- 10th day) (Table 2). The animals treated with mucoadhesive nanoparticles with ENOXA showed in the 10th day a minor wound (0.034 cm^2) compared with the group treated with free ENOXA (0.091 cm^2), being statistically significant (p=0.0238) according the Mann-Whitney test.

Table 2: Macroscopically evaluation of the wound size in the animals of different groups

| Groups                  | D4             | D7             | D10            |
|-------------------------|----------------|----------------|----------------|
| Free-ENOXA gel          | 64.6% ± 6.7    | 34.9% ± 8.8    | 9.2% ± 5.9     |
| Sham                    | 70.3% ± 10.3   | 46.2% ± 12.4   | 18.5% ± 2.5    |
| Pure gel                | 80.6% ± 13.9   | 35.9% ± 10.9   | 9.1% ± 6.1     |
| Empty nanoparticles gel | 58.4% ± 12.6   | 26.0% ± 5.8    | 3.5% ± 2.7     |
| ENOXA-nanoparticles gel | 69.8% ± 13.9   | 28.4% ± 12.8   | 4.7% ± 2.7     |

The animals hemogram presents all the values in the normal range according Melo et al [17] (Table 3), evidencing that no collateral effects was present due to the use of enoxaparin in the animals (normal number of platelets). The enzymes also presents concentrations in the reference range as describe by Melo et al [17]. The mean of the results ± SD is evidenced in the table below (Table 3).
Table 3: Results of the hemogram and enzymatic evaluation of the animals

|        | WBC (k/µL) | RBC (M/µL) | Hb (g/dL) | HCT (%) | Plt (k/µL) | ALT (U/L) | AST (U/L) | Urea (mg/dL) | Creatinina (U/L) |
|--------|------------|------------|-----------|---------|------------|-----------|-----------|--------------|-----------------|
| Média  | 5.23       | 7.02       | 13.70     | 39.10   | 555.03     | 45.5      | 135.9     | 51.5         | 0.45            |
| DP     | 1.87       | 0.62       | 1.06      | 3.34    | 63.80      | 3.5       | 15.3      | 3.6          | 0.03            |

It was not found significant differences in catalase activity, the groups presents values close having a mean of 0.01031 (k/gHb/min) ± 0.0007542.

The TBARS and nitrite analysis revealed a higher concentration of both in the groups treated with enoxaparin, suggesting a increase in the pro-oxidants concentrations by the use of enoxaparin, being statically significant according ANOVA test (p= 0.0022 for TBARS e p< 0.0001 for nitrite), as show the Table 4.

Table 4: Evaluation of stress oxidative markers TBARS e NO\textsubscript{2} in plasm

| Groups                        | µmol NO\textsubscript{2}/µg de ptn | MDA (nM/mg ptn) |
|-------------------------------|-----------------------------------|-----------------|
| Gel free-ENOXA                | 32.1 ± 11.7                       | 0.000268 ± 0.000076 |
| Sham                          | 24.1 ± 9.8                        | 0.000109 ± 0.000075 |
| Pure gel                      | 25.4 ± 11.8                       | 0.000202 ± 0.000076 |
| Empty nanoparticles gel       | 12.4 ± 6.1                        | 0.000055 ± 0.000027 |
| ENOXA-nanoparticles gel       | 27.0 ± 6.3                        | 0.000494 ± 0.000285 |

The anti-Xa activity was not detected, showing enoxaparin did not reach the bloodstream, having only a local effect in the lesion area, offering no adverse risk in the concentration tested for topical application (2mg/g gel).

3.3 Cytotoxicity of free enoxaparin, empty and encapsulated nanoparticles

Free enoxaparin showed toxicity in fibroblasts (3T3) evaluated through MTT (42.5% of toxicity in the concentration of 100 µM) and this is observed in the figure 1A. However, when the enoxaparin is encapsulated in nanoparticles its toxicity decreases a lot (5% of toxicity in the concentration of 100 µM), presenting a significative value through Mann-Whitney test (p=0.0095) evidencing the protector effect of nanoparticles with the drug. The figure 1B presents the non toxicity of the polymers used in the preparation of the nanoparticles showing no toxicity until 200 µM of PCL, demonstrating a interesting drug delivery system.

![Figure 1](image-url)
4. Conclusions

Our results showed no toxicity in vitro. There was an increase in the concentration of the oxidative stress markers in the groups treated with ENOXA. This study demonstrated an interesting formulation based on mucoadhesive nanoparticles with enoxaparin that improved wound healing. Furthermore, the formulation showed a good effect on wound healing in diabetic animals, indicating a promising treatment for ulcers.

5. Acknowledgments

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