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In vitro Biocompatibility and Genotoxicity Assessment of a Gentamicin-Loaded Monoolein Gel Intended to Treat of Chronic Osteomyelitis

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Abstract: The aim of the study was to assess *in vitro* the biocompatibility and the genotoxicity of a gentamicin-loaded monoolein gel intended to treat of chronic osteomyelitis. Indeed, we are developing biodegradable implants based on monoolein and gentamicin. The results of formulations, physico-chemical characterization of the formulated implants and *in vitro* release kinetic of gentamicin from implants were encouraging. As biocompatibility and absence of genotoxicity are the prerequisites for safe use of implants, we performed *in vitro* hemolysis, cytotoxicity and, genotoxicity tests. Hemolysis was evaluated by incubating human erythrocytes in direct contact with the implant whereas cytotoxicity was evaluated by 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) assay using fibroblasts and macrophages. Alkaline comet Assay was used to evaluate genotoxic potential of the implants. From these *in vitro* assays, the implant based on monoolein and gentamicin showed no genotoxic potential and has satisfactory biocompatibility.

Key words: Biocompatibility, genotoxicity, monoolein, gentamicin, implant

INTRODUCTION

Chronic osteomyelitis is an infection of bone and its marrow. Known since antiquity, it is characterized by progressive inflammatory destruction and new apposition of bone (Lew and Waldvogel, 1997). Chronic osteomyelitis is most common with some diseases like sickle cells disease, diabetes and rheumatoid polyarthritis (Habibou *et al*., 1999; Ader *et al*., 2004; Springer *et al*., 2007). It is frequent in Africa and some developing countries. For example, the frequency of chronic osteomyelitis is about 5% of the diseases in the orthopaedic and bone surgery Service of Ouagadougou Academic Hospital / Burkina Faso (Nacoulma *et al*., 2007).

Chronic osteomyelitis treatment often fails because it is difficult to achieve therapeutic drug levels at the site of infection by systemic administration without damage. Therefore, pharmaceutical forms able to deliver bactericidal drug levels directly at the site of infection while maintaining low systemic levels are studied (Scott *et al*., 1988; Garvin *et al*., 1994; Zhang *et al*., 1994; Désévaux *et al*., 2002). Poly(methyl methacrylate) (PMMA) beads containing gentamicin have been employed clinically to prevent or treat osteomyelitis since 1970s (Trippel, 1986). However, PMMA-based delivery systems present deficiencies. PMMA beads are not degraded *in vivo*. They have to be surgically removed later

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and replaced by new beads or a bone substitute. To avoid costly and painful surgery, several biodegradable controlled antibiotic-release devices are being developed. Currently, biodegradable polymers such as poly(D,L-Lactide), poly(D,L-lactide-co-glycolide) are used as devices (Zhang *et al*., 1994). Upon these delivery systems, the free (unencapsulated) drug is released quickly in a phenomenon termed burst effect (Mauduit *et al*., 1993; Schmidt *et al*., 1995). Our alternative was to use a biodegradable and bioadhesive monoglyceride (monoolein) to make a sustained-release formulation of the antibiotic (gentamicin sulfate). The results of formulations, physico-chemical characterization of the formulated implant and, *in vitro* release kinetic of gentamicin were encouraging; particularly for the implant containing 80% monoolein (glyceryl monooleate), 15% water and 5% gentamicin sulfate (Ouédraogo *et al*., 2008). In spite of these encouraging studies, there is no biocompatibility study of the formulated implant.

So, in the present study, *in vitro* hemolysis, cytotoxicity and, genotoxic tests were carried out to assess the safe use of the implant that we are developing.

MATERIALS AND METHODS

Materials

The assessed product was gentamicin-loaded monoolein gel composed of 80% monoolein (glyceryl monooleate), 15% water and 5% gentamicin sulfate. It was a liquid crystalline gel and became very viscous in contact with aqueous body fluids at 37°C. It was characterized as sustained-release implant (Ouédraogo *et al*., 2008). It was sterile and apyrogenic. The chemical structure of glyceryl monooleate is shown in Fig. 1.

For the hemolysis test, a solution of 0.9% chloride sodium, distilled water, human blood, the implant, glass tubes containing 0.5 mL of a solution of 0.129 M sodium citrate (Beliver Industrial Estate, Plymouth, UK) and UV-Visible spectrophotometer (S1000 SECONAM Esaco International, France) were used. Venous blood (5 mL) were obtained from the antecubital fossa of ten informed healthy volunteers whose hemoglobin is normal (Hemoglobin AA) and ten informed volunteers whose hemoglobin is abnormal (hemoglobin SS). Immediately after venopuncture, 4.5 mL of blood were placed into each tube containing sodium citrate.

Chinese Hamster (*Cricetulus griseus*) fibroblasts V79-4 and mouse (*Mus musculus*) macrophages P388D1 cells lines were used for cytotoxicity and genotoxicity tests. The cells lines were acquired from American Type Culture Collection (ATCC) bank.

Hemolysis Test

Hemolytic activity of the implant was evaluated according to the method of Roy Chowdhury *et al*. (2004). The anti-coagulated blood was diluted with the solution of chloride sodium 0.9% in the proportion of 1:9. For checking the hemolysis, 0.2 mL of diluted blood was added to 10 mL of distilled water. Since distilled water is known to cause large-scale rupture of Red Blood Cells (RBC), the Optical Density (OD) count of this solution was taken as positive control referred to as OD (positive). Similarly, for negative control, 0.2 mL of diluted blood was added to 10 mL of the

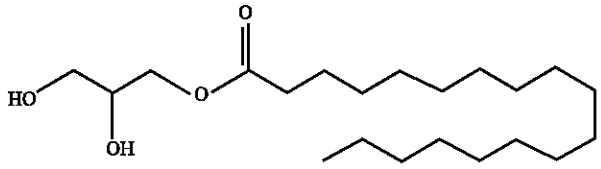

Fig. 1: Chemical structure of glyceryl monooleate

chloride sodium 0.9% (w/v). The OD count of this solution was taken as negative control referred to as OD (negative). Having obtained the two standard OD, the OD of the material (implant) was obtained in similar lines: 200, 300, 400 and 500 mg of the implant were respectively taken in test tubes containing 10 mL of chloride sodium 0.9% (w/v) and then 0.2 mL of diluted blood was added to the test tube and mixed gently. The OD of the sample is referred to as OD (test).

The mixtures were incubated at 37°C for 60 min. The OD of the incubated solution was measured in the UV-Visible spectrophotometer at 545 nm wavelengths.

The hemolytic activity was determined by the following equation:

$$\text{Hemolysis (\%)} = (\text{OD test-OD negative/OD positive-OD negative}) \times 100$$

For each blood sample from the 20 volunteers, the experiment was performed in triplicate.

Cytotoxicity Test

Cytotoxicity was evaluated by the direct contact method, using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) colorimetric assay.

Fibroblasts experimental culture medium (GIBCO, invitrogen Co, UK) was Dulbecco's Mod Eagle Medium supplemented with fetal bovine serum, streptomycin, penicillin and glutamine. Macrophages were cultured in RPMI medium supplemented with penicillin, streptomycin, glutamine, Hepes Buffer and fetal bovine serum (GIBCO, invitrogen Co, UK). Cells were cultured at 37°C in a humidified incubator equilibrated with 5% CO_2. Cells were harvested by means of a sterile trypsin-EDTA solution (0.05 M trypsin, 0.02 M EDTA in normal Phosphate Buffered Saline, pH 7.4) from the culture flasks, resuspended in the experimental cell culture medium and diluted to 125000 cells mL^{-1}. This cell suspension (0.2 mL) was seeded in each well of the microplate (96 wells). The microplate was incubated for 24 h at 37°C in a humidified incubator equilibrated with 5% CO_2. After this period, dissolved implant in methanol (at non-toxic concentration) was distributed in wells at concentrations ranged from 0.009 μg mL^{-1} to 37.5 μg mL^{-1}. A similar experiment was done with the solvent of the implant. The dilutions were done in the culture medium. As negative control, only experimental culture medium was put in contact with cells. In order to verify a possible interference between implants and reagents, implants were put in wells without cells.

Microplates were then incubated for 48 h at 37°C in a humidified incubator equilibrated with 5% CO_2. After the incubation period, culture medium was discarded and replaced with 0.2 mL $well^{-1}$ of MTT solution. At the end of the incubation period, the MTT solution was removed and replaced by 0.2 mL dimethylsulfoxide (DMSO) per well, in order to dissolve formazan. The wells were swirled for 30 min until the purple color was uniform. The Optical Density (OD) of each well was read on a spectrophotometer for microplates (Labsystems, Finland) at 540 nm. The OD values obtained were averaged. The cells viability was expressed by comparing the OD mean of the sample to negative control one (which, by definition, is 100%).

The cytotoxicity test was performed in triplicate.

***In vitro* Genotoxicity Test**

The genotoxic potential of the implant was investigated by using the comet assay. The comet assay was performed under alkaline conditions according to the procedure of Singh *et al*. (1988) with slight modifications. Fibroblasts V79-4 and macrophages P388D1 suspensions were respectively mixed with the implant (37.5 μg mL^{-1}) dissolved in methanol (1% v/v) and incubated at 37°C for 48 h. In order to evaluate a possible interference of the solvent of the implant (methanol) on the test, a control test was also performed by treating cells with diluted methanol (1% v/v). A negative control was performed with the culture mediums.

At the end of treatment, the cells were washed, trypsinized and re-suspended in fresh medium. They were then centrifuged (2000 rpm) for 2 min. Liquefied agarose (300 μL) was then added to a

15 μL aliquot of cells suspensions (containing approx. 2, 000,000 cells mL^{-1}). The samples were applied to a microscope slides precoated with agarose. The slides were then covered with a coverslip. After 20 min at 4°C, the coverslip was removed and the slides were immersed for 60 min at 4°C in lysis solution containing 89 mL of lysis stock solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, ~8 g NaOH to obtain a pH of 10, 890 mL of deionized water and 1% sodium lauryl sarcosinate), 1 mL Triton X-100 (Merck) and 10 mL DMSO. The slides were then placed in an electrophoresis chamber in a chilled bath and maintained submersed in alkaline running buffer (pH > 13) (5 mL 200 mM EDTA, 30 mL 10 N NaOH and 965 mL of deionized water at 4°C) for 40 min, to examine for single strand breaks. The slides were electrophoresed for 20 min at 25 V and 300 mA. The slides were then covered with neutralizing solution (0.4 M Tris-HCl, pH 7.5) for 15 min at 22°C. Finally, the slides were submersed in absolute ethanol for 10 min at 22°C. All the procedures were carried out under low light to avoid extra DNA damage by radiations. The slides were stained with ethidium bromide (20 μg mL^{-1}) and examined with fluorescence microscopy.

The DNA damage was expressed as Tail DNA%, where;

$$\text{Tail DNA (\%)} = \frac{\text{Tail DNA}}{\text{(Head DNA+ Tail DNA)}} \times 100$$

The mean value of the Tail DNA% in a sample was taken as an index of DNA damage in this sample.

Statistical Analysis

Statistical analysis was performed using GraphPad PRISM version 2.01 (GraphPad Software Inc., USA). Values of $p<0.05$ were considered significant. After testing data for normal distribution student's t paired test was used to compare at each dose, hemolysis mean as a function of type of erythrocytes.

Kruskal-Wallis's test followed by the Dunn's multiple comparison test was used to compare Tail DNA% of untreated cells, cells treated by the diluted solvent and ones treated by the implant.

RESULTS

Hemolysis Test

Hemolysis induced by the implant was a linear function of the implant doses. At 200 mg/100 mL dose of the implant, hemolysis was almost non-existent for the two types of erythrocytes. Hemolysis observed with abnormal erythrocytes at high doses were statistically higher than normal erythrocytes ones ($p<0.05$) (Fig. 2).

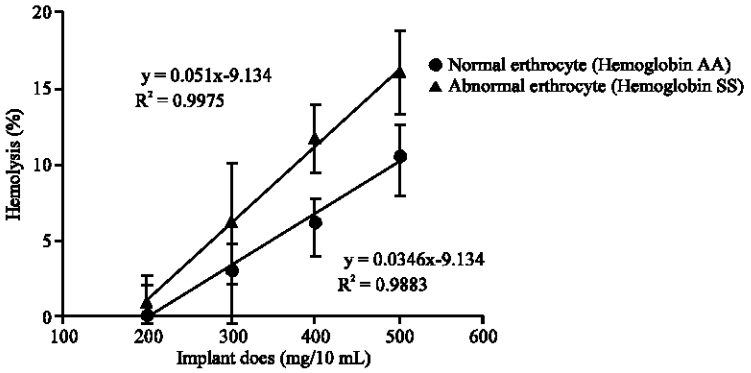

Fig. 2: Hemolysis percentage as a function of implant dose; the results are presented as mean±SD of 10 individual analysis of RBC integrity in 3 different experiments

Table 1: Tail DNA (mean±SEM) percentage as function of cells and product

	Negative control	Diluted methanol (1% v/v)	Implant
Fibroblasts V79-4	14.5±1.2	18.6±3.0	13.4±1.3
	(n = 187)	(n = 24)	(n = 139)
Macrophages P388D1	15.8±1.0*	10.3±0.8	9.2±1.0
	(n = 202)	(n = 181)	(n = 130)

Kruskal-Wallis's test followed by Dunn's post-test when $p<0.05$; *Negative control, diluted methanol, implants 1, 2 Tail DNA% comparison ($p<0.001$)

Cytotoxicity Test

The viability of unexposed cells (fibroblasts and macrophages) was similar to the viability of the cells exposed to the implant and diluted methanol. It was about 100%. The optical density of wells containing implants without cells was around zero.

***In vitro* Genotoxicity Test**

The tail DNA percentage mean was below 20 for unexposed fibroblasts and treated ones (Table 1). There was statistically no difference between the two values of tail DNA percentage mean. As for macrophages cells line, the tail DNA percentage mean was about 10 for the macrophages treated by diluted methanol (implant solvent) and by the implant. When not exposed, the tail DNA percentage of cells was surprisingly about 15%. It was statistically higher than treated macrophages one ($p<0.001$).

DISCUSSION

Biosafety tests such as cytotoxicity and genotoxicity are aimed at excluding the severe harmful effects of implants. In addition to cytotoxicity test, the hemolysis test is an important parameter in testing the biocompatibility of materials on organisms (Roy Chowdhury *et al*., 2007).

Toxic substances may induce membrane damage and impairment of metabolic activity in the cell. The main consequences of these events are cells lysis and death. As erythrocytes are among the first cells that come into contact with the formulated implant *in vivo*, cells lysis was evaluated by hemolysis test. In addition to erythrocytes which hemoglobin was normal (AA), erythrocytes with hemoglobin SS were used in the hemolysis test because patients (affected by chronic osteomyelitis) whom implants are intended, often have sickle cells disease (Habibou *et al*., 1999). At 200 mg/10 mL, implant did not provoke erythrocytes lysis. Indeed, mean of hemolysis percentage did not statistically differ from zero ($p<0.001$). This concentration was chosen as the highest concentration of implant which could be obtained in the blood during an implantation *in vivo*. Thus, the implant is compatible with normal and abnormal erythrocytes at therapeutic doses. However, it can cause minor hemolysis at over-doses. As shows (Fig. 1), hemolysis induced by the implant increased with the dose of implant. The highly hemocompatible doses of implant (i.e., when hemolysis percentage is less than 5 (Chowdhury *et al*., 2003)) were about 351 mg and 277 mg/10 mL diluted blood respectively for the normal and abnormal erythrocytes (Fig. 1). Therefore, Abnormal erythrocytes (hemoglobin SS) were more sensitive to the implant effect than normal erythrocytes ($p<0.05$). This higher sensitivity to the implant could be explained by the taint of erythrocytes with hemoglobin SS. However, the implant was highly compatible with both types of erythrocytes. Indeed, a study reported that Tween 80®, a typical non-ionic surfactant used for intravenous administration, induces 82.8% hemolysis at a concentration of 100 mg/10 mL (Lee *et al*., 2003) whereas implant did not provoke any hemolysis at this concentration.

In addition to hemolysis test, cytotoxicity test represents the initial phase in testing the biocompatibility of implants (Roy Chowdhury *et al*., 2007). In addition to fibroblasts which are widely used to test the biocompatibility of implants (Ignatius and Claes, 1996; Vale *et al*., 1997;

Corry and Moran, 1998; Tiozzo *et al*., 2003), macrophages were used in our study. Indeed, macrophages are among the predominant cells that could come into contact with implants *in vivo* (Woodward, 1999).

The cytotoxicity test can be used for predicting the possible skin irritating potential of topical formulation (Thornback-Lecoq *et al*., 1997). The assessed implants did not exhibit cytotoxicity for fibroblasts nor macrophages even at the highest concentration (37.5 μg mL^{-1}). This highest concentration was the saturation limit of implants in the diluted methanol. Thus, the implant could be no irritating when it will be used *in vivo*.

The non-cytotoxicity of the implant confirms the high biocompatibility of the monoolein (the major component of the implant) reported by Ganem-Quintanar *et al*. (2000) and Rowe *et al*. (2003).

As the optical density of wells containing implants without cells was around zero, there was no interference between implants and reagents. Therefore, implants did not affect the measure of optical density. Moreover, methanol did not influence cells viability at the concentrations used to dissolve the implant. So, it could be used at low concentrations to dissolve liposoluble samples during cytotoxicity tests.

The alkaline comet assay was used in this genotoxicity study because it is a sensitive technique to assess the genotoxic potential of compounds (Devaux *et al*., 1997). The highest concentration of implant was used to study genotoxicity. It did not provoke cells death. Indeed, nucleases activated during cell death can confound the detection of direct chemically-induced DNA damage (Henderson *et al*., 1998).

As DNA damage of unexposed fibroblasts and treated fibroblasts were statistically identical (Table 1), the assessed implant did not cause structural changes in the DNA molecule.

Concerning genotoxicity with macrophages, the levels of DNA damage caused by diluted methanol and the implant were similar but were surprisingly less than the one observed with unexposed macrophages ($p<0.001$). Could diluted methanol stimulating macrophages proliferation? Further investigations will be necessary to elucidate this hypothesis.

Implants did not cause DNA damage since they had the same Tail DNA percentage as their solvent during the comet assay. They could not lead to tumour formation when they will be in contact with tissues.

In conclusion, results obtained in hemolysis, cytotoxicity and genotoxicity tests suggested that the implant composed of monoolein and gentamicin are neither hemolytic, nor cytotoxic and have no genotoxic potential. This novel formulation might be safe for further development as an implant intended to treat locally chronic osteomyelitis.

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