**In vivo Biocompatibility and Toxicity Assessment of a Gentamicin-Loaded Monoolein Gel Intended to Treat Chronic Osteomyelitis**

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**Abstract:** Biocompatibility and preliminary toxicity of a novel gentamicin-loaded monoolein gel (implant) intended for the local treatment of chronic osteomyelitis were investigated in mice. The mice, randomly allotted in 3 groups of 10, received respectively a single dose (0.65 mL) of normal saline, monoolein and the gel by subplantar injection. Clinical monitoring and assessment of induced oedema were carried out during 52 days after implantation. A histologic examination of the implantation site was performed at the end of the experiment. Renal and hepatic functions of the implant were also assessed on 52 days post-implantation by using biochemical and histological methods. In mice, no adverse reaction occurred after implantation. Only, a transitional foreign body reaction was observed in mice implanted by the monoolein and the implant. The paw volume of the mice increased within 3 h post-implantation and returned to baseline by 52 days. The liver and kidneys histology at light microscopy and biochemical parameters were similar for all mice. Further investigation is undertaken to detect eventual early damages which could have been resolved with time. Nevertheless, the novel gel is biocompatible and doesn’t show sub-chronic toxicity.

**Keywords:** Monoolein, gentamicin, implant, *in vivo* biocompatibility, toxicity

**INTRODUCTION**

Osteomyelitis is an infection of the bone and its marrow, mainly caused by pyogenic microorganisms such as *Staphylococcus aureus* (Lew and Waldholzplant, 1997). Its treatment is currently carried out by surgical curettage of the infected regions followed by systemic and repetitive administrations of antibiotics such as gentamicin over a long period. Unfortunately, the relatively low blood flow in the bone, the short half-life and systemic toxicity of the antibiotic agents do not always permit to reach therapeutic levels of the drug at the infection site. In order to overcome this difficulty, the use of sustained release implants, which are able to deliver locally sufficient concentrations of antibiotic into the site of infection while maintaining low systemic levels, is more suitable (Garvin et al., 1994; Zhang et al., 1994; Huneault et al., 2004).
That is why, non-degradable gentamicin poly(methylmethacrylate) beads have been clinically employed for preventing or treating osteomyelitis since the 1970s (Trippel, 1986). To avoid the cost, pain and other risks associated with the necessary second surgical intervention for extracting non-biodegradable delivery systems, biodegradable implants, using poly (D,L-lactide) or poly (D,L-lactide-co-glycolide) carriers were investigated (Zhang et al., 1994). Although these polymeric delivery systems allow prolongation of the drug release, they are solid, non-biodegradable and often show marked burst effects due to the high proportion of non-encapsulated drug (Mauduit et al., 1993; Schmidt et al., 1995).

These limitations of the polymeric implants in osteomyelitis management have led us to develop a non polymeric delivery system, consisting of monoolein (glyceryl monooleate)/water liquid crystals, incorporating gentamicin sulfate as an antibiotic drug. This non polymeric system is semi-solid and bioadhesive. It progressively releases the antibiotic for a period of 3 weeks without burst effect.

When a new material is introduced into the market, or an existing material is proposed for a different application, its properties should be investigated (Zafalon et al., 2007). A fundamental understanding of the in vivo degradation phenomenon as well as an appreciation tissue responses play a key role in the development of biodegradable materials for therapeutic. Our previous results demonstrated that the gentamicin-monoolein-water gel was biocompatible in vitro and had no genotoxic potential. Owing to the complexity of body fluids, results of in vitro studies are not always good indicators of in vivo behaviour.

The aim of present study was to investigate in animal the biocompatibility of the novel implant (that we are developing) and to perform its preliminary toxicity assessment.

MATERIALS AND METHODS

Experiments were performed from January to June, 2007 in 3 laboratories: Bioanalytical Chemistry and Toxicology Laboratory of Pharmacy Institute (Free University of Brussels/Belgium), Pharmacology and Toxicology Laboratory (University of Ouagadougou/Burkina Faso) and Histology and pathology Laboratory (Yalgado Ouedraogo University Hospital of Ouagadougou/Burkina Faso).

Experimental Animals

Thirty NMRI male mice weighing 35-39 g at the time of experience were used in the study. They were 12-13 weeks old. A 10-days period of acclimatization was applied before implantation. The mice were reared in polypropylene cages with food and water ad libitum. The room temperature was 20-25°C and the humidity rate was 40-60%. The mice were exposed to alternate cycle of 12 h of darkness and light each. They were randomly allotted into 3 groups of 10. They were fasted for 12 h before the experiment. The ethical guidelines for the investigation of animals used in experiments were followed in all tests.

Implant

The assessed product was a monoolein-water gel containing gentamicin (5%). It was a liquid crystalline gel and became very viscous in contact with aqueous body fluids at 37°C. The gel was a sustained-release implant. It was sterile and apyrogenic.

Implantation Procedure

The right hind paw of the mouse was aseptically cleaned by using ethyl alcohol 70% (V/V). Into the subplantar surface of the right hind paw of groups 1 (negative control group), 2 (test group) and 3 (reference group), a single dose of 0.05 mL of normal saline (NaCl 0.9%), implant and monoolein were respectively aseptically injected.
Clinical Monitoring

The animals were daily and carefully observed for any behavioural change and mortality during 52 days after implantation. The implantation sites were parallel observed for local inflammatory reaction (oedema, redness, heat and pain).

Preparation of Blood Samples

Prior to blood sampling, animals fasted for 12 h. Blood samples were drawn from the jugular vein of each sacrificed animal. The samples were collected in plastic test tubes and allowed to stand for complete clotting. The clotted blood samples were centrifuged at 3000 rpm for 15 min and serum samples aspirated off and frozen.

Host Local Response to the Implant

Assessment of Induced Oedema

The increment of the paw volume was assessed by Winter et al. (1962) method with slight modification. Before injection of normal saline, implant or monoolein, the average volume of the right hind paw of each mouse was registered from 3 readings. After injection of the products, the readings were obtained for each mouse at 3, 6, 24, 48, 72 h and 12, 19, 26, 35, 52 days. The paw volume was registered with the aid of a Pletthysmometer (7150 UGO Basile, Italy). The oedema was expressed as an increase in the volume of paw.

Histologic Examination of the Implantation Site

Fifty-two days after implantation, the mice were killed by cervical dislocation. The two hind paws were rejected and fixed in 10% buffered formalin (Cooper, France) for 72 h. The non-implanted left hind paw was the negative control for each mouse. The paws were dehydrated in a graded series of reagent alcohols. Then they were embedded in paraffin and cut to a thickness of 4 μm. The sections were parallel to the plant of the paw. They were mounted on glass slides and stained with Hematoxylin-Phloxine-Saffron (HPS). The slides were examined under a light microscope by two independent histopathologists. The type of inflammatory cells and the presence of a fibrous capsule were evaluated. The density of inflammatory reaction was graded as follows: 0, no infiltrate; +, minimal infiltrate; ++, moderate infiltrate and ++++, marked infiltrate. Besides, the right hind paws of 3 mice per group were dissected for macroscopic examination.

Preliminary Assessment of Renal and Hepatic Function

The liver and the two kidneys of the sacrificed mice were surgically removed. These organs were immediately fixed in fixed in 10% buffered formalin (Cooper, France) for 72 h. Longitudinal sections at the approximate midline of the organs were performed. Then, they were routinely dehydrated, embedded in paraffin and cut to a thickness of 4 μm. The sections were stained with Hematoxylin-Phloxine-Saffron (HPS).

The renal sections were examined by two independent histopathologists for tubular brush border loss, interstitial oedema, tubular dilatation and necrosis of epithelium. The two independent observers also examined the hepatic sections for histological lesions. A minimum of 10 fields for each kidney and liver slide were examined and assigned for severity of changes using scores on a scale of none (-), mild (+), moderate (++) and severe (+++) damage.

The hepatic and renal functions were assessed by dosing respectively bilirubins and creatinine in serums. Clinical chemistry assays (total bilirubin, direct bilirubin, creatinine) were performed on defrosted serums using a Mira Plus analyses (ABX Diagnostics, Suisse) and appropriate commonly available kits.
Effect of the Implant on Triglycerides Metabolism

Triglycerides concentrations in mice serum were measured after scarification. The automate Mira Plus analyses and triglycerides kit were used for these analysis.

Statistical Analysis

Data are expressed as means±Standard Deviation (SD). All means were treated with One-way ANOVA test followed by Dunnet’s multiple comparison to compare the paw volume before implantation to one after implantation at different time. The same One-way ANOVA test was used to compare the serum bilirubins and creatinine concentrations of 3 lots. Criterion for statistical significance was p<0.05. Statistical analysis was carried out using GraphPad PRISM version 2.01 software (GraphPad Software Inc., USA).

RESULTS

Clinical Monitoring

All animals remained healthy with normal food and water intake as well as behaviour during the study. Neither systemic clinical signs of pain, distress, discomfort nor local adverse reactions (pain, lameness, redness, abscess formation or necrosis) were observed during the post-implantation period. The monoolein or implant immediately formed a semi-solid depot at the site of injection. The implanted (by monoolein and the implant) paws volumes increased to reach a maximum at 24 h post-implantation, then progressively decreased. This oedema disappeared at 19 days post-implantation. It was always strictly limited to the implantation site.

Host Local Response to the Implant

As shown in Table 1, the volume of paws implanted by the monoolein or the implant increased since 3 h after implantation. This increment was maximal at 24 h post-implantation, but decreased after this period. At 19 days post-implantation the implanted (by the gel) paw volume was not significantly different from the one before implantation. The paws volume of the negative control lot transiently increased only at 6 h post-implantation.

Post-mortem macroscopic observation at the site of implantation did not reveal any presence of monoolein and implant, nor fibrous capsule. The surrounded tissues appeared macroscopically normal.

Table 1: Paws volume (mL) of mice before and after subplantar administration of 0.05 mL normal saline, monoolein and implant

<table>
<thead>
<tr>
<th>Post-implantation time</th>
<th>Paws volume (Mean±SD, n=10) of mice implanted by</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal saline</td>
</tr>
<tr>
<td>Before implantation</td>
<td>0.16±0.01</td>
</tr>
<tr>
<td>3 h</td>
<td>0.17±0.01</td>
</tr>
<tr>
<td>6 h</td>
<td>0.19±0.02*</td>
</tr>
<tr>
<td>24 h</td>
<td>0.16±0.01</td>
</tr>
<tr>
<td>48 h</td>
<td>0.16±0.01</td>
</tr>
<tr>
<td>72 h</td>
<td>0.16±0.01</td>
</tr>
<tr>
<td>6 days</td>
<td>0.16±0.01</td>
</tr>
<tr>
<td>12 days</td>
<td>0.17±0.02</td>
</tr>
<tr>
<td>19 days</td>
<td>0.17±0.02</td>
</tr>
<tr>
<td>26 days</td>
<td>0.18±0.03</td>
</tr>
<tr>
<td>35 days</td>
<td>0.16±0.02</td>
</tr>
<tr>
<td>52 days</td>
<td>0.16±0.02</td>
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</tbody>
</table>

*One-way ANOVA test followed by Dunnet’s multiple comparison test. **: Paws volume after implantation versus before implantation (p<0.05), ***: Paws volume after implantation versus before implantation (p<0.01)
Fig. 1: (A): Light micrographs of implanted paw section from a negative control mouse (A1), mouse implanted by monoolein (A2) and by the gentamicin-loaded monoolein gel (A3)
(B): Light micrographs of kidney section from a negative control mouse (B1), mouse implanted by monoolein (B2) and by the gentamicin-loaded monoolein gel (B3)
(C): Light micrographs of liver section from a negative control mouse (C1), mouse implanted by monoolein (C2) and by the gentamicin-loaded monoolein gel (C3)

Haematoxylin-eosin safron staining, magnification 100 x

Note a normal appearance of paw, kidney and liver sections on 52 days post-implantation by monoolein or by the implant and lack of inflammatory cells in the stained sections.

The histologic reaction to monoolein, implant and normal saline was uniform on 52 days after implantation. The surrounding tissues (Fig. 1) were not infiltrated by polymorphonuclear cells, macrophages, multinucleated giant cells, lymphocytes. Their (surrounding tissue) architecture was normal. There was no fibrous capsule.

Preliminary Assessment of Renal and Hepatic Function

The normal morphology of kidneys was preserved. Their histologic structures were similar in the negative control group and groups implanted by the monoolein or implant. The kidneys showed normal glomeruli, no tubular brush border loss, no tubular dilatation, no material in the lumens of tubules and no necrosis of epithelium (Fig. 1). There was no infiltration of inflammation cells in the histologic sections.

The serum creatinine level in negative control group and the tested groups were similar (p>0.05) on 52 days after implantation (Table 2).

The morphology and the architecture of mice liver were similar for all groups. Histological examination of the stained sections from the liver of negative control group revealed that the general well-organized pattern of hepatocytes and blood sinuoids was similar to treated group one (Fig. 1). The morphology of the liver cells was normal. Any sign of hepatocytes necrosis were not observed. The liver of all groups were not infiltrated by inflammation cells.
Table 2: Levels of serum creatinine, direct bilirubin, total bilirubin and triglycerides of mice implanted by the monoolein, the implant and by normal saline on 52 days after implantation

<table>
<thead>
<tr>
<th>Mice treated</th>
<th>Creatinine (μmol L⁻¹)</th>
<th>Direct bilirubin (μmol L⁻¹)</th>
<th>Total bilirubin (μmol L⁻¹)</th>
<th>Triglycerides (μmol L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal saline</td>
<td>37.2±4.1</td>
<td>4.1±1.0</td>
<td>21.3±3.0</td>
<td>2.4±0.2</td>
</tr>
<tr>
<td>Monoolein</td>
<td>39.6±3.7</td>
<td>4.0±0.8</td>
<td>22.2±2.9</td>
<td>2.3±0.4</td>
</tr>
<tr>
<td>Implant</td>
<td>39.4±3.2</td>
<td>4.0±1.5</td>
<td>22.5±2.6</td>
<td>2.4±0.2</td>
</tr>
</tbody>
</table>

Means±SD (n = 10). One-way ANOVA test: p>0.05

The levels of serum direct bilirubin and total bilirubin were similar (p>0.05) for negative control group and tested groups (Table 2).

**Effect of the Implant on Triglycerides Metabolism**

The level of serum triglycerides in negative control group were not different from the one in mice group implanted by the monoolein or the implant (Table 2), that on 52 days after implantation.

**DISCUSSION**

This study reports for the first time an *in vivo* biocompatibility and toxicity evaluation of an implant based on antibiotic-monoolein-water. The study was performed in mice because this animal model facilitates evaluation of a local reaction and is more sensitive to implantation than rat (Van Tilbeuren *et al.*, 2006). Monoolein, recognized as a biocompatible and non-toxic product (Ganem-Quintanar *et al.*, 2000) was used in the study as a reference product. The dose of implant (0.05 mL) by mouse was chosen according to the maximal dose which is susceptible to be used with adult patients.

The increment of the paws volume in mice groups implanted by the monoolein or by the implant during about 12 days could be an acute inflammation at the implantation site. This acute inflammation rapidly decreased after 24 h and is similar for the two implanted groups (Table 1). The lack of other clinical signs of inflammation (redness, pain, heat) (Cotran *et al.*, 1994) could allow us to conclude that the acute inflammation was moderate and tolerable by mice. This inflammatory reaction over the first week post-implantation have been reported in previous studies with other polymers such as poly (orthoesters), poly (esters) and poly (anhydrides) (Bermachez *et al.*, 1993; Ibih *et al.*, 1998; Hooper *et al.*, 1998). All these various polymers are considered as resorbable biocompatible materials.

The paw volume of mice implanted by the monoolein and the implant returned to its baseline respectively on 35 days and between 35-52 days post-implantation (Table 1). The implant and the monoolein would have been completely degraded during this time.

The histological examination of the site of implantation confirmed that the inflammatory reaction to implant and monoolein was transitional. On 52 days after implantation, any sign of chronic inflammation was not found (absence of macrophages, lymphocytes, plasmocytes, neovascularization, fibrous capsule) (Anderson, 1994).

Gentamicin, the antibiotic of the implant is well known to cause nephrotoxicity (Adam, 2003). Nephrotoxicity induced by gentamicin is a complex phenomenon characterized by an increase in serum creatinine, severe proximal renal tubular necrosis and loss of tubular brush border (Whiting and Brown, 1996; Fashola *et al.*, 2000). The implantation of the monoolein and implant did not change the serum creatinine level and renal histology in mice, suggesting no renal toxicity.

The liver is often impaired by many xenobiotics. The serums direct bilirubin and total bilirubin levels were measured as markers of liver dysfunction. They were not influenced by the implantation of the monoolein or implant. The lack of injury was further confirmed by macroscopic examination and light microscopic examination of the stained liver sections.

*In vivo*, the monoolein which is a component of the implant is hydrolysed to glycerol and fat acids (Ganem-Quintanar *et al.*, 2000). These metabolites could influence the metabolism of
triglycerides. An abnormal concentration of triglycerides in serum is known to be associated with cardiovascular diseases (Castelli, 1986; Cambien et al., 1986; Parra et al., 1992). The implantation of the monoolsen or implant did not increase the synthesis of triglycerides.

Although the implant (gel) has likely no harmful effect at long-term, serum chemical investigations and histopathology of the implantation site, liver and kidney could have been performed earlier to detect eventual early damages and how they resolve with time. Nevertheless, this eventual acute toxicity would be moderate and reversible since sub-chronic toxicity study didn't reveal damages.

In conclusion, the novel gentamycin-loaded monoolsen gel is well tolerated and has acceptable biocompatibility at long-term. The host foreign body reaction is moderate, transitional and limited to the implantation site. It was demonstrated that the gel is degradable in vivo and has neither nephrotoxic nor hepatotoxic effects at long-term. Further investigation is undertaken to detect eventual early damages which could have been resolved with time.

ACKNOWLEDGMENTS

The authors would like to thank CUD/CIUF, APEFE and CGRI (Belgium) for their financial support.

REFERENCES


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