In vivo biocompatibility evaluation of injectable nano-hydroxyapatite/Poloxamer 407 based formulations. Part 1

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ABSTRACT

The subcutaneous rat tissue reaction of five experimental Poloxamer 407/nano-hydroxyapatite based thermo-gelling formulations was investigated. These formulations were prepared in an attempt to be injected as a solution through the root canal, where they undergo a transition into a gel inside the bone lesion. Histopathological evaluations were made 7 days and 30 days after injection. The tissue and inflammatory reactions were graded according to ISO 7405:1997 standard. Data were statistically analyzed using the Non-Parametric Kruskal–Wallis test and Wilcoxon signed rank test. Results revealed insignificant differences in scoring of inflammatory response between the tested groups (at p=0.975). The inflammatory response scoring for the all tested formulations was limited to mild and moderate inflammation after 7 days. A statistically significant decrease in inflammatory scoring and necrosis of the tested groups after 30 days was found. It could be concluded that the tested formulations are biocompatible.

Key words: Biocompatibility, nano-hydroxyapatite, Poloxamer 407, periapical lesions buprofen

Introduction

Conservative treatment of local defects in bone arising as a result of trauma, surgery or infection should be considered to alleviate the need for the more invasive and destructive line of treatments. Periapical lesions of endodontic origin are among the causes of these bone defects. Several therapies are focused on treatment of the damaged tissue by using biocompatible materials (Katebzadeh et al., 1999, Hauman and love 2003)

Biocompatibility is the ability of the material to perform in a specific application without eliciting a harmful immune or inflammatory reaction. Depending on the material composition, different tissue responses may result. If the tested material is nontoxic and biologically active then it will integrate with the surrounding tissue. However, if it is biologically inactive, it may be encapsulated by a fibrous capsule. On the other hand; if the material is toxic, rejection and localized death of the surrounding tissue can occur (Hisham et al., 2010).

Ceramics used within the biomedical field are classified as either bioinert or bioactive. Calcium phosphates, bioglasses and glassceramics are bioactive bioceramics group of materials. They are defined as being osteoconductive (supporting bone growth) or osteoinductive (stimulating bone growth); they have a compositional similarity to the mineral phase of bone. All types of bioceramics are osteoconductive, but not all are osteoinductive. Hydroxyapatite (HA) has the advantage of being osteoinductive, and is typically used for coating biomedical implants to induce bone regeneration. Synthesis of HA at the nano-level would have superior functional properties which would have a greater impact on implant-cell interaction in the body environment (Hisham et al., 2010).

Another important characteristic of HA is the possibility of incorporation of other components on its structure, such as metallic ions (Ingrid et al., 2010), and other drugs as ibuprofen, thus promoting alterations on important physicochemical properties with impact on the biological response to this biomaterial.

Triblocks copolymers [poly(oxyethylene) – poly(oxypropylene) –poly(oxyethylene)] are the most commonly encountered thermosensitive systems in the pharmaceutical field (Wei, Xu et al., 2002). Poloxamer 407, a member of these polymers, is very often used because it allows the formation of transparent, colorless, easily washable gels with water which are moreover little irritant for the skin and the mucous membranes (Koffi, Agnely et al. 2006). This type of polymer has a gelation temperature below body temperature, making it possible to prepare easily spreadable liquid preparations which gellify at body temperature (Koffi, Agnely et al., 2006).
Poloxamers consist of poly(oxyethylene) and poly(oxypropylene) units, with the general formula poly(oxyethylene)x-poly(oxypropylene)y-poly(oxyethylene)x (Vadnere, Amidon et al. 1984). The phenomenon of thermogelation produced in poloxamer is perfectly reversible and is characterized by a gelation temperature. Nano-hydroxyapatite loaded with ibuprofen and formulated in a thermo-gelling Poloxamer 407 based formulations were prepared in an attempt to be injected as a solution through the root canal, where they undergo a transition into a gel inside the bone lesion. These injectable gels will deliver the loaded materials; (nano-hydroxyapatite and ibuprofen), to the bone lesion enhancing the healing of periapical lesions without the need for surgical intervention.

The ultimate objective of this animal study in rats was to evaluate the subcutaneous rat tissue reaction of these experimental formulations.

Materials and Methods

Materials tested:

Two nano hydroxyapatite formulations were used in this study. The prepared formulations include nano-hydroxyapatite (HA NPs) and nano-hydroxyapatite particles loaded with 5% ibuprofen (HA NPs-ibuprofen). The powder particles have an average size of 100 nm. Five in-situ gelling Poloxamer 407 gels were prepared as illustrated in table (1). A sixth group; (GP6) where the needle of an empty syringe was introduced in the rat’s skin with no material injection was used as a control group. The tested Poloxamer preparations were free flowing liquids below 25°C and converted to gels at about 30°C.

Table 1: The weight percentages of the components of the prepared in-situ gelling Poloxamer gels:

<table>
<thead>
<tr>
<th>Formula code</th>
<th>Components (% w/w)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Poloxamer 407 (HA NPs)</td>
</tr>
<tr>
<td>GP1</td>
<td>18</td>
</tr>
<tr>
<td>GP2</td>
<td>18</td>
</tr>
<tr>
<td>GP3</td>
<td>18</td>
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<tr>
<td>GP4</td>
<td>18</td>
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<td>GP5</td>
<td>18</td>
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<tr>
<td>GP6</td>
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</tbody>
</table>

In vivo biocompatibility test:

Ten male Wister rats weighing 250-270 g were used as the animal model. The animals were housed in a temperature-controlled environment with water and food ad libitum. The experimental protocol was reviewed and approved by the Ethical Committee at the National Research centre, Egypt. This work is considered to be the first part of the research project conducted in the National Research Centre, Cairo, Egypt. The rats were anesthetized with diethyl-ether (El-Nasr Pharmaceuticals Chemicals Co., Cairo, Egypt) and the anesthesia method used was anesthetic chamber induction.

The dorsal skin of the animals was shaved and cleaned with 10% iodine solution. Using a glass template, 6 circles were demarcated on the dermis of each rat leaving 2 cm between each circle. Using a syringe, 0.1 ml of each formulation was injected subcutaneously into the 5 circles. For the control group, the needle of an empty syringe was introduced in the 6th circle, but no material was injected. Evaluation was done 7 days and 30 days after injection. After each examination period, 5 animals from experimental groups were sacrificed by anesthetic overdose. The dorsal skin was shaved and tissue specimens were excised with a scalpel, stored in 10% formalin solution for 48 hrs, and thereafter embedded in paraffin blocks using standard procedures and serially sectioned at 4µm.

For each tissue specimen, a section was obtained and stained with Hematoxylin and Eosin (H & E). Stained sections were examined in a blind fashion in order to estimate the tissue response adjacent to the injected materials. Five sections belonging to the central area of each specimen were analyzed at different magnifications of a CX21 Olympus microscope (Tokyo-Japan).

According to ISO 7405:1997 standard, the tissue and inflammatory reactions were graded as follows:

0= None: No inflammatory cells infiltration
1= Mild: Scattered chronic inflammatory cells without tissue changes
2= Moderate: Focal inflammatory cell infiltration with tissue changes but without necrosis
3= Severe: Severe infiltration of inflammatory cells
4= Abscess: Abscess formation.

For necrosis, scoring was: 0= absent and 1= present.
Statistical analysis:

Data were statistically analyzed using the Non-Parametric Kruskal–Wallis test to study the effect of different materials on the inflammation and necrosis. Wilcoxon signed rank test were used to study the effect of time within each group. Statistical analysis was performed with IBM® SPSS® (SPSS Inc., IBM Corporation, NY, USA) Statistics Version 21 for Windows.

Results:

Histopathological findings:

Similar results in the form of sparse inflammatory reaction and absence of necrosis were noted in the histological specimens of groups GP1, GP3, GP5, and GP6 after 7 days. No granulation tissues were noted in any of the previously mentioned groups; however a number of histological specimens showed fibrous tissue condensation and mild angiogenic activity, figure (1). On the other hand, after 30 days, these groups showed similar results in the form of negligible sporadic inflammation in some specimens, figure (2). Mild eosinophilia was also noted in most specimens examined during this experimental period as shown in figure (3).

In contrast, histological sections of GP2 and GP4 after 7 days showed some tissue necrosis and granulation tissue reaction with the appearance of multinucleated giant cells figure (4).

Histological specimens of GP2 and GP4 after 30 days revealed ectopic calcifications in the subcutaneous tissues. The calcific masses were encircled by multinucleated foreign body giant cells, thin fibrous rim and few chronic inflammatory cells. Mild eosinophilic reaction was detected in the vicinity of the calcific stacks, figure (5).

![Fig. 1: Subcutaneous tissue response after 7 days; GP1 (A), GP3 (B), GP5 (C) and GP6 (D). Note the mild inflammatory reaction (I), angiogenesis (A), and fibrous condensation (F). (H&E, 40X)](image)

![Image](image)
Fig. 2: Subcutaneous tissue response after 30 days GP1 (A), GP3 (B), GP4 (C) and GP6 (D). Note the absence of inflammatory reaction in all the examined specimens. (H.E., 40X)

Fig. 3: The eosinophilic reaction seen in most of the examined specimens during 30 days interval. (H&E, 200 X)
Fig. 4: Subcutaneous tissue response after 7 days GP2 (A) and GP4 (B). Note the necrosis (N), Granulation Tissue Reaction (G) and foreign body multinucleated giant cells (arrows). (H&E, 40X)

Fig. 5: Subcutaneous tissue response after 30 days in GP2 (A) and GP4 (B). Note the calcifications in the subcutaneous connective tissues. (H&E, 40X), Inset of ectopic calcifications (H&E, 200 X).

Results of statistical analysis:

The Kruskal–Wallis non-parametric one-way ANOVA test comparing the inflammatory response to different tested groups is shown in figure (6). Results revealed insignificant differences in scoring between the tested groups (at p=0.975).

Comparing the effect of time on the inflammatory response between the tested groups, the non-parametric Wilcoxon signed rank test showed a highly significant difference in scoring (at p≤0.05) which decreased after 30 days for all the tested groups. Table (2)

![Boxplot for the scoring of inflammation between different groups.]:

Fig. 6: Boxplot for the scoring of inflammation between different groups.
Table 2: Frequency of inflammation scoring after 7 days and 30 days for the different materials used and Wilcoxon Signed rank test.

<table>
<thead>
<tr>
<th>Inflammation Scoring</th>
<th>7 days</th>
<th>30 days</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>GP1</td>
<td>0</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>GP2</td>
<td>0</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>GP3</td>
<td>0</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>GP4</td>
<td>0</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>GP5</td>
<td>0</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>GP6</td>
<td>0</td>
<td>5</td>
<td>0</td>
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</tbody>
</table>

As regards the scoring of necrosis between different groups the Kruskal–Wallis non-parametric one-way ANOVA showed an insignificant differences in scoring (at $p=0.066$) for different materials used.

Comparing the frequency of necrosis scoring after 7 days and 30 days for the different tested groups, statistical analysis showed a highly significant differences in scoring (at $p \leq 0.05$) which was decreased after 30 days for the all tested groups. Table (3)

Table 3: Frequency of necrosis scoring after 7 days and 30 days for the different materials used and Wilcoxon Signed rank test.

<table>
<thead>
<tr>
<th>Necrosis Scoring</th>
<th>7 days</th>
<th>30 days</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>GP1</td>
<td>5</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>GP2</td>
<td>2</td>
<td>3</td>
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</tr>
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<td>GP4</td>
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<tr>
<td>GP5</td>
<td>4</td>
<td>1</td>
<td>5</td>
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<tr>
<td>GP6</td>
<td>5</td>
<td>0</td>
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</table>

Discussion:

Periapical lesions are considered among the most frequently occurring pathologies found in alveolar bone (Ricucci et al. 2006). Because the necrotic pulp cavity is relatively inaccessible to the immune response, it becomes a reservoir of infection. The establishment of an inflammatory response is believed to be an attempt to prevent the spread of infection to periapical tissues. When faced with a periapical lesion that persists after root canal treatment, a periapical surgery or extraction might be the only treatment options (Torabinejad et al. 2009).

However, there are no current available intracanal medicaments that could enhance periapical bone regeneration. The preparation of an injectable medicament to be injected through the prepared root canal into the periapical lesion having the ability to enhance the healing of periapical lesions could increase the success rate of endodontic cases with periapical lesions.

The aim of the present study was to assess the soft tissue biocompatibility of experimental thermo-gelling formulations by quantifying the host reaction at their interface. For this purpose, the severity of the early inflammatory reactions to Poloxamer 407/nano-hydroxyapatite, Poloxamer 407/ nano hydroxyapatite particles loaded with 5% ibuprofen, Poloxamer 407/ nano hydroxyapatite/ ibuprofen, Poloxamer 407/ ibuprofen thermo-gelling formulations together with Poloxamer 407 used, as a control, was assessed. In this study; nano-hydroxyapatite was used to imitate bone mineral crystals which are nano-sized with a very large surface area (Hisham et al., 2010).

Poloxamer 407 was used very early for the manufacture of topical dosage forms and hydrogels intended for regional delivery of anti-inflammatory drugs (Tomida, Shinohara et al., 1987) and drugs intended for ophthalmic (Mansour et al. 2008), injectable (Dhanikula, Dhanikula et al., 2008), rectal ( Koffi, Agnely et al. 2008) and percutaneous administration (Nair and Panchagnula 2003). It is also known for its excellent compatibility with other chemicals, its high capacity of solubilizing various active ingredients and for allowing prolonged release of the active ingredient (Morishita, Barichello et al., 2001). Poloxamer 407 was tried as a drug delivery system in this study as they could be conveniently injected as a solution through the root canal, where they undergo a transition into a gel inside the bone lesion with its favorable residence time.

The events at the interface between a biomaterial and the adjacent tissue are the direct result of the cellular, chemical, physiological and mechanical reactions evoked by the presence of these materials (Jui- et al.,1997)

In the present study inflammatory host response of subcutaneous rat tissue was judged histologically taking the amount of necrosis and the infiltration with immuno-inflammatory cells into account.

Previous studies indicated that once a biomaterial is introduced into the body, a sequence of events including injury, blood-material interactions, provisional matrix formation, acute inflammation, chronic inflammation, granulation tissue development, foreign body reaction, and fibrosis/fibrous capsule development occurs. The acute inflammatory response with biomaterials usually resolves quickly, usually less than one week, depending on the extent of injury at the implant site. Following acute inflammation, chronic...
inflammation is identified and is usually of short duration and is confined to the implanted biomaterial site (James Anderson et al., 2008).

With biocompatible materials, early resolution of the acute and chronic inflammatory responses takes place followed by granulation tissue identified by the presence of macrophages, the infiltration of fibroblasts, and neovascularization in the new healing tissue. Granulation tissue is the precursor to fibrous capsule formation and granulation tissue is separated from the implant or biomaterial by the cellular components of the foreign body reaction; a one- to two-cell layer of monocytes, macrophages, and foreign body giant cells (FBGC) (James Anderson et al., 2008).

These findings are consistent with the results of the present study, where inflammatory reaction with absence of necrosis was noted in the histological specimens of the control groups after 7 days. The same pattern of results was detected for Poloxamer 407/ ibuprofen and Poloxamer 407/ nano hydroxyapatite particles loaded with 5% ibuprofen formulations indicating a normal tissue response for these formulations after 7 days. However the presence of fibrous tissue condensation and mild angiogenic activity in a number of histological specimens of these groups indicating the critical processes in repair process that provides the necessary nutrients and participating cells for healing. The mild eosinophilia noted in most specimens and the statistically significant decrease in inflammatory scoring (at p≤0.05 for these groups after 30 days; indicates the biocompatibility of such formulations where no harmful immune or inflammatory reaction was detected with time.

In some histological specimens with Poloxamer 407/nano-hydroxyapatite and Poloxamer 407/ nano hydroxyapatite/ ibuprofen formulations, results of this study detected the presence of ectopic calcifications, encircled by FBGC, and thin fibrous rim with few chronic inflammatory cells. This is a hallmark histiological feature of chronic inflammation, accompanied by FBGC-mediated biomaterial degradation (Amy et al., 2003). It also may indicate the bioactivity of the incorporated nano- hydroxyapatite.

Results of the current study showed different tissue reaction for Poloxamer 407/ nano- hydroxyapatite particles loaded with 5% ibuprofen, compared to the other two nano-hydroxyapatite containing formulations. Such finding indicates that the incorporation of ibuprofen inside the nanoparticles produced alterations on the physicochemical properties of the nano- hydroxyapatite at the tissue/material interface.

Results also showed that the inflammatory response scoring for all of the tested formulations was limited to mild and moderate inflammation. Additionally the statistically significant decrease in necrosis scoring after 30 days for the different tested groups is an indication of the biocompatibility of the tested formulations where no harmful immune or inflammatory reaction was detected with time.

Conclusions:

Based on the obtained results, it could be concluded that all the prepared formulations are biocompatible. The incorporation of ibuprofen inside the nanoparticles produced alterations on the physicochemical properties of the nano- hydroxyapatite at the tissue/material interface. The successful results of the biocompatibility will be taken into consideration for the future work on animal dog model for bone enhancement.

References


