Studies on hemolysis properties of medical \(\alpha\)-calcium sulfate hemihydrate

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ABSTRACT

Objective: The as-provided \(\alpha\)-calcium sulfate hemihydrate bone repair material exhibits superior degradation properties and osteogenic property to the traditional calcium sulfate bone repair materials. Its effects on the blood and the biocompatibility were studied to ensure its safety for the bone implantation application.

Method: The hemolytic tests were conducted to evaluate the blood compatibility of the \(\alpha\)-calcium sulfate hemihydrate. The extracts of \(\alpha\)-calcium sulfate hemihydrate with different concentration reacted with 2% rabbit red blood cell suspension. The existence and morphology of the red blood were observed through naked eye observation and microscope observation. The hemolytic ratios were calculated through the absorbances using a ultraviolet spectrophotometer.

Result: It was observed by naked eye that the red blood accumulated at the bottom of the test tubes and the upper liquids were clear, colorless and transparent in the experimental sample. The microscope observation exhibited that the cells of the experimental samples had the normal morphology, no deformed and broken blood sell existed. The hemolytic ratios of the experimental sample were all less than 5%. The hemolytic ratios were increased with the increase of the concentration of the extracts.

Conclusions: The hemolytic ratios of the medical \(\alpha\)-calcium sulfate hemihydrate accorded with the standard ISO/TR7405-1984(E). It could be concluded the material would not cause acute hemolysis. The osteogenic property exhibited dose dependence.

1. Introduction

Calcium sulfate is a kind of excellent bone repair material except for its several shortcomings of too fast degradation, weak strength of setting bulks and poor bioactivity. Our group developed a new method to modify \(\alpha\)-calcium sulfate hemihydrates to allow it to grow along the grain planes which degrade slower. It was proved that the obtained \(\alpha\)-calcium sulfate hemihydrates exhibit excellent degradation, stable calcium ion releasing rate and good for ingrowth of blood vessels and osteoblasts\cite{1,2}.

Biocompatibility, the property of the response of the live organs to the non-active material, is important for biomaterials\cite{3}. The evaluations of biocompatibility of biomaterials mainly include blood compatibility evaluations and histocompatibility evaluations. Among them, the hemolysis test is the method to evaluate the blood compatibility of materials and in vitro hemolysis activity, in which the effect of the testing specimen on blood cells can be sensitively detected\cite{4-7}. In this study, in order to ensure the \(\alpha\)-calcium sulfate hemihydrates can be safe to human body, the hemolysis test was conducted to evaluate its biocompatibility preliminarily to provide references for experimental scientific research and clinical application\cite{8}.

2. Materials and methods

2.1. Materials and main instruments

The involved materials and main instruments included: medical \(\alpha\)-calcium sulfate hemihydrates provided by Hainan Trauma and Disaster Rescue Key Laboratory, male New Zealand rabbit of 1.9 kg, electronic balance, ultraviolet spectrophotometer, constant temperature water bath, centrifuge (Hainan Medical College Central Laboratory).
2.2 Experimental methods

2.2.1 Preparation of leaching solutions of α-calcium sulfate hemihydrates

α-calcium sulfate hemihydrates powders were mixed with physiological saline at a ratio of 0.1 g/mL. The mixtures were put into penicillin bottles and settled in a 37 °C water bathing for 72 h, shaken every 24 h. Then the mixture were centrifuged at 2 000 r/min for 5 min, the supernatants were separated as the leaching solutions of α-calcium sulfate hemihydrates. The obtained leaching solutions were diluted into different concentrations of 25%, 50%, 75% and 100% by adding physiological saline with labels of A, B, C, D.

2.2.2 Preparation of red blood cell suspensions

10 mL blood was taken by ear vein from a New Zealand rabbit, put into a baker and stirred using a glass rod for 10 min to remove fibrinogen. After that, 100 mL physiological saline were added into the blood and mixed without foaming, centrifuged at 1 500 r/min for 5 min to take the precipitated red cells. The washing was repeated for more than three times until the supernatant was colorless. The obtained red cells were finally mixed with physiological saline to form 2% red cell suspension.

2.2.3 in vitro hemolysis testing

Six 10 mL clean glass tubes were taken and labeled as 1, 2, 3, 4, 5 and 6. (1) tubes 1-4 were responding to specimen groups of A, B, C, D. (2) tube 5 was for negative control group E. (3) tube 6 was for positive control group F. Firstly, 2.5 mL red cell suspension were added into six tubes. Then, 2.5 mL specimen groups of A, B, C, D were added into tubes 1-4. 2.5 mL physiological saline and 2.5 mL distilled water were added into tube 5 and tube 6 separately. All the tubes were shaken to make the mixture homogeneous. A bit of liquid was taken from each tube for blood smear. The smears were observed under a optical microscope to observe the rupture of red cells. At another hand, all the tubes were settled in air for 2 h followed by naked eye observation. Finally, all the samples were mixed homogeneously and centrifuged at 750 r/min for 15 min. The supernatants were taken and their absorbance was measured using a UV spectrophotometer at a wavelength of 545 nm. The measurement was repeated 3 times for a average value. The hemolysis rate were calculated according to the following formula: \( Z(\%) = \frac{(At - Anc)}{(Apc - Anc)} \times 100\% \), (Z: RBC hemolysis ratio, At: experimental group absorbance, Anc: negative control group absorbance, Apc, positive control group absorbance)

3. Result

The hemolysis phenomenon of each sample group was observed by naked eye as shown in Figure 1. It is found that in the positive control group of tube 6, the upper liquid showed transparent and red deepening from the top down. It is because the red cell was broken to release heme and hemoglobin and the fragment of the broken red cells deposited at the bottom. This phenomena indicated that hemolysis occurred. While in the experimental groups of tube 1-4 and the negative control group of tube 5, the supernatants were colorless and transparent and at the bottom of tubes exhibited deep red. It is because of the red cells all deposited at the bottom, shown no hemolysis.

Figure 1. Naked eye observation of hemolysis phenomenon of sample groups (tubes of 1, 2, 3, 4 were 25%, 50%, 75% and 100% sample group. Tube 5 was the negative control group, tube 6 was the positive control group).

Figure 2. Microscopic observations ((1).negative control group, (2).100% sample group, (3).positive control group).
The absorbances results and the calculate hemolysis rates were listed in Table 1. The sample groups with various concentration (25%, 50%, 75%, 100%) had hemolysis rates of 1.085%, 1.756%, 2.634% and 3.099% respectively. It can be seen that the hemolysis rates increased as the concentration of leaching solution increased. Moreover, all the values of hemolysis rates were below 5%. According to ISO/TR7405-1984 (E)[12], it can be concluded that the used α-calcium sulfate hemihydrates cannot cause acute hemolysis.

Table 1.
Absorbance results and calculated hemolysis rates of experimental groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>Absorbance</th>
<th>Hemolysis Rate %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experimental group A</td>
<td>0.034</td>
<td>0.031</td>
<td>0.030</td>
<td>0.032±0.02</td>
<td>1.085</td>
</tr>
<tr>
<td>Experimental group B</td>
<td>0.038</td>
<td>0.036</td>
<td>0.042</td>
<td>0.039±0.03</td>
<td>1.756</td>
</tr>
<tr>
<td>Experimental group C</td>
<td>0.050</td>
<td>0.044</td>
<td>0.049</td>
<td>0.048±0.03</td>
<td>2.634</td>
</tr>
<tr>
<td>Experimental group D</td>
<td>0.050</td>
<td>0.053</td>
<td>0.057</td>
<td>0.053±0.04</td>
<td>3.099</td>
</tr>
<tr>
<td>Control group Negative</td>
<td>0.018</td>
<td>0.019</td>
<td>0.022</td>
<td>0.020±0.02</td>
<td>-</td>
</tr>
<tr>
<td>Control group Positive</td>
<td>1.083</td>
<td>1.083</td>
<td>1.085</td>
<td>1.084±0.01</td>
<td>-</td>
</tr>
</tbody>
</table>

4. Discussion

The hemolysis test is the fundamental method to evaluate the blood compatibility and acute hemolysis activity of materials. It can sensitively detect the influence of test sample on the red cells. It also can be used as an initial screening test for a supplement of in vitro cytotoxicity test[13]. When the materials interacted with blood, the component of hemolysis ingredients may cause broken of red cells and release of hemoglobin and result in the increase of free hemoglobin in the plasma. As a result, once hemolysis took place, it can be observed that the supernatants were in red color by Naked eye and red cells deformed and ruptured by microscope.

The absorbance of experimental groups were also measured using a UV spectrophotometry, which has the advantages of ease to handle and high accuracy. When the monochromatic radiation penetrated the supernatants of the mixture of 2% red blood cell and leaching solution of α-calcium sulfate hemihydrates, the energy were absorbed. The absorbance was direct proportional to the concentration of free hemoglobin and the thickness of the liquid layer (optical path length). The more hemolysis of red blood cells, the higher concentration of hemoglobin in supernatants, the more absorption of radiation. Thus, the absorbance would increase with the increase of the degree of hemolysis. In this study, the calculated hemolysis rates exhibited that the used α-calcium sulfate hemihydrates can not cause acute hemolysis.

Moreover, all of the hemolysis rates of all the sample groups were below 5% (ISO standard). The hemolysis rates increased with the increase of concentration of sample groups, indicating a dose-dependence. It is because of the degradation of α-calcium sulfate hemihydrates which α-calcium sulfate hemihydrates ionizes calcium ions and sulfate ions. When the concentration of calcium ions in the supernatants reached to a certain concentration, it would reduce the erythrocyte membrane composition, change the morphology, enhance the viscosity, and decrease the membrane lipid fluidity and membrane mechanical properties of red cells resulting in the decrease of the deformation ability of red cells. There was a dose-dependent between the calcium ion concentration and the deformation ability of red cells. Therefore, in this hemolysis test, a certain hemolysis rate existed and it increased as the concentration of sample groups increasing. It is also shown that it should take caution of the dose of α-calcium sulfate hemihydrates during the clinic applications.

The average hemolysis rate of α-calcium sulfate hemihydrates was 2.144% (below 5%), conformed to the ISO standard. There was a dose-dependent between the hemolysis rate and the concentration of calcium sulfate hemihydrates. The material was suitable to be used as bone implants as the aspect of hemolysis, but the other biological performance still needs further evaluation.

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