An *in vivo* study on the reaction of hydroxyapatite-sol injected into blood

HIDEO AOKI, H. AOKI, T. KUTSUNO
Tokyo Bioceramics Institute Co. Ltd.

WEI LI
West China University of Medical Sciences

MOTOO NIWA
The Nippon Dental University

In order to identify the possibility of hydroxyapatite-sol being used as a drug carrier and absorbent, an *in vivo* experimental study was performed. Pure hydroxyapatite microcrystals were synthesized by reaction of high purity $Ca(OH)_2$ and H_3PO_4 solutions while using an ultrasonic homogenizer. Hydroxyapatite-sol was prepared by dispersing hydroxyapatite microcrystals into physiological salt solution. The hydroxyapatite-sol in different concentrations was injected into veins of both 25 Wistar rats and 5 Beagle dogs. The medium lethal dose was determined as 160 mg/kg. By observing the change of O_2 and CO_2 gas partial pressure, it was considered that the main cause of death by hydroxyapatite-sol injection was due to the blockage of capillaries. When one-sixth amount of the medium lethal dose was injected into the veins of the dogs, the value of phosphorous increased but calcium and magnesium kept stable. LDH, CPK, GOP and GDT values dramatically increased in 30 min after injection, however, one day after injection, the values returned to normal. Repeated experiments by similar methods were continued on same animals for 2 years in two-week intervals, the results in every experiment were almost same, no chronic damage or permanent side effects were discovered in the two years experiment. According to the results above, it was suggested that the hydroxyapatite-sol could be applied as a drug carrier into blood by using a small amount less than one-sixth of the medium lethal dose.

1. Introduction

Since the success in sintering hydroxyapatite in 1970s, various kinds of biomaterial made of hydroxyapatite have been developed worldwide. Among them, dental implants made of sintered hydroxyapatite only or metal coated with hydroxyapatite, artificial hip joints of titanium alloy stems coated with hydroxyapatite on the surface are most popular [1]. However, uncalcined hydroxyapatite has not been applied as any biomaterials yet. In this study, hydroxyapatite—sol (HA-sol) made of hydroxyapatite microcrystals without being calcined was developed. In order to investigating the possibilities of hydroxyapatite-sol to be applied as a drug carrier and absorbent, the medium lethal dose, changes of pH value and partial gas pressure, and changes of blood components were examined after injection into veins by an animal test.

2. Materials and methods

2.1. Materials

Microcrystals of hydroxyapatite were synthesized by dropping 0.3 mol/l H_3PO_4 solution into 0.5 mol/l $Ca(OH)_2$ with 99.999% high purity suspension solution while using an ultrasonic homogenizer at 20 kHz. The crystal sizes averaged 0.05 μm in length, and 0.02 μm in width and thickness. The gel-like suspension solution made of hydroxyapatite microcrystals was diluted to a concentration of 14.7 mg/ml by physiological salt solution when it was actually used for injection. Otherwise, specific surface areas of hydroxyapatite microcrystals that constituted hydroxyapatite-sol were measured by BET method and transmission electron microscope. Secondary sizes by aggregation of the microcrystals were determined by a scattering laser diffraction method and optical microscopic observations.

2.2. Methods

Twenty-five Wistar rats and five Beagle dogs were used as experimental animals. Hydroxyapatite-sol in various concentrations was injected into veins of both rats and dogs. The medium lethal dose was determined by injection to 25 rats. After the medium lethal dose of the sol was determined (160 mg/kg), the sol in amount of one-sixth of the medium lethal dose (26 mg/kg) was

injected into the veins of 5 dogs. The changes in blood component, including partial gas pressure, serum ions and enzyme level such as LDH, GOT, GPT, GPK and ALP were examined by general biochemical methods. The study was continued on same animals for 2 years by repeating the similar experiments in two-week intervals.

3. Results

3.1. Hydroxyapatite-sol

Fig. 1 shows a transmission electron microphoto of ultrasmall crystals constituting hydroxyapatite-sol. The crystals were plate-like in shape, less than 0.1 μm, and with a mean length of approximately 0.05 μm, thickness of 0.01 μm and wideness of 0.015 μm. The surface area was 67 m^2/g. The secondary particle sizes aggregated from primary particles of the ultrasmall hydroxyapatite crystals in the sol were determined as 1–10 μm by a scattering laser diffraction method. Fig. 2 shows an X-ray powder diffraction pattern of the sol. Although each peak was line-broadening, all of these peaks were identified as from hydroxyapatite. Halo of the dotted area was owing to water diffraction.

3.2. The medium lethal dose (LD_{50})

Twenty-five Wistar rats weighing 240–250 g were divided into 5 groups, each group kept 5 rats. Different amounts of hydroxyapatite-sol were injected into the veins of the rats. A physiological salt solution was used as a control. Fig. 3 shows the results after injection. When 320 mg/kg of the hydroxyapatite-sol was injected into the veins, all of the 5 rats ceased breathing in a few minutes after injection. In the case of an injection of 226 mg/kg, 1 of the 5 rats survived. In the case of injection with 160 mg/kg, 4 of the 5 rats survived. In the case of injection with 113 mg/kg hydroxyapatite crystals, 2 of the 5 rats survived. In the case of hydroxyapatite-sol injection with 80 mg/kg, all of the rats were survived with normal condition. From the data above, the medium lethal dose was calculated as 160 mg/kg by Probit Analysis method [2].

3.3. O_2, CO_2 partial gas pressure and pH changes

Quantity changes of O_2, CO_2, HCO_3^-, CO_2 and pH in blood were measured after injection of one-sixth medium lethal dose (26 mg/kg) of hydroxyapatite-sol. The result was summarized in Fig. 4. Immediately after injection, the partial gas pressure of O_2 decreased from 88.3 mmHg to 37.0 mmHg. After 1 h, however, the value of O_2 partial gas pressure recovered to a normal value, while the values of partial gas pressure in CO_2, HCO_3^- and TCO_2 decreased from 35.7 to 25.5 mmHg, from 23.4 to 17.6 mmol/l and from 24.4 to 18.4 mmol/l, respectively. Six hours after injection, the values of CO_2 and HCO_3 recovered to normal values. The pH value slightly decreased immediately after injection, but recovered to near-normal level in one hour and completely recovered to normal in 10

3.4. Change in blood components and enzymes

Fig. 5 shows, when one-sixth of the medium lethal dose, e.g. 26 mg/kg of hydroxyapatite-sol was injected into veins, the value of phosphorous increased in 30 min after injection and recovered to normal level in one day, while the calcium and magnesium in the blood did not change apparently. On the other hand, when 4 ml/kg of a physiological salt solution was injected into the veins of rats and dogs, no change in the blood components was observed.

Fig. 6 shows, when 26 mg/kg of hydroxyapatite-sol was injected into veins of dogs, the changes in enzyme level were measured for one week. The LDH, CPK, COP

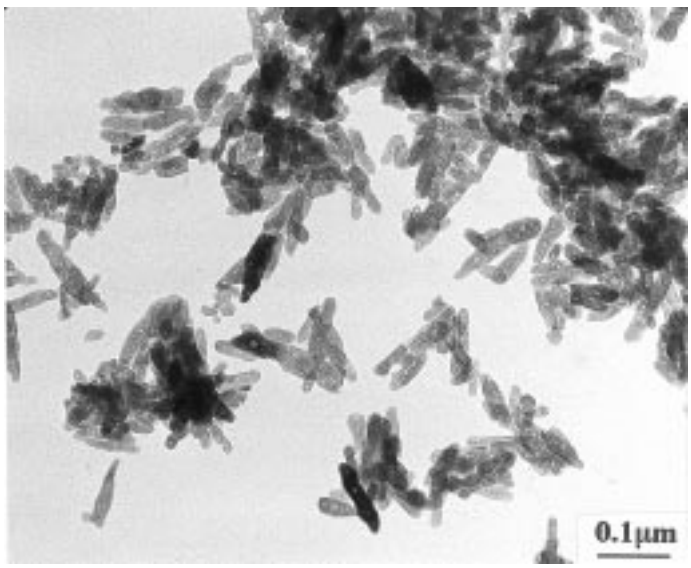

Figure 1 High purity hydroxyapatite microcrystals.

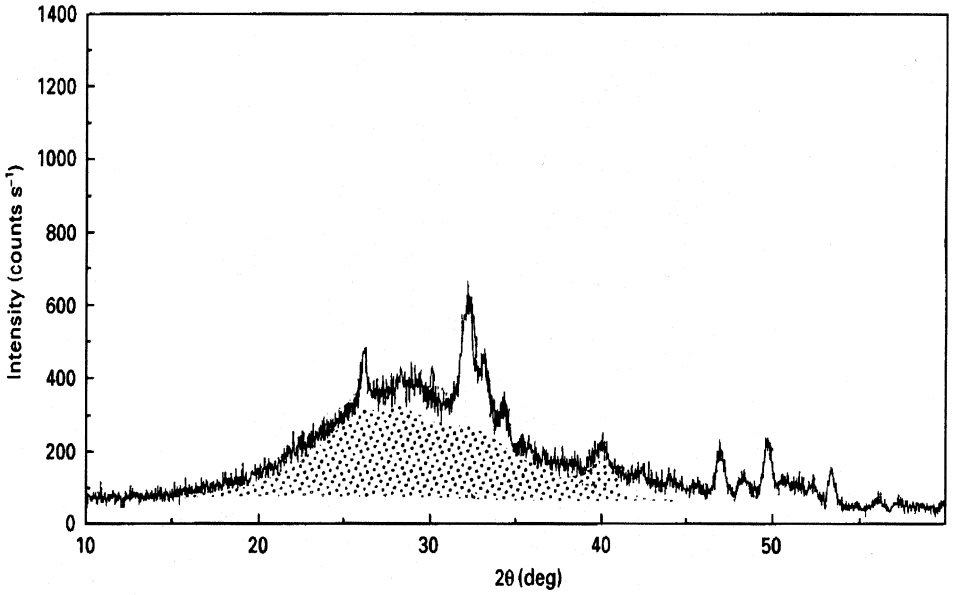

Figure 2 X-ray powder diffraction pattern of the hydroxyapatite-sol.

and GTP drastically increased immediately afterinjection and recovered to normal in one day, while the ALP level did not change apparently during the first 6 h, but it increased two times in 1 day after injection.

4. Discussion

4.1. Hydroxyapatite-sol

Although the research and application of calcined hydroxyapatite have continued for the past 25 years, the uncalcined hydroxyapatite hasn't been much concerned by material scientists. There are many special characteristics that were worthy to study existed in hydroxyapatite, such as its adsorbent characters, the interaction with HIV and cancer cells, the effects on osteoporosis disease and so on. There were some authors started their interest on this field and published their works that concerned tissue reactions [4, 5], cell reactions [6, 7], blood reactions [8] and coating method [9, 10] with hydroxyapatite-sol.

It is well known that hydroxyapatite is synthesized by a neutral reaction of $Ca(OH)_2$ and H_3PO_4 solutions at a room temperature [3]. Similar to those of usual hydroxyapatite, the crystal sizes, so called primary sizes, are less than 0.1 μm. Hydroxyapatite-sol in a concentration of 14.7 mg/ml is a liquid like milk in consistency and can be used for injection using a syringe. Hydroxyapatite crystals suspending in solution are usually aggregated to secondary crystals of which the sizes are in the range of 5 to 30 μm. In this study, the primary crystal sizes of hydroxyapatite-sol are less than 0.1 μm, however, the secondary sizes are less than 10 μm, because larger particles which were more than 10 μm

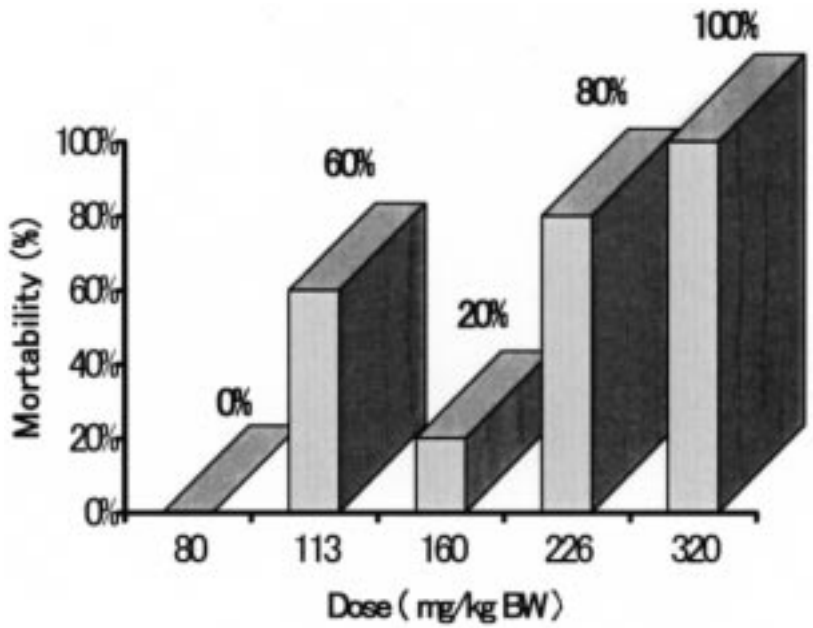

Figure 3 Dose-mortability relationship.

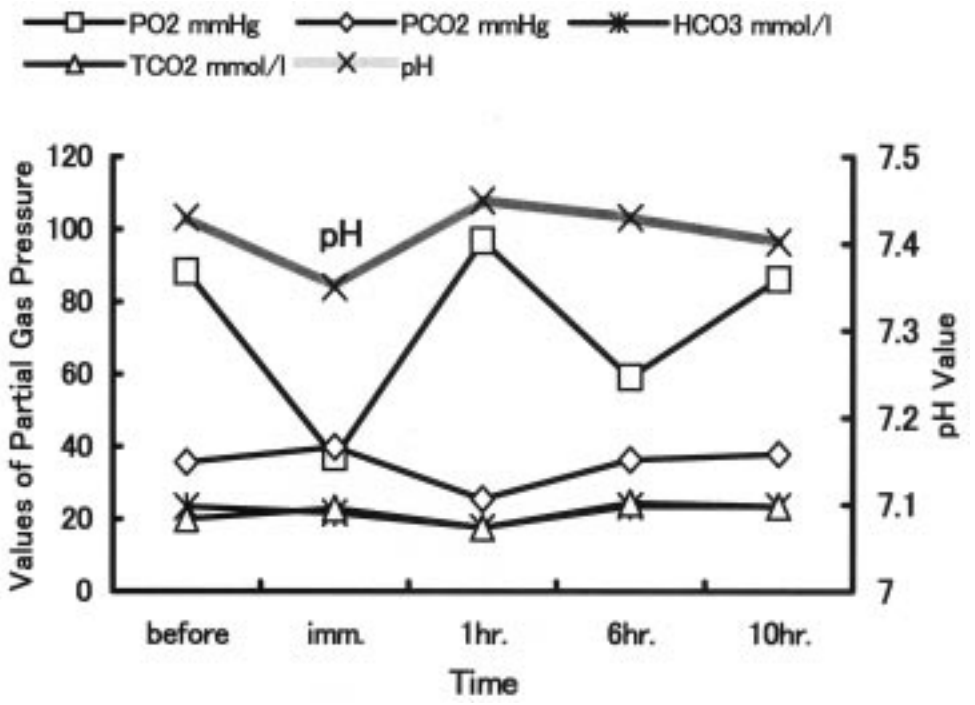

Figure 4 Partial gas pressure and pH change after injection.

were fine-grained by ultrasonic effect [5]. The average surface areas of three materials, hydroxyapatite micro-crystals constituting hydroxyapatite-sol, amorphous calcium phosphate synthesized by an usual solution reaction without using an ultrasonic homogenizer, and calcined hydroxyapatite powder heated at 800C, were about 100, 76.8, and 25.4 m^2/g, respectively. Apparently the surface areas of hydroxyapatite- sol were larger than that of hydroxyapatite synthesized without using an ultrasonic homogenizer. It is suggested that hydroxyapatite-sol synthesized under using an ultrasonic homogenizer were more effective in applying as a drug carrier because of their larger surface area.

The Ca/P ratio of hydroxyapatite suspending in sol was approximately 1.7, slightly higher than 1.67 of the stoichiometric hydroxyapatite. These crystals consisted of a small amount of carbonate ions substituting for PO_4 sites in the hydroxyapatite structure. The amount of carbonate oxide was determined as 1.03 wt% with a chemical analysis, so-called Conway method. By the results of analyzing the amount of carbonate oxide and comparing the Ca/P ratio of pure hydroxyapatite, the higher Ca/P ratio of hydroxyapatite crystals constituting hydroxyapatite-sol was proven as the result of substituting carbonate ions for phosphate ions in the hydroxyapatite composition.

4.2. The medium lethal dose

The medium lethal dose of hydroxyapatite-sol was determined as 160 mg/kg from the results of an animal test using Wistar rats. On the other hand, the medium

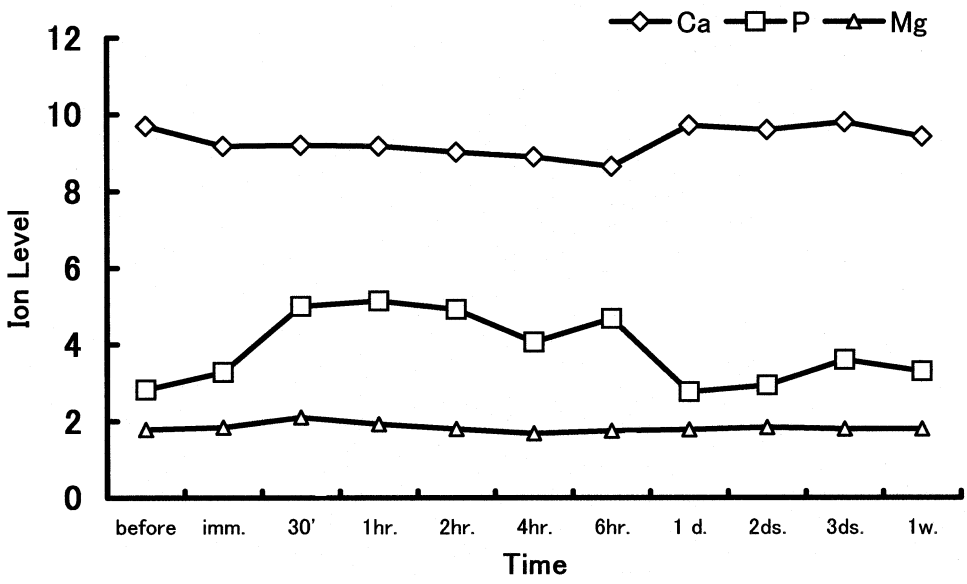

Figure 5 Ions change in blood after injection.

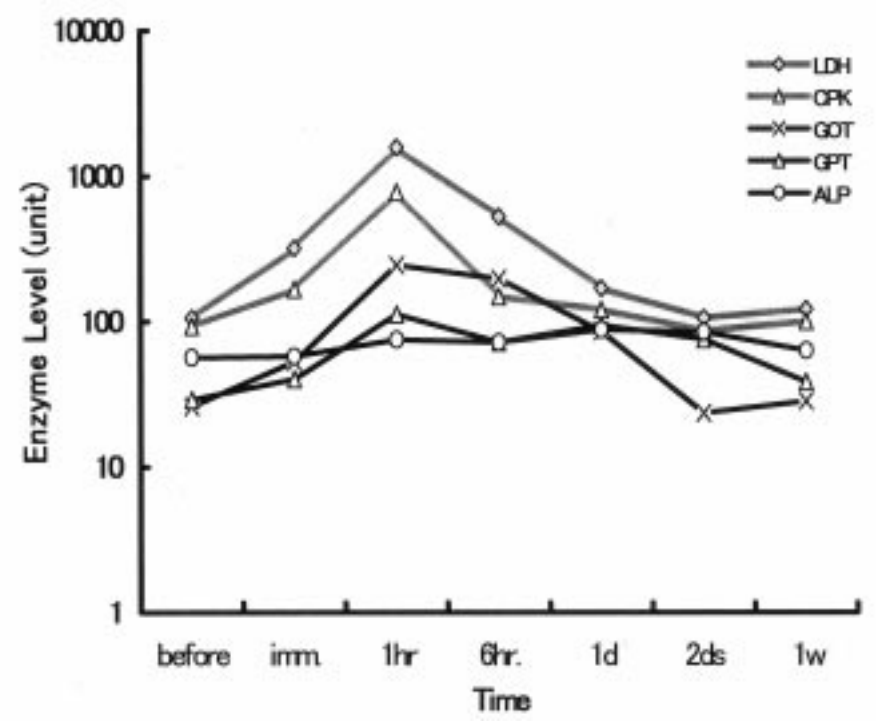

Figure 6 Change in enzyme level after injection.

lethal dose of usual hydroxyapatite suspension would be smaller due to the aggregation of primary particles to larger secondary particles in sizes. The medium lethal dose would change a little according to particle sizes and concentration of hydroxyapatite-sol, injection speed and age of animals. For example, the medium lethal dose of hydroxyapatite-sol would decrease by injection of concentrated solution or in rapid speed. From our previous study [11] and the phenomenon of decreasing in O_2 and CO_2 partial gas pressure in blood, It was considered that the main reason for death caused by hydroxyapatite-sol injection was due to blockage of the capillaries.

4.3. O_2, CO_2 partial pressure and pH change

Partial gas pressure changed clearly after hydroxyapatite-sol injection. PO_2 drastically decreased immediately after injection and recovered to normal level after 1 h while the PCO_2, $PHCO_3$ and TCO_2 didn't change immediately, but they decreased while the PO_2 recovered to normal level in 1 h. The pH values in blood slightly decreased immediately after hydroxyapatite-sol injection, then recovered to a slightly higher value after one hour, and recovered to a perfect normal level after 10 h. Although the mechanism of these changes in phenomenon is not clear, it indicated that the capillaries in lungs were temporarily blocked by crystals suspending in hydroxyapatite-sol. It is also considered that the hydroxyapatite-sol would adsorb calcium, hydrogen phosphate, and other ions in blood, and bond to proteins, enzymes, lipids, and saccharides.

4.4. Change in blood components and enzymes

When 26 mg/kg (4 ml/kg) of hydroxyapatite-sol was injected into the veins, the level of phosphorous increased in 30 min after injection and recovered to normal level after one day, but the calcium and magnesium in the blood did not change apparently. The levels of sodium and chlorine would not be affected by the injection of hydroxyapatite-sol [3]. The enhancement of phosphorous level in blood might be considered as being caused by the releasing of phosphorous ions from hydroxyapatite, or the exchanging of ions between hydroxyapatite and blood components, or the changing in permeability of cell membranes caused by stimulation of hydroxyapatite-sol.

Thirty minutes after injection, the LDH values dramatically increased and the CPK, GOP and GPT values also increased with time. The enzyme values recovered to normal level after one day. But ALP values did not change until 1 day after injection, after 1 day the ALP values increased over 2 times. On the other hand, when 4 ml/kg of a physiological salt solution was injected into the veins of animals, no change in the blood components was observed.

The mechanism of changes in enzyme values was not clearly understood. Most of the enzymes concerned in this study were mainly existed in cytoplasm. The increasing of enzyme value in serum indicates the motivation of cell activity or increasing of cell death, which causing the releasing of enzymes into serum. The following reasons might be concerned with the enzymes releasing into blood. 1. Capillaries blockage caused death of cells by decreased O_2 pressure. 2. Vast amount of hydroxyapatite crystals entering into cells by phagocytosis caused the death of large amount of cells. 3. Environment changed by the alteration of calcium, phosphorous and $PHCO_3$ and TO_2 values caused an increasing of cell death. 4. The enhancement of cell activities of macrophagocyte caused by stimulation of hydroxyapatite in blood.

Even though the injected amount of hydroxyapatite-sol was as high as one-sixth of the medium lethal dose, almost all the index returned to their normal levels from one day to one weeks after injection. This study were continued on same animals for 2 years by repeating the similar experiments in two-week intervals, but all the results in every experiment were almost same as above. It seemed that the injection of hydroxyapatite-sol in one-sixth of the medium lethal dose into blood would not cause chronic damages or any permanent side effects to the living body.

According to the results above, it was suggested that the hydroxyapatite-sol could be applied as an effective drug carrier in blood by using a small amount less than the medium lethal dose.

5. Conclusion

1. The medium lethal dose by injection of hydroxyapatite-sol into vein of rats and dogs was determined as 160 mg/kg. The main reason for death by hydroxyapatite-sol injection was due to blockage of the capillaries.

2. By injection of sixth of the medium lethal dose into veins, the blood components, including pH, O_2 and CO_2 partial gas pressure, serum ions and enzymes changed apparently. But after one day, they returned normal. Repeated experiments by similar methods for 2 years showed that the hydroxyapatite-sol would not cause chronic damage or permanent side effects to the living body by injecting less than one-sixth of medium lethal dose into blood.

3. Hydroxyapatite-sol could be considered to apply as an effective drug carrier into blood by using a small amount less than one-sixth of the medium lethal dose.

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