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RESEARCH ARTICLE

The Effectivity of Porous Hydroxyapatite loaded with Gentamycin on Chronic Tibial Osteomyelitis in Rabbit Models

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Abstract

Introduction: Osteomyelitis management with debridement, guttering, and systemic antibiotics often do not give satisfactory results due to the inability of antibiotics to reach the infection site. Another method of antibiotic delivery in the form of beads are deemed ineffective as it requires two separate surgeries. Combination of local antibiotics with absorbable porous hydroxyl appetite scaffolding and antibiotic carriers have not been studied. Methods: we conducted pre-test and post-test control group in a rabbit model of osteomyelitis. Ten rabbits divided in control group and the treatment group (n=5). We injected Staphylococcus aureus in the rabbit tibia, forming the osteomyelitis model, and subsequently performed treatment for osteomyelitis. In the control group, we performed debridement and administered ceftriaxone injection for 4 weeks, whereas in the treatment group, we added the combination of porous hydroxyapatite and gentamicin. Afterwards, we performed clinical assessment, x-ray examination, culture, and histopathology. Results: radiologically, tibia cortical thickening scores improved in the treatment group compared to the control group (p=0.48) as well as histopathological osteomyelitis evaluation score (p=0,009). Clinically, there were improvements in the swelling scores (n=5) of the treatment group compared to control group, but no significant statistically (p=0.053). In culture, there were no significant difference between the two groups (p=1.00).Conclusion: Combination of porous hydroxyapatite and gentamicin as a local treatment of osteomyelitis of the rabbit tibia osteomyelitis models improved radiological and histopathological scores and also clinically compared to existing standard treatment procedures for chronic osteomyelitis.

Keywords: *Animal model, Ceftriaxone, Chronic osteomyelitis, Gentamycin, Guttering, Osteomyelitis, porous hydroxyapatite.*

Introduction

Osteomyelitis is a bacterial infection on bone tissue [1,2]. In United States, incidence of osteomyelitis is approximately 1 in 5000 children. Bones that are prone to develop osteomyelitis in decreasing order are tibia (50%), femur (30%), fibula (12%), humerus (3%), ulna (3%), and radius (2%) [3]. Around the world, incidence of osteomyelitis is reported to be higher in developing countries owing to higher incidence of motor vehicle accidents, open fractures, and delayed treatment of contaminated which leads to osteomyelitis [3,4].

Experts have found a new route system for local antibiotic delivery with HA scaffold impregnated with antibiotics.[5]Ha scaffold impregnated with antibiotics releases its contents slowly and thus eliminate the need for a second surgery. Ha scaffold fills the defect and resorbable [6, 7]. Hydroxyapatite (HA) is a synthetic graft of ceramic compound

with similar structure to human bone, both physically and chemically [8]. HA scaffolds come in different size such as porous and nano-composite which affects its efficacy. Until recently, the use of porous HA is yet to be studied, even in animal model. This study aims to determine the efficacy of porous HA combined with antibiotics for chronic osteomyelitis in rabbit model.

Material and Methods

This is an experimental study involving treatment and control groups, each groups is composed of five New Zealand rabbits. The rabbits used as subjects weighted 2,500-3,500 grams each without any injuries on any extremities other than the treatment. Rabbits who were pregnant or dead were excluded from this study. Each rabbit underwent medical check-up including weight and temperature. The rabbits were then divided into treatment and control group with 5 rabbits in each group. The protocol can be seen in Fig. 1 below.

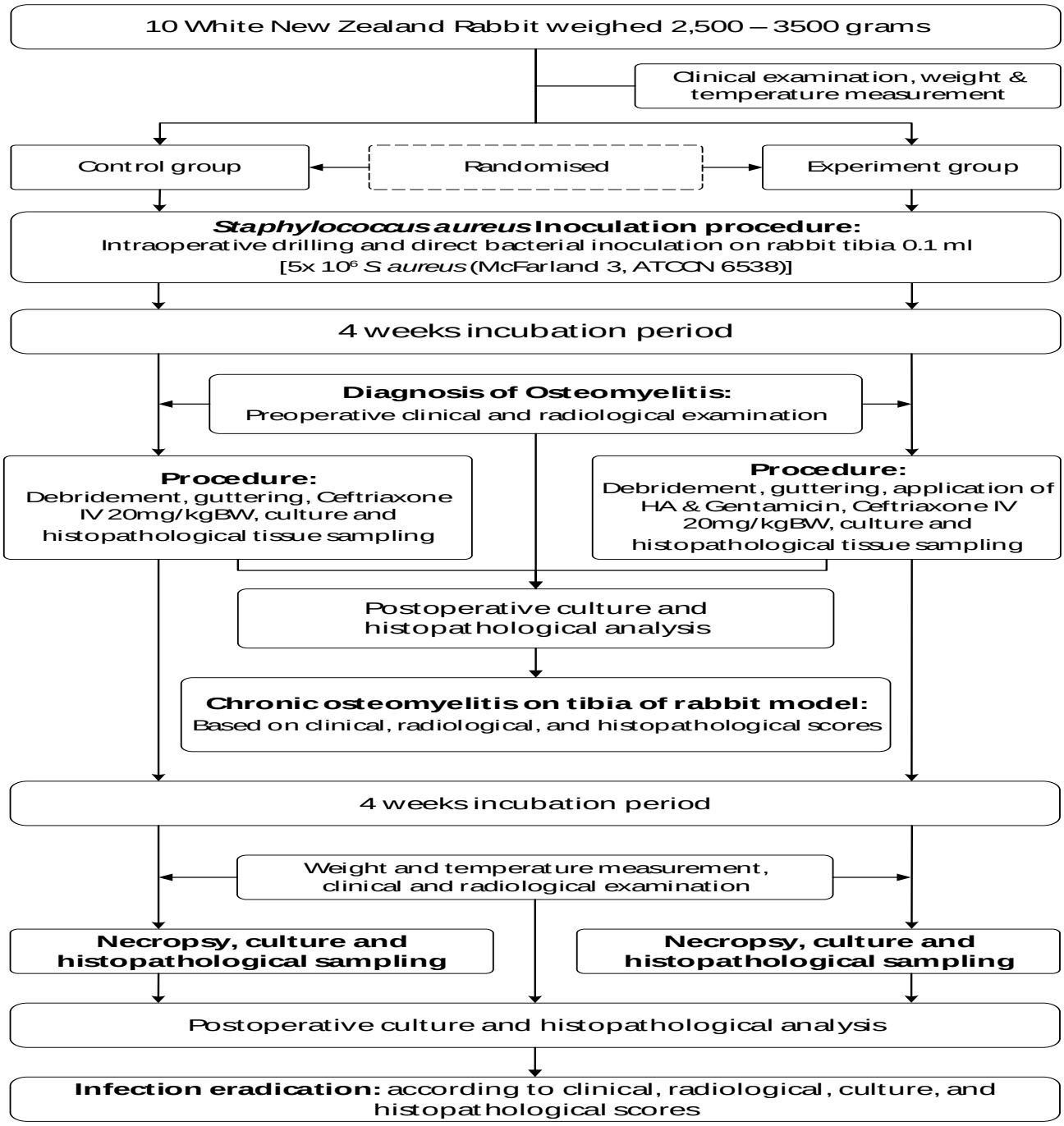

Figure 1: Study protocol

Inoculation of Staphylococcus Aureus into Rabbit Tibia

The rabbits were put on examination table. An aseptic and antiseptic procedure was done on right thigh with 70% alcohol which followed by ketamine administration (40 mg/kg intramuscular). Response to anesthethic agents were carefully examined and the anesthesia is considered to be succesful after pupils constriction and decreased respiratory rate. The rabbits were put into left lateral decubitus facing away from the operator. The fur was shaved and prepared with 70% alcohol and betadine. A 1-2cm incision was made along the right thigh penetrating the skin, fascia, on to the tibial bone.

Four holes with 1.5 mm diameter were drilled on each bone followed by injection of 0.1 ml (5 x 10^6 S. aureus in a syringe) and prompt coverage with bone wax. The surgical wound was sutured layer by layer and bandaged. The rabbits were then returned to their cage and was given ketoprofen 3mg/kg intramuscularly every 12 hours for 3 days.

The rabbis were incubated in individual cages in controlled environment (18-21^{o}C) for four weeks. They were given commercial food (5 grams/ 100 body weight/ day) and sufficient water. Every three days the rabbits were examined for weight measurement, motoric activity and motion, appetite, signs of local or systemic infection, and tempature measurement by timpanic membrane thermometer.

Debridement and Application of HA and Gentamicin on Rabbit Tibia

Control Group

The rabbits were prepared in a similar way as the inoculation process. After being anesthetized, the rabbits were put into left lateral decubitus facing away from the operator. A 2 cm longitudinal incision was made on the previous surgical incision penetrating the skin, fascia, on to tibia bone.

Samples were taken from adjacent infected wound with sterile cotton buds which were then planted into sterile agar media for culture. Debridement was performed and a 1 x 0.5 cm gutter was made through the cortex with drill. Soft-tissue and debris from drilling were soaked into 10% formaldehyde and sent for histopathological analysis. Surgical wound was cleansed with 0.9% NaCl and sutured layer by layer. Rabbits were given ketoprofen 3 mg/kg intramuscularly every 12 hours for 3 days and ceftriaxone 20 mg/ kgBW/ day (divided into 2 doses) intramuscularly for 4 weeks.

Treatment Group

The rabbits were prepared in a similar way as the inoculation process. After being anesthetized, the rabbits were put into left lateral decubitus facing away from the operator. A 2 cm longitudinal incision was made on the previous surgical incision penetrating the skin, fascia, on to tibia bone. Samples were taken from adjacent infected wound with sterile cotton buds which were then planted into sterile agar media for culture. Debridement was performed and a 1 x 0.5 cm gutter was made through the cortex with drill. Soft-tissue and debris from drilling were soaked into 10% formaldehyde and sent for histopathological analysis.

Surgical wound was cleansed with 0.9% NaCl, and HA porous previously soaked in 0.1% gentamicin was placed in the gutter. The wound was sutured layer by layer. Rabbits were given ketoprofen 3 mg/kg intramuscularly every 12 hours for 3 days and ceftriaxone 20 mg/ kgBW/ day (divided into 2 doses) intramuscularly for 4 weeks.

The rabbis were incubated in individual cages in controlled environment (18-21^{o}C) for four weeks. They were given commercial food (5 grams/ 100 body weight/ day) and sufficient water. Every three days the rabbits were examined for weight measurement, motoric activity and motion, appetite, signs of local or systemic infection, and tempature measurement by timpanic membrane thermometer.

Radiological Examination

Ten rabbits underwent x-ray examination in radiology unit of veterinary hospital, Bogor Institute of Agriculture (BIA). Anteroposterior and lateral radiographs of right tibia were taken 1 day preoperative and 4 weeks postoperative. The radiographs were analysed by animal radiologist.

Rabbit Euthanasia and Sampling for Culture and Histopathological Analysis

Four weeks after debridement, the rabbits were put into examination table and prepared similar to previous surgeries.

However, this time the rabbits were injected with anesthetic agents until respiratory failure and deemed in a state of cardiac and respiratory arrest.

After euthanasia procedure, the rabbits were placed in left lateral decubitus facing away from the operator, and prepared for surgery. A 3-cm longitudinal incision is made along the previous surgical wound penetrating skin, fascia, onto the tibia. Tissue samples from adjacent infected wound were obtained with cotton buds which were then planted in a zigzag fashion in sterile media agar for culture purpose. Osteotomy was then performed 1 cm toward proximal and distal end and the tissue was soaked in 10% formaldehyde for histopathological analysis. Surgical field was then cleansed with 0.9% NaCl until considered clean and sutured layer by layer. Surgical wound was closed and the rabbits were incinerated.

Clinical Examination, Culture and Histopathological Analysis

Clinical examinations include infection confirmation by the presence of swelling, abscess or fistula formation. Assessment was done semi-quantitatively. On plain radiograph, sclerosis, osteolysis, sequestrum, or involucrum can be seen. Plain radiographs will be staged and scored accordingly by observing the periosteal reaction and cortical destruction (osteolysis) as seen in Table 1 and 2.[9] Radiographs were analyzed by two independent radiologists from BIA. Assessment was done semi-quantitatively.

Table 1: Clinical and radiological stages of osteomyelitis (adapted by Pineda et al)[9]

Stage	Clinical	Radiological
1	No abnormality	No abnormality
2	Swelling around the incision site	Cortical thickening > 10% or destruction of normal architecture of tibia >10%.
3	Swelling involving proximal tibia	Cortical thickening >50% or destruction of normal architecture of tibia >50%.
4	Swelling involving >50% tibia	Cortical destruction of more than 75%
5	Swelling across the whole tibia	Cortical thickening > 100% or destruction across the whole tibia
6	Lesions with abscess formation or fistula	Articular invasion or destruction of tibial anatomy

Table 2: Clinical and radiological scoring of osteomyelitis (adapted by Pineda et al)[9]

		Treatment	Control
Clinical	Normal	0	0
	Swelling around the incision site	3	3
		2	2
		0	0
	Swelling involving proximal tibiaSwelling involving >50% tibia	0	0
	Swelling across the whole tibiaAbsesLesions with abscess formation or fistula	0	0
Radiological	Normal	0	0
		5	5
	Cortical thickening/destruction> 10%	0	0
	Cortical thickening/destruction> 50%	0	0
	Bone destruction> 75%	0	0
	Cortical thickening/destruction 100%	0	0
	Articular invasion or destruction of tibial anatomy		
Culture	Positive	0	1
	Negative	5	4
Histopathological	HOES		
	7,5	1	1
	8	3	1
	8,5	1	1
	9	0	1
	9,5	0	1

Histopathological analysis was performed by a pathologist from BIA. Histopathological findings for osteomyelitis include the presence of granulocytes or lymphocytes and evidence soft-tissue necrosis. The assessment was made semi-quantitatively using Histopathological Osteomyelitis Evaluation Score (HOES) [10]. HOES criteria evaluate bone necrosis, soft-tissue necrosis, granulocyte infiltration, new bone formation, and lymphocyte or macrophage infiltration. Each item is scored from 0 (no abnormality) to 3 (severe, on all 3 field of view). The result is then divided into no indication of osteomyelitis, acute osteomyelitis, chronically

florid osteomyelitis, chronic osteomyelitis, and subsided osteomyelitis.

Data Analysis

Data analysis was done using Statistic Program for Social Science version 18.0. To determine the difference of infection eradication between control and treatment group, the following studies were conducted: (1) clinical, radiological, and cultures between the groups were analyzed using Fischer's Exact test; (2) histopathological comparison within the groups were analyzed using Mann-Whitney test; (3) clinical and histopathological comparison within the groups were analyzed using Wilcoxon test; (4) radiological and culture within the groups were analyzed using McNemar's test; (5) for difference in weight and temperature paired t-test was done.

Results

To obtain rabbit osteomyelitis model in tibia, after 4 weeks of inoculation of S. aureus the rabbits underwent clinical, radiological, and histopathological examination. The inoculation process was successfully conducted in every rabbit in both the treatment and control groups. The baseline characteristic of rabbit model is shown on Table 3.

Table 3: Clinical, Radiological, Culture and Histopathological comparison between Treatment and Control groups

			Treatment	Control	p-value
Clinical	4 weeks after inoculation	Swelling around incision site	3	3	1,000 [a]
		Swelling of proximal tibia	2	2	
	4 weeks after treatment	Normal	5	2	0,053 [b]
		Swelling around incision site	0	2	
		Swelling of proximal tibia	0	1	
Radiological	4 weeks after inoculation	Normal	0	0	-
		Cortical thickening>10%	5	5	
	4 weeks after treatment	Normal	4	0	*0,048[a]
		Cortical thickening>10%	1	5	
Culture	4 weeks after inoculation	Positive	0	1	1,000[a]
		Negative	5	4	
	4 weeks after treatment	Positive	1	1	1,000[a]
		Negative	4	4	
Histopathological	4 weeks after inoculation (average)	7,5	1	1	0,278[b]
		8	3	1	
		8,5	1	1	
		9	0	1	
		9,5	0	1	
	4 weeks after treatment (average)	5	4	0	**0,009[b]
		6	1	1	
		6,5	0	1	
		8	0	1	
		8,5	0	2	

During the incubation period, there was a decline in appetite and weight loss occurred with an average of 140 grams. No rabbit died during the incubation period. The rabbits could urinate and defecate normally, remained active and responded well to interaction. Average weight of subjects in treatment group was lower at the time of inoculation and 4 weeks after inoculation compared to control group. However, 4 weeks after treatment, the average weight on treatment group increased and even surpassed the control group. The difference between both groups were not statistically significant at the time of inoculation (p=0.116), 4 weeks after inoculation (p=0.222) and 4 weeks after treatment (p=0.576). During the 8-week incubation period, the average temperature of rabbits remained stable, in range of 38.3-39.4^{o}C. The average weight and temperature of rabbits during the incubation period are shown in Fig. 2 and 3, respectively.

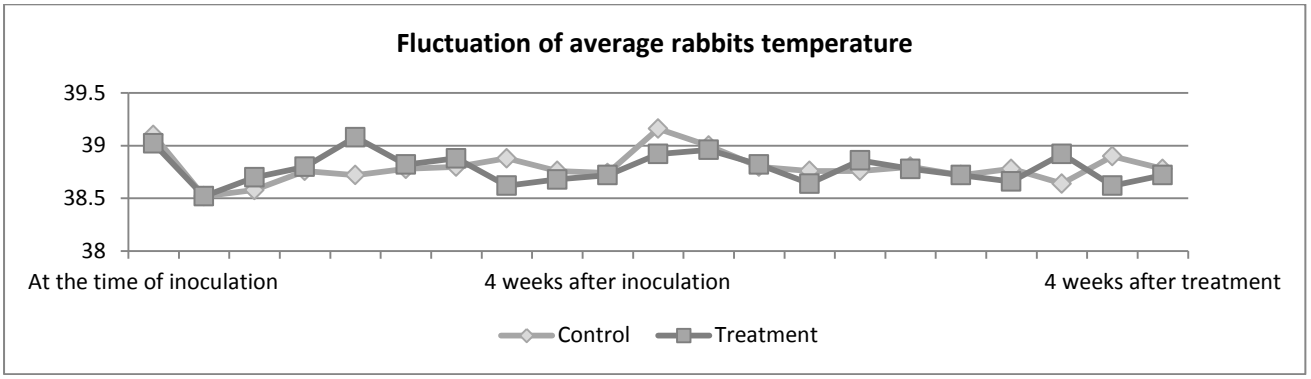

Figure 2: Fluctuation of average rabbits' temperature at the time of inoculation, 4 weeks after inoculation, and 4 weeks after treatment

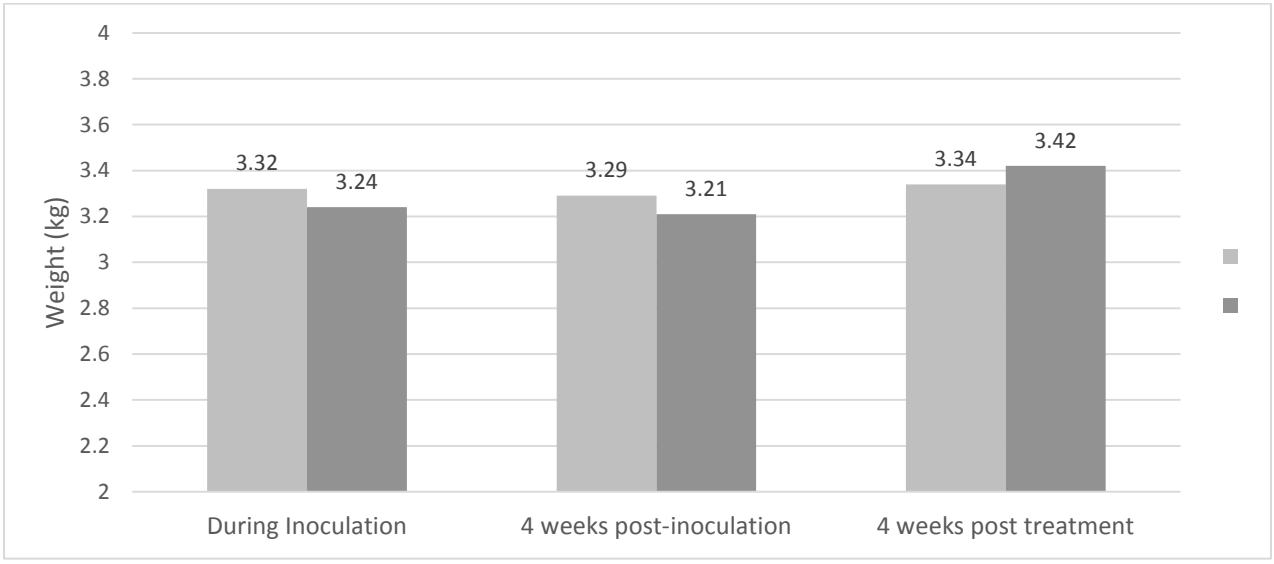

Figure 3: Comparison of rabbits' weight at the time of inoculation, 4 weeks after inoculation, and 4 weeks after treatment

All the rabbits underwent clinical, radiological, culture, and histopathological examination. From clinical examination, 4 weeks after the inoculation, both the treatment and control groups showed clinical signs of osteomyelitis (p = 1.000, Fischer's Exact test). After treatment, all the rabbits in treatment group returned to normal state, while the rabbits in control groups still showed signs of osteomyelitis, however, this was not statistically significant (p=0.053, Mann-Whitney U test).The clinical features after inoculation, and after treatment for experiment and control groups are shown in Fig. 4 and 5.

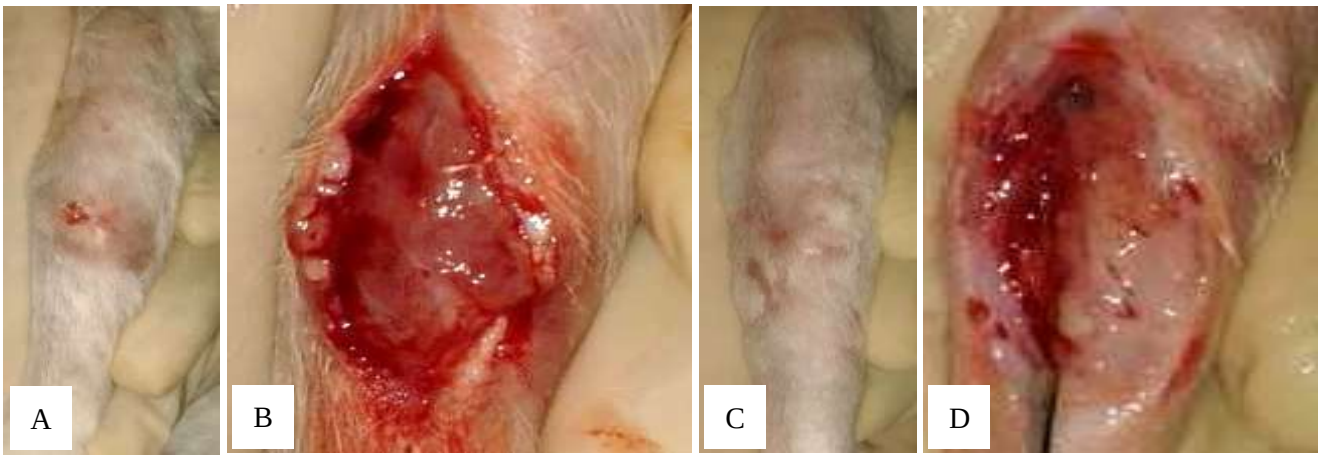

Figure 4: Clinical appearance at 4 weeks after inoculation of S. aureus. We can see fistula (A) on incision site and exudate underneath the fistula after incision (B). All the rabbits in both groups showed swelling on incision site (C) and subcutaneous exudate (D).

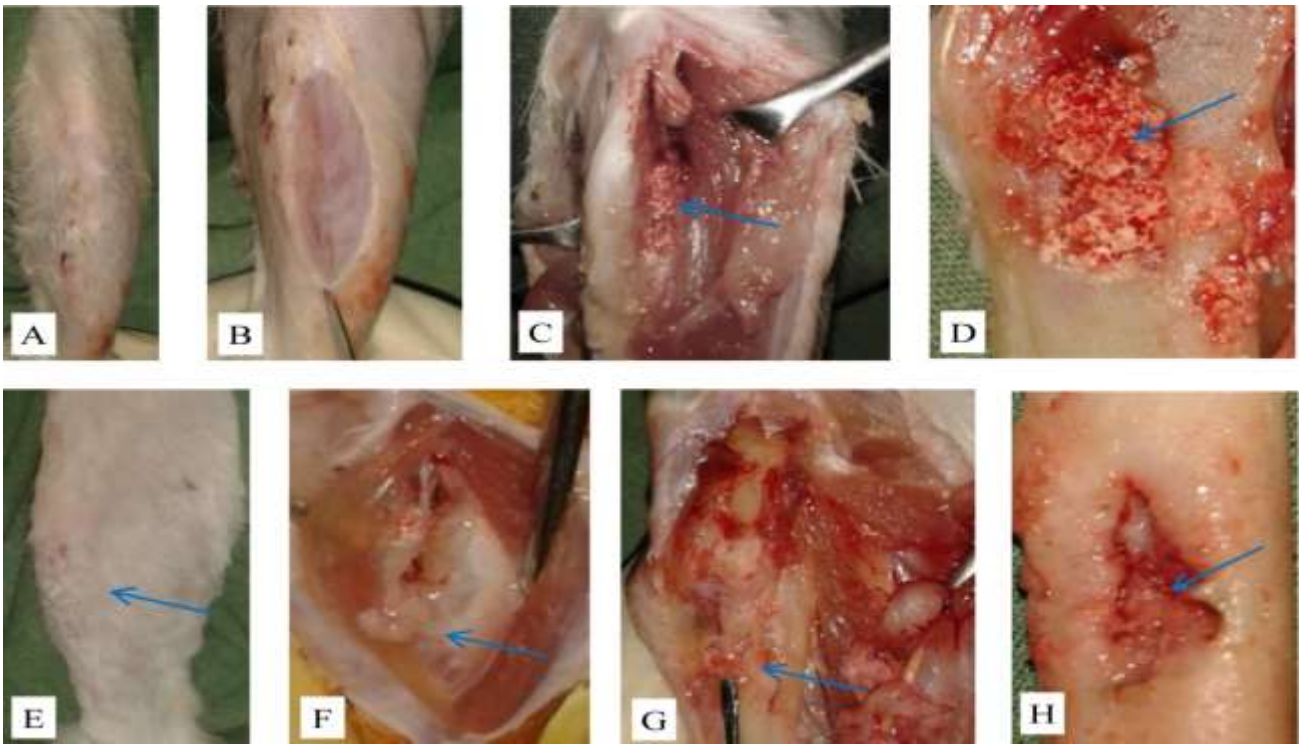

Figure 5: Comparison of clinical features after treatment. The treatment group [A] showed no sign of swelling, while the control group showed sign of swelling [E]. In subcutaneous region of rabbits in treatment group, there was no sign of inflammation [B] while the control group showed yellowish mucoid tissue [F] with thicker soft-tissue compared to treatment group. IN figure [C] and [D], we can see defects filled with unresorbed synthetic bone graft, while in the control group the defects were filled with fibrotic scar.

From radiological examination, after 4 weeks of inoculation, all the rabbits in both treatment and control group showed more than 10% of cortical thickening. However, 4 weeks after treatment, only one rabbit in the treatment group showed radiological sign of osteomyelitis while the rest returned to normal. On the other hand, the rabbits in the control group still showed signs of osteomyelitis at 4 weeks after treatment as shown on Fig.6 ($p = 1.000$; Fischer's Exact test.

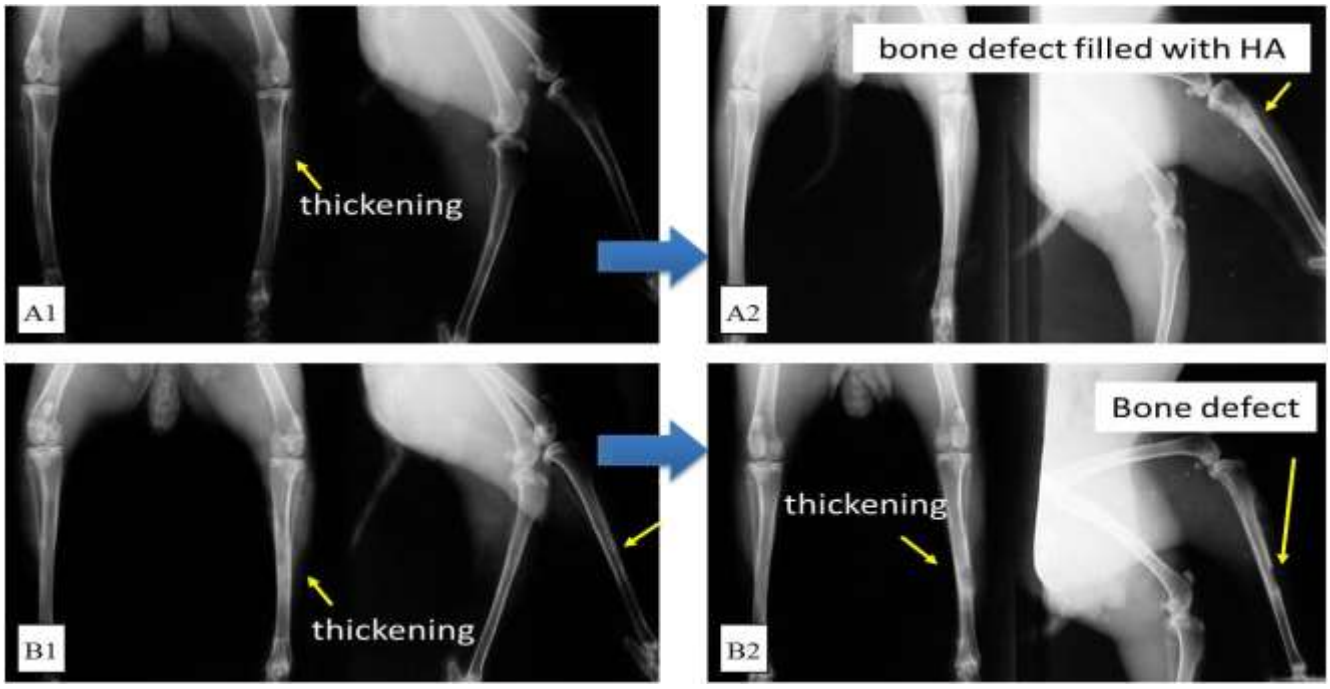

Figure 6.:Radiologic analysis of rabbit tibia after treatment in both control and treatment groups. (A1 and B1) both have osteomyelitis on tibia, (A2) treatment group shows bone healing, while (B2) control group shows bone defect on tibia.

Culture from samples taken from surgical procedure showed only one rabbit from control group had positive result. Four weeks after treatment, one rabbit in both groups showed positive result, while the rest had negative result. This was not statistically significant (p = 1.000; Fischer's Exact test).

Based on histopathological analysis on 10 samples taken from surgery, all samples met the criteria for chronic osteomyelitis after 4 weeks of inoculation as shown on Fig. 7 (HOES score > 6 on both groups). Both groups were comparable at four weeks after inoculation as shown on Fig.8 and 9, respectively for treatment and control groups (p = 0,278; Mann-Whitney U test). This result was from two independent observers.

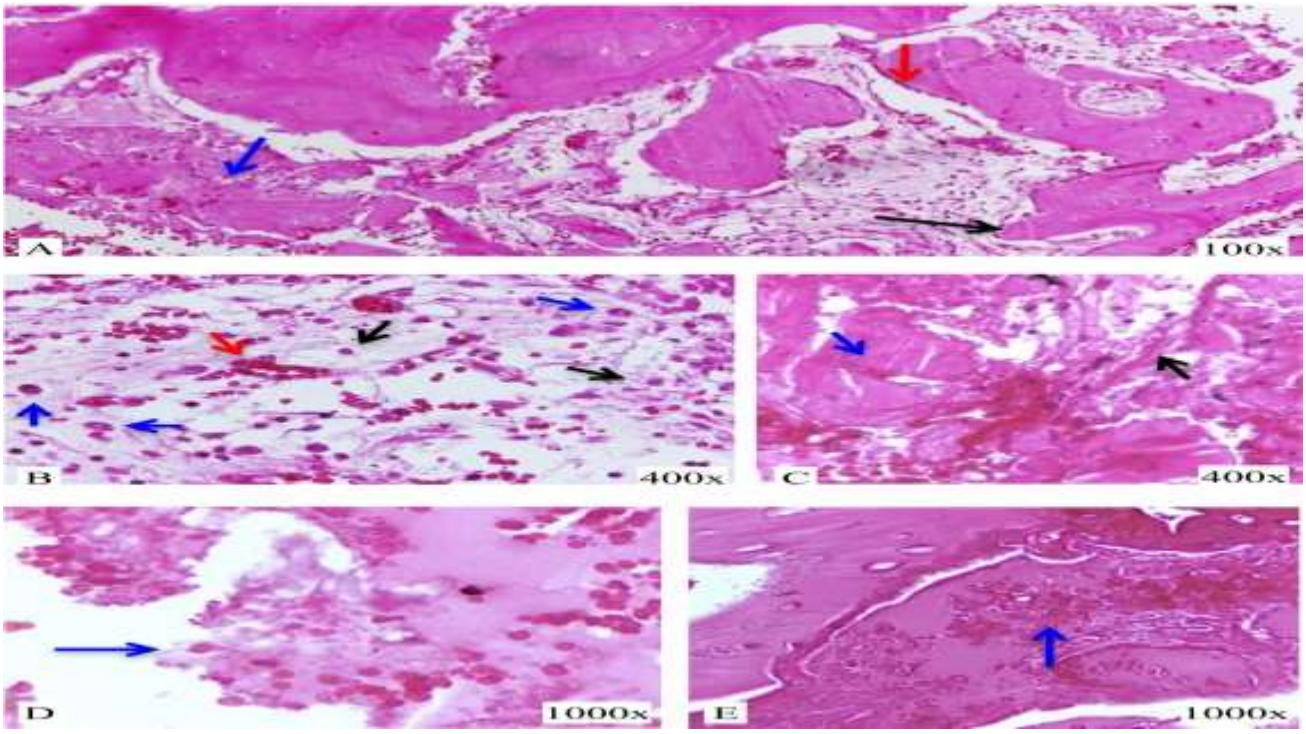

Figure 7: Histopathological analysis of tibia after 4 weeks of inoculation of S. aureus (HE staining) On 100x magnification (A) necrotic bone area (blue arrow), surrounding soft-tissue with abundance of PMN cells (black arrow) and new bone formation (red arrow) can be seen. On 400x magnification (B), granulocyte with lobulated nucleus (blue arrow), lymphocytes (black arrow) and vascularization with erythrocytes (red arrow) can be seen. Figure (C) depicts bone necrosis (blue arrow) and soft-tissue necrosis (black arrow). On 1000x magnification, (D and E) appearance of cocci with necrotic surroundings can be seen

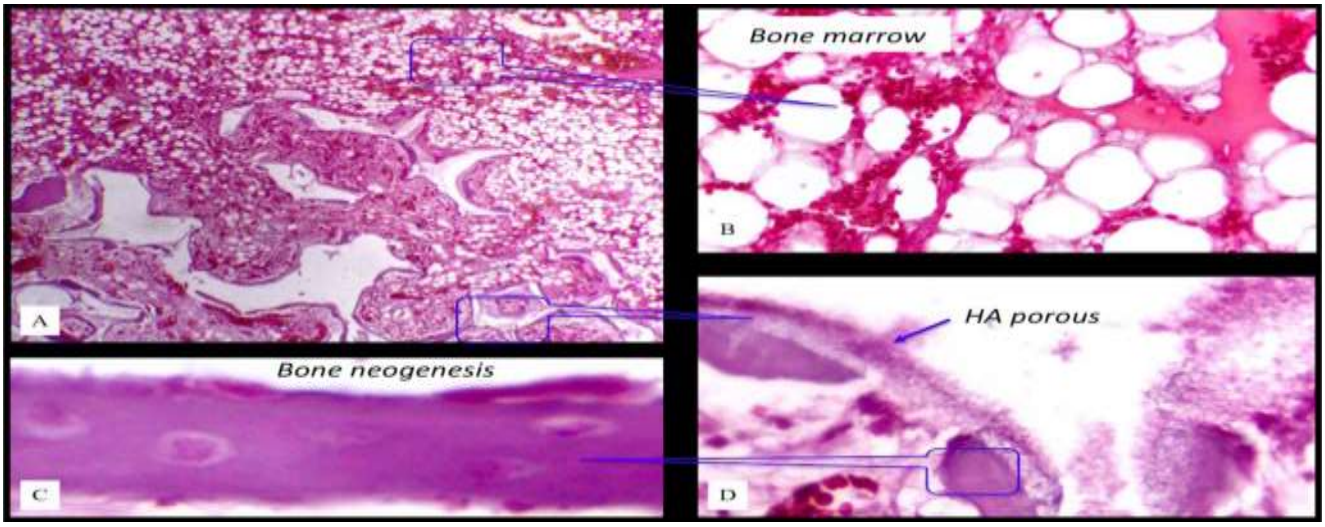

Figure 8: Histopathological analysis of rabbit tibia after treatment in treatment group (HE Staining). On 100x magnification (A), bone marrow (B) with HA content and new bone formation with HA inside (C and D)

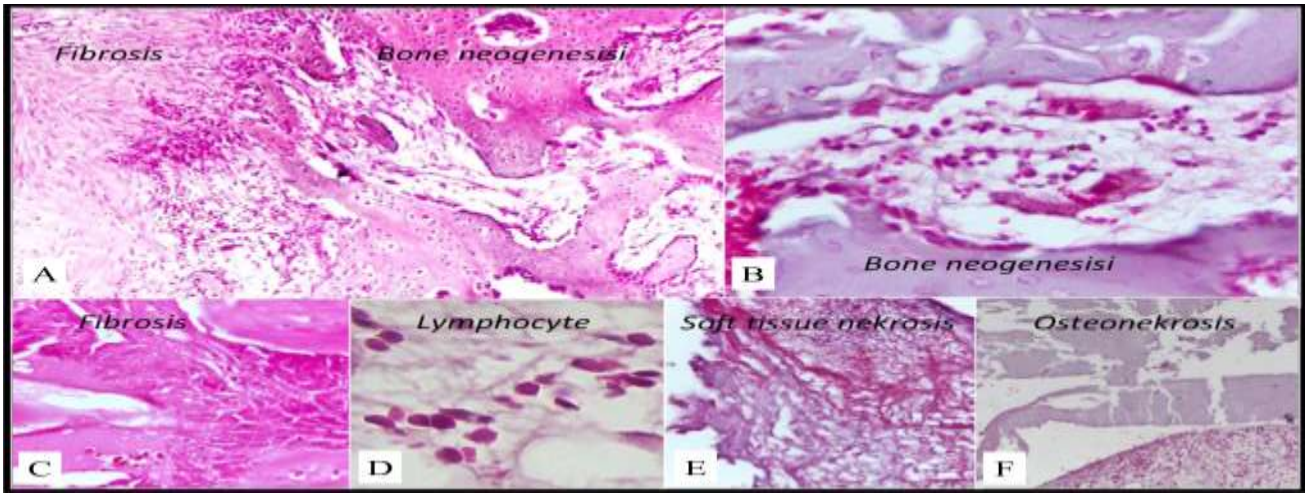

Figure 9: Histopathological analysis of rabbit tibia 4 weeks after treatment in control group (HE staining). On 100x magnification (A), we can see the border of new bone and fibrotic tissue with granulocyte in between. Amidst the new bone formed are granulocyte infiltration (B and D), fibrotic areas (C). Soft-tissue necrosis is characterized by the absence of intact cells (E) as in bone tissue (F).

Four weeks after treatment, 4 rabbits in control group still had HOES score > 6 which means that they had osteomyelitis, This was statistically significant (p = 0.009; Mann-Whitney U test). The clinical, radiological, culture and histopathological results are shown on Table 3.

Table 3: Clinical, Radiological, Culture and Histopathological comparison between Treatment and Control groups

			Treatment	Control
Clinical	4 weeks after inoculation	Swelling around incision site	3	3
		Swelling of proximal tibia	2	2
	4 weeks after treatment	Normal	5	2
		Swelling around incision site	0	2
		Swelling of proximal tibia	0	1
Radiological	4 weeks after inoculation	Normal	0	0
		Cortical thickening>10%	5	5
	4 weeks after treatment	Normal	4	0
		Cortical thickening>10%	1	5
Culture	4 weeks after inoculation	Positive	0	1
		Negative	5	4
	4 weeks after treatment	Positive	1	1
		Negative	4	4
Histopathological	4 weeks after inoculation (average)	7,5	1	1
		8	3	1
		8,5	1	1
		9	0	1
		9,5	0	1
	4 weeks after treatment (average)	5	4	0
		6	1	1
		6,5	0	1
		8	0	1
		8,5	0	2

Discussion

Generally, bone is relatively resistant to infection. However, trauma, surgery, foreign body insertion or implants accompanied by contamination disrupts bone integrity and contribute to infection.[11,12]Organisms

causing bone infection produce extracellular material and biofilm layer which blocks antibiotics delivery.[13]Thus, prophylactic antibiotic on bone graft is expected to prevent bacteria colonization on its surface [14].
A study by Itokazu et al. [15] found that hydroxyapatite block could carry antibiotics by means of centrifugation which releases slowly on an in vitro study. This biocompatibility was also previously reported and proven effective in human.[16,17]Another study by Krisnapiboon reported that hydroxyapatite composite had good biocompatibility as a carrier for gentamicin, phosphomycin, imipenem, and amphotericin B [18].

Another study reported that porous HA releases its antibiotic contents slowly and continuous over 2-week period and additional polylacticglycolid acid extends antibiotics release for up to 4 weeks, even surpassing the bactericidal concentration needed to eliminate the pathogen.[19,20]The antibiotic effect only occur locally and does not add to systemic effect. Combination of gentamicin and HA does not interfere with osteoblastic proliferation or cell morphology and thus serve simultaneously as a good basis for bone regeneration and infection eradication [18].

Osteomyelitis is diagnosed clinically by the presence of unspecific symptoms such as fever, shivering, lethargy, and irritability, particularly in acute phase. In this study, the temperature of test subjects was still within normal limits during the observation period. The method of measurement by tympanic membrane thermometer immediately after rabbits were taken out of their cage contribute to this, as the rabbits were kept on controlled environment with 23^{o}C temperature. Ideally, temperature is measured through anus, however, we wanted to keep the rabbits in as minimal stress as possible.

On all study subjects, criteria for osteomyelitis were met. Clinically, the rabbits showed swelling in tibia. Swelling is an early response to infection as a result of inflammatory process and increased vascularization around the infection site. This swelling can extend to proximal and distal portion of tibia, and even to articular surface.

According to Pineda et al[9], early signs of infection on bone is periosteal reaction , swelling around infected bone, cortical thickening, lytic lesion, cortical erosion, osteopenia, loss of trabeculae and new bone formation in second or third week, as detected on plain radiograph. In our study, radiological examination on all subjects revealed cortical thickening >10% around inoculation site which suggest periosteal reaction.[21,22] However, radiological examination only has 43-75% sensitivity and 75-83 specificity in diagnosing osteomyelitis [9].

The gold-standard for osteomyelitis diagnosis are culture and histopathological results.[23] Only one subject had positive culture result. This negative culture results may in part due to inadequate sample volume so that they do not grow on agar media. A study by Mikus et al[24] showed that culture is unreliable for osteomyelitis diagnosis as 20% of negative culture result subsequently yield positive result on histopathological analysis.

In this study, the treatment group showed significant improvements in clinical, radiological, and histopathological. These results were based on treatment strategy which includes surgical debridement, systemic antibiotic therapy for 4-6 weeks, and local application of antibiotics, as was previously reported by Sanchez.[20]

After 4 weeks of inoculation, all subjects had HOES score >6 which indicates chronic osteomyelitis. At the time of analysis, all subjects in the treatment group had HOES score below 6, which signals improvement of osteomyelitis, and this was statistically significant ($p = 0.009$; Mann-Whitney U test). As Tiemann et al. [10] suggest, histopathological analysis serves as the basis to determine the acuteness of the disease. According to the same study, it is also noted that in chronic osteomyelitis, culture results do not always meet with histopathological results.

Surgical debridement aims to convert a necrotic milieu into well vascularized environment so as to aid antibiotic delivery and shorten its duration.[25] In situ implantation of local antibiotics along with scaffold is thought to eliminate bacteria in and around lesion and reduce dead space. These combination produce lower serum

antibiotic concentrations compared to serum concentrations after systemic administration, thereby reducing toxicity associated with side effects. The use of definitive antibiotic was based on culture and sensitivity test of S. aureus to ceftriaxone and gentamicin.

Porous HA acts as a biodegradable substance absorbed over 3-6 months period [26] Mustafa [19] reported slow and continuous gentamicin release from porous HA over a two-week period. This feature simultaneously aids bone regeneration and elimination of infection.

Evaluation of infection elimination was based on clinical, radiological, culture, and histopathological analysis. However, histopathological analysis was the primary means to determine infection elimination, as reported by Atkins [27].

Clinical, radiological, and culture served as supportive means. Swelling is also present in soft tissue infection or it may subside after acute phase. In acute phase, radiological examination is of little significance as changes only appears after 2-3 weeks.[28] Several bone tumors and fracture healing may mimic osteomyelitis on plain radiograph [5,29]. Positive culture result indicates definite presence of bacteria, however negative result does not rule out the absence of infection as other factors also contribute to culture results. Based in these factors, we established histopathological analysis as the primary means to determine osteomyelitis. Histopathological analysis also provides insight of improvement at cellular level.

Conclusion

As far as we are aware, this study is the first in vivo study which determines the efficacy of gentamicin and porous HA combination for treatment of chronic osteomyelitis. We generated a rabbit model for chronic osteomyelitis using S.aureus for our study, which is the most common invading organism in human. The diagnosis of osteomyelitis is made based on examinations likely done on patients according to hospital standards which results in a better accuracy. Treatment strategy performed in this study was performed similar to treatment for human, and thus our study serves as a basis for clinical trial with human subjects.

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