Mechanism of novel enoxaparin sodium high viscosity bone cement reducing thrombosis by regulating the expression of CD40 in endothelial cells

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Research Article

Keywords: Thrombosis, Enoxaparin sodium, Bone cement, CD40, Endothelial cells

Posted Date: March 29th, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1377847/v2>

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Abstract

Objective: PMMA bone cement develop local thrombi, to solve that,our study found that ES-PMMA bone cement,a new kind of material,could reduce local thrombosis.We took a simple and reproducible animal model to confirm the reducing and preliminarily explored the molecular mechanism.

Methods: On the basis of carotid artery vein extracorporeal shunt, the New Zealand rabbits were modeled into three groups: Sham group, PMMA group and ES-PMMS group. There were 10 rabbits in each group. Four hours after modeling,collecting the experimental samples,and comparing the amount of thrombosis between those groups. The collected vascular tissue samples were quantitatively detected for endothelial cell related thrombomodulin.

Results: There were thrombosis in PMMA group and ES-PMMA group,which didn't exist in Sham group. The weight of thrombosis in PMMA group was $0.00732 \pm 0.00089 \text{g/cm}$, The weight of thrombosis in the ES-PMMA group was $0.00554 \pm 0.00077 \text{g/cm}$ $P < 0.001$. We found that the expression of CD40, a related protein that can regulate thrombosis in vascular endothelial cells, was significantly lower in the ES-PMMA group than that in the PMMA group using RT-qPCR as well as Western blot.

Conclusion: Compared with PMMA bone cement, ES-PMMA bone cement can reduce local thrombosis by decreasing the expression of thrombus associated regulatory protein CD40 in vascular endothelial cells.

Background

Bone cement has been used for a long time in the clinic, with the continuous development of joint replacement, operation related complications and adverse events gradually emerged; as one of the important and serious complications, the incidence rate of bone cement implantation syndrome is as high as 28% [1], although its exact mechanism has not been fully described so far, at present, it is believed that it is mainly caused by the following ways, including allergic reaction, pulmonary embolism, complement activation and histamine release, combined with the increase of pulmonary vascular resistance, ventilation/perfusion mismatch, which eventually leads to acute hypoxia, right ventricular failure and cardiogenic shock [2].Although pulmonary embolism is rare in joint replacement, the mortality rate is very high. According to relevant reports, the incidence of pulmonary embolism after joint replacement can reach 0.2% – 0.4% [3].

The current research focuses on the toxic reaction of bone cement particles in the blood, and the thrombosis result from activating the human complement system and the activating of human coagulation system because of thermal effect [4]. Thrombus shedding can lead to lower extremity venous thrombosis or pulmonary embolism, whether the physical and chemical properties of bone cement can directly lead to local thrombosis has not been studied.

This study verifies that high viscosity bone cement can produce local thrombosis in animal models, and confirms that enoxaparin sodium high viscosity bone cement, a new material, has the ability to reduce

local thrombosis compared with ordinary high viscosity bone cement, and explains the preliminary molecular mechanism of how this material reduce local thrombosis, which is described in detail below.

Material Method

Animal model, grouping and experimental material process

Six month old male New Zealand rabbits (Wangdu Tonghui animal breeding Co., Ltd., animal Certificate No. : 210426) (approved by the medical ethics committee of the Third Medical College of Hebei Medical University, ethics acceptance No. z2021-007-2), weighing $2.5 \pm 0.5\text{kg}$, was satisfactorily anesthetized and maintained with 20% urethane, and the anesthetic effect was satisfactory; Take the supine position and establish the rabbit carotid artery vein extracorporeal shunt as described in the literature [5]. Modeling rabbits into three groups: Sham-operated group(FS group)(n = 10), ordinary high viscosity polymethylmethacrylate bone cement (PMMA) group (Con group) (n = 10) and new enoxaparin sodium high viscosity polymethylmethacrylate bone cement (ES-PMMA) group (M group)(n = 10). In M group, added 8000 axaiu enoxaparin sodium powder (Chengdu Baiyu, China) to 40g PMMA bone cement (Heraeus, Germany) for premixing [6], and then mixed with bottled liquid monomer for modulation, and then mixed with bottled liquid monomer for modulation, Then placed them under high-power scanning electron microscope (SEM, Hebei Medical University electron microscopy center, Hitachi, S-3500N), and compared the differences in the characterization of bone cement between different groups. Glue The bone cement of different groups with surgical silk thread, and make sure that bone cement completely and evenly covered. Then, placed them in the shunt, and extend about 1cm into the blood vessel through the shunt to make it contact with the vascular endothelium, And keep circulating for 4 hours. We placed nothing in the shunt in FS group, and modeled the same procedure to other two groups. The approximate process of modeling and obtaining samples is shown in Fig. 1, and the characterization of PMMA and ES-PMMA is shown in Fig. 2.

Sample collection

Four hours after modeled, collecting samples and observing results, taking out the bone cement thrombus sample from Con group and M group, calculating the amount of thrombus which is attached to the bone cement strip, storing the vascular tissue sample which is in contact with the bone cement and proceduring the same process in the FS group in $-80\text{ }^{\circ}\text{C}$ refrigerator, and detecting the thrombus related regulatory protein of vascular tissue endothelial cells by RT-qPCR, Western blot and immunofluorescence.

RT qPCR was used to detect the mRNA expression level of thrombus associated protein in endothelial cells in vascular tissue.

According to the manufacturer's instructions, total RNA of different groups was extracted with Trizol reagent (Invitrogen, USA), and the mRNA was reverse transcribed with primescript RT reagent kit (Takara,

Japan). SYBR premix ex Taq Kit (servicebio Wuhan) was used for quantitative real-time polymerase chain reaction on iq5 real time PCR amplification instrument of Applied Biosystems (Applied Biosystems USA). 94 °C for 4min, 94 °C for 30s, 60 °C for 30s and 72 °C for 30s, a total of 40 cycles. Each sample was subjected to three repeated amplification reactions and mRNA analysis. The primer sequences of polymerase chain reaction were: CD31 F: TCCTACGATGCCAGGTCTGA R: CATTTCCGGCATGGGAATGGC; Vascular endothelial cell adhesion molecule VCAM-1 / CD106 F: GCCCTTTGGAGGTTGGAGAA R: GAACTGGTAGACCCTCGCTG; Endothelin endothelin F: TGA TCCCAGAGAGGACGTG R: CTCCTGGACGGCTACAATCC; Thrombomodulin F: TTCCTCTGCGAGTTCCCCTT R: CGTAACAGGTCAGCTCCAAG; CD62p F: AGTGTGTAGCTGTCCAGTGC R: AGTCACCAAAGGGATGCGAG; GAPDH F: TGG AATCCACTGGCGTCTTC R: TCATGAGCCCCTCCACAATG. Pass 2^{-ΔΔCT} method was used for quantitative analysis of relative mRNA and histogram was drawn. The relative expression of the gene was normalized to the expression of GAPDH.

Western blot analysis

Washing blood vessel samples of different groups with ice cold PBS and then lysing with Ripa (beyotime, China) containing protease inhibitor. The protein lysate was separated in different percentages of SDS-PAGE and then transferred to polyvinylidene fluoride (PVDF) membrane (millipore, USA). The membrane was incubated with the primary antibody containing CD31 (Abcam, UK), CD40 (Abcam, UK), CD62P (Abcam, UK), CD106 (Abcam, UK), endothelin (Abcam, UK) and thrombomodulin (Abcam, UK) in a 4 °C shaking table overnight, and the membrane was incubated in the corresponding secondary antibody for 1.5 hours. The film was placed in the Western LightningTM Chemiluminescence Reagent color developer for 30 s, the film was placed in the exposure box immediately, and the exposure film was exposed to 1 min in the darkroom, then developed and fixed, and analyzed by LabWorksTM gel imaging and analysis system (UVP, USA). GAPDH was used as internal reference control.

Tissue immunofluorescence

The frozen vascular tissues of different groups were balanced for 1 hour, the slice thickness was 8 μ m. Placed in Triton X-100 (mass fraction 0.5%) for 10 minutes, blocked with 4% goat serum albumin bas, primary anti-cd40-antibody (Abcam, UK) 1:200, overnight at 4 °C, rinsed with PBS for 3 times, secondary anti Sheep anti rabbit IgG antibody (Abcam, UK) 1:400, rinsed with PBS at room temperature for 2 hours, dried and observed and photographed under fluorescence microscope, Image Pro Plus software carries out semi quantitative analysis on the image and calculates the fluorescence intensity.

Data analysis

All experiments were carried out three times. The results were expressed as mean ± standard deviation. T-test was used for comparison between groups. Statistical analysis consists of SPSS software (version 17.0). $P \leq 0.05$ is considered statistically significant. $P \leq 0.05$ is marked as *, $P \leq 0.01$ is marked as **, and $P \leq 0.001$ is marked as ***.

Results

Electron microscopic characterization of PMMA and ES-PMMA.

8000 axaiu ES and 40g PMMA powder were used in the M group of the experimental group. Under the electron microscope, it can be seen that the gap between the particles on the surface of the ES-PMMA is filled with syrup like substance, which is considered to be the adhesion of enoxaparin.

Analysis of thrombus results in different samples

Four hours after the establishment of animal model, we recorded that the mass of thrombosis in Con group was $0.00732 \pm 0.00089\text{g/cm}$ and that in M group was $0.00554 \pm 0.00077\text{g/cm}$, $P < 0.001$, with statistical difference. There was no thrombosis in FS group in our study.

Differential expression of endothelial cells and thrombus associated proteins in vascular tissue

The vascular endothelial cells in Con group and M group which is in contact with bone cement were collected for RT-qPCR, Western blot and immunofluorescence detection. Meanwhile, detecting FS group's vascular endothelial cells by those same ways. Relevant studies have shown that thrombosis is related to inflammation, hypercoagulable state, vascular endothelial cell injury or functional regulation. The related proteins expressed by endothelial cells are related to thrombosis. The main proteins are CD62p, CD40, CD31, endothelin, thrombomodulin and CD106. We detected the mRNA expression levels of six thrombus related proteins in the samples by RT-qPCR. The results are shown in Fig. 3. We found that the expression of CD62p, CD40 decreased in group M compared with Con group, $P < 0.01$, and the expression of CD40 decreased significantly in group FS compared with Con group, and $P < 0.001$, which is statistically significant. There was no statistical difference in other indexes between the Con groups and M group, $P > 0.05$.

The expression of these six proteins was detected by Western blot. As shown in Fig. 4, the protein expression of CD31 decreased in group M compared with that of Con group, $P < 0.05$, and the protein expression of CD40 decreased, $P < 0.01$, which was statistically significant. There was no significant difference in other indexes between the two groups.

CD40 fluorescence staining of vascular endothelial cells

Given that CD40 mRNA and protein expression was significantly lower in M group compared with Con group, immunofluorescence staining was performed again compared with CD40 expression in those three groups, and the results are shown in Fig. 5, Base magnification: $\times 200$

Discussion

After the application of high viscosity bone cement in clinical practice, the application of this operation is accompanied by the complication of bone cement implantation syndrome (BCIS) [7, 8], with the incidence rate as high as 28%. One of the causes of BCIS is the embolism caused by bone cement implantation, including pulmonary embolism and lower limb venous embolism [9]. However, whether the physical and chemical properties of bone cement itself can cause local thrombosis that results in lower limb venous thrombosis, pulmonary embolism has not been reported.

We simulated the local operating environment of surgery and studied whether the material of bone cement could form thrombosis around it with a quantitative measurement index. Since bone cement is dispersed in the animal body after the establishment of animal models of hip and knee replacement, and it is very difficult to collect tissue samples around bone cement quantitatively, we simulated the microenvironment related to bone cement in surgery. In joint replacement, the surrounding environment in contact with bone cement is the arteriovenous and small blood vessels around the joint cut off during the operation, the small blood vessels in the medullary cavity and the blood circulating in the blood vessels. Main reasons of thrombosis include slow endovascular blood stream, blood high condensation state, platelet activation, endothelial cell injury or abnormal expression of thrombosis related regulatory proteins. High viscosity of bone cement can affect the blood vessels and blood compositions flowing through it. We established New Zealand rabbit carotid artery vein extracorporeal shunt, secondly, implanted silk-bone cement strip to simulate the microenvironment in which blood flowing through the surface of bone cement, so that it could contact the inner wall of blood vessels to simulate its influence on tiny blood vessels. Through this model we can quantitatively observe whether physical and chemical properties of high viscosity bone cement can cause the formation of local thrombosis.

Our group have developed a new kind of material by mixing PMMA with ES, and explored the best ratio [6] of 8000 axaiu in ES: 40 g PMMA finished product, this kind of state of bone cement on the basis which releases heparin sodium to anticoagulated when meets the biomechanical strength.

The properties and lower release volume of bone cement can theoretically reduce the formation of local thrombosis without causing massive bleeding. We verified whether it could reduce the formation of local thrombosis. In the animal model, we found that thrombosis was attached around three high viscosity bone cement strips in the Con group. We recorded the amount of thrombosis, and the result was $0.00732 \pm 0.00089\text{g/cm}$. The amount of thrombus in the experimental group was $0.00554 \pm 0.00077\text{g/cm}$. Although the experimental group cause thrombosis, the number of thrombosis was less than that of ordinary high viscosity bone cement, and the difference was statistically significant.

Thrombosis is mainly caused by blood hypercoagulability, platelet activation, injury of vascular endothelial cells or abnormal expression of clot-related proteins [10–14]. The cause of local thrombosis caused by bone cement is that bone cement directly contacts with blood components or acts on vascular endothelial tissue. In this study, the vascular tissues which is in contact with bone cement were selected to compare the influence of ES-PMMA and PMMA on the expression of thrombosis related proteins in vascular endothelial cells, and illustrated the difference of the influence of these two bone cement on

thrombosis by measuring the expression of these proteins. Among many proteins expressed by endothelial cells, endothelin [15], vascular endothelial cell adhesion molecule [16], CD62p [17, 18], CD31 [19], thermoregulatory protein [20, 21], and CD40 [22, 23] are closely related to thrombosis. We detected the differential expression of endothelial cells and thrombus related proteins by RT-qPCR and Western Blot, and found that the expression of CD40 in M group was significantly lower than that in Con group, which was statistically significant. Immunofluorescence method detected that the fluorescence value of CD40 in M group was lower than that in Con group. In addition, relevant studies have proved that CD40 in endothelial cells has a regulatory effect on thrombosis [24]. CD40 is one of the molecules in the tumor necrosis factor receptor superfamily. The effect of enoxaparin sodium is mainly played by anticoagulant factor α [25], which belongs to a serine protease [26], and serine protease exists in digestive enzyme and complement system [27, 28]. The complement system is composed of complement inherent components, complement regulatory proteins and complement receptors [29]. Some proteins in complement receptors are expressed in vascular endothelial cells [30], while CD40 is expressed in B cells, mononuclear macrophages, dendritic cells and vascular endothelial cells [31]. Therefore, we found that ES-PMMA bone cement reduce local thrombosis by regulating vascular endothelial CD40 protein expression.

Conclusion

In summary, using a simple and reproducible animal model, we proved that PMMA produce local thrombosis, and ES-PMMA can reduce local thrombosis compared with PMMA. Our study elucidated that ES-PMMA, a new kind of material, can reduce local thrombosis compared with PMMA by regulating the low expression of CD40 protein on endothelial cells of vascular tissue.

Abbreviations

PMMA
Polymethylmethacrylate
ES
Enoxaparin sodium
SEM
Scanning electron microscopy

Declarations

Acknowledgements

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics declarations

Ethics approval and consent to participate

All experiments were performed in strict accordance with the guidelines for care and use of laboratory animals and were approved by the medical ethics committee of the

Third Medical College of Hebei Medical University, ethics acceptance No. z2021-007-2.

The study was carried out in compliance with the ARRIVE guidelines.

Authors' contributions

Study conception and design: Sang, Qi. Acquisition of data: Sang, Hao, Ding. Analysis and interpretation of data: Sang, Sun. Drafting of manuscript: Sang, Shen. Critical revision: Fu & Qi. All authors read and approved the final manuscript.

Funding

This study was supported by the Government Funded the Training of Outstanding Talents in Clinical Medicine and Basic Research Project.

Competing interests

This material has not been published and is not under consideration elsewhere. The authors declare that they have no competing interests. All authors have read and contributed to the submitted manuscript, and there is no conflict of interest among the authors.

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Figures

Figure 1

a: enoxaparin sodium (ES) and high viscosity bone cement powder samples; b: Extracorporeal shunt and prepared silk thread bone cement strip; c: Spare arteriovenous vessels of exposed New Zealand rabbits; d: Established animal model; e: Take blood vessel samples in contact with bone cement strips; f: Intravascular bone cement thrombus samples.

a

b

Figure 2

a is the PMMA, which shows that there are a lot of gaps between the bone cement particles, b is the ES-PMMA, which shows that the surface of the bone cement

particles is covered with syrup-like substances, which we use as enoxaparin attachment.

Figure 3

mRNA expression of CD31,CD40,CD62p,CD106, Endothelin, Thrombomodulin, and showing decreased CD62p,CD40 expression in M group compared with Con group, P <0.01, significantly decreased CD40mRNA expression in FS group compared with Con group, and P <0.001.

Figure 4

Expression level of CD31,CD40,CD62p,CD106, Endothelin, Thrombomodulin proteins, decreased expression level of CD31 and CD40 in M group compared with Con group(p <0.05), and significantly decreased expression level of CD40 in FS group compared with Con group(p <0.01).

Figure 5

Compared with Con group, the fluorescence brightness of vascular endo-thelial cell CD40 was significantly lower in M group and FS group.

Supplementary Files

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