COMPARATIVE ASSESSMENT OF THE METHODS OF CHEMICAL STERILISATION OF DEMINERALISED BONE MATRIX

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Aims

This work was aimed at finding components of preserving solutions that are most optimal for the preservation of demineralised bone matrix (DBM).

Materials and methods

The osteoinductive properties of DBM sterilised by various chemicals (chlorhexidine, catamine AB, antiseptic complex, and performic acid) were studied. Experiments were conducted on sexually mature rats aged 3–4 months. Thigh bones were removed from the animals, cleared of soft tissues, sterilised, and decalcified. The resulting DBM was freed from the bone marrow and washed with running water for 2–2.5 hours to pH 7.2, and then sterilised by various chemical agents. The sterilised DBM was implanted intramuscularly in the anterior thigh surface of recipient rats aged 3–4 months.

Results

Demineralised bone matrix sterilised with chlorhexidine and catamine AB has no osteoinductive activity, therefore these substances in the studied dosages cannot be used for its preparation. Antiseptic complex (polymyxin, furazolidone, sorbic acid) and performic acid reduce the osteoinductive properties of DBM in the sterilisation process, and antiseptic complex reduces them to a greater extent. The most suitable chemical agent for DBM sterilisation is performic acid.

Conclusions

Allogeneic unsterilised DBM of rats has pronounced osteoinductive properties. Various methods of chemical DBM sterilisation usually reduce the osteoinductive properties of the graft. Performic acid has the least ability to reduce the osteoinductive properties of DBM and can be recommended for sterilisation of demineralised bone matrix.

Keywords: preservation, storage, preparation, transplantation of organs and tissues, biological graft.

INTRODUCTION

Wide implementation of osteoplastic surgeries in clinical practice is aimed to obtain such grafts that would exert a constant osteogenesis effect in a relatively short time. There is an acute necessity in the development of better methods of preservation of graft tissues for transplantation, especially those that have a locomotor or mechanically framework structure. The search for an inductive basic structure brought researchers to the organic part of the bone – bone matrix [1, 2]. Long-term studies successfully finished with the discovery of the inducing osteogenesis factor revealed in decalcified bone matrix (DBM), that is, bone morphogenetic protein. It was known long before its discovery that this factor was thermolabile and degraded after the demineralization of bones with nitric, trichloroacetic, chromic, osmic acids, alkalis, UV rays, and gamma irradiation. The high osteoinductive activity of the demineralized matrix was also confirmed by other researchers. DBM is more immune inert than allograft bone [3]. Experimental transfer of DBM for bone defect repair showed its practical equivalence to autografts [4]. Application of DBM in clinical practice for repairing the defects of the skull and maxillofacial bones, different injuries and diseases of the locomotor apparatus allowed the specialists to recommend it for the implementation in practice. However, the issues of mass preparation of DBM are still acute, especially, when it comes to the development of methods of sterilization of demineralized bones. The application of physical methods of sterilization of DBM is excluded because they destruct the osteoinductive factor. For this reason, chemical sterilization is the only possible option. The methods of chemical sterilization should be tested thoroughly because, under the influence of certain chemical substances, the osteoinductive activity of DBM can be also affected [5].

The choice of chemical sterilization of DBM that would preserve its properties is a complicated task. Some studies

showed that a 0.5% solution of formalin for the sterilization and conservation of DBM slowed down the osteogenesis [6]. Other authors proposed to sterilize DBM with an antiseptic complex that included kanamycin, sulphosalicylic acid, honey, and formalin [7]. The authors presented clinical results of the application of DBM sterilized with this antiseptic complex but they did not report its influence on the osteoinductive properties of DBM [7, 8]. Significant experimental material showed that nonsterile DBM treated with silver salts at a concentration of 10^{-4} M became sterile and antiseptic and preserved these properties without a reduction of osteoinductive properties.

The study was aimed to compare several methods of chemical sterilization of the demineralized bone matrix.

MATERIALS AND METHODS

The present study focused on the osteoinductive properties of DBM sterilized with different chemical substances (chlorhexidine, catamine AB, antiseptic complex, performic acid) for the choice of an optimum method of sterilization. The tests were performed on mature rats aged 3-4 months. After the euthanasia of animals, femoral bones were removed, cleaned of soft tissues and placed into a 2.4% solution of hydrochloric acid for decalcification. The flask with the solution and grafts was placed on the mixer in the refrigerator (the time of sterilization was 24 hours, the time of cleansing in the saline solution was 30 minutes). Decalcification lasted for 45 minutes at +2 – +4°C. The process of decalcification was controlled with X-ray imaging and Kossa histochemical reaction. The obtained DBM was cleaned from the bone marrow and cleansed with running water for 2-2.5 hours to pH = 7.2. Further, it was sterilized with different chemical agents:

- 0.75% solution of chlorhexidine bigluconate;
- 0.5% saline solution of catamine AB (time of sterilization 24 hours, then cleansing in a sterile physiological solution for 30 minutes);
- antiseptic complex (ASC): 200,000 units of polymyxin, 0.05 g of furazolidone and 0.2 g of sorbic acid per 100 ml of saline solution (time of exposure – 24 hours);
- performic acid (time of sterilization – 30 minutes).

In all the cases, sterilization was performed at +2 – +4 °C.

The transfer of sterilized DBM to 3-4-month-old recipient rats was performed intramuscularly from the anterior surface of the thigh.

Totally, 4 series of tests were conducted. The authors transferred DBM sterilized with chlorhexidine, catamine AB, antiseptic complex, and performic acid. In the control series, DBM prepared in sterile condition was transferred (K). Each series included 20 operated animals. In 30, 60, 90 and 120 days after the surgery, 4-5 rats from each series were euthanized. On these days, X-ray was performed and processes of osteoinduction were studied by histological methods (van Gieson and hematoxylin-eosin staining).

Experiments on animals were conducted according to the "European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes" (Strasburg, March 18, 1986), ETS No. 123 and the Decree of the Ministry of the Russian Federation No. 199H "Good Laboratory Practice" dated April 1, 2016.

RESULTS

The study results showed that after the sterilization by the studied methods, DBM was 100% sterile. In the control series, the histological study revealed the areas of absorption of DBM on Day 30. Along the edge of the formed cavities, osteoblast chains and newly formed bone were seen. The cavities primarily contained myeloid bone marrow. In 60 days, the areas of absorptions and the volume of induced bone increased. Medullary cavities contained myeloid and fatty bone marrow. By the 3rd month, the process of resorption of DBM continued increasing and areas of newly formed bone tissue calcified. In 120 days, DBM was fully absorbed and substituted with a newly formed bone. On Day 30, X-ray images revealed a weak shadow of the graft with distinct boundaries. On Day 60, the intensity of shadow increased and its structure was inconsistent. In 90-120 days, the graft shadow acquired a consistent structure with distinct boundaries. However, the shadow intensity was weaker than in the normal bone of the recipient.

Series I and II of the experiment had the same morphological and radiological data. The formation of new bone tissue was not observed at any stage. X-ray images of DBM did not have shadows within the post-implantation period. On Day 30, histological tests in Series III revealed osteocyte-free DBM with areas of absorption and calcification. By Day 60, the processes of resorption and calcification of DBM enhanced. Osteogenesis was observed in a few cases and in a low volume. In 90-120 days, the absorption of DBM and the volume of calcificates continued increasing. Some areas of osteogenesis were observed in the specimens obtained after a 3-month observation. In 120 days, there was no newly formed bone tissue and the number of calcificates was insignificant. In this series of the experiment, the X-ray images revealed the shadow of the graft with indistinct boundaries in 60 days. Its intensity increased by Day 120 after the surgery. In Series IV, on Day 30, DBM grafts started to have foci of absorption. Activation of cellular elements, like fibroblasts and osteoblasts, was observed along Haversian canals and lines of adhesion that had some ingrown vessels. There were separate areas of induced bone tissue. By Day 60, the absorption of DBM enhanced. Stripes of newly formed bone tissue, calcificates, along the edges of the formed cavities started appearing. Myeloid bone marrow appeared in the formed cavities. In 90 days, the absorption of DBM was more expressed, the processes of induction of bone tissue corresponded to the degree of expression of 2-month observation.

The formed cavities contained myeloid and fatty marrow. The samples obtained after a 4-month observation had more expressed absorption and focal calcificates along the edges of the formed cavities. Some areas contained induced osteogenic tissue. X-ray images taken 30 days after the surgery did not show the graft. By Day 60, a weak shadow of the graft with indistinct boundaries and uneven structure appeared. In 90-120 days, the intensity of the shadow increased, the boundaries of the graft were distinct but did not reach the intensity of the bone in the recipient (Table 1).

It should be noted that non-sterilized DBM (control test series) underwent intensive resorption and provided the osteoinductive effect after the ectopic implantation into the muscle. A different picture was observed in the post-implantation period in the matrix that underwent chemical sterilization. Thus, DBM sterilized with chlorhexidine and catamine AB did not exert the osteoinductive effect. Probably, these substances fully inactivate bone morphogenetic protein responsible for the induction of bone tissue. DBM sterilized with an antiseptic complex was characterized with active resorption. However, osteogenesis was observed only 2 months after the surgery, only in some cases and in minor volume. Calcification was intensively increasing during the period of observation. DBM implants sterilized with performic acid exerted osteoinductive effect during the period of observation (1 month). By Day 60, myeloid bone marrow started to appear in the formed cavities.

In 90 days, the appearance of fatty bone marrow was observed. The volume of the induced tissue was larger than in the previous test series but lower than in the control group. Calcificates were also detected during all the periods of observation.

CONCLUSIONS

The results of the present study showed that allogenous non-sterilized DBM in rats had osteoinductive properties. Connective tissue elements that penetrate the graft during the process of resorption differentiate for ontogenesis. DBM sterilized with performic acid preserved the osteoinductive properties but they were reduced. DBM that was sterilized with an antiseptic complex was characterized by an even lower degree of osteoinduction. When it comes to the technique of the surgery, it should be noted that precise apposition of the donor and recipient bone parts is required because a larger area of contact and the firmness of fixation of the graft provide the quick formation of osteotylus and the restoration of blood circulation in the graft, which significantly influences the transformation of the donor bone. It was shown that DBM sterilized with chlorhexidine and catamine AB did not exert osteoinductive activity, so these substances in the studied dose could not be used for its sterilization. The antiseptic complex and performic acid reduce the osteoinductive properties of DBM. The antiseptic complex had the highest effect on the osteoinductive properties of DBM. The most suitable chemical substance for the sterilization of DBM was performic acid.

Table 1

Transfer of DBM sterilized with chlorhexidine, catamine AB, antiseptic complex and performic acid

Test series	Methods	Days			
		30 days	60 days	90 days	120 days
Series I (chlorhexidine)	Histology	Regeneration of bone tissue was not observed	Regeneration of bone tissue was not observed	Regeneration of bone tissue was not observed	Regeneration of bone tissue was not observed
	Osteoinduction	Was not observed	Was not observed	Was not observed	Was not observed
	X-ray	No DBM shadow	No DBM shadow	No DBM shadow	No DBM shadow
Series II (catamine AB)	Histology	Osteocyte-free DBM with areas of absorption and calcification were observed	Processes of resorption and calcification in DBM were enhancing	Absorption of DBM was enhancing, the volume of calcificates was increasing	Absorption of DBM was enhancing, the volume of calcificates was increasing
	Osteoinduction	Low volume	Low volume	Few areas of osteogenesis	Regeneration of bone tissue was not observed, the number of calcificates was significant
	X-ray	No DBM shadow	Graft shadow with indistinct boundaries	Graft shadow with indistinct contours	The intensity of shadow was increasing
Series III (antiseptic complex)	Histology	Osteocyte-free DBM with areas of absorption and calcification were observed	Processes of resorption and calcification in DBM were enhancing	Absorption of DBM was enhancing, the volume of calcificates was increasing	Absorption of DBM was enhancing
	Osteoinduction	Osteogenesis was observed not in all cases and in low volume	Osteogenesis was observed not in all cases and in low volume	Few areas of osteogenesis	Regeneration of bone tissue was not observed and the number of calcificates was significant
	X-ray	No DBM shadow	Graft shadow with indistinct boundaries	Graft shadow with indistinct boundaries	The intensity of shadow was increasing
Series IV (performic acid)	Histology	DBM grafts had foci of absorption along Haversian canals and lines of adhesion. Activation of cellular elements like fibroblasts and osteoblasts with ingrown vessels	Absorption of DBM enhanced, stripes of newly regenerated bones and calcificates were observed along the formed cavities	Absorption of DBM was more expressed, processes of induction of bone tissue corresponded to the degree of expression after 2-month observation	More expressed absorption was observed. Focal calcificates were seen along the borders of the formed cavities
	Osteoinduction	Separate areas of the induced bone tissue	Myeloid bone marrow in the formed cavities	Myeloid and fat bone marrow was observed in the formed cavities	Induced osteogenic tissue was observed in separate areas
	X-ray	The graft was not visualized	Weak shadow of the graft with indistinct boundaries and inconsistent structure	The intensity of shadows increased, graft boundaries were distinct but did not reach the shadow intensity of the bone recipient	The intensity of shadows increased, graft boundaries were distinct but did not reach the intensity of the bone recipient

Any surgery that involves preserved homological tissues can be associated with serious complications. Thus, thorough control for the preparation and sterilization should be performed. Besides, a search for new improved methods of graft preservation, surgical techniques, and solution of tactical, technical, biological, and immunobiological problems associated with the transfer of homografts is needed. During the post-operative period, patients should be provided with treatment, care, and constant observation by surgeons and other specialists.

The methods of conservation of organs and tissues are important in transplantology and can be considered adjuvant therapy and a stage of transplantation. The prepared grafts are more viable and resistant to infection when fixed properly. They retain better and faster without significant transformation.

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CONFLICTS OF INTEREST
The authors declare no conflict of interest

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