Liver Tissue Decellularization as a Promising Porous Scaffold Processing Technology for Tissue Engineering and Regenerative Medicine

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© **M.M. Bobrova,** Clinical Research Assistant;
L.A. Safonova, Clinical Research Assistant;
O.I. Agapova, Researcher, Bionanotechnology Laboratory;
M.E. Krasheninnikov, PhD, Senior Researcher, Cellular Technology Laboratory;
M.Yu. Shagidulin, MD, PhD, Head of the Department of Experimental Transplantology and Artificial Organs;
I.I. Agapov, DSc, Professor, Head of Bionanotechnology Laboratory

Academician V.I. Shumakov Federal Research Center of Transplantology and Artificial Organs,
Ministry of Health of the Russian Federation, 1 Stchukinskaya St., Moscow, 123182, Russian Federation

The aim of the investigation was to study mechanical and biological properties of decellularized liver tissue when used as a porous matrix in regenerative medicine.

Materials and Methods. Three groups of liver samples were prepared by decellularization using a perfusion solution with different concentrations of Triton X-100. The vascular network was visualized by perfusion of 0.5% blue dextran solution. We used histological tissue staining, optical microscopy and scanning electron microscopy. Human hepatocarcinoma cell line Hep-G_2 was used to assess the proliferative cell activity on the obtained matrix.

Results. Decellularized rat liver was prepared by perfusion of sodium phosphate buffer via the portal vein, the buffer containing the following detergents: Triton X-100 of different concentrations and sodium dodecyl sulfate. Decellularization of whole organ does not lead to changes in the specific structure of the tissue scaffold, the vascular network also does not damaged. Decellularized liver with 3% Triton X-100 solution has the highest tensile strength and elasticity. Microparticles with a mean size 200 μm were prepared from decellularized liver matrix. There was investigated cell compatibility for hepatoblastoma cell line Hep-G_2. The cell compatibility was significantly higher on microparticles from decellularized liver scaffold with 3% Triton X-100 solution.

Conclusion. Decellullarization-produced liver matrix was found to preserve the native three-dimensional structure of liver tissue and vascular network. Decellularized matrix is biocompatible. It maintains the adhesion and proliferation of human hepatocarcinoma cell line Hep-G_2 and has mechanical properties appropriate for surgery.

Key words: decellularization; decellularized liver; extracellular matrix; cell microcarriers.

Liver transplantation is the only radical method to treat severe hepatic failure, though currently the number of such surgeries is limited due to acute shortage of available donor organs. Waiting time and high cost of conventional liver transplantation cause the necessity for searching an alternative, thrifty and effective strategy of liver transplantation [1]. Hepatocyte transplantation is one of modern therapy methods. However, despite optimistic and encouraging experimental and clinical results, there is still a great deal of problems to be solved, among these are the development of immune response when using allo- and xenogenic cells, and immunosuppressant need, as well as the development of techniques to encapsulate isolated hepatocytes, or treat them with enzymes to extend their operation time in body [2, 3].

Decellularization of tissues and organs is a promising technique to generate matrix materials for tissue engineering and regenerative medicine. The technique will help processing an extracellular hepatic matrix with

For contacts: Bobrova Maria Mikhailovna, e-mail: mariabobrova.msu@gmail.com

vessels that can be used to prepare liver grafts [4]. The advantage of decellularization is the retention of liver matrix structure, the organ vasculature, liver capsule, and bile ducts. Recellularized liver matrix supports specific biological hepatic functions including albumin and urea secretion, and provides mechanical properties typical for a native liver. Such liver grafts can be implanted in a donor organ providing its functional activity with minimal ischemic damage [5].

The aim of the investigation was to study mechanical and biological properties of decellularized liver tissue when used as a porous matrix in regenerative medicine.

Methods

Liver decellularization. The experiments were carried out on male Wistar rats (250–350 g). All Animal Care Procedures were carried out in accordance with the protocols approved by Committee on Animal Care and Usage of Ministry of Health Care and Social Development of the Russian Federation, Order No.755 dated 12.08.1977, and Declaration of World Health Organization (Helsinki, 2000).

An hour before surgery the rats were injected with heparin, 200 µl, to reduce thrombosis, and 30 min later they were given an angioprotector, Trental, 200 µl, as, and before the operation the rats were anesthetized with Thiopental, 200 µl for general parenteral anesthesia. All drugs were given intraperitoneally. The portal vein was cannulated, the liver being removed from a rat's body. The liver was first perfused by sodium phosphate buffer, 200 ml, at a rate of 150 ml/h to free the organ from blood using a peristaltic pump. Three types of liver samples were prepared: for liver decellularization we used three perfusion types with lysing reagents: group 1 — perfusion solution contained sodium phosphate buffer, 500 ml, 0.1% sodium dodecyl sulfate, 1% Triton X-100; group 2 — sequential perfusion using two solutions containing sodium phosphate buffer, 500 ml, 0.1% sodium dodecyl sulfate, with the difference that the first solution was added 1% Triton X-100, while the second — 2% Triton X-100; group 3 — sequential perfusion using three solutions containing sodium phosphate buffer, 500 ml, 0.1% sodium dodecyl sulfate, with the difference that the first solution was added 1% Triton X-100, the second — 2% Triton X-100, and the third — 3% Triton X-100. The perfusion rate was 150 ml/h. Detergents in solutions were eliminated from the liver using sodium phosphate buffer.

Matrix vasculature analysis. Decellularized liver was stained to visualize the vascular network by perfusion of 0.5% blue dextran solution, its molecular weight being 2 MDa, at the rate of 150 ml/h.

Histological study of liver matrix. We prepared cryosections of liver matrix, 14 µm thick using cryotome Leica CM 1900 UV (Leica Microsystems GmbH, Germany). For this purpose matrix in formalin was kept in a fridge at –70°C for 1 h followed by hematoxylin and eosin staining to reveal nucleated cells. Hematoxylin was applied on a sample for 10 min and water washed, after that eosin was applied for 1 min and washed by water. The samples were dehydrated by incubation in two portions of 96% ethanol for 2 s followed by final dehydration by their incubation in two portions of 100% xylene for 5 s. The obtained stained samples were embedded in Canada turpentine.

Liver matrix structure was studied using an optical microscope Carl Zeiss Axiovert 25 (Jena, Germany) with a camera Axio Cam HRC (Carl Zeiss, Germany).

The analysis of liver matrix mechanical properties. The properties of a decellularized liver were analyzed using a tension testing machine Zwick/Roell BZ 2.5/TNIS (Zwick GmbH & Co. KG, Germany). Samples 5 cm×5 mm in diameter were prepared. The thickness of the samples was measured and taken into account in calculation. The samples were put in clips of testing machine. The preloading was 0.05 H. Further, the samples were tested by tension at the rate of 50 mm/min. We measured the values of two parameters: tensile strength (MPa) and elasticity or elongation in percentage of an initial sample length. The obtained findings of strength-elongation curves were statistically processed using a program TestXpert (Zwick GmbH & Co. KG, Germany).

Preparation of microparticles from decellularized liver matrix. To prepare microparticles we sheared a decellularized liver by surgical scissors, put into a test tube and brought the volume up to 15 ml by adding a solution of 15% glycerol in sodium phosphate buffer, pH=7.4, and incubated for 20 min followed by centrifugation for 10 min at 8,500 g. The precipitate was pestled in liquid nitrogen by a precooled pestle and mortar for 5 min. The result particles were put into a clean and precooled test tube to bring their volume up to 25 ml by 70% ethanol with stirring, and filtered through the double gauze to get rid of large coarse tissue particles. The resultant suspension was centrifuged for 10 min at 450 g. Supernatant was removed, and the precipitate was added 70% ethanol, 25 ml, and centrifuged for 10 min at 450 g, the supernatant being removed. Further, the precipitate, 10 ml resulted from centrifugation was added 70% ethanol, 10 ml, resuspended, centrifuged for 5 min at 450 g, followed by supernatant removal, the precipitate was again added 10 ml of 70% ethanol. The procedure was repeated twice. The precipitate was resuspended, the result suspension containing microparticles of decellularized liver matrix, their mean size being 200 µm. Fractionation of particles was controlled visually by an optical microscope.

Analysis of liver matrix microparticle structure by scanning electron microscopy. Decellularized liver samples for scanning electron microscopy were fixed in 2.5% glutaric aldehyde in sodium phosphate buffer for 2 h in the dark at room temperature. Then fixed samples were washed 5 times within 5 min in sodium phosphate buffer followed by dehydration in high-proof ethanols, and 30-minute incubation in every ethanol with increasing

concentration. After that the samples were transferred into acetone. The samples were dried in a critical point dryer HCP-2 (Hitachi Ltd., Japan). Dried samples were covered by a gold layer, 20 nm thick, inside the argon atmosphere at ion current 6 mA and pressure 0.1 mm Hg using IonCoaterIB-3 (Eiko Engineering, Japan), and analyzed by a scanning election microscope Camscan S2 (Cambridge Instruments, Great Britain), its resolution being 10 nm, and operating voltage 20 kV. Software MicroCapture (SMA, Russia) was used for image acquisition.

Proliferative activity analysis of the cells on decellularized matrix. The experiment was carried out in 2 ml test tubes. To study proliferative activity we used three different substrates: 1) particles prepared from group 1 decellularized liver matrix samples; 2) particles prepared from group 3 decellularized liver matrix samples; 3) cytodex-3 was used as a positive control. Each test tube was added 50 µl of carriers, DMEM, 100 µl, HEPES, 20 mmol/ml, 10% fetal bovine serum and gentamicin, 40 µg/ml. Incubation medium contained $5.3 \cdot 10^4$ cells of human hepatocarcinoma cell line Hep-G_2. The cells were incubated in an incubator at 37°C in 5% CO_2 with stirring for 9 days.

Proliferative activity was assessed by MTT assay on experiment day 3, 6 and 9. Each tube was added 50 µl of a fivefold solution of 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT), which is a substrate for mitochondrial dehydrogenases functioning in living cells, and incubated in an incubator at 37°C in 5% CO_2 for 4 h resulted in the formation of insoluble deep blue formazan crystals [6]. The tubes were centrifuged for 15 min at 885 g. Supernatant was removed, formazan precipitate being dissolved in dimethyl sulfoxide. A stained solution was transferred in a 96-well plate. Optical density was recorded at wavelength 540 nm by Picon (Picon Incorporated Company, Germany; Uniplan, Russia).

Data processing. The findings were statistically processed by ANOVA, p=0.05 being taken as a significant point.

Results and Discussion. Decellularization is a technique based on the removal of cells and most components of the main histocompatibility complex from tissue of the whole organ due to perfusion with detergent solutions [7]. We prepared a rat liver free from hepatocytes due to the perfusion by increasing concentration detergents (Figure 1). Triton X-100 concentrations for liver decellularization were decided upon according to the previously reported data [8–10], and in our work under a single study for the first time we compared three different concentrations of Triton X-100 in a washing buffer to prepare a decellularized sample. To assess the preserved vascular network functioning we stained decellularized liver matrix by intravenous injection of blue dextran (See Figure 1). All concentrations of Triton X-100 used were found to preserve vasculature.

The integrity of organ vascular architecture is one of key advantages of the technique, since the underlying problem of all grafts is its adequate vascularization. That is why decellularization can be considered a promising technique to solve the problem of transplant vascularization support. One more advantage of the

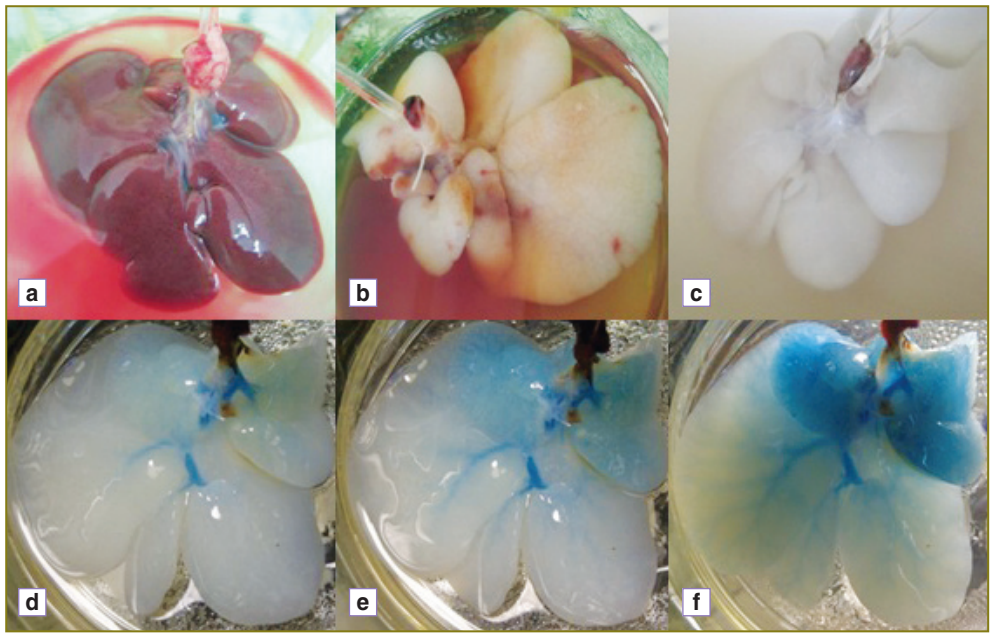

Figure 1. Decellularized liver: (a) liver perfused by sodium phosphate buffer to become free of blood; (b) liver perfusion by a lysing reagent (group 1 sample); (c) liver wash free from detergents; (d) the liver 6 min after starting staining; (e) the liver 7 min after starting staining; (f) the liver 9 min after starting staining

method is possible recellularization of liver matrix by adult hepatocytes and progenitor cells via *in vitro* culture perfusion after decellularization with preserved structural and functional features of native capillary network [11].

For liver matrix structure assessment we studied cryosamples after hematoxylin and eosin staining. The analysis showed after perfusion the samples to preserve their three-dimensional architecture and natural structural membrane matrix. Histology revealed neither nuclear nor cytoplasmic staining in decellularized matrix (Figure 2). Removing cells and their components from organ makes it possible to avoid an immune response when using products from decellularized matrix. Thus, decellularization enables to fabricate bioengineering structures exhibiting minimum immunogenicity. Decellularized matrix provides the necessary microenvironment for cells, which has an effect on their morphology, differentiation and proliferation [12], and, therefore, when populated by cells the matrix is likely to provide the necessary environment to recover the organ functional activity, since its native structure has been completely preserved. There were found no differences in the structure of matrices decellularized by solutions with different concentrations of Triton X-100, so no findings of group 1 and 2 samples were presented.

Mechanical properties of extracellular matrix such as tensile strength and elasticity are significant biophysical parameters, since they can influence cell morphology, their proliferation and differentiation [13]. In addition, mechanical properties of the samples are key indicators of product feasibility in surgery. Therefore, we studied the alteration of mechanical properties of liver matrix depending on the decellularization technique and the concentration of detergents. To record the indices of material tensile strength (MPa) and elasticity (elongation percentage) the samples were tested on a tensile machine Zwick/Roell BZ 2.5/TNIS. The Table demonstrates the findings of the experiment.

Thus, group 3 decellularized liver samples appeared to be the most strong and elastic. According to literature [15], extracellular liver matrix comprises the following components: structural proteins (collagen, elastin), adhesive proteins (fibronectin, laminin, tenastin), glycose aminoglycans, proteoglycans, glycoproteins. Due to different water solubility of extracellular matrix components in a washing buffer, the qualitative and quantitative composition of the matrix can change after organ decellularization resulting in the alteration of matrix physical properties.

Microparticles, 200 μm in mean size, were fabricated from decellularized liver matrix. Suspension of such particles can be used as a hepatocyte carrier to recover the liver by injections. To reveal three-dimensional structure of microcarriers we studied microparticle of decellularized liver samples by a scanning electron microscope (Figure 3).

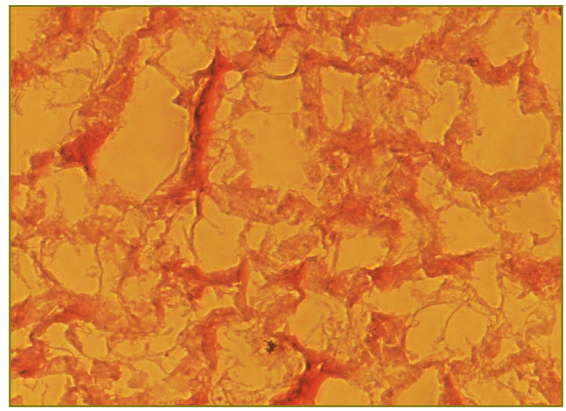

Figure 2. Extracellular liver matrix (group 3 sample); hematoxylin and eosin staining. Specific structure of liver matrix is preserved, histology revealed no nuclei (×200, phase contrast)

Tensile strength and elasticity indices of different liver matrix samples (standard deviation values for 5 independent measurements are shown)

Samples	Strength (MPa)	Elasticity (%)
Decellularized liver, group 1	0.16±0.03	64.25±4.65
Decellularized liver, group 2	0.18±0.06	55.33±10.0
Decellularized liver, group 3	1.11±0.04	77.50±5.79
Native liver	0.27 [14]	—

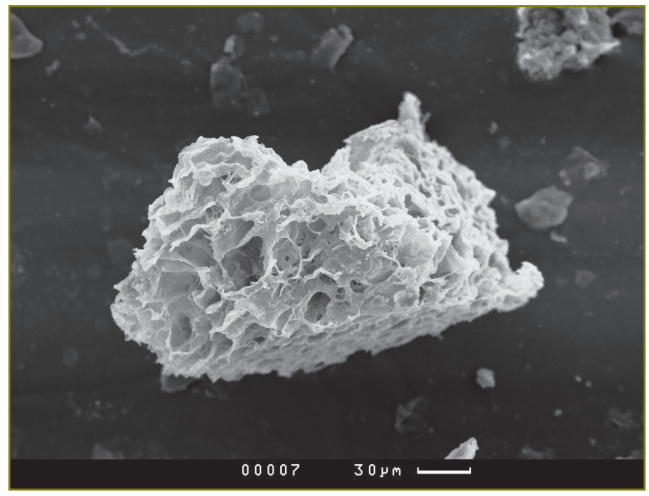

Figure 3. Scanning electron microscopic imaging of decellularized liver matrix microparticle (3 group sample)

According to scanning electron microscopy findings, the matrix is characterized by a ramified system of pours, their mean size being 20 μm. Such porous structure is optimal for hepatocyte adhesion [16]. Thus, liver decellularization results in the preservation of porous matrix structure that makes it possible for cells to penetrate and migrate deep into matrix contributing to the reconstruction of three-dimensional structure of native liver tissue.

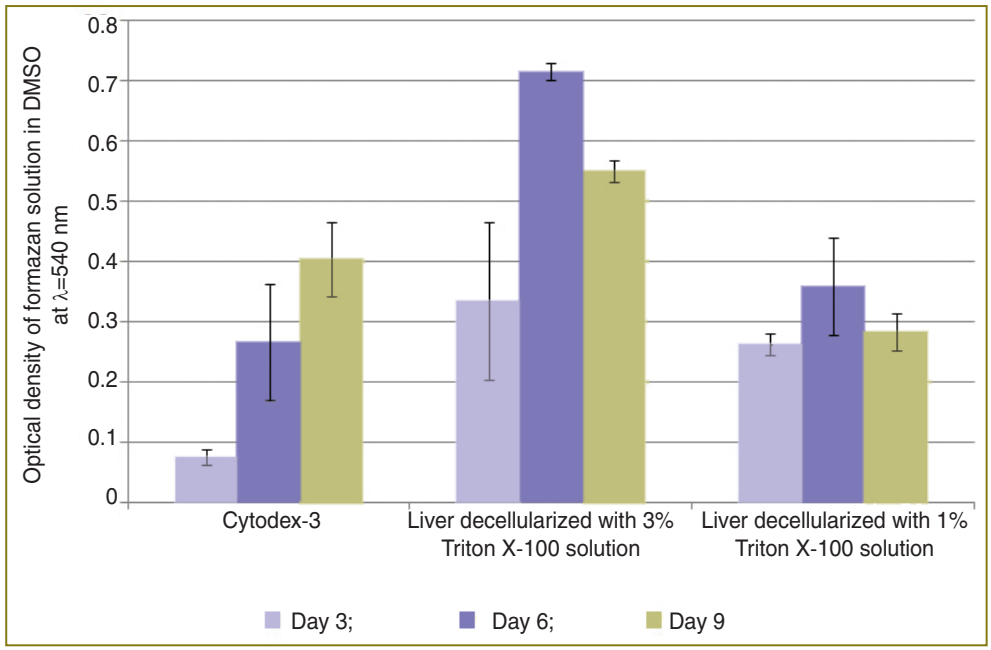

Figure 4. Data on proliferative activity of Hep-G_2 cells on different carriers on experimental day 3, 6 and 9 (standard deviation values for 5 independent measurements are shown); DMSO: dimethyl sulfoxide

Cell proliferative activity was assessed by the example of human hepatocarcinoma cell line Hep-G_2. Cytodex-3, a commercial microcarrier, was used as a positive control, it consisting of microspheres, their average diameter being 175 μm, covered by denatured collagen. 3 group decellularized liver matrix particles were found to have the highest proliferative activity (Figure 4). Higher proliferative activity may be due to qualitative and quantitative alteration in matrix composition, since extracellular matrix components have different solubility in a detergent solution. When Triton X-100 with this concentration is perfused, for cells there can be accessible previously screened sites to bind to receptors promoting hepatocyte adhesion, RGD-sequences on fibronectin and collagen [17]. Moreover, 3% Triton X-100 perfusion can result in the release of growth factors related to extracellular matrix components, e.g., hepatocyte growth factor in native conditions related to perlican [18].

Such micromatrices can be used as cell carriers for hepatocyte transplantation, or as the material for injection therapy, since the architecture of extracellular matrix and the composition of extracellular matrix proteins are supposed to be preserved that enables to create native environment for hepatocytes.

Organ decellularization is a promising technique of regenerative medicine in modeling both full-size bioengineering constructions such as an organ transplant, and in some other fields: 1) engineering of specific microcarriers for cells; 2) development of hydrogels from lyophilized decellularized tissue by its pepsin treatment followed by dissolving in sodium phosphate buffer; 3) fabrication of coverings from the solution of intercellular matrix components resulted from enzymatic degradation of lyophilized decellularized tissue [19].

Microcarriers can be used in transplantation as auxiliary material in dry form or in the form of suspension for injections as a minimal intervention. For example, microparticles from decellularized skin matrix are used for skin regeneration [20].

Traditionally used hepatocyte transplantation has practical limits, mainly due to the difficulties related to obtaining sufficient amounts of functioning hepatocytes necessary for therapeutic efficacy [21]. Moreover, primary hepatocytes easily lose viability and their functions in both *in vitro* culture, and also after transplantation [22]. One of the methods to solve these problems is hepatocytes cultured on hydrogel from extracellular liver matrix or the covering of artificial carrier surfaces by liver matrix components for further hepatocyte culturing and transplantation [23, 24]. Extracellular matrix components as a part of a product create microenvironment favorable to hepatocytes, and similar to that of hepatocytes in native tissue. Such functional carriers are developed for liver tissue engineering, cell-based therapy and transplantation. Moreover, coverings made from decellularized liver matrix and a matrix-based hydrogel provide more effective adhesion and proliferation of hepatocytes compared to classical matrices, e.g., commercial collagen and Matrigel [25].

Conclusion. The study of biological and mechanical properties of decellularized matrix of Wistar rat liver derived by detergent perfusion of liver via the portal vein aimed at the assessment of further application of

the matrix in regenerative medicine showed that three-dimensional structure of liver matrix and vasculature are preserved after decellularization. Histological study revealed neither cell nuclei nor cytoplasmic staining in decellularized liver matrix. When human hepatocarcinoma Hep-G_2 cells were cultured on decellularized liver matrix, their proliferative activity was significantly higher than that on cytodex, an artificial microcarrier. The best indices of all the parameters under study were recorded in the decellularized liver samples resulted from sequential washing by solutions containing Triton X-100 with concentrations 1, 2 and 3%.

In the course of the research work we selected the optimal parameters of decellularized liver of Wistar rats. The prepared matrix has properties close to the matrix of native liver tissue that enables to solve the problems related to the recovery of liver structure and function. Moreover, decellularization can be applied to prepare both the whole decellularized matrix and also microparticles, which can be used by noninvasive treatment techniques and support liver functions. Decellularized liver matrix and microparticles derived from the matrix can be considered unique constructions with native liver tissue structure that makes it possible to use them in regenerative medicine and tissue engineering.

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Conflicts of Interest. The authors have no conflicts of interest to declare.

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