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# PRODUCTION OF ORGAN EXTRACELLULAR MATRIX USING A FREEZE-THAW CYCLE EMPLOYING EXTRACELLULAR CRYOPROTECTANTS

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## Abstract

Biologic scaffold materials composed of the extracellular matrix (ECM) are typically derived by processes that involve decellularization of tissues or organs. All decellularization methods result in the ECM architecture disruption and a potential loss of surface structure and composition. Freeze-thaw processing effectively lyses cells and permits to diminish amounts of chemical lysing agents henceforth. However, it also causes certain disruptions of the ECM ultrastructure. In order to diminish these adverse effects we suggested using extracellular cryoprotectants (namely 5% trehalose) to preserve the ECM molecular network without impeding the cell lysis. The original optimization of the perfusion-mediated decellularization method to comprise the single freeze-thaw processing cycle and subsequent perfusion with chemical agents' solution is presented here.

**Keywords:** Tissue Engineering, Extracellular Matrix, Decellularization, Freeze-Thaw Cycle, Extracellular Cryoprotectants, Trehalose.

## INTRODUCTION

Parenchymal organ engineering through repopulation of extracellular matrix (ECM) – including xenogenous or cadaveric ones – with recipient's own stem cells may provide the solution to the problem of graft histocompatibility and a deficit of donor organs in clinical transplantology (2).

Three-dimensional scaffolds control cell functions and promote the formation of new tissues and organs (1). The scaffolds provide initial support to seeded cells, localize the cells in the proper spaces, provide physical and biological cues for adhesion, migration, proliferation and differentiation (5), and assemble the propagated cells and secreted substances into functional tissues and organs. The scaffolds are gradually being replaced by cells' own products during the regeneration. Therefore they should be biocompatible, biodegradable and provide appropriate signals for the seeded cells to coordinate the process. (3).

Moreover, ECM modulate signal transduction by binding of extracellular signaling molecules such as growth factors, cytokines, etc. to cell-surface receptors that trigger events inside the cell (2).

The ECM is composed of an interlocking mesh of fibrous proteins, glycoproteins, proteoglycans and glycosaminoglycans. The molecular composition and 3D microstructure of ECM is specific for every tissue (4).

Tissue scaffolds are obtained by the selective removal of cells whilst preserving the

intercellular substance. Cells are removed by the disruption of intercellular junctions, cellular, nuclear and organoid membranes, which results in the exposure, lysis and subsequent washing out of the cellular content. ECM obtaining methods are numerous and diverse, including mechanical (shaking, ultrasound destruction, grinding) physical (high hydrostatic pressure, a freeze-thaw cycle), chemical (hyper-, hypotonic, acid and alkaline solutions, detergents, organic solvents, chelating agents), enzymatic (proteases, lipases and nucleases), etc. To extend the decellularization degree several different methods are used simultaneously. The main flaw of all the above methods is that they tend to damage the intercellular substance as well (though to a lesser extent), which results in adverse effects on the composition, biological activity, and biomechanical property of remaining ECM. Recently a number of methods for preparing the whole parenchimatose organ ECM have been reported, which combine a complete removal of cellular materials for decreased immunogenicity while also minimizing tissue disruption to retain the native matrix structure with the maximum maintenance of the mechanical properties (1, 3, 10).

The goal of this research was to determine whether extracellular cryoprotectants (prior to intercellular ones) can have a beneficial effect on the microstructure and composition of the extracellular matrix during decellularization.

The freeze-thaw processing effectively lyses cells within tissues and organs and even multiple freeze-thaws do not significantly increase a loss of ECM proteins from the tissue. The freeze-thaw processing effect on the mechanical properties is minimal for loadbearing mechanically robust tissues (dermal, submucosal, ligamentous, cartilaginous etc.). Yet even a single freeze-thaw cycle does produce certain disruptions (fractures) of the ECM ultrastructure by ice crystals and should therefore be used only when such effects are acceptable in the final ECM product (7).

Tissue scaffolds derived from parenchymatose organs are delicate and the preservation of their 3D microstructure is especially important for subsequent recellularization. In order to improve the preservation of organ scaffolds during the freeze-thaw processing we decided to use extracellular cryoprotectants (ECC). Contrary to penetrating intracellular cryoprotectants, ECC are able to prevent the intracellular ice formation during freezing only in suspensions of solitary cells. In tissues, where cells adhere to ECM and each other, the osmotic effect of ECC (11) may even slightly facilitate the cell membrane rupture, so its protective effect extends basically to the intercellular substance. Among all the other ECC for this pilot research we chose trehalose because it may also stabilize ECM proteins (8, 9) under stress conditions of the decellularization process. Forestall, the very first concentration to be estimated (5% w/v) has shown quite a satisfactory quality of ECM obtained.

## MATERIALS AND METHODS

The experiments were run with strict adherence to EU Directive 86/609/EEC on Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes. The study was performed on 34 rat livers divided into 3 groups (10 animals each), and four as a control group.

***Pretreatment with cryoprotectants***

Before decellularization, prior to freezing by placing in refrigerator at -25°C overnight, ten livers from Group I were subjected to the 30 min PBS perfusion (10 ml/min at ambient temperature), ten livers from Group II were perfused with 10% v/v solution of the penetrating cryoprotectant glycerol (10 ml/min at ambient temperature) in PBS for 30 minutes, and ten livers of Group III – by 5% w/v solution of Trehalose dihydrate in PBS (10 ml/min at ambient temperature) for 30 minutes.

***Decellularization protocol***

After defrosting, livers from all three experimental groups were subjected to decellularization in a closed-loop perfusion system by perfusion with 1% w/v solution of sodium dodecyl sulfate (SDS) and 0,02% w/v EDTA in PBS for 24 hours at a flow rate of 10 ml/min at ambient temperature with triple perfusate replacement (every 8 hours). After decellularization, the resulting scaffolds were washed at the same flow rate (10 ml/min) by 1 liter of PBS in order to wash off the residual decellularizing agents, lysosomal enzymes, tissue detritus and other unwanted substances. Two livers from the control group were decellularized in this manner without the preceding freeze-thaw processing, and two other livers (the immunohistochemical control group)

were subjected to a histological examination right after the isolation.

***Histological analysis***

All tissue blocks were embedded in paraffin (HISTOMIX®)) on a Tissue processing center TPS 15 Duo (MEDITE) according to a standard program.

Embedding the tissues in paraffin blocks was conducted on Tissue Embedding System TES 99 (MEDITE). From each liver a three tissue samples were arbitrarily selected. Tissues in paraffin blocks were cut using a microtome pfm Slide 2003 (PFMMEDICAL) to the desired thickness 4 μm and affixed to glass slides.

The tissue specimens were imaged using a Leica DMR microscope at magnifications from x50 to x630. The degree of decellularization and protein retention of the scaffold materials, as well as determination of cells' remnants, tissue edema and fractures, was assessed by routine Mayer's hematoxylin-eosin (H&E; J.T. BAKER) and Van Gieson's (VG; J.T. BAKER) staining.

***Immunohistochemical analysis***

In the following steps all slides with adequate assumed ECM quality have been subjected to immunohistochemical analysis on fibronectin, laminin-β1, elastin, collagen XVIII (endostatin), and nidogen (two slides from each tissue sample). Those glycoproteins belong to a variety of substances that support essential role for both functional the structural characteristics of ECM (1) and could be crucial for potential recellularization success.

Deparaffinization, rehydration and antigen unmasking of all paraffin-embedded sections was processed by Declare® (Cell Marque™). N-Histofine® Simple Stain MAX PO (MULTI) (Nichirei Biosciences, Inc.) was used for antigen retrieval.

Immunolabeling was performed with primary rabbit polyclonal antibodies to elastin (Abcam), laminin Ab-1 (Thermo Scientific), endostatin (ABBIOTEC), nidogen (Santa Cruz Biotechnology, Inc., USA) and monoclonal rabbit antibody to fibronectin (Epitomics).

After endogenous peroxidase blocking by 3% solution of H2O2 in absolute methanol, sections were incubated with Histofine® Simple Stain MAX PO and primary antibodies.

The reaction products were detected using N-Histofine® DAB-3S kit (Nichirei Biosciences, Inc.) chromogen/substrate system.

## RESULTS

***Control group***

The control group specimens appear to be irregularly colored (Figure 1a). The histological examination reveals some irregularity of decellularization showing signs of tissue oedema, microruptures, numerous areas of poor decellularization with tissue detritus and even separate entire regions of retained hepatocytes (Figure 1b). The better decellularized areas are localized primarily near *porta hepatis* (Figure 1c).

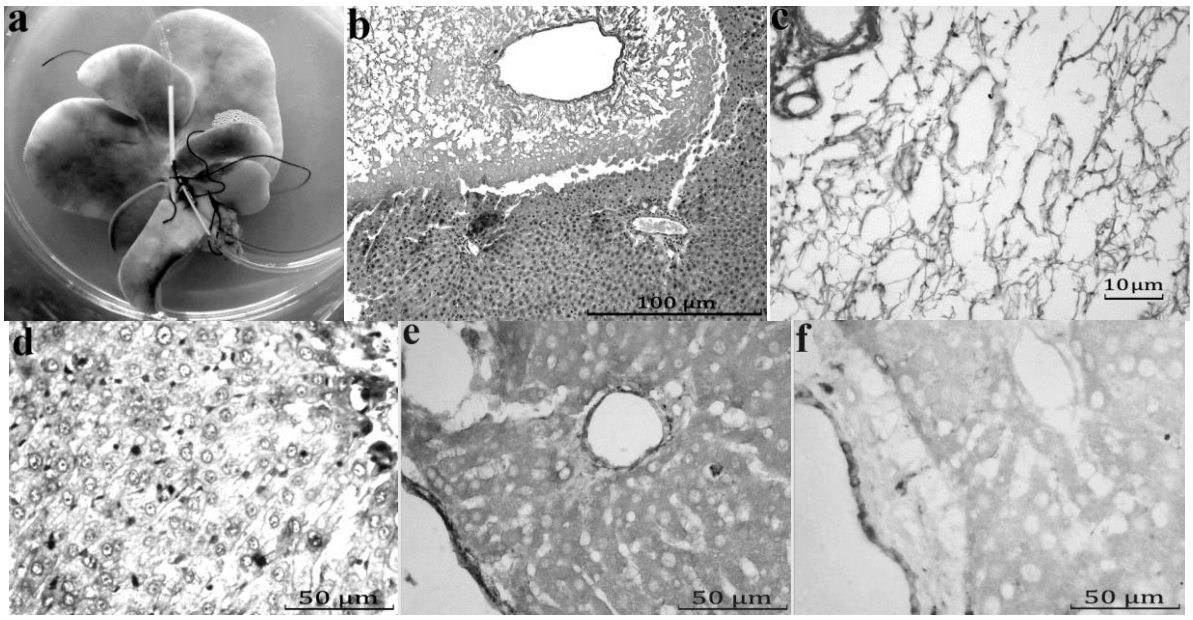


**Figure 1**. *Control group*: (a) irregular coloring (macrophotograph); (b) tissue oedema, areas of poor decellularization, tissue detritus, retained hepatocytes (H&E, x10); (c) decellularized area near *porta hepatis* (VG, x63).Immunohistochemical localization of: d) fibronectin (x40); e) elastin (x40); f) endostatin (collagen XVIII) (x40).

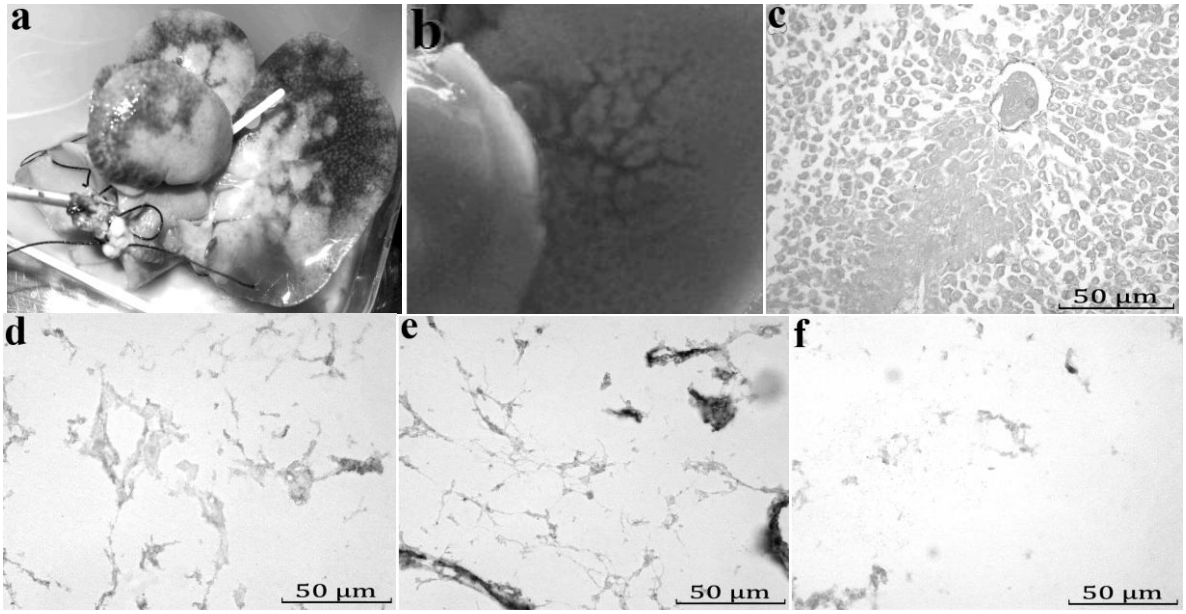


**Figure 2**. *Group I*: a) irregular coloring, impaired structure (macrophotograph); b) capsular and parenchymal ruptures (macrophotograph, close view); c) tissue oedema, poor decellularization, tissue detritus & retained hepatocytes (H&E, x40). Immunohistochemical localization of: d) fibronectin (x40); e) elastin (x40); f) endostatin (x40)

Generally, the decellularization results in the control group can be described as unsatisfactory, though the immunohistochemical evaluation demonstrates good specific staining for fibronectin (Figure 1d), elastin (Figure 1e) and collagen XVIII (Figure 1f).

***Group I (freezing without cryoprotectants)***

All Group I specimens (freezing without cryoprotectants) express reduced volume, irregular coloring, impaired structure (Figure 2a) and numerous capsular and parenchymal ruptures (Figure 2b). The histological examination reveals severe decellularization irregularity (not shown), tissue oedema, numerous areas of poor decellularization with tissue detritus and islets with retained hepatocytes (Figure 2c).

The immunohistochemical evaluation shows a good preservation of ECM glycoproteins, though the matrix fiber structure is rarefacted (Figure 2d-f).

Generally the quality of decellularization in Group I is even worse than in the control group.

***Group II (freezing with glycerol)***

Group II specimens (freezing with glycerol) show an absence of fractures, almost

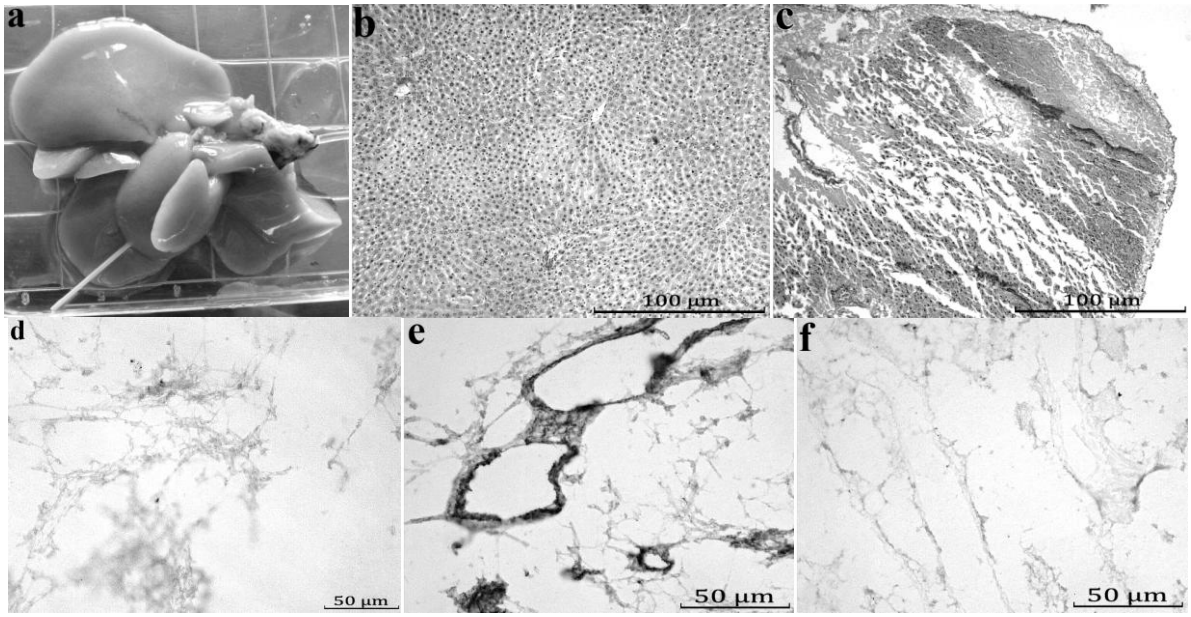


**Figure 3.** *Group II*: a) opaque light brown liver coloring (macrophotograph); b) area of almost intact liver tissue (H&E, x10); c) tissue oedema, some detritus along with retained hepatocytes (VG, x10). Immunohistochemical localization of: d) fibronectin (x40); e) elastin (x40); f) endostatin (x40)

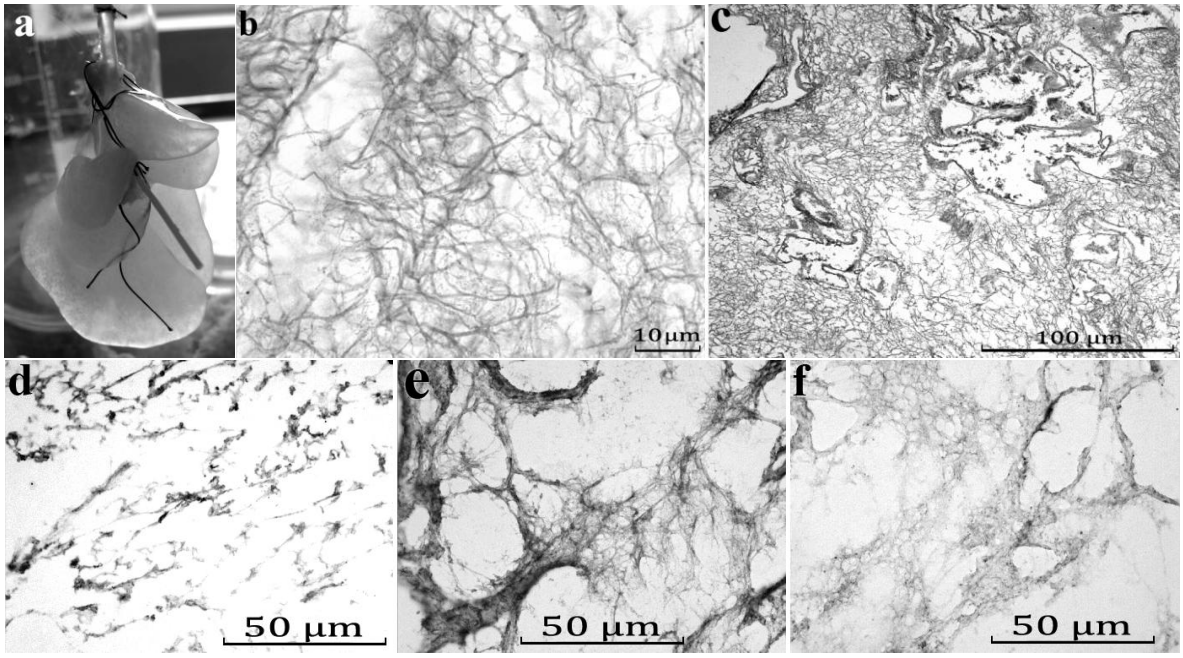


**Figure 4.** *Group III*: a) almost transparent uniform structure (macrophotograph); b) basically retained ECM structure (VG, x63); c) solitary microruptures (H&E, x10). Immunohistochemical localization of: d) fibronectin (x40); e) elastin (x40); f) endostatin (collagen XVIII) (x40).

uniform opaque light brown coloring (Figure 3a), which indicates a poor decellularization quality. The histological examination reveals the decellularization irregularity showing areas with almost intact liver tissue (Figure 3b) that interleave with zones of tissue detritus with some retained hepatocytes and tissue edema (Figure 3c). The immunohistochemical evaluation displays a certain decline in the amount of immunostained ECM glycoproteins, as well as signs of scaffold fractions and rarefaction (Figure 3d-f).

Generally, the decellularization quality in Group II can be considered to be the worst among all the series of the experiment.

***Group III (freezing with trehalose)***

Visually, all Group III specimens (freezing with trehalose) are almost transparent, have the uniform structure with a clearly visualized vascular network inside the lobes (Figure 4a). The histological examination reveals that the ECM structure has been basically retained (Figure 4b,c), with solitary microruptures (Figure 4c). There are no signs of cellular elements, tissue edema or detritus. The immunohistochemical evaluation displays levels of immunostaining of ECM glycoproteins fibronectin (Figure 4d) and endostatin (Figure 4f) comparable to those of the control group. The elastin level is insignificantly lower, however the microstructure of matrix fibers is retained.

Therefore we consider the decellularization quality in Group III as adequate.

## DISCUSSION

We suppose that the reasons for tissue detritus and retained abundance of hepatocytes in Group I specimens should be the numerous ruptures in the vascular network that prevent decellularizing solution from contacting peripheral hepatic tissues. Poor decellularization quality in Group II is apparently explained by a cryoprotective effect of glycerol (11) that penetrates into the hepatic cells and partly protects them from a cleavage during freezing and defrosting. We suggest that in this case a prolonged perfusion should be needed to obtain a better decellularization quality. To check this hypothesis, beyond the limits of the experiment, we have conducted a prolonged perfusion (36 hours) of the Group II liver and obtained the results close to those of Group III.

This indicates that pretreatment with any cryoprotectant type before the freeze-thaw processing may have some minor advantages, while the use of extracellular cryoprotectants (not necessarily trehalose – other non-penetrating solutes might be equally or maybe even more effective), that protect extracellular matrix and at the same time do not impede cells' cleavage, improves organ scaffolds preservation in decellularization protocols comprising freeze-thaw processing. Thus it widens the field of

freeze-thaw processing application not only for scaffolds from mechanically robust tissues.

Questions may arise why we purposely omitted any nucleic acids detection systems and did not pay attention to the possible residual DNA presence, the absence of which should be a mandatory part of any decellularization protocol, suitable for practical application (3, 6 10).

The reasons for this are as follows:

- Unlike conventional incubation with agitation, perfusion methods turn out to be more effective for removal of residual DNA (bearing in mind that endogenous nucleases are being released from cellular compartments during the decellularization process) (3, 6).
- No visible traces of nuclear material could be detected when applying H&E staining in Group III ((Figure 4c).
- We consider the danger of residual DNA-RNA presence to be a bit overestimated in relation to organ ECM, given the fact that they should be necessarily recellularized before transplantation.
- Fetal bovine serum (FBS), which is used in the vast majority of culture media (which are used for subsequent recellularization), contains enough nucleases to remove residual DNA and RNA at a concentration as low as 2.5% (6). Even recipients' own serum could potentially be used for this purpose in order to avoid any immunological complications. If the serum-free media are to be used, it would not be too difficult to apply some exogenous ribonuclease/deoxyribonucleases. But it is a very special case, which is irrelevant to this stage of our work.

Thus, given the fact that any kind of blood serum (except of inactivated), regardless of which detergent was employed for initial treatment, effectively removes residual DNA/RNA from the detergent-treated tissues without impacting extracellular matrix mechanics (6), we decided not to carry out the quantification of residual DNA in current study since it does not affect the results.

It is noteworthy that the impact on different ECM glycoproteins of tiny residual glycerol and trehalose amounts, remaining after predecellularizational washout, turned out to be, in our view, surprisingly dissimilar. A certain decline in the amount of immunostained glycoproteins was predictable (3, 10), so we didn't expect the immunostaining levels of fibronectin and endostatin (collagen XVIII) in Group III to be close to those of the control group.

The reason of this "result diversity" we consider the abovementioned trehalose protein stabilizing potential (8, 9). However, we have no generally accepted criteria for comparing the quality of preservation of ECM components using immunohistochemical staining, and diverse existing non-histochemical methods (3, 11-15) are cumbersome, labor-intensive and have a very little relevance to the subject of our study. So our finding is just a value judgment with no quantitative proofs, requiring further clarification.

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