

Light-emitting diode effects on combined decellularization of tracheae. A novel approach to obtain biological scaffolds¹

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ABSTRACT

PURPOSE: To obtain a decellularized tracheal scaffold associating traditional approaches with the novel light-emitting diode (LED) proposal.

METHODS: This study was performed with New Zealand adult rabbits weighing 3.0 - 4.0 kg. Different protocols (22) were used combining physical (agitation and LED irradiation), chemical (SDS and Triton X-100 detergents), and enzymatic methods (DNase and RNase).

RESULTS: Generally, the cells surrounding soft tissues were successfully removed, but none protocol removed cells from the tracheal cartilage. However, longer protocols were more effective. The cost-benefits relation of the enzymatic processes was not favorable. It was possible to find out that the cartilaginous tissue submitted to the irradiation with LED 630nm and 475 nm showed an increased number of gaps without cells, but several cells were observed to be still present.

CONCLUSION: The light-emitting diode is a promising tool for decellularization of soft tissues.

Key words: Tissue Engineering. Biocompatible Materials. Quantum Dots. Trachea. Rabbits.

Introduction

Tracheal lesions whose length is more than 50% (around 6 cm) in adults and a third of the trachea in small children require curative treatment¹⁻⁸. In many cases, endoluminal therapies followed by surgical procedures are required, resulting in high costs to healthcare systems^{4,9}. Failures in repairing such lesions often occur and there aren't any suitable tracheal replacements yet¹⁰ as these still cause lots of complications such as migration and displacement, degradation and failure in materials, chronic bacterial infection, obstruction by exuberant granulation tissue, stenosis, necrosis, anastomotic failure, hemorrhage due to erosion of blood vessels, need for immunosuppression for life, lack of donor sources, and lack of vascularization and epithelialization⁴.

The biological scaffolds from decellularized organs and tissues have been widely used in pre-clinical studies with animals and in clinical applications in humans.¹¹ There are some benefits in using these natural materials¹², such as biocompatibility and feasibility to be implanted. However it is necessary to guarantee the decellularization in order to avoid an immunologic response in the host¹³, while preserving the remaining extracellular matrix¹¹. The physical methods such as freezing, direct pressure, sonication, and agitation disrupt the cell membranes and release their content, favoring the following washing process for the removal of the cell content; nonetheless, these methods usually prove inefficient to reach a full decellularization process, so they should be combined with other methods, such as the chemical ones¹¹.

Nonionic detergents have been widely used in decellularization protocols due to their fairly mild effects on tissue structures; triton X-100 is the principal nonionic detergent used in decellularization protocols¹¹. The most commonly used ionic detergents are sodium dodecyl sulfate (SDS), sodium deoxycholate and triton X-200¹¹. Even though these detergents have been employed in such processes, it is well accepted that trace amounts of DNA and RNA may remain in the decellularized tissue¹¹. Therefore, enzymatic treatment with DNases and RNases are required, leading to high costs¹, which has encouraged us to search for alternative solutions.

In this sense, this paper focuses at the use of Light Emitting Diode (LED), a semiconductor device that emits light, as a decellularization agent¹⁴⁻¹⁶. Even though a cell is usually able to overcome small disturbances of the redox balance, larger-scale disturbances may be lethal to the cells¹⁷⁻¹⁹, such the apoptosis caused by oxidative stress resulting from LED irradiation¹⁷. Our research group has been interested in suitable scaffolds for tissue engineering for a long time^{12,20,21}. This paper describes different

techniques of cell removal from tracheae as a proposed three dimensional scaffold, by associating traditional approaches with the novel LED proposal.

Methods

Animals and housing conditions

The use of such animals was approved by the Ethics Committee on Animal Experimentation, in accordance with the ethical principles adopted by the Brazilian College of Animal Experimentation, protocol number 751. This study was performed with 22 New Zealand adult rabbits weighing 3.0 - 4.0 kg. The rabbits were maintained in a controlled ambient and received standard pellet food and water *ad libitum*.

Briefly, the animals were anesthetized with intramuscular injection of ketamine and xylazine²²⁻²⁴. After trichotomy, antisepsis and isolation by sterile fields in the anterior neck, a medium cervicotomy was performed taking into account the skin and subcutaneous cellular tissue. With the aid of retractors, the trachea was exposed from the larynx until the thorax entrance. After the transection of the trachea at 1cm from the larynx and 1cm from the thorax entrance, the retro tracheal structures were detached, and the animals were sacrificed with a lethal intravenous injection of pentobarbital.²⁵

Decellularization protocols

The 22 obtained tracheae were used for 22 different decellularization protocols. All the protocols, were submitted to 180 rpm agitation at C24 Incubator shaker (New Brunswick Scientific™) at 37°C. The mechanical agitation was used simultaneously with chemical treatments in order to aid cell lysis and the removal of cell debris¹¹. The basal solution of the protocols was 0.02% EDTA, 1% antibiotic-antimycotic in 10 ml of phosphate buffered saline solution¹³. The control tracheae were submitted to agitation in basal solutions only.

First set of decellularization protocols (traditional methods)

The first set of decellularization protocols was developed based on the findings of Elder *et al.*¹³. The tracheae used were freshly collected, and physical (agitation in basal solution), chemical (2% SDS, and 2% triton X-100) and enzymatic methods (type I DNase and RNase) were associated. No LED was employed in this set of protocols. The enzymatic solutions had 0.5 mg/mL DNase

Type I, and 50 mg/mL RNase¹³. Each protocol was performed for 8h and 24h to analyze the influence of decellularization time (Figure 1). After such treatments, the tracheae were washed for 2h in phosphate buffered saline solution under agitation at 37°C¹³, submitted to routine histological processing, stained with hematoxylin and eosin (H&E) and analyzed at a microscope (Axiostar®, Zeiss™, Germany), as described elsewhere¹².

Second set of decellularization protocols (LED based methods)

In the second set of protocols, physical methods (agitation in basal solution), chemical methods (2% SDS and 2% triton X-100) and LED were associated. The tracheae were submitted to a single nonionizing electromagnetic irradiation with LED on continuous mode with 475 nm or 630 nm wavelengths with a dose of 15 J/cm² at a total exposure of 10 minutes. Following LED exposure, tracheae were exposed to chemical action with SDS or Triton-X 100 (Figure 2). After such treatments, the tracheae were washed during 2h in phosphate buffered saline solution under agitation at 37°C¹³, prepared to the histological analysis, as described¹².

| Sample | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|------------------|-----------------|---|---|---|---|---|---|---|---|---|----|
| Physical Methods | Agitation | | | | | | | | | | |
| | 2% SDS | | | | | | | | | | |
| Chemical Methods | 2% Triton X-100 | | | | | | | | | | |
| | Dnase 1/RNase | | | | | | | | | | |
| Time | 8 hours | | | | | | | | | | |
| | 24 hours | | | | | | | | | | |

FIGURE 1 - First set of decellularization protocols. Different physical methods (agitation) and chemical methods (2% SDS and 2% triton x100) were associated, with or without an extra enzymatic method. Each protocol was performed for 8h and 24h to analyze the influence of decellularization time. Light gray boxes refer to control of reactions (1 and 2); dark gray boxes refer to the employed protocols (3 to 10). Painted boxes represent methods that were employed during these protocols.

| Sample | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|------------------|-----------------|---|---|---|---|---|---|---|---|---|----|----|----|
| Physical Methods | Agitation | | | | | | | | | | | | |
| | 2% SDS | | | | | | | | | | | | |
| Chemical Methods | 2% Triton X-100 | | | | | | | | | | | | |
| | LED 475nm LED | | | | | | | | | | | | |
| LED | 630nm LED | | | | | | | | | | | | |
| | 8 hours | | | | | | | | | | | | |
| Time | 24 hours | | | | | | | | | | | | |

FIGURE 2 - Second set of decellularization protocols. Different physical methods (agitation) and chemical methods (2% SDS and 2% triton x100) were associated, with or without a single nonionizing electromagnetic irradiation with LED. Each protocol was performed for 8h and 24h to analyze the influence of decellularization time. Light gray boxes refer to control of reactions (1, 2, 7, 8); dark gray boxes refer to the employed protocols (3 to 6 and 9 to 12). Painted boxes represent methods that were employed during these protocols.

Results

Decellularization protocols - Gross morphology

No matter the diversity of the protocols, the tracheal segments got opaque, pearl-colored, harder to the touch, and non-friable after the chemical treatments (Figure 3B). The tracheae treated with 2% SDS detergent presented a characteristic not identified in the tracheae treated with 2% triton X-100. Systematically, the tracheae treated with SDS got harder at the end of the treatments, which determined a semi-circular (C-shaped) presentation (Figure 3C), when compared to the tracheae treated with Triton-X 100 (Figure 3D).

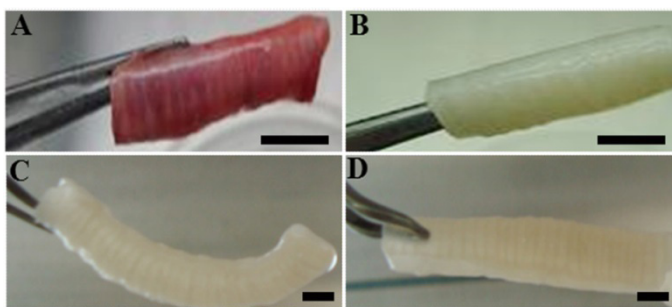


FIGURE 3 - Macroscopical characteristics of the rabbits' tracheal segment. (A) Tracheal segment after surgically removed. No decellularization protocols were employed. (B) Tracheal segment after decellularization treatments. Note that no matter the diversity of the protocols, the tracheal segments got opaque, pearl-colored, harder to the touch, and non-friable after the chemical treatments. (C) Tracheal segment treated with 2% SDS. The tracheae treated with SDS got harder at the end of the treatments, which determined a semi-circular (C-shaped) presentation. (D) Tracheal segment treated with 2% triton x100. The tracheae treated with triton detergent did not present the C-shaped characteristic identified in the tracheae treated with SDS. Scale Bar = 1cm.

First set of decellularization protocols (traditional methods) - Histology assessment

The first set of protocols aimed at identifying the contribution of the different detergents in the presence or absence of enzymes under the action of time. There was an internal disruption of the cartilage matrix, but the chondrocytes nuclei remained inside the cartilage lacunae. Not even longer treatment exposure (24 h) completely removed the nuclei inside the inner cartilage tissue (Figure 4B,D,F,H). However, the cells naturally present at the fibrous perichondrium were successfully removed (Figure 4C).

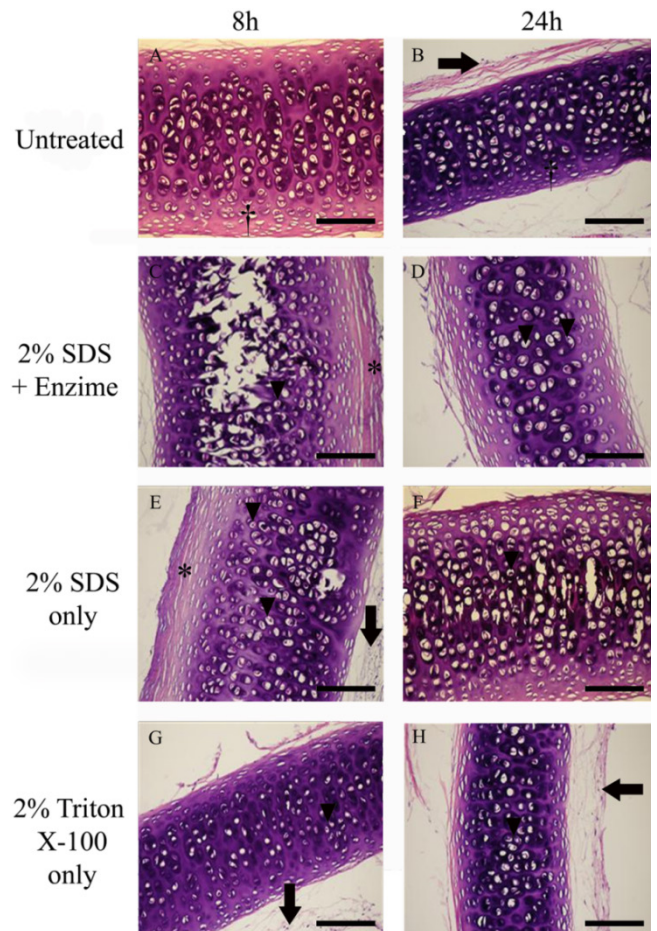


FIGURE 4 - Histological sections of the tracheal rings after the first set of decellularization protocols. The tracheas were submitted to the decellularization protocols, fixed in 10% buffered formaldehyde, embedded in Paraplast™, sectioned at a microtome and stained with H&E. All sections were analyzed at x20 zoom. (A) Untreated tracheal segment were submitted to agitation in basal solutions only during 8h. (B) Untreated tracheal segment were submitted to agitation in basal solutions only during 24h. (C) Tracheal segment treated with 2% SDS and enzyme during 8h. White spaces inside the cartilage matrix can be visualized due to the histological processing artifact. (D) Tracheal segment treated with 2% SDS and enzyme during 24 h. (E) Tracheal segment treated with 2% SDS without enzyme during 8h. (F) Tracheal segment treated with 2% SDS without enzyme during 24h. (G) Tracheal segment treated with 2% triton x100 without enzyme during 8h. (H) Tracheal segment treated with 2% triton x100 without enzyme during 24h. All scale bars = 100µm. Morphological aspects: (*) Perichondrial area after decellularization. Note that the decellularization protocols remove all nuclei in this area. (†) Cellular perichondrium of the untreated trachea. Note the normal morphological aspects of the perichondrium. (‡) Fibrous perichondrium. Note the presence of fibroblasts nuclei and well-organized and thick type I collagen fibers on the untreated trachea and remaining nuclei at the triton and SDS treated trachea. (▼) Hyaline cartilage area with the presence of remaining nuclei due to dense extracellular matrix. Note that no protocol was able to remove chondrocytes from their lacunae.

Second set of decellularization protocols (LED based methods) - Histology assessment

The cartilaginous tissue submitted to LED irradiation with 475nm and 630nm wavelength, with a dose of 15J/cm² presented several lacunae without cells, although there is remaining nuclear material in such decellularized cartilaginous tissue (Figures 5 and 6).

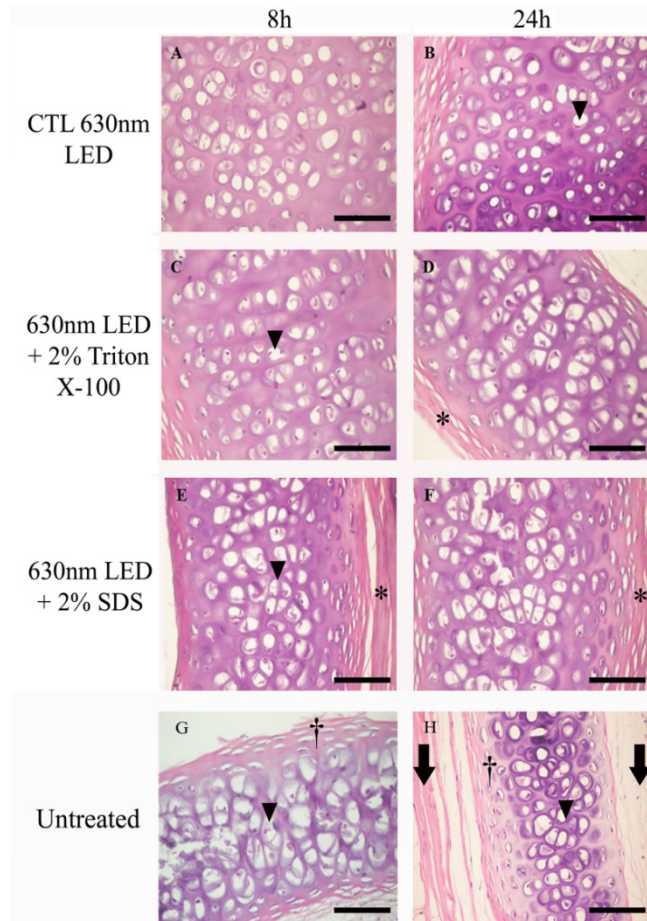


FIGURE 5 - Histological sections of the tracheal rings after the second set of decellularization protocols. The tracheas were submitted to the decellularization protocols, fixed in 10% buffered formaldehyde, embedded in Paraplast™, sectioned at a microtome and stained with H&E. All sections were analyzed at x20 zoom. (A) Tracheal segment treated with 630nm LED during 8h. (B) Tracheal segment treated with 630nm LED during 24h. (C) Tracheal segment treated with 630nm LED + 2% triton x100 during 8h. (D) Tracheal segment treated with 630nm LED + 2% triton x100 during 24h. (E) Tracheal segment treated with 630nm LED + 2% SDS during 8h. (F) Tracheal segment treated with 630nm LED + 2% SDS during 24h. (G) Untreated tracheal segment were submitted to agitation in basal solutions only during 8h. (H) Untreated tracheal segment were submitted to agitation in basal solutions only during 24h. All scale bars = 100µm. Morphological aspects: (*) Perichondrial area after decellularization. Note that the decellularization protocols remove all nuclei in this area. (†) Cellular perichondrium of the control trachea. Note the normal morphological aspects of the perichondrium. (‡) Fibrous perichondrium of the control trachea. Note the presence of fibroblasts nuclei and well-organized and thick type I collagen fibers. (▼) Hyaline cartilage area with the presence of remaining nuclei due to dense extracellular matrix. Note that no protocol was able to remove chondrocytes from their lacunae.

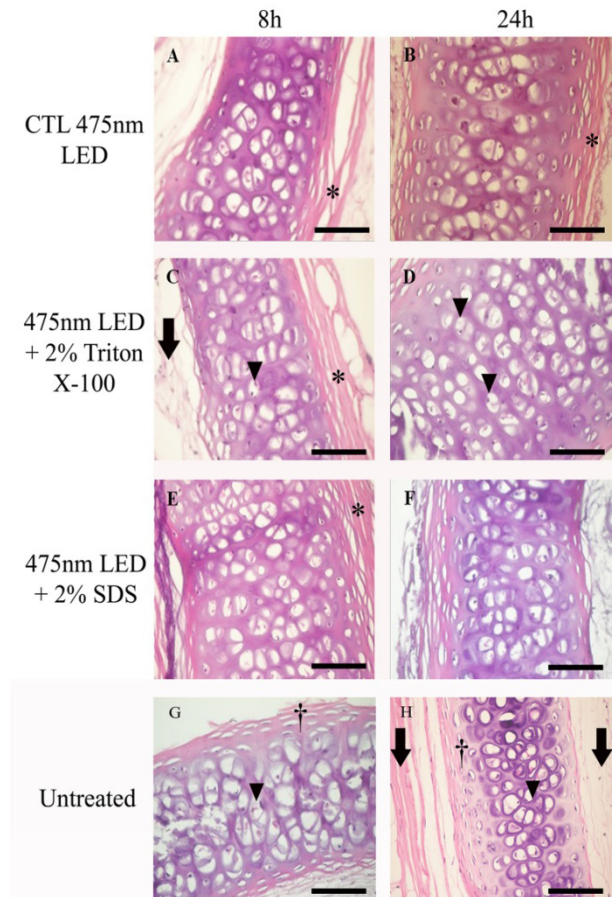


FIGURE 6 - Histological sections of the tracheal rings after the second set of decellularization protocols. The tracheas were submitted to the decellularization protocols, fixed in 10% buffered formaldehyde, embedded in Paraplast™, sectioned at a microtome and stained with H&E. All sections were analyzed at x20 zoom. (A) Tracheal segment treated with 475nm LED during 8h. (B) Tracheal segment treated with 475nm LED during 24h. (C) Tracheal segment treated with 475nm LED + 2% triton x100 during 8h. (D) Tracheal segment treated with 475nm LED + 2% triton x100 during 24h. (E) Tracheal segment treated with 475nm LED + 2% SDS during 8h. (F) Tracheal segment treated with 475nm LED + 2% SDS during 24h. (G) Untreated tracheal segment were submitted to agitation in basal solutions only during 8h. (H) Untreated tracheal segment were submitted to agitation in basal solutions only during 24h. All scale bars = 100µm. Morphological aspects: (*) Perichondrial area after decellularization. Note that the decellularization protocols remove all nuclei in this area. (†) Cellular perichondrium of the control trachea. Note the normal morphological aspects of the perichondrium. (‡) Fibrous perichondrium of the control trachea. Note the presence of fibroblasts nuclei and well-organized and thick type I collagen fibers on the untreated trachea and remaining nuclei only at the triton treated trachea. (▼) Hyaline cartilage area with the presence of remaining nuclei due to dense extracellular matrix. Note that no protocol was able to remove chondrocytes from their lacunae.

Discussion

Regarding the first set of protocols, our findings does not corroborate with Elder *et al.*¹³ when they describe the action of the SDS and Triton x100 detergents as an effective process of decellularizing articular cartilage¹³ with complete lyses of

the chondrocyte nuclear membranes and glycosaminoglycans removal; however, they applied the decellularization protocols on tissue engineered constructed cartilage that share the same remaining extracellular matrix tensile properties of a starting immature bovine cartilage (10–15% of adult tissue). Therefore, their protocols may not have the same positive results if applied to native cartilage, which have much higher glycosaminoglycans and collagen content. Moreover, it was possible to verify that using the enzymes in the employed concentrations does not contribute in the decellularization protocols. To overcome this, an increase in the amount of nucleases could cause better results¹³. Specific literature, though, indicates that high amounts of enzymes should be used in order to get satisfactory results¹.

It was previously reported that the employment of SDS causes some destruction of the cartilage tissue, due to the fact that SDS is an ionic detergent, which is efficient in the solubilization of cytoplasmatic and nuclear cell membranes, tends to denature protein by disrupting the protein-protein interactions, can remove glycosaminoglycans content, and collagen^{11,26}. The nonionic detergents, like triton x100, act more mildly on the tissue structures and have the disruption of lipid-lipid and lipid-protein interactions as a mechanism of action¹¹. They do not alter the functional conformation since the protein-protein interactions of the tissue remain intact¹¹. Triton x100 is less harmful to glycosaminoglycans when compared to the other decellularization agents^{26,27}. In our experiments, the apparent destruction of the remaining extracellular matrix seen at Figure 4 (C,E,F) are not due to the detergent exposure, but a histological processing artifact.

It was not before the early 1980s that the effects of the electromagnetic radiation in molecular and cellular levels started being investigated²⁸. Scientific papers conducted with LASER during the 1980s paved the foundations for the understanding of the molecular mechanisms concerning the effects of the interaction of light with biological tissues²⁸.

Several *in vitro* studies described the effects of Laser and low-intensity LED on the biological tissues reporting an increase in the rates of cell proliferation and synthesis of mRNA and DNA, and an increase in the activation of the mitochondrial functional through structural changes. Consequently, the ATP synthesis also increases¹⁹, and so does the variation of the intra and extracellular pH, the metabolism acceleration²⁹, and the rate of epithelial cell proliferation³⁰, which causes biophysical changes that may lead to an increased rate of exchanges in the cell membrane³¹.

More recent *in vitro* studies show that the action of low-intensity Laser can also be applied to repair and regenerate cartilages due to the biostimulation in the production of the cartilage matrix³². As well as that, they show that the interactions of the electromagnetic waves with the biological tissue cause the cells to warm up a little, including their mitochondrial membrane, leading to oxidative stress and triggering biochemical reactions that speed up the metabolism by increasing oxygen consumption³². A cell is usually able to overcome the effects of stress provided that the disturbances of the redox balance are small, restoring normal intracellular balance with positive stimulatory effects on the cells; larger-scale disturbances, however, may be lethal to the cells¹⁷⁻¹⁹. In this sense, we hypothesized that a higher dose, like the dose chosen for this research (15J/cm²), could induce cell death, and consequently to contribute to the decellularization process.

Literature shows that irradiation with 630nm +/- 20nm LED with doses lower than 10J/cm² stimulates cell proliferation^{33,34}. However, higher doses than 10J/cm² were used to induce cell death, as demonstrated by Karu.¹⁹ Some work described that the tissues irradiated with 475nm +/- 20nm LED with doses higher than 10J/cm² also causes cell damage^{33,34}. Our findings confirm such results, which makes this protocol a new biotechnological method efficient in decellularizing natural scaffolds. The presence of chondrocytes could be tolerated during the matrix remodelling and scaffold recellularization with the cells from the receptor animals³⁵.

Conclusion

The light-emitting diode is a promising tool for decellularization of soft tissues.

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