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Original Article

Use of autologous platelet-rich plasma for skin graft preservation: an experimental comparative study

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Abstract: Objective: In this experimental study, we aimed to determine whether platelet-rich plasma (PRP) is a suitable preservative for dermo-epidermal grafts. An additional objective was to investigate how long grafts can be stored without biological degradation. Methods: We compared pig skin graft preservation using PRP versus saline solution and crystalloid Custodiol®, which is used for hypothermic preservation of organs for transplantation. Grafts (10 × 10 mm) were placed on gauze impregnated with one of the tested solutions, and stored for 3, 7, 11, and 15 days at a constant temperature of 4°C. We evaluated a total of 240 pig skin samples: 120 by histopathology and 120 by fluorescence optical microscopy. Results: Overall, Custodiol® solution appeared to be the best medium for preservation of dermo-epidermal grafts, with beneficial properties manifested on days 7 and 11. Although we expected PRP to be a better preservative than saline, this was not confirmed by our results, as we found no significant difference between these two media. In fact, by day 3, the histopathological results were better with standard saline solution than with PRP. On day 15, with each tested solution, some samples showed histological changes that are incompatible with graft viability. Conclusion: Overall, Custodiol® appears to be the best medium for dermo-epidermal graft preservation. Moreover, the present findings suggest a maximum graft storage time of 11 days in all of the tested solutions. We do not recommend using grafts stored for 15 days, due to isolated signs of graft biodegradation with all solutions.

Keywords: Skin graft, preservation, platelet-rich plasma, saline, Custodiol®

Introduction

Autologous dermo-epidermal grafts are used as a basic treatment for burns and extensive skin defects. Such grafts are a valuable biological material that can be used immediately after collection or preserved under specific conditions. The most common method for short-term storage is to wrap the skin graft in saline-soaked gauze, followed by storage in a refrigerator at a constant temperature of 4°C [1, 2]. This method is economical, as saline solution is inexpensive, as well as simple and not technically challenging. A limitation of this method is that skin grafts stored in saline at 4°C should optimally be used within 10 days of collection [3, 4].

The literature describes other various methods of preserving skin grafts, including the possibility of their storage in platelet-rich plasma (PRP) [1-3]. PRP contains over 30 biogenic factors [1, 5] that affect cell proliferation and differentiation, facilitate tissue remodeling, and accelerate or improve wound healing. For medical applications, PRP is generated from autologous blood, and has an optimal platelet concentration of 2.5- to 5-times higher than the standard platelet concentration in peripheral blood [6, 7].

In the present experimental study, we aimed to determine whether PRP is a suitable preservation fluid for skin grafts, and to compare it with both the traditionally used saline solution and Custodiol® crystalloid, which is used for hypo-

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thermic preservation of organs intended for transplantation [8]. We also examined how long skin grafts can be stored before exhibiting biological degradation.

Material and methods

Experimental animal

The experimental animal was a female domestic pig (*Sus scrofa domestica*) weighing 50.5 kg. This choice was made because pig skin and human skin share very similar characteristics and histological structures [9-11], and because xenogenic dermo-epidermal grafts from pigs have historically been the most commonly used material for temporary defect dressing in extensive burns [11, 12]. The experiment was carried out in accordance with the Act for the Protection of Animals Against Cruelty (No. 246/1992 of the Collection of Laws of the Czech Republic, as amended) and ethically approved by the Subject Committee of the Faculty of Military Health of the University of Defense (registration number 292126/2021-1457).

Graft harvesting

The surgical procedure was performed with the animal placed under general anesthesia. Premedication was administered using ketamine (15 mg/kg IM; Narkamon; Zentiva, Prague, Czech Republic) azaperone (1.0 mg/kg IM; Stresnil; Janssen, Beerse, Belgium) and atropine (0.02 mg/kg IM; Hoechst-Biotika, Martin, Slovakia). Venous access was ensured by insertion of a central venous catheter via the *vena jugularis interna.* After orotracheal intubation, the animal was connected to an anesthesia machine (Cirrus-Trans; Datex-Ohmeda, GE Company, Fairfield, CT, USA) and ventilated with controlled volumetric ventilation. Total sedation was maintained using a titrated combination of intravenous midazolam (0.05-0.1 mg/kg/h; Dormicum; Roche, Prague, Czech Republic) and propofol (2-4 mg/kg/h; Diprivan; Astra Zeneca, Cheshire, United Kingdom). Perioperative analgesia was provided using metamizole (Novalgin; Aventis Pharma, Frankfurt N. M, Germany), at a continuous administration rate of 5 mg/kg/h. Volumotherapy was provided by crystalloids (Infusio Hartmanni; Medicamenta, Vysoke Myto, Czech Republic). Oxygen saturation, ECG, and $ETCO_2$ were monitored throughout the procedure.

After previous preparation of the surgical field, dermo-epidermal grafts were collected under aseptic conditions using an electrodermatome (Aesculap; Aesculap Inc., B. Braun Company, USA). Because pig skin is thicker than human skin, the grafts had a thickness of 0.5 mm [10]. Grafts were collected from the animal's back and sides, after removal of the bristles by shaving. Immediately after collection, the grafts were processed into individual samples of 10 × 10 mm, placed on gauze under sterile conditions, and then washed with 1 ml of preservative solution. A total of 240 samples were prepared in this way. Due to the large wound area, after surgery, the animal was euthanized using T61 (Intervet International B.V., Boxmeer, the Netherlands).

Preservative solutions

We compared three solutions for hypothermic preservation (at 4°C): the standard medium for storing excess skin grafts, saline (sodium chloride 0.9%; B. Braun, Melsungen AG, Melsungen, Germany); autologous PRP derived from pig blood; and the reference medium, Custodiol® (Custodiol®-CE; Dr. Franz Köhler Chemie GmbH, Bensheim, Germany), which is also referred to as Bretschneider HTK solution. Custodiol® is a preservative solution primarily intended for the perfusion and washing of donor kidneys, liver, pancreas, and heart, before and immediately after removal from the donor body.

Preparation of autologous platelet-rich plasma

PRP was prepared using a centrifuge and tube set (TropoCells™; Estar Technologies Ltd., Israel). Venous blood was collected from the animal perioperatively, in a vacuum tube with anticoagulant (adenine citrate dextrose) and separation gel (10-μm pore size). A total of 300 ml of blood was collected. From 10 ml of venous blood, we obtained 2-3 ml of PRP. After 10 minutes of centrifugation at 3600 rpm, the erythrocytes, platelet-poor plasma (PPP), and platelets were separated (**Figure 1**). PPP (3 ml) was collected, and the remaining plasma was mixed with platelets - thereby creating PRP, which was separated using a special filter tube and evenly applied to the surface of each sample using a syringe.

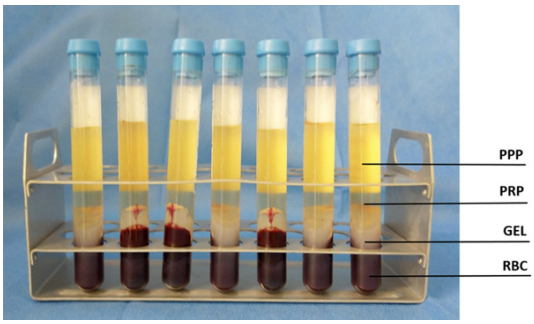

Figure 1. Tubes after centrifugation with separated layers. A centrifuge and tube set (TropoCells™, Estar Technologies Ltd., Israel) were used to prepare the PRP. PPP, platelet-poor plasma; PRP, platelet-rich plasma; GEL, separation gel; RBC, red blood cell.

Method of sample preservation

A total of 240 samples were prepared and divided into two basic groups: 120 samples were evaluated by histopathology, and 120 samples by fluorescence microscopy. From each group, and for each day (days 3, 7, 11, and 15), 30 samples were prepared: 10 with saline, 10 with PRP, and 10 with Custodiol®.

Histopathological evaluation

A total of 120 skin samples were histopathologically evaluated after storge in saline, PRP, or Custodiol® for 3, 7, 11, and 15 days. Samples were fixed in fixative solution with 10% formaldehyde for at least 24 hours. From each specimen, one transverse section was prepared, histologically stained with hematoxylin-eosin, and then microscopically examined. We evaluated changes in the epidermal layer (*stratum basale epidermis* and *stratum spinosum epidermis*) and changes in the dermis (*stratum papillare dermis*), which were scored as 0-3, with a higher number indicating more significant changes (**Table 1**).

Optical fluorescence microscopy

A total of 120 samples were evaluated by optical fluorescence microscopy. On days 3, 7, 11, and 15, the native samples were processed to a size of 5 × 5 mm. Then the samples were incubated for 30 min at 4°C in a staining solution, comprising DMEM FluoroBrite medium supplemented with 10% fetal bovine serum, antibiotics to prevent bacterial contamination, Hoechst 55542 fluorescent dye to stain all nuclei independently of the cell's condition, and ethidium homodimer to stain dead cell nuclei (**Figure 2**).

After staining, the samples were fixed on a slide and examined using an optical microscope (Spinning Disk Nikon CSU-W1). Fluorescent colors were excited using laser wavelengths of 405 nm and 561 nm. Sample were scanned along the Z-axis, in seven parallel planes, with a step of 10 µm. The resulting two-color confocal images were analyzed using a custom Python script. Subsequently, the numbers of nuclei were counted for both channels in each plane, for all locations, and for all logs and days.

Evaluation by fluorescence microscopy is considered a suitable diagnostic method for evaluating the viability of vein grafts, tissue cultures, and liver cells [13-15], and is also used in diagnostic dermatology [16-18]. However, this method was found to be sub-optimal for assessing skin tissue cell viability in our study. This may have been due to non-specific staining (colors also captured in the extracellular matrix) (**Figure 3**), lack of staining in deeper layers of tissue, or nucleus fragmentation during apoptosis. Cell nucleus fragmentation may have occurred naturally during cell deaths in our experiment, resulting in insufficient or diffuse tissue staining. This may have resulted in the omission of some dead cells from our analysis, because they did not match the parameters selected for segmentation.

We also tested a different fluorescent dye, Sytox Green, instead of ethidium homodimer, but this change did not result in improved specificity when staining dead cells. Diffuse signal areas were still observed in the sample, with color also captured in the extracellular matrix. We report these negative results here because other studies [13-15] have evaluated this method as appropriate, but our present experience with a large sample size does not support its suitability.

Statistical analysis

After excluding samples analyzed by fluorescence microscopy, the histopathological measurements were analyzed. For statistical analysis, Kruskal-Wallis (KW) analysis of variance (ANOVA) was used to assess the variability of linear statistical models, enabling the evalua-

Table 1. Scoring of histological changes in individual layers of the specimen

	0	1	2	3
Stratum basale epidermis	Normal findings	Vacuolation of keratinocyte cytoplasm	Apoptosis of keratinocytes (pyknotic nuclei, apoptotic bodies)	Complete disintegration (agitation of intercellular junctions, up to blistering)
Stratum spinosum epidermis	Normal findings	Mild hypereosinophilia	Severe hypereosinophilia (>50% of epidermal thickness)	Disorganization (disarray), maturation disorder
Stratum papillare dermis	Normal findings	Edema	Disorganization (nuclear lysis of cells)	

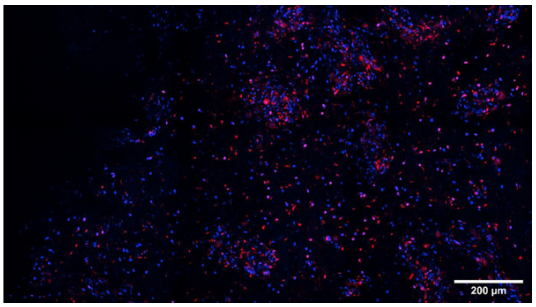

Figure 2. Skin graft sample evaluated by fluorescence optical microscopy. Hoechst 55542 fluorescent dye was used to stain all nuclei independently of the condition of the cell, and ethidium homodimer was used to stain dead cell nuclei. Viable nuclei are blue. The nuclei of dead cells are red. Bar = 200 µm.

tion of multiple factors: preservative solutions (3 levels) and days (4 levels). If the KW method demonstrated significance (P<0.05), a post-hoc test was added to determine which levels differed from each other. For this purpose, we used the non-parametric Dunn's test with Bonferroni modification of the significance level. Data were processed using NCSS 2021 software (Statistical Software 2021 NCSS, LLC, Kaysville, Utah, USA).

Results

Histopathological assessment

Various structural changes occurred over time in all 120 skin samples, with all preservative solutions. In the upper coria (dermal papillae), edema was observed in some of the samples, with some cases exhibiting disorganization and breakdown of the cellular elements. In the basal layer of the epidermis, the vast majority of samples showed degenerative changes (vacuolation of cytoplasm) or apoptotic disappearance of some keratinocytes, and occasionally even disorganization and loosening of intercellular junctions, with intraepithelial blister formation. In the upper layers of the epidermis, a fraction of samples exhibited hypereosinophilia of keratinocytes, which had conspicuously different tincture properties compared to in the basal parts. These changes were further assessed as mild (<50% thick) or extensive (>50%), and occasionally as impaired epithelial stratification.

The histopathological measurements were used for statistical analysis, and preservation solutions were compared according to the days of preservation. At three days of preservation, the samples in saline (KW 0.00246) showed better results than those preserved in PRP (P<0.01), with no significant difference from Custodiol®. On day 3, samples in all three preservative solutions exhibited only slight changes - including vacuolization of keratinocyte cytoplasm in the *stratum basale epidermis*, slight hypereosinophilia and zonation of epidermis in the *stratum spinosum epidermis*, and edema in the dermal layer - or the findings were normal, without structural changes (**Figure 4**).

On day 7, samples preserved in Custodiol® (KW 0.00079) showed the best results, significantly differing from saline and PRP (both P<0.01). Some samples showed changes on day 7, including up to apoptosis of keratinocytes in the *stratum basale epidermis*, up to severe hypereosinophilia in the *stratum spinosum epidermis*, and edema in the dermal papillae (**Figure 5**).

On day 11, samples preserved in Custodiol® (KW 0.00023) again showed the best results, significantly differing from saline (P<0.001) and PRP (P<0.05). The changes observed on day 11 were similar to those on day 7, but with greater frequency.

On day 15, we did not observe any significant differences between solutions (KW 0.919). In

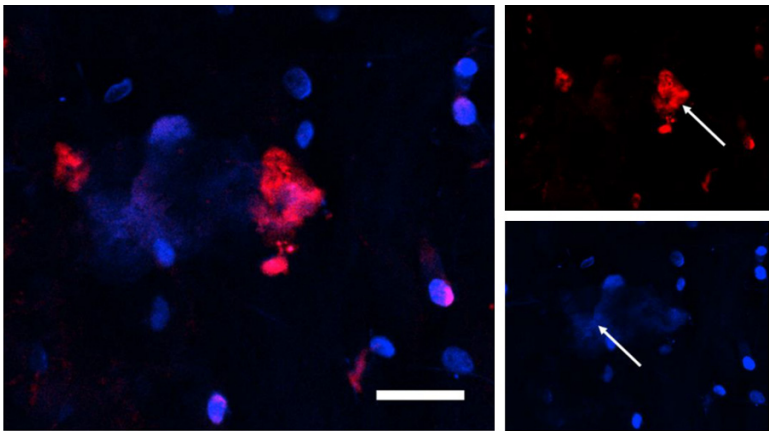

Figure 3. Skin graft sample evaluated by optical fluorescence microscopy, with indications of non-specific staining. Reasons for the non-specific staining may include that both colors (red and blue) were also captured in the extracellular matrix, insufficient staining of deeper tissue layers, or fragmentation of nuclei during apoptosis. White arrows indicate the area that exhibits both colors. Bar = 25 µm.

all tested solutions, a portion of samples exhibited severe changes, including complete disintegration (i.e., disruption of intercellular junctions by blistering) in the *stratum basale epidermis*, disorganization in the *stratum spinosum epidermis*, and decay of cell nuclei in the *stratum papillare dermis*.

Comparison of individual solutions

The obtained data are summarized and graphically represented in **Figure 6**. Overall, among the tested solutions, Custodiol® appeared to be the best medium for the preservation of dermo-epidermal grafts. The advantageous properties of Custodiol® primarily manifested on days 7 and 11. We did not observe any significant benefit of PRP compared to saline or Custodiol®. In contrast, by day 3, the standard saline solution showed better results than PRP. On day 15, some samples in all three solution groups exhibited severe histological changes that are incompatible with graft viability including samples 1 and 7 in Custodiol®; samples 2, 3, and 9 in PRP; and samples 9 and 10 in saline. These findings indicate that the type of preservative used does not affect the lifespan of the graft, and that the maximum storage time for grafts is 11 days in any of the tested solutions.

Discussion

Autologous dermo-epidermal grafts are a valuable biological material, especially for the treatment of extensive burns. As the removed tissue may not all be used immediately, it must be handled conscientiously. Skin grafts that remain unused after skin autotransplantation may be utilized later - for example, if a transplanted graft fails, or on an injured area that was not ready for transplantation at the time of harvesting [19-21].

There are multiple ways to preserve skin grafts. Skin tissue for allogeneic transplants is cryopreserved with liquid nitrogen at -80°C for long-term storage in tissue banks, with 10% dimethyl sulfoxide or 15% glycerol used as a cryoprotectant. Another method of long-term preservation is the storage of vital allogeneic grafts in concentrated glycerol. However, both of these methods have disadvantages: they require special equipment, involve a complex process, and are very expensive [12, 22, 23].

Saline is the most commonly used preservative solution for short-term storage solution of skin grafts, even though it does not seem to empirically be the ideal solution for such storage, and there are solutions with better properties. Most workplaces use the saline storage method for both economical and practical reasons. The disadvantage of storing grafts in saline is the relatively early reduction in the number of viable keratinocytes. Knapik et al. reported that keratinocyte viability decreased by up to 50% after 3 days of storage, which may affect graft attachment [4]. However, in an experimental study using rat skin, a 50% loss of keratinocyte viability did not occur until 4 weeks of storage [24]. In another *in vivo* study using full-thickness human skin grafts, a 90% loss of keratinocyte viability was reported after 10 days [25]. Overall, these studies indicate high variability in the viability of cells preserved in saline solution. The practical impact of the available data is the current recommendation that skin grafts be stored in saline for a maximum of 10 days before use. Grafts stored in this way for 10 days do not show changes affecting the overall viability of the graft [3, 4].

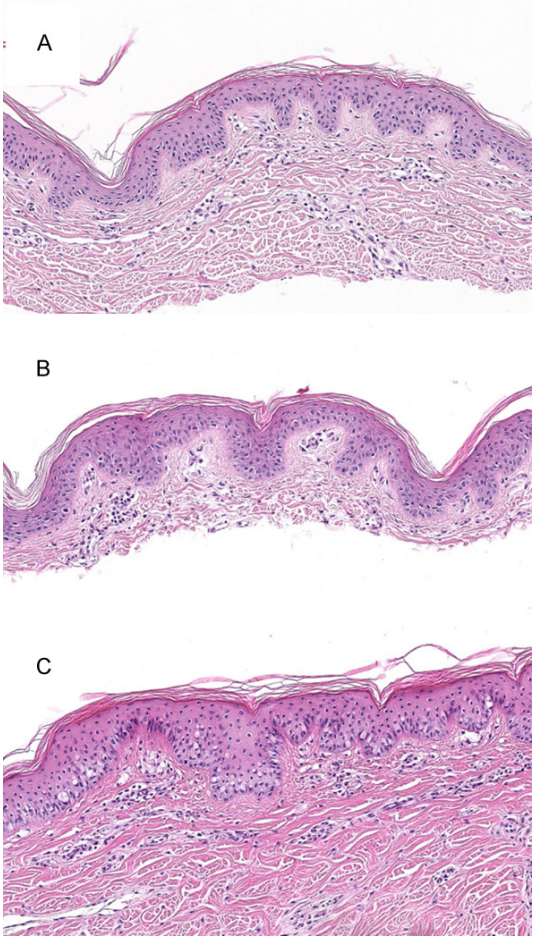

Figure 4. Skin graft samples after 3 days of preservation, evaluated by optical microscopy with hematoxylin-eosin staining, at 20× magnification. (A) Sample preserved in saline, showing discrete changes in the upper corium (focal edema of papillae) and basal layer of the epidermis (vacuolization of cytoplasm in some keratinocytes), and adequate findings in higher layers of the epidermis. (B) Sample preserved in Custodiol®, showing mild hypereosinophilia of the epidermis, and findings in the corium and basal layer similar to in (A). (C) Sample preserved in platelet-rich plasma (PRP), showing edema of dermal papillae, vacuolization of most of the keratinocytes in the basal layer, and slight hypereosinophilia of the higher layers.

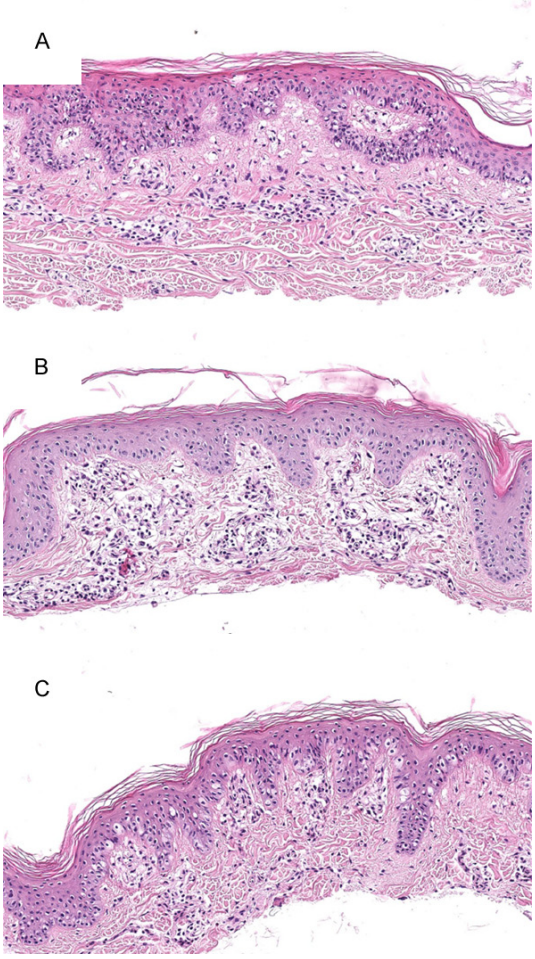

Figure 5. Skin graft samples after 7 days of preservation, evaluated by optical microscopy with hematoxylin-eosin staining, at 20× magnification. A. Sample preserved in saline, showing edema of dermal papillae, vacuolation and occasional apoptosis of basal layer keratinocytes, and extensive hypereosinophilia of the upper layers. B. Sample preserved in Custodiol®, showing dermal papillae edema and vacuolation of part of the basal layer of keratinocytes, with normal findings in the upper layers of the epidermis. C. Sample preserved in platelet-rich plasma (PRP), showing edema and focal disintegration of cellular elements of the corium, extensive degenerative changes in the basal layer (up to disruption of intercellular junctions), and slight hypereosinophilia of the upper layers.

Efforts are being made to develop the most efficient method to ensure that skin graft cells remain viable for as long as possible during storage at 4°C. To this end, several types of preservative media have been clinically tested.

In 1993, Fahmy et al. performed an *in vitro* study with human skin. They investigated Ready Mix [RM$^+$; composed of Dulbecco's Modified Eagle Medium (DMEM) and Ham's F12 media in a 3:1 ratio] and found that it performed significantly better than the other media used to preserve dermo-epidermal skin grafts, specifically Hartmann's solution, Marshall's solution, and saline. After 30 days, the average percentage of viable keratinocytes was 60%. However, RM$^+$ had the disadvantage of a high purchase price [25].

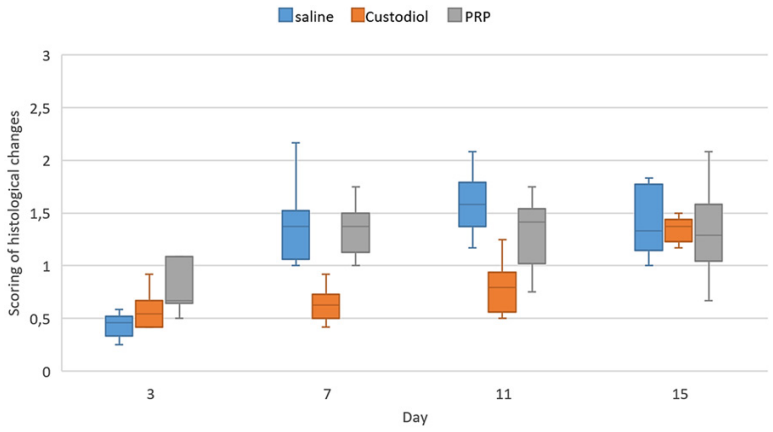

Figure 6. Graphical comparison of individual solutions and scoring of histological changes after the indicated days of preservation.

In another study, Boekema et al. compared DMEM/Ham's F12, tissue culture solution Roswell Park Memorial Institute (RPMI), and saline within a group of 15 patients. They found that RPMI showed better results and may be an alternative solution for graft preservation; however, their findings must be verified in further research [26].

Turhan-Haktanır et al. compared RPMI with saline and amniotic fluid (AF). They reported that RPMI and AF performed significantly better than saline but found no significant difference between RPMI and AF. Due to its nutrient content, growth factors, and antimicrobial properties, AF has clear potential as a suitable solution for storing skin grafts. However, its use may be limited by ethical restrictions and legal norms in some countries [27].

In another experimental study, skin tissue from rats was stored in coconut water, which may be a suitable preservative solution due to its osmolality, pH, and composition of macro- and micronutrients and trace elements. That study aimed to compare solutions that are used to preserve transplanted organs. Specifically, the authors compared coconut water with Ringer's lactate and Belzer's solution, which were used to preserve organs for autotransplantation (spleen, ovary, and skin graft) during 6 hours of storage in a refrigerator at 4°C. The results indicated that coconut water can be used as an alternative to preservative medium. However, a limitation of that study was the short preservation time of a few hours [28].

Overall, it is clear that several solutions are more suitable than saline for storing skin grafts. Although the reported graft storage times varied, all studies have shown a gradual loss of graft viability with an increasing number of storage days [25-28].

In 2000, Cetin et al. compared the useful lifetime of skin grafts stored in saline and plasma. Plasma was selected as a preservative because, as part of the internal environment, plasma contains more factors for graft nutrition than saline isotonic solution. The authors reported that after 30 days of storage, the percentage of surviving keratinocytes was higher in plasma compared to in saline and concluded that plasma is a better environment for storing skin grafts than saline. They also pointed out the possibility of transmitting congenital diseases through allogeneic plasma, and thus suggested that plasma should be autologous [29].

The same rationale can be used to support the use of PRP, which is the fraction of autologous blood having an optimal platelet concentration of 2.5- to 5-times higher than the whole blood content and containing a number of advantageous factors in its granules. Growth factors in PRP include platelet-derived growth factors (PDGFaa, PDGFbb, and PDGFab), transforming growth factor β (TGFβ1 and 2), endothelial growth factor (EGF), vascular endothelial growth factor (VEGF), and insulin-like growth factor (IGF-1). These growth factors modulate cell proliferation, differentiation, angiogenesis, and chemotaxis. Dense granules contain numerous bioactive factors - including serotonin, histamine, dopamine, calcium, and adenosine - which can increase membrane permeability and modulate the inflammatory response. All of these factors have demonstrable effects on wound healing, which support the possibility that PRP could be a suitable preservative solution and prolong the duration of short-term skin graft preservation [30-34].

Keskin et al. demonstrated that PRP influenced the storage time of skin grafts, showing a sig-

nificantly longer graft lifespan with storage in PRP than with storage in saline, especially during the first 8 days. Their PRP storage solution was obtained by mixing 2.5 ml of PRP and 2.5 ml of saline, which they tested on full-thickness skin graft samples with a diameter of 3 mm. Moreover, their samples were not placed on gauze impregnated with the solutions, but rather were placed in sterile containers with 5 ml of solution. This protocol may explain why their samples in saline were affected by maceration, since PRP has a higher viscosity [1]. In another study, Gokkaya et al. reported that the samples stored in PRP had better final appearance, when examined macroscopically, although the final storage time did not significantly differ between samples stored in PRP versus saline [3].

These previous findings led us to think that PRP might be a preferable alternative to skin graft storage in saline. However, our present findings in a relatively large sample did not confirm this expectation. We did not observe a significant difference between PRP and saline, and saline yielded better results on day 3.

In our present study, the best results were obtained using Custodiol® solution. This solution was tested because it is used in standard clinical practice in our department (University Hospital Hradec Kralove) for hypothermic preservation of transplanted organs. Custodiol® contains potassium chloride, potassium hydrogen-2-ketoglutarate, histidine, tryptophan, magnesium chloride, calcium chloride, and mannitol ions. In contrast, saline comprises only Na^+ and Cl^- ions. Custodiol® has a pH of 7.4-7.45 at 4°C and osmolality of 300 mOsm/kg, making it almost identical to the internal environment. Its higher content of ions, together with a low temperature, may be argued to create a better environment, extending the preservation time of skin grafts compared to other solutions. However, the disadvantage of Custodiol® is its purchase price, which is significantly higher than that of saline or PRP. However, in the case of PRP, we must consider the additional costs of purchasing a centrifuge and tube sets.

Despite multiple studies, including our present experiment, there is still no clearly superior option for how to best preserve skin grafts. The currently reported data should be verified in further studies, especially *in vivo*.

Conclusion

Our present histological evaluation study revealed that Custodiol® crystalloid showed the best properties for preserving porcine skin grafts, with significant differences from the other two tested media, especially on days 7 and 11 of preservation. Thus, Custodiol® can be considered an alternative solution for skin graft preservation. The disadvantage is its high purchase price compared to standard saline solution. We expected that PRP would be a better preservative than saline; however, this was not confirmed, and no significant difference was observed between PRP and saline. Moreover, our results suggest that the maximum storage time for grafts is 11 days, when using any of the solutions tested in this research.

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Disclosure of conflict of interest

None.

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