

Evaluation of the impact of decellularization and sterilization on tensile strength transgenic porcine dermal dressings

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Purpose: The aim of this paper was to evaluate which method of acellularization and sterilization is optimal, in the meaning of which processes have the least impact on the deterioration of mechanical properties of porcine tissues used for xenogeneic applications. *Methods:* The static tensile probe was conducted for 80 skin specimens obtained from transgenic swine, which are used as a wound dressing for skin recipient. Obtained data were subsequently analyzed with the use of statistical methods. *Results:* It was found that Young's modulus for the samples after the sterilization process for the dispase substance and the mixed method (SDS + trypsin) were statistically significantly changed. In the case of dispase, Young's modulus value before the sterilization process was 12.4 MPa and after the value increased to 28.0 MPa. For the mixed method (SDS + trypsin) before the sterilization process Young's modulus value was 5.6 MPa and after it was increased to 6.3 MPa. The mixed method (SDS + trypsin) had the slightest effect on changing the mechanical properties of the samples before and after the sterilization process. *Conclusions:* It was confirmed that different methods of acellularization and the process of sterilization have an influence on the change of mechanical properties of the skin of transgenic swine. In the authors' opinion, the mixed method (SDS + trypsin) should be recommended as the best one for the preparation of transgenic porcine dermal dressings because it ensures a smaller probability of dressing's damage during a surgical procedure.

Key words: skin tissue, burn wounds treatment, xenogeneic grafts, static tensile test, statistical analysis, reconstructive surgery

1. Introduction

Skin is an organ which is essential for the proper functioning of the whole organism. On one hand, it provides the body with protection against the external environment, on the other hand, it enables contact with the surroundings. The skin is highly exposed to the impact of external factors, thus its main role is a protective function. Thanks to such features as resistance, elasticity and semi-permeability, it protects the human organism from mechanical injuries, infections, the loss of physiological liquids and harmful radiation [5]. Moreover, it plays an important role in the organism's metabolism of fat and vitamins as well

as water and electrolyte balance. The skin also takes part in immunological processes, thermoregulation of the body and resorption. All these activities are disturbed as a result of burns, which cause not only changes in the scope of anatomy and physiology of the damaged organisms but also lead to endocrinological and immunological disorders. Among all kinds of wounds, burn wounds are characterized by the biggest disorders of all repair factors and, therefore, they are the most difficult to treat [19]. Nowadays, the most common way of treatment involves the application of biostatic (deprived of live cells) grafts obtained from human corpses or transgenic (genetically modified) swine. The process of preparation of biological dressings encompasses acellularization (removal of cells),

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sterilization and storage inadequate conditions. At present, xenotransplantation is becoming more and more popular giving hope in the treatment of serious and extensive skin loss in a situation when there is a lack of human skin, both autologous and allogeneic [3]. The usefulness of the application of xenogeneic grafts has been confirmed by numerous clinical investigations [2], [4], [24], [25]. Taking the above into consideration, the researchers search for optimum methods of storage and sterilization that would have the smallest impact on the decreasing mechanical properties of grafts. Before homing of the skin with the donor's cells, the skin must be properly prepared. The preparation process consists of the removal of the donor's cells with simultaneous preservation of the structure of the extracellular matrix and sterilization. There are many methods of the removal of cells (in other words, decellularization or acellularization) which most often include chemical, biological or mixed procedures [6], [8], [22].

The selection of the best method of the cell removal from tissues or organs depends on many factors, such as tissue cellularity, density, thickness or the content of lipids. The fact that should be borne in mind is that each reagent will cause modifications of the composition of the extracellular matrix and have an impact on certain disturbances of the ultrastructure. Therefore, it is advisable to attempt the minimization of possible undesirable effects [6].

Literature includes works concerning the determination of mechanical properties of the skin [1], [12], [13], [20], [21]. There are also many works dealing with the application of the determined mechanical properties of various biological structures to the process of modelling [7], [9], [10], [15], [18], [23]. However, there is a lack of literature which would confirm the investigations aiming at the determination of mechanical properties of skins subjected to the procedure of acellularization and sterilization. It seems necessary to conduct research which would define what exactly influences the changes of mechanical properties – whether it is a type of acellularization method or the application of the process of sterilization. The developed skin grafts often have weak mechanical properties, which makes them vulnerable to damage during the transplantation procedure. Also, after the transplantation, they are exposed to the action of local shear and tensile forces [14]. Taking the above into account, the aim of the study was to evaluate the methods of decellularization of dressings from transgenic pigskin by determining and comparing the mechanical properties of the examined dressings. Such comparison is aimed at determining which of the decellularization

methods will ensure the best mechanical properties of dressings for surgical procedures.

2. Materials and methods

2.1. Specimens preparation

Within the framework of this work 80 specimens obtained from transgenic swines were tested. The dermis should be harvested from the animal just right after euthanasia. All samples for the tests were taken from the ridge of the pig maintaining the same arrangement of collagen fibres to exclude their influence on the measured mechanical properties.

In the first stage, the harvesting site should be shaved with an electric shaver to remove the hair. Next, the site should be disinfected with a skin disinfectant (e.g., skinsept, octanisept) and dermatome should be set at the optimal depth to remove the entire epidermis with as little dermis as possible. After harvesting the epidermis, the target fragments of the dermis should be harvested at the same site dermatome as well (set at the maximum depth). The dermis shall be used in further procedures and, before its application in further stages of the procedure, it can be stored in water for injections as low as $-80\text{ }^{\circ}\text{C}$. They were subjected to procedures of the removal of cells from the skin. This procedure is called decellularization. In order to remove cells from the tissue of animal origin, four procedures may be applied:

1. Chemical procedure (SDS, Triton X).
2. Enzymatic procedure (Trypsin, Dispase).
3. Physical procedure (Liquid nitrogen).
4. Mixed procedure – a two-stage procedure to combine the enzymatic method (first stage) and chemical method (second stage).

Chemical procedures

In the chemical method of removing cells from pig skin, two different reagents were applied:

1. The first method applied Triton-X reagent in the concentration of 3%: in the first stage, 3% solution was prepared by dosing 30 mL of 10% Triton-X solution to be diluted in 70 mL of H_2O .
2. The other method of chemical removal of cells from pig skin applies a 0.1% solution of SDS (sodium dodecyl sulphate). In the first stage, a 1% SDS solution is prepared by dosing 1 g of the substance to be diluted in 100 mL of water for injections. After

preparing the 1% solution, it should be diluted 10-fold to obtain a 0.1% SDS solution.

Enzymatic procedure

The enzymatic procedure applied the ready-to-use concentrated reagent Triple 1x or 2.4 U/mL dispase. The prepared solutions must be filtered through 0.22 μm filters. Such a filter should be fixed on a syringe after the solution of relevant concentration has been drawn into the syringe. Then the solution must be filtered into the target container. Pigskin should be harvested with a dermatome according to the above-described protocol, resulting in dividing the skin into fragments of predefined dimensions. Each skin fragment should be washed with water mixed with detergent so that blood clots, residual tissues and other contaminants are removed. To degrease the skin, it should be washed with an alcohol solution and then skin fragments should be washed with water to remove the detergent and alcohol. In the next stage, the skin must be immersed in a container filled with the solution and left for 24 h for shaking in 4–8 °C. Afterwards, each skin fragment should be soaked in water to remove the solution. The washing should continue until the decellularisation reagent is entirely removed.

Physical procedure

The physical method requires freezing in liquid nitrogen of temperature -196 °C. Pigskin should be harvested with a battery-supplied dermatome as described above, resulting in dividing the skin into fragments of predefined dimensions. Skin fragments should be divided into equal pieces of 7×20 cm and then placed in 50 mL falcon tubes and tightly locked to obtain an anaerobic environment. Three cycles of freezing in liquid nitrogen are required with the specimens placed in liquid nitrogen for 4 hours each time. Between the cycles, incubation should be held at a temperature from 20 to 37 °C for at least 5 minutes and preferably 10 to 30 minutes. Next, the falcon tubes with the skin should be incubated as described above in ambient temperature for 48 hours.

Mixed procedure

In the first stage, cells are removed from tissues according to the paragraph *enzymatic procedure*, applying one of the enzymes – either trypsin or dispase. The next stage involves the *chemical procedure*, according to the above-described protocol applying one of the chemical reagents: either Tritin-X or SDS. The efficiency of the decellularization process for each method

should be verified by histopathologic preparation with haematoxylin-eosin staining.

Haematoxylin-eosin staining procedure

A fragment of the harvested pigskin should be bathed in a tray filled with alum haematoxylin for 7–10 minutes and then processed by bathing preparations in a tray with distilled water. Next, the preparations must be rinsed under tap water for 15 minutes and then bathed again in the tray with distilled water. After this stage, the preparations are washed in a tray filled with 1% eosin Y with one drop of acetic acid added and finally washed in the tray with distilled water again. In order to verify the degree of removing the chemical solution from the preparation, the skin specimen should be checked for the presence of chemical substances in the preparation. After the process of decellularisation, half of the samples are to be subjected to the process of radiation sterilization (35 kGy) in compliance with the valid procedures of the Tissue Bank. Sterility of the tissues should be confirmed microbiologically. The specimens must be stored at a temperature of -20 °C prior to mechanical tests, samples were thawed in saline at room temperature 21 °C for 20 minutes. The same procedure should be used before the implantation process in the operating room.

2.2. Uniaxial tensile testing

The tests were conducted by means of a testing machine MTS Insight 2. This is an electromechanical machine used for static tests and serves the purpose of low-force measurements in the range up to 2 kN. The tests were carried out on samples with a width of 20 ± 1.97 mm, length 120 mm, thickness 0.49 ± 0.05 . The width of the samples was measured by a certified calliper and the thickness of the samples was measured with a thickness gauge. All samples tested were stored for a period of 2 months from the time of collection. Next, the prepared specimens were subjected to a tensile test after the endings of the samples had been fixed by means of special grips. The ends of the sample were wound on specially prepared rolls for this type of testing, which were later placed in the holders of the testing machine. Those activities aimed to prevent the spontaneous slipping of the specimens from the clamps and to eliminate the phenomenon of concentration of stresses at the place of the sample's fixation. Prior to testing, an initial distance between the clamps was measured as well as the thickness and width of the specimens were determined. Next, the

samples were stretched in the direction of the longitudinal axis (Fig. 1) in quasi-static conditions at a velocity of 5 mm/min [1], [12]. The room in which the investigations were carried out had a constant temperature of 21 °C. The results were recorded at a frequency of 10 Hz. As a result of the carried out tests, the value of Young’s modulus was determined on the basis of the stress and deformation plot in the proportional range. The value of Young’s modulus corresponds to the tangent of the angle of inclination of the segment proportional to the axis of deformation. An exemplary graph is shown in Fig. 2. The presented graph depicts two areas of toe region and linear region, like in [16], and is very similar to the periodontal biline characteristics. In the case of the analyzed material toe region reaches about 1/3 of the total strain range, which maps the viscoelastic properties of the biological material. With regards to this range, mainly elastin fibers are responsible for deformation, while collagen fibers stand for the region analyzed by the

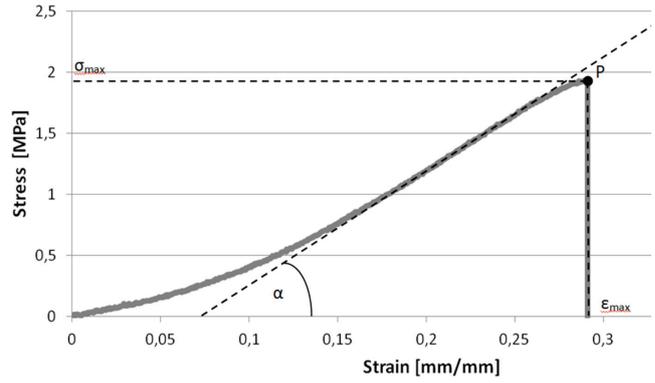


Fig. 2. An exemplary graph from static extension test

authors. It was assumed that the mechanical properties of the tested material were determined for a linear region that corresponds to the model of a linearly elastic material. The value of the maximum stress σ_{max} and strain ϵ_{max} was read at point “P” which is marked in Fig. 2.



Fig. 1. A method of fixation of a sample in the clamps of the strength testing machine

3. Results

3.1. Data obtained in experimental tests

Obtained results of mean values of stresses together with staggered deviations for samples before and after sterilization are shown in Fig. 3. The highest values of the mean stress of samples before sterilization were observed for trypsin (3.9 MPa) and for SDS (3.8 MPa). Other substances showed similar values (in the range of 2.2–2.6 MPa). The lowest values of stress were obtained in the case of the application of a mixed

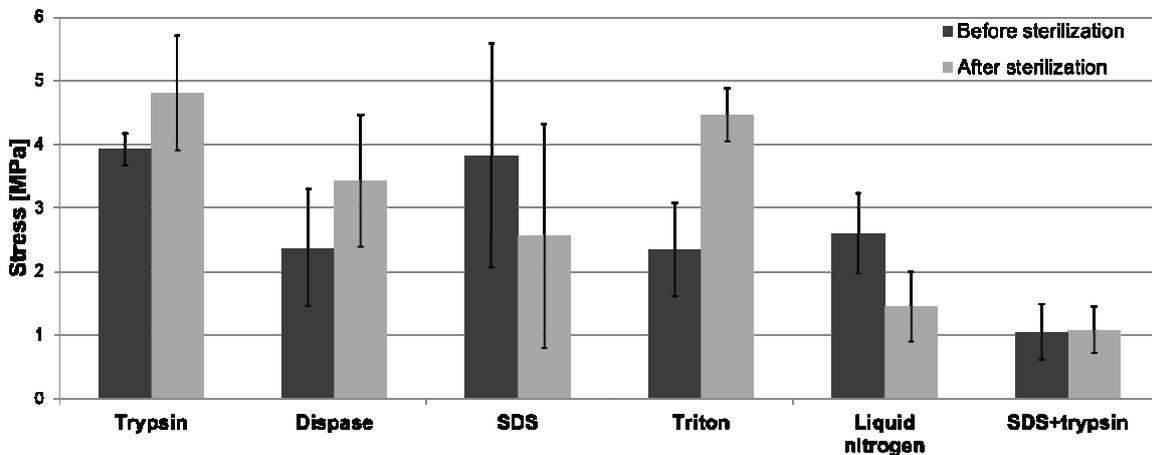


Fig. 3. Mean values of stress

method (SDS and trypsin – 1.0 MPa). Specimens subjected to the process of sterilization revealed the highest mean stress in the case of the application of trypsin (4.8 MPa) and Triton (4.5 MPa) and the lowest mean stress in the case of the mixed method (1.1 MPa) – similarly to the results obtained before sterilization. After sterilization, mean stresses rose for trypsin (by 0.9 MPa), dispase (by 1.1 MPa), Triton (by 2.1 MPa) and SDS + trypsin (only by 0.03 MPa).

Mean values of strain for samples with standard deviation before and after sterilization are presented in Fig. 4. The values of mean strain for specimens before sterilization slightly differ from one another, being still within the range from 0.17 mm/mm (trypsin) to 0.23 mm/mm (SDS and liquid nitrogen). Moreover, specimens after sterilization showed the highest mean strain in the case of trypsin (0.18 mm/mm) and the lowest in the case of dispase (0.12 mm/mm). The comparison of the results obtained for samples before and after sterilization (fig. 4) revealed higher strain

values before the application of the process of sterilization in the case of: dispase (difference 0.09 mm/mm), SDS (0.09 mm/mm), Triton (0.05 mm/mm), liquid nitrogen (0.08 mm/mm), SDS+trypsin (0.02 mm/mm). A slight increase in the strain values after sterilization took place only in the case of trypsin (0.01 mm/mm).

Before the process of sterilization, it was observed that the mixed method (SDS + trypsin) resulted in the obtainment of the lowest mean Young’s modulus (5.6 MPa), whereas the use of trypsin resulted in the highest that mean value of the modulus of longitudinal elasticity (17.3 MPa). After the procedure of sterilization, similarly to the observations made before sterilization, it was noted that mean Young’s modulus was the lowest in the case of applying the mixed method (6.3 MPa), but the highest in the case of using dispase (28.0 MPa). The process of radiation sterilization had an impact on the increase of the mean elasticity modulus in the case of dispase (by 15.7 MPa), SDS (by 1.5 MPa), Triton (15.6 MPa), SDS and trypsin (0.8 MPa) (Fig. 5).

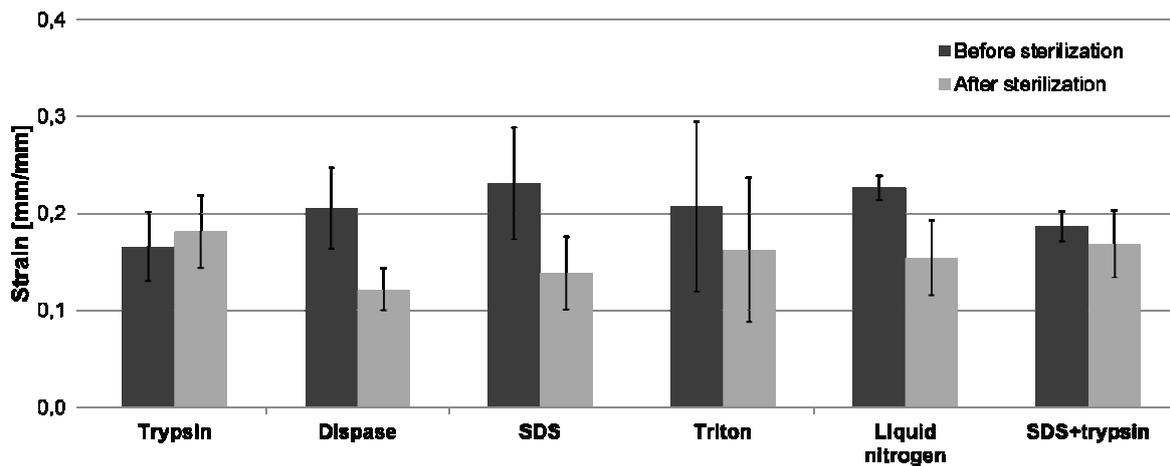


Fig. 4. Mean values of strain

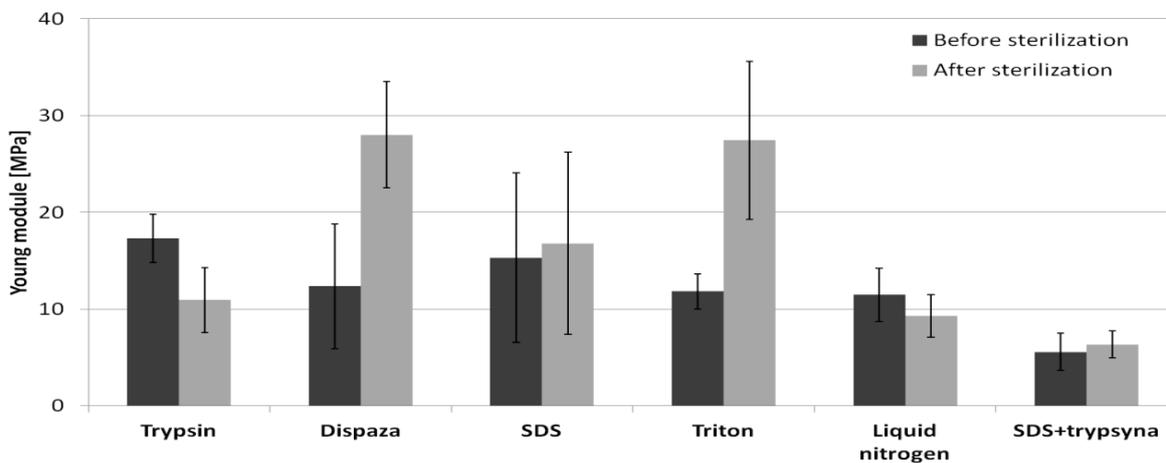


Fig. 5. Mean values of Young's modulus

3.2. Statistical analysis

3.2.1. Data analysis: reagent, sterilization, strain

The obtained test results were subjected to statistical analysis in STATISTICA software. Prior to the performance of the statistical analysis, all assumptions of the ANOVA test were checked. Due to the fact that the analyzed variables are measurable and independent, the researchers checked if the assumptions of the distribution regularity (Fig. 6) and homogeneity of variances were satisfied.

Verification of the assumption of homogeneity of variances was performed by means of Brown–Forsythe’s test, where the strain was the dependent variable and a reagent was the grouping variable (Table 1).

In the case of the analysis where a reagent was the grouping variable, the condition of homogeneity of variances was also satisfied $p = 0.154$ ($p > 0.05$). The assumption of homogeneity of variances was verified by means of Brown–Forsythe’s test also in the case of strain being the dependent variable and sterilization being the grouping variable (Table 2).

The condition of the variance homogeneity was also satisfied $p = 0.883$ ($p > 0.05$). After verification of the assumptions of the ANOVA analysis, a statisti-

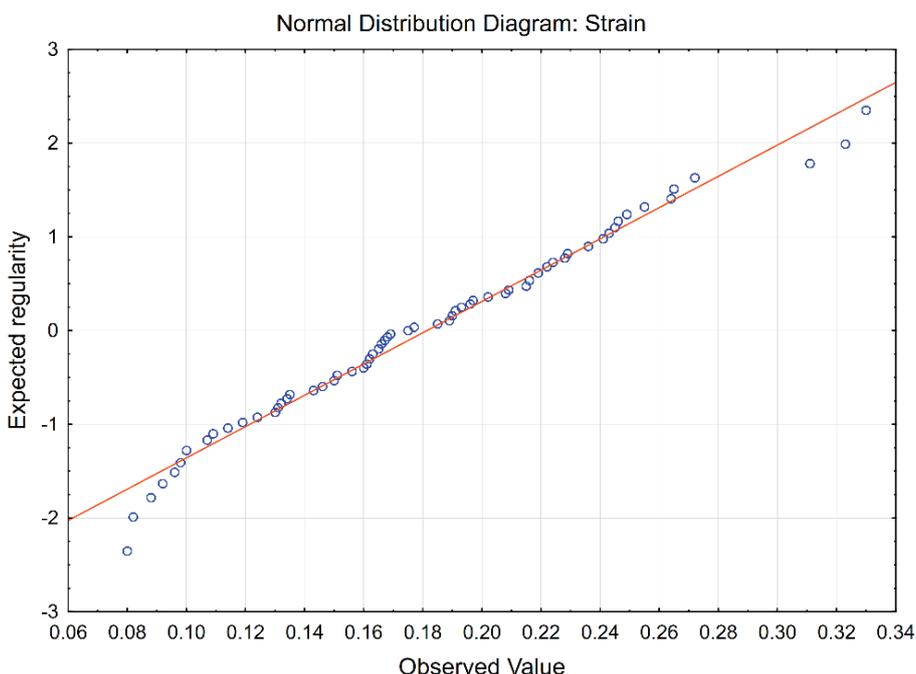


Fig. 6. Normal distribution of analyzed specimens

Table 1. Verification of the assumption of homogeneity of variances by means of Brown–Forsythe’s test, variable grouping reagent

Brown–Forsythe’s test for homogeneity of variances. Marked effects are significant at $p < 0.05$								
Variable	SS effect	df effect	MS effect	SS error	df error	MS error	F	p
Strain	0.016	7	0.002	0.088	63	0.001	1.594	0.154

Table 2. Verification of the assumption of homogeneity of variances by means of Brown–Forsythe’s test variable grouping sterilization

Brown–Forsythe’s test for homogeneity of variances. Marked effects are significant at $p < 0.05$								
Variable	SS effect	df effect	MS effect	SS error	df error	MS error	F	p
Strain	0.000021	1	0.000021	0.064	69	0.0009	0.022	0.883

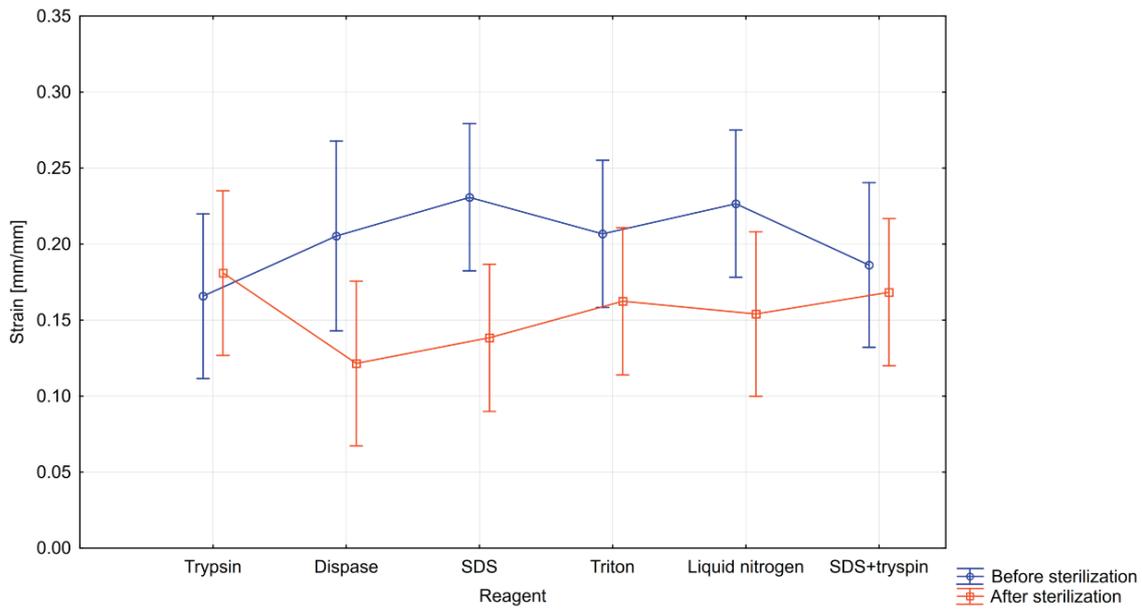


Fig. 7. Graphics interpretation of the interaction effect

cal analysis was conducted. On the basis of the performed analysis, it was ascertained that there were no statistically significant differences in the obtained strain values irrespective of the type of reagent applied $p = 0.971$, ($p > \alpha$, $\alpha = 0.05$). However, statistically significant differences occurred in the strain values in the case of the applied sterilization process $p = 0.0002$, ($p > \alpha$, $\alpha = 0.05$). Moreover, it was determined that between factors, such as “reagent” and “sterilization” there was no interaction $p = 0.372$, ($p > \alpha$, $\alpha = 0.05$).

Diagram (Fig. 7) presents strain values for different reagents before and after the sterilization process. As may be seen, the samples which were not subjected

to the sterilization process are characterized by higher strain values.

3.2.2. Data analysis: reagent, sterilization, Young’s modulus

The statistical analysis was conducted also in order to assess the impact of the type of reagent and sterilization on the values of Young’s modulus.

Due to the fact that the analyzed data were not subjected to a normal distribution (Fig. 8), the comparison of the values of Young’s modulus was conducted by means of the non-parametric Kruskal–Wallis test.

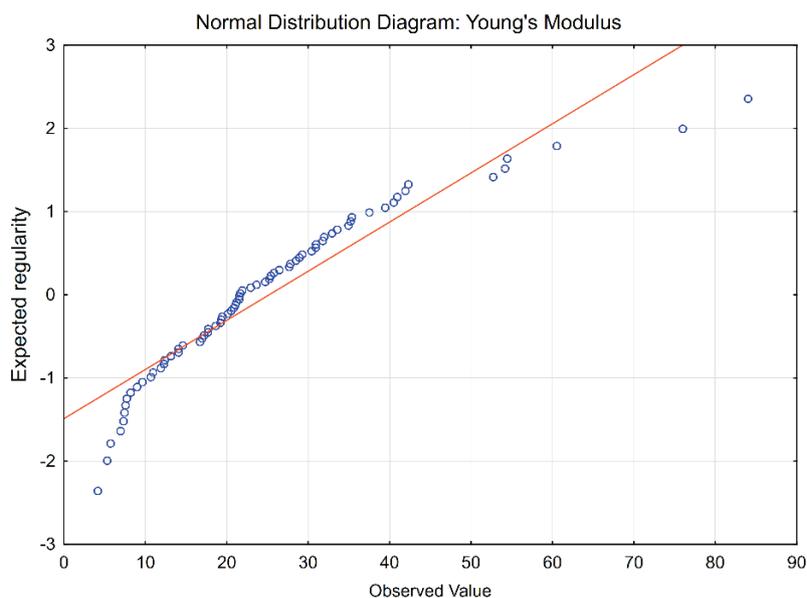


Fig. 8. Normal distribution of analyzed samples

Samples before sterilization

The results of the analysis performed by means of the Kruskal–Wallis test are presented in Table 3. The applied reagent constituted an independent variable, whereas Young’s modulus was a dependent variable. The result of the Kruskal–Wallis analysis equalled $H = 13.18$. The level of probability was $p = 0.0678$ ($p > \alpha$). On the basis of the analysis, it could be deduced that there were no statistically significant differences in the results obtained from Young’s modulus irrespective of the substance applied.

Samples after sterilization

Prior to the analysis of the results from samples after sterilization, it was necessary to define again a grouping variable (in this test it is a reagent) as well as a dependent variable (to which Young’s modulus values were attributed). The result of the non-parametric test equalled $H = 19.11$ and the level of probability $p = 0.0079$ ($p < \alpha$). It might be thus concluded that there were statistically significant differences in the obtained results of Young’s modulus depending on the substance used. Verification aimed to check be-

Table 3. Results of multiple comparisons

Dependent variable: Young’s Modulus	Value p for multiple comparisons; Young’s Modulus. Independent (grouping) variable: Reagent. Kruskal–Wallis test: $H(7, N = 35) = 19.11, p = 0.0079$					
	1 (Trypsin) R: 20.5	2 (Dispase) R: 29	3 (SDS) R: 17.6	4 (Triton) R: 25	5 (Liquid nitrogen) R: 9	6 (SDS +trypsin) R: 4.8
1 (Trypsin)		1	1	1	1	0.63
2 (Dispase)	1		1	1	0.16	0.01
3 (SDS)	1	1		1	1	1
4 (Triton)	1	1	1		0.56	0.05
5 (Liquid nitrogen)	1	0.16	1	0.56		1
6 (SDS + trypsin)	0.63	0.01	1	0.05	1	

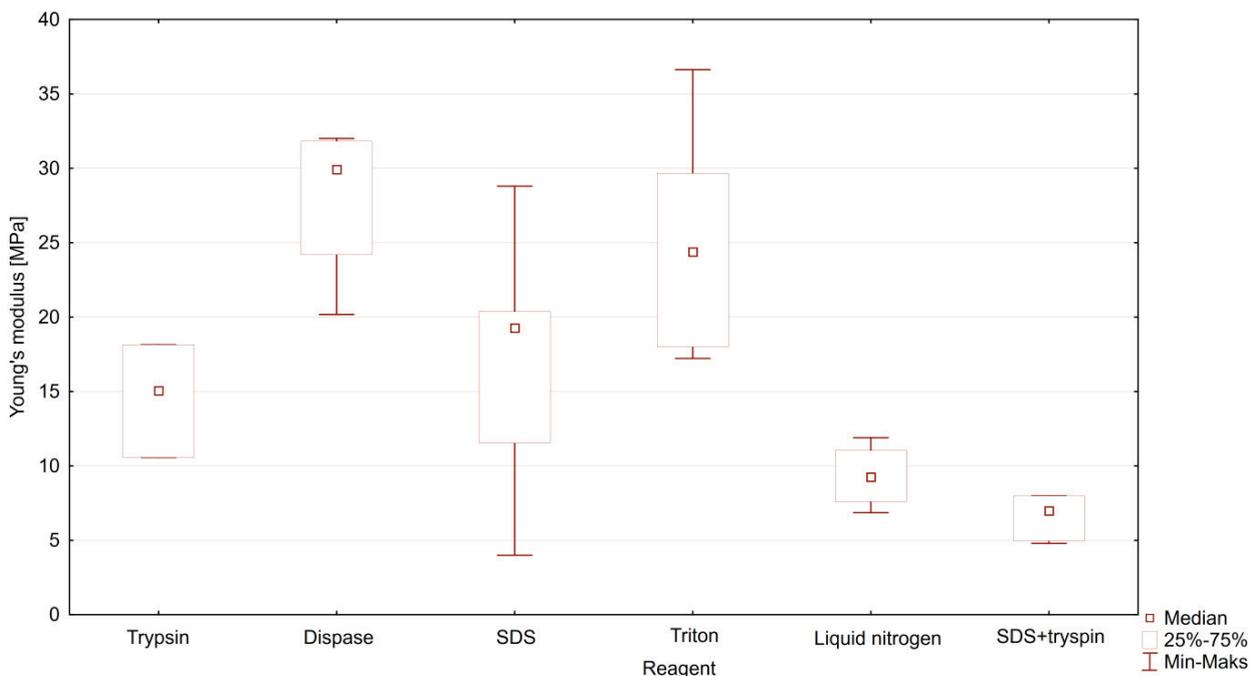


Fig. 9. Graphic representation of results for pig samples after sterilization using different substances

tween which reagents there were statistically significant differences. The verification was conducted by means of multiple comparisons.

The table above presents the results of multiple comparisons performed for samples after sterilization. On this basis, it might be observed that in the case of substance 2 (dispase) and substance 6 (SDS + trypsin) the level of probability $p = 0.01$, in that case, $p < \alpha$. It should be thus concluded that there were statistically significant differences. The analysis of comparisons for the rest of the substances showed the level of probability $p > \alpha$, which means that there were no statistically significant differences between the rest of reagents.

Analyzing diagram in Fig. 9, it can be observed that the highest average value of Young's modulus was obtained for the substance Dispase (28.0 MPa), while the lowest value of the average Young's modulus was obtained for the mixed method (SDS and trypsin – 6.3 MPa). There were also statistically significant differences between the two reagents.

4. Discussion

Assessment of mechanical properties of the skin is crucial for both regenerative surgery and plastic surgery. The research on mechanical properties of the skin of transgenic swine may contribute greatly to the development of xenotransplantation. Knowing the impact of methods of acellularization and sterilization on the mechanical parameters of skins, it is possible to develop methods of preparation of transgenic dermal dressings in a better way. Apart from the processes of acellularization and sterilization, the mechanical properties of the skin are obviously also affected by individual features as well as the place of taking the samples. The mechanical properties of the skin are subjected to changes depending on the direction of the sample's incision in relation to the orientation of the Langer's lines and the system of collagen fibres [1], [16], [17].

With this in mind, the samples prepared for the study were collected from the same anatomical sites of each pig while maintaining the same arrangement of collagen fibres. The temperature of storing the specimens also has an essential influence on the change of their mechanical parameters. The gradient of the stress-strain curve decreases when the temperature increases. That is caused by stretching and sliding of collagen particles in the cross-linking of collagen fibres. Due to the increase of temperature, the organized collagen structure undergoes a trans-

formation into a form with randomly located particles, which results in the reduction of stiffness [11], [12]. Therefore, as a part of this study, all samples were stored at the same temperature ($-20\text{ }^{\circ}\text{C}$) for a period of 2 months. The samples were then thawed in saline at $21\text{ }^{\circ}\text{C}$ for 20 minutes. This treatment was aimed at the possible effect of temperature on collagen fibres was the same for all samples. The study did not include a control group on skin samples prior to the decellularization and sterilization process because the basic goal of the conducted research was the evaluation of decellularization methods. However, the determined mechanical properties are only quantitative comparison parameters.

Appropriate mechanical properties of prepared transgenic porcine dermal dressings are an essential element in the process of operative treatment of burn wounds. Surgeons point out to the fact that the implanted dermal dressings are prone to damage during regenerative and plastic surgeries. That results from the process of dermal dressing preparation, which has a considerable influence on the change of their mechanical properties. That is the reason why preservation of the best material parameters of the skin after decellularization and sterilizations of such significance. The authors of this publication undertook the task of evaluation of the impact of decellularization and sterilization methods on the mechanical properties of dermal dressings.

On the basis of results obtained from the investigations, it was ascertained that the very process of acellularization does not significantly influence the mechanical parameters of the tested dressings. However, the combination of the process of acellularization with the procedure of sterilization has a considerable impact on the worsening of the mechanical properties of the tested specimens. Taking the requirements of a surgical procedure into account, it would be advisable that the dressings obtained after acellularization and sterilization have the highest strain values and therefore the lowest Young's modulus. On the grounds of the conducted statistical analysis of the obtained test results, it was ascertained that the value of Young's modulus for the samples after sterilization in the case of the substance of dispase and the mixed method (SDS + trypsin) was subjected to change in a statistically significant way. In the case of dispase, the value of Young's modulus before the process of sterilization amounted to 12.4 [MPa], whereas after sterilization it increased up to 28.0 [MPa]. For the mixed method (SDS + trypsin), the value of Young's modulus equalled 5.6 [MPa] before sterilization, while after the process of sterilization it minimally rose to 6.3 [MPa].

The mixed method (SDS+trypsin) had the smallest impact on the change of specimen mechanical properties before and after the process of sterilization. The difference between values obtained for Young's modulus and strain amounted to approximately 12% and was the smallest in comparison with other substances. Dermal dressings prepared by means of the mixed method are characterized by a low value of Young's modulus and a relatively high level of strain both prior to and after the process of sterilization. Such mechanical properties are desirable in the case of medical procedures of plastic and regenerative surgeries due to the fact that such dressings will be less vulnerable and prone to damage during a surgical procedure.

5. Conclusions

Within the framework of these investigations, the researchers carried out uniaxial tensile tests on dermal specimens coming from transgenic swine whose gene conditioning the formation of an antigen α Gal (α 1,3-galactosyltransferase) had been knocked out as well as proteins of surface cells had been modified (gene 1,2-fucosyltransferase of a human being). The total number of 80 specimens was tested by means of 8 methods of acellularization. Half of the tested samples were subjected to the process of radiation sterilization (35 kGy) in compliance with the valid procedures of the Tissue Bank. Sterility of tissues was confirmed microbiologically. On the basis of the conducted tests, it was confirmed that different methods of acellularization and the process of sterilization have an influence on the change of mechanical properties of the skin of transgenic swine. In six out of eight applied acellularization methods (Trypsin, SDS, Triton, Liquid nitrogen), the differences in obtained values were not statistically significant. However, in the case of the mixed method (SDS + trypsin) and dispase, statistically significant differences were observed. From the point of view of the requirements of the procedures of reconstructive and plastic surgeries, more favourable mechanical properties were obtained for the mixed method (SDS+trypsin) because it enables the obtainment of the lowest value of Young's modulus, at the same time maintaining a relatively high level of strain. In the authors' opinion, this method should be recommended as the best one for the preparation of transgenic porcine dermal dressings because it ensures a smaller probability of dressing's damage during a surgical procedure.

Conflict of interest

The authors declare that the article content was composed in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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