

METHOD FOR INACTIVATING XENOANTIGENS IN BIOLOGICAL TISSUES

The present invention relates to a method for inactivating xenoantigens in biological tissues, in particular for inactivating xenoantigens in tissues that can be used to manufacture bioprosthetic substitutes, intended for use in the human or veterinary clinical field.

In particular the invention relates to a method for ensuring the inactivation of xenoantigens in connective tissues that are native and/or fixed, heterologous or homologous, in particular of the alpha-Gal epitope, in particular in cardiovascular tissues through the use of biological activities identified in phenolic compounds, polyphenolic compounds and derivatives thereof.

The production of bioprosthetic substitutes is currently a market undergoing major expansion. The clinical improvement of surgical procedures, the decrease in post-surgical complications, the development and management of new immune-modulating medicines, combined with a deeper knowledge of the interaction mechanisms between graft and host, all contribute to facilitating where possible the use of biological prostheses constituted by animal or homologous tissue. In this sense, one sector that is representative but non-limiting is the cardiovascular sector, especially in terms of the social and health impact that the established practice of cardiac valve replacement can cause.

Biomedical technology is capable of developing and surgically applying for replacement purpose, valve prostheses that can imitate the opening and closing function of dysfunctional native valves.

The ideal valve prostheses should be capable of allowing a trans-valve flow that can overlap that of the analog original, healthy valve, ensure a long lifetime and not generate hemolytic or thrombogenic effects.

The valve substitutes that are most often used are biological prostheses derived from xenogeneic tissues, in particular from pig valves or

valves produced with bovine or equine pericardium.

Such valve prostheses and substitutes have the disadvantage that they encounter degenerative processes of calcific dystrophy and/or deterioration with breakage of the cusps, exhibiting a greater sensitivity toward the onset
5 of endocardial infections. For the purpose of improving their mechanical characteristics, decreasing their intrinsic antigenicity, and enabling their preservation, they are usually treated with cross-linking/sterilizing chemical agents such as, for the purposes of non-exclusive example, glutaraldehyde. In addition they can be subjected to treatments according to decalcification
10 or detoxification protocols.

The term "xenogeneic tissue" means a tissue that belongs to an organism of a species other than human; such materials have specific surface antigens that are tolerable inside the species of origin, but which are incompatible when implanted in humans where, if not adequately treated,
15 they are capable of triggering the activation of the complement cascade with platelet aggregation, producing a situation similar to that occurring in the case of a blood group incompatibility.

Such phenomenon is known by the term "hyperacute rejection". The principal cause of the onset of such mechanism is the presence of the alpha-
20 Gal xenoantigen. This epitope is a di-galactoside (galactose-alpha1,3-galactose) present on membrane glycoproteins and glycolipids (primarily of endothelial cells), as well as on different cell types such as monocytes, granulocytes and red blood cells and in important tissue districts such as the myocardial and bone regions. Such crucial antigen is constitutively
25 expressed in all mammals, except in the higher primates and humans.

The human body, from birth, expresses antibodies directed against such epitope as a result of continuous stimulation by the intestinal bacterial flora.

Today, the biocompatibility of the xenogeneic tissues intended for use
30 in manufacturing bioprotheses is obtained by treating with the

aforementioned glutaraldehyde.

Despite such procedure, the alpha-Gal epitope has been shown to remain responsive in currently marketed valve substitutes, causing, after the implant, an increase in the anti-alpha-Gal antibodies circulating both in
5 pediatric patients and in adults.

Furthermore, the antigen-antibody complex that is formed appears to be directly involved in promoting the deposition of calcium salts, favoring the formation of episodes of calcific dystrophy of the valve.

The aim of the present invention is to provide a method for
10 inactivating xenoantigens in biological tissues that is capable of overcoming the limitations of conventional treatments.

Within this aim, an object of the invention is to provide a method that can be applied to connective tissues that are native and/or fixed, heterologous or homologous, which can be used for manufacturing
15 bioprosthetic substitutes, for use in the human or veterinary clinical field.

A further object of the invention is to provide a method for inactivating xenoantigens in biological tissues that is adapted to ensure the inactivation of the alpha-Gal epitope in cardiovascular tissues.

Another object of the invention is to provide a method for inactivating
20 xenoantigens in biological tissues with which to inactivate the above mentioned epitopes thus ensuring an effective treatment that can be applied to the various different types of bioprosthetic substitutes currently on the market.

Another object of the invention is to provide a method that does not
25 favor, after an implant, an increase in circulating anti-alpha-Gal antibodies.

Another object of the invention is to provide a method that does not promote the deposition of calcium salts, therefore limiting the formation of episodes of calcific dystrophy of the valve.

Another object of the invention is to provide a method that can be
30 carried out with conventional devices and machines.

This aim and these and other objects which will become better evident hereinafter are achieved by a method for inactivating xenoantigens in biological tissues, particularly in tissues that can be used to manufacture bioprosthetic substitutes and/or in bioprosthetic substitutes that are already prepared and intended for human or veterinary clinical use, characterized in that it entails the following steps:

- providing a solution based on phenolic compounds, polyphenolic compounds or derivatives thereof, for the inactivation of at least part of the xenogeneic epitopes from said tissues;
- 10 - incubating the samples to be treated in the various solutions based on phenols/polyphenols in controlled conditions;
- subjecting the treated tissues to a series of washes.

The invention also relates to a connective tissue obtained with a method for inactivating xenoantigens in biological tissues according to the invention as described above, characterized in that it has at least some of the antigen component in inactive form.

The invention also relates to a use of connective tissue, obtained with a method for inactivating xenoantigens in biological tissues according to the invention as described above, for the manufacture of bioprosthetic substitutes and/or parts of bioprosthetic substitutes that are already prepared, for use in the human or veterinary clinical field.

The invention also relates to a kit for carrying out a method for inactivating xenoantigens in biological tissues according to the invention as described above, characterized in that it comprises at least:

- 25 - one or more containers containing the buffer in which the most suitable dose of phenolic compounds, polyphenolic compounds or derivatives thereof is to be dissolved;
- one or more containers containing the dose of phenolic compounds, polyphenolic compounds or derivatives thereof in powder form to be combined with the buffer;
- 30

- one or more containers containing the washing buffers;
- an instruction booklet containing the description of the timings and modes of application of the procedure.

Further characteristics and advantages of the invention will become better apparent from the detailed description that follows of a preferred, but not exclusive, embodiment, of the method for inactivating xenoantigens in biological tissues according to the invention. In the accompanying drawings:

- Figure 1 is a view of the results, in percentages, of the application of a method according to the invention in a first variation of application thereof;

- Figure 2 is a view of the results, in percentages, of the application of a method according to the invention in a second variation thereof;

- Figure 3 is a view of the results, in percentages, of the application of a method according to the invention in a third variation thereof;

- Figure 4 is a view of the results, in percentages, of the application of a method according to the invention in a fourth variation of application thereof.

DEFINITIONS

The term "phenolic compounds" refers to molecules characterized, at least in part thereof, by the presence of an aromatic nucleus (benzene ring) bound to one or more hydroxyl functional groups. The above mentioned compounds include, for the purposes of non-exclusive example: simple phenols (molecules with a single benzene ring and containing only hydroxyl groups as substituents, e.g. phenol and hydroquinone), phenolic aldehydes (containing both the phenolic group and the aldehyde group, e.g. aldeide vanillica), phenolic acids (e.g. cinnamic acids), phenylamines (amphoteric molecules containing a weakly acidic group and a strongly basic group, e.g. phenylalanine), phenol compounds (the phenolic ring is bound to another benzene ring or to other heterocyclic compounds that have

hydroxyl/lactone/ketone functional groups, e.g. coumarins and xanthenes), flavonoids (made up of two benzene rings connected by a chain with three carbon atoms that constitutes an oxygenated heterocyclic ring, e.g. catechins, flavonons, flavones, chalcones, flavanonols, flavanols, leucoanthocyanidin, anthocyanidin), phenylpropanoids (characterized by the presence of an aromatic ring with an aliphatic side chain with three carbon atoms, es. hydroxycinnamic acids) and tannins. In the present invention the terms "phenols" and "polyphenols" can have the same meaning, and can be used together or to substitute for each other for the set aims.

10 The term "xenoantigen" refers to molecules of animal origin that can be recognized by the immune and can induce an antibody/immune-mediated/inflammatory response in the human host organism. In the present invention the terms "xenoantigen", "antigen", "xenogeneic antigen", "epitope" and "crucial antigen" can have the same meaning, and can be used
15 together or to substitute for each other.

The term "connective tissue" comprises among others: vessels, cardiac valves, tendons, ligaments, pericardium, muscular fasciae, dura mater, tympanic membrane, intestinal submucosa, cartilage, adipose tissue and bone tissue.

20 The term "fixed" tissues comprises tissues subjected to the action of chemical or biological agents such as for the purposes of non-limiting example: glutaraldehyde, formaldehyde and quercitin.

The term "fixed" tissues comprises tissues that, subjected to the action of chemical or biological agents, develop intra-tissue cross-links with the function of stabilizing protein, lipid and cell structures as well as lowering
25 the potential antigenic action of the host. In the description of the present invention, the terms "fixed" and "cross-linked" can describe a same type of treatment and/or have the same meaning and can be used together or to substitute for each other.

30 The term "heterologous" tissues means tissues of non-human origin.

Such tissues can be presented for clinical use as native or non-treated, instead of being subjected to treatments that boost their regenerative properties (such as, for the purposes of purely illustrative example, decellularizing procedures or procedures for coating/absorption of pro-regenerative/preservative substances for the cell component). In the description of the invention, the term "heterologous" can have the same meaning as "xenogeneic", and they can be used together or to substitute for each other.

The term "homologous" tissues means tissues of human origin. Such tissues can be presented for clinical use as native or non-treated, instead of being subjected to preservative treatments (such as, for the purposes of purely illustrative example, cryopreservation) or treatments that boost their regenerative properties (such as, for the purposes of purely illustrative example, decellularizing procedures or procedures for coating/absorption of pro-regenerative/preservative substances for the cell component).

The term "decellularizing procedures" means all individual or multiple treatments that use, as non-limiting examples, saline solutions (hyper-, iso- or hypo-tonic), detergent solutions (ionic, non-ionic or zwitterionic) and enzymes, and the purpose of which is the partial, selective or total removal of the cell component present in the original tissue.

The term "bioprosthetic substitutes" identifies biological devices that are adapted to substitute a missing part of the organism (a limb, an organ or a tissue) or to integrate a damaged part, intended for human or veterinary clinical use. In the description of the present invention, the terms "bioprosthetic substitutes", "bioprostheses", "biological prostheses" or "device" can have the same meaning, and can be used together or to substitute for each other.

The term "knockout animal for the alpha-Gal antigen" means an animal in which the gene that encodes for the alpha-galactosyltransferase enzyme has been silenced. Such enzyme is responsible for attacking the

membrane glycoproteins and lipoproteins of the alpha-Gal epitope. Its absence causes the production of tissues that completely lack the epitope in question and which in this respect are entirely comparable to the tissue of the human body. In the present invention, knockout animal vascular tissues
5 for the alpha-Gal antigen have been used as an absolute negative control.

Below are some non-limiting examples of application of the method according to the invention.

A method for inactivating xenoantigens in biological tissues according to the invention, particularly for tissues that can be used to
10 manufacture bioprosthetic substitutes and/or in bioprosthetic substitutes that are already prepared and intended for human or veterinary clinical use, comprises the following steps:

- providing a solution based on phenolic compounds, polyphenolic compounds or derivatives thereof, for the inactivation of at least part of the
15 xenogeneic epitopes from such tissues;
- incubating the samples to be treated in the various solutions based on phenols/polyphenols in controlled conditions;
- subjecting the treated tissues to a series of washes.

The method also comprises a subsequent procedure of assessing the
20 effective inactivation of the alpha-Gal epitope by way of comparison of treated/untreated tissues and knockout porcine tissues for the gene of the alpha-galactosyltransferase enzyme.

Such a procedure can be provided for example as disclosed in Italian patent no. 0001409783 and in EP2626701.

25 The biological tissues are constituted by connective tissues which can be native, or native and fixed, or fixed.

The biological tissues can be heterologous or homologous.

The antigenic epitope is constituted by alpha-Gal antigen.

The controlled conditions of the incubation step comprise at least one
30 treatment at the temperature of $40 \pm 2^\circ\text{C}$.

The phenolic compounds, polyphenolic compounds or derivatives thereof for the inactivation of at least part of the xenogeneic epitopes from such tissues are constituted by derivatives of cinnamic acid, tannin and oleuropein.

5 In particular, and by way of example, the cinnamic acid derivatives are constituted by caffeic acid.

In particular, and by way of example, the tannin derivatives are constituted by tannic acid.

10 In particular, and by way of example, the oleuropein derivatives are constituted by hydroxytyrosol.

In particular, and by way of example, at least one phenyl derivative of cinnamic acid is constituted by caffeic acid.

In particular, and by way of example, at least one phenyl derivative of tannin is constituted by tannic acid.

15 In particular, and by way of example, at least one phenyl derivative of oleuropein is constituted by hydroxytyrosol.

A method for inactivating xenoantigens in biological tissues according to the invention, meaning tissues that can be used to manufacture bioprosthetic substitutes, is applied, by way of non-limiting example of the invention, to the inactivation of the alpha-Gal epitope in tissues constituting the following models of bioprosthetic substitutes:

- Hancock II TM Porcine Heart Valve (mod. T510, Medtronic Inc., Minneapolis, USA) indicated in the figures as 'HANCK';
- Freestyle [®] Aortic Root Heart Valve (mod. 995, Medtronic Inc., 25 Minneapolis, USA) indicated in the figures as 'FREE';
- Carpentier-Edwards S.A.V. (mod. 6650, Edwards Lifesciences LCC, California, USA) indicated in the figures as 'SAV';
- Carpentier-Edwards Perimount Plus (mod. 6900P, Edwards Lifesciences LCC, California, USA) indicated in the figures as 'PERI';
- 30 - CardioCel Cardiovascular Patch (mod. C0404, Admedus Regen Pty

Ltd, Perth, Australia) indicated in the figures as 'CC'.

The method for inactivating xenoantigens in biological tissues, and particularly for inactivating alpha-Gal epitopes in bioprosthetic substitute samples, is described below in the details of an embodiment.

5 Tissue samples are taken from the bioprosthetic substitutes as per the above mentioned models currently available on the market. Such samples are weighed damp after light filter paper blotting (range 30-50 mg) and cut into small pieces in order to increase its exposure surface.

For each bioprosthetic substitute, 4 different sets of samples are
10 prepared (n=8 for each set).

Each set will be subjected to a different method.

4 different solutions are prepared based on phenolic derivatives, corresponding to 4 different applicative variants of the method according to the invention, to which the samples will be subjected in a final volume of
15 5ml, specifically:

- method T1: caffeic acid at a concentration comprised between 5mM and 50mM (in the invention the 20mM concentration was adopted) / buffer of sodium phosphate with 600 ± 50 U/ml of Tyrosinase in a ratio of [1:20];

- method T2: caffeic acid at a concentration comprised between 5mM
20 and 50mM (in the invention the 20mM concentration was adopted) in 0.2 ± 0.1 M of NaOH;

- method T3: tannic acid at a concentration comprised between 0.1M and 1.5M (in the invention the 1M concentration was adopted) in a sodium phosphate buffer;

25 - method T4: hydroxytyrosol at a concentration comprised between 0.3mM and 10mM (in the invention the 6mM concentration was adopted) in 0.2 ± 0.1 M of NaOH.

These solutions are left to act under moderate but constant stirring for a total of 12 ± 2 hours at the temperature of $40\pm 2^\circ\text{C}$.

30 At the end of the incubation, the samples are subjected to two washes

with isotonic solution, of 15 minutes duration each, and a third washing in a dedicated buffer (TP) of 15 minutes duration.

The assessment of the presence of any epitopes still active on the surface of the treated samples is based on a modification of the illustrated
5 method by the inventors and described in Italian patent no. 0001409783 and in EP2626701.

Briefly, the treated and washed tissue samples are placed in test tubes to which TP buffer is added up to a final volume comprised between 1000uL and 1500uL.

10 Then a monoclonal mouse antibody, directed against the alpha-Gal epitope, is added (in the present example this is an IgM clone called M86), at the preferable concentration of [1:50] v/v and the whole is incubated for 120±10 minutes at 37±2°C under constant but moderate stirring.

At the end the samples are subjected to centrifugation at 14,750 x g
15 for 30±5 minutes at ambient temperature.

During the incubation with the M86 antibody, a 96-well plate is prepared, in which the bottom of the wells is lined with 100uL per cell of alpha-Gal/serum albumin at 5ug/ml in phosphate buffer. The plate thus prepared is incubated for 60±10 minutes at a temperature comprised
20 between 30°C and 40°C, although it is preferable to stabilize everything at 37°C. Then 3 washes are performed with 300uL per well of phosphate buffer at ambient temperature.

The first washing is left to act for 5 minutes, the two subsequent washes for 3 minutes.

25 The blocking is done with 300uL per well of serum albumin, followed by incubation for 60±10 minutes at ambient temperature, in darkness. Subsequently 3 washes as above are performed.

For each individual well, 100uL of supernatant, taken after centrifugation from each treated sample, are added.

30 The samples are loaded into the plate, each type of sample occupying

the wells of an entire column. There follows incubation of the plate for 120±10 minutes at a temperature comprised between 30°C - 40°C, although it is preferable to stabilize everything at 37°C.

Then 3 washes as above are performed and 100uL per well is added
5 of a solution of secondary antibody (rabbit polyclonal anti-mouse) conjugated with peroxidase enzyme (the ideal solutions of such antibody have been found to be [1:1000], [1:500] and [1:100], preferably the intermediate one [1:500] was adopted).

The plate is then incubated again for 60±10 minutes at a temperature
10 comprised between 30°C - 40°C, although it is preferable to stabilize everything at 37°C.

Then 3 washes as above are performed. 100uL is added per well of a development solution for the peroxidase enzyme, followed by incubation of the plate for 5±1 minutes in darkness.

15 Subsequently 50uL is added per well of a stop solution constituted by H₂SO₄ 2M, and the plate is then read in a plate reader at 450 nm.

The percentage inactivation of the epitope in question can be determined by way of comparison between the number of epitopes obtained: in a control tissue constituted by vascular tissue of knockout animals for the
20 alpha-Gal antigen, in untreated bioprosthetic tissues and in the tissues subjected to the various treatments as described above.

It is clear from Figure 1 that the method in its variation of application T1 displays a marked variability of effect according to the various different bioprosthetic tissues treated.

25 The T1 method has shown a markedly lower efficacy than the other protocols studied, showing as its best result an inactivation limited to about 43% of the epitopes.

In Figure 2 it can be seen that the method in its variation of application T2 with cinnamic acid derivatives only displays an excellent
30 inactivating action against the antigen, with inactivation percentages

comprised between 90% and 98%, similarly to the result shown by the method in the T3 variation (Figure 3, inactivation percentages comprised between 80% and 100%) and T4 variation (Figure 4, inactivation percentages comprised between 89% and 95%).

5 The invention also relates to a connective tissue obtained with a method as described above.

Such connective tissue is characterized in that it has at least some of the antigen component in inactive form.

10 The invention also relates to a use of the connective tissue as described above, for the manufacture of bioprosthetic substitutes and/or parts of bioprosthetic substitutes that are already prepared, for use in the human or veterinary clinical field.

The invention also relates to a kit for carrying out a method for inactivating xenoantigens in biological tissues as described above.

15 Such kit comprises at least:

- one or more containers containing the buffer in which the most suitable dose of phenolic compounds, polyphenolic compounds or derivatives thereof is to be dissolved;

20 - one or more containers containing the dose of phenolic compounds, polyphenolic compounds or derivatives thereof in powder form to be combined with the buffer;

- one or more containers containing the washing buffers;

- an instruction booklet containing the description of the timings and modes of application of the procedure.

25 The envisaged use of the kit is aimed at the autonomous treatment of bioprosthetic substitutes that are already prepared, with a method according to the invention as described above, useful for health facilities such as clinics and hospitals.

30 In practice it has been found that the invention fully achieves the intended aim and objects.

In particular, with the invention a method has been devised for inactivating xenoantigens in biological tissues, and in particular of the alpha-Gal epitope in tissues intended for the production of bioprosthetic substitutes for clinical and/or veterinary use.

5 Furthermore, with the invention a method has been devised for inactivating the above mentioned antigens, thus ensuring an effective treatment that can be applied to the various different types of tissue bioprostheses currently on the market.

Therefore, with the invention a method has been devised that is
10 potentially capable of not causing, after an implant of a tissue treated with such method, an increase in the anti-alpha-Gal antibodies circulating.

Moreover, with the invention a method has been devised that is capable of limiting the deposition of calcium salts, therefore not favoring the formation of episodes of calcific dystrophy of the valve.

15 Last but not least, with the invention a method has been devised that can be carried out with conventional devices and machines.

The invention, thus conceived, is susceptible of numerous modifications and variations, all of which are within the scope of the appended claims. Moreover, all the details may be substituted by other,
20 technically equivalent elements.

In practice the components and the materials employed, provided they are compatible with the specific use, and the contingent dimensions and shapes, may be any according to requirements and to the state of the art.

The disclosures in Italian Patent Application No. 102015000078236
25 (UB2015A006019) from which this application claims priority are incorporated herein by reference.

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CLAIMS

1. A method for inactivating xenoantigens in biological tissues, particularly in tissues that can be used to manufacture bioprosthetic substitutes and/or in bioprosthetic substitutes that are already prepared and
5 intended for human or veterinary clinical use, characterized in that it entails the following steps:

- providing a solution based on phenolic compounds, polyphenolic compounds or derivatives thereof, for the inactivation of at least part of the xenogeneic epitopes from said tissues;

10 - incubating the samples to be treated in the various solutions based on phenols/polyphenols in controlled conditions;

- subjecting the treated tissues to a series of washes.

2. The method according to claim 1, characterized in that said biological tissues are constituted by native, or native and fixed, or fixed
15 connective tissues.

3. The method according to one or more of the preceding claims, characterized in that said biological tissues are heterologous or homologous.

4. The method according to one or more of the preceding claims, characterized in that said antigenic epitope is the alpha-Gal antigen.

20 5. The method according to one or more of the preceding claims, characterized in that said controlled conditions comprise at least treatment at the temperature of $40\pm 2^{\circ}\text{C}$.

6. The method according to one or more of the preceding claims, characterized in that said phenolic compounds, polyphenolic compounds or
25 derivatives thereof for the inactivation of at least part of said xenogeneic epitopes from said tissues are constituted by derivatives of cinnamic acid, tannin and oleuropein.

7. The method according to claim 6, characterized in that said derivatives of cinnamic acid are constituted by caffeic acid.

30 8. The method according to claim 6, characterized in that said tannin

derivatives are constituted by tannic acid.

9. The method according to claim 6, characterized in that said oleuropein derivatives are constituted by hydroxytyrosol.

10. The method according to one or more of the preceding claims,
5 characterized in that at least one phenyl derivative of cinnamic acid is constituted by caffeic acid.

11. The method according to one or more of the preceding claims, characterized in that at least one phenyl derivative of tannin is constituted by tannic acid.

10 12. The method according to one or more of the preceding claims, characterized in that at least one phenyl derivative of oleuropein is constituted by hydroxytyrosol.

13. Connective tissue obtained with a method according to one or more of the preceding claims, characterized in that it has at least part of the
15 antigen component in inactive form.

14. Use of connective tissue according to one or more of the preceding claims for the manufacture of bioprosthetic substitutes and/or parts of bioprosthetic substitutes that are already prepared, for use in the human or veterinary clinical field.

20 15. A kit for carrying out a method for inactivating xenoantigens in biological tissues according to one or more of the preceding claims, characterized in that it comprises at least:

- one or more containers containing the buffer in which the most suitable dose of phenolic compounds, polyphenolic compounds or
25 derivatives thereof is to be dissolved;

- one or more containers containing the dose of phenolic compounds, polyphenolic compounds or derivatives thereof in powder form to be combined with the buffer;

- one or more containers containing the washing buffers;

30 - an instruction booklet containing the description of the timings and

modes of application of the procedure.

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ABSTRACT

A method for inactivating xenoantigens in biological tissues, particularly in tissues that can be used to manufacture bioprosthetic substitutes and/or in bioprosthetic substitutes that are already prepared and intended for human or veterinary clinical use. Such method entails the following steps:

- providing a solution based on phenolic compounds, polyphenolic compounds or derivatives thereof, for the inactivation of at least part of the xenogeneic epitopes from such tissues;
- incubating the samples to be treated in the various solutions based on phenols/polyphenols in controlled conditions;
- subjecting the treated tissues to a series of washes.

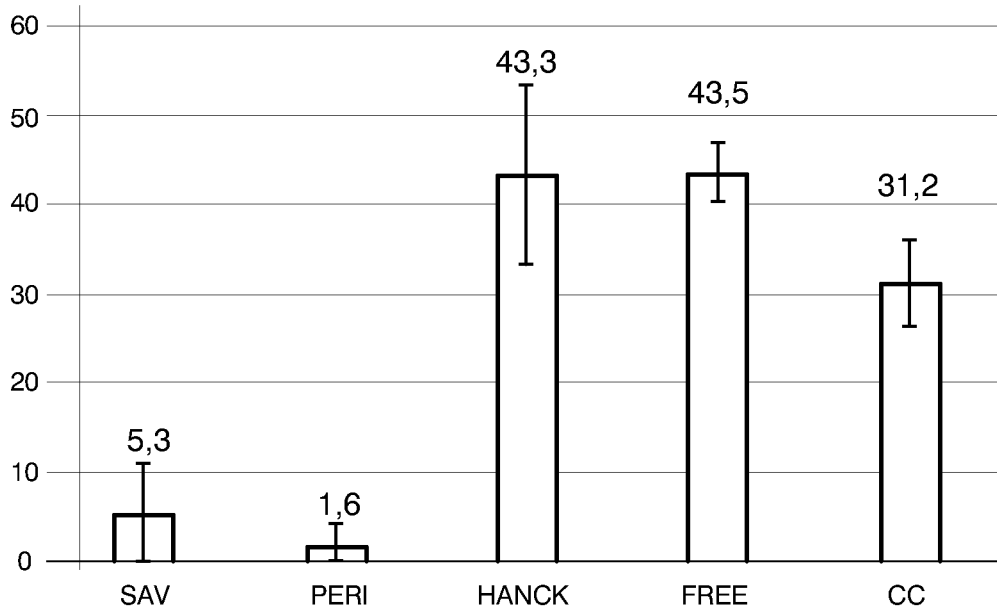


Fig.1

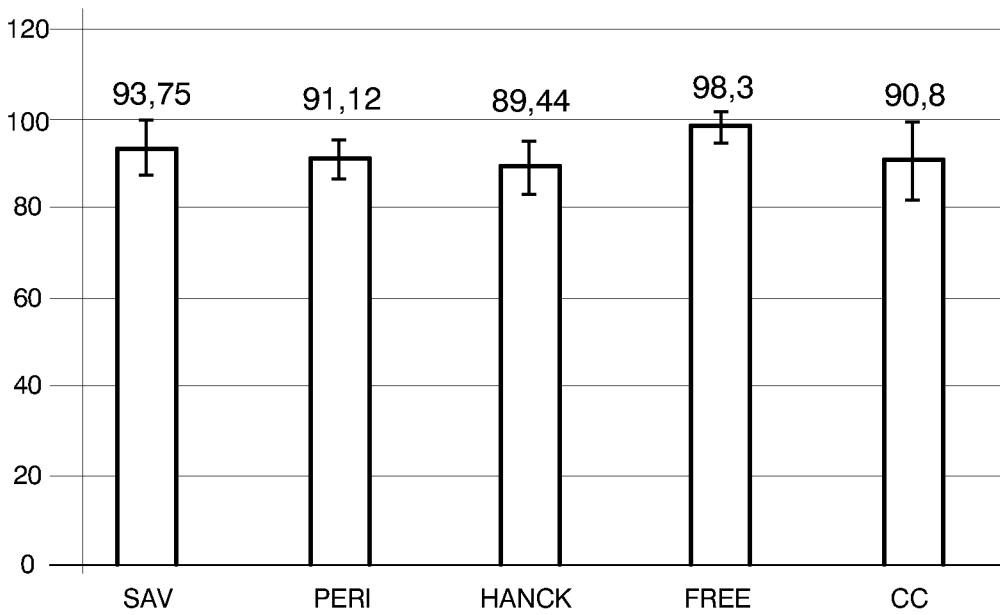


Fig.2

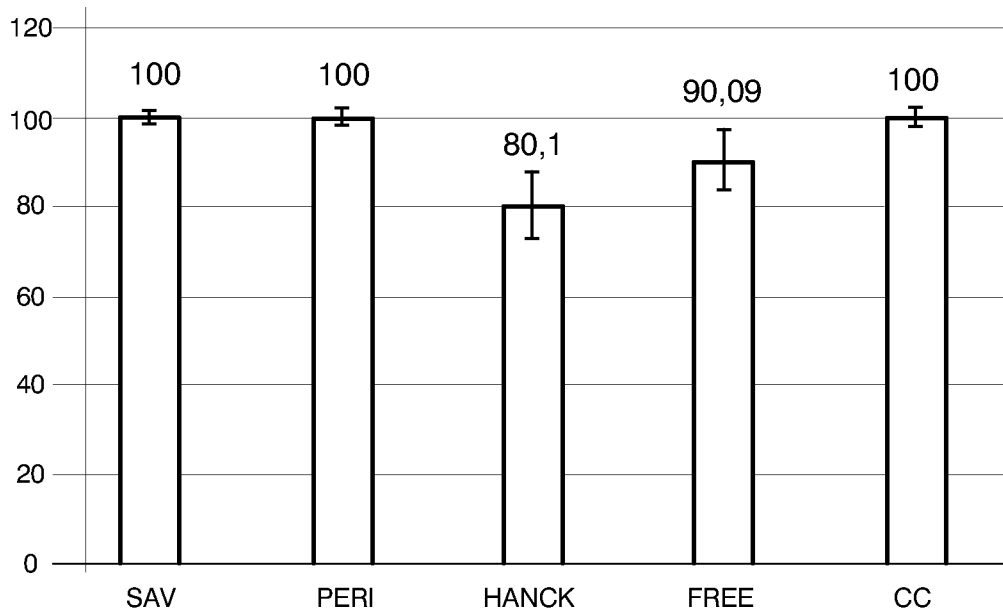


Fig.3

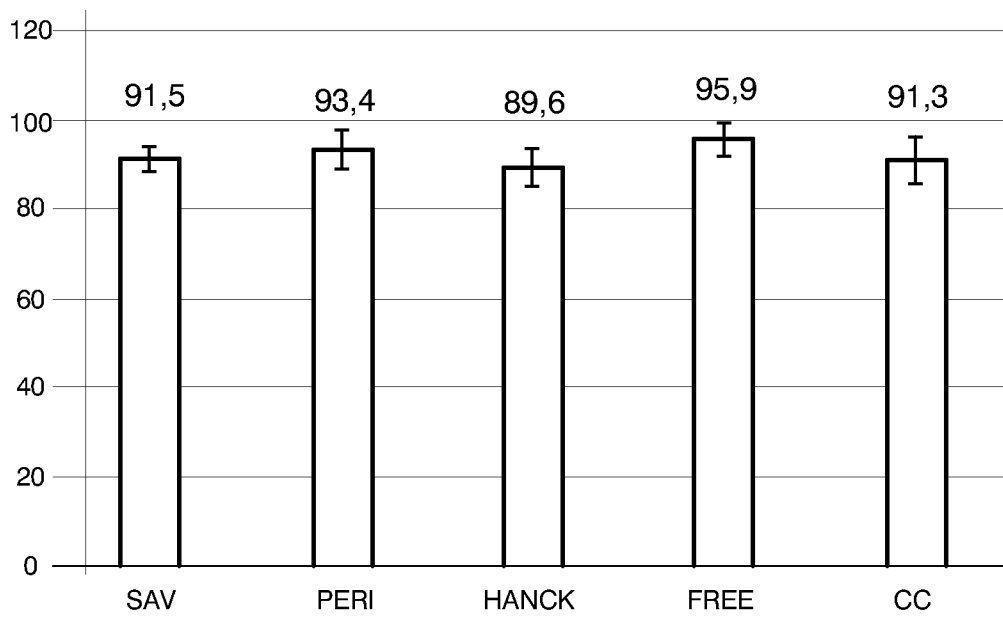


Fig.4