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Histological and mechanical evaluation of antifreeze peptide (Afp1m) cryopreserved skin grafts post transplantation in a rat model

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ABSTRACT

The objective of this study was to evaluate the use of Afp1m as a cryopreservative agent for skin by examining the transplanted skin histological architecture and mechanical properties following subzero cryopreservation. Thirty four (34) rats with an average weight of 208 ± 31 g (mean ± SD), were used. Twenty four (n = 24) rats were equally divided into four groups: (i) immediate non-cryopreserved skin autografts (onto same site), (ii) immediate non-cryopreserved skin autografts (onto different sites), (iii) skin autografts cryopreserved with glycerol for 72 h and (iv) skin autografts cryopreserved with Afp1m for 72 h at −4 °C. Rounded shaped full-thickness 1.5–2.5 cm in diameter skin was excised from backs of rats for the autograft transplantation. Non-cryopreserved or cryopreserved auto skin graft were positioned onto the wound defects and stitched. Non-transplanted cryopreserved and non-cryopreserved skin strips from other ten rats (n = 10) were allowed for comparative biomechanical test. All skin grafts were subjected to histological and mechanical examinations at the end of day 21. Histological results revealed that tissue architecture especially the epidermal integrity and dermal-epidermal junction of the Afp1m cryopreserved skin grafts exhibited better histological appearance, good preservation of tissue architecture and structural integrity than glycerolized skin. However, there was no significant difference among these groups in other histological criteria. There were no significant differences among the 4 groups in skin graft mechanical properties namely maximum load. In conclusion, Afp1m were found to be able to preserve the microstructure as well as the viability and function of the skin destined for skin transplantation when was kept at −4 °C for 72 h.

1. Introduction

Skin one of the largest organs in the body performing a principle role as a barrier against the aggressive surrounding environment. As well as, it avoids the entry of foreign chemicals and micro-organisms, excessive water loss from the aqueous interior, and also offers both stiffness and strength to overcome various mechanical loading. Further duties include sensation, temperature control and insulation. Achieving all these functions requires both stability and flexibility. Yet, the macroscopical and biomechanical properties of the skin can be vulnerable through different factors such as medical or cosmetic treatment, trauma, and diseases [43,55].

Fresh skin grafts including cadaver allograft that are used for many surgical purposes is still to be considered as the 'golden standard' [6]. Unfortunately, the use of fresh skin grafts is severely obstructed by their limited availability [5].

Short preservation of skin grafts for delayed application is still considered as a basic approach in reconstructive and plastic surgery and even in burn. The most common phenomena facing preserved skin grafts even with the presence of a rich oxygenated nutrient medium, is the ischemic necrosis that happens and continue to due to the difference in the diffusion speed between the tissue periphery and the central cells. As a result of this, the toxic metabolites cannot be removed quickly enough and the nutrients and oxygen cannot diffuse fast enough to supply the cells. Therefore, the more effective way for keeping the viability during storage of skin grafts is by reducing the tissue

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Table 1
Microscopic criteria for assessment skin graft performance.

Microscopic parameters	Scores		
	0	1	2
Epidermal integrity	Destroyed	Partial	Normal
Epidermal-dermal junction	Discontinued	Partial	Normal
Collagen organization	Amorphic	Disturbed	Normal
Fibroblast presence	< 10	10–20	> 20
Graft adherence (%)	< 25	25–75	> 75
Leukocytes infiltration	Intense	Similar to control	No leukocytes

temperature thus reducing the various nutritional needs, the cellular metabolic rate and metabolite production [24].

Temporary storing tissue at freezing or refrigerated temperatures is a very common short time technique that are used for preserving tissues before using them in a clinical or experimental setting. This kind of storage could have some effects on the mechanical properties of the stored tissues [15].

It is generally accepted that a primary mode of injury experienced by the tissues that are subjected to subzero temperatures is associated with the transformation of intracellular water from the liquid to the solid crystalline state, *i.e.* intracellular ice formation [34]. All approaches for cryopreservation aim to overcome the biological, chemical, mechanical and thermal stresses of ice crystal formation and recrystallization associated with subzero cryopreservation which affect deeply on the viability and efficiency of tissues used for transplantation and grafting.

To keep good tissue viability, tissue banks around the world have their own techniques for storage and preservation of skin grafts, but the most useful procedures that are commonly in use include: refrigeration at 4 °C, glycerolpreservation with high concentration of glycerol, and cryopreservation at subzero temperatures [4,22,23].

Antifreeze proteins (AFPs) have the ability to protect many organisms from freezing in subzero temperature environments. Since their discovery 45 years ago [56], there has been a growing interest in their various cryoprotective ability as a result of their well-known to sustain the super-cooled state of body fluids through preventing growth of ice. Secondly, they possess capability of inhibiting recrystallization, and thirdly, antifreeze proteins can assist as plasma membrane protections when temperature is low [11,26,54]. Whereas, Anti-freeze peptides are kind of antifreeze molecules that are usually derived from native AFPs and used in various scientific applications. They consist of small number of amino acids not less than 25 comparable to other types of AFPs that are frequently contain increased numbers of amino acids. This kind of antifreeze agents can act as useful antifreeze tool to inhibit ice crystals formation, decrease recrystallization, and reduce thermal hysteresis (TH) value in various scientific applications. The benefits of these peptides over large proteins are their simpler structure, the relatively easy synthesis procedure, reduction of the TH value, enhancement and sustain cold tolerance for a short period of time [38,49].

Afp1m is peptide fragments derived from the parent AFP1 of Antarctic yeast known as Glaciozyma Antarctica (G. Antarctica). This yeast has eight different genes that express various types of AFPs; only one AFP gene has been completely characterized (UniProtKB accession code D0EKL2). The predicted secondary structure of G. Antarctica AFP consisting of four α-helices and three β-strands. The α-helical region of the native AFP has been suggested to be responsible for the inhibition of

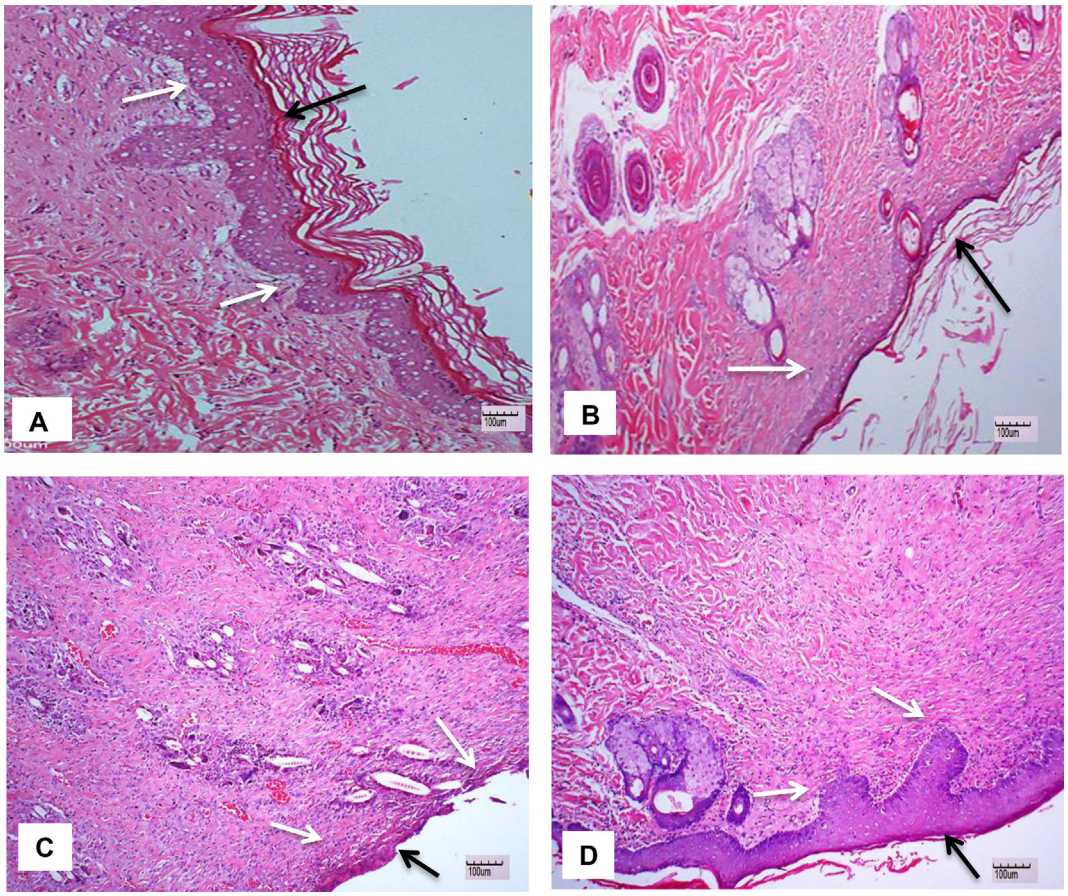

Fig. 1. Photomicrographs showing the skin architecture particularly the epidermal integrity (black arrow) and dermal-epidermal junction (white arrow) of skin graft in group 1, fresh skin autograft from the same site (A); group 2, fresh skin autograft from different site (B); group 3, skin autograft cryopreserved with glycerol for 72 h (C); group 4, skin autograft cryopreserved with Afp1m for 72 h (D) (H&E).

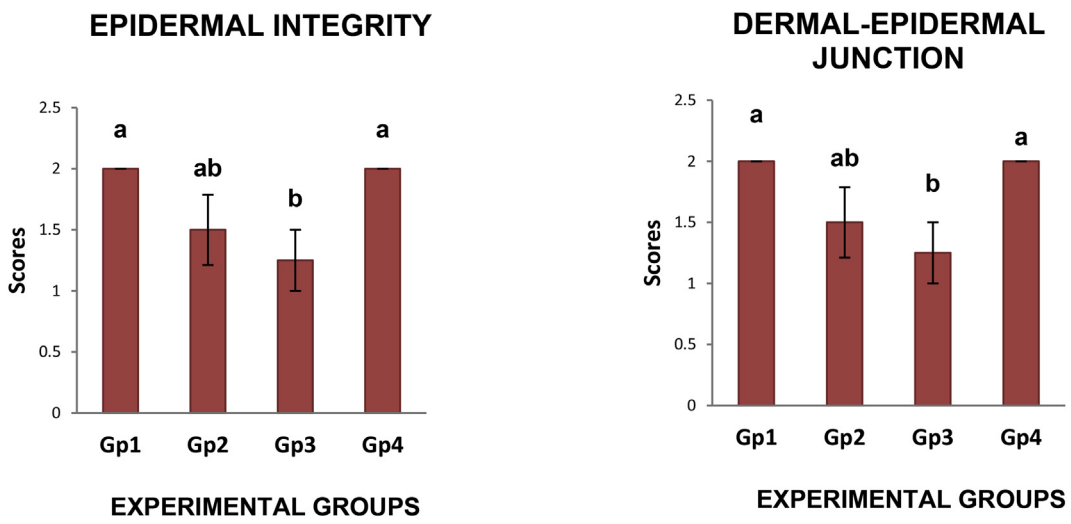

Fig. 2. The comparison in epidermal integrity and dermal-epidermal junction in skin grafts 21 days post transplantation among four experimental groups. Gp1-immediate skin autografts from the same site; Gp2 -immediate skin autografts from different site; Gp3-skin autografts cryopreserved with glycerol for 72 h; Gp4 -skin autografts cryopreserved with Afp1m for 72 h. Means with the different letters indicate significant difference (One-way ANOVA). Data were presented as means ± S.E.M.

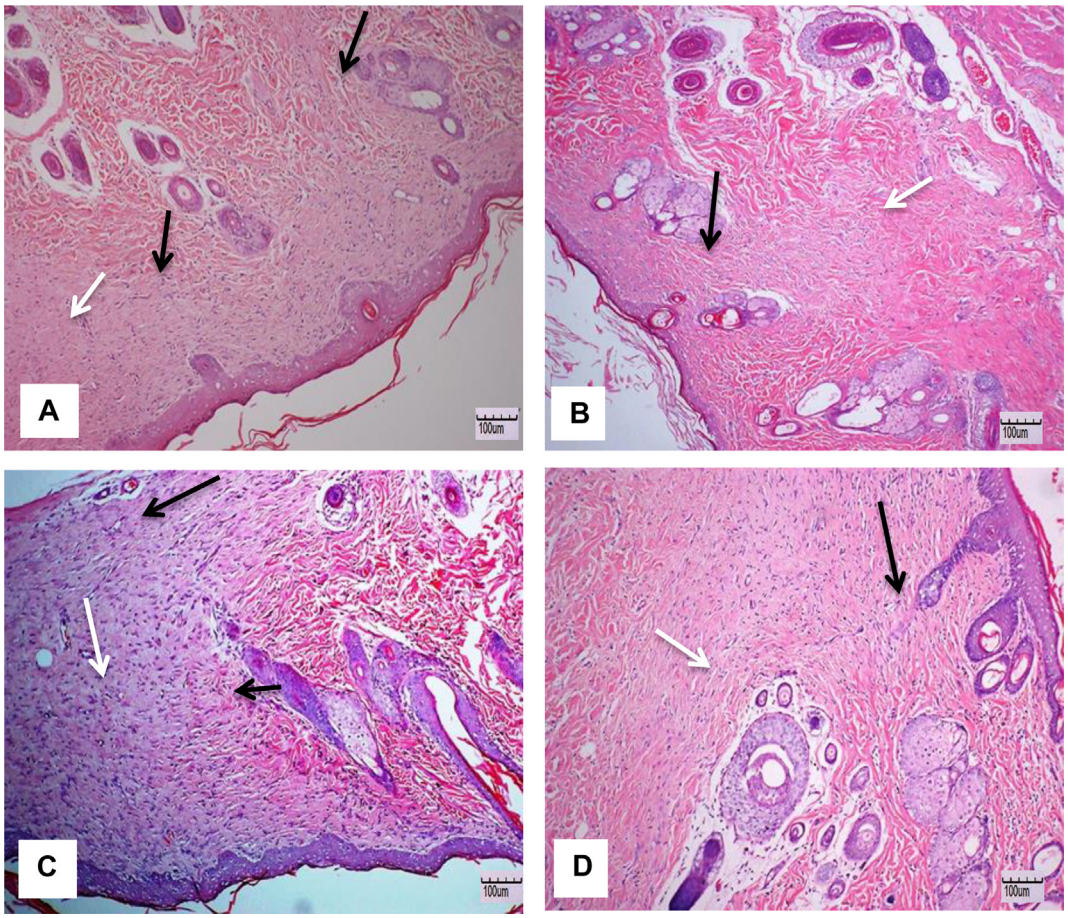

Fig. 3. Photomicrographs comparing the graft adherence to the underlining bed in various skin grafts after 3 weeks transplantation. Complete disappearance in interface between the graft and the wound bed (arrow) with replacement by fibroblasts and collagen deposition under the graft (white arrow) under the graft. A-immediate skin autografts from the same site; B- immediate skin autografts from different site; C- skin autografts cryopreserved with glycerol for 72 h; D-skin autografts cryopreserved with Afp1m for 72 h (H&E).

ice crystal growth. Afp1 is the first helix sequence derived from the protein AFP1. Afp1m is the modified version peptide intended based on the sequence of peptides 1 by replacing Leu19 with Glu [49].

The cryoprotective properties of Afp1m on various dermal, epidermal and hypodermal histological criteria of skin, as well as, its effects on mechanical properties of skin grafts following transplantation were studied and discussed in this article.

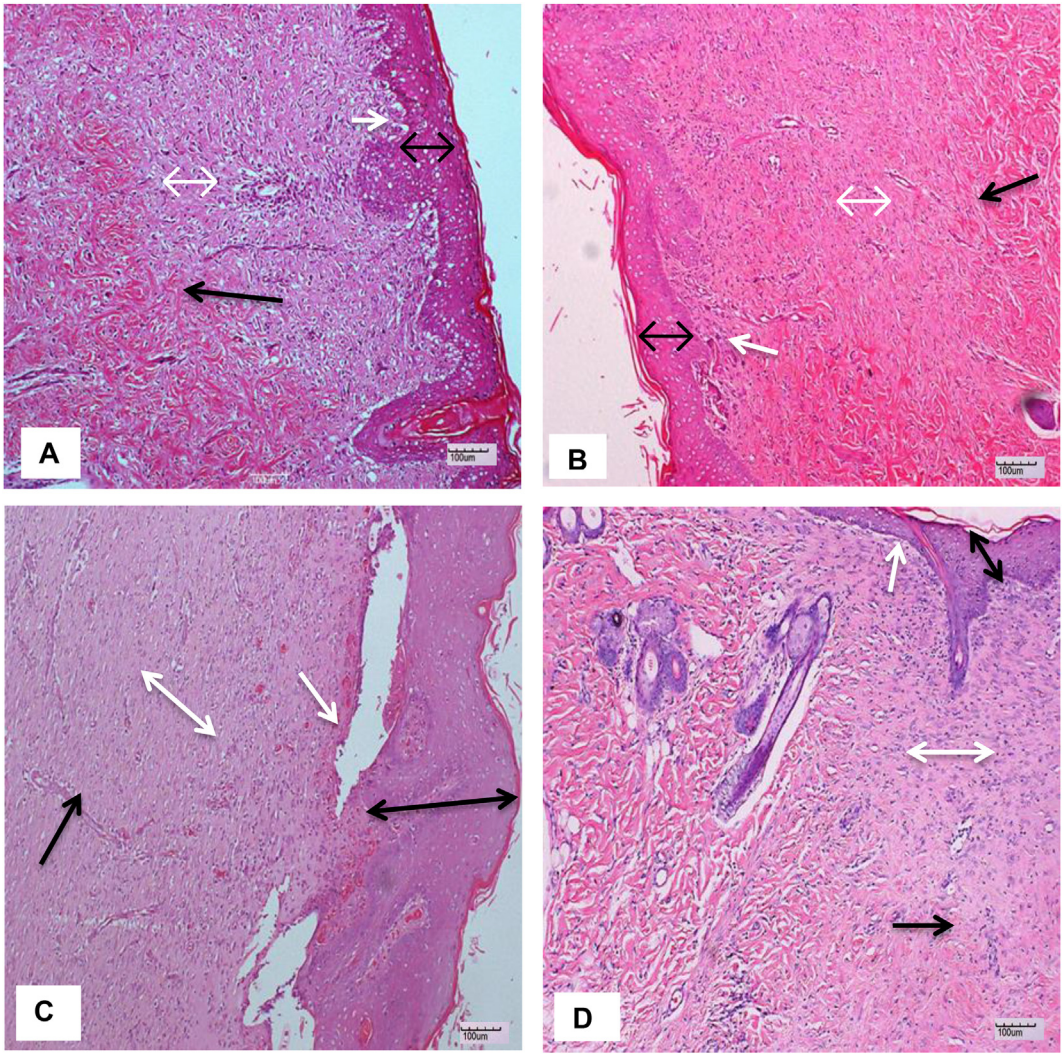

Fig. 4. Photomicrographs showing epidermal hyperplasia (black double-head arrow), fibrosis under epithelium with increase in connective tissues (white double-head arrow) besides decrease in adnexal structures (black arrows), and rich capillary network (white arrow). A-immediate skin autografts from the same site; B-immediate skin autografts from different site; C- skin autografts cryopreserved with glycerol for 72 h; D-skin autografts cryopreserved with Afp1m for 72 h. (H&E).

2. Materials and methods

2.1. Experimental design

Thirty four female rats with an average weight of 208 ± 31 g (mean ± SD), were used for histological and mechanical evaluation of skin grafts following 3 weeks skin transplantation. Rats were anesthetized with an intramuscular injection of Ketamine (70 mg/kg) and xylazine (7 mg/kg). Rats (n = 24) were equally divided into four groups: Group (i): immediate fresh skin auto graft (onto same wound site) (n = 6), Group (ii): immediate fresh skin auto graft (onto different wound site) (n = 6), Group (iii): delayed skin auto graft using cryopreserved glycerolized skin (n = 6), and Group (iv): delayed skin auto graft using Afp1m cryopreserved skin. Rounded shaped full-thickness skins with the diameter of 1.5–2.5 cm were excised from the backs of rats. None-cryopreserved (group 1 & 2) or cryopreserved auto skin graft (group 3 & 4) were placed onto the wound area and stitched. Grafts were covered with Vaseline gauze (Shaoxing Zhende surgical dressing Co. Ltd. Shaoxing, China) and adhesive bandages. The bandage was changed every 2–3 days until the end of experiment [32]. At the end of 21 day all rats were humanely sacrificed using 200 mg/kg intraperitoneal injections of sodium pentobarbitone (Dolethal®, Vetoquinol S.A. France) following the University institutional animal care and use committee approval for skin samples collection and subsequent histological and mechanical studies.

Additional ten rats (n = 10) were used for non-transplanted cryopreserved and non-cryopreserved skin strips comparative mechanical test. Stripes of skin samples of 50 mm in length and 5 mm in width [53,61] that are parallel to long axis were excised from the middle of the rats' back.

2.2. Preparation and cryopreservation of skin grafts

Immediately following the collection of the circular skin grafts from the rats, the samples were soaked in normal saline in a sterile container with 1% penicillin/streptomycin. Streptomycin 100 µg/ml and penicillin 100 IU/[7,10,36]. After that skin grafts were transferred into a sterile cryogenic 5 ml vial containing 5 mg/ml of Afp1m or 85% of glycerol [27,39] separately and were cryopreserved at −4 °C for 72 h. Samples were thawed for 2 min by immersion into a 37 °C water path [22] then repeatedly washed in physiological saline for up to 60 min [37,44] prior to the transplantation as detailed below. Afp1m of 5 mg/ml used to cryopreserve skin strips for 72 h at −4 °C had exhibited the best cryoprotective properties during other *ex vivo* study (unpublished data).

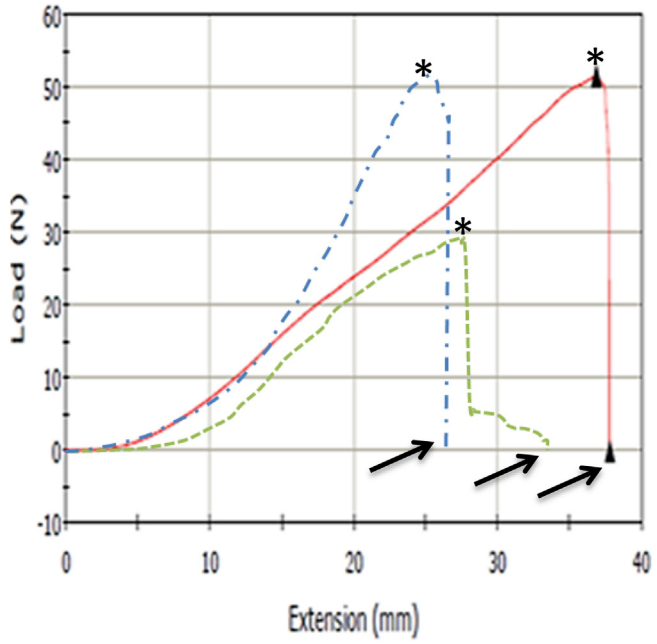

Fig. 5. Graphical presentation for the comparison in strain-stress curve of non-transplanted skin to assess the maximum load of the non-cryopreserved and cryopreserved skin grafts before transplantation. Dash line-non cryopreserved skin; dash dot line- Glycerol cryopreserved skin for 72 h; solid line- Afp1m cryopreserved skin for 72 h. Asterisk shows the maximum bearing point; black arrow shows the ultimate modulus of elasticity (mm). Graphs and data were generated using computer system linked with the Bluehill® material testing software package for Universal Testing Systems v: 2.8 (Instron®, 2008).

2.3. Surgical procedure

The rats were kept on the surgical table over a heating pad in a sternal recumbency state (Conair Corp, East Windsor, USA) to maintain the body temperature. Circular shaped full-thickness skin grafts with size of 1.5–2.5 cm in diameter were surgically harvested from the back of each rat with a no.15 scalpel blade [14,62].

In group 1, the harvested skin graft was immediately sutured onto the same wound defect of each rat by 4-0 nylon sutures (ETHILON, Johnson & Johnson Medical N.V., Belgium). In group 2, harvested skin graft was immediately sutured onto a new wound defect of the same size created adjacent to the first one. The first wound defect was immediately closed by primary closure using simple interrupted suture pattern. In group 3, the harvested skin graft was cryopreserved for 72 h at −4 °C in glycerol while the wound defect was immediately closed by primary skin closure using simple interrupted suture pattern. Three days later, a new circular full-thickness wound defect on the back of the rat was created and the cryopreserved skin graft was sutured onto it. In Group 4, the same steps as in group 3 were repeated except the skin was cryopreservated with 5 mg/ml of Afp1m instead of Glycerol. Grafts were covered with Vaseline gauze (Shaoxing Zhende surgical dressing Co., Ltd., Shaoxing, China) and adhesive bandages [14,22].

2.4. Post-operative care

Animals were individually housed following surgery to minimize trauma and interference with surgical sites. They were observed daily by monitoring personnel for signs of wound complications, anorexia, pain or dehydration [46]. Tramadol HCl (50 mg; Biolab Co., LTD, Thailand) was administered subcutaneously at 4 mg/kg once as a post-operative analgesia.

2.5. Evaluation of skin grafts post transplantation

2.5.1. Histological evaluation of skin grafts healing

Following rat's sacrificed at day 21. The skin grafts were placed in containers with 10% neutral-buffered formalin for 24 h for fixation. Specimens were fixed, embedded in paraffin, sectioned, stained with hematoxylin–eosin, and examined with light microscopy [3].

Each histological section was assessed microscopically for evaluation of skin grafts microstructure features using a scoring system described in (Table 1) [4,7].

2.5.2. Biomechanical evaluation

The mechanical properties (maximum load, tensile stress and strain at maximum load, and elasticity) for skin grafts cryopreserved with 85% of glycerol or 5 mg/ml of Afp1m for 72 h at −4 °C before and after 21 days transplantation were examined.

Stripes of dorsal skin samples that are parallel to long axis starting from thoracic area downwards to the pelvic area were excised from the midline rat back at the site of skin grafts. All these stripes had the same geometric dimensions: 50 mm in length and 5 mm in width [53,61]. Then the skin strips were stored at a room temperature in 0.9% normal saline that has been changed up to 4 times until the time of examination (no longer than 5 h) [61], prior to the biomechanical tests. While the normal non transplanted skin strips were first kept in cryopreservation with Afp1m and glycerol for 72 h at −4 °C, then sample were thawed for 2 min by immersion into a 37 °C water path [22] then at room temperature until fully thawed. Then they were stored at a room temperature in 0.9% normal saline just as described above. The samples were tested using an Instron machine (Model 3365) with 5kN capacity (Instron®, Corp, USA).

Uniaxial stretching test was carried out on stripes of skin grafts from each experimental group for the following properties:(i) maximum load (N), (ii) the tensile stress at the maximum load (MPa), (iii) the tensile strain (ultimate strain %) at maximum load, and (iv) the ultimate modulus of elasticity (maximum extension) mm [19,53,61].

The uniaxial tests involved stretching of the samples at a constant rate of 5 mm/min until they broke [61]. For this purpose, the samples were fixed tightly between the two holding grips of the machine, cranial–caudal orientation was preserved and the skin graft area of the experimental groups was centrally positioned within the testing unite and the ends of each skin stripe were fixed between sand-paper to prevent slipping, the machine preloaded to 0.005 N, held for 120s. Then, a constant increase in force was subjected until the specimens tore and can no longer hold more tension [9]. The data were generated in the computer system connected with the Bluehill® material testing software package for Universal Testing Systems v: 2.8 (Instron®, 2008).

2.6. Statistical analysis

Data obtained from the results were analyzed with SAS (V 20.00, Inc., Chicago, IL, USA). All procedures were performed at 95% confidence level. The score data was evaluated using One-way ANOVA. To determine significance between experimental groups, Duncan's multiple range test were performed and considered significant when P values were less than 0.05 ($P < 0.05$).

3. Results

3.1. Histological results

Post transplantation skin graft take in rats was histologically examined and evaluated at the end of day 21. The results showed that the tissue architecture especially the epidermal integrity and dermal-epidermal junction of fresh and Afp1m cryopreserved skin grafts in groups 1, 2, and 4 exhibited better histological appearance, good preservation of tissue architecture, and structural integrity than glycerolized skin

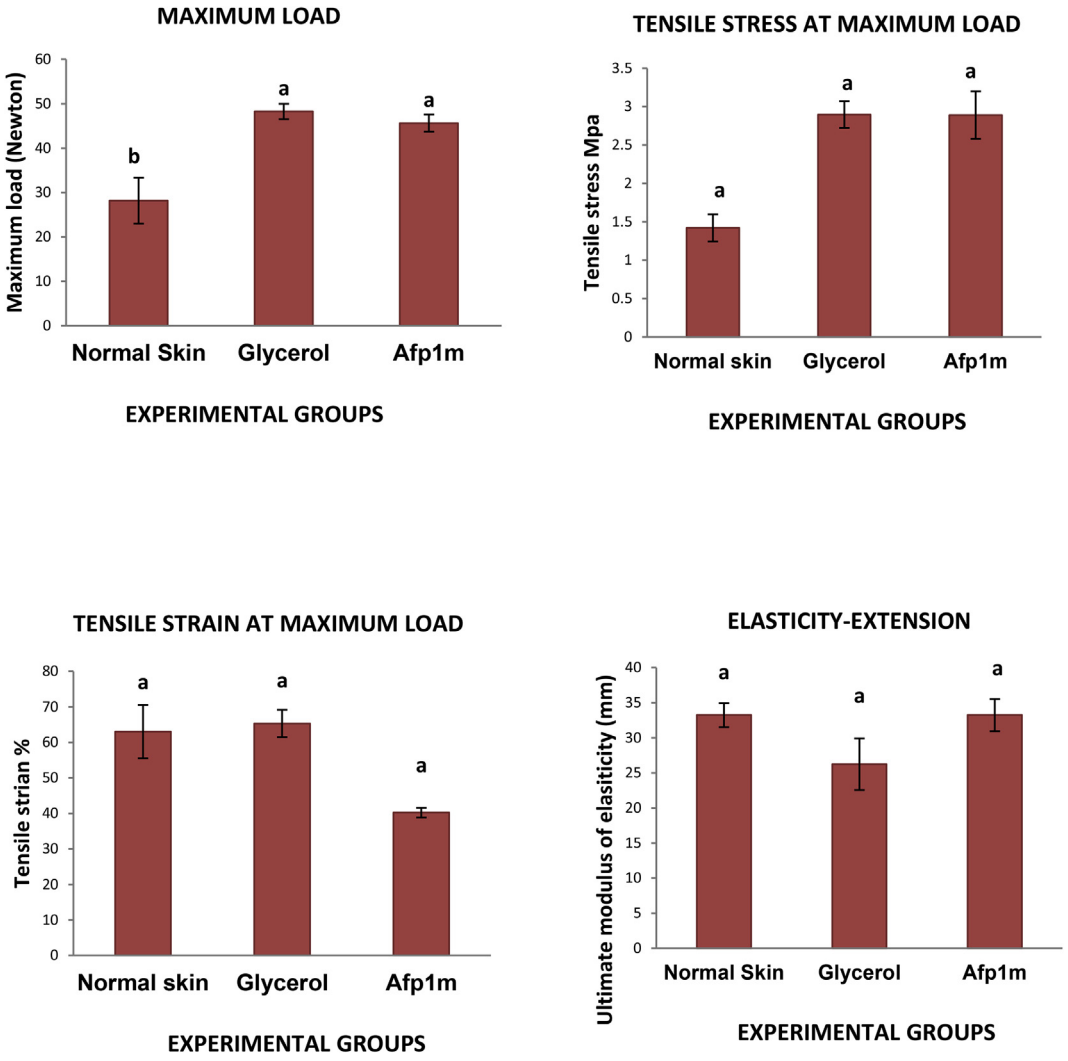

Fig. 6. The mechanical properties of normal skin, Glycerolized and Afp1m cryopreserved skin strips before transplantation. Data were presented as means ± S.E.M. Means with the different letters indicate significant difference.

grafts (group 3). The epidermal integrity was partially loss in glycerolized skin grafts as compared to both fresh and Afp1m skin grafts. The dermal-epidermal junction also showed partial discontinuity in transplanted skin grafts of glycerolized–cryopreserved as compared to both fresh and Afp1m cryopreserved skin grafts (Fig. 1). Statistical analysis showed significant differences among these groups in both epidermal integrity and dermal-epidermal junction (Fig. 2).

After 3 weeks of transplantation, the histology revealed no significant difference in collagen fiber organization, fibroblast presence, and leukocytes infiltration within the grafted area for all groups. There was good adherence between transplanted skin graft and wound beds for all groups. Complete disappearance of histological demarcation between graft and the wound bed with fibroblasts and collagen deposition under the graft was observed after three weeks (Fig. 3).

No statistical significant difference in collagen fiber organization, fibroblast presence, leukocytes infiltration, and graft adherence observed among all groups. Besides that, there were epidermal hyperplasia that was abundant and more extensive in glycerolized skin grafts comparable to fresh and Afp1m groups. In addition, there was fibrosis under epithelium with increase in connective tissues, decrease in adnexal structures. The capillary network undersurface of the skin grafts was presence, a well-developed epidermis and the collagen was loosely arranged and their fibers formed a sub epidermal network in all groups (Fig. 4).

3.2. Mechanical results

Assessment the effect of 72 h of subzero cryopreservation with Afp1m and glycerol on the mechanical properties of the skin was performed using comparison in strain-stress curve of the normal non-transplanted skin. It displayed that the maximum load (Newton) for normal non-cryopreserved, Afp1m, and glycerol cryopreserved were 28.17 ± 5.17, 45.63 ± 1.92, and 48.26 ± 1.73 respectively. While the ultimate modulus of elasticity (mm) were 24.25 ± 1.79, 33.25 ± 2.28, and 26.25 ± 3.68 respectively (Fig. 5).

The statistical results showed that there was a significant difference ($P < 0.01$) in maximum load between the non-transplanted fresh skin and the cryopreserved skin graft groups. However, there was no significant difference in tensile stress at maximum load, tensile strain at maximum load, and ultimate modulus of elasticity among all groups (Fig. 6).

The comparison in strain-stress curve of the transplanted skin after 3 weeks transplantation among both fresh, glycerolized and Afp1m groups exhibited that the maximum load (Newton) for these groups were 19.94 ± 0.51, 19.73 ± 2.86, 26.68 ± 3.20, and 23.39 ± 3.49 respectively. While the ultimate modulus of elasticity (mm) were 31.25 ± 2.95, 30.75 ± 1.54, 27.25 ± 3.09, and 36.5 ± 2.25 respectively (Fig. 7).

The data analysis showed no significant difference in maximum

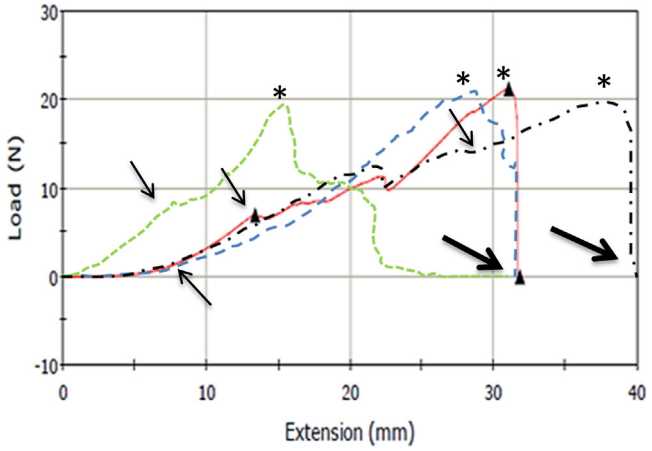

Fig. 7. Graphical presentation the strain-stress curve to assess the maximum load ability of skin grafts after 21 days transplantation. Gp1 (long dash line)-Immediate skin autografts from the same site; Gp2 (solid line) - Immediate skin autografts from different site; Gp3 (dash line) - Skin autografts cryopreserved with glycerol for 72 h; and Gp4 (dash dot line) - Skin autografts cryopreserved with Afp1m for 72 h. Black narrow arrows indicate the weakest point of union between skin grafts and wound beds that indicate the initial beginning of rupture; thick arrow shows the ultimate modulus of elasticity (mm); asterisk shows the maximum bearing point between the skin grafts and wound beds at the start of complete rupture. Graphs and data were generated in the computer system linked with the Bluehill® material testing software package for Universal Testing Systems v: 2.8 (Instron®, 2008).

load, tensile stress at maximum load, tensile strain at maximum load, and ultimate modulus of elasticity among the experimental groups (Fig. 8).

4. Discussion

The histological results showed that the tissue architecture specifically the epidermal integrity and dermal-epidermal junction [16] of fresh and Afp1m cryopreserved skin grafts in group was well preserved than glycerolized skin grafts. The integrity of epidermal layer was moderately damaged in glycerolized skin graft as compared to both fresh and Afp1m skin grafts. There was also a somewhat separation in the dermal-epidermal junction of the glycerolized–cryopreserved transplanted skin grafts as compared to that in both fresh and Afp1m cryopreserved skin grafts in groups. The statistical analysis of the histological features also showed that there were significant differences in both these two histological criteria among the four experimental groups. These significant differences in epidermal integrity and dermal-epidermal junction among the experimental groups could be explained by the fact that the skin grafts in group 1 and 2 were fresh in addition to the immediate transplantation to the wound bed. This produced better histological scoring as the fresh skin graft is always considered the gold standard skin substitutes [6]. For the Afp1m cryopreserved skin grafts, the finding could explain the protective effects of Afp1m that preserve the structural integrity of skin grafts by inhibiting both ice crystal formation, and recrystallization besides acting as a plasma membrane protector [54]. All these properties worked together in preventing the damage of the cell and skin architecture when a temperature falls to subzero temperature. The cryoprotective ability of AFPs to preserve the histological criteria following transplantation of other organs rather than skin has also been reported previously [1,40,41].

The skin architecture of the glycerolized-cryopreserved skin grafts in both epidermal integrity and dermal-epidermal junction were poor preserved and more destroyed than both fresh and Afp1m cryopreserved skin grafts. This could be clarified through the damaging effect of cryopreservation of these skin grafts at −4 °C. Subzero cryopreservation led to loss of about 95% of intracellular water, and a significant elevation of electrolyte concentrations in both extra and intracellular media. As well as, ice crystal formation in the intracellular spaces as glycerol do not have the strong ability to prevent ice crystal formation as Afp1m do, so all these factors deform and compress cells and even destroy skin grafts structures of glycerolized skin grafts [2].

The well-known assumption about glycerol ability to dehydrate the skin by osmosis and diffusion out of the cells and skin matrix was refuted by a study that instead suggested the converse occurs i.e. glycerol enters the skin and sequesters the water [47]. Following using of high concentration of glycerol for banking of non-viable skin allografts, the skin still contained about 11% w/w-tissue of water [30]. Nevertheless, the dehydration following the glyserolisation process could to some extent adversely affect the microstructure of the skin graft.

The well preserved skin architecture especially in epidermal structure and integrity of the Afp1m cryopreserved skin grafts as compared to glycerolized cryopreserved skin grafts makes the Afp1m transplanted skin grafts could perform better in most of their crucial roles [7,8]. As a result of the presence of a good preserved tissue integrity, it could be assumed that these Afp1m cryopreserved skin grafts will display an effective role in reduction the absorption of various chemicals from the external environment, controlling and limitation the passive loss of water from the body, and defensing the body against the microbial infection. This also preserves their ability in protection the body from the effects of ultraviolet light by the presence of melanocytes in addition to its role in giving the skin its color. The well preserved epidermal layer also could play a vital immunity role as result of the presence of Langerhans cells which are dendritic immune cells that considered as the epidermal immune barrier besides its role in contact allergy [55].

At the end of day 21 after transplantation, the histological results showed no significant difference in plane of separation between skin graft and wound bed among fresh, Afp1m cryopreserved, and glycerolized cryopreserved skin grafts in all treatment groups. There was a good graft adherence to the underlining bed in all these groups shown by to complete disappearance in the line of separation between the graft and the wound bed with replacement by fibroblasts and collagen deposition under the graft on day 21 [50]. The well adhesion between the skin grafts and the wound bed is crucial for their successful take [7].

There was also no significant difference in leukocytes infiltration, and collagen fiber organization in all experiment groups. The presence of inflammatory cell infiltration within the transplanted skin grafts in all experimental groups could be considered as a normal histological phenomena following transplantation of either split or full-thickness skin grafts [12,28]. Their presence may be due to the host response that takes place at the junction between host tissue and skin graft. Whereas, the existence of dense collagen fibers tissue could be occurred as a result of the presence of these inflammatory cells. These cells induced extensive inflammatory reaction that may contribute directly or indirectly to a robust fibrotic response [12]. It is also well known that as wound healing progresses; collagen fiber density increases [45].

Besides the above microscopic criteria, the histopathology of skin grafts from all experimental groups indicated that there was an epidermal hyperplasia. This hyperplasia [21] was more abundant with glycerolized cryopreserved skin grafts comparable with those in fresh and Afp1m cryopreserved skin grafts. This histological phenomena suggests relatively low-quality of glycerolized skin grafts. The presence of hyperplasia may be due to excessive synthesis of collagen, decreased matrix degradation or both [12]. Other microscopic criteria include fibrosis under epithelium with increase in connective tissues besides decrease in adnexal structures. The presence of all these histological features could be considered as a normal consequence that is usually take place during the full-thickness skin grafts [28]. It had also been noticed the presence of capillary network undersurface of the epidermis [12.60] in this study. The presence of these good nutrient blood vessels could explain the well adherence and the complete disappearance of the separating plane between the skin grafts and the wound bed at the end of week 3 of grafting in all experimental groups. As a result of presence

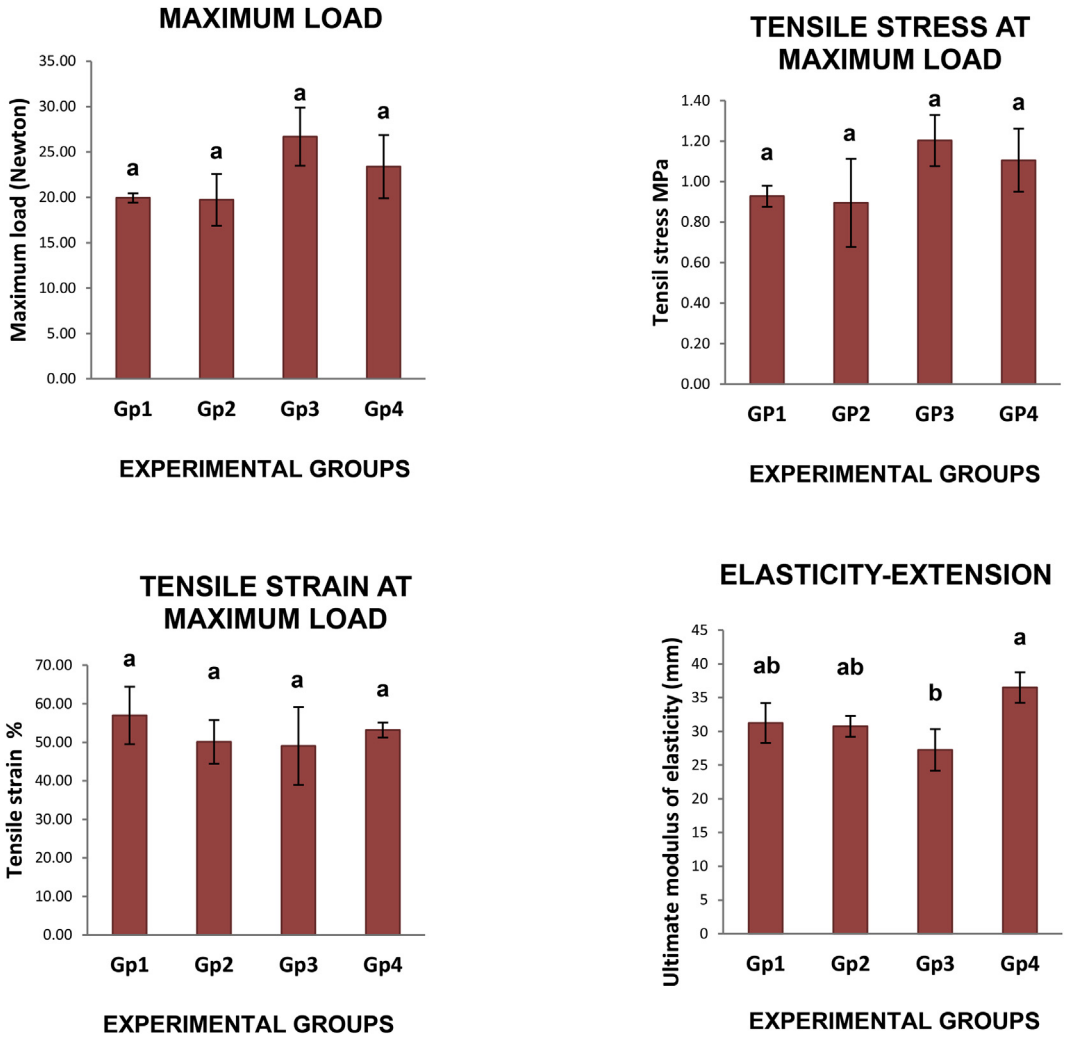

Fig. 8. The mechanical properties of transplanted skin grafts among experimental groups at the end of 21 days. Gp1-immediate skin autografts from the same site; Gp2-immediate skin autografts from different site; Gp3-skin autografts cryopreserved with glycerol for 72 h; Gp4-skin autografts cryopreserved with Afp1m for 72 h. Data were presented as means ± S.E.M. means with the different letters indicate significant difference.

of a well nutrient capillary network after 21 days of skin grafting there was a well-developed epidermis. There was almost clearly defined epidermis with many of the intact morphological features. Dermal substitute degraded gradually and fibroblast lined up in order with infiltration of inflammatory cells, the collagen was loosely arranged and their fibers formed a sub-epidermal network [60]. The presence of these histological criteria may be reflected the normal consequence that is usually happen during the full-thickness skin grafts [28].

The temporary storing of tissue at freezing or refrigerated temperatures is a very common short time technique that are used for preserving tissues before using them in a clinical or experimental setting. This kind of storage could have some effects on the mechanical properties of the stored tissues [13,15,17,29,35,42,51,52,58]. Mechanical evaluation of skin properties can be done by using various stress-strain models [33]. This study showed no significant difference in the mechanical properties including maximum load, tensile stress and ultimate strain at maximum load, and ultimate modulus of elasticity among the four experiment skin grafts groups. This could be explained by the presence of more fragile tissue components during skin grafts healing process. These fragile tissue components within experimental groups were more dominants than other tissues that made these healed skin grafts more sensitive to possible adverse effects. As a result of this fragility, skin graft wounds in all groups broke partially from the weakest point of adhesion between the skin graft and wound bed as a result of lower wound strength and extensibility [18]. This rupture that happened between skin graft and wound bed during uniaxial stretching test could be explained by the fact that collagen fibers in the healed scar tissue never become as organized as in the intact dermis [57]. Besides that, the breaking strength after healing never equals the strength of the skin. Breaking strength during the first week is only 3%, and then it increases up to 20% after 3 weeks. While it reaches up to 80% of its uninjured counterpart at 3 months later after wounding, but never reaches 100% [31]. In addition, the injured wound is remodeled over a long period of time, affecting its mechanical behavior [25]. The stabilization and net deposition of collagen fibers in the wound area are the main controlling factors in the mechanical properties of a healing wound [32].

The mechanical properties especially extensibility elasticity modulus of the Afp1m cryopreserved skin grafts was significantly different from glycerolized cryopreserved skin grafts. This could be due to its ability to inhibit ice crystal formation extracellular thus preventing the damage of extracellular matrix. On the other hand, the formation of ice crystals within the extracellular matrix of the glycerolized cryopreserved skin grafts led to damaging effects on these matrix causes the decreased in the their mechanical properties [20,59]. The formation of ice within extracellular matrix would cause damaging effects to this matrix leading to altering the mechanical characters of the tissue. The ability of Afp1m to inhibit ice crystal formation and to prevent re-

crystallization made the Afp1m cryopreserved skin grafts possessing the highest extensibility elasticity modulus [59]. While the glycerolized group showed the least elasticity modulus as compare to fresh and Afp1m groups. This finding could be due to damaging effects of ice crystal formation during cryopreservation at −4 °C [48].

The present study also showed the cryopreservation of skin with Afp1m had possible beneficial effects on the mechanical properties of the skin that make it stronger in comparison with the normal non cryopreserved skin, before and after skin transplantation. The current result showed there was significant different in mechanical properties between the normal skin and Afp1m cryopreserved skin especially in the maximum load. However, there was no significant different in other mechanical properties of the Afp1m cryopreserved skin after transplantation in comparison of the fresh skin transplantation. With each different group was subjected to the exactly similar rehydration treatment process, any net effect caused by the residual cryopreservative compound on the mechanical properties in cryopreserved skin groups could be very minimal.

On the other hand, the maximum load was much higher in non-transplanted normal skin stripes as compared to skin graft wound stripes. This finding could be due to the difference between the sources of skin strips of both experimental groups. The first group, their skin stripes were taken from the skin of the back in the normal animals. While the second group, their skin stripes were collected from the skin grafts wounds at the back of each animal of the treatment groups. It is well known that wound strength and extensibility are lower than those found in normal skin [18].

5. Conclusion

Afp1m had beneficial protective cryopreservative ability for subzero skin tissue cryopreservation that preserves the tissue microarchitecture, viability and function following transplantation. The preservation of most histological features of the skin tissue as well as the mechanical properties of normal skin even after transplantation makes Afp1m at 5 mg/ml can serve as suitable sole cryopreservative agent for skin tissue short term storage at −4 °C for 72 h.

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