Theoretical and Experimental Cryobiology

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STERILIZATION AND LOW TEMPERATURE EFFECTS ON REGENERATIVE POTENTIAL OF HYALURONIC ACID

Due to its physical properties and pharmacological activity, hyaluronic acid (HA) has considerable potential for use in cryobiology and cryomedicine. The aim of the study was to create a method for sterilizing aqueous solutions of HA that does not reduce its regenerative properties, and to study the effect of low temperatures on their preservation. For the sterilization of aqueous solutions of HA, a gentle sterilization regimen — tyndallization — was proposed, which at the same time ensures the sterility of the solutions and does not affect their regenerative properties. The effects of tyndallization and low temperatures on the preservation of the regenerative properties of 1 and 2% aqueous solutions of HA of different molecular weights: low molecular weight (LMW HA) (<100 kDa) and high molecular weight (HMW HA) (>2000 kDa) was studied in an animal model of excision wound healing. It has been shown that low temperatures do not change the regenerative properties of HMW HA and LMW HA (even in the thermocycling mode), which opens up wide possibilities for use in cryobiology and cryomedicine.

Key words: *hyaluronic acid, low temperature exposure, preservation of regenerative properties, sterilization.*

Hyaluronic acid (HA) is known to be one of the universal and unique molecules in nature, present in all living organisms including bacteria [9, 11, 26, 33]. HA is a linear glycosaminoglycan composed of repeating disaccharide units, such as D-glucuronic acid and N-acetyl-D-glucosamine, bound by β-1,4 and β-1,3 — glycosidic linkages [2, 9, 11, 23, 26].

The average HA amount in adult human body is about 12—15 g, and it is mostly concentrated in skin, eye vitreous body, umbilical cord, synovial fluid of joints, intervertebral discs, embryonic mesenchymal tissues; it is also present in heart valve, lungs, tendon sheath, aorta and prostate [2, 9, 11, 26, 28]. In addition, HA plays an important role in fertilization [22].

HA molecule possesses biocompatible, biodegradable, non-immunogenic, non-thrombogenic, hydrophilic characteristics. It participates in a number of physiological and pathological processes and in a wide spectrum of pharmacological activities. In particular, HA was shown to have significant antioxidative properties due to its reaction with oxygen-inclusive free radicals. HA is recognized for its wound repair effect due to the ability to stimulate inflammatory signal promoting in its turn cell proliferation and migration. Hyaluronic acid is also acknowledged for its ability to prevent adhesion and scar formation [31, 36]. HA molecule is widely known for its regenerative characteristics [4, 7, 9, 15, 31, 35, 43], anti-inflammatory [10, 37—39, 41], immune modulating [9, 26, 35], anti-can-

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cerous [2, 9, 19, 29], anti-diabetic activity [13, 30], as well as anti-aging [2, 9, 26], antioxidative, recovering [9, 15, 21, 31, 35] and cosmetic [3, 5, 31, 33] effects.

HA molecule is considered to be mainly extracellular substance; however, it was also found inside aorta smooth muscle cells in perinuclear space during pre-mitosis and mitosis. HA molecule was noted in cytoplasmatic structures as well [2, 8, 12, 18]. However, HA intracellular characteristics are yet completely investigated. There is a theory postulating the HA control in cell proliferation and inflammation [12, 18]. Such fields as esthetic medicine and cosmetology are considered undoubtful leaders in HA application [3, 42].

HA biological functions were found to greatly depend on the polymer molecular weight [6, 32, 33, 38]. High molecular weight HA (HMW HA > > 2,000 kDa) possesses both depositing and antioxidative properties, remains longer in tissues, skin surface and mucous membranes. HMW HA was noted to suppress cell proliferation and migration of substances towards an inflammation location.

Medium molecular weight HA (MMW HA 100—1000 kDa) showed to initiate synthesis of own endogenous HA, to promote wound regeneration [35] and stimulate cell division. HA of such molecular weight is widely used in cosmetic industry, eye drops, remedies for skin burn treatment, to prevent formation of adhesions after surgeries.

Low molecular weight HA (LMW HA < < 100 kDa) penetrates easily into deep skin layers, it is efficiently absorbed into digestive tract, promotes appropriate migration of water and substances. LMW HA was found to stimulate regeneration in blood capillaries. It has been successfully used to treat inflammations in joints and organs of urogenital system. In addition, LMW HA is used during cosmetic procedures and contouring, and is included in health and beauty products.

Recently HA has increasingly become the focus for profound cryobiological investigations, that is necessitated by studying the low temperature effect on HA regenerative properties preservation [1, 25, 27].

HA polymer is known to be a natural linear polysaccharide, which varies from other representatives of this class by the binding level of water molecules. One HA molecule is able to bind water volume in average 10 000 times higher than its own. HA ability to retain water is conditioned by large amount of hydroxyl groups, thereby promoting hydrogen bonds formation. As a result, in addition to high water solubility the HA molecule possesses high viscosity level even at low polymer concentrations. Availability of such HA characteristics encourages scientists to consider the opportunity of creating HA-based cryoprotective media of biological material preservation.

Providing the initial sterility for the components in cryoprotective solutions is notorious to be a fundamental requirement, especially when using cryopreserved biomaterial in practical medicine, as the basement for wound coverage with an amplified regenerative potential.

Creating the proper sterilization method for HA aqueous solutions, as well as studying the low temperature effect on the preservation of HA regenerative characteristics have been the aim of the investigation.

MATERIALS AND METHODS

The investigation protocol (protocol No. 5, November 22, 2023) was approved by the Bioethics Commission of the Institute for Problems of Cryobiology and Cryomedicine of the National Academy of Science of Ukraine (IPCC NASU, Kharkiv). The experiments were carried-out according to the regulations of the Ukrainian Law «On the Protection of Animals Against Cruelty» (No. 3447-IV of February 21, 2006), as well as in conformity with the European Convention for the Protection of Vertebrate Animals Used for Research or Other Scientific Purposes (Strasbourg, 1986).

The experiments were performed in male Balb/C mice of the same age (6 months) and of relatively identical body mass (25—30 g). The animals were kept under standard Animal House conditions at IPCC NASU. The ability to preserve regenerative properties by HA solutions was investigated by using the model of excision wound repair in animals. Human physiology simulation and prognosing certain therapeutic results have been the purpose of such an excision wound regeneration model in animals. Skin shrinkage is known to be the main mechanism of wound regeneration in rodents, while in human being this process is characterized by skin re-epithelization and granulation tissue formation. Concerning this fact, we used splinting to

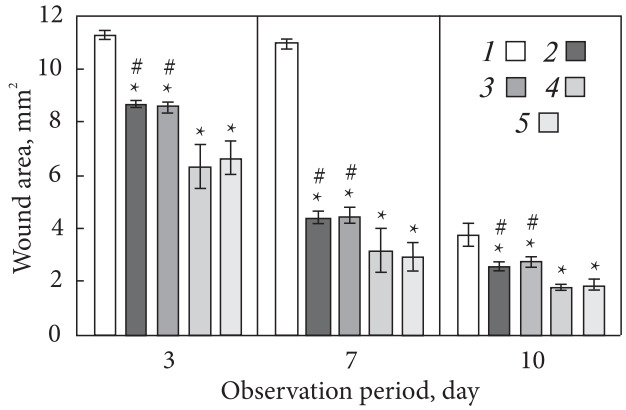

Fig. 1. Effect of HA aqueous solutions on the dynamics of wound surface recovery: *1* — the control; *2* — 1% HMW HA solution; *3* — 2% HMW HA solution; *4* — 1% LMW HA solution; *5* — 2% LMW HA solution; * — differences are significant relatively to the control indices, $p < 0.05$; #— differences are significant relatively to LMW HA indices, $p < 0.05$

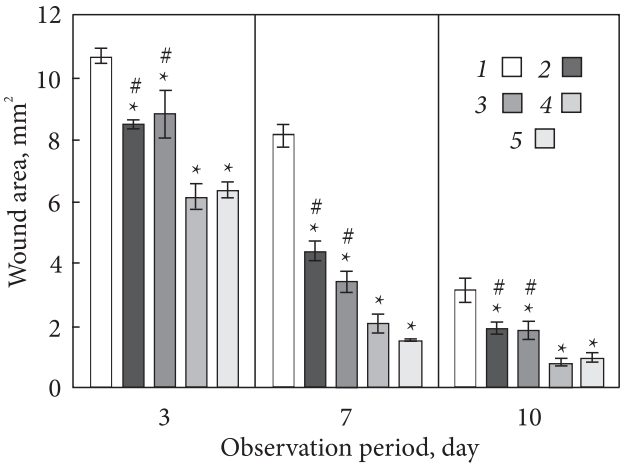

Fig. 2. Dynamics of wound surface recovery following treatment with HA aqueous solution sterilized by tyndallization mode 1: *1* — the control; *2* — 1% HMW HA solution; *3* — 2% HMW HA solution; *4* — 1% LMW HA solution; *5* — 2% LMW HA solution; * — differences are significant relatively to the control indices, $p < 0.05$; # — differences are significant relatively to LMW HA indices, $p < 0.05$

minimize wound shrinkage and thereby stimulate granulation and re-epithelization, in order to approach the mice wound recovery model to that of human. In the experiment we used 1mm width silicone rings, while their inner diameter corresponded to the wound diameter. Splinting ring was closely attached to the wound surrounding area and fixed with medical glue.

For anesthesia «Xylazine» (Alfasan, Netherlands) and «Zoletil-100» (Virbac, France) preparations were intra-abdominally injected in the dosage of 0.1125 and 0.375 mg per animal, correspondingly. Atropine sulfate in the dosage of 0.0025 per mouse was subcutaneously administered 15 min prior to narcosis with the aim of premedication (GNCLS Experimental Plant Ltd, Ukraine). The procedure was followed by shaving the area of the *dorsum interscapularis* and subsequent skin surface treatment with an antiseptic. Two round 5mm excisions were simultaneously accomplished by using skin biopsy scalpel. Wound regeneration dynamics in experimental animals was observed within the 10 days period with daily photo fixation of the recovery. The control group comprised the animals with naturally recovering wounds. Wound surface square was counted using ImageJ 1.54 open-source software (National Institute of Health, USA). The obtained results were shown in graphs, displaying the dependance of the day of complete wound recovery upon the studied parameter. At the end of experiment the animals were back to their normal physiological state.

We used 1 and 2% HA (Bang&Bonsomer, Finland) aqueous solutions both with low (10—100 kDa) and high molecular weight (>2,000 kDa). The solutions were emerged into 5ml volume cryovials (Nunc, USA).

The mice were divided into 5 groups:

- natural wound regeneration (the control group);
- wound treatment with 1% HMW HA solution;
- wound treatment with 2% HMW HA solution;
- wound treatment with 1% LMW HA solution;
- wound treatment with 2% LMW HA solution.

Each group comprised 5 experimental animals. The overall number of 150 mice there was used during the investigation.

Tyndallization as the most gentle heat intermittent sterilization type, was chosen for HA aqueous solutions sterilization. This method was coined by British scientist G. Tyndalle especially for the nutritive media, whose components decompose at temperatures above 100 °C, solutions of vitamins, amino acids, etc. The method is based on heating the fluids up to 70—100 °C, usually during the period of 1 hour, for 3 to 5 times with 24 hours intervals. Such a heating protocol provokes the death of only vegetative cells, leaving the spores viable. Substrate temperature maintaining at an optimum level within 24 hours was shown to stimulate spores growing, and their death during subsequent heating. The following protocols were studied:

• Protocol 1 — the sample was heated at 60 °C for 60 min, thermostated afterwards at 37 ± 1 °C for 24 hours to allow spore forms to grow into vegetative ones. The procedure was repeated 5 times;

• Protocol 2 — the sample was heated at 80 °C for 60 min and subsequently thermostated at 37 ± 1 °C for 24 hours to allow spore forms to grow into vegetative ones. The procedure was repeated 3 times;

• Protocol 3 — the sample was heated at 100 °C for 30 min, subsequently thermostated at 37 ± 1 °C for 24 hours hours to allow spore forms to grow into vegetative ones. The procedure was repeated 3 times.

Aqueous solutions were frozen by emerging the cryovials into liquid nitrogen, and followed by thawing in a water bath at 37 °C. For thermocycling the cryovials with experimental solutions were three times emerged into liquid nitrogen, which was followed by thawing in a water bath using the temperature of 60 °C.

The findings were statistically assessed using «Origin 9.1» software (OriginLab Corp., USA) using Mann-Whitney non-parametric criterion. The data were shown as M ± m, where M represents an average meaning, while m is a standard deviation, the differences at $p < 0.05$ were considered as significant.

RESULTS AND DISCUSSION

The increased attention of researchers, clinicians and cosmetologists to HA and HA-based complex preparations is related to its inherent powerful regenerative properties. Hyaluronic acid is used for the production of injectable preparations, modern biologically active wound dressings and in regenerative medicine under conditions of sterilization of primary HA solutions.

Preservation of HA regenerative properties was later investigated using the model of wound regeneration in experimental animals in comparison with the control (natural wound healing). This index was found to depend on HA molecular weight (HMW HA, LMW HA), HA concentration (1, 2%), sterilisation of HA aqueous solutions using the mentioned earlier tyndallization regimens, freeze-thawing of HA aqueous solutions, especially when using the thermocycling mode.

At the first stage of investigation the manifestation of regenerative characteristics was studied dependently on HA molecular mass (HMW HA, LMW HA) and its concentration (1, 2%). Wound

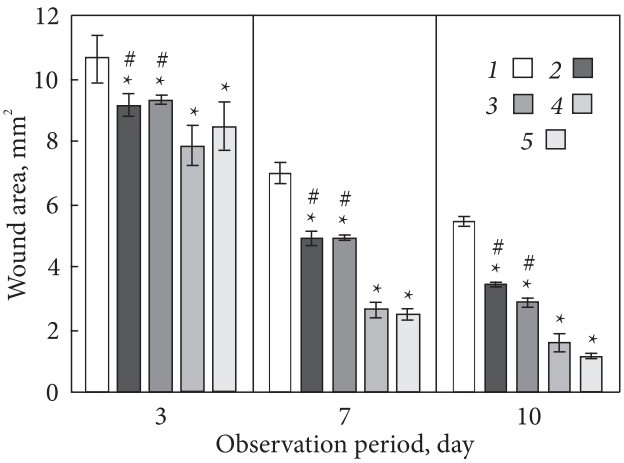

Fig. 3. Recovery dynamics of wound surface treated with HA aqueous solution sterilized by tyndallization mode 2: *1* — the control; *2* — 1% HMW HA solution; *3* — 2% HMW HA solution; *4* — 1% LMW HA solution; *5* — 2% LMW HA solution; * — differences are significant relatively to the control indices, $p < 0.05$; # — differences are significant relatively to LMW HA indices, $p < 0.05$

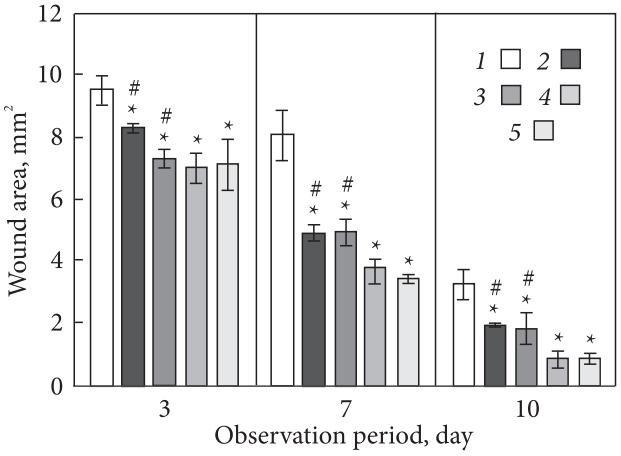

Fig. 4. Recovery dynamics of wound surface treated with HA aqueous solution sterilized by tyndallization mode 3: *1* — the control; *2* — 1% HMW HA solution; *3* — 2% HMW HA solution; *4* — 1% LMW HA solution; *5* — 2% LMW HA solution; * — differences are significant relatively to the control indices, $p < 0.05$; # — differences are significant relatively to LMW HA indices, $p < 0.05$

surface in the control animals was treated with the correspondent HA aqueous solution. Five animal groups (cited earlier) were used in the experiment ($n = 5$ in each group). Dynamics of wound surface regeneration by the 3rd, 7th and 10th days is demonstrated in the Fig. 1.

Analyzing the dynamics of wound regeneration in the control group and in experimental animals, as well as the application of both HMW and LMW HA over the excision wound surface, we may con-

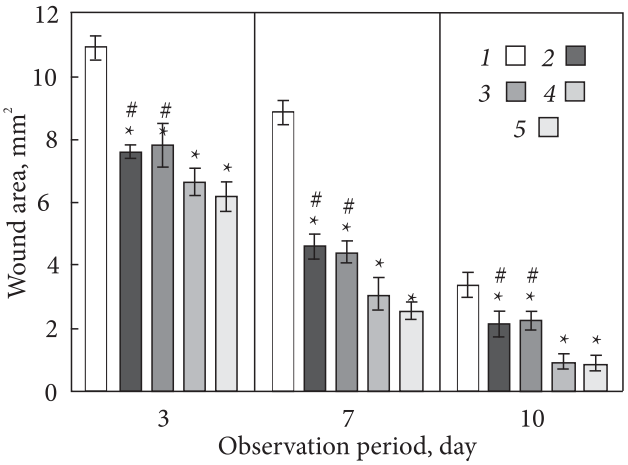

Fig. 5. Recovery dynamics of wound surface treated with HA following freeze-thawing: *1* — the control; *2* — 1% HMW HA solution; *3* — 2% HMW HA solution; *4* — 1% LMW HA solution; *5* — 2% LMW HA solution; * — differences are significant relatively to the control indices, $p < 0.05$; # — differences are significant relatively to LMW HA indices, $p < 0.05$

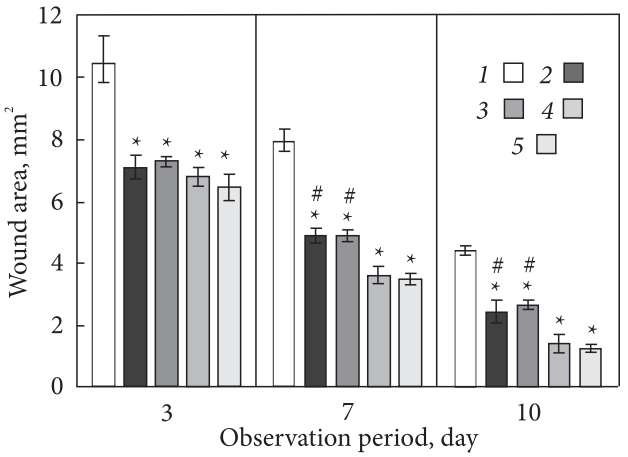

Fig. 6. Recovery dynamics of wound surface treated with HA subjected to thermocycling: *1* — the control; *2* — 1% HMW HA solution; *3* — 2% HMW HA solution; *4* — 1% LMW HA solution; *5* — 2% LMW HA solution; * — differences are significant relatively to the control indices, $p < 0.05$; # — differences are significant relatively to LMW HA indices, $p < 0.05$

clude manifested regenerative properties displayed by HA. Regenerative properties in low molecular HA were noted to be more manifested, presumably because of the presence of small HA chains, characterized by better interaction surface, thus promoting an enhanced regeneration.

Hence, for stable therapeutic results of HA preparations and guaranteeing their regenerative effect, it is important to use HA with the same molecular mass indices, *i.e.*, of low molecular range.

According to the experimental data we may conclude, that both 1 and 2% HA aqueous solution showed relatively the same regenerative properties, while the 2% solution was easier to apply due to its higher viscosity.

At the second stage of investigation, we studied HA regenerative properties following its sterilization. Maintaining the initial HA molecule structure and preserving hyaluronic acid regenerative properties have been the major criteria for selecting the proper sterilization protocol for HA aqueous solutions. There are recommendations on avoiding heating such solutions over 40—50 °C, especially in the case of the further use in regenerative medicine and cosmetology [24, 34]. Concerning this fact, for sterilization of HA aqueous solutions we decided to use the method of tyndallization, which was found to be the most perspective technique for biological material sterilization with the aim of its further industrial use.

Preservation of regenerative properties in both HMW and LMW HA was investigated by using HA 1 and 2% aqueous solutions subjected to the mentioned tyndallization protocols. Five groups of animals were used in the experiment ($n = 5$ in each group). Observation results on the dynamics of wound surface regeneration following treatment by HA aqueous solutions, sterilized according to 1—3 tyndallization protocols, are shown in Figs 2—4, correspondingly.

According to obtained experimental data we may conclude, that tyndallization of HA aqueous solutions (both HMW and LMW HA) does not disorder their regenerative characteristics. However, low molecular HA solutions were found to manifest a higher rate of regenerative properties preservation. In addition, wound surface regeneration in animals after the application of both HMW and LMW HA, subjected to tyndallization protocols 2 and 3, was noted to be slower than in protocol 1. Such a result is thought to be caused by a higher temperature used during tyndallization protocols 2 and 3. The same pattern was observed in the experiment both with 1 and 2% HA solutions. Therefore, tyndallization procedure according to protocols 2 and 3, was found to slightly reduce regenerative properties both in HMW and LMW HA aqueous solutions.

Obtained data were shown to confirm a positive effect of HA aqueous solutions upon the wound surface regeneration dynamics. Similar to the pre-

vious investigation, complete wound surface regeneration was noted by the days 7—8th, whereas the same index in the control group animals was not observed even by day 11. Thus, tyndallization used for HA aqueous solutions, was shown to guarantee both their sterility and preservation of HA initial regenerative properties.

Special attention was dedicated to studying low temperature effect upon the preservation of initial regenerative properties by HA aqueous solutions from the aspect of their molecular weight and concentration. Thermocycling known as one of the stressful mode of low temperature impact was specifically examined. We should note, that thermocycling manifestations might occur during long-term low temperature storage of biological material and during its transfer as well.

In the series of experiments on evaluating low temperature effect, experimental mice were divided into 5 groups (n = 5 in each of them). HA solutions for wound treatment underwent a freeze-thawing. Analogous groups of animals were formed to study preservation of regenerative properties in HA aqueous solutions subjected to thermocycling.

The experiment results are demonstrated in Figs. 5 and 6, showing positive dynamics in wound surface regeneration followed HA aqueous solutions application comparing to the control group. Obtained results let us claim that low temperatures do not reduce HA regenerative properties, even after following thermocycling. In addition, experimental data were shown to respond to the tendencies observed during previous investigations, *i.e.*, there were found significant regenerative properties both in 1 and 2% LMW HA and HMW HA. LMW HA possessed more manifested regenerative properties comparing to HMW HA aqueous solutions.

Wound surface square by day 10 following application of HMW HA subjected to freeze-thawing, made (2.143 ± 0.377) and (2.266 ± 0.257) mm^2 for 1%- and 2% solutions, correspondingly (Fig. 5). After the use of LMW HA, the studied indices made (0.936 ± 0.181) and (0.881 ± 0.193) mm^2 for 1%- and 2% solutions, correspondingly; while the control group index was (3.35 ± 0.367) mm^2.

In HMW HA subjected to thermocycling, the wound surface square by day 10 made (2.438 ± ± 0.343) and (2.66 ± 0.111) mm^2 both for 1 and 2% solutions, correspondingly (Fig. 6). For LMW HA such indices made (1.39 ± 0.25) and (1.23 ± ± 0.063) mm^2, correspondingly. Control group index made (4.431 ± 0.1) mm^2.

Thus, experimental results of the sample immersion into liquid nitrogen let us declare, that HA regenerative properties are preserved more efficiently when using milder freeze-thawing techniques.

Due to HA ability to bind large amounts of free water molecules, and thus to reduce cell damage because of its mechanical factor, hyaluronic acid may be eventually used in cryopreservation as a component for cryoprotective solutions.

CONCLUSIONS

1. HA was shown to be a perspective basement for biological wound covers. In order to accelerate regenerative processes, the use of LMW HA is recommended for wound surface treatment.
2. Sterility of HA aqueous solutions, being the major requirement for the further clinical application, was shown to be achieved by using tyndallizaion technique as sterilization protocol. Protocol 2 (sample heating at 80 °C) was shown to be the optimum beyond all three studied tyndallization protocols.
3. Low temperature effect was found not to influence either LMW or HMW HA regenerative properties (even when using thermocycling). This fact has been opening wide opportunities for HA application in cryobiology and cryomedicine.

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ВПЛИВ СТЕРИЛІЗАЦІЇ ТА НИЗЬКИХ ТЕМПЕРАТУР НА РЕГЕНЕРАТИВНИЙ ПОТЕНЦІАЛ ГІАЛУРОНОВОЇ КИСЛОТИ

Завдяки своїм фізичним властивостям та фармакологічній активності гіалуронової кислоті (ГК) має неабияку перспективність використання для потреб кріобіології та кріомедицини. Метою дослідження було створення методу стерилізації водяних розчинів ГК, які не призводять до зниження її регенеративних властивостей, та вивчення впливу низьких температур на їх збереженість. Для стерилізації водяних розчинів ГК запропоновано режим щадної стерилізації — тіндалізації, який водночас забезпечує стерильність розчинів та не впливає на її регенеративні властивості. На моделі загоєння ексцизійної рани у тварин досліджено вплив режиму тіндалізації та дії низьких температур на збереженість регенеративних властивостей 1 та 2% водяних розчинів ГК різної молекулярної маси: низькомолекулярної (НмГК) (<100 кДа) і високомолекулярної (ВмГК) (>2000 кДа). Показано, що низькі температури не змінюють регенеративні властивості ВмГК та НмГк (навіть в режимі термоциклування), що відкриває широкі можливості використання в кріобіології і кріомедицині.

***Ключові слова*:** гіалуронова кислота, вплив низьких температур, збереженість регенеративних властивостей, стерилізація.