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RESEARCH OF REPARATION PROPERTIES OF LIPOSOMAL FORM OF BIOGENIC LIPIDS

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Abstract

Liposomes are obtained on the basis of the Baikal seal lipids. In experiments in vitro ability to restore membranes of erythrocytes by created liposomes is established. On the basis of created liposomes new transdermal remedy in the form of gel is developed. In experiments in vivo it is shown that the developed gel possesses expressed ability to heal wounds at reproduction of various models of skin damages at rats. The possible reason of wound-healing effect of investigated remedy is that seal lipids contain highly active w-6 and w-3 polyunsaturated fat acids in the parities showing therapeutic effect.

Key words: *liposomes, polyunsaturated fatty acids, Baikal seal, skin regeneration*

1. INTRODUCTION

Recently in many research centers a wide field of operations of fundamental and applied sciences is developed, directed to extensive studies of such natural bioactive compounds as lipids. In present-day concept based on the results of deep structurally functional researches (Shvets, 1997, Efremenko, 2001), lipids and permolecular cellular formations – biological membranes – play a large part in functioning of basic biochemical mechanisms in cutis. Due to increasing requirements of pharmaceutical and cosmetic industries an actual problem of an accessible source of raw materials selection and working out of optimal biotechnological production processes of natural lipid preparations is taken place. Natural origin preparations are different from synthesized chemical compounds by its perfect formula that includes an optimal correlation of micro- and macroelements, vitamins and irreplaceable fatty acids.

One of the ways of maximum medical effect achievement and minimization of side effect of chemotherapeutic preparations is using liposomes. Liposomes, microcapsules from natural or synthetic phospholipids, in many respects turned to be the almost ideal carriers for transport of medicinal connections in an organism. The idea of using vesicles is not new for purposeful transport of bioactive substances, and preparations on liposome basis have been widely adopted in medicine all over the world. Efficiency of liposome influence is caused by possibility of their penetration into fabrics. As a result of interaction of a cell membrane and a liposome membrane the modification of lipid structure of a cell membrane and a reparation of microdamages is taken place. On the other hand, liposome vesicles are universal containers for transportation simultaneously lipophilic substances which join in membranes, and hydrophilic substances which are incorporated in internal volume (Alving, Swartz, 1991; Cevc, 1996; Schreier, Boustra, 1999).

As it is known, the size of liposomes, a structure of lipid layer and correlation of a liposome/lipid influence on therapeutic value liposome system. During last 15-20 years the interest to lipid preparations containing such bioactive components as natural phospholipids, neutral lipids or free fatty acids increases (Bergelson, 1987; Barsukov, 1998, Chiara Sinico et al., 2005).

At liposome creation, lipids of vegetative raw materials and sea hydrobionts are widely used in structure of which polyene fatty acids are prevailed. Tissues of the Baikal seal (hypodermic fat and liver) are perspective sources of bioactive ω-6 and ω-3 polyunsaturated fatty acids which are found out in 3,19:1 (in a liver) and 0,77:1 (in fat) proportions (Radnaeva et al, 1999).

One of directions in working out liposome preparations is designing transdermal medical products. The problems connected with creation of methodical approach to working out a compounding and perfection of biotechnology of their creation are thus solved. The present work is devoted to research of reparative properties of the liposomes, containing fat of a seal as a source of polyunsaturated fatty acids, and an estimation of wound healing actions of the medical product developed on their basis.

2. MATERIAL AND METHODS

Animal material

Fat (hypodermic fat) and a liver of Baikal seal *Pusa sibirica Gmel*. are received according to TC (Technical Conditions) 9281-017-02069473-2001. In experiments on studying pharmacological and pathomorphological characteristics of efficiency of liposome agents action, white outbred rats of both sexes weighting 170-250g from nursery of branch №5 SNC «Institute of biophysics of federal government "Medbioekstrem" at Ministry of Health of Russia, Angarsk city, were used. Animals were located in standard conditions of the vivarium on usual diet due to International standards (Principles of Laboratory Animal Science, 1993).

2.2. Methods of researches

2.2.1. Mixture excretion of egg phospholipids

Phospholipids were extracted from egg yolks by classical method of Folch. Egg yolks (10 pieces) were carefully separated from fiber and homogenized during 10 minutes with 400 ml of acetone. Homogenate was left in a cold room for the night. A sediment was separated by centrifugation. Processing by acetone was repeated, then a thin layer of sediment was put on a filtered paper and dried up till the disappearance of a smell of acetone (1-2 hours). The dried up powder was filled up with 200 ml of chloroform-methyl spirit mix (1:1). The extracting of phospholipids was stirring on a mechanical rocking chair during 30 minutes. The solution was centrifuged at 3000 rpm during 20 minutes. Centrifugates was separated and kept. The sediment processing was repeated, centrifugates were united and evaporated on a rotary vaporizer. After evaporation a mix of phospholipids was dissolved in 50 ml of petroleum ether and in 1000 ml of acetone, mixed and left for the night. The sediment was filtered on a funnel of Byuhner, dissolved in 50 ml petroleum ether and sedimentation was repeated. The sediment was washed out by 200 ml of acetone.

2.2.2.Mixture excretion of seal's liver

Phospholipids were extracted from seal's liver by the method offered by Kreitis M. (Kreitis, 1975). To 6-7 g of liver 3 ml of water and 30 ml of mix chloroform-methanol (1:2) were added and the tissue was homogenized in homogenizer with knives during 2 minutes at room temperature. Homogenate was centrifuged, supernatant was decanted and the sediment was re-extracted by 38ml of chloroform-methanol-water mix (1:2:0.8) in homogenizer during 2 minutes. The tissue was separated by centrifugation, the united supernatants were diluted by 20 ml of chloroform and 20ml of water, water-methanol and chloroform phase was divided by settling in the funnel. The bottom chloroform layer was concentrated on the rotary vaporizer at 30-35°C. The sediment was dissolved in 10ml of chloroform. For phospholipids receipt fivefold acetone volume was added to the solution of summary

lipids in chloroform and the mix was kept 2-3 hours at 0°C, the fell sediment was separated by centrifugation and dissolved in chloroform.

2.2.3. Preparation of liposome structures

The liposome structures were made out of the phospholipids mix with the seal's fat added, in the concentration 60% by method, offered by Bangham A.D. et al. (Bangham, Horne, 1964). With the help of pipette the solution of phospholipids in organic dissolvent into the thick-walled retort were added. The dissolvent on the rotary vaporizer was evaporated so that to give an opportunity for phospholipids to distribute on the wall of retort in the form of thin pellicle. The solution was added, containing 0.25M sucrose, 0.01M buffer, 0.001M EDTA, pH 7.4 and the mix was processed during 7 minutes by ultrasound on dispersant (Ultrasonic dispersant) in water-cooling at frequency 22 kHz.

2.2.4. Preparation of gel with liposomes

The buffer solution was added into the retort with lipids distributed in the form of thin pellicle with previously added α-tocopherol acetate in concentration 1%. It was stirred up on mechanical rocking chair for 30 minutes at room temperature. The mix was insonified on dispersant for 7 minutes at 4°C. The received liposome structures were heated till 40°C and mixed up with previously alloyed on water bath and refrigerated till 40°C PEG (poly-ethylene glycol) 1000 and 6000 (9:1) for getting essential consistence in correlation 1:1 accordingly. It was homogenized up to homogeneous mass and packed up.

The choice of poly-ethylene glycol as gel basis, is caused by its neutrality, hygroscopicity, physiological indifference, ease release of medicinal substances. Poly-ethylene glycol doesn't undergo to contamination by microorganisms, possesses weak bactericidal property with dehydrate action, also doesn't disturb gas exchange of a cutis, little toxic, doesn't cause stimulating action on tissue, easy washed, stable to light, moisture.

2.2.5. Estimation method of liposome biological activity

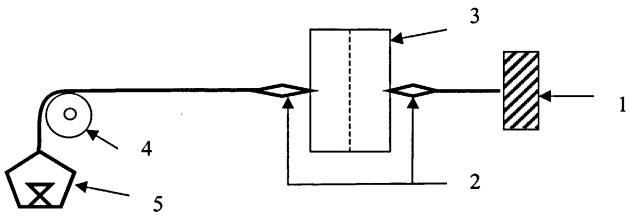
2.2.5.1. Evaluation of membrane stabilizing activity

Evaluation of membrane stabilizing activity of researched preparation was made on the degree of erythrocytes hemolysis, caused by the reagent of Fenton (peroxide hemolysis), and addition of distilled water (osmotic hemolysis) (Kovalev et al, 1986). The components, forming part of Fenton reagent, were used in optimal concentrations, which cause full lysis of 1% erythrocyte suspension: $FeS04x7H_20$ – 0.01 mg/ml; H_2O_2 – 0.2 mg/ml (in converting on 100% solution of hydrogen peroxide). Both components individually don't cause lysis. Hemolysis was considered in 24 hours. For reproduction of osmotic hemolysis to erythrocyte suspension equal volume of distilled water was added. The hemolysis degree was measured in 24 hours on optical density at 540 nm. As probation solution has its own color and turbidity, the additional tests on preparation color in appropriate dosing were put.

2.2.6. Reproduction method of wound cutis damage.

2.2.6.1. The reproduction of linear musculocutaneous wound.

Linear cutaneous wounds were made in the following way: under barbamil narcoses (70 mg/kg) the linear wound was made in the area of rats' back with the sterile scalpel up to own fascia of 5 cm length (Klyuev, 1989). Equally-spaced three stitches were put in, which brought the edges of made wound together (Fenchin, 1979). At the end of experiment on the 8 day the wound-tensiometric by Fisenko (2000) with the help of device was made (picture 1).



Picture 1 – the scheme of device for tensiometric measure of scar strength.

1 - support; 2 – clamps; 3 – flap with wound; 4 – trochlea; 5 – platform with weight.

For determination of scar strength after linear cutaneous wound healing the animals were killed due to momentary decapitation under ether anaesthetic, the parts of cutis with the healed wounds 1cm wide, 3 cm length were cut. One end was fixed in the upper point with special clamp, lower end was fixed with another one with the platform with weight, the mass of which was gradually increased up to scar rupture. The largest weight for scar rupture was registered.

2.2.6.2. The reproduction of plane musculocutaneous wound

Plane musculocutaneous wound was reproduced under anaesthetic (70 mg/kg) by excision of fasciocutaneous flap with the square 400 mm² in the area of rats' back on previously cut areas (Ponomareva-Astrahantseva, 1954). To the edges of the wound for equal initial wound size the aluminium frames were sewn (Kaplan et al, 1989). The wound-healing effect of the researched medicine was made due to monitoring of animals, wound state, period of wound healing. With the aim of objective research of wound process planimetric, cytological and histological wound researches were held (Shalimov et al, 1989).

2.2.6.3. The reproduction of chemical burn

The chemical cutis burn in the area of rats' back was due to subcutaneous introduction of 0.5 ml of 9% acetic acid under anaesthetic (70 mg/kg) on previously cut areas of cutis (Oivin, Shetel, 1961). The derivable wounds were standard, their sizes mustn't prevail 400 mm². On the 3 day necroses-ectomy of affected areas was made, after that researched agent was used.

2.2.7. Methods of wound healing

Wound healing was started after musculocutaneous wounds, then every day 7 mg of the researched medicines were put on. For a control there were used animals, cured with mix of poly-ethylene glycol with buffer.

2.2.8. Methods of evaluation of wound healing effectiveness

Evaluation of dynamics of wound processing was determined due to clinical observation, planimetric, cytological and histological research methods. During observation period the state of wounds, quality and granulation maturity, character of wound separated parts, state of surrounding tissues, periods of epithelization and total healing were evaluated.

2.2.8.1. Evaluation of wound square due to planimetry

A transparent film was put on wound and circuits of wound were outlined with ink. With mass 1cm² of film, the square of wound was calculated by a formula:

$$S = (S_1 \times M) / M_1,$$

where S – wound square; S_1 – square of 1 cm^2; M_1 – mass of 1cm^2 of film; M – mass of outlined film (Fenchin, 1979).

2.2.8.2. Pathomorphological research methods

For pathomorphological research the areas of wounds with bordering intact tissues were fixed in 10% solution of formalin. Deparaffined histological cuts, made on a sledge microtome, were painted with hematoxylin-eosin or picrofucsin by Van Gison (Merkulov, 1969). Photographing of cutis mounts was made with the help of microscope with computer equipment.

2.2.8.3. Cytological research methods

Cytological wound researches were held due to method of "wound imprint" (Pokrovskaya, Makarov, 1942). Wound surface was touched with previously defatted glass, here on the glass the imprints of joined cells are left. The preparations were dried, then fixed in ethanol-aethir mix during 15 minutes, then it was painted with hematoxylin-eosin. The quantity of neutrophilic granulocyte were marked in cytogramma: (+) – separated neutrophilic granulocyte 5-10 in field of view; (++) – small clusters; (+++) – substancial clusters; (++++) – clusters of leucocytes.

2.2.6.4. Statistical research methods

Digital findings were worked out statistically with the use of density-free (Beili, 1964) and parametritic (t-Student's – with the help of computer program Jandel SigmaPlot32) criteria. Significance of findings differences were calculated in groups with probability $p<0.05$.

3. THE RESULTS OF RESEARCH

3.1. The choice of optimal concentration of natural polyunsaturated fatty acids in liposome by the evaluation of membrane stabilizing activity

Working out transdermal medicine, which possesses wound-healing action, the most important index is membrane stabilizing activity of its basic active component. As it is known, membranes play top role both in structural organization and in functioning of all cells – they form intracellular compartments, take part in regulation of all connections and interactions, which are effected between their external and internal sides (Bergelson, 1987).

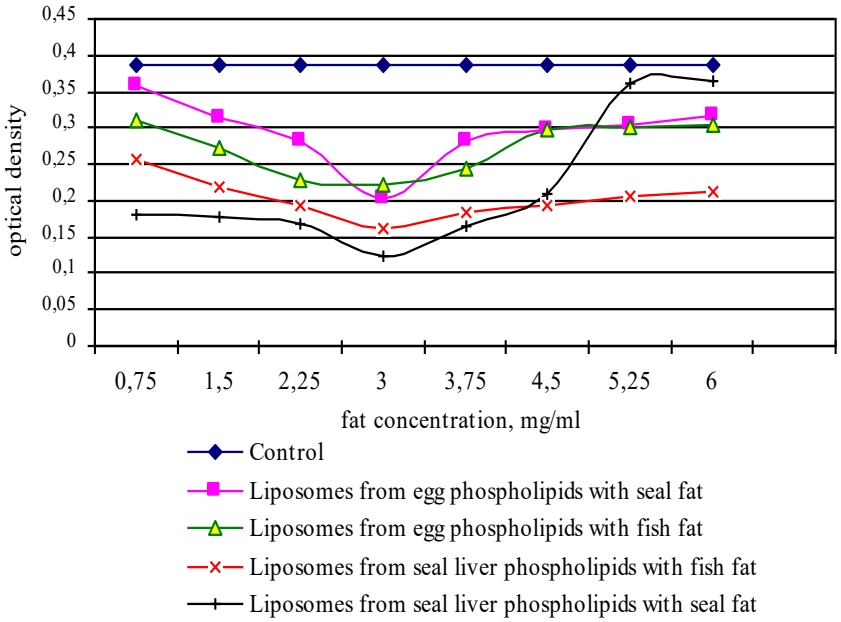
Most of lipids in membranes are represented by phospholipids, glykosfingolipids and cholesterol. In lipid bilayer fatty-acid chains are oriented in parallel to each other, as the result quite tough structure is made. Depending on fatty-acid composition of phospholipid bilayer of membrane of formed blood elements, deformability and plasticity of erythrocytes and platelet may differ.

Modern practice has shown the availability of using natural phospholipids as membrane formative elements in liposome. Phospholipids are natural for an organism by the substances, capable to be included into metabolic process and utilize in the organism. Phospholipids of cellular membranes usually contain at least one polyunsaturated fatty acid, which has at least one double connection in cis-acting, thus non-receding fluidity, the so called diluting or "fluidized" membrane property is caused. The fluidity of membrane has an influence on its functioning. With the increased fluidity the membrane becomes more penetrable for water and other small hydrophilic molecules, and the conditions for diffusion of integral protein are created.

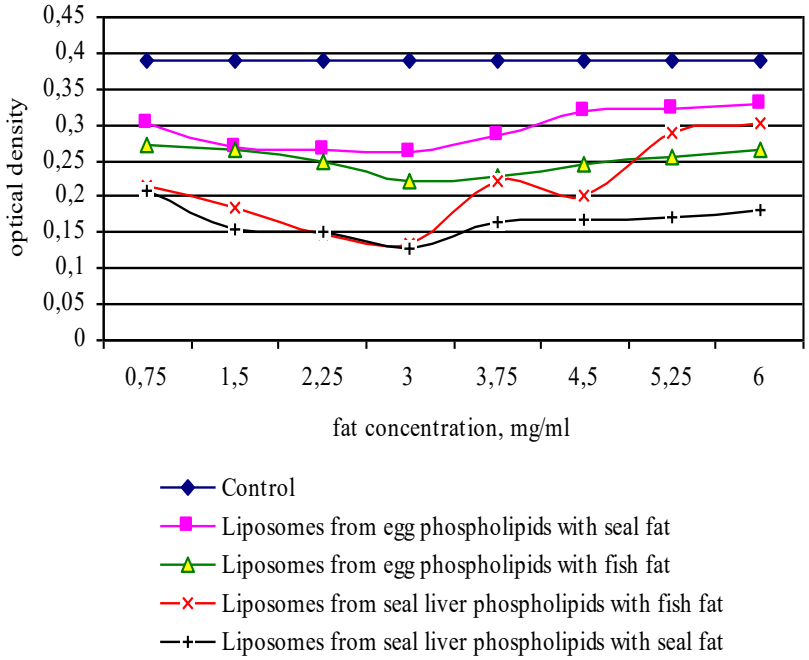
The unsaturation degree of high fatty acids influences on membrane fluidity. It turned out, that ω -3 of polyunsaturated fatty acids more influences on increasing fluidity, than polyunsaturated fatty acids of

ω -6. In this way the tissues of the Baikal seal (hypodermic fat and liver) are the perspective sources of polyunsaturated fatty acids. Polyunsaturated fatty acids change mesomorphic state of membrane, the mobility of lipids and protein molecules in lipid membrane bilayer increases. Changes of phase membrane state influence on the processes of membrane transport, on systems of membrane information transfer, on activity of membrane-combined ferments.

In the experiment liposome out of phospholipids of the seal's liver with the seal's fat adding in concentrations from 0.75 up to 6.00 mg/ml converting to fat, were added to the erythrocytes suspension. For comparison of researching agents in the experiment were used liposomes from egg phospholipids with seal's fat adding and liposomes from phospholipids of seal's liver with pharmacopeia preparation – cod liver oil. The results of researches are presented on pictures 2 and 3.



Picture 2 – Liposome influence with different concentration of seal's fat on osmotic hemolysis of erythrocytes



Picture 3 – Liposome influence with different concentration of seal's fat on peroxide hemolysis of erythrocytes

As it is seen from presented findings liposomes, prepared out of all natural lipids, have membrane-stabilizing action because of decrease of osmotic hemolysis from 5% to 8% in different variants in comparison with control. Also decrease of peroxide hemolysis under researched agents influence from 18% to 67% in comparison with control was taken place. The most relief of both osmotic and peroxide hemolysis of all researched liposome was registered in 3 mg/ml dose.

It turned out that liposome influence made on the basis of seal's liver phospholipids with seal's fat adding to prevention of osmotic hemolysis was more effective than liposome action on the basis of egg phospholipids and liposome with pharmacopeia preparation – cod liver oil, that was received in the same concentration. Similar effect was observed at reproduction of peroxide hemolysis.

Thus, it is shown that introduction of bioactive natural lipids which present in seal's fat and liver, into liposome composition leads to increasing of membrane-stabilizing activity of workable liposome agent. The most effective concentration of fat 3 mg/ml was used in further experiments.

3.2. Evaluation of wound-healing liposome activity with seal's fat adding

At the evaluation of wound-healing effect of workable liposome agent subject to basic pathogenesis factors of wound damage development, there were used the models of linear and surface musculocutaneous wound and model of chemical cutis burn.

3.2.1. Liposome agent influence on healing of linear cutaneous wound

After operation to animals of experimental groups gel on poly-ethylene glycol basis which contains liposome structures was put on wound in 7mg dose. Wounds of animals of control group were cleansed with poly-ethylene glycol mix with buffer solution. Later on dressings were held 1 time a day during all period of the experiment.

The experiments have shown that in animal group, which wounds were cleansed by gel with liposomes, to the 8 day after operation there weren't wound abscess. Whereas in control group 6 rats of 10 received wound abscess.

Effective indices of local impact of medicines on wound, which point on healing degree in regenerative period of wound process are edges approaches and then strengthening of cicatrix. That's why at the reproduction of linear wound to the 8 day after operation strengthening of cicatrix on rupture was determined. Measuring was held due to wound-tensiometric method. The findings of tensiometria are resulted in table 1.

Table 1 – Influence of gel with liposomes on strengthening of white rats cicatrix (n=10)

Group of animals	Strengthening of cicatrix, kg
Control	0.303±0.030
Gel with liposome	0.360±0.030*

Notice: * - significant deviation of value in appropriate groups from value in control group of animals ($p \leq 0.05$).

It is determined that strengthening of cicatrix of experimental group on the 8 day was 19% higher than indices of wound-tensiometria in control group. It says about positive influence of liposome agent on strengthening intercellular structures during regenerative processes in defective tissues. As histomorphological researches have shown, after 8 days from the beginning of the experiment in reproduction of linear wounds, cured by medical gel with liposomes, granulation tissue under epithelium looked more mature, than tissues of another group. There fibrous structures in the tissue prevailed. In border-line tissue around saved hair follicles a new epithelium was proliferated, and it formed multistage structure.

Histological research of wound defect, and also the results, which were received at measuring strengthening of the formed cicatrix tissue on the place of wound, have shown that during curing wound damages by gel with liposomes, there formation, maturation and fibration of granulation tissue are taken place earlier. It indicates stimulating influence of workable transdermal medicine on tissue regeneration at linear wound cutis damage.

3.2.2. The influence of gel on the liposome basis on healing of plane musculocutaneous wound

The plane wound was reproduced in aseptic conditions by accepted method. For evaluation of the dynamics of wound healing on the 3, 7, 14 and 21 day the square of wound surface was determined by planimetric method (Ponomareva-Astrahantseva, 1954). Within specified period of the experiment the material for cytological research of wound surface was taken. The findings in planimetric research of the plane wound are presented in table 2.

Table 2 - The influence of liposome agent on square change of the plane musculocutaneous (mm^2) of experimental rats (n=10)

№	Periods of the experiment	Groups of animals	
		Control	Gel with liposomes
1	3	389.9±12.3	237.7±21.8*
2	7	313.6±10.1	141.4±15.1*
3	14	129.2±10.4	67.9±7.2*
4	21	108.2±9.7	53.9±6.4*

Notice*- significant deviation of value in experimental group from value in control group of animals ($p \leq 0.05$).

As it is seen from the table 2, using gel with liposomes during all the period of experiment have an evident wound-healing effect on rats. On the 3, 7, 14 and 21 day of research essential decrease of wound square of the animals of experimental group in comparison with the evaluated index of the animals of control group in appropriate periods on 39, 55, 47 and 50% was noticed.

Cytological research of wound imprints has shown that on the 3 day of the experiment a lot of neutrophilic leukocytes were marked of both control and experimental groups. Here in experimental group the quantity of destructive forms of leukocytes was 9.5% less than in control group. On the 7 day of the experiment in wound imprints of rats a decrease of neutrophilic leukocytes in both control and experimental groups is marked. In experimental group of animals occurrence of fibroblasts is marked. On the 14 day in wound imprints of experimental group with the use of gel with liposomes fibroblasts and fibrocytes were prevailed, whereas findings of type of cells in control group were appeared only on the 21 day of the experiment.

Thus, is it determined that liposomes further to structural reorganization of neogenic cutis after aseptic tissue damage, and thus hasten processes of wound healing in early periods.

3.2.3. Influence of gel with liposome on wound healing induced by chemical burn

Clinical observations of wound surfaces condition of experimental rats after reproduction of the model of chemical burn were held on the 9, 17 and 25 day. During these periods wound squares were measured, wound condition, quality and mature of granulations, character of separated wound, condition of surrounding tissues, periods of epithelization and healing were marked. The results of measuring in changing the wound square after chemical burn are presented in table 3.

Table 3 - Influence of liposome agent on change of wound square (mm^2) after chemical cutis burn of rats (n=10)

№	Periods of the experiment, days	Groups of animals	
		Control	Gel with liposome
1	9	526±31	314±31*
2	17	413±39	292±27*
3	25	341±12	223±20*

Notice*- significant deviation of value in experimental group from value in control group of animals ($p \leq 0.05$).

As it is seen from table 3, on the phone of using gel with liposomes acceleration of the dynamics of wound closing after chemical burn was marked, and a decrease of wound surface square of experimental group on the 9, 17 and 25 day indicates about it. On the 9 day the wound square on the experimental group has decreased on 40%, on the 17 day – on 29%, on the 25 day – on 35% in comparison with the control group.

4. CONCLUSION

Correlation ω-6: ω-3 polyunsaturated fatty acids is of great importance for metabolic processes in norm and at development of wound damages. In seal's fat there are a lot of ω-3 polyunsaturated fatty acids, which suppress working-out of inflammatory eicosanoids from ω-6 fatty acids that allows to keep a balance of an organism on actions of damaging factors.

Received findings show that natural polyunsaturated fatty acids in liposome form have an evident capacity to stabilize the damaged membranes of cells. Evidently it happens due to change of phase condition of biomembranes with the increase of their fluidity. In the experiments of reproduction of wound cutis damages an evident wound-healing action of researched liposomes is established, that is determined by the bioactive lipids participation in reparative regeneration of connecting tissues structures in posttraumatic process.

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