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IAEA-R-1347-F

Radiation preservation of biological tissues with special emphasis on immunological alterations, (part of a coordinated programme on radiosterilization of medical products and biological tissues)

15 March 1973 - 30 September 1976

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January 1977

FINAL REPORT

CONTRACT NUMBER 1347/RB

TITLE OF PROJECT: Radiation sterilization of biological tissues with special emphasis on immunological alterations (Part of a co-ordinated programme on radiation sterilization of medical materials and biological tissues).

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TIME PERIOD COVERED: 1973 - 1976

ABSTRACT

A method suitable for clinical use and preparations of tendon and skin preserved by lyophilization and radiosterilization have been elaborated and certain characters of the final preparations were investigated.

Twenty-four hours after excision tendon tissue had been lyophilized at +10 °C temperature for two days, wrapped into a plastic bag and sterilized by 2,5 Mrad ^{60}Co gamma irradiation. Sterility of the final preparations was controlled by the usual microbiological methods. The preparation could without any change keep its properties at room temperature for at least abt. a year. Prior to implantation it should be bathed in a physiological saline for about 12 hours.

Aims of the experiments on dogs and rats were to follow the fate of the preparation after implanting into the recipient.

Preparations were transplanted into mongrel dogs. The fate of the graft was detected by histological and electronmicroscopical investigations. We demonstrated that the implanted preparation was desorganized and was absorbed within six weeks. During this time, however, cells from recipients'

own tendon's end migrate into the preparation and parallel to graft desorganization a new tendon tissue is organized from host's own cells.

Graft desorganization kinetics were examined in rats. Rat tendon labelled *in vivo* with ^{35}S was implanted into rats according to previously elaborated method and the decrease of radioactivity in the graft was tested in intervals after the operation. Forty days after implantation the graft could be demonstrated only in tracks. On the basis of these data it seemed probable that implanted tendon served as a matrix for the organization of the new tendon tissue. Experiments on dogs indicated that the time necessary for the full clinical recovery is approximately six weeks. At this time no difference between the movement of operated or control animals was found.

No signs indicating graft rejection or other immune reaction were found in the course of experiments. Experiments were carried out on mongrel dogs, therefore, graft and host compatibility can hardly be expected. In many instances tendon preparations originating from two various donors were implanted into the same animal within half-a-year, or a year. Immune reaction, however, did not appear not even after the second operation, either.

Since no human tendon can be obtained in a quantity meeting clinical demands, clinical examinations were made on pig-tendon preparations. Until now, pigs' Achilles tendon preparations prepared according to the above mentioned method have been in clinical use. Patients recovered without difficulties in every case.

Skin preparations were prepared of pig-skin. The skin was sliced to 0,3 mm thick sections with the help of a dermaton and was lyophilized to 5 per cent humidity. Sterilization was carried out with 3 Mrad irradiation. Microbiological control of the final preparations took place as usual.

The immunological properties of the preparations were also studied. Antiserum against native skin was produced in rabbits. It was demonstrated by indirect haemagglutination that pig-skin antigenity was decreased by approximately two order of magnitudes during the preservation process. Similar results were obtained from experiments carried out on in vivo according to Kossowska. On the basis of our results it seemed possible that mainly the lyophilization was effective for decreasing the antigenity. It has been shown that skin preparations have a short lasting bacteriostatic effect. The extract of the preparation was tested. The multiplication of Staphylococcus aureus in culture was inhibited by the extract of preparation. The effect of the extract was studied on the growth of mixed bacteria culture obtained from burned wounds and the bacteriostatic effect was demonstrable in this case, too.

The clinical application of skin preparations has been used for nearly two years. It is effectively used as a biological dressing for the covering of wound surface in case of burn injuries. According to clinical experiences the application of a pig-skin preparation is more advantageous than that of other wound covering methods. Wounds treated with the preparation have less protein and electrolyte loss than in case of other methods, there is a few occurrance of either infection or further complications and the surface suitable for skin transplantation and epithelium recovery took place earlier.

INTRODUCTION

Aims of the experiments were to produce sterile tendon and skin preparations which kept under humble circumstances are ready for practical, ie. clinical use.

Experiments were motivated by the frequent need of the preparation of this kind in traumatology, since tendons, skins and surrogates are used in the clinical practice in a large quantity even nowadays. During the preparation of the products we wanted to elaborate a few inexpensive and relatively easy methods and laboratory analyses indicated the applicability of the preparations.

In case of tendon tissue the fate of the implanted preparation was studied in the host organ and experiments carried out on mongrel dogs were to discover if there was any immune reaction induced by the implantation of the preparation (1, 2).

The immunogenicity of the skin preparations was investigated and the clinical applicability was also studied (2, 3, 4, 5).

Tendon tissue

The production of the preparation

Preparations were made of human and dog Achilles tendons. Twenty-four hours after excision tendons were lyophilized at +10 °C temperature for two days. Dry preparations, wrapped in a double-wall plastic bag were exposed to ^{60}Co gamma radiation sterilization in a Noratom apparatus (240 R/sec) at +4 °C temperature. Irradiation dose was 2,5 Mrad.

Sterility test

The germ number of samples was counted before irradiation. Samples (approx. 1 g tissue) were shaken in 20 ml, 0,1 % peptone water for 120 min. Washing liquid was parted and studied separately as parallel samples. The liquid was filtered by a HAWP 04700 type 0,45 μ membrane filter. Filtrates were placed onto Oxoid CM325 agar-plates and incubated under aerobic and anaerobic conditions at +32 °C temperature. The rate of the average contamination of the samples was 58 colony-forming units/g. The sterility of irradiated samples was tested according to the prescriptions of Pharmacopea Hung.VI. on Saboraud liquid medium (Oxoid CM147) on mycotic culture medium at +25 °C, as well as on thyoglycollate medium (Oxoid CM173) at +32 °C temperature by 14 days incubation.

The efficiency of the irradiation sterility was in any case controlled on Bacillus pumilus E 601 and Bacillus spaericus C$_1$A sporular test-preparations according to the method suggested by NAU.

The fate of the preparation after implantation

The organization of the preparation was studied in experiments carried out on dogs (1)

Tendon preparations were implanted after keeping them for one to eleven months. Storing did not make any influence on operational results. Prior to implantation preparations were bathed in physiological saline overnight. Mechanical properties of the rehydrated preparation were good, it was not fragile, cords did not evulse during operation.

Tendon preparations were implanted as a substitute into mongrel dogs of excised Achilles tendon. A piece of tendon as large as two or three cm was excised from the middle part of the Achilles tendon while this part was substituted by the implant of rehydrated preparation according to the modified Bunnel operation method.

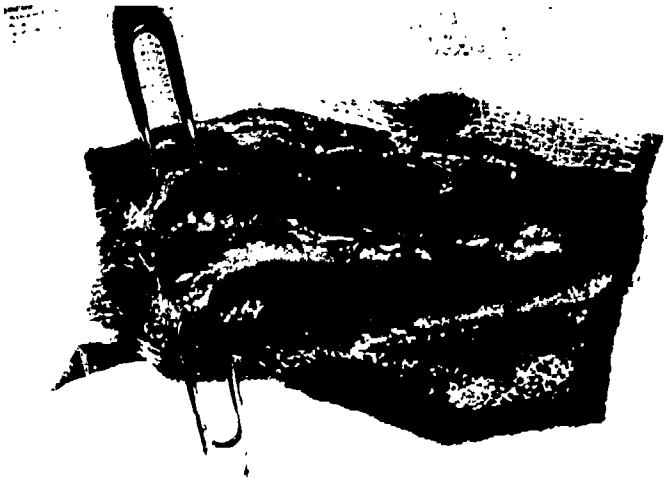


Fig.1 Implant distinguishable from recipient's own tendon

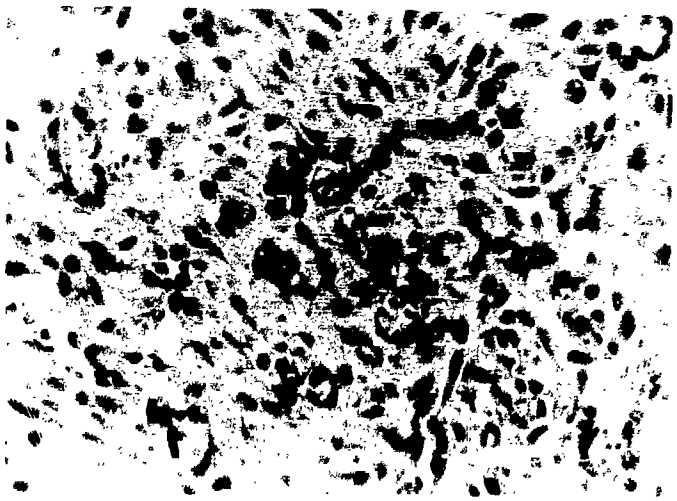


Fig.2 Visible inflammatory infiltrated granulation tissue

After operation graft was excised from animals of a group for histological and electronmicroscopical analysis in various intervals while the regeneration of the function of movement and the full period of recovery were observed on control animals. Animals tried to use their operated legs within three to four weeks after operation. Full functional recovery can be observed within eight to ten weeks after operation, animals' running and walking manner showed no divergence as compared to controls. Observations proved recovery as a definite and no sort of complication was experienced in the function of the operated legs within the period of observation lasting four years.

The investigation on what happened to the implanted piece of tendon started on the fourth day after operation. At this time the operated leg was slightly swollen and graft could even macroscopically be distinguished from recipient's own tendon, nevertheless, there was a demonstrable connection between implanted and the original tendon, as well (Fig.1).

On the histological section of this part of the implemented tendon the structure of the original tendon shows a peculiar change: the proportion of the cells to the collagenous fibre bundles shifts in favour of the cells. The increased number of cells do not correspond to the characteristic, flattened tendon cells of the control tendon but rather they seem to be fibroblasts and young fibrocytes. This phenomenon is presumably connected with the increased regenerating activity of the original tendon.

In the implanted tendon a mosaic-like histological picture developed. While some parts still showed the structure of the tendon tissue,

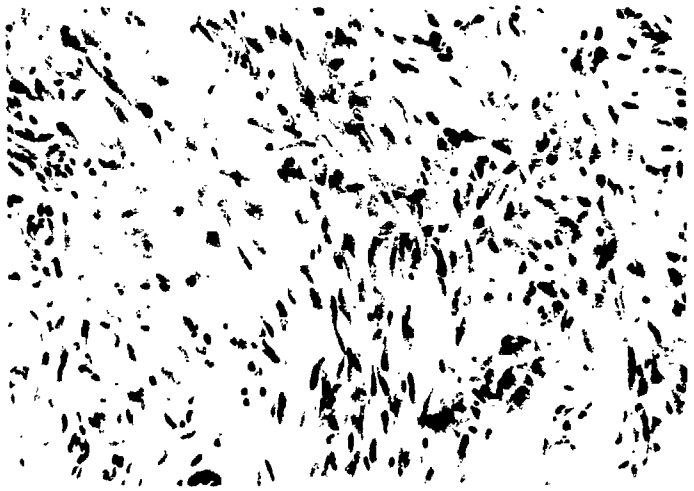


Fig.3 Irregular spots of young, cell-rich connective tissue showing initial fibre formation

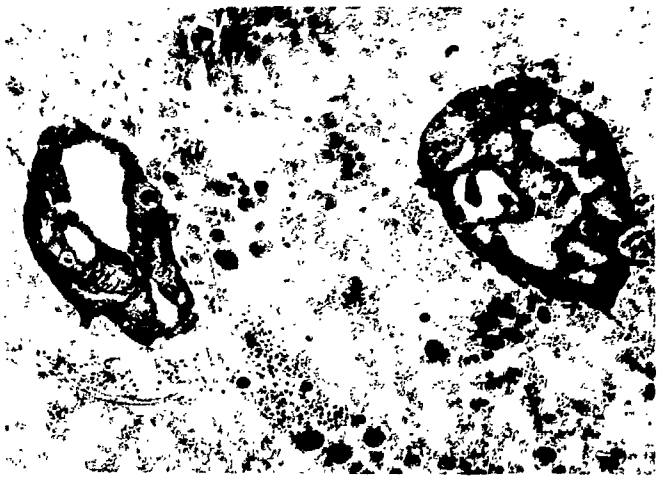


Fig.4 Visible thin, young fibres of the implant

Fig.5 Renewal of collagenous fibrils

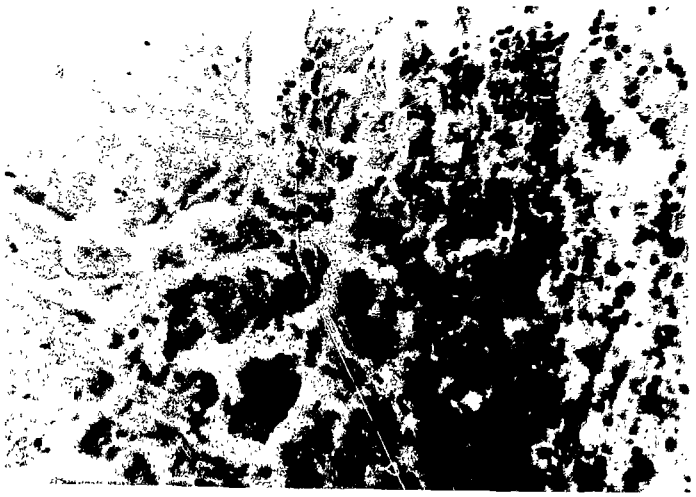


Fig.6 Part of implant surrounded by granulation tissue

others showed nuclear staining. In some places inflammatory infiltrated granulation tissue can be seen (Fig.2), while elsewhere irregular spots of young cell-rich connective tissue showing initial fibre formation could be detected (Fig.3).

The electron-microscopic preparation is prepared from the border zone of the implanted tendon near the original one. In the intracellular amorphous material can be seen both the original fibres originating from the implant and the newly formed fibres originating in the host. The fibres of the implant started to disintegrate. They are stained unequally; some can hardly be seen, but some still thin, young fibres appear in their place (Fig.4).

In Fig.5 one can see the renewal of the collagenous fibrils. When using a higher magnification, the laminosis material around the cells proves to consist of newly formed collagenous fibrils.

On the histological patterns obtained from the middle part of the implant, poorly stained, dead cell-nuclei and disintegrating collagenous fibres can be seen. This part is surrounded in each direction by granulation tissue (Fig.6).

The sections show that the renewal of the implant starts axially, from the border zone of the original tendon and the implant. From the original tendon, interstitial cells flow into the implant and the new fibrin fibres are formed from these cells. But - as is shown in the last section - in the middle parts of the implant, although only to a lesser extent, the formation of the new tendon tissue can be seen to encircle near the edges of the implant. These regeneration processes seem to take place rather rapidly, newly formed capillaries

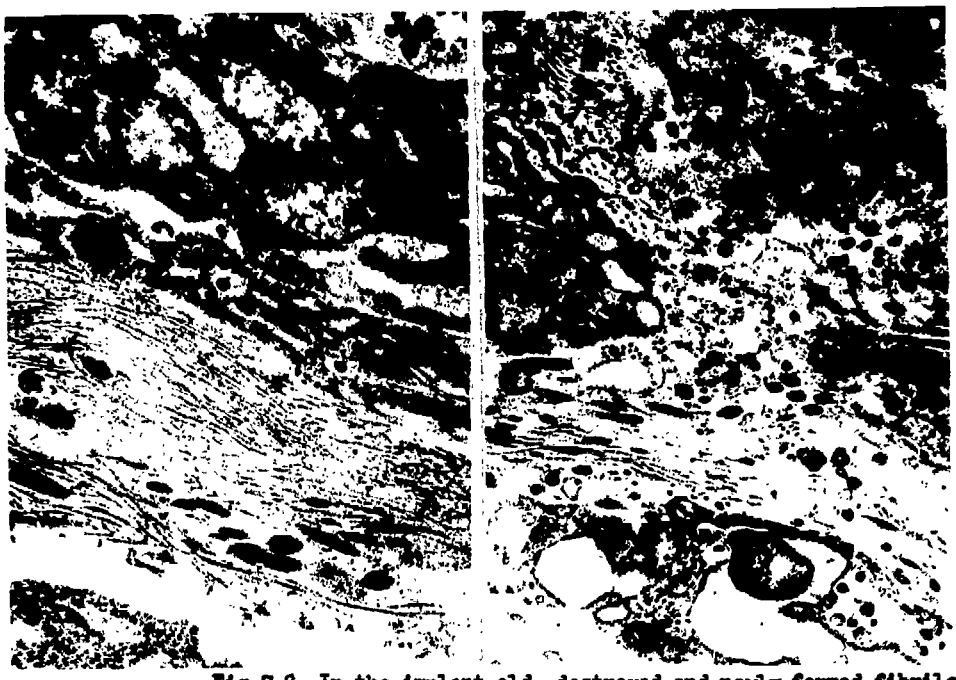


Fig.7-8 In the implant old, destroyed and newly formed fibrils can be seen together

Fig.9 Strong connection between implant and recipient's tendon after 3 weeks

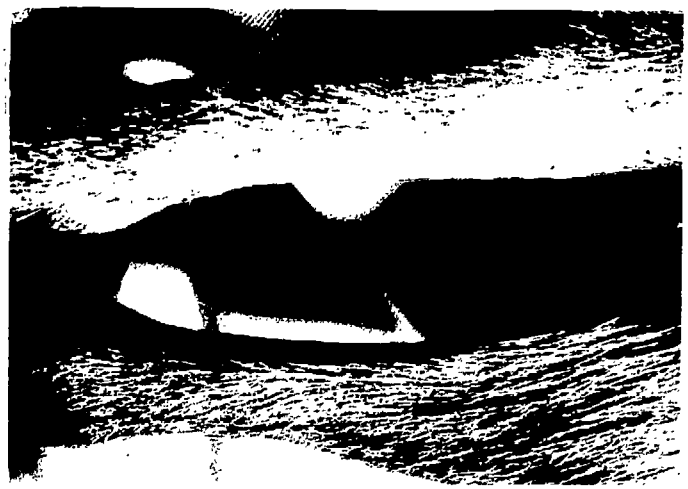


Fig.10 No macroscopic difference between operated and intact legs

Fig.11 Vascularization can be seen on the surface of the implant

being detectable in the implant already 2-3 weeks after operation. Three weeks after operation the re-organization of the implant is still in progress. In the implant, both old, destroyed and newly formed fibrils can be seen together (Figs 7 and 8).

Three weeks after implantation a strong connection between the implant and the recipient's own tendon can be observed macroscopically (Fig.9). A slight swelling can be seen in the implant. The implanted tendon can be easily distinguished from its environment. Strong inflammation reaction or signs of rejection cannot be detected.

Three to four weeks after operation the function of the operated limb recovered. The dogs used their operated limbs, although carefully. Eight to ten weeks after operation they could stand on their hind paws, and jump, which means that total functional recovery could be observed. At this time the operated limb could not be distinguished from the intact one by naked eye.

No difference can be seen macroscopically between the tendon implant and the recipient's own tendon (Fig.10). However, compared with the bradytrophic character of the tissue there is a significant vascularization observed on the surface of the implant (Fig.11). After two months the original and the implanted tendons show only slight differences histologically. The implanted tendon tissue is microscopically richer in cells than the normal one and is young in character; the majority of the cells are not typical, flattened tendon cells. The vascularization of the regenerated tendon is satisfactory. Some capillaries run parallel to the longitudinal axis of the tendon, but capillaries enter also from the edges (Fig.12). Later the swelling of the implant decreases and the increased vascularization ceases six months after

Fig.12 Capillaries run parallel to the longitudinal axis of the tendon, but, as shown here, they also enter from the edges

Fig.13 Macroscopically a slight thickening distinguishes implant from contralateral Achilles tendon

the operation. Macroscopically, only a slight thickening distinguishes the implant from the contralateral Achilles tendon (Fig.13).

After six months the histological pattern of the implanted tendon does not differ from that of the control tendon.

Destruction and organization of implanted preparations were investigated by the application of a labelled tendon. Experiments were made on Wistar R/Amsterdam x Long Evans F_1 hybrid rats.

Rats weighing 130 to 150 g were treated with $Na_2\,^{35}SO_4$ (specific activity 10,4 mCi/mg) altogether twice μCi/rat intraperitoneally every three day. Following the second injection animals were decapitated and both-sided Achilles tendons prepared.

After lyophylization, radiosterilization and rehydration, tendons were supplanted the excised Achilles tendon of recipient rats. Radioactivity of tendon preparations was measured before and after implantation in various intervals.

The radioactivity of the implanted preparation decreased after operation in the function of time (Table I).

Measuring data confirm the conclusion drawn from histological and electron-microscopical investigations that the implanted graft parallel to the organization of the new tendon tissue metabolized within approx. six weeks.

TABLE I

Time	DPM/mg fresh tendon [x] (average ± S.E.)
Before operation	94 ± 0,91
After operation	
1 day	88 ± 1,7
19 days	58 ± 2,1
40 days	19 ± 1,5

[x] Radioactivity of ^{35}S rat tendon before and after grafting

Immunology experiments

Studies on the immunological properties of the preparation is very important from the viewpoint of the applicability of lyophilized and radiosterilized tendon preparations.

Literature data indicate that the tendon tissue is not immunogenic. These data, however, refer to the native tendon tissue and it may happen that the tendon becomes antigenic owing to some eventual changes of the collagenous structure induced by lyophilization and high dose of irradiation. This urged us to study our preparations also by immunological methods. Experiments reported on in this paper were performed on mogrel dogs, histo-compatibility between donors and recipients, therefore, was hardly expected.

Despite of the above, no signs indicating rejection or some other immunogenic property of the graft were observed in neither of our 21 experiments. During four years observation period there were 6 cases when the same dog received implant preparation originated from various donors into the contralateral limb within half, or one year after the first operation. Once, the operation was repeated four times when preparations originating from four various donors were implanted into the same dog within one-and-a-half year.

In this instances the first transplantation may be considered as a sensitization while the second one as a provoking. Since no visible to the naked eye immune reaction was induced by the second operation either, we may suppose that the lyophilized, radiosterilized can-tendon has any immunogenic property.

The immunogenic properties of fresh and lyophilized-radiosterilized human-tendon have also been investigated, in vitro.

infection of further complications and the surface suitable for skin transplantation and epithelium recovery took place earlier.

Neither fresh, nor prepared tendon tissue made any stimulating or cytotoxic effect in lymphotyle cultures. Experiments carried out so far proved that human-tendon preparations had no immunogenic properties, either.

The clinical application of tendon preparations

It is rather difficult to obtain human tendon-tissue in a volume of meeting clinical requirements. To this end, preparations were made of the Achilles tendon of pigs according to the above mentioned method. Preparations are tested clinically in the National Institute of Traumatology. Preparations for tendon supplement were used in five instances. The first operation took place more than one-and-a-half year ago, patients recovered without complication.

The skin

Production of the preparation

Pig skin frictioned with propylenglycol was sliced to 0,3 mm thick sections, cut into 10-30 cm large pieces, lyophilized at +10 ^{o}C temperature to 5 per cent humidity. Packing and radiosterilization of the preparations took place as stipulated above with the only exception that the dose of sterilization was 3 Mrad. Final preparations can be kept at room temperature for at least a year without changing properties.

Sterility test

The sterility test of the samples and the control on the efficiency of radiosterilization were performed according to the method used for tendon tissue testing. The average germ-number of skin washed off with propylenglycol was, in the average of 3 experiments 612 colony-forming units/sq.cm before and 312 c.f.units/sq.cm.after lyophilization.

Antigenicity of the preparations

As is well known from the literature the skin is strongly antigenic, to this end immunological characters of the preparations should in the application of skin-preparations be taken into special account. Since, in the course of clinical application it is most often necessary to use the preparation for the treatment of the same patient over and over again, therefore, one must count with the possibility of sensitization, too.

The change of the antigenic character of the skin was examined in vitro and in vivo in the process of the preparation.

a) Indirect haemagglutination test

Rabbits were immunized by native suspension prepared from skin and the antigenicity of the skin preparation measured by an indirect haemagglutination body. (Table II)

TABLE II

The antigenicity of native, lyophilized and lyophilized-radiosterilized pig skin to antiserum produced by native skin

Antigenic (skin)	Dilution of antiserum			
	10	100	1000	10 000
Native	+	+	+	+
Lyophilized	+	+	-	-
Lyophilized-radiosterilized	+	+	-	-

The natine skin was agglutinated in 1:10 000 while that of lyophilized and final preparation in 1:100 dilution.

of three cm was excised from the middle part of the achilles tendon while this part was substituted by the implant of rehydrated preparation according to the modified Bunnel operation method.

On the basis of the data it seems that the antigenicity of the skin decreases or changes by lyophilization.

b) Lymphoidic proliferation

The fundamental principle of this process is that the proliferation induced by antigenic stimulus initiates in the lymphoidic system in proportion as the immunogenity of the injected material. Tha same method was applied by others, too who investigated preserved tissue preparations (5). Rats were once treated with a native, lyophilized or prepared from the final preparation suspension in the rate of 1 mg skin protein/rat. Six days after treatment rats were decapitated and regional lymph-nodes excised from the place of the injection. ^{3}H-thymidine was added to the lymphocyte suspension prepared from lymph-nodes and DNA radioactivity was measured.

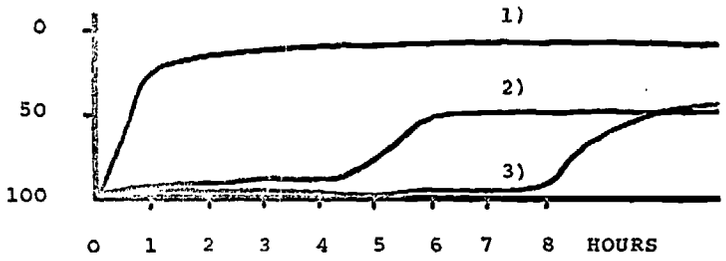
Some data registered in two representative experiments are figuring in Table II. Deducting control value and taking ^{3}H-thyamidin incorporation registered in native skin treated rats, as 100 per cent, the immunogenicity of the skin after lyophilization decreased to 34 per cent and to 21 per cent after lyophilization, radiosterilization (Table III).

TABLE III

^{3}H-thymidin uptake by regional lymph-node lymphocytes challenged by various pig-skin preparations

Groups	DPM/10^{6} lymphocytes	
Exp.	1	2
Control (untreated)	2000	2100
Native skin	4100	5800
Lyophilized skin	3200	2900
Lyophilized and radiosterilized skin	2800	2500

TRANSMISSION, per cent



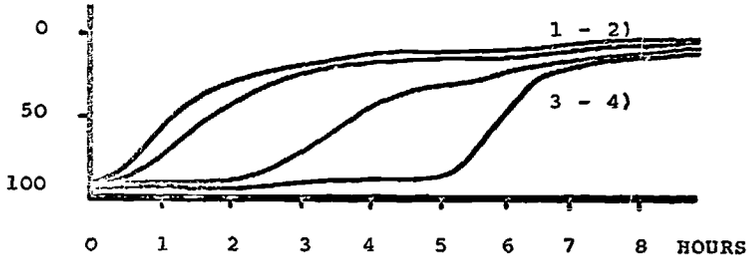
SYMBOLS: 1) Multiplication curve of bacteria in the untreated medium

2) Multiplication curve of bacteria in the medium treated by lyophilized pig-skin

3) Multiplication curve of bacteria in the medium treated by lyophilized-radiosterilized pig-skin

Fig. 14

TRANSMISSION, per cent



SYMBOLS: 1 - 2) Multiplication curve of bacteria in the untreated medium

2 - 3) Multiplication curve of bacteria in the treated medium by lyophilized-radiosterilized pig-skin

Fig. 15

Experimental results were very similar to that of indirect haemagglutination testing results, i.e. the immunogenicity of the native skin decreases during the processing and this change is primarily due to lyophilization.

The bacteriostatic effect of lyophilized, radiosterilized skin preparations

This biological property of the preparation was examined on Staphylococcus aureus bacteria cultures. We extracted the skin preparations treated differently with the medium itself and sterilized by filtration. Treated mediums were inoculated by the same number of bacteria. The multiplication of the number of bacteria was measured on basis of transparency in biophotometer within 24 hours. (Fig.14)

According to results the lyophilized preparation prevented the growth of bacteria during four hours, while the lyophilized and radiosterilized preparation did it for about twice as long. We investigated the effect on mixed bacteria also got from burned injuries (Fig.15).

We observed the baczeriostatic effect of the extract of our preparation even in this cases. In the treated cultures bacteria began to multiplicate in the fifth hour.

Clinical application of skin preparations

To prevent the loss of water, electrolyte and protein of burned patients allogenic and xenogenic skin, native or preserved by different methods are generally used. The biological dressing prevents furthermore the bacterial invasion of the sick organism (6,7,8,9,10,11,12).

The grafts have been rehydrated for ten minutes in sterile physiological saline solution without antibiotics before application. After this, they have been placed on the surface of the injury directly, where they adhered perfectly. The clinical application is simple, so this preparation is suitable for immediate care of mass burns. For such a treatment a large quantity of preparations is required, because the biological dressing must be changed in every 3-5 days.

According to our investigations, in case of superficial burns of second-degree skin grafts prepared by us have been applied, the recovery of epithelium is fast.

The pictures show the recovery of a burn of second-degree by the application of our xenogenic preparation(Figs.16, 17, 18).

On the following pictures the treatment of the burned hands of a patient can be seen. On this part of the body the successful treatment is not easy because the developed epithelium is usually of bad quality that is atrophic. This epithelium is sensitive to trauma and subject to scaring which prevents the proper function of the hands (Figs. 19, 20, 21, 22).

In the third serie we show the treatment of a patient who was burned in 50 per cent of third-degree (Figs.23, 24, 25).

Summary of clinical application

According to clinical observations the application of the preserved skin-graft induced a rapid epithelization of burned injuries of second-degree. We found out that the time of recovery was shorter, the developed epithelium was of better quality

than that produced by other treatments. In case of deep and large burns the applied grafts prevented the loss of body fluids and the infection of the burned injury, consequently the development of bacteriaemia. With this preparation a granulation tissue can be produced suitable to take the autograft. The application of the preparation results in the purification of the injury both in case of superficial and deep infected burns.

An expressed bacteriostatic effect has been observed also by other authors and this effect attributed to the change of pH and local protective reactions (13). On the basis of our outstanding results we suppose that the preparation has a direct bacteriostatic effect (Figs.26, 27, 28, 29).

Fig.16 The infected burn-surface before treatment

Fig.17 Removal of xenogenic preparation graft on the sixth day. The burned surface is totally epithelized

Fig.18 The recovered surface six months after the injury. The patient has been treated with xenogenic graft for six days without administration of other drugs

Fig.19 The freshly burned surface of the hand before removal of the necrotized tissue

Fig.20 Application of biological dressing on wound surface

x Radioactivity of ^{35}S rat tendon before and after grafting

Fig.21 The wound surface after the second change of dressing on the sixth day

Fig.22 An excellent recovery can be seen on the injured surface. The recovered epithelium can be easily wrinkled, it is elastic. It differs from the surrounding epithelium only in colour here and there. The picture was taken hanlf a year after the injury

lyophilization.

Fig.23 The application of xenogenic graft on the large burned surface. The aim of application is, beside saving life the production of granulation surface for autotransplantation

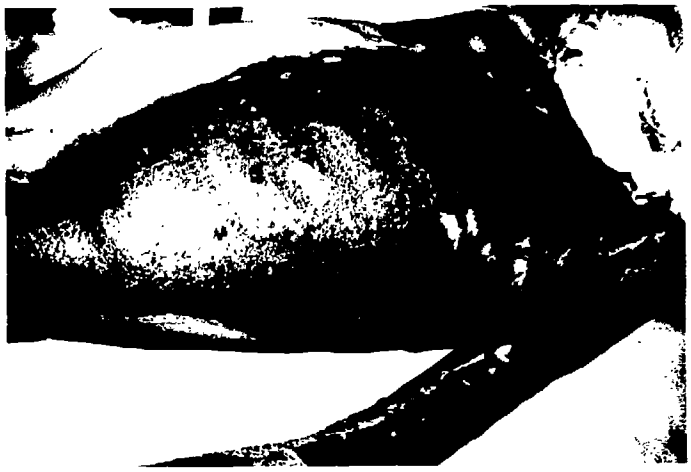


Fig.24. The treated surface is ready for autotransplantation with a high quality of granulated surface

Lyophilized and radiosterilized skin	2800	2500

Fig.25 Autotransplantation performed in consecutive steps. Because of the inavailability of proper skin (donor) tissues some area of the injury has been covered temporarily with the xenogenic preparation

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