

**„VICTOR BABEŞ” UNIVERSITY OF MEDICINE AND PHARMACY
FROM TIMIŞOARA
FACULTY OF MEDICINE
DEPARTMENT XI PEDIATRICS**

CIORNEI BOGDAN



PhD THESIS ABSTRACT

**THE VIABILITY OF USING NILE TILAPIA FISH SKIN
XENOGRAFTS AS A TEMPORARY COVERING IN THE
HEALING PROCESS OF PARTIAL THICKNESS BURNS**

Scientific Coordinator

PROF.UNIV.DR. BOIA EUGEN SORIN

**Timișoara
2024**

Table of Contents

LIST OF PUBLISHED SCIENTIFIC PAPERS	Error! Bookmark not defined.
LIST OF ABBREVIATIONS AND SYMBOLS	Error! Bookmark not defined.
LIST OF FIGURES	VII
LIST OF TABLES	Error! Bookmark not defined.
DEDICATION	Error! Bookmark not defined.
ACKNOWLEDGEMENT	XIII
INTRODUCTION	3
GENERAL PART	Error! Bookmark not defined.
1. SKIN ANATOMY	Error! Bookmark not defined.
1.1. THE EPIDERMIS.....	Error! Bookmark not defined.
1.2. THE DERMAL-EPIDERMAL JUNCTION	Error! Bookmark not defined.
1.3. THE DERMIS	Error! Bookmark not defined.
1.4. THE HYPODERMIS	Error! Bookmark not defined.
1.5. SKIN VASCULATURE.....	Error! Bookmark not defined.
1.6. SKIN INNERVATION.....	Error! Bookmark not defined.
1.7. PILOSITY	Error! Bookmark not defined.
1.8. THE NAILS	Error! Bookmark not defined.
1.9. ADNEXAL GLANDS OF THE SKIN.....	Error! Bookmark not defined.
2. BURNS.....	Error! Bookmark not defined.
2.1. WOUND EVALUATION.....	Error! Bookmark not defined.
2.2. BURN PHYSIOPATHOLOGY	Error! Bookmark not defined.
2.3. BURN MANAGEMENT.....	3
2.4. OREOCROMIS NILOTICUS(NILE TILAPIA)	Error! Bookmark not defined.
2.5. ANIMAL MODEL	Error! Bookmark not defined.
SPECIAL PART	5
1. SCOPE.....	5
2. MATERIALS AND METHODS.....	Error! Bookmark not defined.
2.1. ETHICS APPROVAL.....	Error! Bookmark not defined.
2.2. XENOGRAFT PREPARATION AND EVALUATION	Error! Bookmark not defined.
2.3. EXPERIMENTAL STUDY.....	48
3. RESULTS.....	8
3.1. XENOGRAFT EVALUATION	8
3.2. HEALING POTENTIAL EVALUATION	Error! Bookmark not defined.
4. DISCUSSIONS.....	84
CONCLUSION	99
BIBLIOGRAPHY	Error! Bookmark not defined.2
ANNEX I	Error! Bookmark not defined.
PUBLISHED ARTICLES <i>IN EXTENSO</i>	Error! Bookmark not defined.

INTRODUCTION

Burns are a traumatic pathology with hard repercussions to combat, both for the patient and for the medical institutions involved in their treatment. The World Health Organization estimates that about 11 million people are affected annually by burns, of which 180,000 people die annually as a result of these incidents.[1]. Burns can be caused by objects or substances that release thermal energy, by friction, chemical, by electricity or by electromagnetic or ionizing radiation. Their classification is made according to the affected body surface as well as depending on the depth of the injury.[2].

The treatment is complex, medical and surgical, addressing in the first phase the elimination of the cause and resuscitation of biological functions, and in the second part it focuses on the prevention of infections and restoration of tissues devitalized following the trauma. This treatment is well to be carried out in specialized Plastic Surgery centers, which have all the conditions necessary to fulfil this purpose. These include the existence of an Intensive Care facility to care for large burns, which means a large burn coverage area (>40% body surface), specialized apparatus intended for wound care, adequate sanitary materials and not least medical personnel trained in accordance with the latest treatment guidelines.

The skin or integumentary system, is an important part of the human body, an organ according to whose conformation many aspects of the life of an individual can be intuited. It determines the personality and character of the person who possesses it, in addition it says a lot about its ethnicity, about the activities that they attend, whether they are of a professional or recreational nature and is an indicator according to which interpersonal relationships are realized.

Anatomically, on its largest surface, the integumentary system consists of the epidermis, dermis and the hypoderm or subcutaneous cell tissue. Each of these 3 layers has distinct origins and characteristics, which together complement all these functions of the skin[3].

BURN MANAGEMENT

Medical

The approach to the treatment of burns takes into account several factors, among which we can enumerate the age of the patient, which depends on the total body surface, the area affected by the injury, the existence of airway burns and/or smoke inhalation, the coexistence of associated pathologies, the type and degree of the burn.

Early local treatment involves removing the causative agent, any clothing that can maintain thermal energy or block the blood vessels if edema is formed. The second step is to cool

the affected area with water to a temperature below the normal body, but not cold, to stop the phenomena of heat convection. Thus, the pathological process is inhibited, and this action also helps to reduce pain. It should be remembered that there is a possibility of hypothermia following this action, so after this operation patients must be covered with clean blankets or with anti-burn film that is found in the first aid kits.

The Parkland formula involves the use of the Ringer lactate crystalline solution, consisting of sodium, potassium, calcium, chlorine, as well as L and D isomers of lactate, mixed in an isotonic solution, administered according to the formula with 4ml/kg/% of affected surface. Half of this amount is administered in the first 8 hours and the next half in the other 16 hours.

In addition to the early treatment of shock, the treatment of lesions begins from the first phase of contact with healthcare. It aims at extinguishing the traumatic process by cooling the wound with water, cleaning the area affected by the necrosis resulting from the trauma, preventing infection of the wounds and covering them for regeneration.

Silver Sulfadiazine is a product in the form of cream, the most widespread topical product used in the prevention of infections and treatment of burned wounds. It consists of an antibiotic in the class of sulfonamides, sulfadiazine, which together with silver ions have antimicrobial effects lasting 24 hours. It has the greatest effect on *Pseudomonas* and *Enterobacteriaceae*, but is not limited to them. Although easy to use, this compound reduces healing, and penetration is limited to the epidermal layer[4–7].

SURGICAL TREATMENT

Surgical treatment of burns is represented by the totality of surgical gestures performed in order to cover the wounds and to promote skin regeneration. In this group we include daily toilet, necrotic tissue debridement, excision and grafting.

Debridement in case of first-degree burns is not necessary, the resulting debris shed themselves. In the case of superficial burns (SPT) the debridement of debris and blisters is sufficient. For burns affecting the intermediate and deep dermis, besides those exceeding this level, several techniques are available which include tangential debridement with a Watson or Goul scalpel, electrodermatome or hydrosurgical appliances. As far as grafting is concerned, the gold standard is the use of an autograph harvested from an area with healthy skin, that afterwards goes through a perforation process that increases its covering area, as well as allowing the penetration into the interior of ointments and the evacuation of blood and debris.

Other covering solutions are represented by cadaveric allografts or pork skin xenografts. Although allo- and xenografts were designed with the aim of acting as permanent bandages, most

of the time they are actually temporary bandages that contribute to the initiation of healing. One of the reasons why this happens is poor adherence to the wound bed.

OREOCHROMIS NILOTICUS (NILE TILAPIA)

Tilapia is a name that encompasses more than 100 species of fish in the family of *Cyclids*. They are freshwater fish whose habitat is limited to shallow rivers, ponds, lakes, rivers and several species that live in muddy, standing waters. Historically, they are of particular importance in East Africa where in terms of fish farming activity evidence of the use of these fish as food dates back to ancient Egypt.

The collagen content of the different constituent components of the skin of these species has been determined by different methods of chemical extraction and purification. The type of collagen in most tissues was type I collagen, but it appears that in the skin type III is found in higher amounts in younger specimens. The use of collagen from these species for the treatment of human diseases is intensively studied, but further research is needed in order for its use to be considered feasible[8–12].

To the best of our knowledge, judging from an extensive review of the literature, Alves et al. are the only authors whom have published studies regarding the use of decellularized Nile Tilapia fish skin in the treatment of human burn wounds[13–16]. In one phase II clinical trial comparing the healing outcomes of the patients undergoing treatment with Tilapia fish skin against silver sulfadiazine dressings, they had 32 patients enrolled in the test group. Their study showed better healing times, lower use of analgetics and fewer dressing changes [17].

The potential of fish-derived collagen and peptides in the management of burnt or surgical wounds has been investigated via in vitro studies. These researchers have mostly focused on the in vitro use of a mixture of chemicals, namely chitosan and marine peptides generated from tilapia collagen electrospinning.

SPECIAL PART

1. SCOPE

The aim of this scientific paper is to bring to light aspects related to the use of xenografts from the species of fish *Oreochromis Niloticus* (Nile Tilapia) used in the surgical treatment of burns. Until recently, this type of treatment was not under the scrutiny of the medical world, except in the form of press articles, which often acclaimed the favorable results, without showing the

possible shortcomings of the method. In any case, there was no medical basis for initiating such treatment, other than experimental.

This thesis seeks to study several aspects of a potential surgical repair treatment. To complete this experimental study it was necessary to several objectives, among which are: determination of the viability of the harvested tissue, decontamination and sterilization of grafts, their application and maintenance in place, determining the time necessary for healing through subsequent histological studies.

2. MATERIALS AND METHODS

2.1. ETHICS APPROVAL

The use of these animals has undergone the necessary steps for approval by the Scientific Research Ethics Commission of UMF Victor Babeş Timișoara, in compliance with the ethical norms and research principles, respectively Approval No. 61/30.08.2021 rev 2_19.09.2023. The study complies with the provisions of Directive 2010/63/EU of the European Parliament (Official Journal of the EU, Annex IV, page L276/72) transposed into national legislation by Law no. 43/2014, as well as the ARRIVE Guidelines v 2.0. An approval was also requested from the Veterinary Health and Food Safety Directorate (Avis No.012/31.07.2023) pursuant to the provisions of Government Ordinance No.42.2004 on the organization of veterinary and food safety activities, approved with amendments and additions by Law no.215/2004 with subsequent modifications and supplements and veterinary sanitary rules and measures.

2.2. XENOGRAFT PREPARATION AND EVALUATION

Harvesting and sterilization

The following procedures were inspired by the experience of Alves et.al.[18] and adapted to local conditions and logistics.

Table 1 Sterilization steps[86]

Groups	Intervention	Time
1	Fat and muscle scrapping and 0,9% saline wash (Natural state)	N/A
2	2 x 2% Clorhexidine baths + Solution: 50% Glycerol - 49% Saline (0,9%) 1%PMF (Penicillin+Metronidazol+Fluconazole solution)	2 x 30 minutes 24 Hours at 4 ⁰ Celsius
3	Solution: 75% Glycerol - 24% Saline (0,9%) 1% PMF	3 Hours at 37 ⁰ Celsius
4	Solution: 99% Glycerol - 1% PMF	3 Hours at 37 ⁰ Celsius

Determination of collagen content

A necessary requirement for the establishment of the next route was the determination of collagen content in the samples. Previous studies conducted by other researchers highlights data that support the hypothesis that the fish's skin is abundant in collagen type I and III.

The process of evaluating collagen components of type I and III under polarized light was carried out using histochemical analysis by titration of Sirius red picrate. The above-mentioned images were subsequently subjected to an automatic image analysis methodology using the Icy Bioimage Analysis program [75].

2.3. EXPERIMENTAL STUDY

This experimental study required the use of an appropriate animal model with human-like characteristics so that the results of the study could be easily translated into human patients.

A. Selection and well-being of subjects

The study subjects were six 6-week-old Large White x Landrace female pigs, donated to this experiment by Smithfield Ferme. Following their arrival in the experimental center, the pigs were lodged in groups in concrete floor-covered barracks, using hay as a support, benefiting ad libitum of water and cereal-based nutrition for 10 days in order to reduce the degree of stress and accommodation in the new home. We used the Standard Operating Procedures from the Faculty of Veterinary Medicine, Timisoara Experimental unit [19,20].

B. Burn infliction

A round device with a diameter of 2 cm made of copper was used for the burning procedure, to which a welded aluminum handle covered in wood was attached for thermal insulation. The target temperature was 110 degrees Celsius. This temperature was achieved by contact of the copper plate with a heated dehorning device and its monitoring was carried out with the help of a contact thermometer.

According to the instructions of Andrews and Cuttle[21] 2 types of wounds were attempted, one that affects the superficial dermis and the other that affected the deep dermis, achieving a grade IIA(SPT) and grade IIB burn(DPT). For fulfilling this end-point it would take an exposure of 10 seconds and, 20 seconds respectively at the temperature of 110 degrees Celsius.

C. Lot separation

A total of 14 burn wounds were made with the device mentioned on each of the 6 gilts, as follows:

Table 2 Study lot grouping

2 burn control wounds						
1x10s			1x20s			
	7 days		14 days		21 days	
Control	1x10s	1x20s	1x10s	1x20s	1x10s	1x20s
Experimental	1x10s	1x20s	1x10s	1x20s	1x10s	1x20s

Each sample was subjected to seven length measurements to assess the main endpoints: dermal depth (DD), burn depth (BD) and complete collagen destruction (CDD). The length of maximum injury depth (LMD) and length to maximum depth at the side edge of the epidermis (LTMD) were identified as secondary points of interest.

Weekly samples were collected by excising tissue in full-thickness layer approximately 5mm from the edges of the defect. The harvested specimen were fixed in 10% formaldehyde and two central representative sections were incorporated into paraffin.

A scoring system adapted after the one described by Guo et al was applied to all 72 study samples in order to evaluate the healing process with a qualitative method.

The statistical tests used were descriptives, Welch test, one-way Anova, Repeated Measures Anova, Correlation, Liniar and Logistic Regression, Cronbach's alfa and Principal component analysis.

3. RESULTS

3.1. XENOGRAFT EVALUATION

The application of the Student t test to compare the evolution of type I collagen between the two species showed a statistically significant difference between ON and OM at the starting point (natural, $p = 0,017$, $ON < OM$) and at the final stage of sterilization (99% glycerol, $P = 0,022$, $OM < ON$).

In the case of ON type, it was found that in this group the collagen content of type I collagen was higher as the concentration of the sterilization solution increased ($p < 0,001$). In OM fish, significant differences were found between the unsterilized samples and those treated with 99 % glycerol ($p < 0,001$), as well as between the samples with 50 % and 99 %.

The t test results showed a significant difference in the average amount of type III collagen between the two species in the Natur group. More specifically, OM had a concentration that is 40% higher than ON (natural, $p < 0,001$).

A significant difference in type III collagen concentrations was observed at each sterilization stage for ON, with the exception of the comparison of 50% glycerol and 75% glycerol ($p = 0.065$) and 75% against 99% glycerol ($p = 0.427$). The collagen concentration was higher in the more sterilized samples.

With regard to OM, the study found significant differences in type III collagen concentration between different sterilization steps. In particular, the comparison between Natur and 99% glycerol showed a statistically significant difference ($p=0.005$), with a higher concentration of type III collagen in the more sterilized samples.

3.2. HEALING POTENTIAL EVALUATION

The average dermal depth in group 10s was $1800.50 \mu\text{m}$ ($\text{SD}=195.04 \mu\text{m}$), while the average in group 20s was $1646.73 \mu\text{m}$ ($\text{SD}=163.22 \mu\text{m}$) ($p < 0.01$). We further analyzed the mean values of raw determinations of maximum burn depths with the same Welch test and observed a significant difference between the two exposure periods ($p < 0.01$). No statistically significant difference was found in the comparison of the raw depth of complete collagen fiber denaturation between the two groups ($p=0.078$).

Having discovered a significant difference in dermal depth between the two groups, we calculated two additional variables: (raw burn depth divided by dermis depth) multiplied by 100 (BD/DD %), and (depth full of collagen destruction divided with dermis depth) multiplied by 100 (CDD/DD%). The analysis of the BD/DD% formula demonstrated a statistically significant difference ($p < 0.01$) in dermal burn involvement between group 10s (average: 85.61%, 95% CI= 80.62 to 90.61) and group 20s (average: 123.71%, 95% CI= 114.91 to 132.50).

The author's personal contribution in this part of the study is related to the development of a simple, reproducible and consistent animal model for burn studies. Another contribution is the appreciation of the impact of the lesion by calculating the percentage of total collagen denaturation in the total burn depth at 1 day after exposure.

Repeated measures Anova analysis of dermal thickness irrespective of exposure time, where the between subjects factor was type of treatment and the within subjects factors were raw mean dermal thickness values collected at each of the three weeks of sampling, showed that there is a significant difference between treatment groups overall ($F=4.38$, $p=0.048$). Within subjects effects showed that there is a significant difference between the values observed across

the three time-points ($F=88.22$, $p<0.001$), and a significant effect of the interaction of type of treatment and week of sampling ($F=3.3$, $p=0.046$).

One way analysis of variance was computed in order to assess the differences between each treatment group at each time-point separately, first with both exposures included, and then separately. In this way we discovered a pattern in which at the first and last week of treatment, the thickness of the dermis tends to be significantly greater in the experimental treatment group.

Repeated measures Anova analysis of raw epidermal defect, irrespective of exposure time, where the between subjects factor was type of treatment and the within subjects factors were raw epidermal defect values collected at each of the three weeks of sampling plus the measures collected at 1 day after burn induction, showed that there is a significant difference between treatment groups overall ($F=5.69$, $p=0.026$).

The same repeated measures Anova of epidermal defect (Ep_defect) taking into account the exposure time showed that for the 10 second group, there was a significant difference between treatment groups across the four measurement time-points ($F=5.72$, $p=0.038$), yet for the 20 second exposure group there was no difference observed between treatments ($F=0.88$, $p=0.370$).

Epidermal thickness was another variable taken into account during this study. It was measured only at the last stage of sampling, giving us only 24 samples to analyze. The analysis shows that the mean thickness of the dermis after three weeks of treatment is significantly greater in the experimental group compared to the control. This result was seen even in the 10 second exposure group.

Next, we used a semi-quantitative approach to determine if there is a difference between the two proposed treatments. We evaluated, two main parameters of healing; the state of the dermis and that of the epidermis. In the third week we've seen a significant effect of the type of treatment on the outcome of the total score $F=8.732$, $p=0.007$. When we split the data according to exposure times, the type of treatment had a significant effect in the third week of sampling $F=6.77$, $p=0.026$ in the 10s group. Repeated measures Anova of the Total scores of the dermis component of healing revealed that again there is a significant difference between the 3 weeks with a value for $F(1.94, 42.80)=78.66$ $p<0.001$, yet the overall interaction with the type of treatment was deemed insignificant $p=0.629$. Simple main effects test shows for week 3, the epidermal score for the experimental treatment had higher scores in comparison to the control group $F=21.24$, $p<0.001$.

Further correlation analyses were performed between dermal measurements, either parametric or qualitative and, the results showed that there is a fair and significant correlation

between raw dermal thickness across the three weeks with the dermal criteria that comprised the total dermis score. Unidimensional reliability analysis performed on the scoring variables showed a Cronbach's $\alpha=0.533$ with the adipocyte variable computed as a reverse-scale item as it was initially taken into consideration. Frequentist individual item reliability showed that dropping of the adipocyte coverage factor would increase the Cronbach's α to 0.814.

4. DISCUSSIONS

Apart from the fact that all samples in the experimental group at 3 weeks of treatment showed complete epidermal coverage, in comparisons to 8 out of 12 samples in the control group, the Tilapia group expressed overall higher epidermal scores with a median of 8 compared to a median of 6 for the control.

The limitations of our study are related to the relatively small sample size, the fact that only one pathologist examined the histologic samples, and the fact that the samples received only H-E staining. Additionally, there was no use of any immunohistochemical measurements, which would have allowed for an additional thorough evaluation.

Another limitation was the fact that only H-E staining was used to evaluate the healing process. Other options would have been the use of anti-keratin 14 or anti proliferating cell nuclear antigen staining to follow epidermal migration, Masson Trichrome or anti-collagen antibody staining for the visualization of collagen deposition, anti-FGF staining for fibroblast migration and activity[22].

CONCLUSION

Regarding the process of obtaining the xenograft, the study can conclude that the production, sterilization, packaging and long-term storage of the Tilapia skin grafts is feasible, does not require sophisticated processes and is easily reproducible for scientific endeavors.

The study revealed that during treatment, dermal thickness seems to follow a sinusoidal evolution during the 3 weeks, with a peak in the second followed by a decrease in the third. On the other hand, in the experimental group dermal thickness seems to maintain a stable thickness, with no significant modification between the second and third week. The author cannot predict if this aspect is of any meaningful impact on the healing process.

In all One-way Anova tables that sought to compare the two treatment groups, the results found that there is a significant difference between the treatments mostly in the 10 seconds exposure group, suggesting that the feasibility of the treatment may be higher for a milder burn.

Going further, the analysis of total healing scores was significantly greater in the experimental group.

In summary, this doctoral thesis has revealed that the use of Nile Tilapia skin xenografts, can be a viable alternative to the standard treatment of partial thickness burns. The results of this experimental study are unequivocal, the methods well design and performed and, to the best of the authors knowledge, they are unique. Further, more extensive analyses must be performed and reviewed in order to have a better understanding of the implications of this treatment.

LIST OF PUBLISHED ARTICLES *IN EXTENSO*

Bogdan Ciornei, Adrian Vaduva, Ioan Hutu, Bianca Cornelia Lungu, Daniel George Bratu, Diana Popescu, Vlad-Laurentiu David, Florin-George Horhat, Eugen Sorin Boia- Experimenting with pig-based skin model for burns. Testing of mean literature findings; *Chirurgia*, vol 119, Issue 4, 10 pages, 2024. DOI: 10.21614/chirurgia.3008 I.F: 0,8

Bogdan Ciornei, Vlad Laurentiu David, Diana Popescu, Eugen Sorin Boia, "Pain Management in Pediatric Burns: A Review of the Science behind It", *Global Health, Epidemiology and Genomics*, vol. 2023, Article ID 9950870, 10 pages, 2023. DOI:10.1155/2023/9950870 I.F: 1,9

Bogdan Ciornei; Adrian Vaduva; Vlad Laurentiu David; Diana Popescu; Dan Dumitru Vulcanescu; Ovidiu Adam; Cecilia Roberta Avram; Alina Cornelia Pacurari; Eugen Sorin Boia - Comparison of Type I and Type III Collagen Concentration between *Oreochromis mossambicus* and *Oreochromis niloticus* in Relation to Skin Scaffolding *Medicina* 2023, 59(6), 1002; DOI: 10.3390/medicina59061002 I.F: 2,6

Ciornei B, David VL, Boia ES. Future prospects in the treatment of pediatric burns. A review of the nile tilapia derived biological options for treating superficial partial thickness burns. *Jurnalul Pediatrului*. 2019 July-December, XXII, (87-88):P14-17 ISSN: 2065-4855 <http://www.jurnalulpediatrului.ro/archive/87-88/87-88-03.pdf>