Inhibitors of thermally induced burn incidents – the examinations of the flammability, TGA, SAXS and SEM methods*)

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Abstract: The examinations of the skin flammability, small-angle X-ray scattering (SAXS) and thermogravimetric (TGA) investigations were all carried out in temperature sufficient for simulating a burn incident. Above methods were used to perform assessment of collagen molecular structure changes in conditions of thermal oxidative stress, whereas the scanning electron microscopy analysis (SEM) was used to illustrate skin surface changes. The changes were observed in the presence of active antioxidants such as L-ascorbic acid, sodium ascorbate and hydrogel of orthosilicic acid $H_4SiO_4 \cdot n\ H_2O$. Presence of these modifiers of the burn process minimizes external effects of simulated burn incidents for model samples of animal skin and burn wound epidermis extracted from the patients. The examinations of the skin flammability were carried out with the limited oxygen index (*LOI*) method. In this study, synergy between orthosilicic acid and L-ascorbic acid and sodium ascorbate into animal and human skin has been shown through an increase in *LOI* values. The treatment by means of L-ascorbic acid affects particular morphological changes in the skin which is visible in SEM method. Skin samples incubated in the solution of the 3.5 %, 5 % L-ascorbic acid, 7 % sodium ascorbate solutions and 7 % orthosilicic acid demonstrate the development of a structure resembling a coherent solid composite. SAXS gives structural information on the assembly of dermal collagen as well as the lamellar organization of stratum corneum (SC) lipids located in the outermost part of the epidermis. Using this technique, two lamellar phases with repeat distance of approximately 4.3 and 6 nm in the SC lipids domains were observed. Moreover, the diameter of the collagen fibrils were extracted. The observed differences in the values of these parameters allowed us to better understand the mechanism of modification of the surface of the burn affected skin and the influence of the modification on the process of skin regeneration.

Keywords: collagen of skin, burn incidents, L-ascorbic acid, sodium ascorbate, orthosilicic acid, *LOI*, TGA, SAXS, SEM.

Inhibitory modelowych incydentów oparzeniowych – charakterystyka palności, badania TGA, SAXS i SEM

Streszczenie: Miejscowa oraz ogólna odpowiedź organizmu na oparzenie termiczne jest złożona. Nie tylko prowadzi do uszkodzenia skóry, ale wywołuje też głębokie długotrwałe zmiany w metabolizmie organizmu. Na podstawie wyznaczonego granicznego wskaźnika palności (*LOI*), wyników analizy termograwimetrycznej (TGA) i małokątowej dyfraktometrii rentgenowskiej (SAXS) oceniano zmiany zachodzące w strukturze kolagenu w warunkach stresu oksydacyjnego, a metodą skaningowej mikroskopii elektronowej (SEM) określano zmiany topografii powierzchni badanych próbek skóry. Modyfikowany aktywnymi przeciwutleniaczami, takimi jak: pochodne witaminy C (np. kwas L-askorbinowy, askorbinian sodu) oraz hydrożel kwasu ortokrzemowego $H_4SiO_4 \cdot n\ H_2O$, kolagen wykazuje zwiększoną aktywność biochemiczną, a obecność niniejszych modyfikatorów procesu oparzeniowego minimalizuje zewnętrzne skutki symulowanych incydentów oparzeniowych.

Słowa kluczowe: kolagen, incydent oparzeniowy, kwas L-askorbinowy, askorbinian sodu, kwas ortokrzemowy, *LOI*, TGA, SAXS, SEM.

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Burn wound healing is a complex biological process of replacing damaged tissue by living tissue. During cutaneous thermal injury several factors contribute to further tissue damage and important of these are the oxygen free radicals [1]. The oxidative damage caused by reactive oxygen species (ROS) to lipids, proteins, sugars, and DNA, as well as a significant decrease in total antioxidant capacity, which protects the organism against ROS activity. At this point the early intervention of antioxidant therapy will significantly help to restore cell mediated immunity, decrease free radical mediated damage and minimize tissue destruction during extensive burn injury [2–4].

The examinations of the skins flammability were carried out with the limited oxygen index (*LOI*) method. In this study, synergy between orthosilicic acid and L-ascorbic acid and sodium ascorbate into animal and human skin has been shown through an increase in *LOI* values [5].

The purpose of this study was to macroscopic evaluation of the effectiveness of antioxidants. The selection of *LOI* method for this type of research was based on the similar research for protein fibers (*e.g.* wool) [6]. In the presented paper, the method of *LOI* flammability testing was used in an unconventional way.

Thermogravimetric analysis (TGA) studies on the impact of ethanol and significance of water to penetration of drugs through the skin after 3rd degree burn are presented in [7–9]. In this paper, the TGA technique was used innovatively to determine the effect of active antioxidants, such as L-ascorbic acid, sodium ascorbate, and the hydrogel of orthosilicic acid $H_4SiO_4 \cdot n\ H_2O$, on thermal dissociation of collagen.

Small-angle X-ray scattering (SAXS) gives structural information on the assembly of dermal collagen as well as on a unique lamellar arrangement of stratum corneum (SC) lipids located in the outermost part of the epidermis [10, 11]. In skin research, SAXS has reported very interesting data concerning to the organization of SC lipids and to evaluate the integrity of SC structure arrangement after different treatments [12–14].

The subject of the previous studies were animal samples (subjected to a burn incident which simulated a 3rd degree burn) – a model of skin burnt *ex-vivo*. The earlier studies included application of the following modifiers of the burn process: hydrogel of orthosilicic acid, fucoidan (sulfonated polysaccharide from brown algae), β-glucan (a component of biologically and therapeutically active biomaterials) [15–17] to perform assessment of collagen molecular structure changes in conditions of thermal oxidative stress. In the present paper we observed how the presence of active antioxidants such as L-ascorbic acid, sodium ascorbate and hydrogel of orthosilicic acid $H_4SiO_4 \cdot n\ H_2O$ minimizes external effects of simulated burn incidents for model samples of animal skin and example burn wound epidermis extracted from the patients.

EXPERIMENTAL PART

Materials

The object of the study included animal samples – a model of skin burnt *ex-vivo*. Prepared skin samples were subjected to a burn incident which simulated a 3rd degree burn. The exact procedure for the preparation of samples for research was presented previously in the study [15, 16] and below in Abbreviations:

– Native animal skin (1A);

– Animal skin samples (A) were warmed up at temperature of 100 °C for 60 s and subsequently incubated in:

3.5 % L-ascorbic acid solution and 7 % orthosilicic acid solutions (3.5ALASi);

5 % L-ascorbic acid solution and 7 % orthosilicic acid solutions (5ALASi);

7 % sodium ascorbate solutions and 7 % orthosilicic acid solutions (7ALASi).

– Animal skin samples (AW, second series) were warmed up at temperature of 100 °C for 60 s and subsequently incubated in:

3.5 % L-ascorbic acid solution (AWSLA);

3.5 % L-ascorbic acid solution and 7 % orthosilicic acid solutions (AWLASi).

– Human burn skin [17] samples (S);

– human burn injury skin [17] samples (S) subsequently incubated in:

3.5 % L-ascorbic acid solution (SLA);

3.5 % L-ascorbic acid solution and 7 % orthosilicic acid solutions (SLASi).

Methods of testing

Scanning electron microscopy analysis

The animal and human skin surface was examined using a JSM-5500LV scanning electron microscope supplied by JEOL. The samples were mounted on aluminum stubs and coated with gold (JFC 1200, JEOL). Secondary (SE) and back-scattered electron (BSE) observations were conducted with an accelerating voltage of 10 kV. Microphotographs were obtained at magnifications ranging from 50× to 1000×.

Method of the limiting oxygen index

The obtained flame retardant effect of skins was evaluated using the method of the limiting oxygen index (*LOI*). A parameter that characterizes the method is the lowest percentage of oxygen in the mixture with nitrogen, at which the test specimen ignites and burns on its own. The measurements were performed in accordance with PN–ISO 4589 standard.

Thermogravimetric analyses

Investigated were performed using a TA Instruments Q500 Thermogravimetric Analyzer. Measurements were

performed in a temperature range from 30 °C to 800 °C with a heating rate of 20 deg/min under a nitrogen atmosphere (flow rate 40 cm^3/min). At a temperature of 800 °C, the inert gas was switched to air (3 min) for burning the organic remnants of the sample.

Small-angle X-ray scattering analyses

SAXS measurements were carried out with the compact Kratky camera, equipped with the SWAXS optical system of Hecus-MBraun (Austria). The Cu target X-ray tube, operated at: U = 40 kV, I = 25 mA was used as a radiation source (λ = 0.154 nm). The primary beam was monochromatized by Ni filter and a pulse-height discrimination. Scattered radiation was recorded in acquisition time of 900 s by means of a MBraun linear position-sensitive detector, model PSD 50. The detector had 1024 channels with a channel-to-channel distance of 52 μm. The SAXS data were collected as a function of the scattering vector $s = 2\sin\theta/\lambda$ where 2θ is the scattering angle and λ = 0.154 nm is the X-ray wavelength.

RESULTS AND DISCUSSION

Antioxidant solutions can be used as therapeutically active biomaterials that speed up the process of wound healing.

This thesis, present in numerous ongoing studies worldwide [18–22], was the basis for this investigation. Burn injuries are complex traumatic events with various local and systemic effects, *i.e.*, flame burns.

Many authors studied the capacity of locally applied vitamin C to stimulate dermal vitamin concentration and to SC penetration of the epidermis [22–27].

A scanning electron microscope (SEM) was used to examine morphological changes to the skin surfaces. The results of SEM are shown in Fig. 1.

Micro- and macroscopic assessment of the skin samples demonstrated the process of burn-cleaning and uncovering the morphology of the epidermal surface and destructive changes in the surface.

It appears that the application of the antioxidant eliminates burn blisters from the skin surface. Skin samples

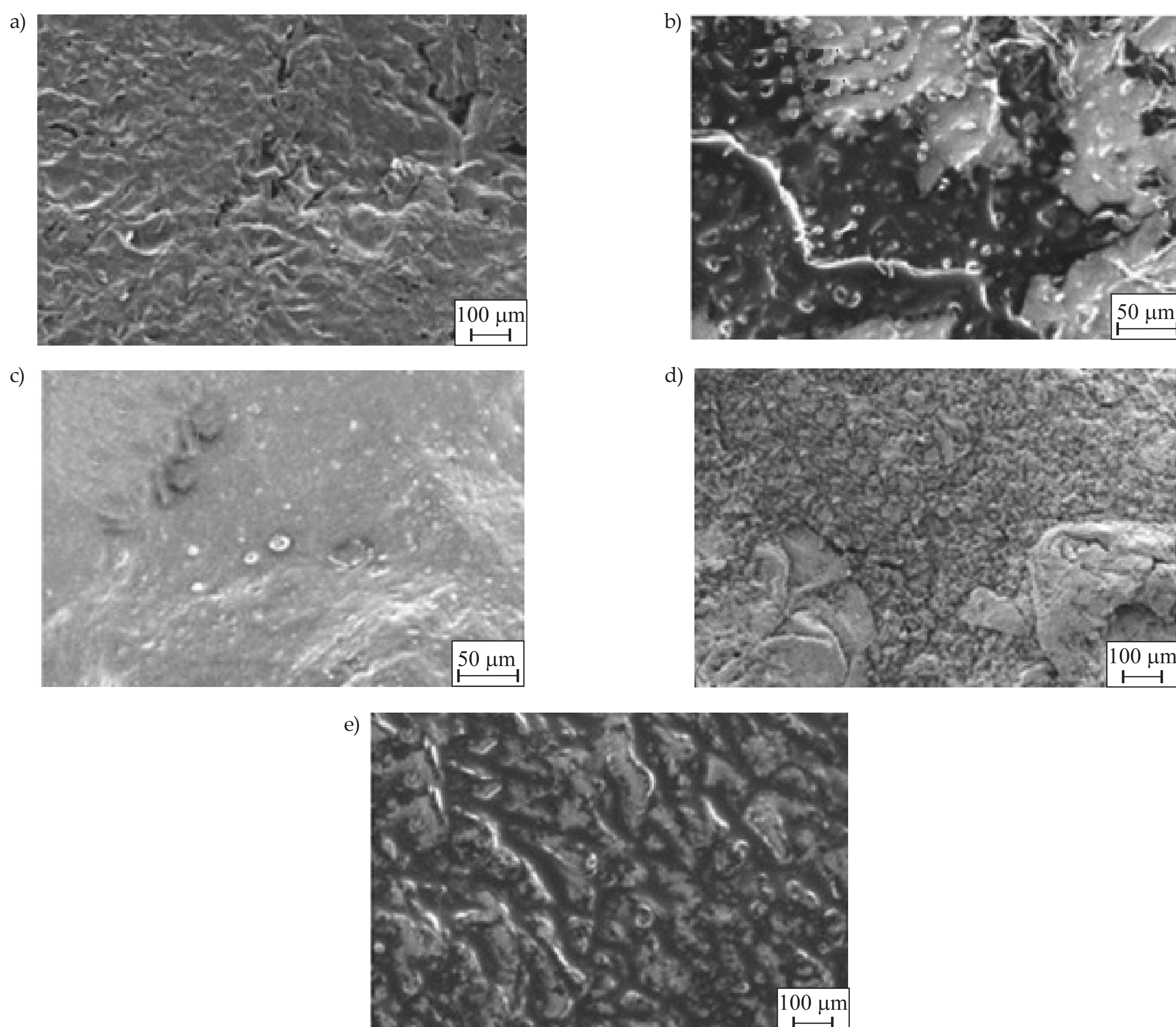

Fig. 1. SEM images of the surfaces of skin samples: a) 1A, b) 3.5ALASi, c) 5ALASi, d) 7ALASi, e) SLASi; magnification 70×, 100×, 300×

T a b l e 1. Results of *LOI* measurements of studied samples

Sample of skin	Limiting oxygen index %
1A	20.9
3.5ALASi	22.0
5ALASi	22.1
7ALASi	22.9

incubated in the solution of the 3.5 %, 5 % L-ascorbic acid, 7 % sodium ascorbate solutions and 7 % orthosilicic acid demonstrate the development of a structure resembling a coherent solid composite (Fig. 1).

A series of samples of animal skins treated in solutions of antioxidants: L-ascorbic acid, a hydrogel of orthosilicic acid and sodium ascorbate, was selected for *LOI* flammability testing. Analyzing the results shown in Table 1, an increase in the *LOI* for samples treated with a mixture of antioxidants is observed, with the highest *LOI* values obtained for a mixture of 7 % sodium ascorbate, and 7 % orthosilicic acid (*LOI* = 22.9 %).

In order to demonstrate the impact of active antioxidants, discussed in the paper, on the process of thermal decomposition of the protein component of skin, TGA studies were conducted. Measurements were carried out in two series. The first one was related to the thermal dissociation process of human skin samples scalded (with fragmentary burn destruction) in an actual fire. The second analyzed the thermal decomposition of samples of animal skin (from chickens) – both raw and after a planned, model scalding incident, which consisted in subjecting the skin to the influence of boiling water for 60 s.

Figure 2 presents a summary of the mass loss curves (TG) and the derivative (DTG) recorded during the thermal decomposition of human skin samples carried out in a nitrogen atmosphere.

Scalded skin sample, not subjected to the treatment by the therapeutic solution of antioxidants exhibits characteristic mass losses associated with: moisture loss (6.4 %), decomposition of fat substances (5.8 %) and, finally, an essential loss corresponding to the thermal dissociation of collagen (63.4 %). On the other hand, the TG curves for scalded skin samples, subjected to a solution of L-ascorbic acid and orthosilicic acid, additionally displayed mass losses in the temperature ranges corresponding to the decomposition of these compounds. The amounts of these losses in the tested samples are varied and correspond to the quantity of sorption of the above acids for scalded skin in a simulated therapeutic process.

The process of the thermal decomposition of animal skins is completely the same, as presented in Fig. 3 for different variants of the samples not treated with the antioxidants and for skins treated with solutions of L-ascorbic acid at different concentrations or sodium ascorbate and 7 % solution of orthosilicic acid.

The maximum rate of decomposition of L-ascorbic acid, expressed by the occurrence of the peaks on differential DTG patterns, is in the range of 197–210 °C, while the temperature range corresponding to the maximum speed of decomposition of the protein component of the skin is in the range 323–343 °C. The maximum rate of decomposition of adsorbed orthosilicic acid, on the other hand, is in the range of 375–418 °C. It should be noted that the above stages of decomposition, both in the case of human and animal skins, are not separate in terms of temperature ranges and overlap to a large extent (DTG signal between them does not reach 0). Generally, the process of thermal decomposition of all tested skins was in the range from approx. 190 °C to less than 500 °C.

In the case of animal skins subjected to a strictly defined burn incident which is repetitive over the population of all tested samples, a detailed analysis of the DTG differential patterns in the temperature range of skin protein component decomposition indicates that for the sample subjected to the tested antioxidants, in particular a solution of orthosilicic acid, the temperature of the maximum weight loss rate corresponding to the protein (collagen) decomposition has moved as much as 20 °C towards higher temperatures. This is the fundamental conclusion of the TGA studies, linking the therapeutic

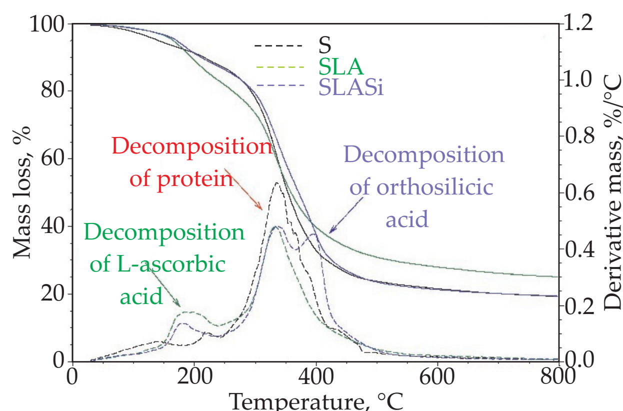

Fig. 2. TG and DTG curves (heating mode 20 deg/min) of selected human skins; visible thermal areas of decomposition: protein, L-ascorbic and orthosilicic acids, respectively

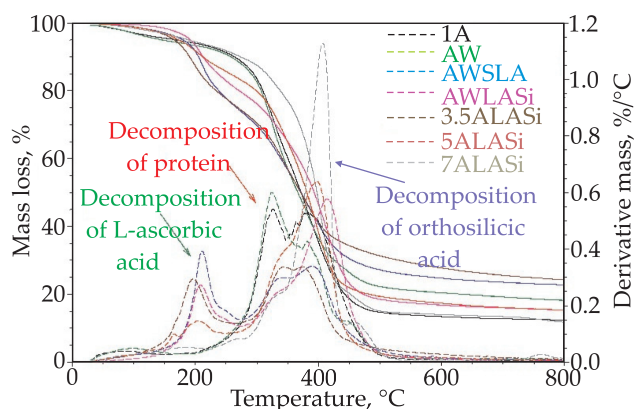

Fig. 3. TG and DTG curves (heating mode 20 deg/min) of studied animal skins; visible thermal areas of decomposition: protein, L-ascorbic and orthosilicic acids, respectively

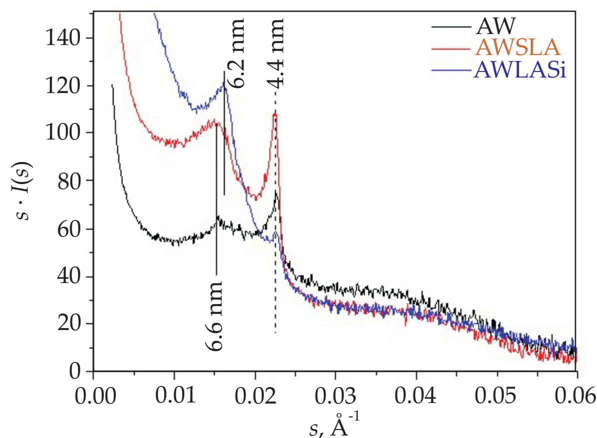

Fig. 4. Comparison of Lorentz corrected SAXS curves of animal skins

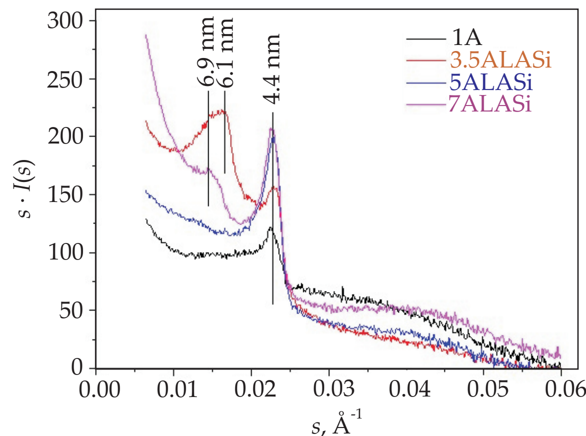

Fig. 5. Comparison of Lorentz corrected SAXS curves of animal skins

impact of orthosilicic acid with delayed thermal degradation of the protein.

Figure 4 shows SAXS patterns for the animal skin samples, which were warmed up at temperature of 100 °C for 60 s (sample AW) and for animal skin samples, which after were warmed up at temperature of 100 °C for 60 s were incubated in 3.5 % L-ascorbic acid solution (sample AWSLA) and in mixture of 3.5 % L-ascorbic acid solution and 7 % orthosilicic acid solution (sample AWLASi). All SAXS profiles exhibit two discrete maxima associated with the lamellar structure of the stratum corneum (SC) which is the uppermost layer of the epidermis. The SC is composed of protein-enriched corneocytes embedded in lipid-rich extracellular matrix. The SC lipids consist mainly of ceramides (CER), free fatty acids (FFA) and cholesterol (CHOL) [12]. Recent SAXS studies revealed that in SC lipids even three lamellar phases can be present with periodicities 13 nm, 6 nm and 4.2 nm, respectively [14]. Both 13 nm and 6 nm phases are formed in lipids containing CER/CHOL mixture, wherein the formation of 6 nm phase requires a higher cholesterol content than the formation of the 13 nm lamellar phase. Furthermore, when the relative amount of cholesterol is very high, the 6 nm phase is the most pronounced one. The presence of short-chain FFA in SC lipids induces the formation of an additional 4.2 nm lamellar phase [12]. On the SAXS curve for the skin AW (Fig. 4) there is a clear peak for the 4.4 nm phase and a very weak peak of 6.6 nm phase. For the skin incubated in 3.5 % L-ascorbic acid solution (AWSLA) intensity of both peaks increases, indicating the regeneration of these lipid lamellar structures in this sample. Addition of orthosilicic acid to the incubation solution (sample AWLASi) results in a further regeneration of the 6.6 nm phase and the loss of 4.4 nm lamellar phase, associated with FFA.

Figure 5 shows the role of L-ascorbic acid in the phase behavior of SC lipids. The SAXS curve of native animal skin (sample 1A) exhibits only one maximum associated with the 4.4 nm lamellar phase. For skins incubated in solutions containing 7 % orthosilicic acid but different amount of L-ascorbic acid intensity of this peak increases indicating the regeneration of the phase rich in FFA. Furthermore, for these samples another maximum, connected with 6 nm CER/CHOL phase, is observed. For the sample incubated in solution containing 3.5 % of L-ascorbic acid this maximum is broad due to the overlapping of two peaks. It indicates on the formation of two slightly different 6.1 nm and 6.9 nm phases. The use of the higher content of L-ascorbic acid in incubation solution causes that these phases do not arise. Hence, the SAXS curve of the sample 5ALASi does not contain the maximum connected with these structures. The SAXS curve of the skin incubated in 7 % sodium ascorbate solutions and 7 % orthosilicic acid solutions (sample 7ALASi) contains two maxima which positions indicate on the existence of 6.9 nm and 4.4 nm lamellar phases in this skin.

In summary, the SAXS measurements revealed a great impact both L-ascorbic acid and orthosilicic acid on the behavior of lamellar phases in SC lipids. This may contribute to better understanding of mechanism governing the regeneration of SC lipids in diseased skin.

CONCLUSIONS

Heat shock and thermal oxidative stress is one of the main causes of pathophysiological alterations observed during burn injury. The observed differences in the values of these parameters (*LOI*, TGA, SAXS, SEM) allowed us to better understand the mechanism of modification of the surface of the burn affected skin and the influence of the modification on the process of skin regeneration. However, in order to make definite conclusions, more tests and analyses are necessary as the regenerative reactions especially in human wounds are dynamic and complex. The above presented study constitutes an example of a comprehensive analytical method (SEM, *LOI*, TGA, SAXS) which can be applied to clinical trial analyses.

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