Pharma Sci-A Comprehensive Method of Identifying Heat Shock Proteins (HSPs)- Pielesz A- University of Bielsko-Biala

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Introduction

Burns are associated with significant inflammatory responses [1]. An acute-phase response is elicited in burns, with increasing plasma levels of interleukin (IL)-6 and tumor necrosis factor (TNF)-a as well as stress hormones: catecholamines and cortisol.

Cytokines such as interleukin 1β (ILlβ) and interleukin 6 (IL6) were significantly elevated in the plasma of patients with thermal injury compared to unburnt control subjects . Both cytokines were highest during the first week after injury and declined over time.

Heat shock response, or stress response, is perhaps the oldest and most conserved form of reaction to stress. Heating cells or organisms generates the expression of a class of proteins known as heat shock proteins (HSP). HSP families include both constitutive and stress-inducible members whose primary function is to interact with native and denatured proteins to prevent the aggregation of aberrantly folded proteins, facilitate the folding of native proteins, facilitate the refolding of denatured proteins, and to aid intracellular protein trafficking.

Wound infections may be caused by endogenous (patient's flora) or exogenous microorganisms (environment, personnel's flora, materials used during surgery and treatment). Most infections are caused by patient's own flora. The most often isolated etiological factors among bacteria are the Gram-positive *Staphylococcus aureus, Enterococcus* sp., Coagulase-negative staphylococci, streptococci and Gram-negative *Pseudomonas aeruginosa* bacilli (often responsible for infecting diabetic foot ulcers and burns). The presence of microorganisms in open wounds is natural and generally does not lead to delayed healing. However, if the interactions between bacteria and the host turn into an infection, the wound condition deteriorates. Moist and abounding in nutrients, a chronic wound makes a perfect environment for multiplication of pathogens. Bacteria may live and reproduce as single cells, although an overwhelming majority of them have the ability to form a biofilm consisting mainly of *Staphylococcus* and *Pseudomonas*. A biofilm is an Organised structure of settled microorganisms (capable of adhering to the surface of a wound, other tissue of a living organism or inanimate elements), surrounded by an extracellular layer of mucus, which is made up of organic and inorganic substances produced by the bacteria. It protects them against adverse environmental conditions (pH, temperature, and radiation), antimicrobial agents (antibiotics) and the host's immune system (antibodies, phagocytes, neutrophils).

The objective of this study was to monitor the effect of temperature on collagen from organic chicken skins; we have used a model in which chicken skin is exposed to a variety of thermal injuries in the presence and absence of human serum albumin. The analyses were carried out after heating the samples to a temperature simulating a burn incident. The experiments were aimed at determining whether heat shock at

physiological/pathophysiological temperatures stimulates the release of HSPs.

Materials and Methods

Preparation of Organic Chicken Skin Samples

Samples of organic chicken skin (an *ex-vivo* burn injured skin model) were collected and determined as previously described. Samples of organic chicken skin were prepared for FTIR spectroscopy by drying them on strips of filter paper (WPS, WS30, and IWS30-40). Samples DPS, DS30, ID30-40, IDS60 were dried in a laboratory dryer at 45°C for three days. In contrast, liquid samples in HSA were frozen at –18°C for 24 hours.

Microbial Analysis

The samples of organic chicken skin were examined. They were exposed to bacteria that can cause nosocomial infections, that is the Gram-positive S. aureus and Gram-negative *E. coli*. Physiological salt (2 cm3) was poured into two sterile test tubes. Using a sterile (red hot) inoculation loop, *E. coli* sample was taken from its culture on enriched agar (used for growing particularly demanding bacteria strains), inserted into one of the test tubes and diluted in the salt. Using a pipette, three drops of the suspension were transferred onto enriched agar; then, using a cooled sterile bacteria spreader, they were spread all over the agar surface. After that, the spreader was sterilized again and a part of skin was placed in the centre of the Petri plate.

The same procedure was repeated for *S. aureus*, which was placed on mannitol salt agar (containing 7.5% NaCl for inhibiting the growth of other bacteria). The Petri plates were subsequently placed in a tube and then kept in a laboratory heater at 37°C for 24 h and 48 h. The samples of organic chicken skin were exposed to the same bacteria: *E. coli* on MacConkey agar (containing salts of bile acids and crystal violet inhibiting the growth of Gram-positive bacteria) and *S. aureus* on mannitol salt agar (with high concentration of NaCl inhibiting the growth of other bacteria). The samples were kept in a laboratory heater at 37°C for 24h. The exact procedure for designation was described in.

Electrophoretic Analysis

The samples were subjected to electrophoresis on a strip of cellulose acetate membrane (CASYS-MINI) in barbital buffer (pH 8.6) at 6 mA, maximum 200 V for 0.5 h. The strips were stained with 0.5% toluidine blue in 3% HOAc solution and then rinsed in distilled water and air-dried. The strips were stained with 0.5% amido black in 5% HOAc solution and then fixed in methanol, HOAc and water (18:4:18); then rinsed in 5% HOAc solution and distilled water, and air-dried. Semi-quantitative analysis of the protein content in the samples was also conducted using GELSCAN v.1.45 software.

IR Spectroscopic Analysis

FTIR spectroscopic analysis was performed using a Nicolet 6700 Fourier-transform spectrophotometer (Thermo Scientific, USA) with OMNIC 7.0 software and equipped on diffusion accessory EasiDiff (Thermo Nicolet Industries) (spectral region: 4000- 500 cm^{-1}, resolution: 4 cm^{-1}, number of scans: 160) of the solid samples (fragments of the samples of pure organic chicken skin or liquid samples in HSA). Spectra of three repacked subsamples of each individual sample were averaged to one spectrum. All spectra were performed using a linear baseline and preprocessed with the Fourier smoothing (Grams 32 AI software, Galactic Industries).

Scanning Electron Microscopy Analysis

Chicken 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 electrons (SE) and back-scattered electrons (BSE) observations were conducted, with the accelerating voltage of 10kV. Microphotographs were taken at magnifications ranging 35×.

Results

Burn injury is a complex traumatic event with various local and systemic effects. Prolonged exposure to temperatures higher than 40°C leads to denaturation of proteins and finally loss of their plasma membrane integrity. This process is rapid and may only take a second when exposed to temperatures higher than 60°C, i.e. flame burns.

In this study acetate electrophoresis (CAE), microbiological procedure and Fourier-transform infrared spectrometry (FTIR) were all carried out after heating the samples to a temperature simulating a burn incident. These tests are illustrated by photos of scanning electron microscopy (SEM) analysis.

The results of scanning electron microscopy analysis are shown in (Figure 1) which is an example of representative analyses of a series of tests. The images reveal clear differences in the morphology of the surface of the samples tested, including even non-specific damage to the skin surface. The differences can be seen for subsequent stages of thermal processing. For samples of skin heated to boiling point for 30s (Figure 1b), and for samples of skin heated to boiling point for 30-40s, then incubated at boiling point for 30s (Figure 1c-1e), the surface is severely damaged, with visible bulges, smoothing and blisters, while (Figure 1e) indicates the smoothing of the skin surface caused by prolonged exposure to high temperature.
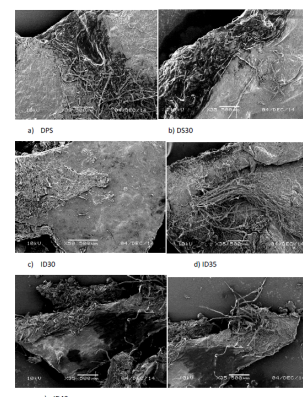

Figure 1. Scanning electron microscopic images of the surface of skin samples: (a) DPS–untreated skin (×35); (b) DS30– skin heated to boiling point for 30s (×35); (c) ID30–skin heated to boiling point for 30s and then incubated at boiling point for 30s (×35); (d) ID35–skin heated to boiling point for 35s and then incubated at boiling point for 30s (×35); (e) ID40–skin heated to boiling point for 40s and then incubated at boiling point for 30s (×35).

Figure 1. Scanning electron microscopic images of the surface of skin samples: (a) DPS–untreated skin (×35); (b) DS30– skin heated to boiling point for 30s (×35); (c) ID30–skin heated to boiling point for 30s and then incubated at boiling point for 30s (×35); (d) ID35–skin heated to boiling point for 35s and then incubated at boiling point for 30s (×35); (e) ID40–skin heated to boiling point for 40s and then incubated at boiling point for 30s (×35).

It is known that following a burn incident, the skin is no longer a mechanical and biological barrier to external factors. All burns carry the risk of infections because bacteria can enter broken skin. Sepsis, or a bloodstream infection, can occur in the most severe cases. This can lead to shock or even death. The resulting necrotic scab is a good medium for the development of microorganisms spreading into the skin and subcutaneous tissue. *Staphylococcus aureus* and coagulase-negative staphylococci (like *S. epidermidis*) are between the most frequently isolated bacteria from burn wounds.

Prolonged exposure to boiling-point temperatures leads to modification of skin samples. The samples of skin heated to boiling point for 30-40 s and then incubated at boiling point for 30s were compared with a reference sample of WPS. However, it turns out that skin damaged in a burn

does not entirely lose its antimicrobial capabilities (Table 1). A severe burn is associated with release of inflammatory mediators which ultimately cause local and distant pathophysiological effects. Mediators including reactive oxygen species and reactive nitrogen species are increased in affected tissue, which are implicated in pathophysiological events observed in burn patients. Both increased xanthine oxidase and neutrophil activation appear to be the oxidant sources in burns. Free radicals have been found to have beneficial effects on antimicrobial action and wound healing. However following a burn, there is an enormous production of reactive oxygen species which is harmful and implicated in inflammation, systemic inflammatory response syndrome, immunosuppression, infection and sepsis, tissue damage and multiple organ failure. Thus clinical response to burn is dependent on the balance between production of free radicals and its detoxification.

Table 1. Resistance of *Escherichia coli* and *Staphylococcus aureus*.

Samples	Microorganisms	
	Escherichia coli	*Staphylococcus aureus*
WPS	+	-
WS30	+	-
IWS30	-	-
IWS35	+	-
IWS40	+	-
DPS	-	-
DS60	+	-
IDS60	-	-

Note: - : Lack of inhibition growth zone; + -zone of the growth inhibition bigger than 1 mm.

Table 1. Resistance of Escherichia coli and Staphylococcus aureus.

Furthermore antimicrobial peptides found in the burnt skin, probably HSP proteins, explain why it is possible for a burn to heal without signs of clinical infection under dressings that have no antimicrobial properties. This can explain the presence of increasing inhibition zones as shown in (Table 1) for samples: WPS, WS30, IWS35 and IWS40.

Other methods such as two-dimensional PAGE have been used to demonstrate that the removal or separation of high abundance proteins enables greatly improved detection of lower abundance proteins. Albumins are the most common serum proteins. Their thermo sensitivity and thermo tolerance was the subject of this study concentrating on cellulose acetate membrane electrophoresis. Albumins are the most common plasma proteins accounting for 55-65% of all blood proteins. They are relatively small molecules with a weight of 66.5-66.9 kDa. It is estimated that following a burn incident, the level of albumin and transferrin falls by as much as 50-70%. Decreasing concentration of constituent proteins (such as albumin) is caused by, among other factors, the loss of proteins as they escape intensively from the capillaries into the extravascular space and the burn wound. During the inflammatory reaction following heat shock, albumin concentration decreases, and it can even convert to lower-weight proteins, possibly caused by complexes-associates forming in the serum. In this CAE study (Figure 2), a significant reduction of albumin concentration was observed during heat stress and associates with lower molecular weight were found. The presence of bands from HSP aggregates is probably related to the secretion of HSPs during the conditioning of samples at boiling temperature for 30-40s and, especially, for 2 minutes (Figure 2). Heat shock proteins, especially the HSP70 family, may be an objective indicator of an individual's tolerance of heat as well as favorably influence the healing of burnt skin. HSP increase correlates with an increase in cardiac output and blood pressure, while reducing the circulating pro-inflammatory cytokines. Local expression of HSPs in burnt tissue was examined, which demonstrated increased concentration of HSP32 and HSP70 in the skin following second- and third-degree burns. The electropherogram of samples in (Figure 2) reveals an additional oligomer band, possibly from HSP32.

Thermal stability is an important property of collagen when used in biomedical. Therefore,

FTIR spectra of chicken skin heated to boiling point for 30-120s and then incubated at boiling point were analysed and compared (Figure 3). Both dried and wet samples were selected. First, in (Table 2), changes of the position of the amide I, II and III bands and absorption ratio, I1235/ I1450 were analysed. The 1720-1600cm^{-1}, 1600-1480cm^{-1} and 1400–1200cm^{-1} region were assigned to amide I, II and III bands, respectively (Table 2). The amide I band (1649-1659 cm^{-1}) in the infrared spectrum is usually assigned to α-helix structure; the two shoulders that appear at 1620 cm^{-1} and 1680 cm^{-1} may be attributed to the intermolecular β-sheet aggregates. These aggregates were identified. The peak of the 1677 cm^{-1} amide band of dry pure chicken skin is markedly shifted to the region of roughly 1692–1699 cm^{-1} for samples of dry chicken skin heated to boiling point for 30-40 s and then incubated in presence of HSA at about boiling point for 30 s, which confirms that heat-induced protein aggregates indeed form the intermolecular β-sheet structure. For wet chicken skin, conversion around 1649cm^{-1} occurs, in the region of 1651-1634 cm^{-1}. The peak of the 1649 cm^{-1} amide band of wet pure chicken skin (Table 2) is markedly shifted to the region of roughly 1634 cm^{-1} for samples of wet chicken skin heated to boiling point for 30-40 s and then incubated in presence of HSA at about boiling point for 30 s. This may be attributed to the intermolecular β-sheet aggregates and increasing of hydrogen bonds. The intensity ratio between the amide III band and the 1470-1450 cm^{-1} band has been used to indicate the triple-helical structure of collagen. Earlier reports indicate that in FTIR spectra of pure collagen, the IR absorption ratio, I1235/I1450 cm^{-1} is 1.00, whereas gelatin membrane (denatured collagen) was reported to have this ratio as 0.59, indicating loss of triple helicity. This loss is indicated by the data in (Table 2). While heating the dry organic chicken skin to boiling point for 60 s (Figure 3)

leads to the disappearance of a wide band in the 1650- 1550 cm^{-1} area, heating the organic chicken skin to boiling point for 120 s leads to the conversion of a band in the 1700–1600 cm^{-1} area, which may be attributed to the intermolecular β-sheet aggregates.
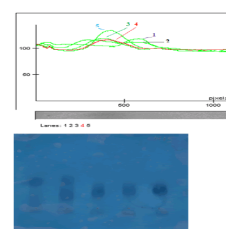

Figure 2. Cellulose acetate membrane electrophoresis and semi-quantitative analysis of HAS samples. From left to right: (a) untreated HSA: (b) HSA in presence of skin heated to boiling point for 30s, and then incubated at boiling point for 30s; (c) HSA in presence of chicken skin heated to boiling point for 40s and then incubated at boiling point for 40s; (d) HSA in presence of chicken skin heated to boiling point for 60s and then incubated at boiling point for 60s; (e) HSA in presence of chicken skin heated to boiling point for 120s and then incubated at boiling point for 120s.

Figure 2. Cellulose acetate membrane electrophoresis and semi-quantitative analysis of HAS samples. From left to right: (a) untreated HSA; (b) HSA in presence of skin heated to boiling point for 30s, and then incubated at boiling point for 30s; (c) HSA in presence of chicken skin heated to boiling point for 40s and then incubated at boiling point for 40s; (d) HSA in presence of chicken skin heated to boiling point for 60s and then incubated at boiling point for 60s; (e) HSA in presence of chicken skin heated to boiling point for 120s and then incubated at boiling point for 120s.

Table 2. The most diagnostic peak (amide I, II and amide III bands) in the IR spectra of dry and wet chicken skin.

Dry samplesfr	Band location			Absorption ratio I_{1235}/I_{1450} cm^{-1}
	1720-1600cm^{-1}	1600-1480cm^{-1}	1400-1200cm^{-1}	
DPS	1756, 1677	1563	1470, 1421, 1383	0.91
DS30	1753, 1676	1560	1464, 1418, 1381	0.92
IDS30	1751, 1692	1560	1467, 1458, 1379	0.85
IDS35	1753, 1698	1568	1469, 1418, 1392	0.77
IDS40	1754, 1699	1567, 1580, 1559	1468, 1418, 1380	0.77
Wet samples	**Band location**			**Absorption ratio I_{1235}/I_{1450} cm^{-1}**
	1720-1600cm^{-1}	1600-1480cm^{-1}	1400-1200cm^{-1}	
WPS	1743, 1663, 1649	1551, 1536, 1519, 1503	1467, 1450, 1412	0.89
WS30	1747, 1662, 1647, 1634	1554, 1538, 1519, 1503	1468, 1453, 1415	0.93
IWS30	1748, 1664, 1651	1553, 1538, 1504	1465, 1453, 1413	0.78
IWS35	1661, 1649, 1635	1551, 1536	1452, 1404	0.67
IWS40	1661, 1634	1550, 1537	1464, 1447, 1407	0.88

Table 2. The most diagnostic peak (amide I, II and amide III bands) in the IR spectra of dry and wet chicken skin.
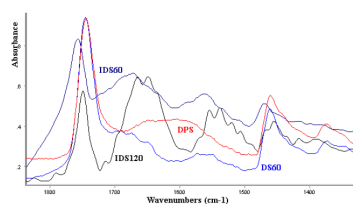
Figure 3. Fragment of FTIR spectra: dry pure chicken skin (DPS); dry chicken skin heated to boiling point for 60s (DS60); dry chicken skin heated to boiling point for 60s and then incubated in presence of HSA at about boiling point for 60s (IDS60); dry chicken skin heated to boiling point for 120s and then incubated in presence of HSA at about boiling point for 120s (IDS120).

Figure 3. Fragment of FTIR spectra: dry pure chicken skin (DPS); dry chicken skin heated to boiling point for 60s (DS60); dry chicken skin heated to boiling point for 60s and then incubated in presence of HSA at about boiling point for 60s (IDS60); dry chicken skin heated to boiling point for 120s and then incubated in presence of HSA at about boiling point for 120s (IDS120).

IR spectroscopy can potentially be utilized to identify areas of tissue affected by early heterotopic ossification as well as areas of tissue that may be predisposed to HO formation. Peaks at 1660 cm^{-1} were also consistent with expected protein bands, including collagen that would be present in bone tissue. The 1450 cm^{-1} band is a combination of protein and lipids CH_2 wagging vibrations; peaks in the bone mineral bands at 958 cm^{-1} (PO_4^{3-}) and 1070 cm^{-1} (CO_3^{2-}) respectively are characteristic of the unique apatite mineralization of bone (Figure 4).
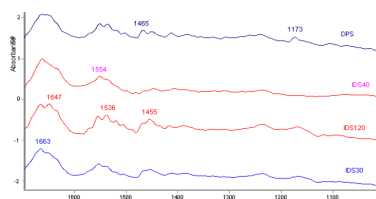

Figure 4. Fragment of FTIR spectra: dry pure chicken skin (DPS); dry chicken skin heated to boiling point for 30s and then incubated in presence of HSA at about boiling point for 30s (IDS30); dry chicken skin heated to boiling point for 40s and then incubated in presence of HSA at about boiling point for 40s (IDS40); dry chicken skin heated to boiling point for 120s and then incubated in presence of HSA at about boiling point for 120s (IDS120).

Figure 4. Figure 4. Fragment of FTIR spectra: dry pure chicken skin (DPS); dry chicken skin heated to boiling point for 30s and then incubated in presence of HSA at about boiling point for 30s (IDS30); dry chicken skin heated to boiling point for 40s and then incubated in presence of HSA at about boiling point for 40s (IDS40); dry chicken

skin heated to boiling point for 120s and then incubated in presence of HSA at about boiling point for 120s (IDS120).

Generally, recording infrared spectra from solutions of pure biochemical compounds using FTIR spectroscopy has been very complicated, but determination of changes in FTIR spectra of liquid samples is fundamental to the discovery of valid biomarkers. In this study, FTIR spectra were recorded from frozen samples of thermally modified serum. Analyzing such samples is complicated; the spectra obtained result in poor signal to noise ratios from liquid body fluids and the strong contribution of water at 1638 cm^{-1}. First, changes of the position of the amide I band were analysed (Table 3 and Figure 5). The most diagnostic peaks in the IR spectra have the locations presented in (Table 3). However, when increasing the concentration of the solution, the different features of the samples can be seen to systematically evolve. The single band at 1652 cm^{-1} observed for serum is separated in an environment modified by the presence of HSPs. The amide I band gradually shifts from 1661 cm^{-1} (Figure 5b) to 1657 cm^{-1} (Figure 5c) and the amide II band at 1550 cm^{-1} becomes better defined. A new band around 1580 cm^{-1} also appears (Figure 5c). Precise analysis of variability within these bands is difficult as the thermo stability of amide bonds is ultimately affected by a variety of factors.

Table 3. The most diagnostic peak (amide I, II and amide III bands) in the IR spectra of the solution of HSA.

Liquid samples	Band location (cm^{-1})
a	1681, 1661, 1643
b	1660, 1652, 1644
c	1662, 1645, 1634
d	1663, 1648, 1634
e	1661, 1643, 1633
f	1652
g	1657, 1644
h	1650, 1637
i	1660, 1643

Note:
(a) The solution of HAS in presence of pure chicken skin
(b) The solution of HAS in presence of the solution of HAS in presence of pure chicken skin;
(c) The solution of HAS in presence of chicken skin heated to boiling point for 30 s and then incubated in presence of HSA in temperature about boiling point for 30 s;
(d) The solution of HAS in presence of chicken skin heated to boiling point for 35 s and then incubated in presence of HSA in temperature about boiling point for 30 s;
(e) The solution of HAS in presence of chicken skin heated to boiling point for 40 s and then incubated in presence of HSA in temperature about boiling point for 30 s;
(f) Untreated concentrated human serum albumin (HSA);
(g) Concentrated HSA in presence of chicken skin (HSAS);
(h) Concentrated HSA in presence of chicken skin heated to boiling point for 5 s;
(i) Concentrated HSA in presence of chicken skin heated to boiling point for 60 s.

Table 3. The most diagnostic peak (amide I, II and amide III bands) in the IR spectra of the solution of HSA.
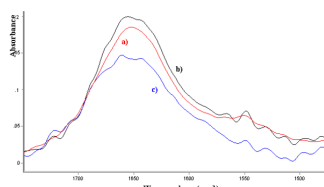

Figure 5. Fragment of the FTIR spectra of liquid samples after freezing: (a) untreated concentrated human serum albumin (HSA); (b) concentrated HSA in presence of chicken skin (HSAS); (c) solution of HSA in presence of chicken skin (HSASS).

Figure 5. Fragment of the FTIR spectra of liquid samples after freezing: (a) untreated concentrated human serum albumin (HSA); (b) concentrated HSA in presence of chicken skin (HSAS); (c) solution of HSA in presence of chicken skin (HSASS).

Discussion

Burn wound healing is a complex biological process involving the replacement of damaged tissue by a living tissue. Preventing adverse metabolic consequences of severe burns is not only a grave clinical problem, but also an interesting analytical issue, which is the subject of ongoing studies as well as of this paper. The paper concentrates on the analytical basis of the body's response to thermal burn, namely the release of HSPs and the question whether it is possible to directly or indirectly identify these proteins using a microbiological procedure, electrophoresis on cellulose acetate and IR spectroscopy.

It is well known that burnt skin is no longer a mechanical and biological barrier for external factors. The resulting necrotic scab is a good breeding ground for the development of micro-organisms spreading deep into the skin and subcutaneous tissue. It was found out that biofilm bacteria can be isolated from 60% of chronic wounds. Atomic force microscopy and electron microscopy revealed the existence of a biofilm in all types of chronic wounds: shin ulcers, pressure ulcers, burns and diabetic foot ulcers. The

presence and the effect of this bacterial biofilm on healing of chronic wounds is currently under investigation, raising controversies among microbiologists and clinicians. By activating a cascade of immune response (including the "oxygen explosion," or release of reactive forms of oxygen with strong antibacterial effect), immune cells cause serious damage to the host's tissues without affecting the biofilm in ways that lead to its eradication. Moreover, the components of the host's cells degraded by the "oxygen explosion" make a perfect nutrient for bacteria and facilitate a rapid restoration of the full structure of the biofilm. Initially, the burnt area is considered free of major microbial contamination. However, Gram-positive bacteria in the depths of sweat glands and hair follicles may survive the heat of the initial injury, and unless topical antimicrobial agents are used, these bacteria heavily colonize the wounds within the first 48 h after injury.

Pseudomonas aeruginosa (21.6%), *Escherichia coli* (13.6%) and *Staphylococcus aureus* (13.2%) are the major strains isolated from burn wounds. According to other authors, *Pseudomonas aeruginosa*, commonly recognized as a microorganism that colonises burn wounds and causes sepsis in burn patients, has recently become less prevalent due to the use of effective and targeted antibiotics. *Staphylococcus aureus* and coagulase-negative staphylococci (like *S. epidermidis*) are between the most frequently isolated bacteria from burn wounds.

Identifying the pathogens responsible for wound bio burden is especially important because the prevalence of multidrugresistant bacteria is increasing, necessitating treatment with appropriate antimicrobial agents. Pathologic alterations of wounds are accompanied by fundamental changes in the molecular environment that can be analyzed by vibrational

spectroscopy. Because of the specificity of Raman and FTIR spectroscopy, they can also be used to evaluate the bio burden of wounds. Differences in the Raman spectral profile of bacterial species as well as bacterial strains are evident [25].

However, it turns out that skin damaged in a burn does not entirely lose its antimicrobial capabilities (Table 1). Antimicrobial peptides found in the burnt skin, probably HSP proteins, explain why it is possible for a burn to heal without signs of clinical infection under dressings that have no antimicrobial properties. This can explain the presence of increasing inhibition zones as shown in (Table 1) for samples: WPS, WS30, IWS35, IWS40 and IWS60.

While serum is one of the most difficult proteome samples to characterize, one of the driving forces in proteomics is the discovery of biomarkers, proteins that change in concentration or state in associations with a specific biological process or disease. Determination of concentration changes, relative or absolute, is fundamental to the discovery of valid biomarkers. One such biomarker of thermal damage to tissue in a burn wound environment is the composition of albumins in serum. Although these studies were conducted on model samples of animal skin, which is a common preclinical standard, the results may find practical consideration in studies on samples obtained from the burn ward.

In the context of protein metabolism, it is the liver that plays the central role in responding to thermal injury. It synthesizes at least seventeen major plasma proteins and is the only source of albumin and α-globulin. Inflammation is the body's defensive response to an agent damaging tissue or organ structures. The inflammatory response has an acute phase ranging from tens of seconds to nearly twelve hours after the injurious stimulus, which subsequently leads to the chronic phase. As part of the inflammatory response or during the

tissue damage, inflammatory cytokines are released, leading to the release of acute-phase proteins. Serum albumin is a negative acute-phase protein. There are five groups of acute-phase proteins in the inflammatory reaction, including albumin and α-2-macroglobulin.

With regard to their molecular masses (KD), HSPs may be divided into the following families HSP27 (belonging to the "small HSP" found in the upper epidermal layer of the skin); HSP32 (increasing when cells are stressed or exposed to toxins or ultraviolet radiation); HSP47, HSP60, HSP70, HSP90 and HSP110. HSP70 is one of the most stress-inducible proteins in the cell, which participate in the regulation of protein synthesis, folding and degradation. HSP response has been associated with increased survival and decreased organ damage. Local expression of HSPs in burnt tissue was examined by Western Blot Analysis, which demonstrated increased concentration of HSP32 and HSP70 in the skin following second- and third-degree burns. The electropherogram of samples in (Figure 2) reveal an additional oligomer band, possibly from HSP32. Conclusions drawn from identifying these bands are consistent with earlier microbiological studies.

FTIR spectroscopy is diagnostically useful to examine the β-sheet structure, which is one of the major structures of the aggregates. Generally, it is well known that protein aggregates induced by heat and chemical denaturant form the intermolecular β-sheet structure. The analysis of β-sheet aggregates and other changes in the supramolecular structure of collagen obtained from model animal tissue, as presented in this paper, once again answers the question of, this time, spectroscopic response to heat shock. Wavelengths analyses between 1500 cm^{-1} and 1700 cm^{-1} revealed two peaks, corresponding to the region of amide bound protein. Recent studies on thermal aggregation process in collagen

suggest that the first step of aggregation consists in a partial opening of the protein native conformation.

This observation for samples of model animal skin also confirms the formation of hydrogen bond between N-H stretch and C=O. In this spectral range, the water overlapping with the amide I band exhibits only a single feature at 1638 cm^{-1}, characteristic of protein-rich samples, and also partially overlapping with the amide II band region (1580-1490 cm^{-1}). Amide I, with its characteristic wavenumbers in the 1600-1700 cm^{-1} range, is mainly associated with backbone C=O stretching vibration. Amide II is generally responsible for the combination of the NH in-plane bending and the CN stretching vibration. The amide III peak is complex because of intermolecular interactions in collagen, consisting of components from C-N stretching and N-H in-plane bending from amide linkages, as well as absorptions arising from wagging vibrations from CH$_2$ groups from the glycine backbone and proline side-chains, indicating that hydrogen bonds were involved in collagen.

In this IR study, while heating the dry organic chicken skin to boiling point for 60s leads to the disappearance of a wide band in the 1650-1550 cm^{-1} area, indicating sample denaturation, heating the organic chicken skin to boiling point for 30-40s leads to the conversion of a band in the 1700-1600 cm^{-1} area ([Figure 3](#)), which may be attributed to the intermolecular β-sheet aggregates. It should be stressed here that for real biological samples, such as animal skin, change trends in the position of bands are discussed as a rule rather than definite location of spectral bands.

Raman and FTIR spectroscopy can provide an objective means of evaluation by monitoring key components of wound bed epithelialization, such as keratin, elastin, and collagen, by identifying and quantifying bacterial load and by detecting

HO ("heterotopic ossification"). HO is defined as the pathological formation of bone in soft tissue, which has been observed following orthopedic surgery, burn injury, traumatic brain injury, and spinal cord injury. These observations also suggest that the process of tissue destruction under the influence of antioxidants can be reversed. The effect of L-ascorbic acid and modified orthosilicic acid as the inhibitors of model burn process was the subject of the authors' previous study.

Determination of changes in FTIR spectra of liquid samples is fundamental to the discovery of valid biomarkers and to the examination of the environment of body fluids in the serum of burn scar tissue. Generally, recording infrared spectra from solutions of pure biochemical compounds using FTIR spectroscopy has been very complicated; although the fingerprint region remains the most informative regarding the molecular composition of the samples and the contributions of water are significantly less. The main limiting aspect of depositing and air-drying the serum remains the inhomogeneity of the resulting deposit. However, the recording of infrared spectra from aqueous solutions, at least in the fingerprint region, is possible and is thus the best candidate for the study of human serum. In this study, FTIR spectra were recorded from frozen samples of thermally modified serum. The single band at 1652 cm^{-1} observed for serum is separated in an environment modified by the presence of HSPs. Identification of this band also confirms earlier microbiological, electrophoretic and spectroscopic tests.

Conclusions

As no model will ever completely replicate clinical human wound healing, it is essential that the model utilized be selected with care. For example, both pig and man have a thick epidermis. Human epidermis ranges from 50 to 120 μm and the pig's from 30 to 140 μm [35]. In

this study, organic chicken skin (an *ex-vivo* burn injured skin model) was analysed. Aggregates of HSP37 proteins were isolated using cellulose acetate electrophoresis. FTIR tests also confirmed the presence of these aggregates resulting from the loss of the triple helicity and forming intermolecular β-sheet structure. In general, it seems that only a comprehensive analysis, such as CAE, IR, microbiological and SEM-microscopic illustration of changes in skin surface-can be regarded as an effective biomarker, an attempt to interpret the physicochemical response to heat shock or stress. Further research will concentrate on finding an effective shielding modifier supporting the process of neutralizing the effects of thermal oxidative stress. And this study can serve as an example of a comprehensive analytical procedure to be used for analyzing clinical trials.

Conflict of Interest

The authors have declared no conflict of interest.

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