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Assessment of Antibiotic Influence on Structural Modifications of Amniotic Membrane by FTIR Spectroscopy

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Abstract: *The amniotic membrane is a readily available biomaterial with an important potential for tissue regeneration in dermatology and ophtalmology, with anti-inflammatory and anti-microbial properties. The extracellular matrix of the amniotic membrane is composed mainly of collagen, fibronectin and laminin. The purpose of our study was to investigate the structural modifications of collagen extracellular matrix of amniotic membrane upon interaction with two different antibiotics, frequently used in surgical and post- surgical procedure, respectively ciprofloxacin and gentamicin. SEM micrographs evidenced the ultrastructure features of dried amniotic membrane, with laminar structure, flexible, transparent, with no blood vesels or nerves. FTIR spectroscopy combined with deconvolution techniques was applied with the aim to determine the extent of denaturation upon treatment with different antibiotics. By spectral analysis, we concluded that gentamicin treatment is more favorable compared to ciprofloxacin, as the denaturation process is reflected by the lower sheet/turns ratio of the secondary structure composition.*

Keywords*: amniotic membrane, natural polymer, FTIR spectroscopy, protein secondary structure*

1. Introduction

The importance of amniotic membrane (AM) in clinical use for tissue regeneration was documented since 1910, when J.W. Davis, used it in skin grafting [1], in the treatment of open wounds. Later, in 1952, B. Douglas reports the use of AM as a natural biomaterial in burns treatment [2], while many researchers reported the use of AM to treat leg ulcers, as well as to treat a continuously widening spectrum of ophthalmic disorders [3, 4]. There are many similarities between AM and the conjunctiva and cornea in terms of its collagen composition and other proteins such as fibronectin and laminin [5]. For this reason, AM have already been proved as a dynamic method for the treatment of the various ophthalmic disorder such as pterygium, shield ulcers, infectious or vernal keratitis, bullous keratopathy, infectious keratitis and so on [6]. Based on its layered structure, AM has unique properties that recommend it for tissue regeneration (including ocular surface reconstruction): antibacterial activity, anti-inflammatory action, promotion of epithelialization, prevention of fibrosis, anti-angiogenic and anti-adhesive actions, pro-apoptotic actions of polymorph nuclear neutrophils [7-10]. The main advantage in using AM for clinical purpose is related to the fact that it can be used by current cryopreservation techniques, which preserves the growth factors and cytokines involved in epithelization, proliferation and differentiation of stromal fibroblasts. According to histological description [11], three distinct layers can be evidenced: 1) the first layer, containing cuboidal epithelial cells that determines the mechanical properties of the membrane; 2) second layer – the basal membrane containing proteoglycans and other proteins with structural function; 3) the covering layer with spongy appearance, composed of mesenchymal cells. As a general aspect, there is a lack of blood vessels and nerves in AM structure. It is well known that cell signaling is influenced by the errors that occurred in the protein structures of receptor molecules and constituent proteins of the extracellular matrix.

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Especially, collagen undergoes many modifications from structural and mechanical point of view, upon interaction with different preservation reactants or antibiotics [6]. It was previously demonstrated that Fourier-transform infrared (FTIR) spectroscopy emerged as a rapid and accurate tool for ex vivo diagnostic field, non-invasive and cost effective [12-14]. The vibrational spectroscopic techniques (FTIR or FT Raman spectroscopy) may offer both quantitative and qualitative information about the structural properties of biological samples and molecular bonds based on the position and intensity of the IR absorption bands, and their modifications due to environmental factors. As the biological samples are very complex systems, the FTIR spectrum is the sum of the synergic participation of sugars, proteins, lipids and nucleic acids [15], the high-resolution information being encoded into the spectrum. Continuous developments in computational bioengineering enable faster and more accurate calculations and discrimination of these components. It is well known that the position of the C=O stretching vibration in the protein backbone (corresponding to amide I absorption band) is very sensitive to the protein folding. The main advantage of FTIR spectroscopy is represented by the possibility to investigate the secondary structure of different proteins either in aqueous state or dried, and hence, is one of the most preferred technique for studying the alterations at molecular level. The modifications of secondary structure are reflected by the percentage ratio of the three common structures: α helix, β sheets, turns and random, while the spectral region of amide vibrational bands are very sensitive to conformational changes in the secondary structure of proteins [12]. So, FTIR spectroscopy by means of second derivative, deconvolution techniques and computational fitting, is a very important tool in order to determine the degree of protein denaturation upon interaction with different antibiotics used in surgical procedure for tissue regeneration.

The main purpose of our study is to assess the structural modifications of collagen extracellular matrix of amniotic membrane upon interaction with two different antibiotics frequently used in surgical and post- surgical procedures, respectively ciprofloxacin and gentamicin.

2. Materials and methods

2.1. Materials

The biological tissue was obtained under strict aseptic conditions, from a patient who had a caesarian delivery at term, with informed consent approved by the institutional board. The chorion and amnion layers were separated by dissection and used for cryopreservation in dimethyl sulfoxide (DMSO) and frozen at -80℃ until further microscopic and spectrophotometric measurement. The preparation method was applied according to a recent protocol described by Gremare et al. [16]. For the FTIR measurement, the cut pieces were allowed to interact with antibiotic solution, for 1 hour, before cryopreservation, respectively ciprofloxacin and gentamicin injectable solution (commercially available from KRKA Company, Slovenia) in a concentration of 40 mg/mL. For the SEM measurement, a small sample was allowed to dry at room temperature, immediately after chorion separation.

2.2. Electron microscopy

Scanning Electron Microscopy (SEM) was applied in order to assess the morphological features of AM both on the surface and the cross section, by using JOEL JSM5510 microscope.

2.3. FTIR spectroscopy

Spectrophotometric measurement were perform using SHIMADZU FT 8400 S (Shimadzu Co., Kyoto, Japan) FTIR spectrophotometer operating in the range 400-4000 cm^{-1}. The small pieces trimmed from AM were lying on the surface of pure Si windows (Nicodom S.R.O., Praha, Czech Republic) and the spectral acquisition condition were: wavelength resolution of 2.00 cm^{-1}, Happ-Genzel apodization and 3 scans/spectrum. Spectral deconvolution and second derivative techniques was apply using Origin 8 software in order to mathematically enhance the resolution of the FTIR spectrum, taking advantage of bands separation and curve fitting procedure [17]. A 9-point Savitsky - Golay function was applied along with a second derivative spectral analysis and curve fitting of amide I band to locate band position. All

second-derivative spectra were baseline-corrected and area-normalized under amide I corresponding range 1700-1600 cm^{-1}. The percentage of secondary structure (α helix, β sheets, turns and unordered) was calculated based on the area under each peak [18].

3. Results and discussions

The gross appearance of a small portion (trimmed) of amniotic membrane, is presented in Figure 1a, while the microscopic details, recorded by SEM, are presented in Figure 1(b, c).

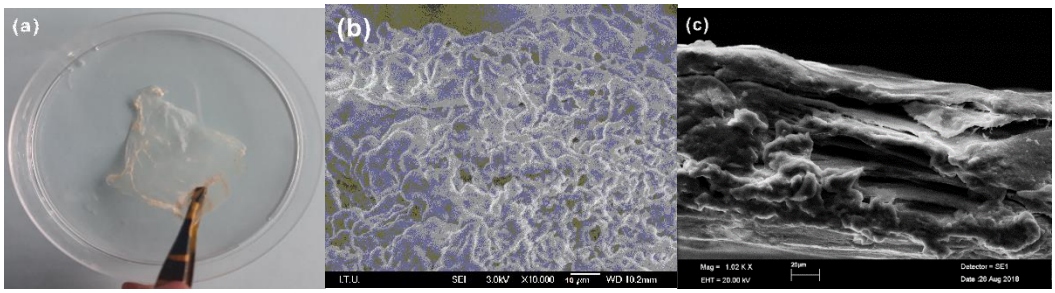

Figure 1. (a) Photographic image of amniotic membrane; (b) SEM micrograph recorded on the surface of amniotic membrane; (c) SEM cross-section image of amniotic membrane

As a general aspect, the basement membrane (amniotic membrane) is flexible and transparent, with no blood vessels or nerves. The microscopic details reveals microvilli at the apical surface of amniotic epithelial cells, in a mosaic arrangement, regularly displayed with homogeneous size. As a result of air-drying process, the appearance of the cells is flattened and compacted. The cross-section image presented in Figure 1(c), revealed the laminar structure of extracellular matrix, composed mainly of collagen fibers which appeared to be well conserved upon the treatment.

In Figure 2, the FTIR spectra of natural, unprocessed, amniotic membrane (air dried) is presented comparatively with the FTIR spectrum of amniotic membrane treated with ciprofloxacin and gentamicin, while in Figure 3 are presented the FTIR spectra of both antibiotics, as supplied from the commercial agent.

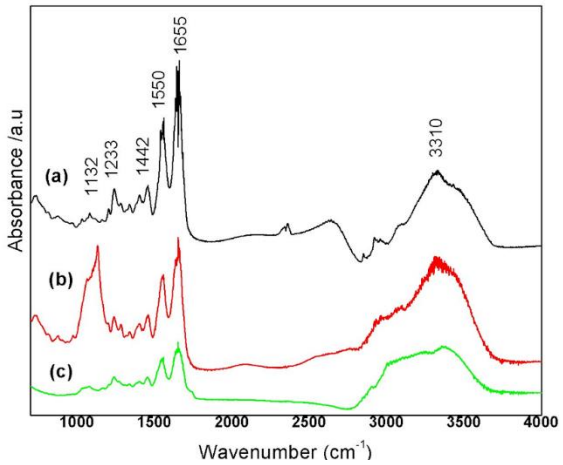

Figure 2. FTIR spectra of amniotic membrane: (a) air dried, natural; (b) after gentamicin treatment; (c) after ciprofloxacin treatment

Being a collagenous tissue, the FTIR spectrum of natural, untreated amniotic membrane reveals the characteristic features of collagen extracellular matrix: amide I (1665 cm^{-1}), amide II (1550 cm^{-1}), amide III (1233 cm^{-1}), CH_2 bending and deformation (1442 cm^{-1}), amide A and C-H bending (3310 cm^{-1}). The

most intense absorption band, amide I, is assigned to C=O stretching mode, while amide II protein absorption band is attributed to the vibration of N–H and C–N bond in stretching mode [19]. The amide III band is a more complex vibrational contribution, combining the stretching vibration of C–N bond and the in plane vibration of N–H bond along with the vibration of C–C bond (stretching mode) and C=O bond (bending mode) [12].

Moreover, the peaks located between 1210–1300 cm^{-1} and around 1100 cm^{-1} are assigned to nucleic acids and phospho-lipids [19]. As a general behavior, after antibiotic treatment, the main fingerprints shifted towards lower wavenumbers, concomitant with reducing their intensity.

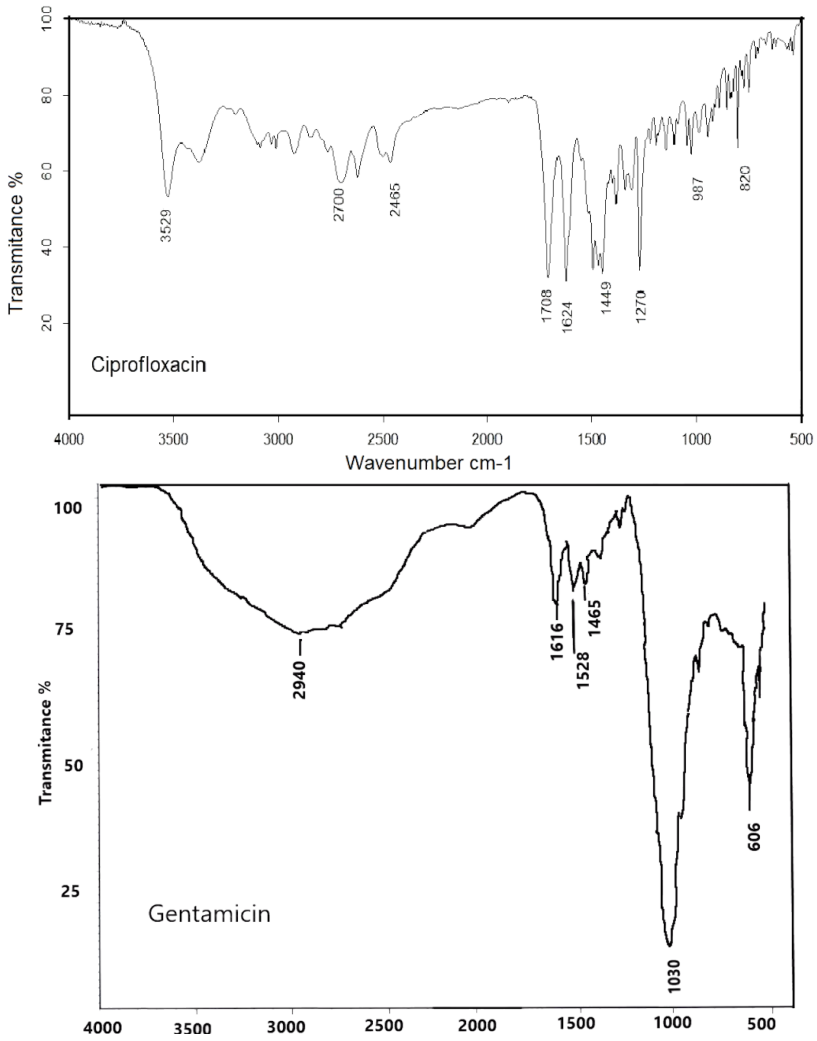

Figure 3. FTIR spectra of ciprofloxacin and gentamicin, as provided from the supplier

As previously reported, the intensity of amide I band of collagen decreased markedly upon denaturation [20]. The moieties of the molecules, namely also functional groups, display one or more absorption infrared bands at specific frequencies, which are also influenced by the surrounding parts of the protein. By comparing the spectral details after antibiotic treatment, as presented in Figure 2, one can observe that ciprofloxacin marker bands are difficult to be distinguished, due to the massive overlapping with the vibrational bands of the membrane itself. By contrary, after gentamicin treatment, the dominant fingerprint at 1030 cm^{-1} can be observed along with the characteristics of the natural membrane. In the same time, the OH stretching bands feature may provide information about the structural modifications induced by changing the hydration level of protein.

Computational processing of the FTIR spectra is required because FTIR spectroscopic features contains thousands of singular spectra. For this reason, a fitting procedure was applied in order to evaluate from the quantitative point of view the structural modifications of collagen matrix, after antibiotic treatment. The most important structural changes can be evidenced through the computational analysis of amide I band, which is commonly used to evaluate different secondary structure elements, taking into account that amide I band is composed of superimposed absorption bands from α helix, β sheets, turns and unordered or random coil contributions [18].

The fitting procedure assumed a Gaussian shape for the amide I band envelope and individual contributions. Each component of the secondary structure corresponds to different C=O stretching frequencies, resulting in different band position. The sum of the areas under each peak represents the total amount of secondary structure in the protein. Computational fitting of amide I vibrational band of natural amniotic membrane and after antibiotic treatment is presented in Figure 4 (a, b, c), while the quantitative assessment of each secondary structure is presented as a diagram in Figure 5.

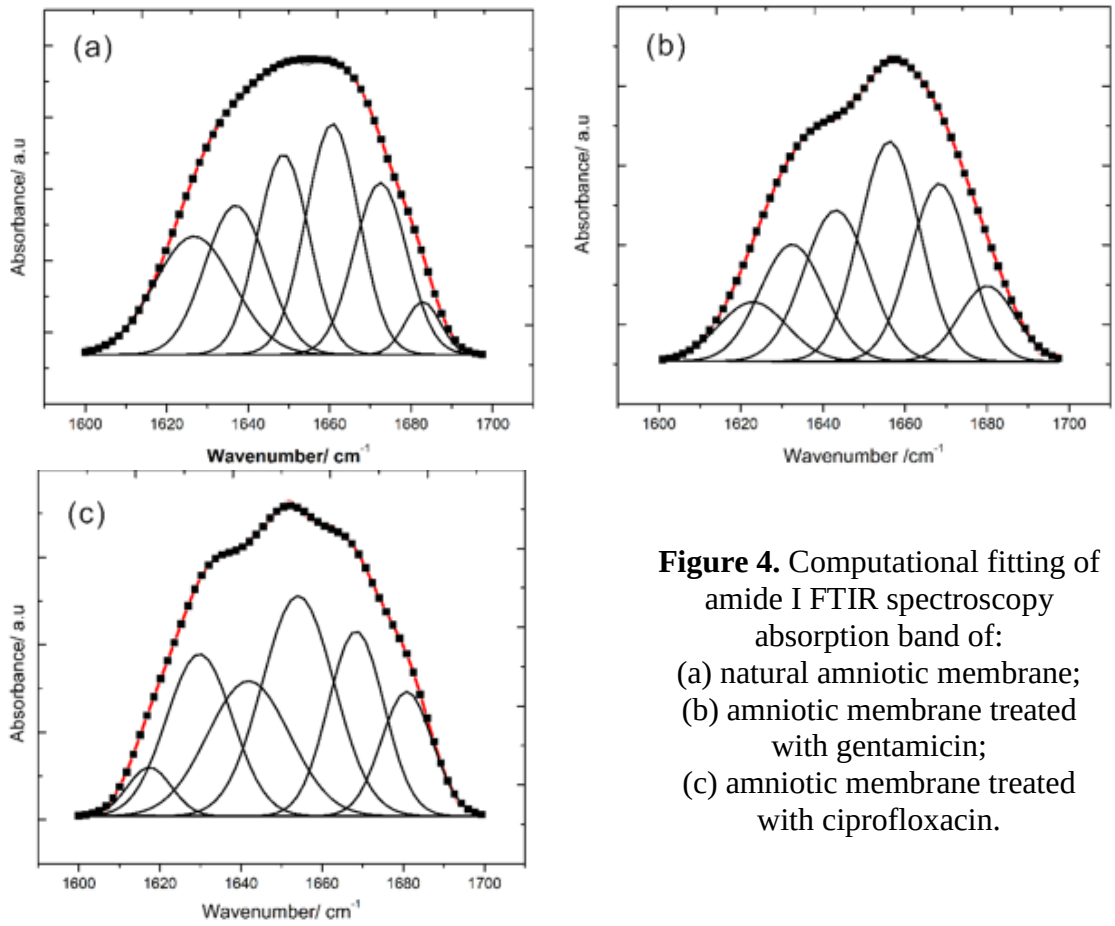

Figure 4. Computational fitting of amide I FTIR spectroscopy absorption band of:
(a) natural amniotic membrane;
(b) amniotic membrane treated with gentamicin;
(c) amniotic membrane treated with ciprofloxacin.

According to the literature [21, 22], FTIR band position of the protein secondary structure are assigned as following:

β -sheet	1633 ± 3 cm^{-1}
random coil	1640 ± 3 cm^{-1}
α helix	1655 ± 3 cm^{-1}
β -turns	1672 ± 3 cm^{-1}
β -turns	1680 ± 3 cm^{-1}.

By analyzing the multi-peak deconvolution of amide I band, one can notice a quantitative decrease of β –sheet structures, accompanied by an increase of less ordered structures, such as turns and random coil. With respect to α helix structure, the ciprofloxacin treatment seems to affect more obviously this structure, compared to the gentamicin treatment. Both secondary structures (alpha and beta) possess NH and CO functional groups forming hydrogen bonds which stabilize the chains, but in this case, α helix is more sensitive as a result of ciprofloxacin treatment. By comparing the quantitative results displayed in Table 1, we can conclude that the gentamicin treatment is more favorable, as the denaturation process is reflected by the lower sheet/turns ratio.

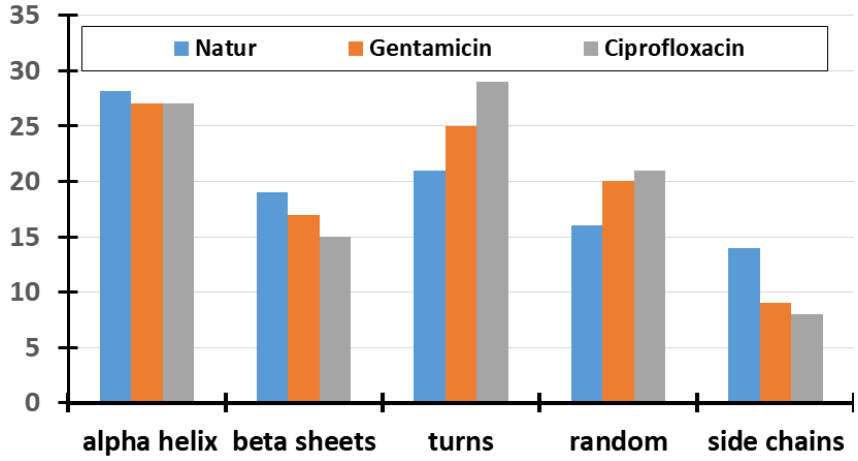

Figure. 5 Quantitative analysis (percentage) based on computational fitting of amide I FTIR spectra recorded on natural amniotic membrane and treated with gentamicin respectively ciprofloxacin

Similar results were obtained by Payne et al. [23] in a study devoted to FTIR spectroscopy of skin collagen, in a temperature-dependent approach, demonstrating the conformational-dependent behavior of amide I components. Upon denaturation of the collagen triple helix, the dominant amide I component (1655 cm^{-1}) decreases, while the 1633 cm^{-1} component becomes more intense. It was noticed a 30 cm^{-1} shift in the position of the amide I band, upon denaturation process. Other fibrillary proteins were found to behave in a similar way. For example fibrinogen adsorption on different hydrophilic or hydrophobic surfaces undergoes conformational changes and denaturation, suggesting a favorable behavior toward hydrophobic surfaces compared to the hydrophilic ones [18]. In this case, the authors concluded that conformational changes occurred in the core of the protein, as interpreted by the quantitative difference in β –sheet and turns percentage. This arrangement might also be responsible for the lacking in the hydration recover of protein, which was often interpreted as the main cause of collagen maturation and ageing.

Our results, even if we do not know whether proteins, lipids, glycomaterials or nucleic acids are involved in the changes of biological functions of the tissue under antibiotic condition, we can conclude that the detected FTIR spectral differences may contribute to a correct approach when choosing the antibiotic for pre or postsurgical treatment. Moreover, we demonstrated that FTIR spectroscopy combined with computational techniques is a noninvasive tool suitable for clinical diagnostic purposes allowing to display irreversible conformational changes of collagen and other proteins in amniotic membrane. In the same time, the results may offer some details regarding to the preservation strategies which plays an important role in preserving the biological properties of amniotic membrane.

4. Conclusions

The main goal of our study was to assess the structural properties of amniotic membrane as a potential natural biomaterial for biomedical applications, including tissue regeneration. Similarities between AM and the conjunctiva and cornea are interpreted especially in terms of collagen composition (mainly collagen type IV) along with other proteins such as fibronectin and laminin. SEM micrographs evidenced the ultrastructure features of dried amniotic membrane, with laminar structure, flexible, transparent, with no blood vessels or nerves. FTIR spectroscopy combined with deconvolution techniques was applied with the aim to determine the extent of AM denaturation upon the treatment with different antibiotics frequently used in surgical and post- surgical procedure. By computational spectral analysis, we can conclude that the gentamicin treatment is more favorable compared to ciprofloxacin, as the denaturation process is reflected by the lower sheet/turns ratio.

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