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Radiolytic Damage to Freeze-dried Human Amniotic Membrane

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Radiation-sterilization at 25 and 35 kGy of freeze-dried human amniotic membranes caused degradative effects in the biologic dressing. The decrease in pH and increase in UV absorption showed that radiation may have caused possible radiolytic changes in this biomaterial. Total nitrogen content, tensile strength, and [H3]water-retention capacity of the irradiated membranes remained invariable. Molecular topography analysis by atomic force microscopy showed radiation-induced defrayment of the collagen fibers, the major structural protein in amnion.

Key Words: radiation processing, human amniotic membranes, atomic force microscopy, wound dressing, tissue banking

INTRODUCTION

Amniotic membrane, the innermost layer of fetal (or placental) membrane, consists of a thick basement membrane and an avascular stroma. It functions to protect the fetus from unwanted maternal insults that may otherwise bring about congenital defects. It has long been recognized that when an incision is performed on the skin of a fetus even during the third trimester, there will be no scar evident after birth. Such phenomenon is dubbed as "scarless fetal wound healing".

The technology of exploiting such "fetal"-feature by applying human amniotic membranes to surgical and burn wounds was incepted as early as 1913 (Sabella 1913). Thereafter, the work by Dino et al. (1966) on the establishment of an amnion bank at the Philippine General Hospital (PGH) became the *first* international publication on human amniotic membrane applications in surgery. Trials on the use of these membranes have established the efficacy of human amnion in treating a wide variety of dermatological disorders. But for reasons that are not very clear, the use of amnion membrane as wound dressing fell out of popularity. Nevertheless, of late, a lot of clinical works have now refocused on amniotic membrane transplantation for managing ocular surface diseases (Dua & Azuara-Blanco 1999; Kruse & Meller 2001; Sippel et al. 2001; Kasparov & Trufanov 2001).

How then does radiation-sterilized human amniotic membrane (RSHAM), a medical product produced at

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Philippine National Research Institute (Figure 1), work as a biologic dressing. Nine specific biological and clinical functions (Table I) have been ascribed to RSHAM based on experience by collaborative researches under the auspice of International Atomic Energy Agency (IAEA 2001) and by several independent research groups (Robson & Krizek 1973; Walker et al. 1977; Bose 1979; Bennett et al. 1980; Faulk et al. 1980; Piserchia et al. 1981; Thomson & Parks 1981, Quinby et al. 1982; Kucan et al. 1982; Haberal et al. 1983). Abundant in amniotic tissues are a myriad of basic fibroblast growth factors (bFGF) and hepatocyte growth factors that are among the major mitogens responsible for up-regulation of epithelial and endothelial cell proliferation. In addition, many types of cytokines present in the amniotic membrane stroma suppress the signaling pathways of TGF-β, IL-1 and IL-2, which reduce inflammation and prevent scarring during wound healing (Na et al. 1999; Koizumi et al. 2000; Hao et al. 2000; Lee et al. 2000; Shinmura et al. 2001). The adhesive property of amnions to epithelial cells is attributed to its basement membrane components. Amniotic membrane is composed mainly of type IV collagen. It also has laminin 1, laminin 5, fibronectin, and collagen VII. In general, the basement membrane side (chorion) is an ideal substrate to anchor epithelial cells (Fukuda et al. 1999).

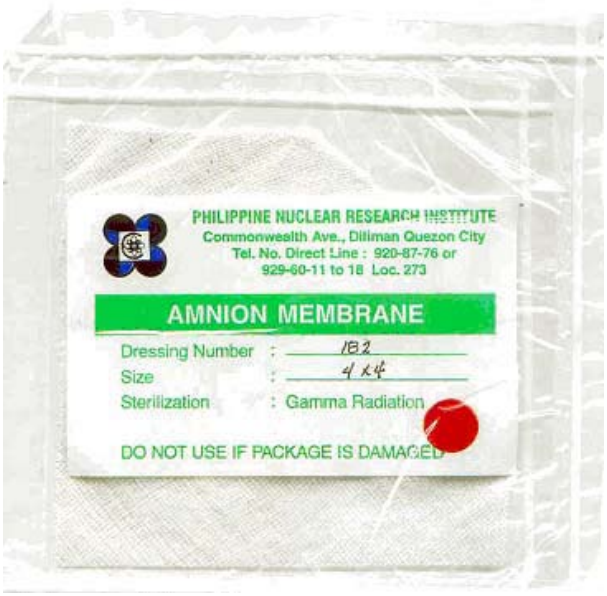

Figure 1. The Radiation-Sterilized Human Amniotic Membrane (RSHAM) produced at the Philippine Nuclear Research Institute (PNRI) and distributed for free to indigent patients in selected government hospitals in the Philippines

Table I. Clinical benefits from the use of amniotic membranes as wound dressings*

Clinical benefits
Decrease in bacterial count of the wound
Reduction of fluid loss
Promotion of healing
Protection of growing epithelium
Tight adherence to the wound surface, increase in mobility and diminished pain
Help in prediction of readiness for grafting
Preparation of skin defects for closure
Decrease in physiological stress for the patient
Stimulation of neovascularization

*Hansbrough 1987

While radiation-processing technology has long been applied in improving the shelf life of human amniotic membranes, little is known about the effects of radiation-sterilization on its wound healing property. Applying either 25 or 35 kGy for radiation-sterilization of the amniotic membranes is not arbitrary, but was determined on the basis of available data (AAMI 1991; ISO 1994). The choice of 25 kGy was based on the predicted bioburden after radiation treatment to achieve a sterility assurance level (SAL) of 10^{-6} for bacteria in biomaterials that will come into contact with immuno-compromised tissues. Recently, HIV-contamination of tissues has been a big issue with regard to the safety of human-sourced materials for tissue banking. There are a number of experimental reports showing that γ-irradiation deactivates HIV in tissue culture and in infected bones with a D_{10} value between 4 to 6 kGy. Therefore, to achieve a SAL of 10^{-6} for HIV, dried preparations of amniotic membranes need to be exposed to at least 35 kGy to safeguard patients against the spread of HIV. This study aims to elucidate further the molecular bases of wound healing activity of RSHAM (Hansbrough 1987**)** by presenting data on its physico-chemical features.

MATERIALS AND METHODS

Collection and Processing of Amnion Samples

One hundred-fifty five (155) placentas were collected from the delivery room of the East Avenue Medical Center, Quezon City from June-July, 2001. Amnion membranes were removed from the bulk of the placenta, and then washed with 5% sodium hypochlorite solution. Clean mats of amniotic membranes were freeze-dried under vacuum at 0.01 psi for 3 h (Heto Holten, Denmark). Amnion membrane samples were divided and cut into desired sizes and some were pulverized in a 10-mm mesh. The samples were then double-packed with polyethylene bags and irradiated at 25 and 35 kGy with a Laboratory-Scale GammaCell (Atomic Energy of Canada, Ltd.).

Sterility Test
Powdered amnion samples (0.5 g each) were aseptically transferred to 2-mL thioglycollate broth. The tubes were incubated at 32°C for 14 d and examined daily for any change. Turbidity in the media indicates presence of microbial contamination. The sample broth was then plated onto a Plate Count Agar (Biotest) and colony growth was checked after 2 d incubation at 32°C in a microbial incubator (Sanyo, Japan). Sterility testing was done in duplicates.

Proximate Analysis
Total nitrogen content of the samples was determined using the Kjeldahl procedure. The pH was measured using a Beckman P50 pH meter. Absorption spectrum was obtained using a Perkin-Elmer Lambda 20/1.0 nm UV/Vis Spectrometer. Moisture content was determined by desiccation in the Sartorious Moisture Analyzer MA 30. These proximate analyses were done in triplicate samples.

Atomic Force Microscopy
Molecular topographic analysis was carried-out using a TMX 2000 Explorer Atomic Force Microscope. Silicon nitride probe tips were used to scan the sample surface.

Water Retention Experiment
Amnion disks of 1.0-cm diameter were used for the water retention experiment. Individual weights of the disks were determined using a Sartorius microanalytical balance. The samples were placed in a 96-well microtiter plate (Dynatech, USA) and 100 μL of [H^3] labeled water was added to each well. After incubation for 2 h at room temperature, amnion samples were transferred to polyethylene scintillation vials and absorbed tritium was measured by liquid scintillation using Wallac 1414 LSC. Mean water retention of the capacity of six replicates was expressed in counts per minute/milligram (cpm/mg)

Tensile Strength Analysis
Determination of tensile strength on dumbell-shaped amnion strips was carried out using the Instron Model 1011 Universal Testing Instrument at ambient conditions. A total of 56 amnion strips samples were tested. Thickness of individual strip was measured.

Statistical Analysis
Single-factor ANOVA at 95% confidence level was employed to determine significant changes in the property of the amniotic membranes.

RESULTS

The irradiated amnion membranes were negative for microbial growth. It was interesting to note that lyophilized amnion samples failed to present neither a change in coloration nor turbidity in the thioglycollate broth. However, some colony growth was observed with the Plate Count Agar with the non-irradiated control samples showing the initial bioburden of the material.

Table 2 presents the mean values of the physico-chemical properties of the amnion samples. Radiolytic change in the amniotic membrane was evident in the dose-dependent decrease in pH. Nevertheless, the slight drop in pH was still within the physiologic range and may not disrupt wound-healing processes. By inspection, we failed to find change in the percentage nitrogen content in the tissues. For both tensile strength and water retention/ absorption tests, no significant change was also seen after irradiation.

Table 2. Some physical properties of irradiated and non-irradiated amniotic membrane.

Dose (kGy)	pH	% Nitrogen	[H3]-Retention (cpm/mg)	Tensile Strength (kg/m2)
0	6.97 + 0.00	1.42 + 0.31	26.87 + 17.07	0.023 + 0.011
25	6.81 + 0.01	1.49 + 0.37	33.46 + 11.26	0.035 + 0.016
35	6.73 + 0.02	1.44 + 0.34	34.55 + 12.70	0.009 + 0.008
p-value	< 0.001*	0.8397	0.7205	0.0933

* significant at 95% level of confidence

Radiolytic UV-vis absorption spectra showed increase in the optical densities as the sample absorbs within the UV region (190-195 nm) for 25 kGy and 35 kGy irradiated amnion extracts. In contrast, water extract of non-irradiated control displayed decreased optical densities towards the UV region (Figure 2).

These radiolytic changes may also be evident with the molecular topography analysis of the amnion samples. Surface matrix of irradiated amnion membranes at 35 kGy appeared to have deeper and more scattered ridges compared to the non-irradiated amnion. However, these may be interpreted by possible effects on sample preparation. Generally, we failed to see drastic differences in the film topographies, and this may corroborate with our results of no significant change in the tritium absorption or tensile property analyses. (Figure 3).

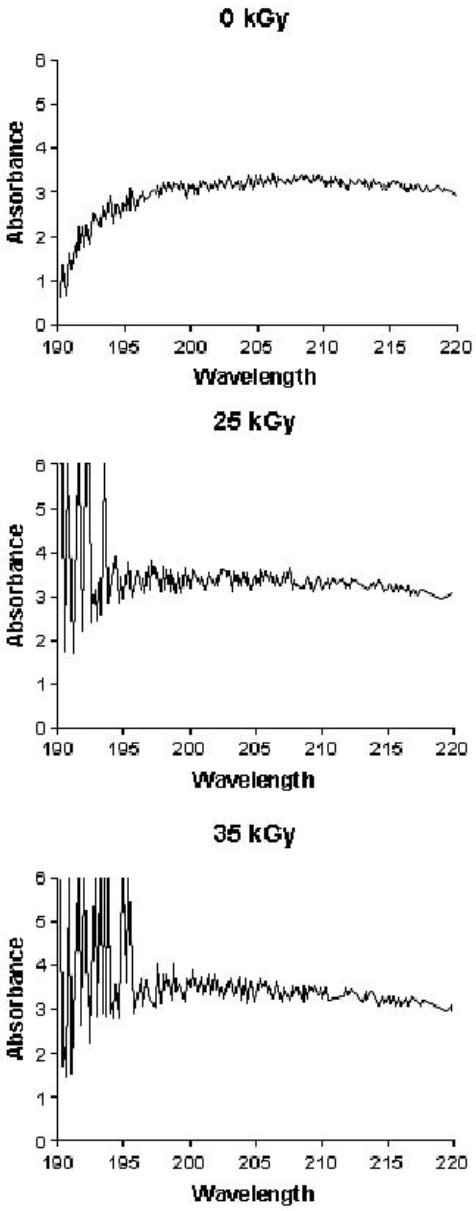

Figure 2. Absorption spectrum of control and irradiated human amnion membranes. After irradiation, amniotic membrane samples were homogenized in PBS buffer. After clearing step, supernatants were normalized for protein concentration and absorption spectra were compared. Increased absorption was observed from 190-195 nM range for irradiated amnion. These peaks were not present originally in the non-irradiated lot.

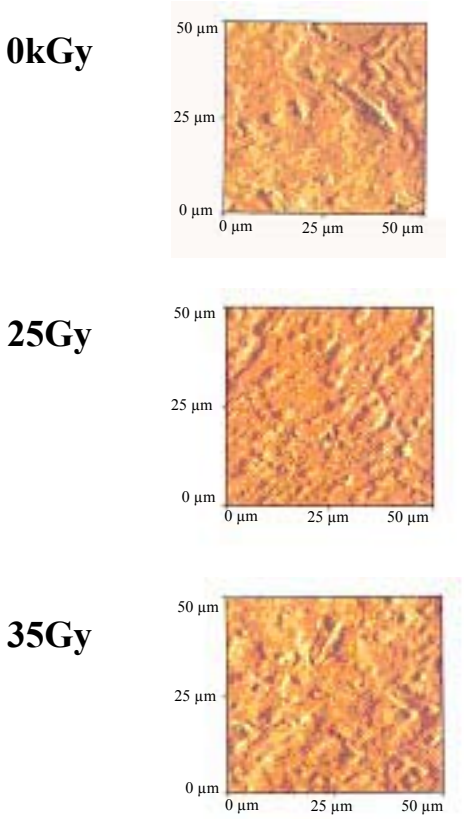

Figure 3. Molecular topography of irradiated and non-irradiated human amniotic membrane viewed under an atomic force microscope. (In a 50x50 μm^2 window, ridges appeared less pronounced, in terms of depth and density, in the control (0 kGy) when compared to irradiated samples. These changes are a subtle indication of micro-damage to the material due to radiation-processing).

DISCUSSION

Applications of allogenic amnion grafts range from wound dressing of severe burns, dermabrasions, and lower extremity ulcer treatments to plastic surgery, laryngology and ophthalmology. In the Philippines, the preservation of these amniotic membranes are done by lyophilization or deep-freezing and subsequent radiation-sterilization at a dose of 25 kGy. From year 2001-2002, over 200 preserved RSHAM allografts (with a total surface area of over 20,000 cm^2) have been prepared at the PNRI tissue bank and distributed to clinics and hospitals within Metro Manila (PNRI 2003). In Warsaw Tissue Bank (Poland), human amnions that are distributed to various clinics are irradiated at 35 kGy (Tyszkiewicz et al. 1999).

Irradiation of biopolymers may result in chemical changes in proteins such as fragmentation, cross-linking, aggregation and oxidation. These are likewise dependent on its chemical nature, physical state and irradiation condition. In general, such irreversible changes happen because of breakage of covalent bonds of the polypeptide chain happening in both random and non-random fashion. Fragmentation involves reaction of α-carbon radicals with oxygen to form peroxyl radicals that decompose to fragment the polypeptide chain. In contrast, proteins may be converted to higher molecular

weight aggregates due to generation of inter-protein cross-linking reactions, hydrophobic and electrostatic interactions, and formation of disulfide bonds. The formation of high-molecular weight aggregates is negligible at low dose range, but increases significantly at higher doses (Woods & Pickaev 1994), such as the range used in human amnion processing.

From the tests performed, we find significant changes in pH, UV-Vis spectra, and matrix topography of irradiated amnion membranes. The acidic shift after irradiation is likely the consequence of the oxidation of amino acids residues and denaturation of the protein components leading to the liberation of some acidic components. The result from UV-Vis spectrophotometry of water-soluble amniotic extracts showed a consistent pattern of radiolytic degradation. There is an increase in optical densities within the ultraviolet region indicating the chemical groups having amino, ethelene or ketone that are released during protein radiolysis. In one end, it can be observed that there was also more noise in the region below 195 nm, which is typical for turbid samples.

With regard to structure, its major protein component would either be fragmented or cross-linked. One possible mechanism for collagen fragmentation is given by the oxidation of proline, followed by cleavage of the Gly-Pro peptide bond (Uchida et al. 1990). In addition to fragmentation, denaturation of collagen may also have effect on its surface. Fibrillar proteins, like collagen, are typified by a heterogenous packing density and its unfolding as a result of free radical reactions would result in a corresponding specific volume increase (Majeska & Dancewicz 1977).

Some physico-chemical changes such as water absorption and tensile strength were also studied to determine clinical functionality of RSHAM. The absorption capacity of the amniotic membrane as measured by retention of [H^3] water preludes proper biological adherence and optimal solvent-accessibility of growth factors. Tensile strength is vital to ensure that the material does not disintegrate prior to and during its application as a wound dressing. For both parameters, we found no significant difference using a highly sensitive tritium absorption technique (Horricks & Peng 1971) and the standard tensitometric analysis (Marin & Sauer 1959). Judging from the results, we suspect that both protein fragmentation and cross-linking reactions may have occurred within the radiation doses used that led to a "cancellation effect" on the structural (in)stability of amnion after radiation exposure. From the present results, we have demonstrated the maintenance of structural integrity of our biomaterial and provided some molecular bases for the prudent choice of irradiating amnions at 25 and 35 kGy, balancing both the concurrent need to achieve product sterility and to avoid excess damage to the biomaterial.

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