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RESEARCH ARTICLE

CHANGES IN THE FUNCTIONAL CHARACTERISTICS OF AMNIOTIC MEMBRANE AFTER GAMMA IRRADIATION.

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Abstract

The effect of different doses of gamma radiation viz. 15, 20, 25 and 30 kGy on the chemical and functional characteristics of the amniotic membrane was studied. The change in the chemical structure of amniotic membranes at high doses of gamma irradiation was evaluated by means of Infrared (IR) Spectroscopy. The degradation of amnion on irradiation with gamma rays could produce a relative variation in IR absorption troughs. This kind of variation was absent in the samples irradiated to doses of 15, 20, 25 and 30 kGy indicating no qualitative change in the material property of amnion. No significant differences in the water absorption capacity and water vapour transmission rate of amniotic membranes irradiated to different doses were observed. The impact of irradiation doses on antibacterial effect of human amniotic membrane in vitro, showed the inhibitory effect of amniotic membrane on three standard bacterial strainsImpermeability of the amniotic membranes to different microorganisms was also not affected at high doses of gamma radiation. Gamma irradiation at doses of 15–30 kGy did not evoke undesirable changes in the functional properties of the amniotic membrane.

Introduction:-

Amniotic membranes are among the most widely used biological dressing. AM are obtained from the human placenta and possess most of the characteristics of an ideal skin substitute. It acts as an effective barrier, has good adherence to wound, is bacteriostatic and has no immunological reaction. AM have been used in a variety of clinical conditions like full and partial thickness burns, skin graft donor sites, chronic venous leg ulcers, pressure sores, leprotic ulcers **(Singh et al., 2004)** and radiation induced ulcers **(Gajiwala and Sharma, 2003),** hysteroscopiclysis of severe intrauterine adhesions **(Ameret al., 2010).**

Safe and effective amniotic membranes are required for clinical applications. However, transmission of infectious agents is one of the risks associated with transplantation of human tissues. The problem is additionally complicated by the possible presence, in human tissues, of pathogenic viruses such as the human immunodeficiency virus (HIV) **(Singh, et al. 2006)**

To avoid the risk of possible transmission of infectious disease from donor to recipient, the donors are screened and the processed membranes are sterilized. Even if the donors have been tested for HIV, there is a slight possibility that a donor may be infected, for instance, because of the ‘‘window period’’, when the donor is already infected but the screening tests are not yet positive **(Singh, et al. 2006).** Some studies carried out on the inactivation of HIV indicate that the conventional dose of 25 kGy used for sterilization is not sufficient to inactivate high quantities of HIV **(Dziedzic– Goclawska, 2000).** Therefore, it would be advantageous to use doses higher than 25 kGy for tissue allografts to exclude the probability of a contamination with viral pathogen of donor origin.

High doses of gamma radiation may evoke undesirable changes in the functional properties of the amniotic membranes. The present study was therefore carried out to evaluate the effect of high doses of gamma radiation on the chemical structure and functional characteristics of processed air-dried amniotic membranes.

Materials and Methods:-

Tissue Procurement:-

Sample Collection and Preparation:-

Human amniotic sacs were collected from operation theatres of three different hospitals. All the donors were prescreened for the presence of transmissible diseases (e.g., HIV, HBV, and VDRL). The membranes were obtained only from clinically acceptable donors (mothers) after their delivery and kept in plastic containers with sterile physiological saline (0.9% NaCl), and preserved temporarily in a 4°C.

Amnion preparation:-

Amnion samples were washed separately with tap water to remove blood debris. The washed samples were washed in sterile isotonic saline for 3 times, ten minutes each. Then washed with 0.05 % sodium hypochlorite for 10 min., finally washed 3 times with sterile water, 10 min each.

In a laminar air flow, the membranes were separated (glossy, translucent and thinner membrane) from the chorion (opaque and thicker) under aseptic condition. The membranes were cut into pieces of approximately 5x5 cm by surgical scissor, cleaned amniotic membrane was stretched across a frame on gauze dressing, so their epithelial surface was upward. The membranes were kept overnight in freezer and then lyophilized in freeze dryier and packaged in polyethylene package until use.

Gamma irradiation:-

Air-dried amniotic membranes were exposed to different doses of γ radiation viz., 15, 20, 25 and 30 kGy at a Co- 60 γ irradiator in NCRRT. Irradiation was carried out at a dose rate of 5.2 kGy/h at room temperature.

Biophysical characteristics of amnion allografts:-

To investigate the biophysical characteristics of amnion, after exposure to different doses of γ radiation tests on thickness, moisture vapour permeability (MVP), were performed water absorption, water vapour transmission rate and microbial impermeability and antimicrobial activity were examined.

Infrared spectroscopy studies:-

The change in the chemical structure of amniotic membranes at high doses of gamma irradiation was evaluated by means of Infrared Spectroscopy. Infrared (IR) spectra of the amniotic membranes exposed to different doses of radiation were recorded using FTIR Spectrometer Model Vertex 70, Bruker optics, Germany. Spectral scanning was carried out in the range of 400–4600 cm_1 and percent transmission was measured. For comparison of the results, the IR spectra of unirradiated amniotic membranes were used as reference.

Water absorption:-

Water absorption capacity was determined to evaluate the effect of different doses of gamma irradiation on the absorbency of the amniotic membranes. The membranes irradiated to different doses of 0, 15, 20, 25 and 30 kGy were immersed in distilled water. The total amount of water absorbed by amniotic membranes was determined by weighing the membranes before and after different time intervals. Excess water on the membranes sample surface was removed with tissue paper. The total amount of absorbed water was calculated and expressed as g/cm^2.

Water vapour transmission rate:-

The water vapour transmission rate (WVTR) of unirradiated and irradiated amniotic membranes was measured using water method, ASTM E96–66. A water-filled container (r ¼ 1.2 cm) was covered with amniotic membrane and incubated at 35 1C. The WVTR was calculated as decrease in weight of the container per square metre area of amniotic membrane covering the container on 24 h basis as follows: WVTR =(G/t)/A, where G is weight loss of the samples (g), t is test time (h), A is effective membrane area (m^2).

Microbial impermeability:-

Impermeability of amniotic membranes irradiated to different doses was tested to various gram positive and gram negative bacteria. Four strains of bacteria, Bacillus, Escherichia coli, Pseudomonas, Staphylococcus and Streptococcus were used.

Pieces of sterilized amniotic membranes were placed on Soyabean Casein Digest Agar plates. Suspensions of bacteria were prepared in sterile water and placed on the opposite surface of membranes and incubated **(Hilmyet al., 1993).** Plates were checked for the growth after 48 h of incubation.

Sulphur content;-The sulphur content of the AM, as a parameter for the collagen solubility of the allograft was measured by atomic absorption spectroscopy/energy dispersive X-ray analyser (EDX-Analysis, (EDX- Model-Oxford, England attached to SEM Model- JSM- 5400, Jaban (Schrageet al., 1993).

Antimicrobial activity of AM:-

Antimicrobial activity of AM was performed in vitro, on three standard bacterial strains of ***P. aeruginosa, E. coli,*** and ***B. subtilis*** in 5 doses of γ radiation. Bacterial suspensions were prepared in isotonic sodium, cultures on nutrient agar plates were inoculated in order to obtain confluent growth, discs of either chorion or amnion were placed on the agar surface partly used for individual experiments. Plates were inspected in order to reveal whether growth had occurred in inhibition zones.

Results and discussion:-

Dried amniotic membrane contains collagen matrix and key bioactive molecules like fibronectin, laminin, glycosaminoglycans and elastin. Fresh and cryopreserved human amniotic membrane has been widely explored as a biological dressing. However, fresh and cryopreserved amniotic membranes are not readily available or require special storage conditions. This investigation was aimed to study the functional and clinical efficacy of air-dried radiation sterilized amniotic membranes.

Every step of the preparation, preservation and sterilization can influence the properties of a biological material. Previous studies showed that sterilization by irradiation and preservation by freeze-drying have a significant impact on histological and biophysical properties of amnion allografts **(von Versen-Hoynck, et al., 2004).**

Use of higher doses of gamma radiation than the conventional dose of 25 kGy may affect the optimum physiological conditions provided by the amniotic tissue for healing. Maximum clinical efficiency of amnion can be obtained only if its functional properties are preserved. In the present study, the effect of different doses of gamma radiation on the chemical and functional characteristics of amniotic membranes was evaluated to ensure the quality and performance in clinical conditions.

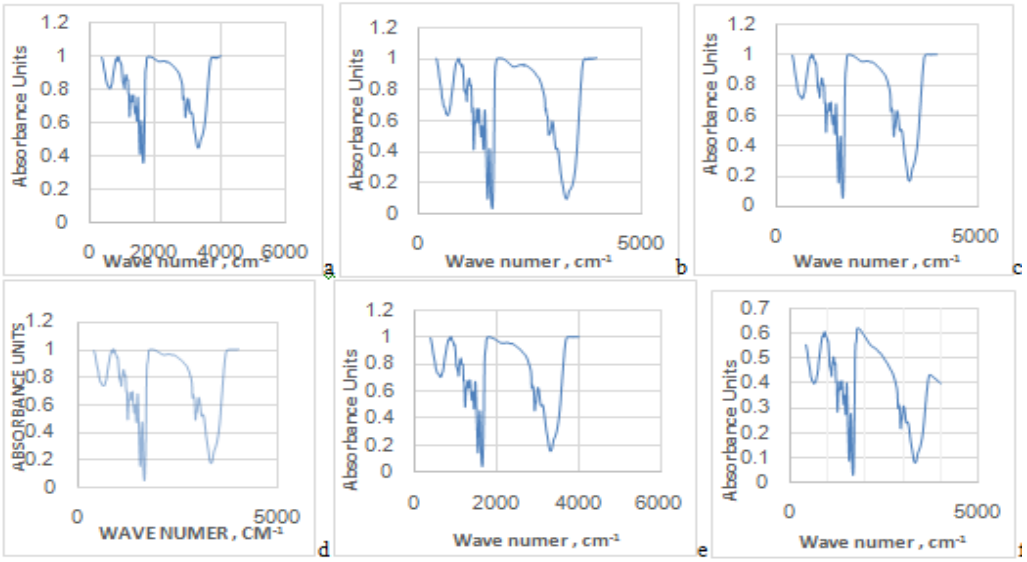

Figure 1:-Infrared spectra of amniotic membranes subjected to: (a) unirradiated control; (b) lyophilized AM; (c) 15 kGy; (d) 20 kGy; (e) 25 kGy and (f) 30 kGy□- radiation.

The membranes exposed to different doses of gamma radiation were characterized by FTIR (Fig. 1). Amniotic membrane being a collagenous material showed the characteristics amide absorption bands at 1655 cm^{-1} (amide I), 1551 cm^{-1}(amide II) and 1239 cm^{-1} (amide III). IR spectral scanning of irradiated amniotic membranes (15, 20, 25 and 30 kGy) as compared to unirradiated amniotic membrane showed no degradation or change in the tissue on

γ irradiation. The degradation of amnion on irradiation with γ rays could produce a relative variation in IR absorption troughs. This kind of variation was absent in the samples irradiated to doses of 15, 20, 25 and 30 kGy indicating no qualitative change in the material property of amnion.

Table 1:-Sulphur content of non-irradiated and irradiated Amniotic Membrane

Elements	Doses					
	A	B	15	20	25	30
Sulphur	6.79	7.64	7.47	6.51	7.78	12.48
CL	93.21	92.36	92.53	93.79	92.22	87.52

The organization of extracellular matrix macromolecules, such as collagens, laminin and fibronectin, plays an important role for physical and biological properties of amnion. The content in sulphur, a principal component of the disulfide bridges in the amniotic matrix, is an indicator for the stability of the allograft. Figure (2)
Quantitative energy dispersive X-ray (EDX) analysis on thin sections of biologic material is a well-established procedure and was used for the determination of the sulphur content of this material **(Schrage** et al., 1993). No change was observed in the sulphur content representing the amount of disulfide bridges in the extracellular matrix and an indicator for the dissolvability of the AM was stable..

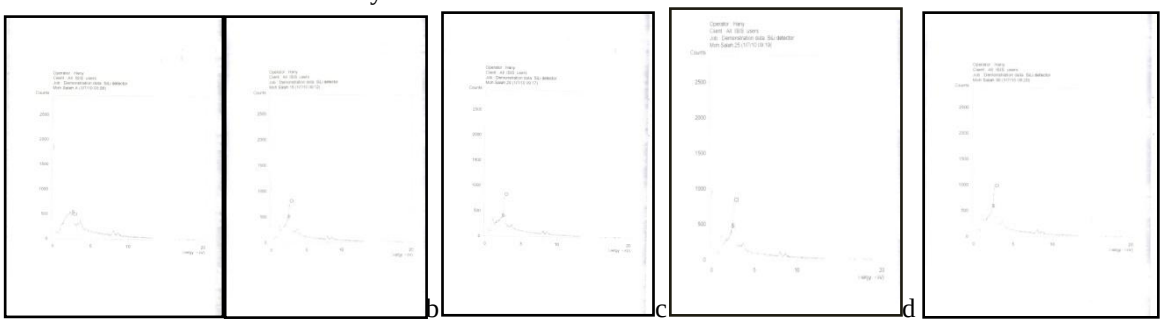
a b c d e

Figure 2:-X-ray (EDX) analysis on thin sections of AM (a) non irradiated, (b), (c), (d) and (e) AM irradiated to 15, 20, 25 and 30 kGy respectively**.**

Table 2:- Impermeability of amniotic membranes after irradiation at different doses of γ radiation.

Bacterial strain	Gram stain	Irradiation dose (kGy)				
		0	15	20	25	30
B. cereas	+ve	+	+	+	+	+
E. coli	-ve	+	+	+	+	+
Ps. Aeurginosa	-ve	+	+	+	+	+
S. aeures	+ve	+	+	+	+	+

Impermeability of amniotic membranes irradiated at different doses was tested to various gram positive and gram negative bacteria. Foure strains of bacteria, *B. cereus, E. coli,Ps. aeurginosa*and *S. aeures* were used.

Pieces of sterilized amniotic membranes were placed on Soyabean Casein Digest Agar plates. Suspensions of bacteria were prepared in sterile water and placed on the opposite surface of membranes and incubated. Plates were checked for the growth after 48 h of incubation.

Amniotic Membrane as dressing must constitute a barrier against external contaminating agents. Impermeability of AM to different bacterial strains, *B. cereas, E. coli, Ps. aeurginosa*and *S. aeures*. Amniotic membranes were found to be impermeable to various bacilli and cocci strains. No effect of high doses of γ radiation on the impermeability of the membranes to microorganisms was observed.

Table 3:-Water vapour transmission rate of amniotic membrane after irradiation at different doses of γradiation.

Time (h)	WVTR				
	Non irradiated	**15 kGy**	**20 kGy**	**25 kGy**	**30 kGy**
1	0.0305	0.0330	0.0300	0.0300	0.0310
4	0.0111	0.0111	0.0111	0.0116	0.0099
8	0.0051	0.0059	0.0060	0.006	0.0052
24	0.0020	0.0020	0.0022	0.0028	0.0017
30	0.0016	0.0018	0.0021	0.0024	0.1700

Amniotic membranes as wound dressings reduce fluid and electrolyte loss and allow exchange of water vapour permitting retention of the appropriate degree of humidity at the lesion surface in order to prevent dehydration and consequent deepening of the wound. Fluid absorption and water vapour transmission by amniotic membrane contribute in creation of the microenvironment conducive to the healing of wounds. Water absorption by amniotic membranes (g/cm^2) exposed to different doses of gamma radiation is presented in Table 4. The rate of water absorption by the membranes was higher initially and reduced drastically with time. No effect of different doses of gamma radiation was observed. WVTR of amniotic membranes after 1,4, 8, 6, 24, and 30 h is presented in Table 3 The results thus indicate that there is no effect on the water absorption and water vapour transmission rate of amniotic membranes onirradiation to 15–30kGy.

Table 4:- Water absorption of AM at high doses of γ radiation

Dose (kGy)	**Weight (g) before**	**Weight (g) before**	**Absorbency**
Control	0.0352	0.4600	0.4248
15	0.0304	0.2076	0.3848
20	0.0544	0.5496	0.4162
25	0.0329	0.4513	0.4184
30	0.0504	0.4632	0.4128

The maintenance of moisture between the wound and the wound dressing and the gas permeability are important criteria for a wound dressing. The maintenance of moisture enables a rapid epidermal healing, because drying of the wound bed can cause necrosis of the new synthesized tissue **(von Versen-Hoeynck, et al., 2008).**

Table 5:- Effect of different doses of γ irradiation on the pH of amniotic membrane.

Dose (kGy)	**pH**
Non irradiated	7.1
15	6.92
20	6.87
25	6.81
30	6.72

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.

Microbial quality of amniotic membrane is one of the most important considerations for its clinical application. As it comes into contact with the open wounds, it needs to be perfectly sterile to avoid contamination as well as transmission of any disease.

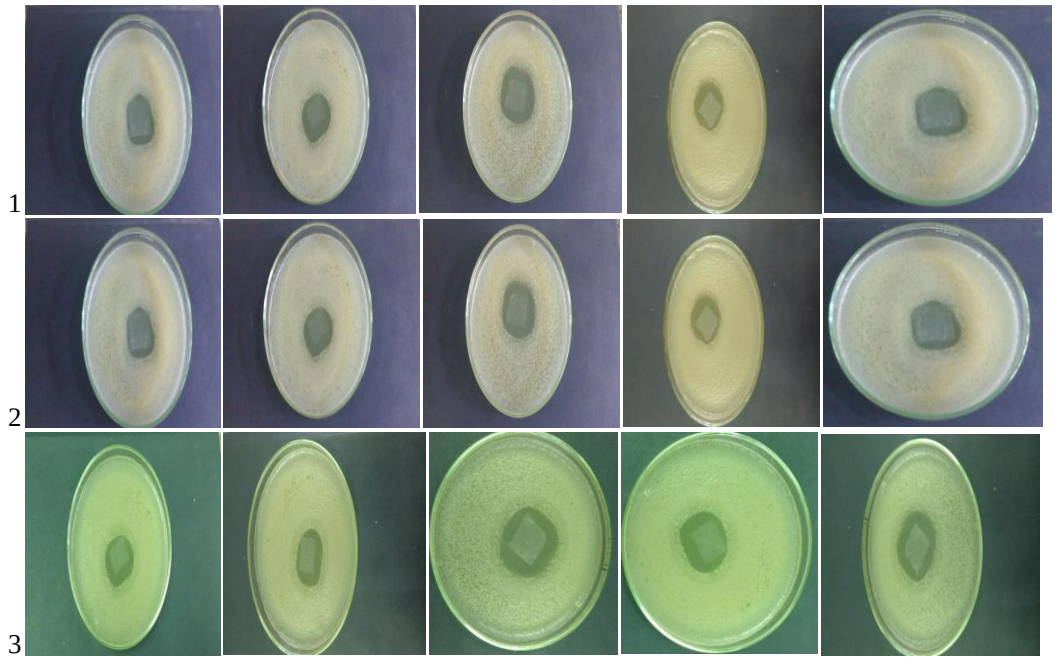

Figure 3:- Antimicrobial of AM (1) *E. coli*, (2) *B. subtilis* and *Pseudomonas aeurogenosa* (a) non irradadited (b) irradiated at 15 kGy (c) irradiated at 20 kGy (d) irradiated at 25 kGy and (e) irradiated at 30 kGy.

The present study was performed to investigate the impact of irradiation doses on antibacterial effect of human amniotic membrane in vitro, showed the inhibitory effect of amniotic membrane on three standard bacterial strains of *P. aeruginosa, E. coli*, and*B. subtilis* in 5 doses of γ radiation, because the inhibition zone was observed in three mentioned strains; in the non-irradiated and irradiated AM at doses, 15, 20, 25 and 30 kGy, no change was observed in the results and diameter of inhibition zone. The present study revealed the inhibitory effect of amniotic membrane on a specific range of standard bacterial strains including E. coli, B, subtilis and P. aeruginosa.It has been demonstrated that amniotic fluidcontains lysozymes, 7S immunoglobulin, b /b globulinand IgA (Kjaergaard et al. or may be due to human b defimsins and elafins**(Dallal et al., 2012)**

Kjaergaardet al. (2001) examined the antibacterial effect of amniotic and chorionic membranes on strains of Streptococcus, Streptococcus, S. aureus, S. saprophyticus, E. faecalis and reported good results in terms of growth inhibition and inhibition zone diameter in Streptococcus Group A, S. aureus, and S. saprophyticus. Their results confirm these observations regarding to the antibacterial effect of amniotic membrane in creating inhibition zone.Antibacterial effect of human amniotic membrane is stable against various radiation doses; therefore the membrane can be used as a biological material with antibacterial effect.

Conclusion:-

There is no change in the infrared spectra of amniotic membranes on gamma irradiation, although the comparative IR spectra of the membranes subjected to steam and dry heat sterilization have shown appreciable changes. The results suggest that there is no significant structural change in amniotic membrane on gamma irradiation at 15- 30 kGy. Water absorption capacity, water vapour transmission rate and microbial impermeability of amniotic membrane remained unchanged at different doses of gamma radiation. The benefit of gamma irradiation at higher doses of 36–50 kGy than the conventional dose of 25 kGy would be in terms of assuring the absence of contaminating viral pathogen of donor origin. Amniotic membranes processed by air-drying are stable and can be irradiated at 36–50 kGy to ensure microbiological safety without compromise to their functional characteristics.

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