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ORIGINAL PAPER

Comparison of structural changes in skin and amnion tissue grafts for transplantation induced by gamma and electron beam irradiation for sterilization

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Abstract Sterilization is an important step in the preparation of biological material for transplantation. The aim of the study is to compare morphological changes in three types of biological tissues induced by different doses of gamma and electron beam radiation. Frozen biological tissues (porcine skin xenografts, human skin allografts and human amnion) were irradiated with different doses of gamma rays (12.5, 25, 35, 50 kGy) and electron beam (15, 25, 50 kGy). Not irradiated specimens served as controls. The tissue samples were then thawn and fixed in 10 % formalin, processed by routine paraffin technique and stained with hematoxylin and eosin, alcian blue at pH 2.5, orcein, periodic acid Schiff reaction, phosphotungstic acid hematoxylin, Sirius red and silver impregnation. The staining with hematoxylin and eosin showed vacuolar cytoplasmic changes of epidermal cells mainly in the samples of xenografts irradiated by the lowest doses of gamma and electron beam radiation. The staining with orcein revealed damage of fine elastic fibers in the xenograft dermis at the dose of 25 kGy of both radiation types. Disintegration of epithelial basement membrane, especially in the xenografts, was induced by the dose of 15 kGy of electron beam radiation. The silver impregnation disclosed nuclear chromatin condensation mainly in human amnion at the lowest doses of both radiation types and disintegration of the fine collagen fibers in the papillary dermis induced by the lowest dose of electron beam and by the higher doses of gamma radiation. Irradiation by both, gamma rays and the electron beam, causes similar changes on cells and extracellular matrix, with significant damage of the basement membrane and of the fine and elastic and collagen fibers in the papillary dermis, the last caused already by low dose electron beam radiation.

Keywords Skin · Xenografts · Allografts · Amnion · Sterilization · Gamma radiation · Electron beam

Introduction

Temporary coverage by biological or synthetic skin substitutes is important for correct and faster healing of burns and other skin defects. An important step in processing of biological tissues is sterilization in order to prevent transfer of infectious diseases (Ghosh et al. 1997). Sterilization is a process eliminating all living microorganisms including bacterial spores, thereby ensuring a safe level of sterility (SAL = Sterility

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Assurance Level). SAL rate can vary depending on tissue/material bioburden as well as on the potential use of the sterilized material. In materials and tissues that come in contact with blood of the patient it is necessary to reach SAL of 10^{-6} (Phillips 2000). This can be reached by exposure of the tissue transplants to physical factors (hot air, saturated water vapors, ionizing radiation, plasma, etc.) or chemical substances (peracetic acid, ethylene oxide, glutaraldehyde, formaldehyde, etc.) (USP 30-NF 25 2007). Tissues during processing in the tissue establishments are decontaminated by a cocktail of antibiotics, which by themselves are not sufficient for sterility assurance. Consequently, their sterilization by physical or chemical methods is needed. For chemical sterilization mostly ethylene oxide, propylene glycol and peracetic acid are used (Booth 1998). The use of chemical substances may lead to persistence of toxic residues in the tissues. It appears more suitable to use sterilization by irradiation, which also may reduce antigenicity of the tissues, it does not cause increase of tissue temperature, it does not leave any harmful residues but can cause biological and mechanical changes (Kearney et al. 1989; Kairiyama et al. 2009). Electron beam, gamma and X-rays may be used, but gamma rays are preferred because of their excellent penetrance into the tissues (Kairiyama et al. 2009). The most common sources of gamma rays are cobalt (^{60}Co) or cesium (^{137}Cs) and the internationally recommended dose for sterilization of medical devices is 25 kGy which dose is also generally recommended for sterilization of tissue grafts (Berk and Ozer 1999). It is possible to use also electron beam irradiation for sterilization but because of lower penetrance it is limited by the thickness of the sterilized specimen (Silindir and Ozer 2009). Electron beam processing involves the use of high energy electrons generated by accelerators. Electron beam radiation has the shortest process cycle of any currently recognized sterilization methods and the products are exposed to radiation for seconds and minutes (Mattern et al. 2005). The main effect of irradiation is ionization of water molecules (indirect effect) with formation of free radicals (H_3O^+ and OH^-). Subsequently hydroxyl radicals cause damage of the DNA of microorganisms and have oxidative effects as well. The reduced water content in the microorganisms causes increase of their resistance to irradiation (Aquino 2012). Less sensitive to irradiation are also prions and some viruses and fungi (Hansen and Shaffer 2001). There are additional factors modifying the sensitivity of pathogens to irradiation, such as temperature, presence of oxygen and water (Aquino 2012).

The present study was oriented to assess the impact of two different types of ionizing radiation on three types of biological tissues and the impact of different doses on structural integrity of the cells and the extracellular matrix.

Materials and methods

Three types of biological tissues grafts (porcine skin xenografts, human skin allografts and human amnion grafts) were procured and processed by standard operating procedures (SOP) of the Central Tissue Bank. Thereafter they have been frozen and kept at −80 °C in deep freezers. The tissue samples assigned for investigation were divided into two groups: the first one was further divided into four subgroups in frozen state packed in special boxes filled with dry ice that were exposed to gamma irradiation doses of 12.5, 25, 35, 50 kGy respectively delivered by commercial Co^{60} irradiation source (Brno, Veverská Bitýška, Czech Republic).The second was processed in the same way into three subgroups that were irradiated by linear accelerator electron beam source (Slovak Medical University, Trenčín, Slovak Republic), beam energy of 7 MeV, with doses of 15, 25 and 50 kGy, respectively. Not irradiated specimens served as controls. The tissues following thawing were fixed in 10 % formalin and processed by routine paraffin embedding technique. Slices 5 μm thick were stained by the following staining methods: hematoxylin and eosin, alcian blue (ALC) at pH 2.5 (mucin staining), orcein (elastic fibers), periodic acid Schiff reaction (PAS reaction—neutral saccharides staining), Mallory phosphotungstic acid hematoxylin, Sirius red and metenamine silver impregnation (collagen fibers staining). The stained specimens were mounted in acryl medium and covered with cover slips.

The slides were evaluated by light microscopy in Eclipse *t*80 microscope (Nikon, Japan) with focus on fibrillar structures.

Results

Each staining detected a different type of morphological changes in particular structures of the irradiated

tissues. Basic staining with hematoxylin and eosin showed in the first group of tissues exposed to gamma irradiation vacuolar degeneration of the epidermal cells in xenografts starting by the dose of 12.5 kGy, whereas in human skin grafts starting by the dose of 35 kGy. Same changes have been observed in the same type of cells after electron beam doses of 15 and 25 kGy, respectively. Morphological changes detected by special staining techniques are summarized in Table 1.

Decomposition of the fine collagen fibers in the papillary dermis was disclosed by Sirius red staining, Mallory's staining and impregnation with metenamine silver. In xenografts and amnion, disintegration of the fine fibers started at the dose of 25 kGy, whereas in allografts after the dose of 35 kGy of gamma irradiation. Same changes were induced in the skin specimens by 15 kGy, and in the amnion membrane wall by 50 kGy of electron beam irradiation. Additional changes were observed in collagen fibers of the basement membrane, where loosening of their density and disruption of the fibers were present. These changes were observed in both xenografts and amnion membranes after gamma irradiation dose of 35 kGy and the by the dose of 25 kGy in the skin allograft. Similar changes were induced by the electron beam with the dose of 25 kGy in the allograft and the amnion membrane and by 15 kGy in the skin xenograft. Orcein staining disclosed damage and disintegration of fine elastic fibers in the papillary dermis of allo- and xenografts after electron beam irradiation dose of 25 kGy. Gamma irradiation induced these changes with the dose of 12.5 kGy in xenografts and 25 kGy in allografts (Fig. 1).

Impregnation with metenamine silver disclosed also condensation of nuclear chromatin in epithelial cells of skin xenografts and of the amniotic membrane already at the lowest doses of both irradiation types, in the allografts these changes appeared after higher irradiation dose (Fig. 2).

Discussion

Assurance of sterility is an important issue in using biological materials in clinical settings. While a variety of sterilization systems are currently available, some of them can elicit negative changes in implanted tissues or in the recipient organism. Chemical agents like ethylene oxide or glutaraldehyde are known to induce a chronic inflammatory response in humans (van Wachem et al. 1994; Kolman et al. 2002; Johnson 2002). One of the most frequently used methods is the sterilization of allografts with ethylene oxide. It is a gas that does not change the biomechanical properties of tissues but leaves cytotoxic and cancerogenic residues which result from its reaction with chlorides. These residues have to be eliminated before application in a time consuming process consisting of several steps (Kearney et al. 1989; Nakheon et al. 2007; Weadock et al. 1996).

Ionizing irradiation has become a routinely used sterilization procedure for tissues assigned for transplantation. Although it is considered as a gentle method for biological materials sterilization, detailed morphological analysis of gamma irradiated tissues documented significant changes in the tissues (Mrázová et al. 2014). The most affected tissue structures

Table 1 Histomorphological changes in the three types of biological tissues after irradiation with gamma rays and electron beam

	Xenografts		Allografts		Amnion	
	Gamma (kGy)	E-beam (kGy)	Gamma (kGy)	E-beam (kGy)	Gamma (kGy)	E-beam (kGy)
Vacuolar degeneration of epithelial cells	12.5	15	35	25	–	–
Nuclear chromatin—condensation	12.5	25	35	25	12.5	15
Disruption and loss of basement membrane density	35	15	25	25	35	25
Decomposition of elastic fibers of papillary dermis	12.5	25	25	25	–	–
Disintegration of fine fibers in the—papillary dermis—amnion wall	25	15	35	15	25	50

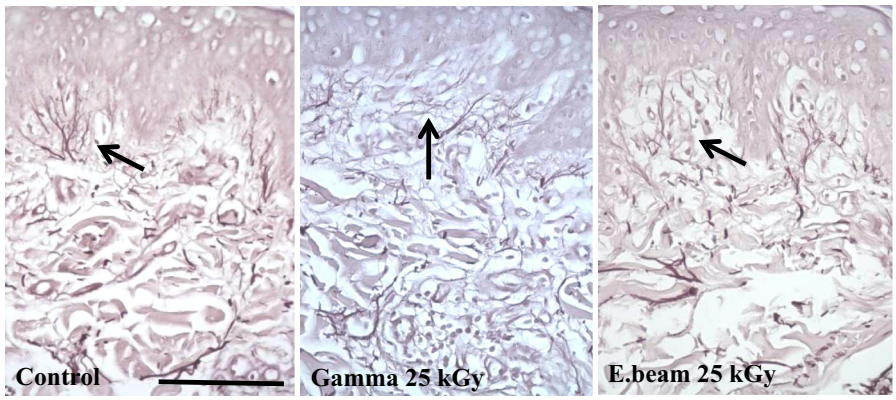

Fig. 1 Porcine skin xenograft evaluated for elastic fibers in the papillary dermis. Irradiation with the dose of 25 kGy of Gamma radiation and electron beam resulted in loss of fine elastic fibers in the papillary dermis (*arrow*). Orcein stain, *bar* = 50 μm

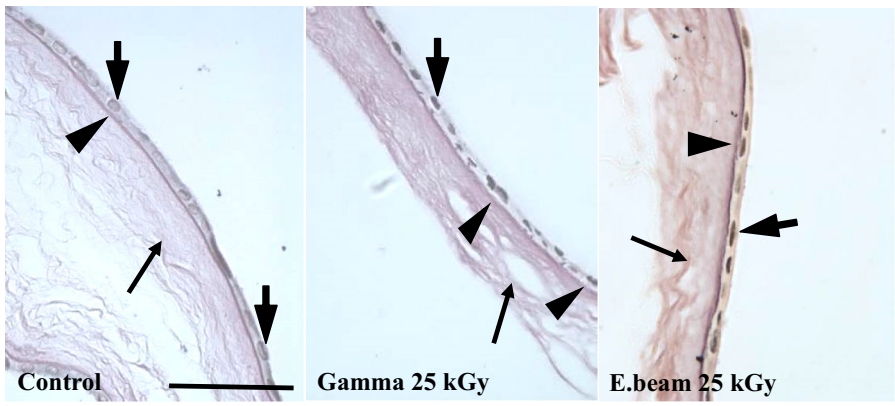

Fig. 2 Human amnion membrane irradiated by Gamma rays and electron beam. Fine collagen fibers in the membrane wall (*arrow*) disappear at the dose of 25 kGy of Gamma radiation and are still preserved at the dose of 25 kGy of electron beam exposure. The basement membrane (*arrowhead*) turns swollen and focally disintegrated. The nuclei of amnion lining cells (*short arrow*) have condensed chromatin after irradiation. Impregnation with metenamine silver, *bar* = 50 μm

are collagens, especially collagen type I. There has been observed reduction of single collagen fibers and formation of abnormal fascicles (Tzaphlidou 2002). The effects of ionizing irradiation can be direct and indirect, depending on the water content in the irradiated tissues. Irradiation in the absence of water may lead to disintegration of collagen polypeptide chains, which is defined as the direct effect. With the presence of water in irradiated tissues the ionizing radiation leads to formation of free radicals and the other products (hydroxyl radical, hydrogen radical, hydrogen peroxide) that cause cross linking of collagen fibers as an indirect effect (Aquino 2012).

There has already been proved that ionizing irradiation for sterilization purposes does change to certain extent some of the properties of the biological skin substitutes. The most important changes include loss of graft viability, irradiation dose dependent impact on their structural integrity and tensile strength, as well as damage or inactivation of important biological substances (such as cytokines and growth factors) contained in the grafts (Rooney et al. 2008). However, many other beneficial properties of the grafts including their barrier functions preventing wound contamination and decreasing both discharge and evaporative water loss from open wounds substantiate their use in situations where temporary coverage of open wounds is indicated (Ge et al. 2011).

Grafts sterilized by radiation can offer less expensive but still beneficial alternatives for wound coverage materials in health care systems with limited resources. Sterilization of graft specimens with gamma radiation

has been widely used in clinical practice. Its negative effect on extracellular matrix and its fibrillary compartment has been well documented (Mrázová et al. 2014; Schmidt et al. 2012), although some authors have reported no significant histological changes after irradiation with the dose of 25 kGy (Rooney et al. 2008). This discrepancy can be explained by the fact, that their conclusions were based on evaluation of specimens only with basic hematoxylin and eosin histological staining.

Sterilization of tissue grafts by high dose electron beam irradiation has shown preservation of good biomechanical properties of tendons and ligaments (Schmidt et al. 2012; Elenes and Hunter 2014). The results of the present work have demonstrated histological changes induced by electron beam that are in several aspects similar to those described after gamma irradiation (Mrázová et al. 2014), although in some of the investigated tissues same changes occurred after higher doses of electron beam than by gamma irradiation. The predominantly affected structures are the fine fibrillary components of the extracellular matrix. The thicker collagen bundles remained unchanged, which might explain the well preserved biomechanical properties of the irradiated tissues (Schmidt et al. 2012).

Our results showed several changes of the cells and the extracellular matrix. In the epithelial cells cytoplasmic vacuolar degeneration and condensation of nuclear chromatin have been observed. These changes are compatible with necrobiotic processes of the cells. For the clinical applications damages of the extracellular matrix are crucial. There were documented mainly changes of the basement membrane and of the fine collagen fibers, including the fine elastic fibers especially in the papillary dermis. All these histomorphological changes were induced by similar irradiation doses of both, gamma and electron beam irradiation. It can be concluded that sterilization of skin and amnion grafts can be equally performed by gamma or electron beam irradiation, based on local availability of the method.

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