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Comparative Study Between Topical Applications of Liposomally Entrapped DNA Repair Enzymes and Thymidine Dinucleotides as Radioprotectors

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ABSTRACT

The delivery of active agents to the skin by liposome carriers recieved great interest during the last three decades. This is based on their potential to enclose various types of biological materials and to deliver them to diverse cell types. Recent work suggests that liposomes as vehicles for topical drug delivery may be superior to conventional preparations. Also, topical application of DNA repair enzymes to irradiated skin increases the rate of repair of DNA potentially damaged cells. Moreover, thymidine dinucleotide is a new skin photo-protective agent against non-ionizing radiation through induction of DNA repair. Gamma irradiation can produce DNA damage in human skin. DNA mutations have an important role in the development of skin cancer and precancerous skin lesions. Albino rats were irradiated with Cobalt-60 gamma radiation with different doses (0.5, 1.5, 3Gy), and were treated by either thymidine dinucleotide or liposomally entrapped DNA repair enzymes topically 24 hours before irradiation.

Evaluation was done histolopathologically by H&E stain. Computerized image analyzer using Masson's trichrome stain was also done. Gamma radiation produced epidermal thinning and dermal inflammatory cells together with collagen fragmentation and clumping in a dose-dependent manner. Comparing between both thymidine dinucleotide and liposomally entrapped DNA repair enzymes pretreated and irradiated rats. Low dose irradiation (0.5Gy) together with previous drugs showed preservation of epidermis with no inflammatory cells and also it maintained the normal architecture of collagen bundles. However, they were ineffective with higher doses. In conclusion our results may suggest that the effects of gamma radiation on the skin at low dose could be minimized by the use of these drugs before exposure.

Keywords: Gamma radiation/ skin/ image analysis/ thymidine dinucleotide/ liposomally entrapped DNA repair enzymes.

INTRODUCTION

The ideal radio-protective as well as photo-protective agents would prevent damaging electromagnetic photons from entering the skin, enhances the skin's ability to repair damage from any absorbed photons, and yet permit tanning. Presently available sunscreens effectively absorb or reflect sunlight, but do not alter the skin's innate repair capacity and do not have any effect on ionizing radiation.

Human skin is a complex organ covering the body's internal systems and organs. It is the body's external interface with the environment, and is considered as a target organ for pollution and also the site of significant absorption of environmental pollutants. About 700,000 new cases of skin cancer

were diagnosed in 1993, and 9,100 people died of cancer; 76% of the deaths were due to skin melanoma. Skin cancers are most closely associated with exposure to ultraviolet radiation (UV) especially UVB (290 to 320 nm). For every 1% decrease in ozone there is a 2% increase in UVB irradiance, and a 2% increase in skin cancer is predicted. It should be pointed out that, the incidence of skin cancer is increasing at an alarming rate, more than 1 million new cases of non-melanoma skin cancer was diagnosed in United States in 2002. Skin cancer risks are expected to peak in the period 2050-2070 due to the long atmospheric lifetime of the ozone depleting substances[(1)].

As mentioned skin cancer is increasing, and sunscreens are apparently underused and misused. Therefore, new drugs are needed, that can be used before and even after sun exposure to reduce skin-cancer formation; such drugs are likely to be of great future value to the normal sun-exposed population, even when sunscreen is used[(2)]. Amifostine is a prodrug of free thiol that may act as a scavenger of free radicals generated in tissues. Amifostine was originally developed as a radioprotective agent in a classified nuclear warfare project[(3)]. Intramuscular administration of caffeine at a dose of 80 mg/kg body weight to the gastrocnemius muscles of Swiss mice 5 min prior to local irradiation (35Gy) of the leg delayed the progression of radiation-induced skin reactions in such animals[(4)].

In guinea pig skin, a thymidine dinucleotides (pTpT) induced tan and DNA repair, which was completely protective against a 6 minimal erythematous dose (MED) UV exposure[(5)]. In the other hand, liposome encapsulation of T4 endonuclease V represents a new drug delivery approach that shuttles enzymes across human stratum corneum and introduces biologically active proteins into living epidermis. Moreover, liposomes are considered, as vehicles for topical drug delivery that may be superior to conventional preparations, which suggests that only a compromised epidermal barrier enables intact liposomes to penetrate the skin. When bacterial DNA repair enzyme, T4 endonuclease V, encapsulated in a pH-sensitive engineered liposome for delivery intracellularly, it increased the rate of repair of sunlight-induced DNA damage in human cells. No adverse effects among patients was detected, this may be due to that T4 endonuclease V delivered by liposomes in a localized manner to the epidermis and does not readily penetrate into the dermis[(6)].

The principal epidemiologic studies of ionizing radiation and skin cancer have all shown that radiation causes basal cell carcinoma but have not found dose-related excess of squamous cell carcinoma or malignant melanoma. The Japanese atomic bomb study indicated that doses of radiation under about 1 Gy confer less risk per unit dose than higher doses do[(7)]. Significant doses of ionizing radiation produce acute skin reaction in human called acute radiodermatitis, which are dose-dependent and reflect damage to the germinative cells of the epidermis and to the cutaneous vasculature. When the dose is about 3.8Gy, it is characterized by erythema, edema, slight burning and pruritic sensation, healing usually occurs within 2-14 days. However, temporary epilation may be also associated, and hair regrowth occurs after 4-12 weeks. Moreover, when the exposure exceeds 10Gy the epidermis exfoliates leaving a denuded dermis, with punched out painful ulcer, with no granulation tissue formation.

Radiation protection standards for the individual exposed to ionizing radiation during daily work have evolved over more than 50 years since the first recommendations on limits by the NCRP and the ICRP. Initial standards were based on the absence of observable harm, notably skin erythema, but have since been modified as other concerns, such as leukemia and genetic effects, became more important[(8)]. When the ICRP made its recommendations in 1977 for dose limits there was no appreciation of the importance of the interaction of ultraviolet radiation (UVR) and X-rays. Both clinical and experimental data showed that the risk of ionizing-radiation-induced cancer is significantly increased by subsequent exposures to UVR. Therefore, risks for sun-exposed areas of skin differ from those that are shielded. The risk estimate for skin cancer is very dependent on the selection of the skin site, the total risks for both UVR exposed and shielded skin is about twice that of shielded skin in non exposed skin areas[(9)]. According to the existing standard, maximal dose to the skin over the career may not exceed 1200 cSv. However, there is high probability of pathology including malignant tumor in delayed period after exposure to 1200 cSv. The maximal dose to the skin

over the space career should not be higher than 600 cSv[10].

MATERIALS AND METHODS

Fifty-four albino rats (Rattus rattus) were irradiated using cobalt-60 (Gamma-cell 220), Atomic Energy of Canada Limited, installed at Radioisotope Department of the Egyptian Atomic Energy Authority, Cairo-Egypt. Cobalt-60 is a radioactive isotope of Cobalt, which the physical ½ life is 5.27 years. It decays by Beta particle and Gamma ray emission. This source provided an average exposure rate of 1 Gy/min in the center of the cage of the machine of irradiation.

Punch skin biopsy 6 mm was taken from each rat of the fifty-four rats. The fifty-four rats were divided into 3 groups (I, II and III) each of them was 18 rats.

Group I: leaved as a control group (without painting).

Group II: painted with Liposomally Entrapped DNA Repair Enzymes, specific plant extract from the nopal cactus "Nopasome" (Ateia AG- Austria).

Group III: painted with Thymidine Dinucleotides(pTpT) (Midland Certified Reagent Company-USA).

Each of the above 3 groups subdivided into 3 subgroups (I0.5, I1.5 and I3), (II0.5, II1.5 and II3) and (III0.5, III1.5 and III3), each of them were 6 rats. 24 hours after painting, irradiation of 0.5 Gy gamma rays using cobalt-60 to subgroups I0.5, II0.5 and III0.5; 1.5 Gy to subgroups I1.5, II1.5 and III1.5; 3 Gy to subgroups I3, II3 and III3.

Skin biopsies were taken from each rat daily after 24 hours of irradiation on the 1st, 2nd, 3rd and 4th day. Punch skin biopsy 6 mm was taken and put in buffered formalin 10% then processing with paraffin, then cut on a rotary microtome into sections 5-7μm thick and stained with Haematoxlin and Eosin (H & E), sections were then examined histopathologically. Masson's trichrome stain was also done in order to demonstrate collagen changes in the rat skin dermis. Evaluation of all skin biopsies were done. Comparative study was done on skin biopsies taken on the 4th day between control subgroups and treated ones.

Image analysis: The data were obtained using Leica Qwin 500 image analyzer computer system (England). The image analyzer consisted of a colored video camera, colored monitor, hard disc of IBM personal computer connected to the microscope, and controlled by Leica Qwin 500 software. The image analyzer was first calibrated automatically to convert the measurement units (pixels) produced by the image analyzer program into actual micrometer units. The area % of collagen fibers in dermis was measured using the color detect menu. The blue staining of collagen by Masson's trichrome was detected and masked by a red binary color. These red areas were measured and their area % in relation to the standard measuring frame of a standard area equal to 29524.44μm^2. Using the measuring field menu the area, area % and standard measuring frame of a standard area equal to 29524.44μm^2 were chosen from the parameters. Ten fields in each specimen were measured and the mean values were obtained using an objective lens of magnification 20, i.e. a total magnification of 200. Using the interactive measuring menu, the epidermal thickness was measured by obtaining 10 readings in each specimen at magnification 200.

RESULTS

Primarily, it should be mentioned that the application of 1.5 Gy in groups I1.5, II1.5 and III1.5 did not show any difference from the results obtained with 0.5 Gy (Groups I0.5, II0.5 and III0.5). Also, the skin biopsies taken daily after 24 hours of irradiation for 4 days showed non significant changes except, in the 4th day of irradiation. It should also be mentioned that the application of Liposomally Entrapped DNA Repair Enzymes give nearly, the same results as Thymidine

Dinucleotides (pTpT).

When the epidermis of rat skin was examined histopathologically after irradiation with 0.5, 1.5 and 3 Gy as a single dose with or without either pTpT or Liposomally Entrapped DNA Repair Enzymes. It was found that irradiation after 4 days caused epidermal thinning, flattening of the rete ridges together with presence of large number of langerhans cells and many pyknotic nuclei (fig.1& 2), which was also demonstrated by computerized image analyzer as a significant epidermal thinning with 0.5 Gy and 3 Gy irradiation of about 14.6% and 37.2% after 4 days respectively as in group I0.5 & I3 (table.1). In the other hand, when pTpT was added there was spongiosis (edema of the epidermis) together with liquifactive degeneration of the basal cell layer.

When computerized image analyzer was done a highly significant increase in the epidermal thickness was demonstrated in the 0.5 Gy of about 52.6% as in group III0.5. Moreover, it was insignificantly decreased with 3 Gy to become slightly lower than the control by 2.38% as in group III3.

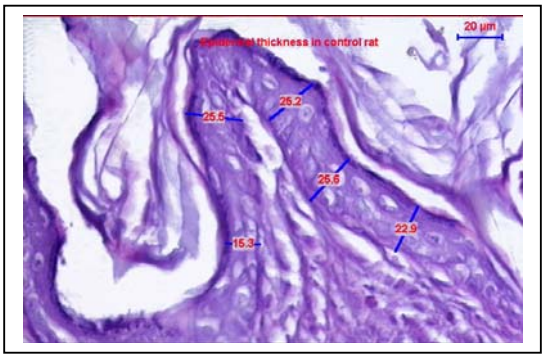

Fig.1. Shows normal epidermis of albino rat skin

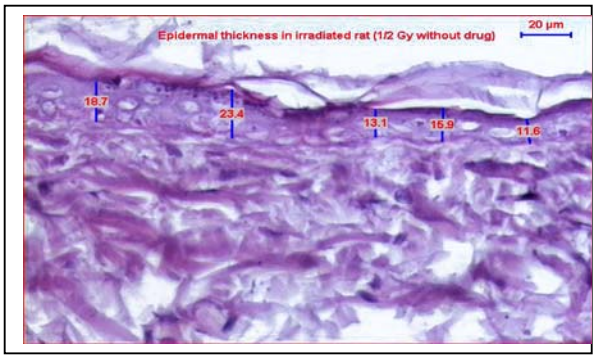

Fig.2. Shows Epidermal thinning 4 days after irradiation.

Table 1. Epidermal thickness

	Rat1	Rat 2	Rat 3	Rat 4	Rat 5	Rat 6	Mean	t-test
Control	**22.141± 6.9**	**22.276±3.18**	**16.877±4.2**	**25.163±8.96**	**25.14±3.91**	**27.115±8.77**	**23.119**	**Control**
Group III0.5	**35.18±4.45**	**34.225±7**	**35.605±10.1**	**34.08±4.98**	**37.314±10.0**	**35.281±4.45**	**35.281*** **	**0.0002556**
Group I0.5	**22.002±5.17**	**18.543±3.44**	**16.284±3.5**	**24.602±2.41**	**17.57±2.83**	**19.37±1.83**	**19.729***	**0.0331761**
Group III3	**29.235±6.56**	**20.247±2.93**	**14.484±4.1**	**21.373±4.42**	**25.367±4.73**	**24.626±5.57**	**22.555**	**0.3712105**
Group I3	**14.92±2.89**	**16.572±1.76**	**13.756±1.88**	**12.423±1.7**	**14.358±3.93**	**14.977±1.65**	**14.501****	**0.0014013**

*, **, *** are the significant differences at t-test < 0.05, < 0.01, < 0.001 respectively

On the other hand, when rat skin dermis was examined histopathologically, irradiation showed fragmentation and clumping of collagen fibers and dilated blood vessels together with intravascular thrombosis. However, in case of pTpT application as in group III0.5 & III3 collagen fibers were healthy with minimal affection. Masson's trichrome stain demonstrated collagen as blue bundles which was stained red by the computer of image analyzer in order to calculate the collagen density in certain areas within the dermis (fig.3 &4).

It was found that irradiation lowered collagen density significantly in a dose like manner by 34.2% and 38.1% with groups I0.5 and I3 respectively. While when pTpT was added, the collagen density was less diminished than the control to by 23.5% and 28.3% respectively as in groups III0.5 and III3

(table.2).

Table 2. Collagen percentage area in the rat skin dermis

	Rat1	Rat 2	Rat 3	Rat 4	Rat 5	Rat 6	Mean	t-test
Control	**39.1±2.4**	**41.05±3.37**	**42.7±3.3**	**40.05±2**	**38.9±3.3**	**38.3±3.3**	**40.05**	**Control**
Group III0.5	**29.023±4.3**	**34.364±2.9**	**29.408±5**	**26.719±4**	**33.484±6**	**30.6±2.5**	**30.6*** **	**0.0002408**
Group I0.5	**27.537±3.9**	**26.362±2.7**	**26.999±3**	**27.712±3**	**28.176±2**	**21.272±3**	**26.34*** **	**2.306E-05**
Group III3	**24.3±3.03**	**21.169±2.32**	**25.703±2**	**31.287±6**	**35.358±8**	**34.419±5**	**28.706** **	**0.0024798**
Group I3	**21.69±5.2**	**21.48±5.318**	**31.35±3.3**	**32.18±3.7**	**17.32±3**	**24.79±2.5**	**24.79*** **	**0.0007223**

*, **, *** are the significant differences at t-test< 0.05, < 0.01, < 0.001 respectively

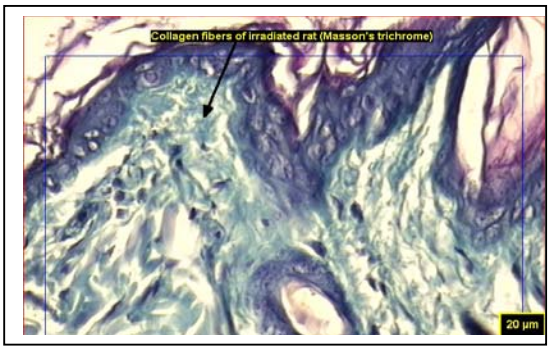

Fig..3. Shows dermis stained by Masson's Stain

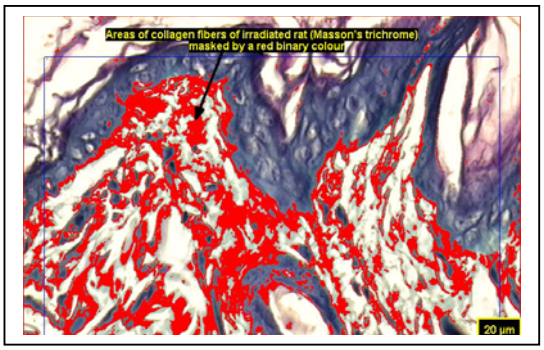

Fig.4. Shows collagen areas masked by red binary color

DISCUSSION

The response of the skin to ionizing radiation has important implications both for the treatment of malignant disease by radiation and for radiological protection. Acute radiation damage to the skin is primarily a consequence of changes in the epidermis; the timing of the peak of the reaction is related to the kinetic organization of this layer.

Recovery of the epidermis occurs as a result of the proliferation of surviving clonogenic basal cells within the irradiated area. The presence of clonogenic cells in the canal of the hair follicle is important, particularly after non-uniform irradiation from intermediate energy beta-emitters. The migration of viable cells from the edges of the irradiated site is also significant when small areas of skin are irradiated[(11)].

Epidermal permeability barrier function is impaired in cases of radiation dermatitis. The functional damage to the stratum corneum induced by ionizing radiation occurs with a delayed course, starting within a mean period of 11 days and reaching maximal values after a mean period of 27 days (range, 13-75 days) [(12)]. Moreover, Goukassian et al., 2002[(13)] reported that pTpT treated skin of all mice showed spongiosis and sunburn cells, which was prominent in Xeroderma pigmentosa diseased mice, consistent with compensatory hyperplasia.

Results confirmed that there is also spongiosis, which increased the thickening of epidermis by 52.6% after 4 days of irradiation when pTpT was previously applied with 0.5 Gy. However, gamma irradiation seems to have a prominent effect on the epidermis as it result in significant epidermal thinning with 0.5 Gy and 3 Gy irradiation of about 14.6% and 37.2% after 4. Large number of

langerhans cells were present in irradiated rat skin and disappeared with pTpT treatment.

Studies in the pig skin, showed that postregenerative phase of hyperplasia reflect a temporary overshoot of cell density above control levels; a subsequent decrease in hyperplasia indicates feedback control of cellular proliferation[14]. Recent work has also established that small DNA fragments, particularly thymidine dinucleotides (pTpT), when added to cultured cells or to intact animal skin, evoke photo-protective responses that include increased melanogenesis (tanning) and induction of gene products involved in DNA repair and antioxidant defenses, leading to enhanced prevention and repair of solar insults[5].

Collagen is known to constitute 70% of dry skin mass, which is either collagen type I, III and VI. Collagen typeI, which comprises 80% of total collagen, decreases by 59% in irradiated skin depending with extent of exposure. UVR is known to cause collagen damage through up regulation of several types of collagen-degrading enzymes called Metalloproteinases[15].

Ionizing radiation is known to cause fattening of the rete ridges and accumulation of edema fluids in the dermis together with fragmentation and clumping of dermal collagen known as radiation dermatitis. Dilatation of dermal blood vessels may also occur giving rise to telangiectasia [16]. Our results confirmed these findings especially with both groups I0.5 & I3. Moreover, although that dermal collagen density in all groups was diminished significantly with a range between 23.5% and 38.1%, but collagen fibers in pTpT & liposomal with DNA repair enzymes treated rats appears healthy with no clumping or fragmentation, which was demonstrated by Masson's trichrome stain. Dilated dermal blood vessels and intravascular thrombosis as in both group III0.5, II0.5, III1.5, II1.5 and III3.

On the other hand, Yarosh et al (2001) [17] confirm the ability of DNA repair enzymes in a liposomal vehicle when applied topically (T4 liposome lotion) for one year daily, to lower the rate of new skin cancers in patients with Xeroderma pigmentosum.

Our findings revealed any that the application of liposomal DNA repair enzymes showed similar results obtained with pTpT with low dose irradiation 0.5 and 1.5 Gy. However, with 3 Gy DNA repair enzymes when applied topically it was ineffective. T4 liposome lotion is designed for post-irradiation application and it must be continued for a longer period in order to notice results.

In conclusion gamma irradiation is known to have harmful effects to the skin, which can be diminished by radioprotective agents. Thymidine dinucleotide (pTpT) and Liposomally Entrapped DNA Repair Enzymes are new photo-protective agents against ultraviolet through induction of DNA repair.

When they were applied topically to rat skin to protect against ionizing radiation an obvious finding were noticed such as preservation of epidermis, minimal inflammatory cells and also it maintained the normal architecture of collagen bundles. So, they can be used as radioprotectors against low dose irradiation and further investigations are needed.

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