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Genetic Protective Role of D-Glucan against Oxidative Stress Induced by Mitomycin and Gamma-Radiation Exposure in Male Albino Rats

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D-GLUCAN is a polysaccharide with multi-branching molecules derived from the cell wall of baker's yeast. It was reported to modulated innate immunity via interaction with membrane receptors on macrophages, neutrophils and natural killer cells and posses potent antioxidant and free radical scavenging capabilities.

The aim of the present study was to investigate chemo-protective and radio-protective effect of D-glucan. Rats were orally (gavages) injected with D-glucan at dose of (20 mg/kg body wt) daily for three weeks. Mitomycin c (MMC) was administered three equal doses (1.5 mg/ kg) day after day for one week (2^{nd} week) before radiation exposure at 3 fractionated doses (2 Gy) day after day in the 2^{nd} week. The investigation were carried out on the days 3 & 10 post radiation-exposure and MMC administration for the determination of bone marrow micronucleus (Mn) frequency and DNA fragmentation in blood lymphocytes.

The results showed that the exposure of animals to MMC and/ or irradiation led to clearly defined DNA fragmentation. D-glucan administration resulted in a significant improvement in lymphocytes DNA fragmentation and amelioration of Mn frequencies at day 3 and more pronounced at day 10 post irradiation. This study indicates that D-glucan has radio and chemo-protective effects against oxidative stress as a result of γ-radiation and/ or MMC exposure. So, D-glucan may be used to reduce the genotoxicity effects of different anticancer drugs and to reduce their unwanted side effects.

Keywords: D-glucan, γ-rays, mitomycin, DNA, micronucleus.

Anticancer drugs used in chemotherapy for tumors and leukemias inhibit proliferation and induce cell death in malignant cells. In addition to is effect, chemotherapy causes sever toxicity in normal cells leading to side effects such as mucositis, hair loss, and myelosuppression (Stahnke *et al.*, 2001).

MMC is one of the most common alkaloids, acting as an antineoplastic agent used to fight a number of different cancers including cancer of the stomach, colon, rectum, pancreas, breast, lung, uterus, cervix, bladder, head, neck, eye and esophagus. It is a potent DNA cross-linker (Siddique *et al.*, 2005). On the other hand ionizing radiation used as radiotherapy which may be used as the primary therapy. It is also common to combine radiotherapy with surgery and / or chemotherapy and/or hormone therapy **(**Mussari *et al.*, 2006). Facing is role, consequence of ionizing radiation exposure could be very serious including DNA damage, micronucleus formation and reduced cell viability (Shmakova *et al.*, 2006 and Sterpone and Renata, 2010). One of the approaches to deal with these problems is to search for suitable anti-mutagens. Over last few years, much attention has been paid to the research of naturally occurring agents that are able to stimulate defence mechanisms of the organism against different oxidative stresses (Bobeck and Calbavy, 2001).

D-glucans consist of linear unbranched polysaccharides of β-D-Glucose like cellulose, but with one 1β→3 linkage for every three or four 1β→4 linkages. D-glucans form long cylindrical molecules containing up to about 250,000 glucose units. D-glucans occur in the bran of grains such as barley and oats, and they are recognized as being beneficial for reducing heart disease by lowering cholesterol and reducing the glycemic response (Chen and Seviour, 2007). According to the free-radical scavenging ability of glucan, no controversial occurred with the anti-tumour activity of the drugs. Moreover, it has been reported that glucan helps in reducing the side effects of conventional chemotherapy and radiotherapy, while at the same time enhancing its effectiveness (Tohamy *et al.*, 2003).

DNA damage caused by ionizing radiation is manifested by single-and double-strand breaks in the sugar phosphate backbone of the DNA molecule, cross-links between DNA strands and also with chromosomal proteins (Dizdaroglu, 1992). Treatment with MMC induces DNA cross-links damage through adding methyl group to guanines in tracts of DNA which leads to inhibition of its replication **(**Tomasz *et al.*, 1987). Selective removal of aziridine function of MMC results in a switch from minor to major groove alkylation of DNA (Nishiyama *et al.*, 1997). Chen and Seviour (2007) and Tohamy *et al.* (2003) studied the mitotic index and Mn frequency after administration of

chemotherapy, their results demonstrated that chemotherapy is a strong chemical agents that can bind and chelate DNA causing its replication inhibition through chromosomal breaks, which resulting in high micronucleus formation through division, while using D-glucan before chemotherapy can offer DNA protection and recorded significant decrease in Mn formation.

The aim of the present study was to investigate chemo-protective and radio-protective effects of D-glucan.

Materials and Methods

Chemicals

D-glucan was purchased from the Vitamin Shoppe Co., North Bergen, USA. MMC, an anticancer drug, isolated from *Streptomyces Caespitosus*, was purchased from Kyowa Hakko Kogyo CO. LTD. Tokyo, Japan.

Radiation source

Caesium-137 unit belonging to NCRRT was used, Atomic Energy Authority, Cairo. ^{137}Cs source offers a dose rate of 0.48 Gy/ min at the time of experiment.

Experimental animals

Ninety six adult male albino rats (120± 10 g) Sprague Dawley strain were obtained from the animal farm of the Egyptian Holding Company for Biological Products and Vaccines (VACSERA), Cairo, Egypt.

Experimental design

The animals were randomly distributed into 8 equal groups, 12 rats for each. Then it categorized as follows: G1: Animals of this group neither received treatments nor exposed to radiation processing. G2: D-glucan treated group, each animal received the proper dose of D-glucan (20 mg/ kg body wt) dissolved in distilled water by gastric intubation's using a plastic syringe with special stainless steel needle as described by Tohamy *et al.* (2003). G3: MMC treated group, rats received intra peritoneal (i.p.) MMC (1.5 mg/ kg body wt) suspended in saline as described by Usui *et al.* (1994). The administration plan was done on 8th, 10th and 12th days, respectively. G4: γ-irradiated group, rats were exposed to fractionated whole body γ-irradiation, delivered as 2 Gy per session, up to total dose of 6 Gy. The irradiation schedule was done at the 8th,

10^{th} and the 12^{th} days, respectively. G5: D-glucan+ MMC group, animals were received D-glucan as described in G2 and additionally they received MMC as described in G3. G6: D-glucan+ γ-irradiated group, animals were received D-glucan as described in G2 as well as they subjected to whole body γ-irradiation as described in G4. G7: MMC+ γ-irradiated group, rats were subjected to whole body γ-irradiation as described in G4 consistent with MMC treatments as described in G3. G8: D-glucan+ MMC+ γ-irradiated group, rats were received D-glucan as described in G2, meantime, they subjected to both MMC treatment and γ-irradiation exposure as described in G3 and G4, respectively.

Collection of samples

Experimental observations were performed at two different time intervals. The first time interval was done three days after ceasing of MMC & γ- irradiation processing. While the second time interval was done ten days after termination of all kind of treatments. Six rats from different animal groups were sacrificed at each time interval. Blood and femoral bone marrow samples were obtained from the experimental animals following normal laboratory procedures and stored at -20 ^{o}C until used.

Determination of DNA fragmentation in blood lymphocytes

DNA fragmentation in blood lymphocytes was determined according to the method described by Sellins and Cohen (1987) which based on the concept that extensively fragmented double-strand DNA can be separated from chromosomic DNA upon centrifugal sedimentation.

Measurement of Mn Frequency

The frequency of micronucleated erythrocytes was evaluated by a technique developed by Schmid (1976) and Feng *et al.* (2000). Percentage of Mn frequency was calculated as follows:

Mn %= (number of cells containing Mn/ total number of cells counted) x 1000.

Statistical analysis

The data obtained in the present work are represented in tables as mean± standard error. Statistical analysis was carried out using two ways analysis of variance (ANOVA) for testing the significance between various treated groups in different time intervals according to Harnett and Horrell (1998). In the

figures, all the treated groups were compared with the control group and the differences were considered significant at $p< 0.05$.

Results

DNA fragmentation in lymphocytes

Fig. 1, 2. represent black and white photograph of agarose gel, stained with ethidium bromide, under UV-irradiation at day 3 and 10, respectively. Track M represent the marker starting with 400 base pair (bp) and ends with 50 bp. Control group in track 1 showed normal DNA with no fragmentation or damage recorded as the DNA stayed in the top of the gel, same results obtained in track 2 (D-glucan treated group).

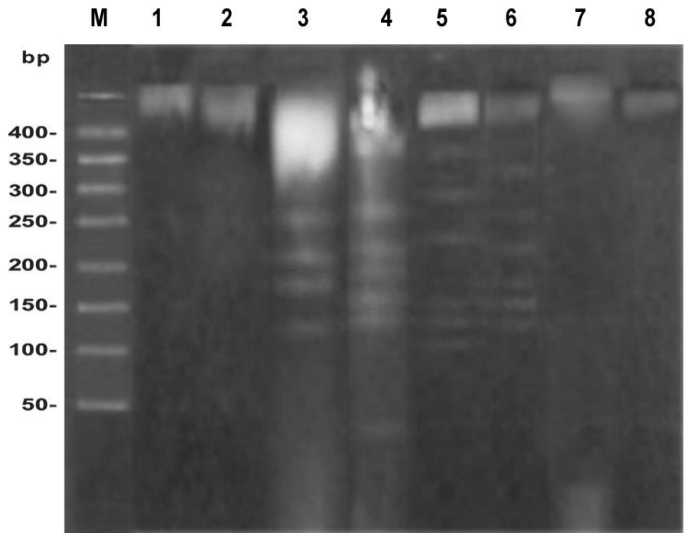

Fig. 1. Effect of D-glucan treatment on DNA fragmentation of lymphocytes in rats subjected to MMC and/ or γ-irradiation at days 3 after radiation exposure.

The groups of rats receiving MMC at track 3 revealed undoubtedly apoptosis measured by DNA-fragmentation, as small fragments travelling farthest from the top to the bottom of the gel at days 3 and 10. Same results obtained in groups of animal exposed to γ-irradiation (track 4) showed complete degradation and damage of the DNA as small fragments travelled farthest on the gel. The treatment with D-glucan before MMC and/or irradiation represented in track 5, 6 were able to reduce the MMC-induced apoptosis and protect DNA from damaged. Track 7 in Fig. 1, 2. represent DNA fragments

from group of rats treated with both MMC and then exposed to γ-irradiation at 3rd and 10th day post-irradiation. This track showed little amount of DNA obtained due to depletion of white blood cells (WBCs) and lymphocytes as a result of combination treatments. On the other hand, track 8 represent quite amelioration in DNA fragmentation recorded after D-glucan administration.

Black and white photograph of an agarose gel, stained with ethidium bromide, under UV-irradiation (Track M): represents the marker. (Track 1): control, (Track 2): D-glucan treated group (Track 3): MMC treated group (Track 4): γ-ray irradiated group (Track 5): groups treated with D-glucan before MMC (Track 6): groups of animal treated with D-glucan before γ-irradiated (Track 7): groups of animal exposed to both MMC then irradiation (Track 8): groups of animal treated with D-glucan before exposure to both MMC and irradiation. Samples that have migrated farthest (tracks 3, 4 & 7) are made up of smaller fragmented DNA than those that have remained near the top of the gel (tracks 1, 2, 5, 6 & 8) at days 3 post irradiation.

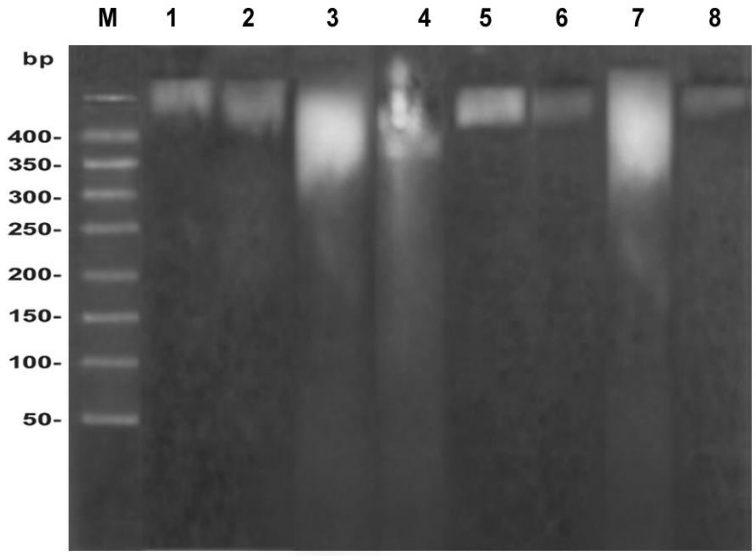

Fig. 2. Black and white photograph of an agarose gel. DNA fragmentation of rats treated with D-glucan before subjected to MMC and/ or irradiation at day 10.

As in Fig. 1. the tracks represented as a following: M represents DNA fragmentation marker and tracks (1-8) represented the tested groups. According to densitometry analysis system, M marker ladder represented by curve with 9

peaks corresponding to 9 bands of DNA fragments ranging from 400, 350, 300, 250, 200, 150, 100, 50 and 20 bp. While, it showed one peak curve in track 1 and 2 which mean no fragmentation corresponding to control and glucan treated group. On the other hand, track 3, 4 and 7 corresponding to MMC, irradiation and combination between both of them respectively. Each track represented with curves recorded several peaks indicating defined damage occurred in DNA. The more pronounced effect found in track 7 (MMC+ Irradiation) as the fragmentation increased. While D-glucan treatment before MMC and/ or irradiation showed significant improvement at day 10 more than day 3.

Bone marrow micronuclei frequency

Tables 1, 2. represent the effect of D-glucan treatment on Mn frequencies of rats subjected to MMC and/ or radiation exposure at the 3 & 10 days post irradiation.

Table. 1. Frequency of Mn in rats treated with D-glucan and subjected to MMC and/ or radiation exposure at day 3 post irradiation.

Groups	Mn Frequency at day 3						Mean± S.E
G1	16/5722 2.8%	14/5635 2.48%	9/4900 1.84%	10/5200 1.92%	12/5400 2.22%	11/4920 2.24%	2.25± 0.15
G2	19/3072 6.18%	13/3300 3.94%	12/2900 4.14%	14/4100 3.41%	15/3200 4.69%	11/3000 3.6%	**(a)** 4.33± 0.41
G3	31/3068 10.10%	19/2100 9.05%	27/2500 9.31%	23/2600 8.08%	34/4000 8.5%	38/3200 11.88%	**(a,b)** 9.49± 0.56
G4	66/4280 15.42%	60/4400 13.64%	50/3000 16.67%	55/3400 16.18%	61/4100 14.88%	59/4000 14.75%	**(a,b,c)** 15.29± 0.44
G5	24/3055 7.85%	25/3400 7.35%	26/3000 8.66%	19/2800 6.79%	29/3600 8.06%	21/3000 7.00%	**(a,b,c,d)** 7.62± 0.61
G6	31/3275 9.47%	29/3300 8.79%	22/2800 10.3%	28/3100 9.03%	29/2800 10.30%	34/3820 8.90%	**(a,b,d,e)** 9.40± 0.28
G7	53/3152 16.81%	49/2600 18.85%	50/3400 14.70%	45/3000 15.00%	39/2400 16.25%	57/2900 19.60%	**(a,b,c,e,f)** 16.87± 0.81
G8	29/2489 11.65%	34/3200 10.63%	24/2190 10.96%	34/2600 13.06%	21/2500 8.40%	35/2600 13.46%	**(a,b,c,d,e,f,g)** 11.36± 0.75

(a) Significant difference from control group.

(b) Significant difference from D-glucan group.

(c) Significant difference from MMC group.

(d) Significant difference from irradiated group.

(e) Significant difference from D-glucan & MMC group

(f) Significant difference from D-glucan & irradiated group.

(g) Significant difference from MMC & irradiated group.

The data in Table 1. revealed that, groups of rats exposed to ionizing radiation (G4) or MMC (G3) and both of them (G7), exhibited significant increase in formation of Mn comparing with control (G1). Irradiated animals (G4) recorded higher frequencies at day 3 after radiation exposure with (15.3%) comparing with control. While groups of animals subjected to MMC alone (G3) recorded (9.5 %) and animals exposed to both MMC+ irradiation (G7) recorded the most manifested increase in Mn frequencies on day 3 with value (16.87 %) comparing with control.

D-glucan treatment before MMC (G5), before irradiation (G6) and before MMC and radiation exposure (G8) showed noticeable amelioration in their Mn frequencies comparing with corresponding groups, irradiated, MMC and combined represented in groups (G3, G4 & G7) at day 3 post irradiation. More improvement was recorded at day 10 post irradiation. Groups of rats treated with D-glucan before MMC and/or irradiation recorded considerable decrease in MN frequency with value (6.3, 10.13 and 9.93) for G5, G6 and G8 corresponding to D-glucan before MMC, D-glucan before irradiation and D-glucan before combination.

Table 2. Frequency of Mn in rats treated with D-glucan and subjected to MMC and/ or radiation exposure at day 10 post radiation.

Groups	Mn Frequency at day 10						Mean± S.E
G1	14/5600 2.50%	7/4100 1.71%	10/5000 2.00%	9/4850 1.92%	13/5000 2.60%	10/4800 2.08%	2.13± 0.14
G2	12/3187 3.77%	20/4050 4.94%	14/3684 3.80%	11/3290 3.34%	16/4090 3.91%	10/2600 3.85%	**(a)** 3.94± 0.22
G3	40/4900 8.16%	38/4500 8.44%	31/3990 7.77%	42/4800 8.75%	29/3000 9.67%	33/2900 11.38%	**(a,b)** 9.03± 0.54
G4	12/1017 11.7%	60/4060 14.78%	54/3100 17.42%	67/4700 14.26%	51/3900 13.08%	50/4000 12.50%	**(a,b,c)** 13.96± 0.83
G5	34/5186 6.56.%	31/5000 6.20%	29/4800 6.04%	22/4050 5.43%	34/4980 6.83%	30/4500 6.67%	**(a,b,c,d)** 6.29± 0.21
G6	29/3229 8.98%	32/3050 10.49%	38/3000 12.67%	30/2940 10.20%	25/2800 8.93%	19/2000 9.50%	**(a,b,d,e)** 10.13± 0.57
G7	26/2808 9.26%	50/3000 16.67%	30/2000 15.00%	39/3000 13.00%	42/2900 14.48%	24/1900 12.63%	**(a,b,c,e,f)** 13.51± 1.04
G8	29/3254 8.91%	32/3050 10.49%	30/3000 10.00%	22/2500 8.80%	27/2900 9.31%	29/2400 12.08%	**(a,b,d,g)** 9.93± 0.51

Legends as in Table 1.

Discussion

MMC cross-links complementary strands of DNA and induces monofunctional alkylation, with attachment to a single DNA strand (Tomasz and Palom 1997). MMC primarily acts as a DNA replication inhibitor, and although monofunctional alkylation is by far the most frequently observed interaction, DNA inter-strand cross-linking is considered to be the most lethal type. Previously two mechanisms were explained by which MMC is chelating DNA through cross-linking and alkylation which require chemical or enzymatic reduction of the quinine function. The primary mechanism of this process involves the C-1 aziridine and the C-10 carbamate groups. Second mechanism is acidic activation of MMC by which DNA alkylation can be produced (Cummings *et al.,* 1995). In this respect the nature of MMC cytotoxicity was described as ability to induce apoptosis through sequences of signals followed DNA alkylation (Crowston *et al.,* 2002 and Kim *et al.,* 2003). Same effect obtained with ionizing radiation which is toxic to living cells because it induces deleterious structural changes in essential biomolecules. A significant part of the initial damage done to cells by ionizing radiation is due to formation of hydroxyl radicals, which reacts with almost all cellular components to induce oxidative damage to DNA, lipid and protein (Sterpone and Renata 2010 and Bertollo, 2010). Moreover about 80% of the total damage to DNA caused by ionizing radiations may result from radiation-induced water-derivative free radicals and secondary carbon –based radicals (Von Sonntage, 1987).

Several studies suggested that certain carbohydrate polymers, such as D-glucan may possess free radical scavenging activity, Slamenova *et al.* (2003). They evaluated the scavenging capacity of D-glucan, and investigated their protective effects against oxidative DNA damage induced by oxidative species which can be generated within the cells: hydroxyl radical (OH·) and non-radical singlet oxygen ($^1O_2$). Finally they contributed the protective action of D-glucan to its free radical scavenging capacity. The most sensitive index of nuclear damage was the micronucleus assay (Xue *et al.,* 1992). Testing chemicals for the ability to induce numerical or structural chromosomal damage is easily accomplished by using the micronucleus assay. In the present study, intra peritonium mitomycin injection induced a significant increase in the micronucleus formation, moreover group of rats exposed to irradiation recorded

more damage than mitomycin injected group as irradiation represents a physical damage to DNA which consider a strong and fixed damage. The most drastic results obtained in group received MMC then exposed to irradiation (combined treatment). These results are in accordance with Tomasz and Palom (1997). In addition, the induction of MN from DNA strand breaks by MMC may be attributed to its ability to form DNA adduct and inhibit replication and to generate free radicals beside formation of highly reactive oxygen species (ROS) by MMC (Chabner and Longo, 2006). Other opinion attributed the formation of Mn after irradiation could be due to emitted photon energy that breaks chemical bonds and produce free radicals in cells. These radicals enhance the damage to DNA and other macromolecules, which increase the risk of cancer **(**Cherry, 2002).

Chorvatovicova *et al.* (1996 and 1998) and Chorvatovicova and Sandula (1995) carried out a series of experiments concerning the anti-clastogenic effect of D-glucan on animals treated with strong mutagen they confirmed the same level of protection whether injected i.p. or intra venous. Their results recorded significant reduction in Mn frequency in chemotherapy treated mice after D-glucan pre-treatment. Moreover, they evaluated the antimitotic activity of D-glucan by investigating the micronucleus frequency and their data showed high significant mitotic index in groups of animal treated with MMC and/or radiation exposure while significantly improved in animals pre-treated with D-glucan.

The present observations recorded that, groups of rats that received D-glucan showed significant amelioration and inhibition of Mn frequency at day 3, which was more pronounced at day 10 post irradiation. Recent studies confirmed the free radical scavenging activity of D-glucan by spin-trap electron paramagnetic resonance (Kogan *et al.,* 2008). The possibility of D-glucan to offer some protection against the genotoxic and cytotoxic effects of chemotherapy was stated by Tohamy *et al.* (2003).

Conclusion

It is concluded to add foods containing D-glucan such as (yeast- barley- oats- wheat and mushroom) to our diet can be a protector against the hazardous effects produced from environmental oxidative stress. Moreover, it could be used as a prophylactic agent against the damaging effects before starting /or during chemotherapeutic regimen.

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الدور الوقائي الوراثي للـ د-جلوكان ضد الإجهاد التأكسدى المستحدث بالميتوميسين و التعرض لأشعة جاما في ذكور الجرذان البيضاء

محمود محمد أحمد و نادية محمد البيه* و شيرين عبد الوهاب منتصر

قسم بحوث البيولوجيا الإشعاعية ، المركز القومي لبحوث وتكنولوجيا الإشعاع ، ص. ب. ٢٩ مدينة نصر ، و *كلية العلوم- جامعة عين شمس.

يتكون الـ د-جلوكان من سلاسل عديدة السكريات و يستخلص طبيعيا من جدار خلايا الخمائر و لها قدرة عالية على تحفيز الجهاز المناعي و ذلك لأن لها مستقبلات على جدران خلايا المناعة الطبيعية مثل المايكروفاج و النيتروفيل و الخلايا الطبيعية المقاتلة.

و كذلك يمتلك كفاءة عالية مضادة للأكسدة و يقدر على اصطياد الشقائق الشاردة.

الهدف من الدراسة تقييم الـ د-جلوكان كواقي من التعرض الكيميائي و الإشعاعى.

تم حقن الجرذان عن طريق الفم بجرعة من الـ د-جلوكان (٢٠ مللى جرام/ كجم) يوميا لمدة ثلاثة أسابيع، و يحقن الميتوميسن بثلاثة جرعات متساوية (١,٥ مللى جرام/ كجم) يوم بعد يوم في الأسبوع الثاني قبل التعرض للإشعاع على ثلاثة جرعات (٢ جراى) يوم بعد يوم في نفس الأسبوع.

تم إجراء القياسات في اليوم الثالث و العاشر بعد التعرض للتشيع و للميتوميسين و ذلك بإجراء اختبار النويات في النخاع العظم و قياس مقدار التكسير في الدنا في خلايا الدم الليمفاوية.

تشير النتائج إلى حدوث تكسير واضح في المادة الو راثية (دنا) بالتعرض للميتوميسين مع أو بدون التعرض للإشعاع المؤين.

وكذلك تم ملاحظة تحسن احصائى معنوي في النتائج لكلا من تكسير الدنا و عدد النويات في اليوم الثالث ويزداد هذا التحسن في اليوم العاشر بعد التعرض.

هذه الدراسة تهدف إلى إثبات أن الـ د-جلوكان يمكن استخدامه كواقي ضد الإجهاد التأكسدى الناتج من التعرض إلى أشعة جاما مع أو بدون التعرض للميتوميسين. لذلك ينصح باستخدامه لتقليل المخاطر الو راثية و الأعراض الجانبية خلال الاستخدام العلاجي للعقاقير المضادة للأمراض السرطانية.