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In vitro Radio Protective Effects of Metformin on Human Lymphocytes Irradiated with Gamma Rays

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Abstract

Radio protective effects of metformin and its ability to alter the spontaneous and induced genotoxic and cytotoxic levels effects on human peripheral blood lymphocytes were investigated in this study. Metformin, a hypoglycemic oral drug, is a biguanide derived from *Galega officinalis* that is widely utilized in controlling type 2 diabetes mellitus. Whole blood samples from 10 healthy donors (5 males and 5 females) were exposed to two doses of gamma-rays (1 and 2 Gray). Lymphocytes in cultures were treated with metformin (10 and 50 μ M) before gamma-irradiation. Cytokinesis-block micronucleus test was used to evaluate the protective effects of metformin on radiation induced genomic damage, cytostasis and cytotoxicity, via scoring micronuclei and nucleoplasmic bridges in once divided binucleated cells as well as counting nuclear division index. The results of the current study revealed that the increase in micronuclei and nucleoplasmic bridges rate is associated with the decrease of nuclear division index in human lymphocytes exposed to gamma radiation in a dose dependent manner. Metformin effectively decreased the rate of spontaneous micronuclei and nucleoplasmic bridges paralleled with the control as well as increased nuclear division index. Moreover, treatment of whole blood samples with metformin (10 and 50 μ M), 2 h preceding to irradiation, remarkably reduced rate of micronuclei and nucleoplasmic bridges accompanied with an increase in nuclear division index rate. The results introduced metformin to be an effective radio protector against DNA damage induced by gamma radiation in human lymphocytes and that it can be used to develop radio protective materials for protection cells of cancer patients from the genomic damage prompted via radiotherapy.

Keywords: gamma ray, metformin, cytokinesis-block micronucleus, lymphocytes

التأثير الوقائي لعقار على الخلايا اللمفاوية المشععة بأشعة كاما في المختبر metformin

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الخلاصة

تم الكشف عن التأثير الوقائي للإشعاعي لعقار metformin وقدرته على تحويل مستوى التأثيرات السامة للجينات الخلايا في الخلايا الليمفاوية في الدم المحيطي البشري. عقار الميتفورمين (metformin) ، دواء يؤخذ عن طريق الفم لخفض السكر في الدم ، وهو مركب biguanid مشتق من *Galega officinalis* يستخدم على نطاق واسع في علاج داء السكري من النوع الثاني. عرضت عينات الدم المأخوذة من 10 متبرعين أصحاء (5 ذكور و 5 إناث) لجرعتين من أشعة كاما (1 و 2 جراي). تم إضافة عقار الميتفورمين (10 و 50 مايكرومولر) للخلايا الليمفاوية قبل التعرض لأشعة كاما. تم استخدام اختبار النواة الصغيرة لتقييم التأثيرات الوقائية للميتفورمين على نسبة الضرر الجيني الناتج عن الإشعاع والسمية الخلوية. عن طريق حساب النوى الصغيرة (MN) micronuclei والجسور النووية (NPB) nucleoplasmic bridges في الخلايا ثنائية النوى بالإضافة إلى حساب مؤشر الانقسام النووي (Nuclear division index) (NDI). أظهرت نتائج الدراسة الحالية أن زيادة معدل النوى الصغيرة والجسور النووية MN و NPB متلازم مع انخفاض مؤشر الانقسام النووي NDI في الخلايا الليمفاوية المعرضة للأشعة كاما وبزيادة الجرعة. عقار الميتفورمين خفض بشكل فعال من معدل النوى الصغيرة والجسور النووية MN و NPB ، بالإضافة إلى زيادة مؤشر الانقسام النووي علاوة على ذلك ، انخفضت نسبة النوى الدقيقة والجسور النووية المصاحبة مع زيادة معدل مؤشر الانقسام النووي في عينة الدم المعاملة بالميتفورمين (10 و 50 مايكرو مولر) لفترة ساعتين قبل التشعيع. أظهرت هذه النتائج أن الميتفورمين يمتلك تأثير وقائي ضد تلف الحمض النووي الناتج عن أشعة كاما في الخلايا الليمفاوية البشرية مما يعزز امكانية استخدامه لتطوير مواد واقية من الإشعاع لحماية خلايا مرضى السرطان من التلف الجيني الناتج عن العلاج الإشعاعي.

1. Introduction

We are always exposed to radiation from several sources, particularly natural sources representing eighty percent of exposure while 20 percent is man made from artificial sources generally from radiation implementation used in medicines [1].

Since the discovery of radiation, lot of research has introduced large information on the effects on health following radiation exposure. According to their occurrence, health effects are classified as early and delayed health effects. Early health effects are evident via diagnosis of clinical syndromes in subjects, which are caused by extensive cell death/damage, such as skin burns, loss of hair and impairment of fertility [2]

In general delayed health effects arise after a long time following exposure. Depending on the radiation dose received these health effects are believed to be initiated through modifications in the genetic material of a cell following the exposure. This has led to an increased frequency in solid tumors and leukemia occurring in the exposed individuals, besides genetic alteration occurring in their offspring [3]. The random energy deposition produced via ionizing radiation causes a wide range of DNA lesions. Ionizing radiation induces damage in the genetic material by direct ionization [4, 5] or through production of hydroxyl radicals that damage the DNA. Hydroxyl-radical attack leads to single-strand breaks (SSBs), double-strand breaks (DSBs), as well as oxidative damage to sugar and base residues, which subsequently can be processed to strand breaks. The aneugenic consequence of ionizing radiation was first documented in 1975 after treating the peripheral blood lymphocytes with gamma-irradiation of 137 Cs at 50 rad [6]. In 1949 Patt *et al.* demonstrated the efficiency of cysteine in the improved resistance of the rats to ionizing radiation [7]. Since then many research have been made to decrease radiation-induced damage by the use of radio protective agents. In view of the fact that radiation-induced cellular damage results primarily because of the impacts of free radicals, It is reasonable to suppose that agents have radical scavenging feature are mostly promising as radio protectors.

A previous research, using different systems, observed that antioxidant capacity dramatically altered in diabetic patients, but less in metformin treated patients [8]. Patrice *et al.* [9] demonstrated that metformin can increase antioxidant protection through a decrease in albumin oxidation.

Metformin is a hypoglycemic oral drug that is a biguanide derived from *Galega officinalis* and is considered a predominant drug recommended in treating type 2 diabetes mellitus. It does not have a direct effect on pancreatic Beta cells and insulin secretion. It modifies plasma glucose through a decrease synthesis of glucose, reduces glucose intestinal uptake besides rise in insulin sensitivity through increased absorption and consumption of peripheral glucose [10]. Furthermore, metformin is recommended in the management of polycystic ovary syndrome and as an assistant therapy for cancer [11].

The cytokinesis block micronucleus cytome (CBMN Cyt) assay in peripheral blood lymphocytes represents one of the reliable techniques with the demanded features of specificity, sensitivity as well as accuracy to be an efficient biological dosimeter of exposure to ionize radiation and genetic susceptibility [12]. Moreover, it has also been utilized to recognize radio protective agents and to verify the effects of nutritional status on susceptibility of the genome-damaging outcome of ionizing radiation [13].

In fact, historically early cytokinesis-block micronucleus (CBMN) assay was limited to evaluating micronucleus (MN) occurrence in binucleated cells but has later grown into a comprehensive “cytome” system for assessing DNA damage, cytostasis and cytotoxicity. Genomic damage scored specially in once divided binucleated (BN) cells which consist of micronuclei (MN), denote a biomarker of chromosome breakage and/or whole chromosome loss, besides nucleoplasmic bridges (NPBs) that signify mis-repair of DNA and/or telomere to telomere end fusions. In addition, nuclear buds (NBUDs) are considered a biomarker of amplified DNA and DNA repair complexes. Cytostatic effects are evaluated through the amount of mono-, bi- and multinucleated cells and cytotoxicity by necrotic and/or apoptotic cell ratios [14]. The aim of current study was to assess the radio protective effects of metformin in peripheral blood human lymphocytes using CBMN Cyt assay.

Materials and Methods

This study was approved by the Ethics Committee of the College of Science, Mustansiriyah University. Peripheral venous blood samples were freshly collected from 10 healthy donors (5 males and 5 females) with ages ranging between 25-35 years. All participants did not consume alcohol and were non-smokers without prior exposure to ionizing radiation for least 3 months.

Soon after blood sample collection, metformin (10 and 50 μM) or metformin solvent (sterile doubled distilled water) were added to each tube and incubated at 37°C. Subsequently, after 2 hours of incubation, samples were irradiated with 1 or 2 Gray of gamma radiation (Gamma chamber 900 Birt India). Then the blood samples were incubated at 37°C for 1 h following irradiation to enable DNA repair. Approximately one half milliliter of each blood sample was mixed with complete culture medium (4.5 ml) (lymphoprime RPMI1640, Capricorn, Germany). The samples were kept in an incubator at 37°C for 72 h .

Blood samples were distributed into groups; as describe below:

Group one (Control): Blood sample was treated with sterile doubled distilled water.

Group two (10 μM Metformin): 10 μM . of metformin was added to blood sample.

Group three (50 μ M Metformin): 50 μ M. of metformin was added to blood sample.
.Group four (Radiation only): Metformin solvent was added to blood sample for 2 h formerly exposure to gamma radiation (2 or 1 Gray)

Group five (10 μ M metformin + Radiation): 10 μ M. of metformin was added to blood sample for 2 h formerly exposure to gamma radiation (2 or 1 Gray).

Group six (50 μ M metformin + Radiation): 50 μ M of metformin was added to blood sample for 2 h before exposure to 2 or 1 Gray [15].

According to the standard protocol described by Fenech [16], after 44h of culture initiation, 6 μ g/mL of cytochalasin-B was added to the cultures and then the cells were collected at 72 h. Cells were mixed with hypotonic solution (KCl, 0.075 M) for 1-3 minutes, subsequently fixing the cells in a solution (3:1 v/v methanol: glacial acetic acid). Samples were stained in 5% Giemsa solution before preparing the slides with air drying technique. Later cells were scored in line with the criteria described by Fenech *et al.* [17].

Slides were analyzed by light microscope under 40 \times and 100x magnifications. One thousands of BN cells were scored for each participants. NPBs and MN were counted in addition. The occurrences of cells with mono-; bi- and multi-nuclear cells were scored to detect cytostatic effects as well as the proportion of multiplying lymphocytes followed different treatments (gamma radiation in the presence and absence of two concentrations of metformin), that can be employed to estimate the nuclear division index (NDI). The NDI was calculated according to the following formulae: $NDI = (M1 + 2 \times M2 + 3 \times M3 + 4 \times M4)/N$, where M1–M4 denote the number of cells with one to four nuclei and N is the total of cells scored.

Statistical Analysis

Statistical analysis was performed with SPSS software (IBM SPSS version 28.0). Data was expressed as mean \pm SE and the difference between groups was assessed using T–test and ANOVA table (Duncan test) [18]. The results were deemed significant if the *P* value was ≤ 0.05 .

Results

The data obtained is summarized in Figures 1, 2 and 3, and Tables 1 and 2. The mean spontaneous MN and NPBs in binucleated cells were found to be 6.40 ± 0.60 and 20 ± 1.06 /1000 binucleated cells respectively. The rate of MN occurrence in binucleated cells largely increased to 29.20 ± 4.59 and 221.0 ± 21.67 after irradiation with 1 and 2 Gray respectively. There was a significant difference between the control and samples exposed to gamma radiation regarding frequency of MN/1000BN cells ($P \leq 0.001$). Also the number of NPBs /1000BN cells increased intensely after gamma-irradiation. This elevation in number of NPBs increased over 10-folds in 2 Gray when compared with the control sample ($P \leq 0.001$).

Blood cells exposure to gamma radiation resulted in reduction of NDI and this value is significantly different in the 2 Gray-irradiated samples when paralleled to the control (untreated samples), 1.18 ± 0.02 and 1.10 ± 0.02 ($P \leq 0.001$) (Figure 3).

Metformin did not produce significant genotoxic effects in lymphocytes cells at 10 and 50 μ M concentrations after 72 h incubation when compared to control, but it significantly increased NDI at 50 μ M concentration ($P \leq 0.001$), as shown in Table 1.

Prior to 2 hours radiation, treatment cells with 10 and 50 μM metformin exhibited a reduction in MN and NPBs number in BN cells when paralleled to radiation-alone group and this decrease in MN score was significant in samples processed with metformin (50 μM) ($P \leq 0.001$), as shown in Table 2. While there were significant differences regarding frequency of NPBs in samples treated with 10 as well as 50 μM prior to irradiation with 2 Gray. Moreover, the NDI values increased to 1.16 ± 0.02 ; 1.18 ± 0.01 ; 1.14 ± 0.01 and 1.18 ± 0.02 in the samples treated with 10 and 50 μM , prior to their exposure to 1 and 2 Gray in that order, paralleled to samples irradiated without treated with metformin 1.13 ± 0.01 ; 1.10 ± 0.02 for 1 and 2 Gray respectively. The results of metformin, regarding the radiation, initiate reduction in NDI effectively improved with increased concentration of metformin from 10 to 50 μM ($P \leq 0.001$) (Tables 1 and 2).

Table 1: The mean \pm S.E of MN; NPBs and NDI in human lymphocytes after different treatments (metformin solvent; 10 and 50 μM metformin; 1 Gray of gamma radiation in presence and absence of metformin)

Parameter	Control	10 μM	50 μM	1 Gray	10 μM Metformin +1 Gray	50 μM Metformin +1 Gray
MN	6.40 $\pm 0.60^a$	4.33 ± 0.6 7 ^a	3.36 ± 0.5 1 ^a	29.20 $\pm 4.$ 59 ^b	22.33 $\pm 1.96^b$	14.67 $\pm 2.$ ^{47c}
NPBs	4.20 ± 1.0 6 ^a	6.22 ± 1.0 6 ^a	2.25 ± 0.4 8 ^a	5.0 $\pm 1.0^a$	3.60 $\pm 1.69^a$	2.50 $\pm 0.50^b$
NDI	1.18 ± 0.0 2 ^a	1.20 ± 0.0 1 ^a	1.23 ± 0.0 1 ^a	1.13 ± 0.0 1 ^a	1.16 $\pm 0.02^a$	1.18 $\pm 0.01^a$

*Different letter indicating significance of differences

MN: micronuclei; NPBs: nucleoplasmic bridges and NDI: nuclear division index.

Table 2: The mean \pm SE of MN, NPBs and NDI in human lymphocytes after different treatments (metformin solvent; 10 and 50 μM metformin, 2 Gray of gamma radiation in the presence and absence of metformin)

Parameters	Control	10 μM Metformin	50 μM Metformin	2 Gray	10 μM Metformin + 2 Gray	50 μM Metformin + 2 Gray
MN	6.40 $\pm 0.60^a$	4.33 $\pm 0.67^a$	3.36 $\pm 0.51^a$	221.0 $\pm 21.$ 67 ^b	190.67 $\pm 26.72^b$	157.50 ± 30.6 0 ^c
NPBs	4.20 ± 1.0 6 ^a	6.22 $\pm 1.06^a$	2.25 $\pm 0.48^a$	61.83 ± 5.8 4 ^b	35.17 $\pm 4.91^c$	23.0 $\pm 6.63^d$
NDI	1.18 ± 0.0 2 ^a	1.20 $\pm 0.01^a$	1.23 $\pm 0.01^b$	1.10 ± 0.02 c	1.14 $\pm 0.01^a$	1.18 $\pm 0.02^a$

*Different letter indicating significant differences.

MN: micronuclei; NPBs: nucleoplasmic bridges and NDI: nuclear division index.

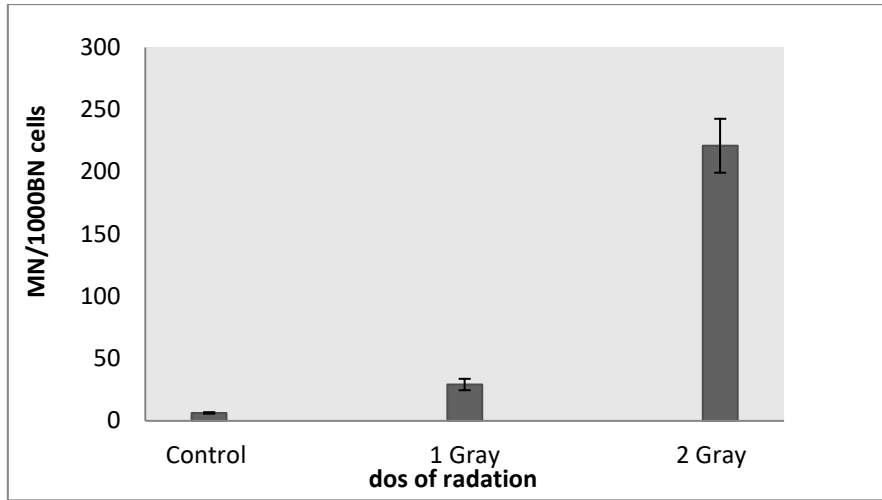


Figure 1: Effects of gamma ray (1 and 2 Gray) on frequency of MN in BN cells. Bars indicate means \pm SE.

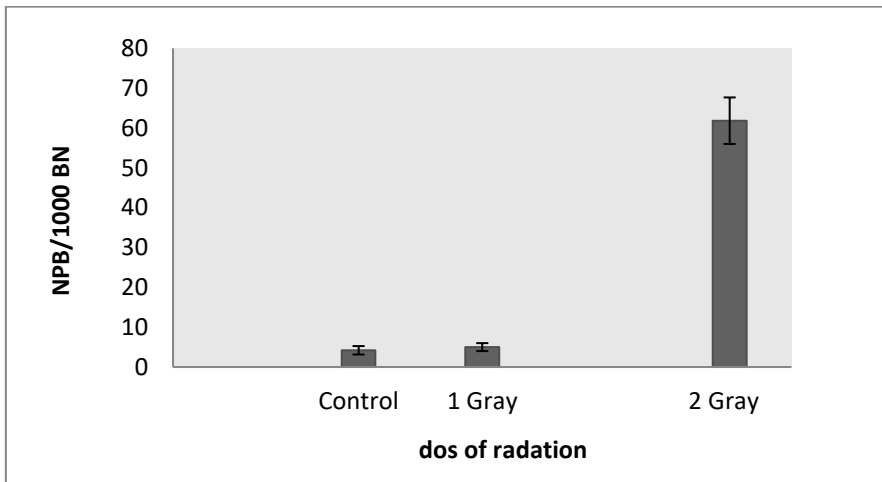


Figure 2: Effects of gamma ray (1 and 2 Gray) on frequency of NPB in BN cells. Bars indicate means \pm SE.

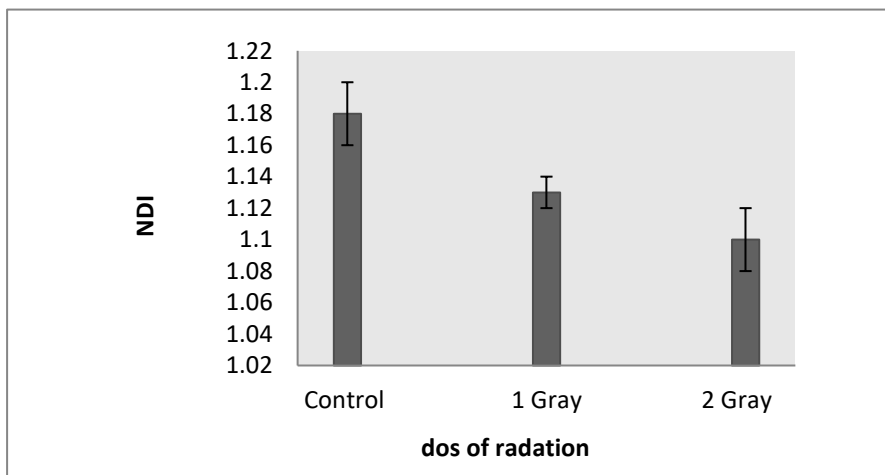


Figure 3: Effects of gamma ray (1 and 2 Gray) on NDI of lymphocytes cells. Bars indicate means \pm SE.

Discussion

The cytome assay can be used for normal cells to determine the intrinsic radio sensitivity of individuals, as well as for monitoring baseline chromosome aberration frequency in unexposed population [19, 20] and those exposed to low-level radiation. The cytome assay was adopted by IAEA (the International Atomic Energy Agency) for ionic radiation biodosimetry and genetic toxicology analysis [13]. This study has revealed that the mean count of MN in sample study (6.40) is substantially in line with the reference value for the general population (6.5 / 1000 BNMN in human population) [21]. The results of present work show an increase in the incidence of MN, NPBs and decrease of NDI in exposed human lymphocytes in a dose dependent manner reflecting genotoxic and cytotoxic of gamma rays doses used in the current study, as shown in Table 1 and 2). This effect has been reported in many other *in vitro* and *in vivo* investigations previously (14). High frequencies of MN were produced by the dose and according to the results of previous studies, the large-sized micronuclei encompass whole chromosomes whereas the small-sized micronuclei include eccentric chromosome fragments (22). Balajee *et al.* [23] used M-FISH technique to examine the chromosome content of MN in lymphocytes generated by several doses of gamma rays. His study concluded that large-sized micronuclei contained materials either from single chromosome or from many chromosomes. They also speculated that the increased frequency of micronuclei with multiple chromosomes at radiation doses exceeding 2 Gray possibly resulted from mis-repair of double strand break of DNA including more than one chromosome, hence resulting in the development and union of eccentric fragments from these chromosomes. Dose-dependent elevation of the score of MN detected in the current study perhaps resulted from amplified mis-repair at high doses of gamma radiation (2 Gray).

In addition, exposure to high dose of ionizing radiation that resulted in the loss of whole or fragments of chromosome harboring important tumor suppressor genes, might initiate chromothripsis, a phenomenon commonly detected in some of cancer cells. These alterations often result in complex chromosome aberrations with changes in numbers of copy of essential genes included in control of cell cycle, repair of DNA besides genomic stability [24].

Furthermore, the results of current study revealed that gamma radiation induced reduction in NDI values. These results suggest that DNA damage induced by ionic radiation mainly initiated apoptotic pathway. According to the results provide by previous studies, radiation-induced apoptosis, not necrosis, via a series reaction of signals led to alteration in expression of more than genes such as p53, Bcl-2 as well as caspase family [25, 26]. Since then many free radical scavengers, in addition to antioxidants, have been detected in order to reduce apoptosis in cells exposed to ionic radiation via alteration in expression of Bcl-2, p53 and Bax genes [27].

The antioxidant activity of metformin detection in many research using different systems, data from several sources has identified that the oxidative stress changed in diabetic patients, but less in metformin treated patients according to another researcher who demonstrated that metformin can alter oxidative stress via increased antioxidant protection by decrease in albumin oxidation [9].

The results of the current study revealed that metformin decreased the rate of spontaneous MN and NPBs associated with increased NDI, thus reducing the spontaneous rate of genotoxic damage, possibly through lowering the oxidative stress [8]. Though, the complete process responsible for this suppressive consequence needs further research. *In vivo* and *in vitro* studies regarding metformin are controversial. While some reports indicated no

genotoxic effects [28], and others [29] have supposed that metformin can produce oxidative stress due to DNA fragmentation. Onaran *et al.* [30] demonstrated that high concentrations of metformin increased cumene hydroperoxide (CumOOH)-induced DNA damage. Moreover, Anedda *et al.* [31] assumed that metformin increased the levels of reactive oxygen species in white adipocytes. The results of present study denote that metformin did not produce a significant genotoxic or cytotoxic effects in lymphocytes cells at 10 and 50 μ M concentrations after 72h incubation, Our results are inconsistent with Cheki *et al.* [15] who showed that treatments lymphocytes with 50 and 10 μ M did not increased MN and NBPs occurrence in lymphocytes. Sant Anna *et al.* [32] also confirmed the non genotoxic activity of metformin using different concentrations of metformin (12.5 - 50.0 μ M). Other research similarly documented the non genotoxic activity of metformin in mice as well as rat bone marrow cells [28, 33]. Conversely, in some other *in vitro* studies using different concentration of metformin demonstrated the genotoxic activity of metformin [34].

The ameliorative role of metformin against genotoxic agents was recognized in previous reports. Mai *et al.* [35] detected the protective role of metformin against genomic damage stimulated by formaldehyde using HepG2 cells. Lee *et al.* [36] detected the protective role of metformin against UV induced damage in human A549 cells, through down regulation of p-chk2, p53 and γ H2AX, also initiating cell cycle arrest and induced DNA repair.

The results of current study demonstrated that the protective effects of metformin on the radiation induced reduction in NDI considerably improved with increased metformin concentration from 10 to 50 μ M (Tables 1 and 2). These results agree with the findings of previous research which concluded that metformin reduced the synthesis of proapoptotic proteins, improved the antiapoptotic proteins and reduced programmed cell death in cardiomyocytes [37]. Furthermore, metformin reduces caspase-9,-6,-3 activations, (ADPribose) polymerase (PARP)-cleavage, besides enhancing programmed cells death through oxidative stress in hepatocytes by initiate of bcl-xl besides suppression of c-Jun N-terminal kinase [38].

The results suggest that metformin drug has radio protector activity against DNA damage induced by gamma radiation in human lymphocytes. Also the present work has extended the knowledge of radio protective materials. Further research that can be used to develop radio protective agents for protecting cells of cancer patients from the genomic damage prompted via radiotherapy, is needed.

Additional experimental investigations are also needed to estimate the alteration in genes expression associated with treating human lymphocytes with metformin that lead to modify genotoxic and cytotoxic impacts of ionizing radiation.

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