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Evaluation of the Protective Role of Vitamin B_{12} on Gamma Radiation Induced Cytotoxicity in Mice

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Abstract. This work was carried out to study the protective role of vitamin B_{12} on the induced cytotoxicity of ionizing radiation as assessed by cytogenetic technique. Vitamin B_{12} (Vit.B_{12}) was injected intramuscularly (i.m.) at a dose of 0.8 mg/kg body weight, 1 hr prior to the exposure of whole body to single dose (2 Gy) gamma irradiation in addition to 2 Gy challenged dose, assessed at 6, 24 and 72 hrs. The frequency of micronuclei (MN) of polychromatic erythrocytes (PCEs), normochromatic erythrocytes (NCEs), the ratio of PCEs/NCEs and mitotic index (MI) of bone marrow cells were evaluated. The results obtained revealed that the administration of Vit.B_{12} pre-irradiation resulted in a significant inhibition in the frequency of radiation induced MN, as well as the ratio of PCEs/NCEs and MI of bone marrow cells. The results also indicated that the dose used of Vit.B_{12} plays a protective role when administered prior to irradiation and assessed at a different times. The best effect on MI was most apparent at 6 and 72 hrs, whereas a 3-fold decrease in the % of MNPCEs was obtained at 72 hrs assay time. It could be concluded that Vit.B_{12} may exert a vital protective role against gamma irradiation.

***Keywords*:** Vitamin B_{12}, Irradiation, Micronuclei, Polychromatic erythrocytes, Normochromatic erythrocytes.

Introduction

A large number of micronutrients (vitamins, essential minerals and other compounds required in small amounts for normal metabolism) are required in the human diet [1]. Folic acid and Vit.B_{12} play an important role in DNA metabolism [2]. Folic acid is required for the synthesis of deoxythymidine monophosphate (dTMP) from deoxyuridine monophosphate (dUMP). Under conditions of folic acid deficiency dUMP accumulates and as a result uracil, instead of thymine, is incorporated into DNA [3]. There is a good evidence suggesting that excessive misincorporation of uracil in DNA not only leads to point mutation but may also result in the generation of single and double-strands DNA breaks, chromosome breakage and micronucleus formation. Folic acid and Vit.B_{12} are also required for the synthesis of methionine and S-adenosyl

methionine, the common methyl donor required for the maintenance of methylation patterns in DNA that determine gene expression and DNA conformation [4]. Deficiencies in folic acid and Vit.B_{12} therefore lead to (i) elevated DNA damage rate and altered methylation of DNA, both of which are important risk factors for cancer, and (ii) an increased level in homocysteine (HC) status considered as an important factor for increased risk of cardiovascular disease [5]. These same defects may also play an important role in inducing developmental and neurological abnormalities. The blood levels of folate and Vit.B_{12} required to prevent anemia and hyperhomocysteinemia are properly determined. However, it is still uncertain whether such accepted levels of sufficiency are in fact adequate to minimize chromosome damage rate and optimize DNA methylation status [6]. A series of studies [7-9] were conducted to investigate the interrelationship between DNA damage in somatic cells and blood status for folate, Vit.B_{12} and HC.

The importance of identifying dietary factors that minimize DNA damage rate is underscored by recent evidence from two epidemiological prospective studies indicating that a reduced level of chromosome damage in lymphocytes is a relevant biomarker of reduced future cancer risk [10, 11]. Most studies have considered the combined action of both folic acid and Vit.B_{12}. The sole effect of Vit.B_{12} has been addressed in few epidemiological studies on men. Preliminary studies in young men [12] indicated that there was a significant negative correlation between the micronucleus frequency in lymphocytes and plasma Vit.B_{12} level. Results from studies in men aged 50-70 years [12] have shown that the micronucleus index correlates negatively with Vit.B_{12} in subjects who are not Vit.B_{12} deficient and that the micronucleus index is significantly and positively correlated with plasma HC status in men who are not folate or Vit.B_{12} deficient. These studies suggested that the plasma levels of HC and Vit.B_{12} that correspond to the minimization of chromosome damage require better definition.

The first study reporting the interrelationship of chromosome damage rate, DNA methylation, HC, folate and Vit.B_{12} status in young Australians was reported by Fenech [13]. No experimental controlled study on the protective role of Vit.B_{12} has been reported. In the present study, the protective effect of the Vit.B_{12} administration before irradiation induced cytotoxicity will be presented.

Material and Methods

Male mice (*Mus musculus domesticus*) of strain SWR/J, each weighing 20-25 gm, were used in this study. The animals were obtained from the Experimental Care Center (ECC) of King Saud University, Riyadh, KSA. The animals were housed in cages and maintained under standard conditions of ventilation, temperature and humidity. The animals were divided into 6 groups, each of 20 mice.

Vit.B_{12} toxicity

Five animals from each group were sacrificed at intervals of 0, 6, 24 and 72 hrs

after the cessation of the treatment. Both femurs were removed and bone marrow was extracted for cytogenetic analysis. Five groups of male mice, each consisted of 10 animals, were used to assess the toxicity of Vit.B_{12}. One group served as control and the other groups each received one of the following doses of Vit.B_{12}: 0.8, 1.6, 2.4 or 3.2 mg/kg body weight injected i.m. daily for three consecutive weeks. The animals were observed for any signs of health deterioration or death and the results were recorded.

Irradiation

Irradiation was carried out using Cobalt-60 source (gamma cell 220-Nordion International Inc., Kanata, Canada) at the Research Center of College of Science, King Saud University, Riyadh, KSA. Each animal received a whole body exposure of 2 Gy and an additional 2 Gy (challenged dose) at a dose rate of of 0.667 Gy/min. Vit.B_{12} was purchased from Sigma and dissolved in saline, and given to animals by intramuscular injection in a concentration of 0.8 mg/kg body weight daily for seven consecutive days.

Combined effect of Vit.B_{12} and radiation on animal survival

Five groups of male mice, each consisted of 10 animals, were used to assess the effect of combined treatment of Vit.B_{12} and gamma irradiation. The first group served as control. The second group was exposed to a single dose whole body 2 Gy gamma irradiation. The third group was injected i.m. with Vit.B_{12} (0.8 mg/kg body weight) one hour prior to 2 Gy irradiation. Group four was exposed to an initial dose of 2 Gy gamma irradiations and another challenging dose of 2 Gy after 6 hours. Group five was exposed to 2 doses of gamma irradiation as in group four except for a pretreatment with Vit.B_{12} (0.8 mg/kg body weight) to the challenging dose of irradiation.

The animals were observed for ill-health symptoms and deaths and the mortality rate was calculated for each group.

Cytogenetic analysis

Bone marrow smears were prepared and allowed to dry overnight. Differential staining to distinguish polychromatic erythrocytes (PCE) and normochromatic erythrocytes (NCE) with May-Gruenwald and Giemsa stains were preformed. The polychromatic erythrocytes (PCEs) stained bluish purple due to their high content of RNA in the cytoplasm. The normochromatic erythrocytes (NCEs) stained orange-yellow and were slightly smaller than PCEs. The micronuclei (MNs) were recognized as deep purple stained bodies in the cytoplasm.

The number of MNs in 500 PCEs were counted for each individual mouse using 100X (oil immersion). The number of NCEs was also counted in the fields of recorded PCEs and scored for MNs and the ratio of PCE/NCE was determined [14-18]. The mitotic index (MI) [19, 20] was also estimated for bone marrow cells under different experimental conditions. A total of 2000 cells were counted per animal from each of the control and treated groups. The number of cells in mitosis from the 2000 cells were also counted. The percentage of M.I. was calculated as follows:

$$MI\% = \frac{\text{Number of cells in division}}{\text{Total number of cells scored}} \times 100$$

Statistical analysis

Student's t-test was applied for the statistical analysis of the cytogenetic results [21].

Results

Toxicity of Vit.B_{12} studies

The results demonstrate that the treatment of mice with Vit.B_{12} at the previous indicated doses 0.8, 1.6, 2.4 or 3.2 mg/kg body weight caused no death during 30 days after the cessation of the treatment. No apparent significant changes in body weight were observed. Also, no morphological changes were noticed during the whole experiment.

Lethality of irradiated mice pretreated with Vit.B_{12}

Figure 1 demonstrates the mortality rate in different animal groups at different time intervals following irradiation. The dose of Vit.B_{12} was equivalent to the human 0.8 mg/kg body weight, and was injected intramuscularly one hour before the irradiation with 2 Gy, in addition to another group pretreated with Vit.B_{12} and irradiated with an additional dose of 2 Gy.

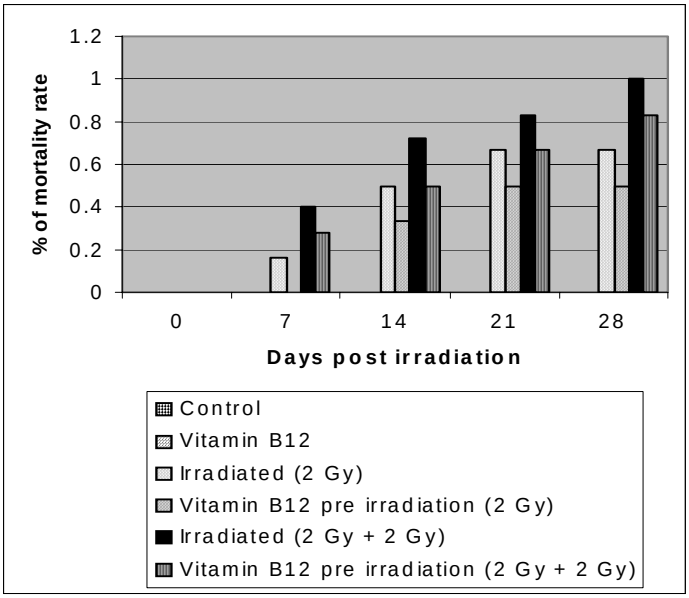

Fig. 1. The mortality rate in different animal groups.

The results demonstrate that 2 Gy irradiation showed a mortality rate of 16.6, 50, 66.6 and 66.6% at 7, 14, 21 and 28 days post irradiation respectively, while the Vit.B_{12} pretreated irradiated group showed a mortality rate of 0, 33.3, 50 and 50%, respectively at 0, 7, 21 and 28 days post irradiation. Additional exposure to 2 Gy challenged dose resulted in an increase in the percentage of mortality to 40, 72.2, 83.3 and 100% at 7, 14, 21 and 28 days, respectively post irradiation, while pretreatment with Vit.B_{12} (0.8 mg/kg b.w.) resulted in a mortality rate of 27.7, 50, 66.6 and 83.3% at the same experimental time respectively, which indicates a lower mortality rate of about 12.3, 22.2, 16.7 and 16.7% at 7, 14, 21 and 28 days, respectively. Morphological observations of the surviving animals in the irradiated groups revealed that all animals showed irritated nose, ears, loss of hairs, swollen feet and lesion tails. Groups pretreated with Vit.B_{12} (0.8 mg/kg b.w.) were normal and did not show any symptoms of sickness.

The results obtained indicated that the optimum radioprotective action of Vit.B_{12} against induced radiation mortality and accompanied morphological changes induced by whole body gamma irradiation was achieved at day 14.

The mitotic index (MI)

Data of the mitotic index of bone marrow cells are presented in Fig. 2.

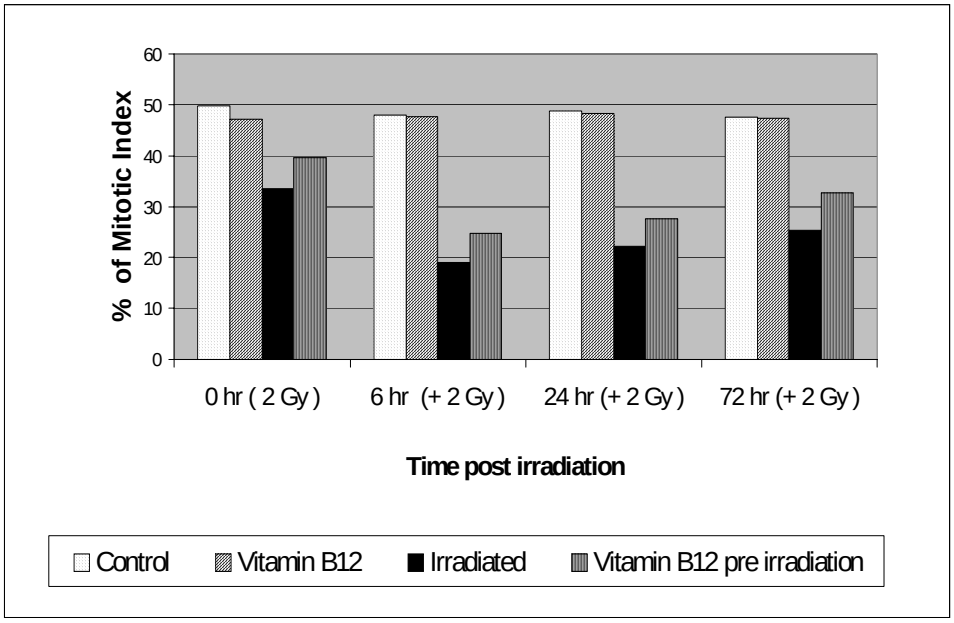

Fig. 2. Changes in mitotic index (M.I.) of bone marrow cells in different experimental groups of mice assessed at 0, 6, 24 and 72 hrs post irradiation.

The control group showed a mitotic index of 49.82% assessed at 0, 6, 24 and 72 hrs, while Vit.B_{12} treated group revealed a mitotic index ranging between 47.22–50%. No significant changes were detected between Vit.B_{12} treated group and the control group at different time intervals.

The frequency of changes in the mitotic index for the irradiated group was 32.9% at 0 hr which indicates a highly significant delay (P_1<0.001) in the MI compared to the control group, while the irradiated group pretreated with Vit.B_{12} showed a highly significant delay (P_2 <0.001) in the mitotic index when compared to Vit.B_{12} treated groups, but less than the effect induced by irradiation alone. Pretreatment of the irradiated group with Vit.B_{12} resulted in a remarkable highly significant recovery 15.7%, (P_3<0.001) when compared to the irradiated group at 0, 6, 24 and 72 hrs.

The additional challenged dose of 2 Gy resulted in a highly significant delay in the mitotic indices compared to the control group at 6, 24 and 72 hrs. Also, irradiated pretreated Vit.B_{12} groups showed a relative recovery of about 22.7, 19.8 and 22.6% at 6, 24 and 72 hrs respectively when compared to the irradiated group.

The micronucleus test

The MNPCEs, MNCEs and PCEs/NCEs data obtained from bone marrow smears are presented in Table 1.

Comparison of the control and Vit.B_{12} treated groups showed no significant differences in the percentage of total MNPCE at different assay times. The irradiated group showed a significant increase in PCEs compared with both control and Vit.B_{12} treated groups. The differences increased steadily to reach 3 folds by 72 hrs. The frequency of PCEs with more than one MN was even higher than 3 folds suggesting that cells with one MN are more liable to more damage than non-affected cells.

Pretreatment of irradiated mice with Vit.B_{12} has resulted in a significant reduction in the percentage of MNPCEs to about 35-45% of those values induced by irradiation alone at 24 and 72 hrs, respectively. The frequency of MNNCEs was greatly increased by exposure to 2 Gy of gamma irradiation and the increase was time dependent as it reached almost 3 folds by 72 hrs. Pretreatment of irradiated groups with Vit.B_{12} caused significantly less damage and resulted in a much lower frequency which reached about 50% of those inflected by radiation alone. The PCE/NCE ratio was similar between the control and Vit.B_{12} treated groups. Irradiation resulted in a significant decrease in PCE relative to NCE. Pretreatment with Vit.B_{12}, resulted in a moderate decrease of PCEs relative to NCE.

Discussion

Assessment of the protective role of Vit.B_{12} has been measured by a number of parameters. Irradiated mice pretreated with doses of Vit.B_{12} showed a better survival

Table 1. Percentages and ratios of MNPCEs in bone marrow cells in different animal groups* assessed at 0, 6, 24 and 72 hrs. post irradiation

Groups of animals	PCEs				NCEs	Ratio PCEs/NCEs
	Total MNPCEs	With 1 MN	With 2 MN	With 3 MN	MNNCEs	
Control						
0 hr	0.155±0.003	0.126±0.004	0.023±0.002	0.006±0.002	0.081±0.007	1.225±0.021
6 hr	0.165±0.007	0.131±0.003	0.029±0.004	0.005±0.002	0.078±0.006	1.246±0.017
24 hr	0.1646±0.008	0.131±0.004	0.026±0.003	0.006±0.006	0.077±0.012	1.253±0.034
72 hr	0.166±0.009	0.132±0.003	0.028±0.004	0.008±0.003	0.071±0.010	1.246±0.030
Vitamin B_{12}						
0 hr	0.150±0.002 P_1>0.05	0.125±0.004 P_1>0.05	0.020±0.003 P_1>0.05	0.005±0.002 P_1>0.05	0.074±0.003 P_1>0.05	1.282±0.013 P_1>0.05
6 hr	0.160±0.006 P_1>0.05	0.130±0.005 P_1>0.05	0.026±0.004 P_1>0.05	0.004±0.003 P_1>0.05	0.069±0.011 P_1>0.05	1.269±0.016 P_1>0.05
24 hr	0.158±0.010 P_1>0.05	0.131±0.005 P_1>0.05	0.023±0.005 P_1>0.05	0.005±0.002 P_1>0.05	0.067±0.006 P_1>0.05	1.286±0.031 P_1>0.05
72 hr	0.162±0.009 P_1>0.05	0.129±0.005 P_1>0.05	0.026±0.005 P_1>0.05	0.008±0.003 P_1>0.05	0.064±0.009 P_1>0.05	1.282±0.013 P_1>0.05
Irradiated						
0 hr	0.199±0.009 P_1<0.001	0.151±0.009 P_1<0.01	0.036±0.003 P_1<0.001	0.0116±0.003 P_1<0.05	0.213±0.011 P_1<0.001	0.773±0.013 P_1<0.001
6 hr	0.248±0.008 P_1<0.001	0.168±0.007 P_1<0.001	0.067±0.008 P_1<0.001	0.013±0.004 P_1<0.01	0.212±0.005 P_1<0.001	0.562±0.006 P_1<0.001
24 hr	0.353±0.010 P_1<0.001	0.235±0.007 P_1<0.001	0.097±0.005 P_1<0.001	0.020±0.004 P_1<0.001	0.208±0.010 P_1<0.001	0.371±0.011 P_1<0.001
72 hr	0.458±0.012 P_1<0.001	0.297±0.010 P_1<0.001	0.123±0.006 P_1<0.001	0.039±0.004 P_1<0.001	0.190±0.006 P_1<0.001	0.358±0.005 P_1<0.001
Vitamin B_{12} pre irradiation						
0 hr	0.165±0.008 P_2<0.001	0.129±0.008 P_2<0.01	0.027±0.005 P_2<0.01	0.009±0.003 P_2>0.05	0.103±0.012 P_2<0.001	1.198±0.055 P_2<0.001
6 hr	0.202±0.004 P_2<0.001	0.148±0.005 P_2<0.001	0.036±0.004 P_2<0.001	0.018±0.003 P_2>0.05	0.104±0.016 P_2<0.001	0.977±0.016 P_2<0.001
24 hr	0.222±0.003 P_2<0.001	0.151±0.003 P_2<0.001	0.047±0.003 P_2<0.001	0.024±0.003 P_2>0.05	0.123±0.009 P_2<0.001	0.730±0.008 P_2<0.001
72 hr	0.250±0.031 P_2<0.001	0.183±0.005 P_2<0.001	0.051±0.002 P_2<0.001	0.029±0.003 P_2<0.001	0.113±0.013 P_2<0.001	0.781±0.017 P_2<0.001

* Each value represents the mean of 5 records ± SD
P_1 the significant of changes from Control value.
P_2 the significant of changes from Irradiated group.
MN micronuclei
NCEs normochromatic erythrocytes
PCEs polychromatic erythrocytes

curve than those exposed to radiation alone with 2 or 2 + 2 Gy (challenged dose). The significant reduction of mortality in response to pretreatment with Vit.B_{12} suggests that the latter has an overall protective role or different vital systems compatible with life. The results suggest that the increased dose of Vit.B_{12} could substantiate the observed protection offered by the tested dose (0.8 mg/kg b.w.). This is in accordance with the contention made by Toohey [6], who questioned the essential dietary dose, which might not be enough to protect against the genetic damage of DNA. This conclusion is also in concordance with the absence of any toxicity of higher doses of Vit.B_{12} tested in this study.

Assessment of radiation hazards can be achieved by measuring MI. Regarding the MI of bone marrow of the irradiated animals, a significant and a considerable decrease in its frequency was observed. This suppression of MI was noted in several studies both *in vitro* and *in vivo* in different animals [22, 23]. In this study, a significant recovery in the MI frequency was noted in the Vit.B_{12} pretreated irradiated group. This recovery in the MI of bone marrow is probably due to the increased rate of proliferation of surviving cells in the bone marrow [24] or to the increased resistance of bone marrow cells to radiation damage. Many studies have shown that ionizing radiation increased the incidence of MN *in vivo* [25, 26]. In the present study, whole body gamma irradiation of mice raised the incidence of MNPCEs very significantly when estimated at 0, 6, 24 and 72 hrs post irradiation. The MNPCE that appeared at 0 hr most likely represents erythroblasts in G2 phase of the cell cycle during irradiation, while the ones that appeared at 24 hrs may be at the G1/S phase of the cell cycle. During the irradiation both hydrogen peroxide and hydroxyl free radicals are found within the cell which can directly or indirectly cause DNA damage [12, 27, 28]. The radioprotective effect of Vit.B_{12} was assessed in this study by estimating the frequency of induced MN in PCEs and NCEs of bone marrow *in vivo*. The protective action of Vit.B_{12} against gamma irradiation depends on the dose and the rate of exposure. Vit.B_{12} plays an important role in DNA metabolism [29] and it is also required for the synthesis of methionine and S-adenosyl methionine, the common methyl donor required for the maintenance of methylation patterns in DNA that determine gene expression and DNA conformation [4]. It has also been reported that Vit.B_{12} plays an important protective role in cervical carcinogenesis [30] and in patients with rheumatoid arthritis [31].

In this study, the administration of Vit.B_{12} did not alter the incidence of MN of PCEs and MCEs. However, the pretreatment of irradiated group with Vit.B_{12} at different time intervals showed a very significant decrease in the frequency of MNNCEs. Similar results [13] were reported, where Vit.B_{12} caused a significant decrease in the MN of NCEs induced by gamma rays [8, 32-34]. The ratio of PCEs to MNCEs decreased significantly at 6 and 72 hrs post-irradiation. This was due to a decline in the number of PCEs relative to NCEs in the bone marrow after irradiation. Pretreatment with Vit.B_{12} prior to irradiation re-established the relative proportion of PCEs/NCEs. Protection of bone marrow tissue by Vit.B_{12} against radiation toxicity is expressed as an increase of

the ratio of PCEs/NCEs [25, 26]. It might be concluded that Vit.B_{12} may exert a vital protective role against gamma irradiation.

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تقييم الدور الوقائي لفيتامين ب١٢ من السمية الخلوية المستحدثة بأشعة جاما في الفئران الصغيرة

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ملخص البحث. تهدف الدراسة إلى معرفة الدور الوقائي لفيتامين ب١٢ على التأثيرات السمية الخلوية للأشعة المؤينة وذلك باستخدام التقنية الخلوية الوراثية. تم حقن فيتامين ب١٢ في العضل بجرعة ٠,٨ مليجرام/كيلوجرام من وزن الجسم وذلك قبل ساعة من التشعيع الكلي للجسم بجرعة ٢ جراي من أشعة جاما بالإضافة إلى جرعة محفزة أخرى (٢ جراي)، ثم تقييمها بعد التشعيع في الأوقات ٦ و ٢٤ و ٧٢ ساعة من الجرعة الأولى مباشرة. تم حساب معدل تكوين النويات الدقيقة (MN) متعددة الصبغيات (PCEs)، وكذلك طبيعية الصبغيات (NCEs) ومعدل النسبة بينهما (PCEs/NCEs) التي أحدثتها التأثيرات السمية الخلوية للإشعاع المؤين ومدى تحسن هذه النسب بواسطة فيتامين ب١٢، كذلك تم حساب معدل الإنقسام الخلوي (MI) لخلايا نخاع العظم.

أوضحت النتائج أن إعطاء فيتامين ب١٢ قبل التشعيع أدى إلى تحسن معدل تكون النويات الدقيقة ونسبة PCEs/NCEs وكذلك معدل الإنقسام الخلوي نتيجة التعرض للإشعاع بقيمة معنوية، كما أوضحت النتائج أن فيتامين ب١٢ بالجرعة ٠,٨ مليجرام/كيلوجرام من وزن الجسم يلعب دوراً واقياً عندما يعطى قبل التعريض للأشعة ويتم تقييمه في أوقات مختلفة. وكان أفضل تأثير واضح على معدل الإنقسام الخلوي (MI) عند ٦ و ٧٢ ساعة لحساب معدل الإنقسام الخلوي، كذلك كان هناك نقص في نسبة تكون النويات الدقيقة متعددة الصبغيات بمعدل ٣ أضعاف عند ٧٢ ساعة من وقت التقييم. ويمكن استنتاج أن لفيتامين ب١٢ دور حيوي وقائي من التأثيرات الضارة نتيجة التعرض لأشعة جاما.