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Study The Lung-Protective Effects of Riboflavin and Cyanocobalamin Against Lung Toxicity-Induced by Cyclophosphamide in Rats

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Abstract

Cyclophosphamide is a cytotoxic alkylating agent, it's used associated with different side effects including lung toxicity through oxidative damage. Riboflavin and cyanocobalamin have lung-protective effects. This study was designed to evaluate the protective effects of both vitamins against lung toxicity induced by cyclophosphamide. Seventy healthy adult albino rats of both sexes were divided into seven groups each group containing ten rats, all groups were treated for seven days then after (150 mg/kg) of cyclophosphamide was injected intraperitoneally at day seven, these groups were divided as follow; Group one: intraperitoneally injected with (1ml/kg/day) of normal saline. Group two: injected intraperitoneally with a single dose of cyclophosphamide (150 mg/kg). Group three: administered orally riboflavin at a dose (10 mg/kg/day) and cyclophosphamide. Group four: riboflavin administered orally at a dose (40 mg/kg/day) and cyclophosphamide. Group five: riboflavin administered orally at a dose (0.1 mg/kg/day) and cyclophosphamide. Group six: administered orally a mixture of riboflavin at a dose (10 mg/kg/day) and cyanocobalamin at a dose (0.1 mg/kg/day) and cyclophosphamide. Group seven: administered orally a mixture of riboflavin at a dose (40 mg/kg/day) and cyanocobalamin at a dose (0.1 mg/kg/day) and cyclophosphamide. On day eight rats were sacrificed and serum was obtained for glutathione and the total antioxidant capacity measurement and lung extracted for the immunohistochemical study; both vitamins significantly ($P<0.05$) increased glutathione and the total antioxidant capacity and improve the immunohistochemical changes in comparison with the cyclophosphamide-treated group, these results indicate the protective effects of both vitamins against cyclophosphamide-induced lung toxicity.

Keywords: Cyclophosphamide, Vitamin B2, Vitamin B12, Lung toxicity

دراسة التأثيرات الوقائية للريبوفلافين والسيانوكوبالامين ضد سمية الرئة التي يسببها سيكلوفوسفاميد في الجرذان

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الخلاصة

سيكلوفوسفاميد هو عامل مؤلكل سام للخلايا ، يستخدم مرتبطًا بآثار جانبية مختلفة بما في ذلك تسمم الرئة الذي قد ينجم عن الضرر التأكسدي. للريبوفلافين والسيانوكوبالامين تأثيرات واقية للرئة. صممت هذه الدراسة لتقييم التأثيرات الوقائية لكل من الفيتامينين ضد تسمم الرئة الناجم عن السيكلوفوسفاميد. تم تقسيم ٧٠ جرذًا بالغًا سليمًا من كلا الجنسين إلى سبع مجموعات تحتوي كل مجموعة على عشرة فئران ، وعولجت جميع المجموعات لمدة سبعة أيام ثم بعد ذلك حقنت ب (١٥٠ مجم / كجم) من سيكلوفوسفاميد داخل الصفاق في اليوم السابع ، تم تقسيم هذه المجموعات على النحو التالي ؛ المجموعة الأولى: الحقن داخل الصفاق ب (١ مل / كغ / يوم) من محلول ملحي عادي. المجموعة الثانية: تحقن داخل الصفاق بجرعة وحيدة من سيكلوفوسفاميد (١٥٠ مجم / كجم). المجموعة الثالثة: تناول الريبوفلافين عن طريق الفم بجرعة (١٠ مجم / كجم / يوم) وسيكلوفوسفاميد. المجموعة الرابعة: الريبوفلافين يؤخذ عن طريق الفم بجرعة (٤٠ مغ / كغ / يوم) وسيكلوفوسفاميد. المجموعة الخامسة: الريبوفلافين عن طريق الفم بجرعة (٠,١ مجم / كجم / يوم) وسيكلوفوسفاميد. المجموعة السادسة: يعطى عن طريق الفم خليط من الريبوفلافين بجرعة (١٠ مجم / كجم / يوم) وسيانوكوبالامين بجرعة (٠,١ مجم / كجم / يوم) وسيكلوفوسفاميد. المجموعة السابعة: يعطى عن طريق الفم خليط من الريبوفلافين بجرعة (٤٠ مجم / كجم / يوم) وسيانوكوبالامين بجرعة (٠,١ مجم / كجم / يوم) وسيكلوفوسفاميد. في اليوم الثامن تم التضحية بالجرذان وتم الحصول على مصل الجلوتاثيون وقياس القدرة الكلية لمضادات الأكسدة والرئة المستخرجة من أجل الدراسة المناعية الكيميائية. كلا الفيتامينان زادا بشكل ملحوظ ($P <$ ٠,٠٥) من الجلوتاثيون والقدرة المضادة للأكسدة الكلية وتحسن التغيرات المناعية الكيميائية بالمقارنة مع المجموعة المعالجة بالسيكلوفوسفاميد ، وتشير هذه النتائج إلى التأثيرات الوقائية لكلا الفيتامينات ضد التسمم الرئوي الناجم عن السيكلوفوسفاميد.

الكلمات المفتاحية: سيكلوفوسفاميد ، فيتامين ب ٢ ، فيتامين ب ١٢ ، تسمم الرئة

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Introduction

Cyclophosphamide (CP) is a cytotoxic alkylating agent which is widely used to treat a wide range of malignant diseases including lymphoma, leukemia, breast cancer [1]; however, its usage is associated with different side effects including lung toxicity, nephrotoxicity, hepatotoxicity [2]. Pulmonary toxicity induced by CP is believed to result from the active metabolite of CP which are phosphoramide mustard and acrolein that may induce oxidative damage to the alveolar tissues [3]. Riboflavin which is known also as vitamin B2 belongs to the group of vitamins that are soluble in water [4], that found in different types of food like cheese, egg, salmon, dairy products [5]. Riboflavin (vitamin B2) is considered an important precursor of two important nucleotides first one is flavin mononucleotide (FMN) and the second one is flavin adenine dinucleotide (FAD) [6]these nucleotides have important roles in different redox reactions [6]. It was found that riboflavin-deficit mice may show a reduction in fatty acid oxidation furthermore deficiency of vitamin B2 may cause anaemia, skin diseases, cardiomyopathy [7, 8]. Cyanocobalamin (vitamin B12) is a water-soluble vitamin [9] found in milk, fish, eggs, and meat [10]. Vitamin B12 (cyanocobalamin) is considered an important cofactor for the metabolism of homocysteine and methylmalonic acid [11], furthermore, its deficiency may be associated with numerous diseases like ischemic stroke, anaemia, disturbed vision [12, 13].The current study aims to evaluate the protective effects of riboflavin and cyanocobalamin against cyclophosphamide-induced pulmonary toxicity.

Materials and Method

Experimental study

Seventy healthy adult albino rats of both sexes with a weight range from 200 to 230 gm were used in the present study, rats of both sexes equally distributed throughout the experimental groups; they were achieved from and kept under controlled temperature in the Animal House of Basrah University's College of Pharmacy. The animals were fed commercial pellets and had free access to the water supply throughout the trial.

Drugs

Five hundred mg of cyclophosphamide vial was provided by Baxter in the United States. Amazing nutrition in the United States provided riboflavin capsules (400 mg). TQ pharma, Japan, provided the cyanocobalamin Tablet (1 mg).

Study design

The healthy experimental albino rats were divided randomly into seven groups each group consist of ten rats as follows:

Group one: Rats in this group were received intraperitoneal (IP) injection of (1ml/kg/day) of normal saline (NS) for seven consecutive days; which represents the control group. Group two: Rats in this group were received intraperitoneal (IP) injection of a single dose of CP (150 mg/kg) [14] on day seven. Group three: Rats in this group were orally administered vitamin B2 at a dose (10 mg/kg/day) [15] for seven consecutive days and one intraperitoneal injection of cyclophosphamide at a dose (150 mg/kg) on day seven. Group four: Rats in this group were orally administered vitamin B2 at a dose (40 mg/kg/day) [15] for seven consecutive days and a single IP injection of cyclophosphamide at a dose (150 mg/kg), on day seven. Group five: Rats in this group were orally administered vitamin B12 at a dose (0.1 mg/kg/day) [15] for seven consecutive days and a single intraperitoneal injection of cyclophosphamide at a dose (150 mg/kg), on day seven. Group six: Rats in this group were orally administered a mixture of vitamin B2 at a dose (10 mg/kg/day) and vitamin B12 at a dose (0.1 mg/kg/day) for seven consecutive days and a single intraperitoneal injection of CP at a dose (150 mg/kg), on day seven. Group seven: Rats in this group were administered orally a mixture of vitamin B2 at a dose (40 mg/kg/day) and vitamin B12 at a dose (0.1 mg/kg/day) for seven consecutive days and single intraperitoneal injection of CP at a dose (150 mg/kg), on day seven.Diethyl ether was used to euthanize rats 24 hours after the end of the management period (i.e., on day 8). Intracardiac puncture yielded 5 ±1 mL of blood, which was collected in special tubes containing gel and clot activator to yield serum for glutathione and serum total antioxidant capacity (TAC) measurement, lungs were extracted and washed with phosphate-buffered saline and then stored in 10% formalin solution for immunohistochemical study.

Immunohistochemical study

The terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay is used to determine apoptosis in lung tissue [16].

Statistical analysis

Statistical analysis was performed by SPSS version 25 for windows software. Data were expressed as mean ± SD. A one-way analysis of variance was used to examine the statistical significance of the differences between the groups (ANOVA). P-values less than 0.05 were considered statistically significant differences.

Results

Table 1 showed that rats intraperitoneally injected with CP at day seven at a dose of 150mg/kg (Group two) resulted in a significant decrease ($P<0.05$) in the serum level of glutathione in comparison with the relevant level in control (Group one). Mean ± SD of the levels of glutathione in the serum for (Group two and Group one) was found to be respectively, 33.4 ± 0.516 and 87.6 ± 0.516. Moreover, (Table 1) illustrated that there was a significant increase ($P<0.05$) in serum glutathione

level in groups treated with vitamin B2 (10mg/kg/day and 40 mg/kg/day) each for one week prior to CP (IP 150mg/kg) (groups three, and four respectively), vitamin B12 (0.1mg/kg/day for one week prior to CP (IP 150mg/kg) (Group five), a mixture of vitamin B2 (10mg/kg/day) with vitamin B12 (0.1mg/kg/day) prior to CP (IP 150mg/kg) (Group six) and vitamin B2 (40mg/kg/day) with vitamin B12 (0.1mg/kg/day) (Group seven) for one week prior to IP injection of 150mg/kg of CP compared to the relevant serum level to (Group two) rats IP injected with CP (150mg/kg). Mean ± SD of serum glutathione levels for groups (three, four, five, six, seven and two) were respectively, 47.4 ± 0.516, 50.9 ± 0.737, 54.1 ± 0.316, 61.3 ± 0.483, 71.5 ± 0.527, and 33.4 ± 0.516.

Table 1. Effects of Vitamin B2 and Vitamin B12 on Serum Total Antioxidant Capacity Level

Group/Treatment	Mean TAC mmole/l ±SD
Group one (control)/ injected IP with 1ml/kg/day NS	1.42 ± 0.0186^{a}
Group two/CP IP 150mg/kg	0.05 ± 0.003^{b}
Group three/ vitamin B2 (10mg/kg/day) prior to CP (IP 150mg/kg)	0.56 ± 0.008^{c}
Group four/ vitamin B2 (40mg/kg/day) prior to CP (IP 150mg/kg)	0.86 ± 0.03^{d}
Group five/ vitamin B12 (0.1mg/kg/day) prior to CP (IP 150mg/kg)	1.03 ± 0.008^{e}
Group six/ vitamin B2 (10 mg/kg/day) plus vitamin B12 (0.1mg/kg/day) prior to CP (IP 150mg/kg)	1.16 ± 0.024^{f}
Group seven/ vitamin B2 (40mg/kg/day) plus vitamin B12 (0.1mg/kg/day) prior to CP (IP 150mg/kg)	1.27 ± 0.017^{g}

Each value represents mean ± standard deviation (SD). Values expressed in small letters (a, b, c, d, e, f, and g) are significantly different ($P<0.05$). Number of animals in each group=10

Immunohistochemistry (TUNEL assay) of rats' lung tissue

A section of rats' lung tissue of (**Group one**) shows normal control lung tissue showing part of lung alveolar shows normal architecture cells (no apoptosis, green- colored cells) as shown in Figure (1A).

Immunohistochemistryical changes in rats' lung intraperitoneally injected with a single dose of CP (150mg/kg) (**Group two**) revealed damages included inflammatory cells are found with nuclear fragmentation (red arrow) and pyknosis (black arrow) with a thickness of the alveolar wall characterized by massive apoptosis as shown in the Figure (1B).Section of rats' lung orally administered of (10 mg/kg/day, 40mg/kg/day) of vitamin B2 (**Group three** and **Group four**; respectively) for seven consecutive days before IP injection of (150mg/kg) of CP and orally administered of (0.1 mg/kg) of vitamin B12 **Group five**) for seven days before IP injection of (150mg/kg) of CP at day seven showed fewer histopathological changes such as inflammatory cell (black arrow) karyorrhexis (red arrow) and destruction of alveolar-like emphysema with infiltration inflammatory cells (red arrow). As shown in Figures (1C, 1D, and 1E) respectively.

In addition to that combination of oral administration vitamin B2 (10, 40mg/kg) with vitamin B12 (0.1mg/kg) for seven consecutive days before IP injection of (150mg/kg) of CP at day seven (**Group six** and **Group seven**) respectively show improvement in lung tissue compared with other treated groups that have noted normal architecture alveolar wall (black arrow). As shown in Figures (1F, and 1G) respectively.

Immunohistochemical study of lung

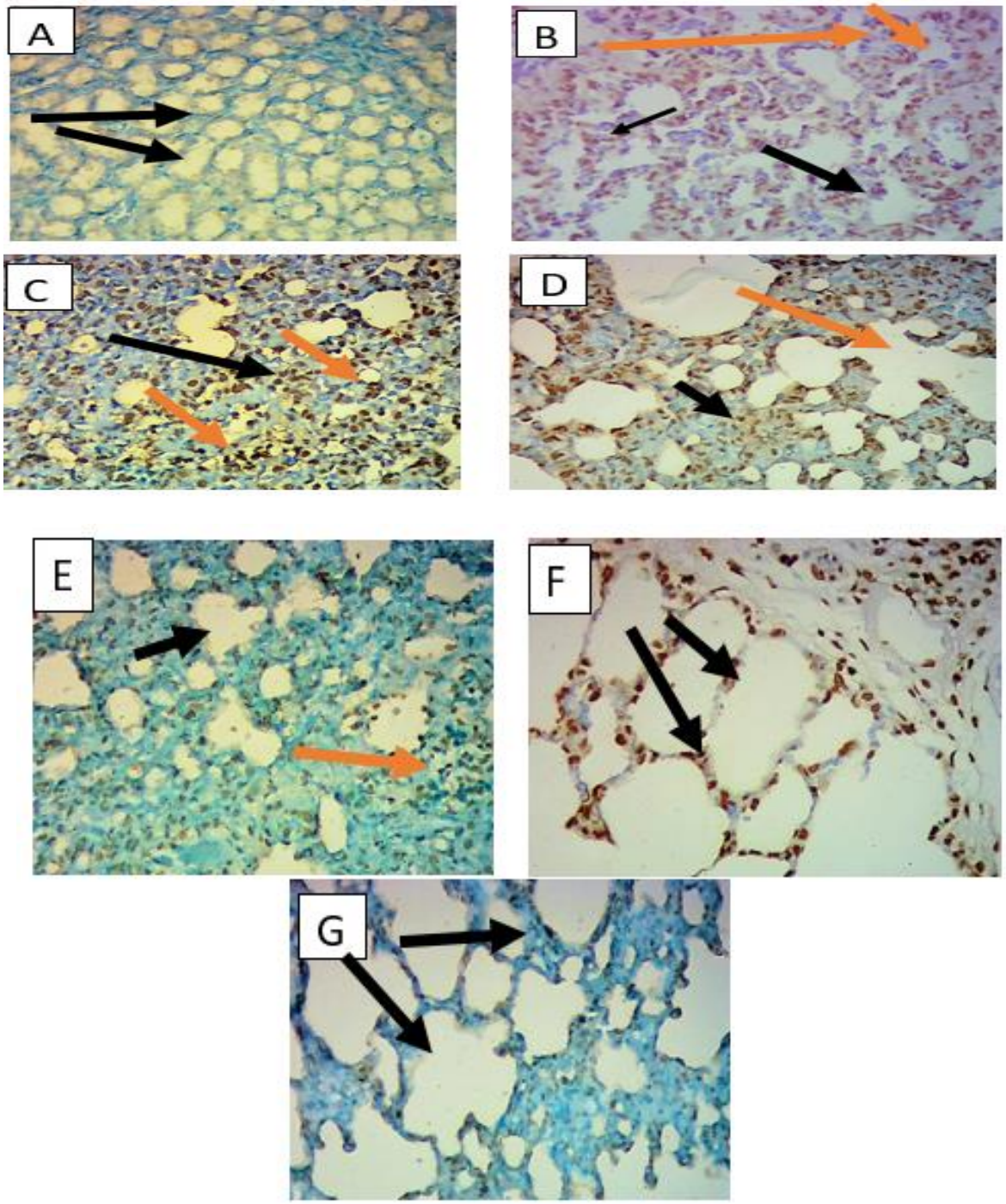

Figure 1. Light micrograph of immunohistochemical changes in lung tissue of rats are presented on the plate. A) the normal control lung tissue showing part of lung alveolar shows normal architecture cells (black arrow). B) lung tissue of rats treated with 150 mg/kg of cyclophosphamide has revealed damages included inflammatory cells are found with nuclear fragmentation (red arrow) and pyknosis (black arrow) with a thickness of the alveolar wall. C) lung tissue treated with 10 mg/kg of Vit. B2 shows fewer histopathological changes such as inflammatory cell found (black arrow) karyorrhexis (red arrow). D) lung tissue treated with 40 mg/kg of Vit. B2 shows destruction of alveolar-like emphysema (black arrow) with infiltration inflammatory cells (red arrow). E) lung tissue treated with 0.1gm/kg of Vit. B12 shows fewer infiltration cells (red arrow) with a normal alveolar wall (black arrow). F) lung tissue treated with 0.1gm/kg of Vit. B12 combination with 10 mg/kg of Vit. B_2 shows improvement in lung tissue compared with other treated groups that have noted normal architecture alveolar wall respectively (black arrow). G) lung tissue of rat treated with a combination of Vit B12 0.1g/kg and Vit B2 40mg/kg shows normal lung tissue (black arrow). TUNNEL stain 40X.

Discussion

In the present study, rats were treated with a single dose of CP (150 mg/kg) **Group two** produce a significant reduction in a glutathione level ($P< 0.05$) compared to **Group one** (control group) this attributed to the biotransformation of CP since, Cyclophosphamide is prodrug required metabolic bioactivation by cytochrome P450 (CYP-450) enzyme system producing 4-hydoxycyclophosphamide which undergo ring-opening to aldophosphamide which decompose spontaneously to phosphoramide mustard (which is responsible for antitumor activity) and acrolein, both metabolites(phosphoramide and acrolein) will induce oxidative damage and free radicals formation, furthermore acrolein will form adduct with glutathione leading to decrease in glutathione level, glutathione considered as the most important intracellular non protein thiol donor group, playing important functions in detoxification of reactive oxygen species, acting as cofactor for other enzymes and regulate DNA synthesis, so cyclophosphamide may result in reduction of glutathione level [17].

Furthermore, pre-treatment with vitamin B2 (10 mg/kg/day, and 40 mg/kg/day) **Group three** and **four** respectively; and pre-treatment with (0.1mg/kg) of vitamin B12 **Group five** and pre-treatment with the combination of both vitamins **Group six** and **seven** before CP injection produce a significant increase in glutathione level this attributed to the role of vitamin B2 as being the source for two important coenzymes which are flavin mononucleotide and flavin adenine dinucleotide [18] which have an important role in the oxidation-reduction reaction, also for the activity of superoxide dismutase and catalase [19], furthermore for conversion of oxidized form glutathione(GSSG) to reduced one (GSH) [15, 20].

Whereas vitamin B12 plays an important role in the maintenance of glutathione level by direct-acting as a superoxide scavenger [21], furthermore vitamin B12 might have a protecting role against (low-grade) inflammation-induced oxidative stress by affecting or modulating the expression of growth factors and inflammatory cytokines [22].

Rats treated with CP (150 mg/kg) **Group two** produce a significant reduction in TAC level ($P< 0.05$) compared to **Group one** (control group) this effect could be explained by damaging effects produced by CP as a result of its metabolism and oxidative stress which induce a reduction in TAC [23].

Furthermore, pre-treatment with vitamin B2 (10 mg/kg/day, and 40 mg/kg/day) **Group three** and **four** respectively; and pre-treatment with (0.1mg/kg) of vitamin B12 **Group five** and pre-treatment with the mixture of both vitamins **Group six** and **seven** respectively; before CP injection produce a significant increase in TAC levels this could be explained by their ability to maintain antioxidant enzyme levels including superoxide dismutase, glutathione level in which vitamin B2 plays an important role [24]; furthermore the role of vitamin B12 as a scavenger for free radicals [21, 25] and its role in modulating the level of the inflammatory cytokines [25].

In the current study, rats injected with a single dose of CP (150 mg/kg) **Group two** shows inflammatory cells that found with nuclear fragmentation and pyknosis with a thickness of the alveolar wall which confirms the effects of CP when compared with **Group one**, however rats pre-treated with different doses of vitamin B2 **Groups three** and **four** and a fixed dose of vitamin B12 **Group five** prior to CP shows fewer histopathological changes such as inflammatory cell, karyorrhexis and destruction of alveolar-like emphysema when compared with **Group two** furthermore; rats pre-treated with combinations of different doses of vitamin B2 and fixed-dose of vitamin B12 **Groups six** and **seven** prior to CP injection shows improvement in lung tissue compared with other treated groups that have noted normal architecture alveolar wall this attributed to the effects of both vitamin B2 and vitamin B12 as the antioxidant activity and their ability to increase the level of glutathione which consist with the finding of *Bashandy SA et al and Moshiri M et al* which founds that vitamin B2 and vitamin B12 have an important role in the maintenance glutathione level and conversion of glutathione from oxidized form to reduced form [26, 27].

Conclusion

Cyclophosphamide has serious lung toxicity side effects, so to reduce this effect, pre-treatment with a combination of vitamin B2 and vitamin B12 may have a beneficial role to reduce this toxic effect.

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Ethical Clearance

In Iraq, the Research Ethical Committee oversees scientific research with ethical approval from the ministries of the environment, health, higher education, and scientific research

Conflict of Interest

There are no conflicts of interest declared by the authors.

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