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Abrogation of 5-Fluorouracil (5-FU) Induced Myelosuppression Model in Sprague Dawley Rats by Carvedilol and Vitamin D as Monotherapy or Double Therapy

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Running title: Abrogating myelosuppression using carvedilol and vitamin D.

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Abstract

Myelosuppression is the most common and seriousside effect of chemotherapy. Oxidative stress, inflammation, and apoptosis are the primary cause of toxicity.This study was designed to evaluate and compare effects of carvedilol and vitamin D when used either alone or together for the abrogation of 5-fluorouracil (5-FU) induced myelosuppression. Carvedilol and vitamin D effects on myelosuppression were not assessed before.This study was carried out on 50 female Sprague Dawley rats divided into five equal groups. The bodyweight and blood film were assessed weekly, vitamin D levels wereassessed at the start and at the end. At the end of the experiment, blood was collected to perform complete blood count(CBC) and assessreduced glutathione (GSH),

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malondialdehyde (MDA) and tumor necrosis factor alpha (TNFα).The right femurs and tibiae were processed for examination of histopathological changes and immunohistochemistry of Bcl-2, while left femurs and tibiae were prepared for bone marrow cell count.Combination of both carvedilol and vitamin D provide a significant decrease in MDA, TNF α and a significant increase in GSH, Bcl-2 expression, with significant improvement in CBC, bone marrow cell count and histopathological finding when compared to other groups.Carvedilol and vitamin D were found to be promising candidates for the abrogation of 5-Fu inducedmyelosuppression. Their combination is superior to treatment by each one alone.

Keywords: Myelosuppression, 5-fluorouracil, carvedilol, vitamin D.

Abbreviations:

(5-FU) 5-fluorouracil

(Bcl-2) B cell lymphoma 2

(CBC) complete blood count

(CMC) Carboxy methyl cellulose

(GSH) Reduced glutathione

(i.p.) intraperitoneal injection

(MDA) malondialdehyde

(PBS) phosphate-buffered saline

(PLTs) Platelets

(RBCs) Red blood cells

(SOD) superoxide dismutase

(TNFα)Tumor necrosis factor alpha

(vit D) vitamin D

(WBCs) White blood cells

(ROS) reactive oxygen species

Introduction:

Myelosuppressionis considered the most common and dangerous adverse effect of cytotoxic cancer chemotherapy that can interrupt the course of treatment. Myelosuppression affects not only the course of chemotherapy but also quality of life in cancer patients (Fernández-Ortega et al., 2012). It can be induced by most of cytotoxic chemotherapeutic agents (Xiao et al., 2017).

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5-Fluorouracil (5-FU) is a pyrimidine analogue cytotoxic drugprescribed for various types of cancers (Bergh et al., 2000; Twelves et al., 2005; Wang et al., 2015). Its mechanism of action is mediated through irreversible inhibition of the enzyme thymidylate synthase, so interrupting DNA replication (Longley, Harkin, & Johnston, 2003). Hematopoietic progenitors are rapidly proliferating so, inhibition of DNA synthesisandincorporation of 5-FU into DNA elicit apoptosis (Li & Slayton, 2013). Also, 5-FU causes an uncontrolled increase in the intracellular reactive oxygen species (ROS) such as superoxide ions and hydrogen peroxide as well as a reductionin the antioxidant capacity marked by superoxide dismutase and glutathione peroxidase (Xiao et al., 2017).

Carvedilol is a nonspecific β-blockerwith α adrenergic blocking activityand it was proved to has unique potent antioxidant and antiapoptotic properties. It is indicated for the inthe therapy of hypertension and heart failure(Kawai et al., 2004). Carvedilol was reported to suppress caspase-9 and subsequent apoptotic pathways (Xu et al., 2014). In addition it was proved to improve the B-cell lymphoma 2 (BCL-2) gene expression, which possess antioxidant and antiapoptotic properties(Abdel-Raheem et al., 2015).

It is well known that vitamin D (vit D) regulates calcium and phosphorus metabolism. However, it also has various roles beyond its role in skeletal homeostasis. It has been reported that it can help prevent chronic diseases like diabetes, cardiovascular disease, and chronic kidney disease through regulation of oxidative stress by the following ways; the enhancementof the antioxidant defense system by inducing the expression of reduced glutathione (GSH), Glutathione peroxidase, and superoxide dismutase (SOD) and inhibiting the expression of NADPH oxidase (Mokhtari, Hekmatdoost, & Nourian, 2017). Also, recent studies showed that vitamin D decreased the apoptosis rate in peripheral blood mononuclear cells (PBMCs) of lupus patients. Also, vitamin D inhibits apoptosis by increasing the expression of Bcl-2 (Tabasi et al., 2015).

1. Material and methods:

2.1. Drugs and chemicals

Carvedilol, Vitamin D, and 5-fluorouracil were purchased from (Multi-Alpha Pharma),(HOLLAND & BARRETT), and (Amriya Pharmaceutical Industries-Egypt); respectively.

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1.2. Experimental animals

50female *Sprague Dawley* rats were purchased from the supplier of the Faculty of Medicine, Tanta University. Those weighing 120– 200 g body weight were used in this experiment. Before the start of the study, they were acclimated for seven daysunder an environment of 22 ± 2, 12 h light/dark cycle, and fed commercial standard chow and tap water ad libitum. The handling of animals and all experimental procedures were conducted in accordance with protocols approved by the institutional "Research Ethics Committee, REC", Faculty of Medicine, Tanta University, Egypt (Approval no. 33219/07/19).

1.3. Induction of a myelosuppression model by 5-FU:

Myelosuppression (in groups 2,3,4 and 5) was induced by 5-FU 150 mg/kgas a single dose by intraperitoneal injection (i.p.)**(Park et al., 2012).**

1.4. Experimental design and treatment protocol

This study was performed on 50 female *Sprague Dawley* rats. The animals were divided randomly into 5 equal groups (10 rats for each).(Group 1) served as the normal control group, received vehicles of saline (i.p) once, in addition to 0.5% CMC and distilled water by oral gavage. (Group 2)served as untreated induced-myelosuppression by 5-FU as a single dose of 150 mg/kg by intraperitoneal (i.p) injection and received vehicles of 0.5% CMC and distilled water by oral gavage. (Group 3)induced-myelosuppression group treated by carvedilol in a dose of 10 mg/kg/day by oral gavage.(Group 4)induced-myelosuppression group treated by Vit D by oral gavage in a dose of 500 IU /kg/day. (Group 5)induced-myelosuppression group treated by both carvedilol and vit D in the same doses mentioned before.

Treatment protocol by CMC and distilled water vehicles (for groups 1 and 2) and by drugs (for groups 3, 4, and 5) was started one week before induction of myelosuppression and continued tothe end of the experiment on the 21st day. Bodyweight (g) was recorded daily and blood film was done on zero point, 7th, 14th and 21st days.

At the end of experiment (21st day), animals were euthanized by the administration of phenobarbital sodium and lidocaine, blood was collected from each animal by intracardiac puncture and divided into two parts; one part was processed for

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complete blood count (CBC) and detection of blood reduced glutathione (GSH), and the other part was left to clot for 10 minutes at room temperature and then centrifuged for 20 minutes at speed of 2000 r.p.m to obtain serum for assaying serum levels of malondialdehyde (MDA) and tumor necrosis factor-alpha (TNF-α). The samples were preserved until assaying at - 80°C.

Then, both femurs and tibiae of each rat were dissected, to clear any red blood cells and clots, all samples were washed with phosphate-buffered saline (PBS) solution (pH 7.4). The right femur and tibia were fixed in 10 % formalin and processed forhistopathological changes and immunohistochemical expression of Bcl-2, while the left femur and tibia were used to count bone marrow cells by hemocytometer.

1.4.1. **The bodyweight (g):**

Body weight of each rat was recorded on zero points, 7^{th}, 14^{th}, and 21^{st} days.

1.4.2. **Leukopenia grade:**

Blood film for all groups was done on zero points, 7^{th}, 14^{th}, and 21^{st} days by puncture of tail vein of the rat, leukopenia is graded by The World Health Organization (WHO) grading system which classifies leukopenia during chemotherapy as follows: absent (grade 0), mild (grades 1), moderate (grade 2), and severe (grades 3 and 4) **(W. Liu, Zhang, & Li, 2013).**

1.4.3. **Counting bone marrow cell:**

The femurs and tibiae at both ends were cut with sharp sterile scissors using a 26-gauge needle and a 10-cc syringe filled with phosphate-buffered solution (PBS) to flush the bone marrow out into a 50 ml Falcon conical tube. Centrifugation of cells at 1,500 rpm for 7 min at 4°C, the cells were resuspended gently by pipetting the cell suspension up and down. Two ml of cell suspension was taken and mixed with 8 ml of PBS for dilution to avoid overcrowding of cells.100 µl of the suspension and 100 µl of PBS were taken in a new eppendorf tube to reach a dilution factor (1) **(X. Liu & Quan, 2015)**.

The full grid on a hemocytometer contains nine squares, each of which is 1 mm^2. Each square of the four corner squares of the hemocytometer contains 16 smaller squares. Using a hand tally counter, the cells were counted in one set of 16 squares. When counting, a system was employed whereby cells are only counted when they are set

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within a square or on the right-hand or top boundary line to avoid counting cells twice. The hemocytometer then moved to the next set of 16 corner squares andcounting was carried on until all 4 sets of 16 corners are counted **(Absher, 1973)**. Cell concentration can be calculated from the following formula:

Total cells/ml= Total cells counted X $\frac{dilution\ factor}{number\ of\ squares}$ **X 10,000 cells / ml**

1.5. Biochemical analysis:

1.5.1. Determination of blood reduced glutathione (GSH) levels:

The assay of reduced glutathione (GSH) levels (mg/dl) was performed using Biodiagnostic Kit (Cat. No GR 25 11)according to the method described by Konard. **(Konrad, Richards, Valentine, & Paglia, 1972).** It depends on the reduction of 5,5` dithiobis (2 - nitrobenzoic acid) with glutathione (GSH) to produce a yellow compound. The reduced chromogen directly proportional to GSH concentration and its absorbance can be measured at 405 nm.

1.5.2. Determination of serum malondialdehyde (MDA) levels:

MDA level was assessedby a kit purchased from Biodiagnostic company (Cat. No MD 2529),through measuring thiobarbituric reactive speciesaccording to the method described by Ohkawa(Ohkawa, Ohishi, & Yagi, 1979)**,**wherethiobarbituric acid reactive product result from reaction of thiobarbituric acid with MDA in a low pH medium at a temperature of 95°C for 30 min, the absorbance of the formed pink product can be measured at 534 nm.

1.5.3. Assay (ELISA) of serum tumor necrosis factor- alpha (TNF-α).

It was measured in serum by rat Enzyme-linked immunosorbent(ELISA) kit obtained from Biodiagnostic Company (Cat. No 201-11-0765) in accordance with the manufacture`s protocol. Measurements of optical density were recorded using a 96-well microtiter plate reader (photometry at 450 nm) and verified against its standardized curve. The results were expressed as nanogram per litre(ng/L).

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1.5.4. **Assay of serum 25(OH) vitamin D (ng/dl)**

Blood was obtained from retroorbital venous plexus by a capillary tube, and allowed to clot for 10 minutes at room temperature and then centrifuged for 20 minutes at speed of 2000 r.p.m to obtain serum for further assay of serum levels of 25-hydroxy Vit D, it was measured on automated fluorescence immunoassay analyzers (Tosoh AIA 1800 ST, Tokyo, Japan) (Kwiecinski, Petrie, & DeLuca, 1989)

1.6. Histological examination of hematoxylin and eosin(H&E) stained sections:

The right femur and tibia were decalcified by 15% EDTA for 5 weeks, the EDTA solution was changed every day then, paraffin sections (4 µm) were prepared and processed for examination of histopathological changes by light microscopeafter staining with H&E.

1.7. Immunohistochemical expression of Bcl-2:

Immunohistochemical staining was performed using Rabbit Anti-Human Bcl-2 monoclonal Antibody (Clone [EPR17509]) obtained from MasterDiagnostica, in accordance with the manufacturer's instructions, 4 m thick sections of right femurs and tibiae were mounted on charged slides, dried overnight at 60 °C, deparaffinized, rehydrated, and heat-induced epitope retrieval boil tissue for 20 minutes at 95 °C using Master Diagnostica EDTA buffer pH 8. After finishing, rinsed with 3–5 changes of distilled water and cooled to room temperature for 20 minutes. Peroxidase solution was used to block for 10 minutes at room temperature. Incubation time for the primary antibody (Rabbit Anti-Human Bcl-2 Monoclonal Antibody Clone EP36) was 20 minutes. Finally, hematoxylin counterstaining and final mounting of the slide. The color intensity was measured using the software (Image J) (**National Institute of Health, Bethesda, Maryland, USA**) **(Schneider, Rasband, & Eliceiri, 2012), the colourintensiy of** Bcl-2 stainingexpressed from zero to 4 grade, cytoplasmic and nuclear staining considered positive, then one-way analysis of variance (ANOVA) test was used to analyze the data statistically, followed by Post-Hoc Tukey‘s test.

1.8. Statistical analysis

All obtained data were tabulated and statistically analyzed using Graph Pad Prism software 5. Shapiro-Wilk test for normality was performed. Data were represented as

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mean ± standard error of the mean (SEM).The significance was determined at values of $P<0.05$**.**

2. Results

3.1. Bodyweight results (g) on zero-points, 7th, 14th, and 21st days: (Fig.1.)

On zero point and 7th day, values of bodyweight didn`t differ significantly between the different studied groups. While on the 14th and 21st days, the untreated group displayed a significant drop in bodyweight value as compared to the normal control group (group 1). Group treated by carvedilol (Group 3)displayed a significant increase in body weight value when compared to the untreated myelosuppression group (group 2). Also, the vitamin D treated group (group 4) displayed a significant increase in bodyweight value when compared to the untreated myelosuppression group (group 2) and a non-significant difference in bodyweight value as compared to the carvedilol treated group (group 3). Group treated by both carvedilol and vitamin D showed a significant rise in body weight value as compared to the untreated myelosuppression group (group 2), Also it showed a non-significant difference in bodyweight value as compared to the carvedilol treated group, as well as vitamin D treated group.

2.2. Leukopenia grade in blood film at zero-point, 7th, 14th and 21st days: (Fig.2)

There was no leukopenia in all groups on zero points and 7th daywhile on the 14th, and 21st days, the untreated myelosuppression group (group 2) revealed leukopenia severe grade on 21st day with a non-significant decrease as compared to leukopenia severe grade on the 14th day. Carvedilol treated group (group 3), the group treated by vitamin D (group 4), and the group treated by both carvedilol and vitamin D revealed leukopenia mild grade on the 21st day with significant decrease as compared to moderate leukopenia grade on the 14th day.

2.3. Complete blood count (CBC) in different studied groups: (Table 1)

RBCs, WBCs and PLTs counts were significantly reduced in the untreated myelosuppression group (group 2) compared to the normal control group (group 1), while the group treated by carvedilol (group 3) and vitamin D treated group (group 4) both revealed a significant elevation in RBCs, WBCs and, PLTs counts when compared to the untreated myelosuppression group (group 2), and there was a non-significant difference

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in RBCs, WBCs and PLTs counts when both previous groups compared to each other. When compared to groups 2, 3, and 4, the group treated with both carvedilol and vitamin D (group 5) had a significant increase in RBCs, WBCs, and PLTs.

2.4. Bone marrow cell count ($x10^4/mm^3$) from right femurs and tibiae of different studied groups: (Fig.3A)

The untreated myelosuppression group (group 2) hada significant lowering in bone marrow cellcount when compared to the normal control (group 1), carvedilol treated group (group 3) and vitamin D treated group (group 4) hada significant rise in bone marrow cell count as compared to the untreated myelosuppression group (group 2) and there was a non-significant difference when compared to each other. Group treated by both carvedilol and vitamin D (group 5) displayed a significant elevation in bone marrow cell count when compared to group 2, group 3, and group 4.

2.5. Results of blood GSH (mg/dl) and serum MDA levels (nmol/ml): (Fig.4A, 4B)

Comparing the untreated myelosuppression group (group 2) to the normal control group (group 1); it revealed a significant reduction in blood GSH levels and a significant elevation in serum MDA levels. In the carvedilol treated group (group 3) and treatment of myelosuppression by vitamin D (group 4); there was a significant elevation in blood GSH levels and a significant reduction in serum MDA levels when compared to the untreated myelosuppression group (group 2), and a non-significant variation as compared to each other. Comparing group 2, group 3 and group 4 to group treated with both carvedilol and vitamin D (group 5); displayed a significant rise in blood GSH levels,and a significant decline in serum MDA levels.

2.6. Results of serum tumor necrosis factor-alpha (ng/L) (TNF-α): (Fig.4C)

The untreated myelosuppression group (group 2) showed a significant increase in serum TNF α level as compared to the normal control (group 1). Myelosuppression treated by carvedilol (group 3), and group treated by vitamin D showed a significant decrease in TNFα level as compared to the untreated myelosuppression (group 2).In the group treated with both carvedilol and vitamin D (group 5); showed a significant decrease in serum TNF α level as compared to group 2, group 3, and group 4.

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2.7.Results of serum vitamin D (vit D) levels (ng/dl) before the start (zero-points) and after the experiment (21st day): (Fig. 5)

Normal control group (group 1); serum vit D level was non significantly different when it was measured before the experiment at zero-points and after at 21st day. In the untreated myelosuppression group (group 2); there was a significant decrease in vit D level after the experiment as compared to its level before at zero points.

2.8.Histopathological examination of H& E stained sections of femoral and tibial bone marrow by light microscope: (Fig.6)

Group 1 (normal control group) (A, B) where (A) showing normal bone marrow cellularity (H & E X100). and (B) Higher magnification showing normal bone marrow cellularity (H & E X 400). Group 2 (untreated myelosuppression) (C, D) where (C) showing a drastically reduced cellular components (H&E X 100). (D) Higher magnification showing drastically reduced bone marrow cellularity (H & E X 400). Group 3 (Carvedilol treated group) (E, F) where (E) showing a mild increase in cellularity (H & E X 100) and (F) Higher magnification showing a mild increase in bone marrow cellularity (H & E X 400).Group 4 (vitamin D treated group) (G, H) where (G) showing a moderate increase in cellularity (H & E X 100). and (H) Higher magnification showing a moderate increase in bone marrow cellularity (H & E X 400). Group 5 (CARV = VitD group) (I, J) where (I) showing almost normal bone marrow cellularity (H & E X 100) and (J) Higher magnification showing almost normal bone marrow cellularity (H & E X 400).

2.9. Results of Bcl-2 immunohistochemical expression in femoral and tibial bone marrow: (Fig.3B&7)

The untreated myelosuppression group (group 2) displayed a significant decline in the estimated color intensity of Bcl-2 when compared to the normal control group (group 1). Group treated by carvedilol (group 3) displayed a significant elevation in the estimated color intensity of Bcl-2 when compared to group 2. Also, the vitamin D treated group (group 4) displayed a significant elevation in the estimated color intensity of Bcl-2 when compared to group 2 and group 3. Treatment of myelosuppression by both carvedilol and

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vitamin D (group 5) showed a significant increase in the estimated color intensity of Bcl-2 as compared to group 2 and group 3 while, there was a non-significant difference in estimated color intensity of Bcl-2 as compared to vitamin D treated group (group 4).

2.10. Correlation of leukopenia grade with GSH, MDA, TNF α levels, and Bcl-2 expression in the untreated myelosuppression group (group 2): (Table.2)

In the untreated myelosuppression group, there was a significant negative correlation between leukopenia grade and blood GSH level & Bcl-2 expression, also there was a non-significant positive correlation between leukopenia grade and serum MDA level, while there was a significant positive correlation between leukopenia and serum TNF α level.

2.11. Correlation of bone marrow cells count with GSH, MDA, TNF α levels, and Bcl-2 expression in the untreated myelosuppression group (group 2): (Table.3)

There was a significant positive correlation between bone marrow cells count and blood GSH level & Bcl-2 expression, while there was a significant negative correlation between bone marrow cells count and serum MDA & TNF α levels.

2.12. Correlation of serum vitamin D levels with GSH, MDA, TNF α levels, Bcl-2 expression, leukopenia grade, and bone marrow cell count in the untreated myelosuppression group (group 2): (Table.4)

The untreated myelosuppression group revealed a significant positive correlation between serum vitamin D levels and blood GSH level, Bcl-2 expression, and bone marrow cells count, while it revealed a significant negative correlation between serum vitamin D levels and serum TNF-α levels & leukopenia grade, and a non-significant negative correlation between serum vitamin D levels and serum MDA level.

3. Discussion

Seeking new approaches for managing myelosuppression is in demand, as all current available treatments are expensive and ineffective.Blood transfusion, growth factors, and bone marrow transplantation are the most common procedures. Neutropenia is the most severe complication of myelosuppression, and blood transfusions are unsuccessful in reversing it. Chemotherapy regimens that induce profound myelosuppression are treated with growth factors. Growth factors, on the other hand, require a large enough pool of hematopoietic progenitors to be successful.

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The present results of the untreated myelosuppression group when compared to the normal control group showed a significant increase in serum level of MDA and TNF alpha, while there was a significant decrease in body weight, GSH, RBCs, WBCs and PLTs count, serum Vit D, bone marrow cell count, Bcl-2 expression and marked hypocellularity in the femoral and tibial bone marrow.

In the present study, according to the blood film performed on zero points, 7^{th}, 14^{th}, 21^{st} days, rats of the "Untreated myelosuppression group" showed grade 4 leucopenia by the day 7^{th} post-injection. Although there is slight recovery started by day 14^{th}, it is non-significant, indicating the importance to start treatment to achieve earlier improvement of the condition. This result is in agreement with previous studies in mice and rats(Kojima et al., 2003; Park et al., 2012; Takano, Tanaka, Aoi, Yahagi, & Fushiya, 2004).

5-FU induced myelosuppression is accompanied by marked loss of body weight **(M. Song, Baik, Hong, & Sung, 2016)**. In the present study, there was a significant decrease in body weights in the untreated myelosuppression group after induction which is in line with the results ofprevious studies**(Lee et al., 2006; M. Song et al., 2016)**. 5-FU injections do significant damage to the gastric epithelium in the stomach and small intestine tissue, resulting in malnutrition, malabsorption, mucositis, and diarrhea, both of which contribute to progressive weight loss. **(M.-K. Song, Park, & Sung, 2013; Sonis, 2010; Sonis et al., 2004)**.

The results of the present study showed a significant decrease in the count of the three hematological parameters WBCs, RBCs and, PLTs in addition to bone marrow hypocellularity in the group of untreated myelosuppression induced by 5-FU that is in line with the previous studies (Taylor et al., 2017; Wang et al., 2015).

The findings of this research support the notion that oxidative stress plays a significant role in the pathophysiology of myelosuppression. Some animals in the untreated myelosuppression group showed signs of oxidative stress in their blood and serum, as represented by a significant decrease in reduced glutathione level and a significant increase in MDA. These results agree with that reported in the previous 5-FU induced myelosuppression model in rats using GSH and MDA as markers of oxidative stress**(Atiq et al., 2019; M. Song et al., 2016)**. Also, in the untreated myelosuppression

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group, we found a significant negative correlation between leukopenia grade and blood GSH level, while a positive correlation between leukopenia grade and MDA level was present, but this correlation achieved no statistical significance. However, this non-significant correlation doesn't deny the causality as it may be attributed to other unknown undetermined factors owing to oxidative stress being controlled by a wide variety of enzymatic and non-enzymatic antioxidant molecules (Suhail et al., 2012).

Furthermore, in the present study the untreated myelosuppression group showed a significant positive correlation between bone marrow cells count and blood GSH level, while there was a significant negative correlation between bone marrow cells count and serum MDA level, these results confirm the entanglement of oxidative stress in the pathogenesis of myelosuppression.

Overproduction of pro-inflammatory cytokines including interferon and tumour necrosis factor (TNF) has been implicated in the activation of hematopoietic stem cells` oxidative DNA damage checkpoint mechanisms **(Baldridge, King, & Goodell, 2011).**Furthermore, oxidative mediators have been shown to activate inflammatory cascades by upregulating transcription factors, such as nuclear factor-kappa B (NF-kB) **(Savoia & Schiffrin, 2007)**, which in turn enhances the expression of pro-inflammatory cytokines, chemokines, adhesion molecules, and inducible nitric oxide synthase in a vicious cycle **(Kleinert, 2004; Tak & Firestein, 2001)**.

The obtained result of the untreated myelosuppression group revealed a marked increase in TNF-alpha which proves that inflammation has a main role in the development of myelosuppression. These results agree with that reported by **Xiao et al, (2017)**(Xiao et al., 2017).

In addition to that, the present work showed a negative significant correlation between bone marrow cells count and levels of TNF-α, and a positive significant correlation between leukopenia grade and levels of TNF-α, this relationship proved the implication of pro- inflammatory cytokines in the pathogenesis of myelosuppression. This finding is in agreement with **Stifter et al. (2005)** who found a negative correlation between bone marrow cellularity and peripheral cytopenia and levels of TNF-α in patients with myelodysplastic syndromes (Stifter, Heiss, Gastl, Tzankov, & Stauder, 2005).

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In the present study, the untreated myelosuppression group showed a significant decrease in Bcl-2 immunohistochemical expression in bone marrow sections indicating that cells undergo programmed cell death "apoptosis" when exposed to chemotherapy, these results agree with previous apoptotic results in myelosuppression models **(K Kontos, Christodoulou, & Scorilas, 2014; Xiao et al., 2017).** 5-FU distributes readily into bone marrow, small intestine, kidney, liver, and spleen (Chadwick & Chang, 1976; Shirasaka, 2009). In the bone marrow, 5-FU is incorporated in the DNA and induces oxidative stress, which is partly responsible for myelotoxicity (Schuetz, Wallace, & Diasio, 1984). The antioxidant potential of hematopoietic stem cells was found to be weakened by 5-FU, and the intracellular ROS content increased significantly. When hematopoietic cells are exposed to ROS, they undergo a DNA damage response, which leads to apoptosis or senescence (Xiao et al., 2017). Also, in the untreated myelosuppression group, we found a negative significant correlation between leukopenia grade and Bcl-2 expression in marrow tissue, while there was a positive significant correlation between bone marrow cells count and Bcl-2 expression which indicated the less and weak expression of Bcl-2 in marrow tissue, the more severe the myelosuppression.

Most of the animals of the untreated myelosuppression group compared to the normal control group in the present study showed a drastic reduction in the cellular components and these marked histopathological changes are in concordance with that described by **Park et al. (2012)(Park et al., 2012)**.

It was demonstrated in earlier studies that bone marrow stromal cells express α and β adrenergic receptors (Kotova et al., 2014). Interestingly, carvedilol significantly inhibited H_2O_2-induced reactive oxygen species formation, apoptosis, and subsequent cell death in BMSCs (M. Chen, Chen, & Lin, 2016).

The present study showed that treatment by carvedilol provided antioxidant, anti-apoptotic, and anti-inflammatory effects as evidenced by a significant decrease in MDA, TNF-alpha, and a significant increase in GSH and Bcl-2 expression when compared to the untreated myelosuppression group.

These results agree with the reported antioxidant and anti-inflammatory effects of carvedilol in rat kidney mitochondria **(Rodrigues et al., 2011)**, reduction of TNF alpha

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and MDA in cadmium-induced cardiotoxicity **(Refaie, El-Hussieny, Bayoumi, & Shehata, 2019).** Furthermore, it decreased MDA and increased GSH in experimental myocarditis **(Yue-Chun et al., 2010)**. Because of the anti-apoptotic effects caused by carvedilol in the present study, other previous studies confirmed the effect of carvedilol on increasing Bcl-2 expression at different tissues including kidney **(Abdel-Raheem et al., 2015; Ahmed, Ebaid, El-Nahass, Ragab, & Alhazza, 2020)** and cardiac tissue **(Zhang, Yang, & Zhang, 2019)**.

These findings were reflected histopathological as a mild improvement of the cellularity of bone marrow, as well as the disease outcome in the form of improvement of the bone marrow cell count and the three hematological parameters (RBCs, WBCs and PLTs) when compared to untreated myelosuppression group. Also, carvedilol ameliorated the decrease in body weight which is in line with previous studies performed on diabetic rats **(Zheng, Li, Gao, Zhang, & Robinson, 2019)**. However, this effect was probably due to decreased disease activity as carvedilol itself has no direct effect on bodyweight **(Eid, Abdelkader, Abd El-Raouf, Fawzy, & Ezz-El-Din, 2016)**.

Patients taking chemotherapy were four times more prone to develop severely depressed levels of vitamin D compared to those not on chemotherapy **(Fakih et al., 2009)**.

However, to our knowledge vit D level in 5-FU induced myelosuppression was not assessed before. So, it is of great interest to assess it in our study, where the untreated myelosuppression group showed a significant decline in Vit D level after induction of myelosuppression by 5-FU as compared to its level before 5-FU injection.

Via activation of the enzyme glucose-6-phosphate dehydrogenase (G6-PDH), which downregulates nitrogen oxide and upregulates superoxide dismutase, vitamin D increases the expression of glutathione peroxidase and affects glutathione formation (SOD). These effects of vitamin D minimize the burden of intracellular ROS. **(Y. Liu, Hyde, Simpson, & Barycki, 2014)**.

Also, hypovitaminosis D and the resulting defective mitochondrial activity also induce and increase inflammation, which is known to cause cellular damage and apoptosis **(da Luz Dias et al., 2018; Morris & Maes, 2014)**. As a result, the anti-

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inflammatory effects of adequate vitamin D levels are of important consideration **(Berk et al., 2013)**.

In this framework, the untreated myelosuppression group in our present study revealed a significant positive correlation between serum vitamin D levels and blood GSH level, Bcl-2 expression, and bone marrow cell count and there was a significant negative correlation between serum vitamin D levels and serum TNF α levels & leukopenia grade. While there was a non-significant negative correlation between serum vitamin D levels and serum MDA level. These findings are in line with a previous study in which significant intercorrelation was reported between serum vitamin D level and apoptotic, oxidative, and inflammatory markers in patients with chronic viral hepatitis C, and the deficiency in vitamin D levels was shown to have a critical role in the pathogenesis of hepatitis via apoptotic, oxidative stress, and inflammatory mechanistic pathways (Gabr et al., 2016).

Our study revealed that the vitamin D treated group when compared to the untreated myelosuppression group, provided antioxidant, anti-inflammatory, and anti-apoptotic activities which is proven by a significant increase in the GSH and Bcl-2 expression and a marked decrease in MDA, TNF alpha levels.

In this context, these results are in line with **BaSalamah et al. (2018),** who proved that vitamin D increase GSH and reduce MDA and TNF alpha levels in rats withrenal and testicular injuries **(BaSalamah et al., 2018)**, Furthermore, vitamin D was recently evaluated to decrease TNF alpha in rat treated with 5-FU **(S.-C. Chen et al., 2020)**.

Regarding Bcl-2 expression in bone marrow sections, there is a moderate increase in Bcl-2 antiapoptotic marker in vitamin D treated group as compared to the untreated myelosuppression group, a result which is consistence with previous reports performed on rats with ischemic-reperfusion hepatic injury and rat model of periodontitis and obstructive pulmonary disease respectively in which vitamin D increased the expression of Bcl-2 **(Han et al., 2019; Seif & Abdelwahed, 2014)**.

Moreover, histopathological examination revealed that the cellularity of the bone marrow was moderately increased in the vitamin D treated group as compared to the untreated myelosuppression group.

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This was reflected in the disease outcome which was proved by the marked increase in the bone marrow cell count and the complete blood count results and improvement in body weight when compared to the untreated myelosuppression group.

When the carvedilol group and the vitamin D treated group were compared to each other, they didn`t show any difference as regards to their antioxidant and anti-inflammatory effects. However, the vitamin D treated group showed superiority in its antiapoptotic effects as compared to the carvedilol treated group, this may be a result of the presence of vitamin D receptors in the bone marrow tissue **(Langub, Reinhardt, Horst, Malluche, & Koszewski, 2000; Paubelle et al., 2020)**. Moreover, as mentioned before that vitamin D deficiency itself contribute to apoptosis (Gabr et al., 2016) so, compensation of this deficiency in the group treated by vitamin D protected from this effect in addition to the antiapoptotic effect of vitamin D itself that abrogated apoptosis induced by 5-FU. On the other hand, the carvedilol treated group and the it D treated group did not show any significant difference in the disease outcome regarding the histopathological finding, CBC, and the bone marrow cell count.

The group treated by both carvedilol and vitamin D showed an evident antioxidant, anti-inflammatory, and anti-apoptotic effects proved by a significant decline in MDA, TNF alpha, and a significant increase in GSH and Bcl-2 expression as compared to the untreated myelosuppression group. Moreover, histopathological examination showed apparently normal bone marrow cellularity that is reflected on the outcome of the disease as improvement of the CBC and increased bone marrow cells count.

When the combination group is compared to the carvedilol treated group, it revealed superiority with regard to anti-inflammatory, anti-oxidant, and anti-apoptotic effects.

On the other hand, when the combination group was compared to the vitamin D treated group, it provided superiority as regards to its anti-inflammatory and anti-oxidant effects. Although, it didn`t show an advantage over the vitamin D group as regards the antiapoptotic effect presented. However, it revealed apparently normal bone marrow cellularity as regards histopathological findings which were reflected on the disease

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outcome proved by a significant increase in CBC and bone marrow cell count as compared to the other treated groups.

4. Conclusion

Carvedilol and vitamin D are promising candidates for the prevention of myelosuppressioncaused by cytotoxic drugs through combined antioxidant, anti-inflammatory, and antiapoptoticmechanisms. Their combination is superior to treatment by each one alone. Moreover, these findings should be confirmed in humans in future clinical trials.

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Conflict of interest

This is a confirmation that there is no conflict of interest in this work.

Author contributions

All the named authors participated sufficiently in this research work according to the specialty and expert of each one. Lamiaa El Ballat, performed most experiments and data analysis, participated in the writing of the manuscript. AmanyAbdin designed the study and helped with writing. EsamLaag performed histopathology, immunohistochemistry, and its statistical analysis and helped in writing. Reem El Kholy was involved inexperimental design, participated in performing experiments, data analysis, writing, and revision. All authors read and approved the final manuscript.

The authors declare that all data were generated in-house and that no paper mill was used.

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(Table 1): Complete blood count (CBC) values in different studied groups

Groups / CBC Values	Group 1 (Normal control) (n=10)	Group 2 (Untreated myelosuppression) (n=10)	Group 3 (CARV) (n=10)	Group 4 (Vit D) (n=10)	Group 5 (CARV + Vit D) (n=10)	One-way ANOVA F value (P value)
Red blood cells (RBCs) ($x10^6$/µl)	7.75±0.29	3.98±0.28 P1 <0.001	5.78±0.36 P2 <0.001	6.01±0.35 P2 <0.001 P3 NS	7.53±0.49 P2 <0.001 P4 <0.01 P5 <0.01	17.35 (P<0.001)
White blood cells (WBCs) ($x10^3$/µl)	8.01±0.51	1.64±0.40 P1 <0.001	4.61±0.26 P2 <0.001	4.86±0.46 P2 <0.001 P3 NS	7.14 ±0.45 P2 <0.001 P4 <0.001 P5 <0.001	33.95 (P<0.001)
Platelets (PLTs) ($x10^3$/µl)	823.2±24.87	137.6±42.45 P1 <0.001	466.3±36.98 P2 <0.001	457.9±34.07 P2 <0.001 P3 NS	676.9±76.01 P2 <0.001 P4 <0.001 P5 <0.01	31.63 (P<0.001)

— **Values expressed as mean± SEM, n= number, NS=non-significant**

— **Tukey test**

P1: Group 2 (Untreated myelosuppression) Vs group 1 (Normal control)

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P2: Group 3 (CARV), group 4 (Vit D) and group 5 (CARV + Vit D) Vs group 2 (Untreated myelosuppression)

P3: Group 4 (Vit D) Vs Group 3 (CARV)

P4: Group 5 (CARV + Vit D) Vs group 3 (CARV)

P5: Group 5 (CARV + Vit D) Vs group 4 (Vit D)

— **CARV = Carvedilol, Vit D= Vitamin D.**

Table (2): Correlation of leukopenia grade with GSH, MDA, TNF alpha levels, and Bcl-2 expression in the untreated myelosuppression group (group 2).

Pearson correlation coefficient (r)		
	Leukopenia grade	
	r	P Value
Blood reduced glutathione (GSH)	-0.889	P <0.001
Serum malondialdehyde (MDA)	0.510	P NS
Serum tumor necrosis factor alpha (TNF α)	0.811	P< 0.001
Bcl-2 expression	0.745	P<0.01

Table (3): Correlation of bone marrow cells count with GSH, MDA, TNF alpha levels, and Bcl-2 expression in the untreated myelosuppression group (group 2).

Pearson correlation coefficient (r)		
	Bone marrow cells count	
	r	P Value
Blood reduced glutathione (GSH)	0.943	P <0.001
Serum malondialdehyde (MDA)	-0.700	P <0.01
Serum tumor necrosis factor alpha (TNFα)	-0.897	P < 0.001
Bcl-2 expression	0.850	P<0.001

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Table (4) Correlation of serum vitamin D levels with GSH, MDA, TNF α levels, Bcl-2 expression, leukopenia grade, and bone marrow cell count in the untreated myelosuppression group (group 2):

Pearson correlation coefficient (r)		
	Serum vitamin D levels	
	r	P Value
Blood reduced glutathione (GSH)	0.830	P <0.001
Serum malondialdehyde (MDA)	-0.579	P NS
Serum tumor necrosis factor alpha (TNFα)	-0.780	P < 0.001
Bcl-2 expression	0.840	P<0.001
Leukopenia grade	0.859	P<0.001
Bone marrow cells count	0.970	P<0.001

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