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# Protective Role of Ginger against Metformin Induced Alteration in Bcl2 Expression in the Spleen of Normoglycemic Albino Rat

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## Abstract

**Background**: The spleen is the largest and most essential organ in lymphoid system for the immunological reactions in the blood. Apoptosis can be defined as a programmed cell death in tissues. The B-cell leukemia/lymphoma-2 (Bcl-2) families are apoptosis regulators. Metformin is a safe drug that can lower blood glucose level, and may be used in non-diabetic patients with polycystic ovarian syndromeor with impaired glucose tolerance. Ginger is a member of ginger family. It has been used in India as a treatment for headache and common cold. It is known as an anti-oxidant agent.

**Aim of the work**: This study has been designed to evaluate the possible pathological effect of metformin on spleen in normoglycemic rats, it's possible effect on Bcl2 expression, and the potential protective role of ginger.

**Material and methods**: Thirty adult male albino rats (200-250gm) were obtained from AL-Nile Experimental Animal Center, Mansoura, Egypt. The animals were divided randomly into 3groups 10 rats each; Control, metformin treated group (200mg/kg orally for 1 month), and metformin-ginger group (200mg/kg /day metformin + 300 mg/kg/day ginger for 1 month). Rats in each group were weighted and sacrificed at the end of the experiment, spleen were dissected. The specimens were used for paraffin sections, stained with hematoxylin-eosin (HE), Masson's trichrome and Bcl2 immunohistochemistry.

**Results**: Sections of ME treated group showed distorted splenic contour, degenerated cells with vacuolated cytoplasm and pyknoticnuclei. Masson trichrome stained sections revealed massive fibrosis in white pulp, red pulp and among cellular cords. The spleen treated with (ME+G) showed normal architecture without distortion, well defined white pulp, red pulp and splenic sinuses. The cellular cord showed lymphocytes and macrophages. Masson trichrome stained sections of the spleen showed minimal fibrosis among cellular cords. The treated group with metformin (ME), showed reduction of Bcl2 expression in all zones. While, the metformin -ginger (ME+G) treated group showed extensive expression in germinal center, mantle layer and marginal zone.

**Conclusion**: From this study, it be concluded that metformin like any other drug may have beneficial curative properties in some medical conditions, however it should used be with caution to avoid its hazardous effect especially in the spleen which is the most vital organ in lymphatic and immune system. Also, ginger is a natural herbal agent could protect from hazardous side effect of metformin.

**Keywords**: Metformin, Bcl2, spleen, ginger

## Introduction

The spleen is the largest and most essential organ in lymphoid system for the immunological reactions in the blood [1]. It contains group of heterogeneous cells, mainly lymphocytes (T and B lymphocytes), hence, it plays a major role in immunity [2]. Apoptosis can be defined as a programmed cell death in tissues. It has a vital mechanism in regulation of T and B lymphocyte maturation in different lymphoid organs after recognition of antigens [3]. The B-cell leukemia/lymphoma-2 (Bcl-2) families are apoptosis regulators. TheBcl-2 and Bcl-2 associated X (Bax)

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protein have been recognized as an essential protein for the regulation of mitochondria permeability and mitochondria-associated apoptotic signaling [4].

Metformin is a safe drug that can lower blood glucose level, and may be used in non-diabetic patients with polycystic ovarian syndrome or with impaired glucose tolerance [5]. Metformin, like any other drug, is associated with side effects; it has been reported to cause lactic acidosis and pancreatitis [6].

Ginger (zingiber officinale) is a member of ginger family. It has been used in India as a treatment for headache and common cold. It was reported that long term use of ginger in the human diet may result in hypoglycemia and hypolipidemia [7]. The Ginger's active ingredients, such as gingerols, are known to be anti-oxidant agents [8]. It was reported that Z. officinale could preserve the ultimate immune function of the immune system as it increases the titer of antibodies like IgG and IgM [9].

This study has been designed to evaluate the possible pathological effect of metformin on spleen in normoglycemic rats, its possible effect on Bcl2 expression, and the potential protective role of ginger.

## Materials and Methods

### Experimental animals

Thirty adult male albino rats (200-250gm) were obtained from AL-Nile Experimental Animal Center, Mansoura, Egypt. The animals were housed in a clean separate cage, 18°C temperature and 45% humidity. They had free access to standard food and drinking water. The experiments were carried out according to the regulations laid down by the Ethical Committee in Faculty of Medicine, Mansoura University.

### Experimental protocol

The animals were weighted and divided randomly into 3 groups 10 rats each.

**Group 1 (CN):** control rats received vehicle only.

**Group 2 (ME):** Metformin treated rats; 200mg/kg/day for one month through oral route, this dose is equivalent to≈1000 mg in average weight human based onReagan-Shaw method; human equivalent dose (mg/kg)=animal dose (mg/kg)×animal (km)/human (km) [10]. Metformin was obtained from a local pharmacy.

**Group 3 (ME+G):** Metformin treated rats; 200mg/kg /day+ ginger(300 mg/kg/day ) for one month, added to the daily diet [11]. Ginger was obtained from Sigma Aldrich Company in a powder form.

### Sacrifice of rats and specimens collection

By the end of the experiment, rats in all groups were anaesthetized with Ketamine (60 mg/kg i.p.), spleen were dissected. The specimens were used for paraffin sections. The slides were processed for staining with hematoxylin-eosin (HE), Masson's trichrome and Bcl2 immunohistochemistry.

For immunohistochemical stain, sections (5µ thick) were used for the activated Bcl2 detection system (Biovision activated Bcl-2 [1:100]). The sections were deparaffinized in Xylene for 1 h. Next, rehydration in 100%, 95%, 80% and 70% alcohol series for2 min each. Then, immersion in distilled water for 5 min, after that, sections were washed in PBS for 10 min and exposed to microwave radiation at 500 W for 10 min in citrate buffer(10 mM, pH 6.0) for antigen retrieval. The primary antibody was applied at 4°C overnight and washed with PBS. The biotinylated secondary antibody was applied, then, incubated with the enzyme conjugate and 3,3-diaminobenzidinetetrahydrochloride. Finally, sections were stainined with Mayer's hematoxylin as a counter stain [12].

### Morphometric Study

The area % of splenic fibrosis was evaluated in Masson's trichrome-stained sections. The blue stained area was measured per unit area in 10 random non overlapping fields. The area % of Bcl2 was measured per unit area in 10 random non overlapping fields.

Morphometric study was done using program NIH Image J program (National Institutes of Health, Bethesda, MD, USA), according to the program instruction.

### Statistical Analysis

Data were expressed as means±SD. To compare the significance between the different groups, analysis of variance (ANOVA) was used followed by post-hoc Tukey test. $P<0.05$ was considered as significant. All statistical analyses were performed by the computer program SPSS (Statistical package for social science) version 22.

## Results

### Histological and Immunohistochemical results

In this study, the effect of ginger powder added to the daily diet on the Metformin treated spleen was compared with Metformin treated spleen in rats. The control group, showed normal architecture of the spleen. The parenchyma of the spleen consisted of white pulp and red pulp. The white pulp revealed lymphocytes arranged around central arteriole. The red pulp composed of cell cords with splenic sinuses (**Figure 1A** and **1B**). The splenic cords contain macrophage, plasma cells, lymphocytes and other blood cells as erythrocytes and granulocytes. Masson trichrome stained sections of the spleen showed minimal fibrosis among cellular cords (**Figure 2A**). The Metformin treated group, there was distorted splenic contour. The white pulp appeared irregular; some cells were degenerated, with vacuolated cytoplasm and pyknoticnuclei (**Figure 1C** and **1D**). Masson trichrome stained sections revealed massive fibrosis in white pulp, red pulp and among cellular cords (**Figure 2B**). The Metformin and ginger treated group, the spleen showed normal architecture without distortion, well defined white pulp, red pulp and splenic sinuses. The cellular cord showed lymphocytes and macrophages (**Figure 1E** and **1F**). Masson trichrome stained sections of the spleen showed

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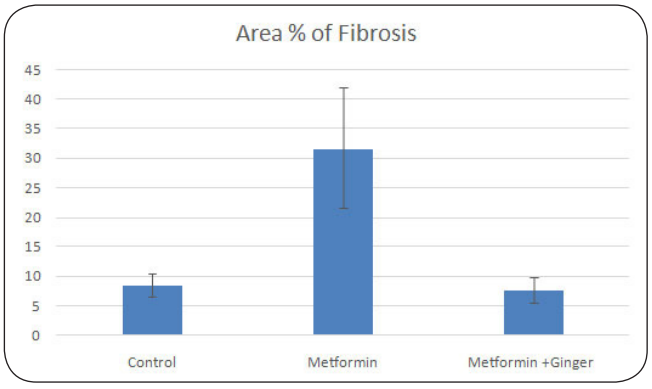
minimal fibrosis among cellular cords (**Figure 2C**).

## Morphometrical results

1- Area % of fibrosis:

| Area % of Fibrosis | Control | Metformin | Metformin+ Ginger |
|---|---|---|---|
| | 8.44±1.91% | 31.83±10.2%* | 7.61±2.16%** |

- *Significant versus Control group.
- ** Significant versus Metformin group.

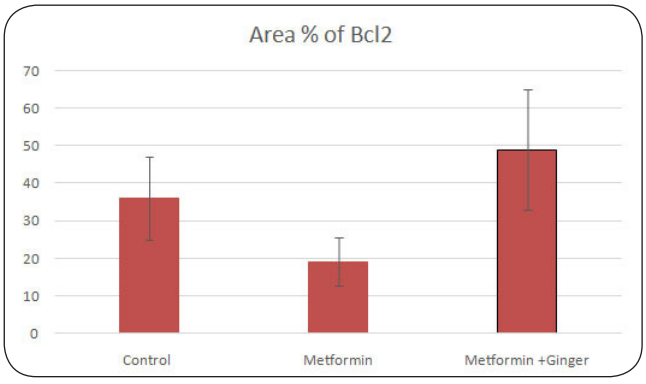



2- Area % of Bcl2 positive reaction:

| Area % of Bcl2 | Control | Metformin | Metformin+ Ginger |
|---|---|---|---|
| | 36% ± 11% | 19% ± 6.4%* | 49% ± 16.1%** |

- *Significant versus Control group.
- ** Significant versus Metformin and Control groups.

In the control group, Bcl2 was expressed in germinal center, mantle layer and marginal zone. The treated group with metformin (ME), showed reduction of Bcl2 expression in all zones. While, the metformin-ginger (ME+G) treated group showed extensive expression in germinal center, mantle layer and marginal zone (**Figure 3**).



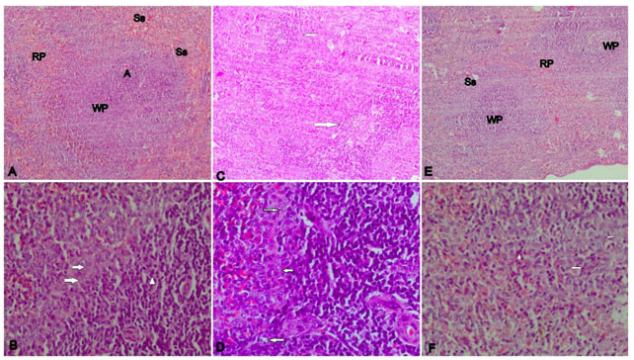




**Figure 1**. **A,B**. Photomicrograph of the spleen in control group, shows the parenchyma of the spleen consisted of White pulp (WP) and Red pulp (RP). The white pulp reveales lymphocytes (arrow head) arranged around central arteriole (**A**). The red pulp composed of cell cords with splenic sinuses (Ss). The splenic cords contain macrophage (arrows)(**A**: H&E X 100, **B**: H&E X 400). **C,D**. Sections of Metformin treated group shows distorted splenic contour, the white pulp appears irregular (arrows). Some cells are degenerated, with vacuolated cytoplasm and pyknotic nuclei (arrows). (**C**: H&E X 100, **D**: H&E X 400). **E,F**. Sections of the spleen in Metformin-Ginger treated group, shows normal architecture without distortion, well defined white pulp (WP), red pulp (RP) and splenic sinuses (Ss). The cellular cord shows lymphocytes (arrow head) and macrophages (arrows). (**E**: H&E X 100, **F**: H&E X 400).

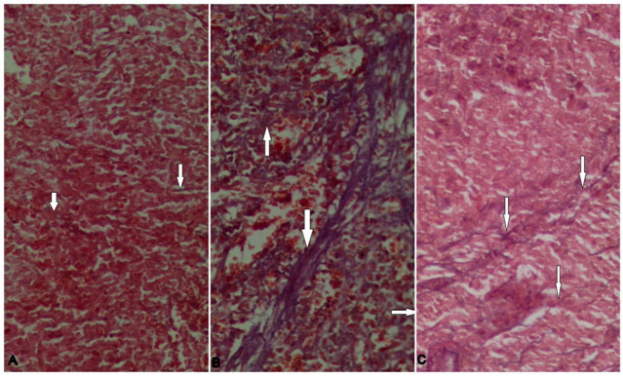



**Figure 2**. **A**. Photomicrograph of the spleen in control group shows minimal fibrosis among cellular cords (arrows).
**B**. Sections of Metformin treated group shows massive fibrosis in white pulp, red pulp and among cellular cords (arrows).
**C**. Sections of the spleen in Metformin-Ginger treated group, shows minimal fibrosis among cellular cords (arrows).
(Masson trichrome X 400).

## Discussion

Metformin is one of the most commonly used drugs for diabetes worldwide. It has been reported recently that metformin may exhibit anticancer effects in ovarian cancer [**13**] might reduce recurrence of prostate cancer in type 2 diabetic patients [**14**].

In this study, the metformin treated group for one month showed distorted splenic contour, the white pulp appeared irregular, and some cells were degenerated, with vacuolated cytoplasm and pyknotic nuclei. In parallel to our result, metformin treatment was reported to alter B cell subsets, suppressing the formation of germinal center, marginal zone and plasma cells in mice [**15**]. This could be due to the fact that metformin is known to affect AMPK (AMP-activated protein

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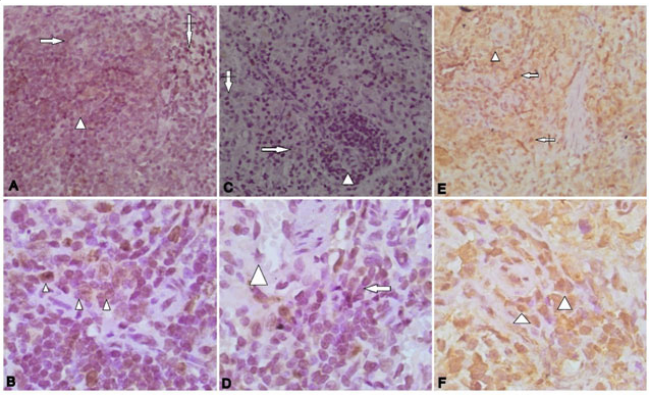


**Figure 3**. **A,B**. Photomicrograph of the spleen in control group, shows normal expression of Bcl2 in germinal center (arrow heads), mantle zone (arrow) and marginal zone (crossed arrow). (**A**: Bcl2 immunohistochemistry X 400, **B**: Bcl2 immunohistochemistry X 1000). **C,D**. Sections of Metformin treated group shows reduction of Bcl2 expression in germinal center (arrow head), mantle zone (arrow) and marginal zone (crossed arrow) (**C**: Bcl2immunohistochemistry X 400, **D**: Bcl2 immunohistochemistry X 1000). **E,F**. Sections of the spleen in Metformin-Ginger treated group, shows extensive expression of Bcl2 in germinal center (arrow heads), mantle zone (arrow) and marginal zone (crossed arrow).
(**E**: Bcl2 immunohistochemistry X400, **F**: Bcl2 immunohistochemistryX 1000).

kinase), mTOR (mechanistic target of rapamycin) and suppress STAT3 (phosphorylation at tyrosine 705) activity in vitro causing suppression of splenic B cells and its subsets [16]. It was suggested that the mitochondrial respiratory-chain is the site for metformin action in human, this causes reduction in cellular ATP, increases the AMP:ATP ratio, and AMPK activation [17]. Another explanation for our finding that it was proven that metformin drug cause vitamin B12 deficiency [18], this could result in reduction of CD4 and CD8 lymphocytes and depressed activity of natural killer cell, all those effects could be treated with vit B12 supplementation [19].

Interestingly, Masson trichrome stained sections revealed massive fibrosis in white pulp,red pulp and among cellular cords in metformin treated group. This comes in contrast to the fact that metformin significantly reduces fibrosis. It was documented that metformin can attenuate the TGF-β1 (Transforming growth factor)-induced fibrosis and collagen accumulation expression in EPCs (Endothelial progenitor cells) in some organs (i.e. heart, liver and lung). However, in that study they did not investigate whether metformin could reduce fibrosis of EPCs in vivo or not [20].

According to our findings, there was significant reduction of area % of Bcl2 (19%±6.4%) in metformin treated group when compared with control group (36%±11%). In the control group, Bcl2 was expressed in germinal center, mantle layer and marginal zone. The treated group with metformin, showed reduction of Bcl2 expession in all zones. In agreement with our finding, it was reported the metformin treatment inhibited spleen germinal center B cells formation in mice, which in turn reduce Bcl2 expression [21]. Also, it was documented that metformin could down-regulate the expression of anti-apoptotic protein Bcl2 expression in the synovial fluid of collagen induced arthritis rat model [22].

In this study, Hematoxylin and eosin-stained sections of the spleen in metformin-ginger treated group showed normal architecture without distortion, well defined white pulp, red pulp and splenic sinuses. The cellular cord showed lymphocytes and macrophages. Masson trichrome stained sections of the spleen showed minimal fibrosis among cellular cords groups. In line with our results, it was documented that ginger in dose of 200mg/kg/day attenuated the hepatocytes degeneration and reduced collagen deposition in CCL4 induced liver fibrosis model in adult male wistar rats [23]. In addition, ginger administration (100mg\kg\day for 8 weeks) improved CCL4 induced hepatic necrosis, reduced collagen deposition and relived thickening and congestion of blood vessels in rats [24].

According to our finding, the ginger treated group showed extensive expression of Bcl2 in germinal center, mantle layer and marginal zone, the area % of Bcl2 expression was 49%± 16.1%, it showed significant increase in comparison to control and metformin treated groups. In contrast to our finding, it was documented that ginger extract significantly decreased expression of Bcl-2 mRNA and protein in the cancer cells of the breast [25]. In addition, there was insignificant effect of ginger on the expression of Bcl-2 in the crypts of normal appearing colonic mucosa in patients at increased risk for colorectal cancer [26]. This contrast with our finding may be explained that ginger may decrease expression in Bcl2 in cancer cells but has no effect on normal cells. However, in agreement with our finding, previous results showed that rats treated with Ginger powder had less apoptosis in the injured intestinal tissues, the expression Bcl-2 protein levels increased [27].

From this study, it be concluded that metformin like any other drug may have beneficial curative properties in some medical conditions, however, it should be used with caution to avoid its hazardous effect especially in the spleen which is the most vital organ in lymphatic and immune system. Also, ginger is a natural herbal agent could protect from hazardous side effects of metformin.

## Competing interests
The author declares that he has no competing interests.

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