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Hematinic and Anti-Anemic Effect of Thymoquinone Against Phenylhydrazine-Induced Hemolytic Anemia in Rats

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Abstract: Herein, the possible hematinic and anti-anemic effects of TQ supplementation therapy was investigated in a rat model of hemolytic anemia induced by Phenylhydrazine (PHZ). Forty eight adult male Wistar rats (16 rats per group) were randomly classified into: normal control group, PHZ group and PHZ+TQ group. PHZ was injected intraperitoneally at 40 mg kg^{-1} on day 0 and 2 additional doses were given at 9 am and 6 pm, on day 1 while TQ (15 mg/kg/day) was given orally. Six rats of each group were sacrificed at days 3, 5 and 8 and their blood samples were collected at each time point for analysis. After 3-5 days of PHZ injection, rats developed acute hemolytic anemia reflected by significant decreases in RBC count, Hemoglobin (HGB) concentration and Hematocrit (HCT) percentage and significant increases in reticulocytes population and serum heme concentration. Interestingly, simultaneous therapy with TQ had significantly reversed these deteriorating effects of PHZ on RBCs, HGB, HCT and heme at each time point of analysis, however, it trended to induce further increase in reticulocytes population. Additionally, TQ therapy significantly reversed the decreases in serum levels of total Glutathione (GSH) and activities of Superoxide Dismutase (SOD) (as indices of antioxidant status) as well as the increases in serum levels of Thiobarbituric Acid Reactive Substances (TBARS) (as indices of lipid peroxidation and oxidative stress) that were induced by PHZ intoxication. The present data suggest the favorable hematinic and anti-anemic effect of TQ therapy on PHZ-induced oxidative stress and hemolytic anemia in rats. This in turn may pave the way to use TQ as part of anemia management. However, further studies are required to confirm this suggestion.

Key words: Hemolytic anemia, phenylhydrazine, thymoquinone, rats, oxidative stress

INTRODUCTION

Anemia is a common blood disorder affecting people of all ages and posing a great threat to global healthcare. There are several types of anemia, many of which are rare but in all cases there is a reduction of number of circulating red blood cells and circulating hemoglobin (Holden and Acomb, 2007). Hemolytic anemia is a form of inherited or acquired anemia results from either intravascular or extravascular RBC destruction (Powers and Silberstein, 2009). It has numerous external and internal causes ranging from relatively harmless to life-threatening. The exposure to many chemicals including the administration of some drugs has been associated with RBC destruction and hemolytic anemia is a part of the clinical syndrome associated with intoxication (Beutler, 2001).

Phenylhydrazine (PHZ) and its derivatives were first given a medical application at the end of the 19th century as antipyretics but the toxic action on red blood cells made their use dangerous. However, this compound seems to be very useful in experimental models studying mechanisms of hemolytic anemia (Berger, 2007). PHZ-induced hemolytic anemia can also be used as a model for the study of haematinic effects of new agents or as a model of reticulocyte research (Biswas *et al*., 2005; Xie *et al*., 2003; Berger, 2007).

Developing therapeutic agents from natural products has renewed the worldwide attention and stimulated new wave of research on the benefits of "Herbal Medicine" as an effective alternative therapeutic tool for various illnesses (Zhao *et al*., 2011). Numerous plants have been tested for their therapeutic potential and among them *Nigella sativa*, commonly known as black cumin is an amazing herb. Several studies indicated that *Nigella sativa* and/or its major bioactive compound 'Thymoquinone (TQ)" has potent antioxidant, anti-inflammatory, cardio-protective, anti-cancer, anti-diabetic, anti-anemic, anti-microbial, cytoprotective and immune-modulatory properties (Aggarwal *et al*., 2008; Alkharfy *et al*., 2011; Ahmad and Beg, 2013; AbuKhader, 2013).

Recently, TQ has shown to counteract the development of disease-induced anemia (Ayinla *et al*.,

2010) as well as in preventing oxidative stress and cell damage in circulating erythrocytes (Harzallah *et al*., 2012). Therefore, the present study was designed to investigate the possible favorable hematinic and anti-anemic effects of TQ supplementation on a rat model of PHZ-induced hemolytic anemia.

MATERIALS AND METHODS

Animals, treatments and experimental design: A total of forty eight adult male Wistar rats, weighing 210±20 g, were used in all the experiments. The rats were housed in metabolic cages with a 12 h light/dark cycle. They had continuous access to food and water during the entire period of experimentation. This study was approved by the Local Animal Care Committee of Umm Al-Qura University, KSA and carried out in accordance with the Guide for the Care and Use of Laboratory Animals published by the US Institute for Labortary Animal Research (1996).

At day 0, rats were randomly classified into the following 3 group (16 rats per group): normal control group, Phenylhydrazine (PHZ) group and Phenylhydrazine+Thymoquinone (PHZ+TQ) group. For induction of the model of hemolytic anemia, PHZ (Sigma-Aldrich Chemical Company; St. Louis, MO, USA) was dissolved in saline and injected by the peritoneal route at 40 mg kg^{-1} body weight on day 0 and two additional injections were given at 9 am and 6 pm, on day 1 as described previously (Moreau *et al*., 2012). TQ (Sigma-Aldrich) was given orally by gastric gavage at a daily dose of 15 mg kg^{-1} body weight.

Blood sampling and analysis: Under diethyl ether anesthesia, 6 rats of each group were sacrificed at days 3, 5 and 8. At each sacrification time point, two blood samples were immediately with drawn from the vena cava of each rat. The first sample was collected into a tube contained disodium salt of Ethylene Diamine Tetra Acetic Acid (EDTA) anticoagulant and used for assessment of the following erythrocyte indices: Red Blood Cell (RBC) count, Hemoglobin (HGB) concentration and Hematocrit (HCT) percentage. These parameters were quantified by the standard hematological measurements using an automatic hematological assay analyzer (Beckman Coulter, USA). The second blood sample was collected into a plain centrifuge tube without any anticoagulant and after centrifugation process and then its corresponding serum was obtained and used for measurement the sera concentrations of heme and the biomarkers of anti-oxidation and oxidative stress.

Concentrations of heme in the sera samples were determined by the Pyridine Hemochrome Method as previously described (Moreau *et al*., 2012) and the heme concentration of each sample was measured from absorbance intensity at 556 nm based on a pyridine hemochrome calibration curve determined from hemin.

The serum levels of total reduced Glutathione (GSH) and activities of Superoxide Dismutase (SOD) (as indices of non-enzymatic and enzymatic antioxidant status, respectively) as well as the concentrations of Thiobarbituric Acid Reactive Substances (TBARS) (as indices of lipid peroxidation and oxidative stress) were determined by using Specific Commercial kits (Cayman Chemical; Ann Arbor, Michigan, USA) and all samples were processed in duplicate and according the manufacturer's instructions.

Statistical analysis: The results were expressed as the Mean±Standard Deviation (SD) and statistical analysis was carried out using SPSS Software, Version 16.0 (SPSS Inc., Chicago, IL, USA). Differences among the groups were investigated using one-way Analysis of Variance (ANOVA) followed by a Student's t-test. $p<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Hematological data showed that there was significant drop in Hemoglobin (HGB) concentrations, RBCs counts and Hematocrit (HCT) values in rats at day 3 and 5 post-injection of PHZ (Table 1 and 2) and these drops in RBCs, HGB and HCT were also accompanied by a significant increases in reticulocytes percentage (Table 1 and 2). At the end of the study (i.e., at day 8) all these hematological adverse effects of PHZ were also observed (Table 3) but at less degree than those at days 3 and 5. On the other hand, treatment with TQ had significantly reversed the deteriorating effects of PHZ on the values of RBCs, HGB and HCT at each time point of analysis, however, it trended to elevate the reticulocytes increasing that occurred secondary to PHZ administration

Table 1: Hematological findings and serum levels of antioxidant and lipid peroxidation parameters at day 3 post Phenylhydrazine (PHZ) injection

Parameters	Normal controls	PHZ group	PHZ+TQ group
RBC (10^6 μL^{-1})	8.52±0.28	4.82±0.65*	7.45±0.21#
HGB (g dL^{-1})	12.21±0.57	6.83±0.43*	11.87±0.62#
HCT (%)	46.22±3.45	30.33±1.22*	41.45±2.11#
Reticulocytes (%)	2.45±0.17	31.00±2.11*	34.06±4.75*
GSH (μmol/mg protein)	20.70±2.10	3.40±0.30*	18.30±1.13#
SOD (U/mg protein)	4.10±0.20	1.03±0.20*	3.70±0.50#
TBARS (nmol/mg protein)	20.50±3.10	1130.50±37.9*	58.70±7.23#

The values are means±SD and n = 6 for each group. *$p<0.05$ versus control group; #$p<0.05$ versus PHZ untreated group. TQ: Thymoquinone; RBC: Red Blood Cell; HGB: Hemoglobin; HCT: Hematocrit; GSH: Total Glutathione; SOD: Superoxide Dismutase and TBARS: Thiobarbituric Acid Reactive Substances

Table 2: Hematological findings and serum levels of antioxidant and lipid peroxidation parameters at day 5 post Phenylhydrazine (PHZ) injection

Parameters	Normal controls	PHZ group	PHZ+TQ group
RBC (10^6 μL^{-1})	8.41±0.71	5.17±0.370*	8.16±0.25#
HGB (g dL^{-1})	12.83±0.67	6.23±0.500*	12.02±0.27#
HCT (%)	45.06±1.18	33.11±1.140*	46.45±1.33#
Reticulocytes (%)	2.83±0.81	27.00±1.310*	32.08±2.33*
GSH (μmol/mg protein)	18.50±1.50	4.20±0.300*	19.20±2.10#
SOD (U/mg protein)	4.80±0.20	1.20±0.200*	4.10±0.80#
TBARS (nmol/mg protein)	21.40±4.20	1008.30±137.4*	45.70±4.70#

Table 3: Hematological findings and serum levels of antioxidant and lipid peroxidation parameters at day 8 post Phenylhydrazine (PHZ) injection

Parameters	Normal controls	PHZ group	PHZ+TQ group
RBC (10^6 μL^{-1})	8.27±0.17	6.27±0.38*	8.78±0.11#
HGB (g dL^{-1})	11.93±0.74	9.18±0.44*	12.13±0.16#
HCT (%)	46.77±2.05	38.45±1.28*	47.03±1.16#
Reticulocytes (%)	2.87±0.83	12.00±0.51*	17.11±1.22*
GSH (μmol/mg protein)	19.44±2.30	8.70±0.90*	22.10±3.20#
SOD (U/mg protein)	4.11±0.80	2.50±0.20*	5.30±0.70#
TBARS (nmol/mg protein)	23.10±2.20	468.00±22.9*	27.60±3.30#

The values are means±SD and n = 6 for each group. *$p<0.05$ versus control group; #$p<0.05$ versus PHZ untreated group. TQ: Thymoquinone; RBC: Red Blood Cell; HGB: Hemoglobin; HCT: Hematocrit; GSH: Total Glutathione; SOD: Superoxide Dismutase and TBARS: Thiobarbituric Acid Reactive Substances

(Table 1-3). Moreover, intravascular hemolysis of RBCs is associated with release of their hemoglobin with subsequent accumulation of a free heme. In support, an increased serum concentration of heme was significantly observed in PHZ-received rats, particularly at days 3 and 5 after PHZ injection and it was significantly reversed by TQ therapy (Fig. 1). In summary, anemia was rapidly induced in PHZ-injected rats and was significantly prevented by TQ supplementation therapy.

One of the major mechanisms by which PHZ induced hemolytic anemia is induction of oxidative stress. Therefore, the levels of GSH (an example of non-enzymatic antioxidant defense mechanism), activities of SOD (an example of enzymatic antioxidant defense mechanism) and concentrations of TBARS (an index of lipid perioxidation and oxidative stress) were measured in the sera samples of all animal groups at days 3, 5 and 8 post-PHZ injection. As demonstrated in Table 1-3, injection of PHZ into rats had associated with significant reduction in serum GSH content and SOD activity as well as marked elevation in serum TBARS content. By contrast, concurrent administration of TQ with PHZ had obviously counteracted these altering effects of PHZ on GSH, SOD and TBARS in all tested sera samples (Table 1-3).

Herein, the anti-anemic effect of Thymoquinone (TQ) supplementation therapy was investigated in a rat model of hemolytic anemia induced by Phenylhydrazine (PHZ). The results reveal that administration of PHZ at

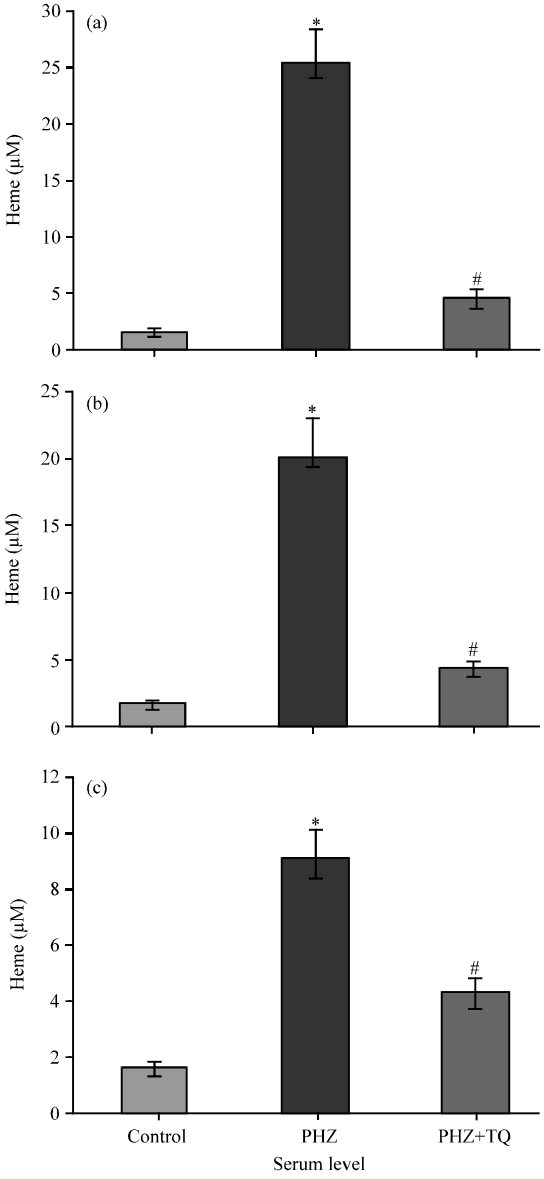

Fig. 1: Serum levels of heme at a) day 3, b) day 5 and c) day 8 post-PHZ injection. PHZ: Phenylhydrazine; TQ: Thymoquinone. The values are means±SD and n = 6 for each group. $p<0.05$ versus control group; #$p<0.05$ versus PHZ untreated group

120 mg kg^{-1} divided into 2 days resulted in development of acute hemolytic anemia reflected by significant drop in HGB concentrations, RBCs count and HCT values, particularly at days 3 and 5 post-injection of PHZ (Table 1-3) and these drops in RBCs, HGB and HCT were also accompanied by a significant increases in reticulocytosis and serum heme concentration (Table 1 and 2 and Fig. 1). These findings are in consistency with those previously reported in other

species regarding PHZ hematotoxicity (Agbor *et al*., 2005; Berger, 2007; Moreau *et al*., 2012). On the other hand, simultaneous treatment of rats with TQ (15 mg/kg/day) had significantly reversed the deteriorating effects of PHZ on the values of RBCs, HGB, HCT and heme at each time point of analysis, however, it trended to elevate the reticulocytes increasing that occurred secondary to PHZ administration (Table 1-3 and Fig. 1). These hematoprotective and anti-anemic favorable effects of TQ have also been reported previously in other disease models. In this concept, Ayinla *et al*. (2010) and Meral *et al*. (2004) showed that treatment with extracts of *Nigella sativa* (the plant from which TQ is derived) significantly reversed the decreasing on RBCs counts, HGB concentrations and HCT% as well as susceptibility of RBC to haemolysis in diabetic rats and diabetic rabbits, respectively. More recently, TQ therapy remarkably attenuated erythrocyte oxidative damage and significantly corrected the decline of RBCs count, HGB concentration and HCT values that were induced in rats with colon cancer (Harzallah *et al*., 2012). Taken together, anemia was rapidly induced in PHZ-injected rats and was significantly prevented by TQ supplementation therapy suggesting the favorable hematinic and anti-anemic effect of TQ.

In the present study, PHZ induced decreases in RBCs count and HGB concentration was accompanied by a marked pheripheral reticulocytosis and treatment with TQ had resulted in a further increase in reticulocyte percentage when compared with normal control rats (Table 1-3). Reticulocytes are immature RBC, develop in the red bone marrow and then circulate in the blood stream and convert into mature red blood cells. Like mature red blood cells, reticulocytes do not have a cell nucleus and they are called reticulocytes because of a reticular (mesh-like) network of ribosomal RNA (Davis *et al*., 1954). In human beings, when there is an increased production of red blood cells to overcome chronic or severe loss of mature red blood cells such as in a hemorrhagic or haemolytic anemia, there is often a markedly high number and percentage of reticulocytes (Mohandas and Gallagher, 2008; Malleret *et al*., 2013). Therefore, the marked increase in reticulocytes that was observed could constitute one of the erythropoietic regenerative responses following PHZ-induced acute hemolytic anemia.

In this study, PHZ-received rats generated high concentrations of free heme and TBARS (an index of lipid perioxidation and oxidative stress) and low levels of levels of both GSH (an example of non-enzymatic antioxidant defense mechanism), activities of SOD (an example of enzymatic antioxidant defense mechanism) in their sera particularly after 3-5 days from PHZ injection (Table 1-3 and Fig. 1). By contrast, concurrent administration of TQ with PHZ had obviously counteracted these altering effects of PHZ on heme, GSH, SOD and TBARS in all tested sera samples (Table 1-3 and Fig. 1). As in malaria, robust hemolysis, high concentrations of free heme and oxidative damage are induced in PHZ-treated animals (Maines and Veltman, 1984; Moreau *et al*., 2012). Acute hemolysis of RBCs is accompanied by a rapid saturation of hemoglobin/heme scavenging and concomitant accumulation of intravascular free heme (Kumar and Bandyopadhyay, 2005). More importantly, this accumulated free hem has toxic effects to cells and organs, promoting lipid peroxidation and the production of Reactive Oxygen Species (ROS) resulting in membrane injury, DNA damage and cell apoptosis (Ryter and Tyrrell, 2000; Khan and Quigley, 2011). Free heme also acts as a pro-inflammatory molecule and heme-induced inflammation is involved in the pathology of diverse conditions (Kumar and Bandyopadhyay, 2005). In this respect, increased concentrations of heme have also been significantly observed in PHZ-received mice (Moreau *et al*., 2012). Also, in agreement with the present data, previous studies demonstrated that induction of oxidative stress and lipid peroxidation is the principal mechanism in PHZ-induced hemolytic anemia (Jollow and McMillan, 2001; Latunde-Dada *et al*., 2006; Berger, 2007; Moreau *et al*., 2012). As the powerful free radical scavenger and antioxidant property of TQ is now well documented (AbuKhader, 2013), the ameliorating effects of TQ therapy against PHZ hematotoxicity could be attributed to its antioxidant activity.

CONCLUSION

The collective data of the present study revealed that TQ supplementation has a hematinic, anti-anemic and antioxidant potential in a rat model of oxidative stress hemolytic anemia induced by PHZ. This in turn may pave the way to use TQ as part in management of drugs and chemicals induced-hemolytic anemia. However, further studies are required to precisely define such anti-anemic property of TQ.

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