Amelioration of cardiotoxic impacts of diclofenac sodium by vitamin B complex

N.A. ABDULMAJEED¹, H.S. ALNAHDI¹, N.O. AYAS¹, A.M. MOHAMED¹,²

¹Biochemistry Department, Faculty of Science for Girls, King Abdulaziz University, Jeddah, Kingdom of Saudi Arabia
²Therapeutic Chemistry Department, National Research Center, Dokki, Egypt

Introduction

Diclofenac Sodium (Voltaren) is one of the most widely prescribed nonsteroidal anti-inflammatory drugs (NSAIDs) in the world. It is used mainly to relieve symptoms across multiple clinical indications, including inflammation, pain, osteoarthritis, rheumatoid arthritis and ankylosing spondylitis¹. NSAIDs are characterized by the ability to inhibit cyclo-oxygenase enzymes, both the COX-1 and COX-2 isoenzymes. COX-1 and COX-2 catalyze the conversion of arachidonic acid to eicosanoids, which play an important role in the maintenance of gastrointestinal, renal, and cardiovascular homeostasis². Nonselective NSAIDs inhibit platelets in a reversible and incomplete manner via COX-1 inhibition³. In recent years, the safety of NSAIDs use in clinical practice has been questioned. The evidence on the increase in cardiovascular risk with the use of NSAIDs is still scarce, due to the lack of randomized and controlled studies with the capacity of evaluating relevant cardiovascular outcomes. However, the results of prospective clinical trials and meta-analyses indicate that these drugs cause important adverse cardiovascular effects⁴-⁵, which include increased risk of myocardial infarction⁶, ischemic heart diseases⁷, heart failure⁸, and arterial hypertension⁹. Over the last years, evidence has accumulated showing that oxidative stress induced by NSAIDs plays an important role in the pathogenesis of cardiovascular disease¹⁰.

In the absence of reliable cardio-protective drugs in allopathic medical practices, B vitamins plays a major and an important role in the management of various cardiac disorders¹¹-¹³.

The B vitamins are water-soluble vitamins required as cofactors for enzymes essential in cell function and energy production. Several studies have been investigated for the therapeutic mechanisms of B vitamins in experimental and clini-
Thiamin (vitamins B1) is a coenzyme for a mitochondrial alpha-ketoglutarate and pyruvate dehydrogenases that are part of the multi-enzyme complexes which form a part of the citric acid cycle. It also acts as a cofactor of transketolase (TK), a reversible cytosolic enzyme that catalyzes the first and last step of the pentose phosphate pathway which plays a major role in the production of NADPH for maintaining cellular redox, glutathione (GSH) levels and protein sulphhydryl groups. Vitamin B1 therapy can counter the risk factors of cardiovascular diseases. It can attenuate the development of streptozotocin-induced diabetes and its complications, such as dyslipidemia and atherosclerosis in rodent models. Vitamin B1 can act directly as an antioxidant, it prevents microsomal lipid peroxidation as well as oleic acid oxidation. Vitamin B1 also prevented cytotoxicity, ATP depletion and formation of ROS. It decreases mitochondrial, protein and DNA oxidative damage induced by the mitochondrial respiratory inhibitors or by iron-catalyzed oxidative damage. Vitamin B1 supplementation protects NADP+-dependent dehydrogenase activities that supplies NADPH which helps in maintaining GSH in the reduced form.

Vitamin B6, the collective name given to pyridoxine, pyridoxamine, pyridoxal and their phosphorylated derivatives, is an essential cofactor for numerous enzymatic reactions. It acts as a cofactor for enzymes involved in decarboxylation, transamination, deamination, racemization and trans-sulfuration reactions. It was used as a therapeutic agent in the treatment of diabetes mellitus, epilepsy and cardiovascular disease. The antioxidant and radical scavenging properties of the B6 vitamin have been previously documented. It has a potential role in reducing oxidative stress markers associated with homocysteine or in preventing ROS formation and lipid peroxidation in a cellular model.

Cobalamins (Cbl; vitamin B12 derivatives) are micronutrients essential as a co-factor for the synthesis of methylcobalamin (MeCbl) and adenosylcobalamin (AdoCbl), the respective co-factors for cytosolic methionine synthase (MS) and mitochondrial L-methylmalonyl-CoA mutase. It has fundamental therapeutic roles in the treatment of different pathological conditions. It modulates the immune response. High doses of Cbl have been used to treat pernicious anemia for decades with no apparent toxicity. Cbl supplementation is beneficial in treating many inflammatory diseases, and can protect against oxidative stress-associated pathologies. Cbl therapy normalizes levels of TNF-α and epidermal growth factor in Cbl-deficient patients. It can act as a second-line of defense when O2•- production overwhelms the SOD protection system, a mechanism by which Cbl can protect cells against oxidative stress. Beside, vitamin supplements containing cyanocobalamin (CN Cbl, vitamin B12) decrease low-density lipoprotein oxidation in both healthy individuals and patients with coronary artery disease.

The current study was undertaken to study the prophylactic potential action of vitamin B complex (vitamins B1, B6 and B12) against risk factors for cardiac tissue damage induced by NSAID, diclofenac sodium toxicity.

**Materials and Methods**

**Chemicals**

All chemicals used were of high analytical grade, product of Sigma and Merck Companies. Kits used for the quantitative determination of different parameters were purchased from Biogamma, Stanbio, West Germany. Vitamin B complex injection (Merck Company) with National Agency for Food and Drug Administration and Control (NAFDAC) registration no. 70/04059/07 containing B1 (100 mg), B6 (100 mg) and B12 (1.0 mg) per 3 ml. Diclofenac sodium (Voltaren) injection (Novartis Company, NAFDAC no. 81-11-80) was used for the study.

**Animals and Treatments**

Animal experiments were performed with approval from the Local Ethics Committee.

50 Wistar male albino rats (120-150 g) were used for the study. The animals were obtained from Experimental Animal Center, King Abdel-Aziz University. The animals were housed in clean polypropylene cages and maintained in an air-conditioned animal house at 20 ± 2°C, 50-70% relative humidity and 12-h light/dark cycle. The animals were provided with commercial rat pellet diet and deionized water ad libitum. Animal utilization protocols were performed in ac-
Amelioration of cardiotoxic impacts of diclofenac sodium by vitamin B complex

Biochemical Cardiac Tissue Analysis

Phosphoglucone isomerase (PGI) activity was measured in a reaction medium containing tris-HCl buffer (0.2M, pH 7.4), fructose-6-phosphate (5 mM), MgCl2 (10 mM), NADP (0.2 mM). The increase in extinction at 340 due to NADPH production was recorded (40). LDH activity was evaluated in a reaction mixture containing tris buffer (50 Mm, pH, 7.5), sodium pyruvate (0.6 mM) and NADH (0.18 mM). The rate of NADH consumption is determined at 340 nm and is directly proportional to the LDH activity41. CAT was determined by monitoring the decomposition of hydrogen peroxide as described by Aebi (42). GR activity was measured by the modified method of Erden and Bor43. The reaction mixture contained the following in the final concentration: 4.1 mM Tris-HCl, pH 7.5, 15 mM MgCl2, 5.7 mM EDTA, 60 mM KCl, 2.6 IU GSSG and 0.2 mM of NADPH in final reaction volume of 1 ml. The reaction was started by the addition of tissue extract containing approximately 100 micro-gram of protein. The decrease in absorbance was monitored at 340 nm. G-6-PDH activity was assayed in a reaction mixture contained triethanolamine buffer (86 mM, pH 7.6), MgCl2 (6.9 mM), glucose-6-phosphate (1 mM), NADP (0.39 mM). The reduction of NADP was followed at 340 nm44.

Statistical Analysis

Data were analyzed by comparing values for different treatment groups with the values for individual controls. Results are expressed as mean ± SD. The significant differences among values were analyzed using analysis of variance (one-way Anova) followed by Bonferroni as a post-ANOVA test. Results were considered significant at $p < 0.05$.

Results

Serum cardiac damage markers, namely AST and, CK-MB, in the normal and different experimental rat groups intoxicated with either low or high repeated dose of diclofenac sodium are shown in Figure 1. The results showed that injection of the two toxic doses of diclofenac induced pronounced increases in these biomarkers compared with normal animals, and the injection of vitamin B complex simultaneously with diclofenac administration significantly down-modulated the deviation in these markers.
The activities of some glycolytic enzymes in rat cardiac tissue including, PGI and LDH in the normal and different experimental rat groups intoxicated with either low or high repeated dose of diclofenac sodium are illustrated in Figure 2. These enzymes were markedly decreased in the cardiac of rats intoxicated with either of the two repeated doses of diclofenac compared with the normal group. Co-administration of vitamin B complex with diclofenac injection markedly ameliorated the alteration in these enzyme activities compared with the intoxicated animals.

The levels of some antioxidant biomarkers namely G6PD, GR and CAT significantly decreased in the cardiac of rats intoxicated with either of the two doses of diclofenac compared with normal animals (Table I). Co-administration of vitamin B complex, greatly up-modulated the
The current study revealed that both repeated doses of diclofenac sodium (1.5 mg/kg and 3 mg/kg) induced cardiotoxicity as indicated by elevations in serum cardiac damage markers, namely CPK-MB and AST and reductions in glycolytic enzymes (PGI and LDH) in cardiac muscles compared with normal animals. The alteration in these enzymes was more evident in rats injected with higher dose of diclofenac which may reflect the severity of damaging impact of this NSAID was dose dependent. The cardiotoxicity impact of diclofenac sodium was previously documented in experimental animal model by biochemical and histopathological studies (45). Some studies demonstrated that metabolism of diclofenac by CYP2C9, CYP3A4 and UGT2B7 is the most critical in diclofenac toxicity assessment due to the formation of reactive metabolites (46). The prognostic value of the studied biomarkers has been proven to be high in terms of predicting adverse cardiovascular events, such as death or myocardial infarction (47-48).

CK catalyzes the transfer of phosphate to adenosine diphosphate, producing adenosine triphosphate, which serves as an energy source for many tissues, including muscle. Three different isoenzymes of CK have been identified: CK-MM, CK-BB, and CK-MB. CK-MB may be regarded as a sensitive and specific marker for myocardial infarction (47). Elevated serum AST level in human and animals has also been previously reported for diclofenac (49-50). CK-MB and AST are released by damaged heart tissue and are frequently used as diagnostic markers for myocardial infarction (51). In 2000, the European Society of Cardiology and the American College of Cardiology published a new definition of infarction based on an elevation of these enzymes as one criterion (52). Levels of cardiac damage markers...
may be elevated as early as 4-6 h following the damage inducing event\textsuperscript{53}. Also, several fold decrease of glycolytic enzymes in liver, kidney and testis was previously reported in rats treated with sod diclofenac which can be taken as toxic manifestation of the drug\textsuperscript{54}.

Co-injection of vitamin B complex to diclofenac intoxicated rats with either of the two repeated doses effectively ameliorated the cardiac function biomarkers. This positive response obtained by the used vitamin complex may attributed to its ability to protect and stabilize cel-
lular membranes by manipulating the diclofenac toxicity. The anti-toxic and the cardio-protective effects of B vitamins was previously ensured. Vitamins B1, B6 and B12 was reported to have beneficial roles in preventing cytotoxicity, formation of reactive oxygen species (ROS), lipid peroxidation and ATP depletion which have the major in tissue injury and cell death. Oxidative damage in the cell or tissue occurs when the concentration of ROS generated exceeds the antioxidant capability of the cell or when the antioxidant capacity of the cell decreases. Some authors demonstrated the involvement of oxidative stress during diclofenac-mediated tissue toxicity. Levels of GSH metabolizing enzymes (GR and G-6-PDH) and CAT are the main determinants of the antioxidant defense mechanism of the cell.

The current study showed that either repeated low or high dose of diclofenac induced oxidative stress in cardiac tissues of rats as evidenced by significant reduction in the activities of reduced glutathione (GSH) metabolizing enzymes, GR and G-6-PDH as well as CAT in cardiac tissues of diclofenac intoxicated rats versus normal healthy ones. This effect was a dose dependent and may consider one of the diclofenac mechanisms induced cardiotoxicity. GR is the key enzyme in the conversion of oxidized glutathione (GSSG) back to the reduced form (GSH). G-6-PDH, a rate limiting enzyme of the pentose phosphate pathway, is required for NADPH generation which is needed for the maintenance of GSH in its reduced form. GSH, a non-enzymatic antioxidant, has an important role in scavenging the electrophilic moieties produced by toxic chemicals and conjugate them to less toxic products. CAT is an antioxidant enzyme widely distributed in all animal tissues. It decomposes hydrogen peroxide and protects the tissue from highly reactive hydroxyl radicals. Thus the less amount of GSH production due to inhibition of its metabolizing enzymes together with reduction of CAT activity in cardiac of diclofenac intoxicated rats may reduce the capacity of the tissue to protect itself from the diclofenac induced oxidative tissue damage. Our result is supported by some authors who stated the reduction in the levels of hepatic and renal enzymatic and non-enzymatic antioxidants of animals injected with diclofenac. Administration of vitamin B complex prevented the cardiac antioxidant depletion which was more evidenced in rats intoxicated with low diclofenac repeated dose. The maintenance of antioxidant capacity in protecting the cardiac tissue against oxidative stress by B vitamins may consider one of its cardioprotective mechanisms. The antioxidant, antioxidative stress and free radical scavenging potential actions of vitamin B1, vitamin B6 and vitamin B12 were previously documented. Beside, vitamin B1 was found to have the ability to protect NADP+-dependent dehydrogenase activities that supplies NADPH which helps in maintaining GSH in the reduced form.

Previous studies showed that metabolic disorder such as hyperglycemia and hyperlipidemia were principle risk factors for cardiovascular disease. In line with other NSAID, the current study demonstrated that injection of rats with either low or high repeated dose of diclofenac caused hyperglycemia and hyperlipidemia which represented by marked increase in serum glucose and lipid profiles including TG, TCh and LDL-C with a concomitant decreased in HDL-C in relation to control ones. This metabolic disorder induced by the used drug was severe in rats injected with high diclofenac repeated dose. The increase in serum glucose in diclofenac treated rats may be reflected that the drug either affected the insulin receptors due to its cytotoxicity and/or it has damaging impact on pancreas. The latter suggestion is supported by previous studies reported that treatment with diclofenac was associated with acute pancreatitis which is the major cause of impaired glucose metabolism and hyperglycemia. However, hyperlipidemia induced by diclofenac injection may be explained on the basis that enhancement of lipolysis by diclofenac, leading to increased concentration of plasma free fatty acids. The stimulating effect of diclofenac on lipolysis may related to its potent inhibitory effect on prostaglandins synthesis that are involved in the feed-back regulation of lipolysis, and mediate the inhibitory effect on lipolysis of lipoprotein lipase activity. High plasma FFAs may consider the major cause of hyperlipidemia through influxing into liver for lipoprotein synthesis and production. On the other hand, it was found that high plasma FFAs have been shown repeatedly to induce insulin resistance which has the principle role of elevated blood glucose and hyperlipidemia. Dresner et al stated that in humans, high plasma concentrations of FFAs decrease insulin receptor substrate-1-associated PI3-kinase activity and glucose transport in...
skeletal muscle. Additionally, some studies revealed that hyperlipidemia (include hypertriglyceridemia, hypercholesterolemia, high of LDL and low of HDL concentrations in blood) is a well-recognized feature of ir and hyperglycemia and has been well documented in animal models and in humans with ir 

Specifically with respect to cardiac disease, hyperglycemia and insulin resistance have been associated with left ventricular hypertrophy and diastolic dysfunction. In addition, varieties of adverse deleterious impacts associated with hyperglycemia have been reported, including direct effects of hyperglycemia, consequences from hyperinsulinemia, or associated metabolic changes such as increased FFA. Hyperglycemia may induce nonenzymatic protein glycosylation, protein kinase C activation, oxidative stress, and increased TNF-α with consequences that may include myocyte apoptosis and fibrosis. Hyperinsulinemia has been associated with collagen deposition and myocardial fibrosis. High free fatty acid levels have cardiotoxic effects including disruption of plasma membrane integrity, elevation of intracellular calcium, and increased sympathetic activity. Beside, previous works demonstrated that acute elevation of plasma FFAs induces inflammation, oxidative stress, activates the nuclear factor-κB pathway, impairs endothelium-dependent vasodilation, and blunts insulin-mediated vasodilation and NO production in humans. Additionally, FFAs could also contribute to endothelial dysfunction by triggering endothelial cell apoptosis and inhibiting cell cycle progression.

In addition, hypercholesterolemia and high level of LDL-C with a decrease level of HDL-C induced in rats by diclofenac injection, may considered other serious risk factors and important early events in the pathogenesis of atherosclerosis in both peripheral and coronary circulation. Lipid compounds and products of their oxidation especially LDL-C accumulate during formation of atherosclerotic lesions. LDL-C functions in the atheroma formation whereas the HDL-C plays an important role in inhibiting the formation of atheroma. The antiatherosclerotic action of HDL consists in its ability to remove cholesterol from vascular wall, stimulate prostacyclin formation and inhibit the synthesis of adhesive molecules. Furthermore, some studies have linked hypertriglyceridemia to higher serum small dense LDL particles, atherothrombosis and impaired endothelial function, the hallmarks of several prevalent cardiovascular diseases as well as their complications. These results may explain the adverse cardiovascular effects with the use of NSAIDs.

Lowering the plasma lipid levels through dietary or drug therapy may be beneficial in decreasing the risk of vascular disease.

Co-injection of vitamin B complex along with diclofenac administration to rats, effectively prevented diclofenac induce metabolic disorder in glucose and lipid profiles. The B complex successfully normalized their levels in rats injected with low dose of diclofenac indicating its hypoglycemic and hypolipidemic effects. This beneficial action of vitamin B complex were previously documented. It was reported that vitamin B1 mitigated metabolic disorders in hypertensive rats through regulating blood glucose, TGs and total cholesterol levels suggesting the deterrence of ir and hyperlipidemia by treatment with vitamin B1. Vitamin B1 therapy can also counter the development of streptozotocin-induced diabetes as well as complications, such as dyslipidemia. Also, the dietary intake of vitamin B6 reduced serum total cholesterol level. Additionally, vitamin supplements containing vitamin B12 decrease LDL oxidation in both healthy individuals and patients with coronary artery disease.

Conclusions

From the current investigation, it can be concluded that the prophylactic administration of vitamin B complex along with diclofenac sodium administration may be beneficial in ameliorating its toxic side effects induced cardiac tissue damage.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

References


Amelioration of cardiotoxic impacts of diclofenac sodium by vitamin B complex


5) Minuz P. Nonsteroidal anti-inflammatory drugs and cardiovascular risk: is prostacyclin inhibition the key event? J Am Coll Cardiol 2008; 52: 1637-1639.


46) DALY AK, AITHAL GP, LEATHART HB, SWAINSBURY RA, DANG TS, DAY CP. Genetic susceptibility to diclofenac-induced hepatotoxicity: contribution of UGT2B7, CYP2C8, and ABC2C2 genotypes. Gastroenterol 2007; 132: 272-281.


56) HICKEY EJ, RAJE RR, REID VE, GROSS SM, RAY SD. Diclofenac induced in vivo nephrotoxicity may involve oxidative stress mediated massive genomic DNA fragmentation and apoptotic cell death. Free Radic Biol Med 2001; 31: 139-152.


Amelioration of cardiotoxic impacts of diclofenac sodium by vitamin B complex