# AZT-Induced Oxidative Cardiovascular Toxicity: Attenuation by Mg-Supplementation

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## Abstract

Cardiovascular effects of chronic AZT treatment on SD male rats (185 g) fed either a normal Mg diet (0.1% MgO) or a high Mg diet (0.6% MgO) were examined. AZT treatment (1 mg/ml drinking water) for 3 weeks led to a 5.5-fold ($0.88 \pm 0.11$ nmol/min/$10^6$ cells, $P < 0.05$) elevation in neutrophil basal activity of $O_2^-$ production versus controls ($0.16 \pm 0.03$ nmol/min, assayed ex vivo as SOD-inhibitable cytochrome $c$ reduction). Concomitantly, plasma 8-isoprostane and $PGE_2$ levels rose 2.1-fold and 3-fold (both $P < 0.05$), respectively, compared to control; however, RBC GSH decreased 28% ($P < 0.02$) with GSSG content increased 3-fold, indicative of systemic oxidative stress. High Mg diet substantially attenuated the AZT-induced neutrophil activation by 70% ($0.26 \pm 0.05$ nmol/min, $P < 0.05$); reduced plasma 8-isoprostane and $PGE_2$ to levels comparable to normal; and RBC GSH was restored back to 92% of control. AZT alone caused moderate, but significant vascular inflammatory lesions in the heart (assessed by H&E staining). Immunohistochemical staining revealed significantly higher (about 4-fold) infiltration of CD11b positive cells (WBC surface marker) in the atria and ventricles of AZT-treated rats. However, these inflammatory pathological markers were minimal in samples of rats treated with AZT plus high Mg diet. Moreover, AZT alone significantly ($P < 0.02$) decreased rat weight gain by 21% at 3 weeks; Mg-supplementation completely prevented ($P < 0.05$) the weight gain loss due to AZT intake. It is concluded that high dietary Mg may provide beneficial effects against AZT toxicity due to its systemic antioxidative/anti-inflammatory properties.

### Keywords

AZT toxicity; Neutrophil activation; Oxidative stress; Cardiac inflammation; Mg-supplementation

## Introduction

Azidothymidine (AZT, Zidovudine) was the first approved nucleoside reverse transcriptase inhibitor (NRTIs) drug showing clinical efficacy against the human immunodeficiency virus (HIV) [1, 2]; it inhibits in vitro replication of some retroviruses including HIV, and is a thymidine analog in which the 3′-OH group is replaced by an azido (–N$_3$) group [3]. Unfortunately, its use has also been associated with considerable toxicity, causing macrocytic anemia, neutropenia, and myopathy [1, 2, 4–6]. Chronic AZT toxicity is generally attributed to damaged mitochondrial mtDNA synthesis resulting in mitochondrial myopathy with ultra-structural changes in heart and skeletal muscle in both animals and

humans [4–7]. However, Szabados et al. [8] reported that short-term treatment of rats with AZT increased reactive oxygen species (ROS) and peroxynitrite production in the rat heart before any significant changes were observed in mtDNA content. These findings suggested that ROS-mediated processes could be important contributors to the cardiomyopathy resulting from AZT treatment. Since AZT therapy may generate or amplify free radical toxicity, this argues in favor of employing antioxidant supplementation [9–11] during AZT treatment. De la Asuncion et al. [9] showed that mice treated with AZT alone experienced both a moderate loss of skeletal muscle total glutathione (GSH + GSSG) and a 5-fold increase in the GSSG/GSH ratio; this index of glutathione oxidation was substantially attenuated by supra-nutritional doses of the antioxidant vitamins C and E. High-dietary supplementation of Mg has been shown to provide beneficial effects against oxidative vascular pathogenesis in diabetes and cyclosporine-induced nephrotoxicity in rats [12, 13]. Others [14] also showed that Mg-supplements attenuated high cholesterol-induced atherogenesis in a rabbit model. Therefore, we sought to determine if a higher supplement of Mg in the diet of rats provided beneficial effects against AZT-mediated cardiovascular and systemic toxicity in vivo.

## Materials and Methods

### Animal Model, AZT Treatment and Diets

All animal experiments were guided by the principles for the care and use of laboratory animals as recommended by the US Department of Health and Human Services and approved by The George Washington University Animal Care and Use Committee. Male SD rats (185 g) were fed either a normal Mg (0.1% w/w MgO) or a high Mg (0.6% MgO, Teklad Lab., Madison, WI) diet. Use of MgO avoids potential confounding effects presented by the counter-ions found in other inorganic and organic Mg salts. AZT was administered orally in drinking water (1 mg/ml) for up to 3 weeks [15]. Plasma Mg levels were determined by atomic absorption spectroscopy (AA).

### Systemic Oxidative Indices and Neutrophil Activity

At sacrifice, whole blood was obtained by cardiac puncture. Plasma 8-isoprostane and $PGE_2$ (as PGE-metabolites) were assayed by EIA kits obtained from Cayman Chemical (Ann Arbor, MI) [16]. RBC reduced (GSH) and oxidized (GSSG) glutathione levels were determined enzymatically by the DTNB-GSSG reductase method [17, 18]. Neutrophils were isolated by a step-gradient centrifugation method [16, 19]. Superoxide anion production from neutrophils ($0.5–0.75 \times 10^6$/ml) with or without phorbol myristate acetate (PMA, 100 ng/ml) stimulation was assayed in a phosphate buffer (pH 7.6) containing 5 mM glucose, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 75 μM cytochrome $c \pm 50$ μg SOD. The superoxide released was estimated as SOD-inhibitable reduction of cytochrome c using the extinction coefficient: $E_{550} = 2.1 \times 10^4 \, M^{-1} \, cm^{-1}$.

### Histochemical Analysis

Cardiac tissue was rapidly excised, rinsed, quickly embedded in OCT compound, and frozen at −70°C until used. CD11b positive cells in the frozen tissue sections (Cryo-sections, 5–10 μm) were visualized by indirect, immunohistochemical staining using mouse anti-rat CD11b antibody (1:500, Chemical International, Temecula, CA) and Vectastain Elite ABC kit immunoperoxidase system (Vector Laboratories Inc., Burlingame, CA) [20]. In the negative controls, the primary antibody was omitted and they were included in all assays. Samples were examined under an Olympus BX60 microscope and multiple digital images were taken with a digital camera (Evolution Colour MP; Media Cybernetics, Silver Spring, MD). CD11b positive cells were counted in the micrographs and quantification was normalized

based on standardized area obtained with Adobe Photoshop. Hematoxylin–Eosin (H&E) staining was also performed for overall morphology assessment.

### Statistics

Data were reported as means ± SEM of five animals per group. Statistical difference was evaluated by Student's $t$-test between groups, and significance set at $P < 0.05$.

## Results

### Neutrophil Activation by AZT and Effect of Mg-Supplementation

It was suggested that AZT treatment may produce systemic production of ROS [8, 10]. Since neutrophils (PMNs) can be a major cellular source for ROS generation, we examined the relative short-term (3 weeks) effect of AZT treatment in rats on the basal free radical activity of PMNs. As represented by Fig. 1, without stimulation, PMNs isolated from untreated rats receiving normal dietary Mg displayed only a low level of basal superoxide anion generating activity ($0.198 \pm 0.071$ nmol/min/$10^6$ cells). However, the activity of the PMNs was markedly and significantly elevated 4.92-fold by AZT treatment in the normal Mg rats ($0.975 \pm 0.11$ nmol/min/$10^6$ cells, $P < 0.01$). Importantly, dietary supplementation with high MgO (6-fold higher than normal dietary level) effectively attenuated this AZT-induced elevation in basal activity by 70% ($0.422 \pm 0.08$ nmol/min/$10^6$ cells); high Mg alone had no significant effect on basal neutrophil activity. As expected, in the presence of PMA, PMNs from the untreated Mg-normal rats were stimulated and generated a 5.5-fold higher superoxide production rate (Fig. 1). However, PMN samples from AZT-treated rats did not respond further to PMA stimulation. Nevertheless, the responses of PMNs from the high Mg + AZT or the high Mg alone groups to PMA challenge were comparable to that of the untreated Mg normal group.

### AZT-Mediated Changes in RBC Glutathione Status

Previously, we showed that AZT at a dose of 0.7 mg/ml (in drinking water) for 21 days produced only a modest (but non-significant) decrease in total glutathione (GSH + GSSG) in mice receiving normal dietary Mg [15]. In the current study, we examined the effect of AZT administration (1 mg/ml for 21 days) on RBC glutathione status (both GSH and GSSG levels). As indicated by Fig. 1a. AZT alone induced a 28% significant ($P < 0.01$) decrease in GSH in the RBCs from normal Mg rats, and promoted a 2-fold ($P < 0.05$) elevation of the GSSG content ($118 + 21$ vs. $58 \pm 14$ nmol/gm Hgb for the control). As a percentage of the total glutathione, GSSG normally constitutes less than 1% ($0.8 \pm 0.2\%$) in control rats; with the administration of AZT, the GSSG rose significantly ($P < 0.01$) to $2.2 \pm 0.4\%$ of the total RBC total glutathione. When supplemented with high Mg, the AZT-induced GSH decrease was significantly attenuated; the GSH level was restored to 96% of normal controls (Fig. 2). In addition, the RBC GSSG contents of the high Mg + AZT rats, either expressed as absolute quantity or as % of total GSH + GSSG, remained within the normal ranges.

### Effects of AZT and High Mg on Systemic Lipid Peroxidation Indices

As a quantitative index of systemic oxidative stress, levels of $F_2$-like isoprostanes, which derive from non-enzymatic peroxidation of polyunsaturated fatty acids [21], were determined by an 8-Isoprostane Enzyme Immunoassay. The results (Fig. 3a) indicate that AZT treatment of normal Mg rats induced a 2.2-fold higher level of 8-isoprostane ($106 \pm 13$ vs. $49 \pm 4$ pg/ml for the controls), and this was significantly attenuated in the high Mg + AZT group. High Mg diet alone had no effect on the basal level of plasma 8-isoprostane. Previously, we found that AZT promoted COX-derived vasoactive prostanoid products such as $TXA_2$ or $PGE_2$ [15]. In current study, we also found that AZT treatment of normal Mg

rats caused a 3.6-fold higher ($P < 0.01$) plasma $PGE_2$-derived metabolites (PGEM). Again, when fed a high Mg-diet, the enhanced PGEM level induced by AZT was attenuated 70% ($P < 0.05$).

### Effect of AZT on Cardiac Morphology/Pathology

Histochemical analysis of cardiac tissue revealed that AZT treatment caused moderate, but noticeable vascular inflammatory lesions in the atria (by H&E staining) and ventricles (Fig. 4b) of AZT + normal Mg control rats. Immunohistochemical staining revealed further infiltration of monocytes/WBCs (4B, based on cell surface marker CD11b positivity) in these hearts. However, the appearance of this inflammatory marker was substantially reduced in hearts from the AZT + high Mg group (Fig. 4c). Using quantitative morphometric analysis, it was estimated that the relative number of CD11b positive cells (brown staining cells per sq. micron) significantly increased 4- to 5-fold in both atria and ventricles from the normal Mg + AZT group compared to the controls. These increases in CD11b positive cells in both atria ($1.70 \pm 0.21$ vs. $0.43 \pm 0.03$ positive cells/sq. micron $\times$ 10,000) and ventricles ($1.55 \pm 0.15$ cells vs. $0.0.34 \pm .032$ positive cells/sq. micron $\times$ 10,000) were almost completely attenuated by high Mg-supplementation in the AZT treated groups (reduced to $0.52 \pm 0.07$ [atria] and $0.34 \pm 0.04$ cells [ventricles]/same area). High Mg alone (without AZT) had no effects on the levels of positive CD11b cells in either atria or ventricles (Fig. 5).

### Effect of AZT and Mg Supplementation on Weight Gain and Circulating Mg Levels

We used changes in animal weight gain rate as a gross indication of drug toxicity. AZT alone (in normal Mg group) significantly ($P < 0.05$) decreased the rate of animal weight gain by 21% ($68 \pm 6$ g vs. $86 \pm 5$ g/3 weeks) (Table 1); however, Mg-supplementation completely prevented ($90 \pm 5$ g, $P < 0.05$ vs. Ctl + AZT) this lower weight gain induced by AZT intake. These differences in weight gain occurred even though the amount of AZT consumed by the normal Mg and high Mg groups were comparable (Table 1). It was worth noting that Mg supplementation alone or with AZT treatment resulted in significantly higher (33%) circulating Mg levels (Table 1).

## Discussion

In this study, we found that AZT alone caused enhanced basal free radical generation from circulating neutrophils in the normal Mg-rats; this elevation was suppressed by high dietary Mg. This elevated ROS generation from neutrophils may contribute substantially to systemic oxidative stress caused by AZT administration. Previous studies reported that AZT administration in rodents resulted in elevated ROS generation in various tissues, such as the heart, skeletal muscle, liver, and endothelium [7–10]. Since the mitochondrion is the main subcellular target of AZT toxicity [9, 22, 23], it is generally believed that this organelle contributed to the bulk of the elevated ROS generation; both superoxide anions and hydrogen peroxide could derive from AZT-induced partial inhibition of the respiratory electron transport chain in the mitochondria [22, 23]. However, in isolated neutrophils, it is presumed that the NADPH-oxidase system is one of the main sources of superoxide anion generation [24]. It remains to be determined if both the NADPH-oxidase and mitochondrial systems of the neutrophils contribute to the elevated superoxide due to AZT treatment. Interestingly, we found that while the neutrophils displayed elevated basal super-oxide activity, their response to PMA stimulation appeared to be blunted. Since PMA activates neutrophil through protein kinase C (PKC) stimulation [25], we speculate that this PKC activation step, which is Ca-dependent, might be impaired. Further study is required to confirm if this signaling cascade might be altered by AZT treatment.

In association with elevated ROS generation from circulating PMNs, and perhaps from other tissues, it was not surprising to observe changes in other indices of oxidative stress: elevated plasma 8-isoprostane levels, decreased RBC GSH, and increased GSSG. In normal Mg rats, increased $PGE_2$ metabolite was induced by AZT treatment. In our previous studies, dietary Mg-deficiency in rodents resulted in systemic oxidative stress accompanied by elevated COX-derived vasoactive prostanoids such as $PGI_2$, TBX, and $PGE_2$ [16, 18–20]. The $PGE_2$ increase may be attributed to a generalized oxidative activation of the endothelium when exposed to AZT. The toxic effects of AZT on the heart were evident from the appearance of micro-lesion formation (H&E staining), which may be due to the well-known AZT-induced mitochondrial myopathy [8, 9]. However, part of the injury may be a consequence of increased infiltration of inflammatory WBCs (macro-phages/monocytes/neutrophils). Once a CD11b-dependent adhesion interaction between neutrophils and myocytes has been established [26], infiltrating WBCs may become activated and induce direct oxidative injury to cardiac myocytes.

Although AZT cardiotoxicity in HIV patients has been well documented [1, 6, 7], the potential beneficial effects of Mg-supplementation has never been explored. In a historical review of patients with coronary artery disease, it was shown that Mg-supplements improved myocardial metabolism, inhibited tissue calcium accumulation and lessened myocardial cell death [27]. In our own study [28], we found that acute Mg supplementation provided significant protection against post-ischemic cardiac dysfunction and oxidative injury. Others have observed that dietary Mg-supplementation significantly lowered blood pressures in hypertensive patients [29] and in spontaneously hypertensive rats [30]. Since it has been reported that AZT (1 mg/ml) could increase systolic blood pressure in the rat [31], the cardioprotective effects observed here might be contributed, in part, by the anti-hypertensive effects of Mg-supplementation.

In this study, we have demonstrated that dietary supplementation with high Mg suppressed the endogenous activation of circulating neutrophils and the associated indices of oxidative stress. Recent evidence strongly suggests that Ca influx is required for both priming and activation of NADPH oxidase activity in neutrophils [24]. In this study, high dietary Mg resulted in significantly higher (33%) circulating total Mg levels. Since Mg is thought to be a natural "calcium antagonist" [32], the anti-calcium effect of high circulating Mg may contribute to suppression of the Ca-mediated priming and activation of NADPH oxidase in neutrophils from AZT-treated rats. At the cardiac tissue level, the beneficial effects of Mg were further manifested by the attenuation of the WBC infiltration and cardiac inflammatory pathology. A study by Asai et al. [33] indicated that Mg-supplementation attenuated macrophage infiltration in the kidney of animals exposed to cyclosporine A, by inhibiting adhesion molecule elevation. In the current study, it may be presumed that the suppressed WBC infiltration in the heart of the high Mg + AZT animals was also a consequence of the higher circulating Mg. Since Ca is required for COX activation and subsequent $PGE_2$ production [34], one may assume that attenuation of this process was also mediated by the anti-Ca effect of high Mg. Interestingly, the protective effect reported here is similar to that reported in an antioxidant supplementation study (vitamin E + C) of an AZT myopathic mouse model [8]. In that study, AZT treatment decreased skeletal muscle GSH and the GSH/GSSG ratio; antioxidant treatment significantly blunted changes in both of these indices [8]. Previously, we showed that the cardiac pathologic effects induced by AZT were potentiated by dietary Mg-deficiency [15]. Using an isolated endothelial cell model [35], we showed that cellular injury mediated by AZT or select AZT-metabolites was iron-dependent, and that subnormal extracellular Mg (<0.5 mM) augmented and higher Mg >1 mM) attenuated this injury. We suggest that high $Mg^{2+}$ may be able to competitively displace "catalytic iron" from membrane phospholipid binding sites, and thus able to prevent site-specific hydroxyl radical formation [35]. The oxidative toxicity of AZT may also be related

to its influence on tissue distribution of prooxidant metals. Pollack and Weaver [36] reported that AZT treatment (oral, 1–2 mg/ml) of normal mice for 1–4 weeks led to 50% elevations in non-heme liver iron content and a 20% increase in peritoneal macrophage iron. They suggested that AZT interfered with heme synthesis, which may cause upregulation of transferrin receptor synthesis leading to increased cellular iron uptake. Our pilot experiment ($N = 3$) indicated that AZT treatment (1 mg/ml drinking water) caused modest increases in hepatic (~10%) and cardiac (~29%) tissue iron content after 3 weeks of exposure. In a different study, we showed that longer treatment (6 weeks) at a lower dose of AZT (0.5 mg/ml) significantly elevated (1.72-fold, $P < 0.05$) rat liver iron content [37].

In conclusion, we believe that the protective effects of Mg may be partially related to its anti-peroxidative properties. This observation may have potential clinical relevance to HIV patients, who frequently exhibit significant hypomagnesemia [38] as well as disturbances in iron metabolism and tissue distribution [39]. The presence of either or both conditions in HIV patients receiving AZT treatment may pose a further risk of oxidative stress due to iron-driven ROS production. While the mechanisms remain to be further explored, this study clearly demonstrates the protective effects of high dietary Mg against AZT-induced oxidative stress, cardiac pathology, and systemic inflammation in rats. Thus, our findings suggest that Mg-supplementation may warrant consideration as an effective adjunct therapy to combat NRTI toxicity in HIV patients.

## Acknowledgments

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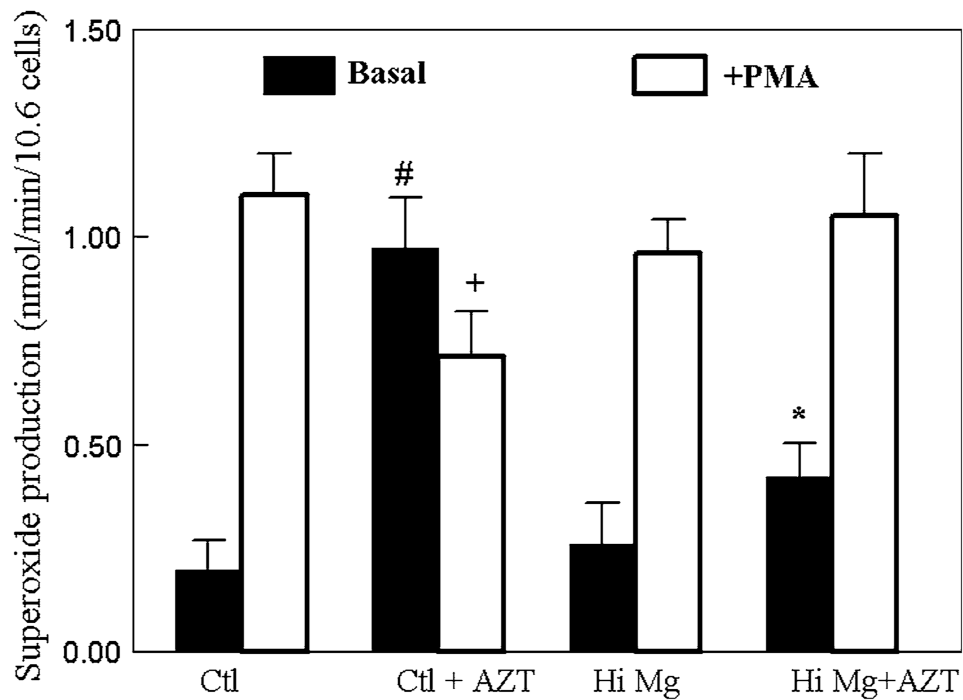
**Fig. 1.**
AZT promotes basal neutrophil $O_2^-$ generation, which is blocked by high dietary Mg-supplementation. Rats receiving AZT (1 mg/ml) in drinking water for 3 weeks were fed either a normal Mg or a 6× higher Mg diet. Neutrophils were isolated from the whole blood by a step gradient centrifugation method. The ability of the neutrophils to generate superoxide anions ±100 nM PMA was determined by SOD inhibitable cytochrome reduction. Values are means ± SEM from five rats; * $P < 0.05$ versus Ctl and Hi Mg + AZT group; # $P < 0.01$ versus Ctl and <0.05 versus Hi Mg + AZT rats
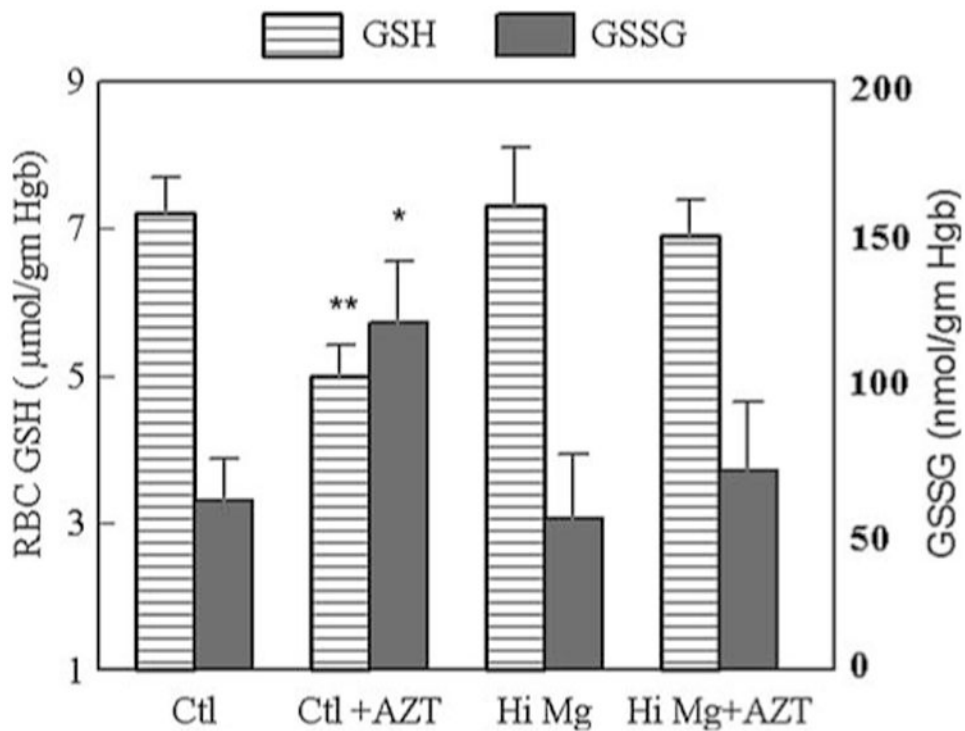
**Fig. 2.**
Changes in the glutathione status in RBCs obtained from normal Mg or high Mg rats ± AZT. GSH and GSSG were determined by the enzymatic "recycling" method. Other conditions are described in Fig. 1. * $P < 0.05$ versus Ctl and Hi Mg + AZT group; [#] $P < 0.01$ versus Ctl and <0.05 versus Hi Mg + AZT rats
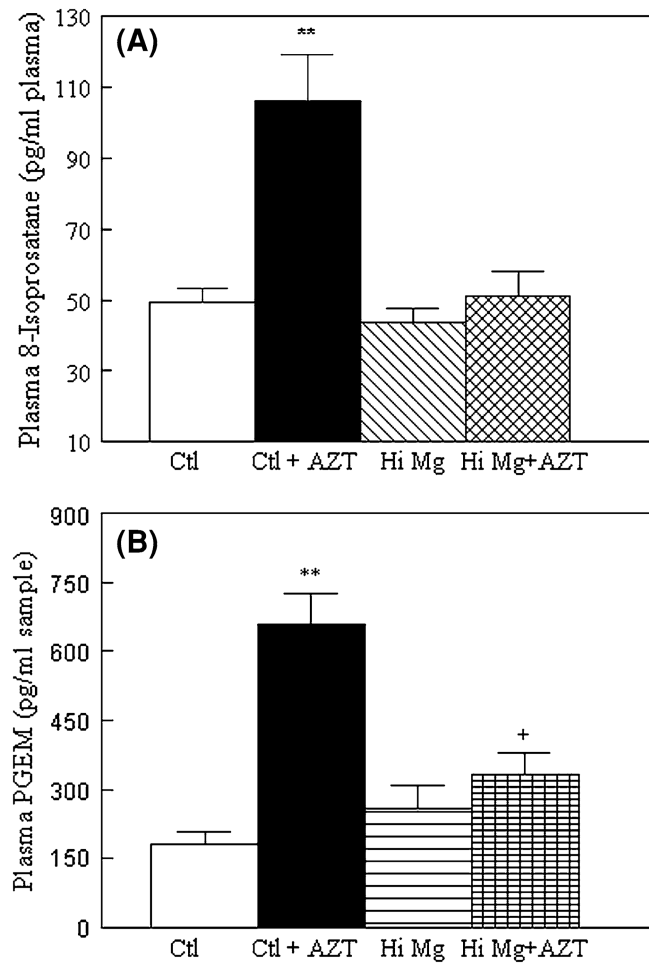
**Fig. 3.**
AZT enhances circulating 8-isoprostane (**a**) and PGE$_2$ (**b**) in normal Mg rats and the attenuating effects of high Mg diet. Both plasma 8-isoprostane and PGE$_2$-metabolites were determined by EIA Immunoassay kits. Other conditions are as described in Fig. 1; * $P <$ 0.05 and [#] $P <$ 0.01 versus Ctl and <0.05 versus Hi Mg + AZT group
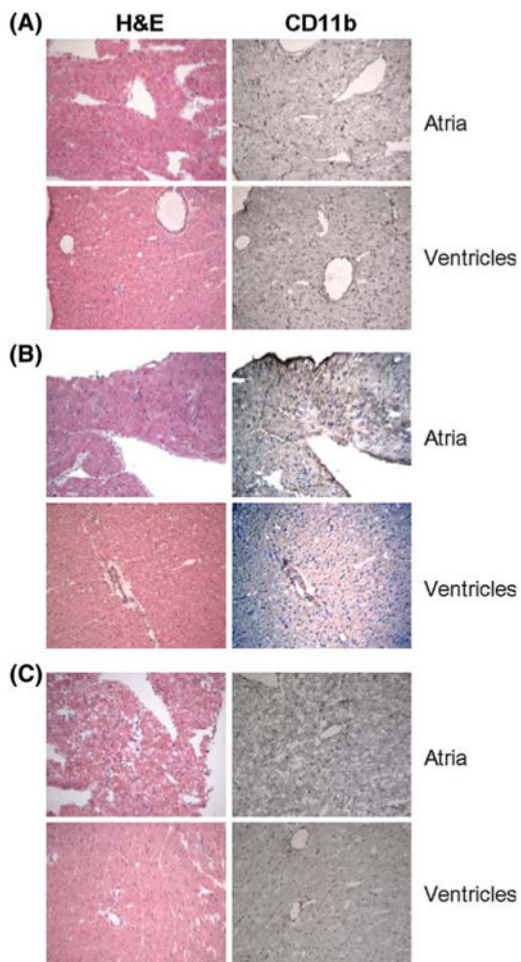
**Fig. 4.**
Micrographs showing H&E (*left*) and immunohistochemical staining (*brown-dark*) for CD11b of atrial and ventricular sections from **a** control, **b** control + AZT; **c** high Mg + AZT treated rat hearts. All tissues were obtained after 3 weeks of AZT treatment. Other conditions are as described in Fig. 1 and in "Materials and Methods" section. Magnification, 20× (*color online*)
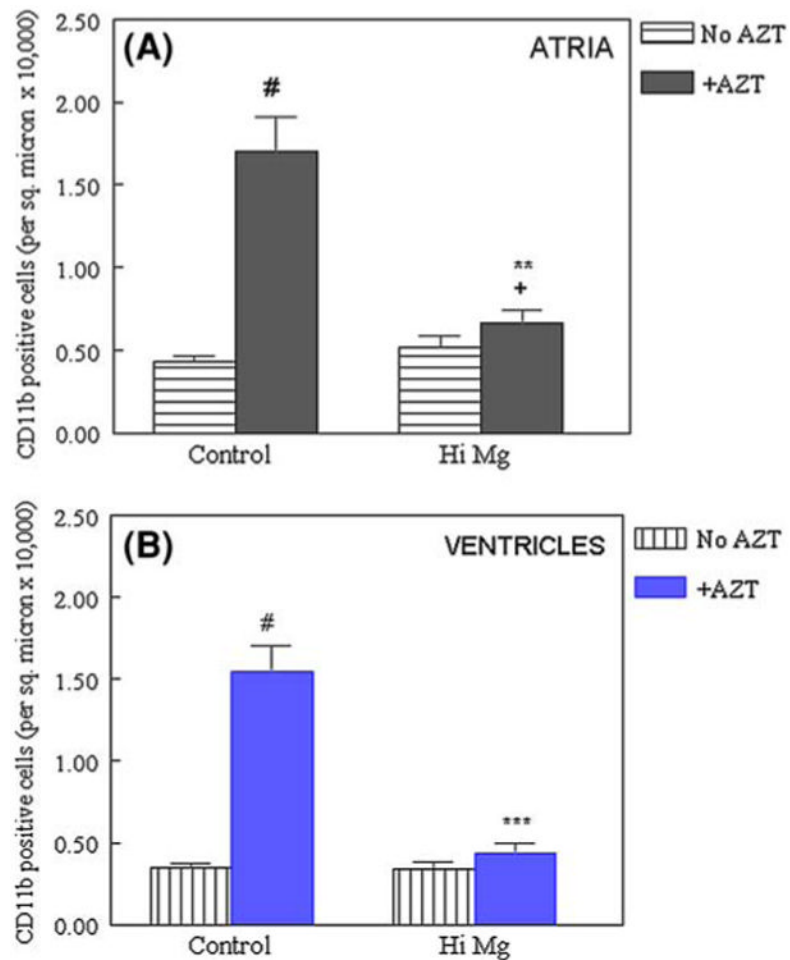
**Fig. 5.**
Numerical density of CD11b positive cells in the **a** atria and **b** ventricles from AZT treated rats with control or high Mg diets. Data are derived from 7 to 8 different fields including all five animals per group; [#] $P < 0.01$ versus Ctl, [+] $P < 0.05$ versus Ctl, [**] $P < 0.01$, [***] $P < 0.001$ versus #

**Table 1**

**Effect of high Mg-diet on plasma Mg and weight gain in AZT-treated rats**

| Rat groups | Initial body wt. (g) | Wt. gained (g/3 weeks) | AZT consumption (mg/kg/day) | Plasma Mg (mM) |
|---|---|---|---|---|
| Normal Ctl | 276 ± 18 | 86 ± 5 | – | 0.796 ± 0.05 |
| + AZT | 265 ± 8 | 68 ± 6 [*] | 95 ± 7.7 | 0.794 ± 0.04 |
| Mg-Suppl. | 274 ± 12 | 87 ± 7 | – | 1.06 ± 0.08 [*] |
| AZT ± Mg-Sup | 279 ± 13 | 90 ± 5 [+] | 98 ± 7.5 | 1.05 ± 0.06 [*] |

Rats were fed either normal Mg diet (0.1% MgO) or Mg-Suppl diet (0.6% MgO) for 3 weeks ± AZT administration (1 mg/ml in drinking water)

Data = means ± SE of five rats

[*] $P < 0.05$ versus Ctl

[+] $P < 0.025$ versus AZT