

# Vitamin B12 Protects Against Hypoxia/Reperfusion Injury in Mouse Proximal Tubule Cells

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## **Abstract**

Acute kidney injury (AKI) is a common syndrome characterized by a sudden decline in kidney function that can potentially lead to death. Ischemia/reperfusion injury (IRI) is the leading cause of AKI and is inevitable during kidney transplants. There is no effective treatment available to treat IRI. Pathways involved in IRI are evidenced to lead to reactive oxygen species (ROS), inflammation, fibrosis, apoptosis, DNA damage response (DDR) and autophagy. Vitamin B12 (B12) or cobalamin, is essential for the human body and is pharmacologically known to scavenge ROS, suppressing inflammation and reverse impaired autophagy that occurs in B12 deficient conditions. To test whether B12 has beneficial effects in IRI, I subjected cultured mouse proximal tubule cells (BU.MPT) to a hypoxia/reperfusion (H/R) procedure and measured transcription of markers for inflammation (*Mcp1*, *Il6*, *Nos2*) and fibrosis (fibronectin), protein markers for apoptosis (*Tgfβ1*, *c-cap3*), and DDR (p.H2AX) induced by hypoxia/reperfusion (H/R). Presence of B12 during the H/R procedure at 0.3μM dramatically inhibited the upregulation of these markers studied and to an increased cell survival. Together, my findings suggest that B12 is a highly promising molecule to prevent/treat AKI.

## Introduction

Acute kidney injury (AKI) is a clinical condition that affects roughly 200,000 people every year in the United States<sup>1</sup>. It is characterized by the sudden decline of renal function, leading to the dangerous accumulation of waste products and chemical imbalance in one's blood<sup>2</sup>. The disease progresses from cellular damage to decreased glomerular filtration rate, leading to kidney failure and in severe situations, it results in death<sup>2</sup>. Despite its frequency and severity, there is limited treatment of AKI and most are still in the development stage. For example, animal studies of  $\alpha$ -melanocyte-stimulating hormone ( $\alpha$ -MSH) have confirmed its anti-inflammatory and anti-apoptotic activities to protect from AKI<sup>1</sup>. However, because its reduction of serum creatinine is not considered an acceptable endpoint for FDA requirements of drug registration, it is not yet an approved treatment<sup>1</sup>.

Many causes can lead to AKI such as severe dehydration, but the leading cause of AKI is ischemia renal injury (IRI) or renal ischemia/reperfusion injury which is unavoidable during kidney transplant<sup>3,4</sup>. During IRI, there are two distinct stages that lead to cell damage. The ischemia phase can be characterized as the inhibition of oxygen flow to the organ, which leads to the accumulation of metabolic intermediates. When reperfusion occurs and oxygen flow is restored to the organ, these intermediates react with oxygen to produce oxygen radicals, namely superoxide ( $O_2^-$ ) and hydrogen peroxide ( $H_2O_2$ ) which leads to uncontrolled oxidation of cells. For example, during ischemia, ATP is catabolized into ADP and AMP, which leads to an abnormally high accumulation of hypoxanthine. When hypoxanthine reacts with oxygen of reperfusion, xanthine oxidase catabolizes hypoxanthine to xanthine which generates  $O_2^-$ , a free radical. Xanthine is further catabolized to uric acid by xanthine oxidase and more  $O_2^-$  is

also generated, which causes excessive oxidative stress on cells<sup>5</sup>.

IRI can be reduced if certain reagents can suppress free radical generation or scavenge them. Allopurinol, an inhibitor of xanthine oxidase, which was used mainly for clinical treatment of gout may also have beneficial effects on ischemia-reperfused kidneys<sup>5, 6</sup>. However, Bussmann et al. reported that Allopurinol did not exert protective effects on the kidneys of rats subjected to ischemia-reperfusion injury<sup>7</sup>, Therefore, there is yet to be a universally successful result of using allopurinol to treat IRI. Superoxide dismutase (SOD), however, could be more effective in treating renal IRI, as it is an  $O_2^-$  scavenger. In 1993, Pollak et al. found that post-operative renal function did not change when human recombinant SOD was administered immediately prior to renal allograft and 1 hour after it in a randomized double-blind trial<sup>8</sup>. In 2001, Yin et al reported that *Sod* gene transduction minimized ischemia-reperfusion–induced acute renal failure<sup>9</sup>. Therefore, SOD plays an inclusive role in renal IRI, as almost no findings have been published regarding the topic since. This also indicates the urgency of finding AKI interventions, as hundreds of thousands of patients experience this life-threatening illness every year.

Vitamin B12 (B12, cobalamin) has been shown to exhibit a SOD mimetic activity which scavenges superoxide free radicals<sup>10</sup>. In a reverse direction, B12 deficiency has been found to result in lower SOD activity in livers of C57BL/6 mice<sup>11</sup>. In addition to its antioxidant effect, B12 has anti-inflammation and autophagy properties, as it is involved in the production of S-Adenosyl Methionine (SAM) <sup>12</sup>, the universal donor to over 100 DNA, protein, and lipid methylation reactions<sup>13</sup>. More specifically, B12 is a cofactor for the conversion of homocysteine to methionine, which is then converted to SAM.

Therefore, the addition of B12 reverses impaired autophagy caused by high concentration of homocysteine resulting from B12 deficiency in mouse astrocytes<sup>15</sup>. SAM additionally inhibits the expression of tumor necrosis factor-alpha (TNF $\alpha$ ), an inflammatory protein induced by lipopolysaccharides (LPS) in human leukocytes.

Based on these previous findings, the first objective of my study was to test whether B12 has a beneficial effect on hypoxia/reperfusion injury and the mechanism(s) involved in the effect of this molecule in vitro.

## **Methods**

**Cell culture, In vitro hypoxia/reperfusion (H/R) experiment:** BU.MPT cells of the mouse kidney proximal tubular epithelial cell (PTEC) line<sup>15</sup>, established by Dr. JS Levine was maintained in DMEM medium supplemented with 10% fetal bovine serum (FBS) and 10 units/mL of interferon- $\gamma$  (IFN- $\gamma$ ) at 37 °C. In preparation for the experiments, cells were grown to  $\geq 90\%$  confluence in a humidified 5% (v/v) CO<sub>2</sub> atmosphere at 39 °C in the absence of IFN- $\gamma$  containing 10% v/v FBS. In this condition, BU.MPT cells behave like primary cultures of mouse kidney PTEC<sup>15</sup>. After confluence, cells were starved for 24 hours with 0 % FBS and were randomly divided into 4 groups. The first group of cells grew in a normal culture condition of 21% oxygen and medium with 10% FBS and 4.5g/L glucose. The second cell group grew in normal culture conditions with 0.3 $\mu$ M vitamin B12. The third cell group grew in a H/R condition and the fourth group grew in a H/R condition with 0.3 $\mu$ M B12. The hypoxia condition culture took place in an anaerobic chamber equilibrated with approximately 1% O<sub>2</sub>, 5%CO<sub>2</sub> and 94% N<sub>2</sub> and media lacking glucose and FBS. After 3 hours under the hypoxia condition, the two experimental cell groups were returned to the normal culture condition with addition of

glucose (4.5g/ml) and FBS (10%) for 1 hour, which was regarded as reperfusion. B12 (0.3 $\mu$ M) was added to medium during 3-hour incubation. At the end of the experiments, medium and cells were collected for analysis. The dosage was chosen based on other B12 literature reports<sup>16</sup> preliminary experiments which confirmed 0.3  $\mu$ M provided significant decrease (50%) of *Mcp1* expression

**Cell viability assay:** Cells were cultured in 96-well plates and received the treatment described above. Then, 10  $\mu$ l of CCK-8 solution (Sigma) was added to each well (1/10 dilution) and the plates were further incubated for 3 hours. The absorbance was measured at 450 nm using a microplate reader (SpectraMax M5 Microplate Reader, Molecular Devices). The mean optical density (OD) of five wells in the indicated groups was used to calculate the percent cell viability according to the following formula:

Percent cell viability = OD treatment group/OD control group.

**Western blot:** Fresh cultured cells were lysed in radioimmunoprecipitation assay buffer (RIPA) buffer and protein concentration was determined by BCA protein assay kit (Thermo scientific, IL). Total protein of 20 to 60  $\mu$ g/lane were subjected to 10%–20% SDS-PAGE, electrotransferred onto PVDF membranes, The antibodies used in the study were: phospho-Histone H2A.X (Ser139) (#9718, 20E3, Cell Signaling), cleaved Caspase-3 (Asp175)( #9664, 5A1E, Cell Signal ), caspase3 (9665; Cell Signaling Technology),p-Chk1 (Ser345; 2348; Cell Signaling Technology),  $\beta$ -actin (13E5; Cell Signaling Technology). The intensity of the targeted protein band was evaluated using Image Studio Software (San Francisco, CA). Individual protein level was quantitated relative to the  $\beta$ -actin level in the same sample and further normalized to the respective control group, which was set as one.

**Quantitative RT-PCR:** Total RNA from cells was extracted using Trizol (Life Technologies, St. Paul, MN) following the manufacturer's instruction. NanoDrop spectrophotometer method and gel electrophoresis was used to check quantity and quality of RNA. mRNA was quantified with TaqMan real-time quantitative RT-PCR (7500 real time PCR system, Applied Biosystems, Foster City, CA) by using one-step RT-PCR Kit (Bio Rad, Hercules, CA) with *18s* as reference genes in each reaction for mouse tissue.

**Statistical analysis:** Experiments were carried out 4 plates ( $P^{100}$ ) per group, and experiments were repeated two times. Data are presented as mean  $\pm$  SEM. Multifactorial ANOVA test was used with the program JMP 12.0 (SAS Institute Inc. Cary, NC). Post hoc analyses were done using the Tukey–Kramer Honest Significant Difference test. Differences were considered to be statistically significant with p values less than 0.05.

## Results

### **Vitamin B12 inhibits hypoxia/reperfusion (H/R) induced inflammation, fibrosis, DNA damage response, and apoptosis in cultured mouse proximal tubule cells.**

To investigate the effects of vitamin B12, experiments were performed on mouse proximal tubule cells<sup>17</sup>. B12 prevented cell death induced by H/R and it had no effect on control cells (Fig. 1A). In addition, the expression of inflammatory marker genes, including *Mcp1* (monocyte chemoattractant protein 1), *Il6* (interleukin 6) and *Nos2* (inducible nitric oxide synthase). In addition, *Tgf $\beta$ 1* (transforming growth factor beta-1,

an apoptosis and fibrosis marker<sup>18</sup>) and *Fibronectin* (a fibrosis marker) was upregulated by H/R whereas the addition of B12 suppressed this upregulation (Fig. 1 C,D,E,F). B12 had no effects on the expression of *Il6*, *Nos 2*, *Tgfβ1*, and *Fibronectin* in the control cells. In contrast, B12 also significantly suppressed the level of *Mcp1* transcript in control cells, which requires further examination to understand its mechanism. (Fig. 1B). On western blots, I found that cleaved- caspase-3(c-Cas3, apoptosis marker) was upregulated by H/R but the addition of B12 decreased its expression. Lastly, p-H2A.X (phosphorylated histone 2AX, hallmark of DDR) protein levels increased by H/R, and the addition of B12 decreased the level of the proteins (Fig. 1G) in H/R conditions, but it had no effects on proteins levels in control cells.

## **Discussion**

In this study, B12 executed protective effects on injuries induced by H/R in mouse proximal tubular cells by significantly decreasing inflammation, fibrosis, DDR and apoptosis induced by H/R. Proximal tubule cells were used for this study because epithelial cell injury associated with H/R is most sensitive in proximal tubule cells in most animal models of ischemia<sup>19</sup>. They are most sensitive to H/R due to limited glycolytic capacity. These cells preferentially utilize lipids over glucose for energy and are rather gluconeogenic. This leads to the inability to maintain normal ATP levels under oxidative stress.

As a response to the injury induced by H/R, cells induce a cascade of inflammatory signals in order to recruit macrophages, neutrophils and other proinflammatory stimuli to the cell<sup>20</sup>. Although inflammation is an important physiological



process to control damage and clear the injured cells, but often it overdoes and causes further damage of tissues. My results demonstrated that B12 treatment during the H/R condition significantly inhibited inflammation, as transcription level of *Mcp1* decreased when B12 was added. Because *Mcp1* is a protein involved in the recruitment, regulation of migration and infiltration of macrophages to the site of injury, decreased expression of this gene leads to suppressed inflammation and protects renal cells from further damage, thus preventing kidney damage. In addition, because of B12's role of inhibiting transcription of the inflammatory response genes *Il-6* and *Nos2* (coding inducible nitric oxide synthase) in H/R conditions, there is evidence that its properties are reno-protective. Lastly, due to B12's capacity of inhibiting inflammation, it is an important treatment to prevent allograft kidney rejection during kidney transplant, as organ rejection is defined by excessive inflammation and this is one of the main causes of ESRD<sup>21</sup>.

In addition to inflammation, fibrosis (or the formation of excess fibrous connective tissue) was suppressed in by B12, as was demonstrated by the decreased transcription level and expression of Fibronectin, the glycoprotein responsible for wound healing through fibrosis<sup>21</sup>. Fibrosis also plays a critical role in IRI as an extension of the inflammation that occurs. The overproduction of Fibronectin and collagen causes fibrosis, and this excessive scar tissue formation significantly impairs cellular function<sup>22</sup>. Taken together, my data regarding inflammation and fibrosis suggests that B12 has potential to be a therapeutic agent for preventing the transition from AKI to CKD.

The data also provided evidence that B12 can reduce DDR. DDR plays a crucial role in cell death during IRI as a result of ROS. The ROS cause excessive oxidative

damage on the cell, including degradation of DNA through oxidation of nucleic bases and DNA strand breakage. The rate of oxidative DNA damage is inversely related to life span of organisms so extreme oxidative stress results in cell dysfunction and death<sup>23</sup>. Therefore, the application of a ROS scavenger is critical for cell health. Because p-H2AX, phosphorylated histone variant 2AX is a marker of DNA damage<sup>24</sup>, inhibited levels of H2AX indicated that there was suppressed DNA damage in the cells with B12.

Apoptosis was measured by cleaved caspase 3 (c-Cas3) and because H/R increased c-Cas3, but the addition of B12 to H/R conditions decreased it, suggesting that B12 inhibited apoptosis in this study. The effect of B12 on  $O_2^-$  is under investigation now.

## **Conclusion**

The current data indicates that B12 may execute beneficial effects in IRI by inhibiting damaging factors/pathways; therefore, it is a promising treatment for AKI. The in vitro data clearly show the B12 inhibits inflammation, fibrosis, apoptosis and DDR in proximal tubule cells. It is also possible that B12 may inhibit these processes in other cell types, like endothelial cells and/or monocytes. Because multiple cell types and multiple factors/pathways are implicated in IRI in vivo, the precise role of individual cell type and/or factor is unclear. A future experimental approach such as studying single cell RNA-seq<sup>25</sup> could provide crucial information to better understand the role of different cells and the mechanism through which IRI occurs and B12 functions. At any rate, although dietary absorption of B12 is complex and limited, there is no toxicity of high dose B12 has been indicated. Further studies on its preventive and therapeutic use for IRI is warranted.

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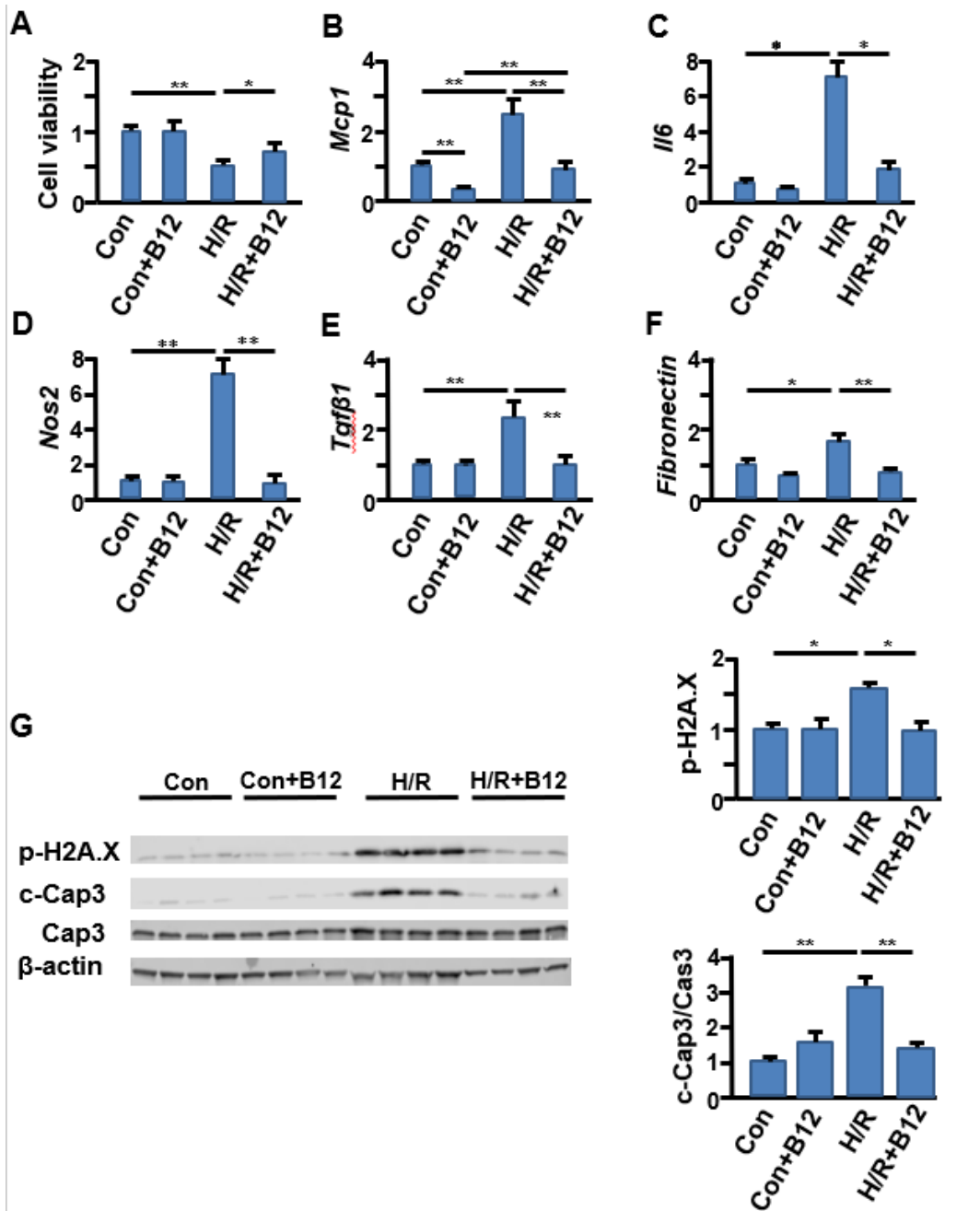


Figure 1. Vitamin B12 increases cell survival and inhibits Hypoxia/reperfusion (H/R) induced inflammation, fibrosis, DNA damage response (DDR) and apoptosis in proximal tubule cells.

A) Relative cell viability measured by Cell Counting Kit-8. B-F) mRNA level of inflammatory and fibrotic genes. G) Western blot (left panel) and densitometric quantitative results (right panel) of p-H2A.X and c-Cap3/Cas3 in cells in four groups of cells. \* $p < 0.05$ , \*\* $p < 0.01$ .  $n = 8$ .

### Legend

con=control. H/R = hypoxia/reperfusion. B12 = vitamin B12.

Mcp1: monocyte chemoattractant protein 1, Il6: interleukin 6, Nos2: inducible nitric oxide synthase, Tgf $\beta$ -1: transforming growth factor beta 1, p.H2AX: phosphorylated histone 2AX variant, c-Cap3/Cas3: cleaved capase3 /caspase-3