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TOPIC HIGHLIGHT

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Dietary supplementation of some antioxidants against hypoxia

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Abstract

The present study aims to clarify the protective effect of supplementation with some antioxidants, such as idebenone (200 mg/kg, *ip*), melatonin (10 mg/kg, *ip*) and arginine (200 mg/kg, *ip*) and their combination, on liver function (T. protein, albumin, alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase), energetic parameters (adenosine triphosphate, adenosine diphosphate, adenosine monophosphate, inorganic phosphate, total adenylate, adenylate energy charge and potential phosphate). The effect on glycolytic and glycogenolytic enzymes (glucose, glycogen, glycogen phosphorylase, pyruvate kinase and phosphofructokinase against hypoxia) was also studied. The drugs were administered 24 and 1 h prior sodium nitrite intoxication. All biochemical parameters were estimated 1 h after sodium nitrite injection. Injection of sodium nitrite (75 mg/kg, *sc*) produced a significant disturbance in all biochemical parameters of liver function, energetic parameters and glycolytic and glycogenolytic enzymes. Hepatic damage was confirmed by histopathological examination of the liver as compared to controls. The marked changes in hepatic cells induced by sodium nitrite were completely abolished by pretreatment with the drug combination, suggesting potential protection against sodium nitrite-induced hypoxia. It could be concluded that a combination of both idebenone and melatonin or idebenone and arginine provides potential protection against sodium nitrite-induced hypoxia by improving biochemical parameters and preserving liver histology.

Key words: Hypoxia; Idebenone; Melatonin; Nitrate/nitrite; Adenosine triphosphate

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INTRODUCTION

Hypoxia, or hypoxiation, is a pathological condition in which the body as a whole (generalized hypoxia) or a region of the body (tissue hypoxia) is deprived of adequate oxygen supply. Variations in arterial oxygen concentrations can be part of the normal physiology, hypoxia in which there is complete deprivation of oxygen supply is referred to as anoxia. Hepatic tissue is quite vulnerable to

hypoxic injury. The morphological expression of hypoxic injury seems mediated by changes in the cortical cytoskeleton[1]. Lemasters *et al*[2] produced hypoxia in isolated, hemoglobin-free, perfused rat liver by reducing the flow rate of oxygen-carrying fluid entering the organ. This caused anoxia in centrilobular regions. In these anoxic areas, structural derangements developed rapidly, characterized by bleb-like protrusions of hepatocyte plasma membrane through fenestrations in the sinusoidal endothelium. Periportal tissue remained normoxic and was completely spared.

Idebenone (hydroxydecyl benzoquinone), a short chain synthetic analogue of coenzyme-Q10 (CoQ-10), is a vital cell membrane antioxidant and essential constituent of the adenosine triphosphate (ATP) producing mitochondrial electron transport chain. It is a potent antioxidant agent and unlike CoQ-10 has the ability to operate under low oxygen tension situations[3].

The pineal gland is the main source of melatonin (N-acetyl-5-methoxytryptamine) in the circulation. It is also produced in small amounts in the retina, gastrointestinal system and by leukocytes[4]. Melatonin (MEL) is tiny[5] and highly lipophilic, and for this reason, it is found abundantly in all parts of the cell. MEL protects the DNA, lipids and proteins against oxidative damage[6,7]. The free radical scavenging and antioxidant effects of MEL have been shown in many studies[8-11].

2-Amino-5-guanidinopentanoic acid (arginine) is an important, versatile and a conditionally essential amino acid. Besides serving as a building block for tissue proteins, arginine plays a critical role in ammonia detoxification, and nitric oxide and creatine production. Arginine supplementation is an essential component for the treatment of urea cycle defects but recently some reservations have been raised with regards to the doses used in the treatment regimens of these disorders[12].

In the present study, we aimed to compare the protective effects of idebenone, melatonin, arginine, idebenone + melatonin and idebenone + arginine against liver injury through histological (hematoxylin and eosin) and biochemical [T. protein, albumin, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP), ATP, adenosine diphosphate (ADP), adenosine monophosphate (AMP), inorganic phosphate (Pi), total adenylate (TA), adenylate energy charge (AEC), *potential phosphate* (PO), glucose, glycogen, glycogen phosphorylase, pyruvate kinase (PK) and phosphofructokinase (PFK) parameters] against hypoxia in ameliorating sodium nitrite induced hypoxia in rat livers.

PRINCIPLES AND TECHNIQUE

Drugs and chemicals

Idebenone, melatonin, arginine and sodium nitrite used in this study were analytically pure products of Sigma-Aldrich Chemical Co., St. Louis, MO, United States. All other chemicals were of the highest analytical grade. Sodium nitrite and melatonin were dissolved in normal saline, while, idebenone and arginine were suspended in 1% gum acacia in normal saline.

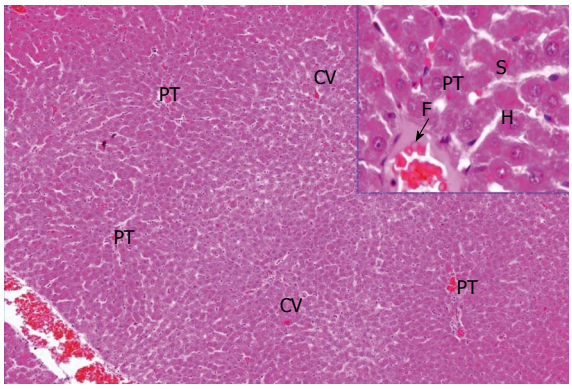

Figure 1 Liver section (hematoxylin and eosin × 100 with lateral magnification × 200) showed the normal structure of liver control.

Animals

Adult male albino rats, weighing 180-200 g obtained from the animal house of King Saud University, were used in this study. They were fed with a standard laboratory diet and tap water ad libitum and housed in cages (ten rats per cage). All animals were kept at standardized laboratory conditions (25 ± 5 ℃, 55% ± 5% humidity, and a 12 h light/dark cycle). One week after acclimatization in the laboratory where experiments were performed, the animals were fasted for 3 h prior to drugs administration. All experiments were carried out according to recommendations of King Saud University of Experimental Animals Ethics Committee which is matched with international ethics for handling of experimental animals. The doses of sodium nitrite, idebenone, melatonin and arginine used in the current study were chosen according to preliminary studies in our laboratory and were matched with those in the literature[13-16] respectively.

Experimental design

Animals were divided into seven groups (n = 10 rats) and were treated as follows: Group Ⅰ normal group (Figure 1) treated with saline; group Ⅱ treated with sodium nitrite only[13] (75 mg/kg, *sc*); group Ⅲ was administered idebenone[14] (200 mg/kg, *ip*); group Ⅳ was injected with melatonin[15] (10 mg/kg, *ip*); group Ⅴ was treated with idebenone (200 mg/kg, *ip*) followed by melatonin (10 mg/kg, *ip*) one hour later; group Ⅵ was treated with arginine[16] (200 mg/kg, *ip*); group Ⅶ was treated with idebenone (200 mg/kg, *ip*) followed by arginine. These drugs were injected 24 h and 1 h before sodium nitrite injection.

Liver tissues homogenate

Liver tissue was homogenized in 0.9 mol/L NaCl (1:10 w/v) for the estimation of glucose, total protein, albumin, ALT, AST and ALP. Liver tissue (0.25 g) was homogenized using 7% trichloroacetic acid for the extraction of adenosine nucleotides according to the method of Wijsman[17]. For estimation of PFK, 0.25 g liver was

Table 1 Liver function, level of energetic parameters, level of some glycolytic and glycogenolytic enzymes in hypoxica rats at different treated groups

Parameters	Control Ⅰ	Hypoxia Ⅱ	Treated-groups					*P* value
			Idebenome Ⅲ	Melatonin Ⅳ	Id + Melatonin Ⅴ	Arginine Ⅵ	Id + arginine Ⅶ	
Liver function parameters								
T.protein (g/dL)	13.4 ± 2.5^{a}	9.2 ± 1.07^{c}	10.26 ± 0.57^{b,c}	10.63 ± 0.6^{b,c}	11.28 ± 0.52^{b}	11.03 ± 1.05^{b}	11.54 ± 0.81^{b}	< 0.001
Albumin (g/L)	68.9 ± 5.4^{b}	56.18 ± 7.5^{c}	68.3 ± 6.68^{b}	84.47 ± 14.2^{a}	68.98 ± 7.1^{b}	67.10 ± 9.90^{b,c}	72.34 ± 9.7^{b}	< 0.01
AST (GOT) (U/L)	9.78 ± 1.18^{a}	4.64 ± 1.25^{c}	8.86 ± 1.32^{a}	8.64 ± 1.04^{a,b}	7.01 ± 1.13^{b}	8.65 ± 1.84^{a,b}	9.64 ± 1.49^{a}	< 0.001
ALT (GPT) (U/L)	9.69 ± 3.67^{a,b}	5.87 ± 0.99^{c}	10.4 ± 1.9^{a,b}	9.28 ± 2.1^{a,b}	7.34 ± 3.93^{b,c}	10.53 ± 1.6^{a,b}	10.78 ± 1.4^{a}	< 0.05
ALP (U/L)	461.98 ± 40.1^{b,c}	623 ± 64.9^{a}	486 ± 62.0^{b}	449.5 ± 47.7^{b,c}	436.3 ± 65.6^{b,c}	400.5 ± 39.3^{c}	418.4 ± 46.2^{b,c}	< 0.001
Level of energetic parameters								
ATP (μmol/mg)	1.47 + 0.11^{a}	0.602 + 0.03^{f}	0.978 + 0.05^{d}	1.03 + 0.07^{d}	1.27 + 0.03^{b}	0.87 + 0.05^{e}	1.16 + 0.05^{c}	< 0.001
ADP (μmol/mg)	0.152 + 0.007^{e}	0.37 + 0.02^{a}	0.24 + 0.01^{b}	0.20 + 0.01^{c}	0.18 + 0.005^{d}	0.26 + 0.02^{b}	0.21 + 0.008^{c}	< 0.001
AMP (μmol/mg)	0.08 + 0.008^{e}	0.188 + 0.01^{a}	0.13 + 0.01^{c}	0.12 + 0.01^{c,d}	0. 108 + 0.008^{d}	0.15 + 0.008^{b}	0.12 + 0.008^{c}	< 0.001
Pi (μmol/mg)	12.82 + 1.9^{a}	5.34 + 0.75^{e}	8.12 + 0.46^{d}	8.47 + 0.36^{c,d}	9.93 + 0.35^{b}	7.84 + 0.31^{d}	9.49 + 0.58^{b,c}	< 0.001
TA	1.70 + 0.12^{a}	1.16 + 0.02^{e}	1.35 + 0.05^{c,d}	1.36 + 0.075^{c}	1.56 + 0.03^{b}	1.27 + 0.03^{d}	1.49 + 0.05^{b}	< 0.001
ATP/ADP	9.65 + 0.64^{a}	1.65 + 0.18^{g}	4.05 + 0.29^{e}	5.06 + 0.039^{d}	7.21 + 0.31^{b}	3.41 + 0.31^{f}	5.64 + 0.26^{c}	< 0.001
ATP/AMP	18.08 + 2.47^{a}	3.21 + 0.26^{e}	7.82 + 0.83^{c}	8.70 + 1.27^{c}	11.72 + 0.97^{b}	5.80 + 0.47^{d}	9.40 + 0.99^{c}	< 0.001
AEC	0.90 + 0.01^{a}	0.68 + 0.01^{f}	0.82 + 0.01^{d}	0.83 + 0.01^{d}	0.87 + 0.01^{b}	0.78 + 0.01^{e}	0.85 + 0.01^{c}	< 0.001
PO	0.77 + 0.15^{a}	0.31 + 0.05^{e}	0.50 + 0.05^{c,d}	0.60 + 0.04^{b}	0.73 + 0.02^{a}	0.44 + 0.06 ^{d}	0.59 + 0.06^{b,c}	< 0.001
Level of some glycolytic and glycogenolytic enzymes								
Glucose (mg/g)	74.28 ± 6.43^{a}	58.5 ± 7.1^{c}	67.2 ± 3.2^{a,b}	63.0 ± 7.0^{b,c}	65.03 ± 5.5^{b,c}	65.82 ± 6.7^{b,c}	67.9 ± 4.8^{a,b}	< 0.01
Glycogen (mg/g)	8.9 ± 1.2^{b}	3.4 ± 0.64^{c}	8.7 ± 1.02^{b}	8.9 ± 0.53^{b}	8.8 ± 0.56^{b}	8.99 ± 0.45^{a,b}	9.98 ± 0.63^{a}	< 0.001
Glycogen- phosphorylase	0.99 + 0.03^{g}	3.00 + 0.12^{a}	1.93 + 0.05^{c}	1.75 + 0.08^{d}	1.38 + 0.11^{f}	2.17 + 0.11^{b}	1.54 + 0.05^{e}	< 0.001
PK (μmol/min per mg)	4.00 + 0.276^{f}	7.81 + 0.16^{a}	5.51 + 0.38^{c}	5.34 + 0.25^{c,d}	4.92 + 0.10^{e}	5.87 + 0.15^{b}	5.11 + 0.12^{d,e}	< 0.001
PFK (μmol/min per mg)	8.16 + 0.48^{e}	32.03 + 2.05^{a}	20.70 + 1.34^{b}	18.43 + 0.52^{b}	10.13 + 1.27^{d}	20.32 + 0.44^{b}	15.13 + 2.74^{c}	< 0.001

Data are expressed as means ± SD of ten rats in each group; a, b, c, d, e, f, g means within columns with no common superscripts differ significantly. Unshared superscript letters between groups are the significance values at $P < 0.05$. Statistical analysis is carried out using one way analysis of variance (ANOVA) using CoStat computer program. AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; ATP: Adenosine triphosphate; ADP: adenosine diphosphate; AMP: Adenosine monophosphate; AEC: Adenylate energy charge; PO: *Potential phosphate*; PK: Pyruvate kinase; PFK: Phosphofructokinase; Pi: Inorganic phosphate; TA: Total adenylate.

homogenized in 50 mmol Tris HCL, 1 mmol disodium ethylenediam-inetetraacetate dehydrate (EDTA) and 5 mmol $MgSO_4$ at pH 8.2 and then centrifuged for 10 min at 0 ℃. The supernatant was used for enzyme assays while 0.25 g tissue was homogenized in 5 mL Tris-HCL buffer pH 7.6 for detection of PK. On the other hand, 0.25 g tissue was homogenized in 100 mmol maleate-NaOH-buffer (pH 6.6) containing 20 mmol NaF, 1 mmol EDTA, 0.5 mg/mL bovine serum albumin and 10 mmol DL-dithothreitol for estimation of glycogen phosphorylase.

Biochemical studies

Glucose was determined colorimetrically at 505 nm by the method described by Trinder[18]. Total protein reacts with Bradford reagent to give a blue complex, which is measured calorimetrically at a wavelength of 595 nm (Bradford[19]). The albumin level was measured according to the method of Doumas *et al*[20] using Randox Diagnostic kits. In a buffered solution, bromocresol green forms with albumin; a green colored complex, its intensity is proportional to the amount of albumin present in the sample. ALT was determined according to the method of Reitman and Frankel[21]. AST was determined according to the method of Reitman *et al*[21]. ALP activity was measured photometrically[22].

Energetic parameter evaluation

ATP was assayed following the procedure of Lamprecht *et al*[23]. ADP and AMP were assayed in a single assay system according to the method of Jaworek *et al*[24].

Calculation of phosphate potential: PO is an alternative index used to indicate the free energy status of the tissues and can be calculated from the concentration of ATP, ADP and Pi according to Van Waarde *et al*[25] PO = [ATP]/[ADP] [Pi].

Calculation of total adenylates: TA = ATP + ADP + AMP.

Calculation of adenylates energy charge: AEC = 1/2 [ADP + ATP]/[AMP + ADP + ATP]. Glycogen content was estimated by the method of Carroll *et al*[26] as the green color formed was read at 610 nm against blank. PK was measured according to the method of King[27]. PFK was estimated according to the method of Zammit *et al*[28]. Glycogen phosphorylase was determined according to the method of Hedrick *et al*[29].

Histological study

Liver samples were removed and placed overnight in fixative containing 10% formalin, liver were paraffin-embedded for hematoxylin and eosin (HE) staining and cut at 5 μm in the longitudinal plane; liver samples from different groups were named as control (Ⅰ), hypoxia (Ⅱ), idebenone (Ⅲ), melatonin (Ⅳ), idebenone + melatonin (Ⅴ), arginine (Ⅵ), idebenone + arginine (Ⅶ). Sections

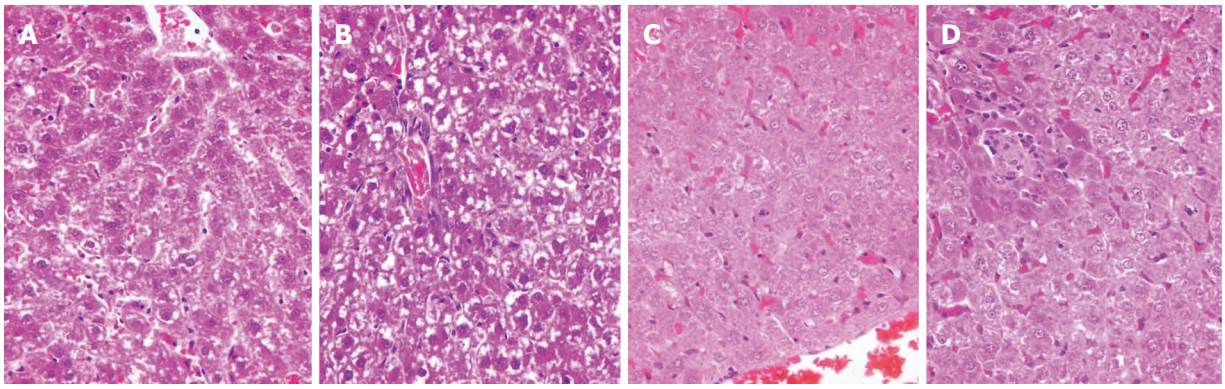

Figure 2 Liver section (hematoxylin and eosin × 200) centrilobular region and periportal region. A: Liver section [hematoxylin and eosin (HE) × 200] centrilobular region; B: Periportal region showed severe morphological changes as a result of giving sodium nitrite; C: Liver section (HE × 200) centrilobular region; D: Periportal region of rats treated with melatonin against sodium nitrite.

were examined under the microscope for the extent of any damage and compared with the control group.

Statistical analysis

The results of biochemical analysis were analyzed using one-way analysis of variance followed by Co-stat computer program. Values of less than 0.05 were regarded as statistically significant.

RESULTS

Biochemical observation

There was a significant reduction in total protein, albumin, AST and ALT of 31%, 18.5%, 52.6% and 39% in the hypoxia group while ALP activity increased in this group (Table 1). All treated groups revealed enhancement of total protein activity of 7.9%, 10.6%, 15.5%, 13.6% and 17% in groups Ⅲ, Ⅳ, Ⅴ, Ⅵ and Ⅶ respectively. Albumin was enhanced by 17.6%, 41.6%, 18.6%, 15.8% and 23.5% in groups Ⅲ, Ⅳ, Ⅴ, Ⅵ and Ⅶ respectively. AST and ALT activities showed changes of 43%, 40.9%, 24%, 41%, 51% and 46.7%, 35%, 15%, 5%, 50.7% in treated groups Ⅲ, Ⅳ, Ⅴ, Ⅵ and Ⅶ respectively. Elevated ALP activity was inhibited by a different amount with different antioxidants.

There was a significant decrease in ATP level in hypoxic rats 59% (Table 1). Treatment of the hypoxic group produced significant amelioration of ATP level by 25.6%, 29%, 45%, 18% and 38% in groups Ⅲ, Ⅳ, Ⅴ, Ⅵ and Ⅶ respectively. When we studied hypoxic rats, significant increases of ADP (143%) and AMP (135%) levels were observed. Upon hypoxia, treated rats showed an improvement in ADP and AMP levels by 85.53%, 111.8%, 125%, 72% and 105% respectively for ADP and by 72.5%, 85%, 100%, 47.5% and 85% for AMP and for the same previous treatments respectively. In addition, there was significant reduction in Pi of 58.34% in the hypoxic group. Treated rats showed a significant amelioration of the effects of hypoxia, by 21.7%, 24%, 35.8%, 19.5% and 32% respectively for idebenone, melatonin, idebenone + melatonin, arginine and arginine + idebenone respectively. At the same time, AEC showed a significant reduction of 24.44%. Significant enhancements of 15.6%, 16.7%, 21%, 11% and 18.9% were observed in treated groups, respectively. Measurements of ATP/ADP, ATP/AMP, PO and TA showed significant reductions in the hypoxic rats of 82.9%, 82%, 59.7% and -31.76%, respectively. Hypoxic groups that received treatment showed significant enhancements in ATP/ADP, ATP/AMP, PO and TA of 24.9%, 35%, 57.6%, 18% and 41%, respectively, for ATP/ADP and of 25.5%, 30%, 47%, 14% and 34%, respectively, for ATP/AMP. Changes in TA detected were 11.18%, 11.76%, 23.53%, 25.29% and 19.41%, respectively, while changes in PO recorded were 24.68%, 37.66%, 54.55%, 16.88% and 36.36% for the same previous mentioned treatments respectively. Glucose and glycogen content showed a marked decrease in the hypoxia group (21% and 61.8% respectively). Treatment with antioxidants changed these decreases to 11.7%, 6.1%, 8.7%, 9.8% and 12.6% respectively, for glucose (Table 1), and 59.9%, 61.7%, 60.7%, 60.9%, and 73.9% respectively for glycogen; while glycogen phosphorylase, PK and PFK enzyme activities showed significant increases in hypoxic rats of 95%, 203% and 292.5% respectively, but these increases were significantly ameliorated after treatment of hypoxic rats with idebenone, melatonin, idebenone + melatonin, arginine and idebenone + arginine. Changes in glycogen phosphorylase were 109%, 126%, 163.6%, 83.8% and 147.5% respectively for each treatment listed above while enhanced levels of PK of 57.5%, 61.8%, 73%, 48.5% and 67.5% were achieved, respectively. Changes in PFK were 138.85%, 166.67%, 268.38%, 143.5% and 207% respectively.

Histological observation

Severe morphological changes were seen in Figure 2A and B particularly in the peri-portal area, compared with Figure 1 control group with normal hepatocyte cells. The hepatocytes were shrunken and vacuolated. The radiating cordlike arrangement of the hepatocytes was disturbed, except in the region around the central veins. Figure 2C and D also showed morphological changes both in the

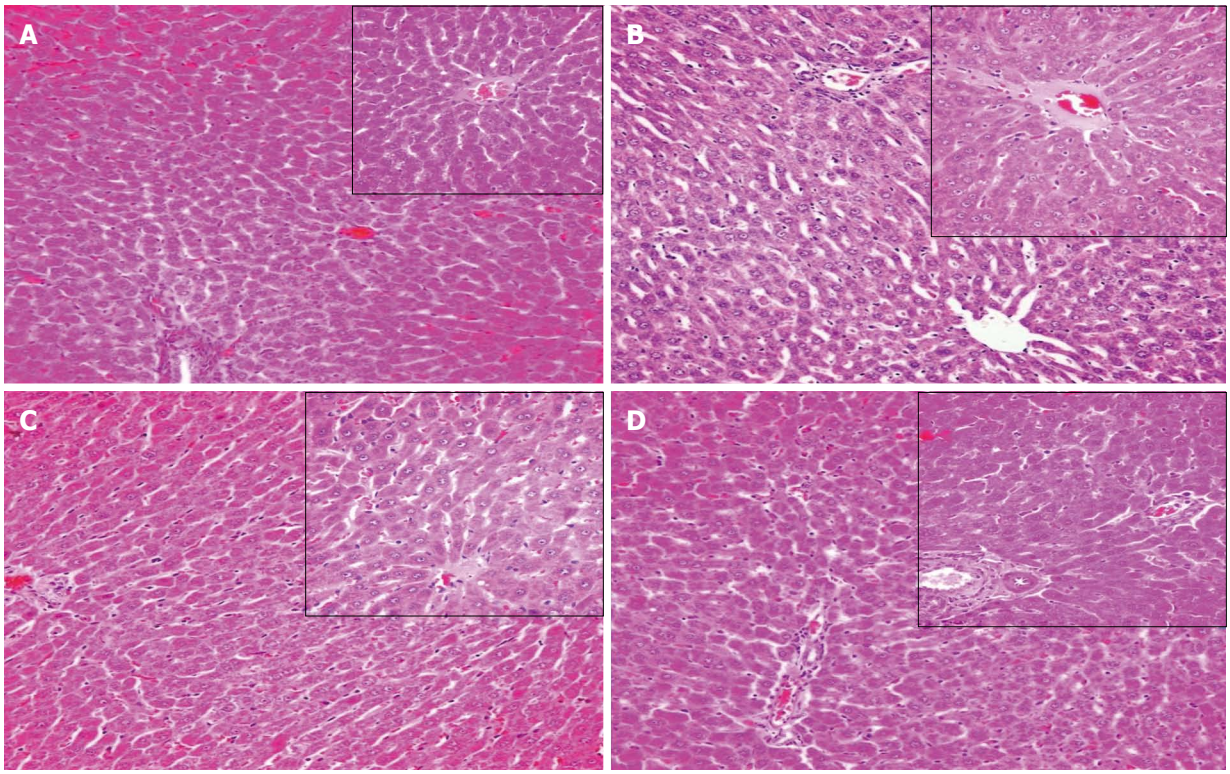

Figure 3 Liver section (hematoxylin and eosin × 200) of rats. A: Liver section [hematoxylin and eosin (HE) × 200] of rats treated with idebenone against hypox ia; B: Liver section (HE × 100 with lateral magnification × 200) of rats treated with idebenone + melatonin against sodium nitrite showing repairing of liver cells; C: Liver section (HE × 100 with lateral magnification × 200) of rats treated with arginine against sodium nitrite revealing normal hepatocyte; D: Liver section (HE × 100 with lateral magnification × 200) of rats treated with idebenone + arginine against sodium nitrite, showing hepatocytes with normal histological.

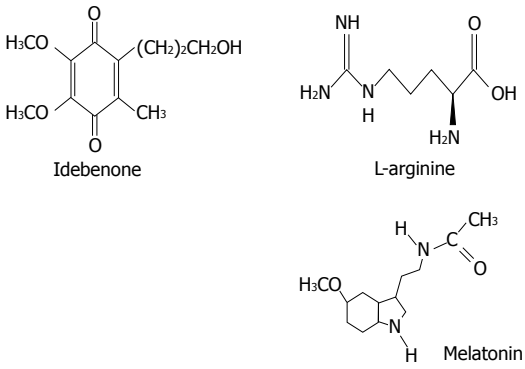

Figure 4 Chemical structures of idebenone, melatonin and arginine.

peri-portal and centrilobular regions. The liver cells were swollen and vacuolated and sinusoidal spaces appeared to be obliterated. Figure 3 did not show any marked difference as compared to the control group.

DISCUSSION

Liver injury causes vascular disorganization and local tissue hypoxia starting early in disease course. In this context, hypoxia acts not only as an aggravating factor of cell damage and inflammation, but also as an inhibitor of liver regeneration, a major stimulus of angiogenesis and fibrogenesis, and a promoter of liver carcinogenesis[30]. Lack of oxygen causes metabolic cell death; increased oxygen concentrations carry a risk for oxidative damage to proteins, lipids and nucleic acids, possibly initializing apoptosis or carcinogenesis[31]. The present results demonstrate a reduction in ATP level in the liver of hypoxic rats, accompanied by an increase in ADP and AMP concentrations. This could easily be correlated with the aerobic-anaerobic transition induced by the hypoxia[32]. The significant reduction in the Pi concentration in hypoxic rats despite the reduced level of ATP could be explained by the fact that, when liver is subjected to metabolic stress, a large amount of phosphate is trapped due to the presence of an abnormally high level of phosphoryl acceptor[25]. The ATP/ADP ratio revealed that the energy utilization reaction was higher than the energy-generating reaction in the low oxygen state and, in turn, this could confirm the impairment of oxidative phosphorylation in the liver and reduced metabolism[33]. The significant decrease in PO during the period of hypoxia confirmed the inhibition of Krebs cycle enzymes and the impairment of the electron transport chain[34]. Liu *et al*[35] reported that hypoxia reduced the level of adenylate energy charge, ATP/ADP and ATP/AMP in collaboration with decreased levels of ATP, ADP and AMP due to an increase in the activity of adenylate kinase enzyme. The significant decrease of TA in hypoxic rats may be explained by the finding of a decrease in the ATP level which is considered to be the main contributing part of TA. In concurrence with the current result, Emerling *et al*[36] found that cells exposed to anoxia (0 mL/L O_2) show a decrease

in ATP levels. The intracellular ATP level might indeed direct AMP catabolism either towards IMP or adenosine. Jyoti *et al*[37] demonstrated that hypoxia activates AMP-activated protein kinase (AMPK) signalling independent of a decrease in both ratios.

AEC is a linear measure of the ratio of ATP concentration to total adenylate concentration, which ranges in value from 1 in the fully charged state to 0. Paradoxically, high values of AEC are often associated with high toxicant exposures, and low AEC values with low exposure. These discrepancies may be caused by the inability of AEC measurements to adequately evaluate cytosolic adenylate concentrations, which are the critical parameters in enzymatic regulation[38].

The present results also demonstrated a significant increase in PK, PFK and glycogen phosphorylase in the liver of hypoxic rats. In parallel results Solaini *et al*[39] found that the AMPK pathway, which causes increases glycolysis, is driven by enhanced catalytic efficiency of some enzymes, including phosphofructokinase-1 and pyruvate kinase. The marked reduction in glucose and glycogen, while a significant increase in glycolytic enzymes PK, PFK was detected in liver tissue of hypoxic rats, may be due to an increase in metabolic activity in liver of hypoxic rats to compensate the inhibition of Krebs cycle caused by hypoxia. The increase in anaerobic glycolysis might be attributed to the activation of PFK due to decreased citrate formation provision of energy due to inhibition of the Krebs cycle and decreased nicotinamide adenine dinucleotide, oxidised form (NAD)/nicotinamide adenine dinucleotide, reduced form (NADH) (NAD/NADH) ratio due to inhibition of mitochondrial oxidation which favours the conversion of pyruvate to lactate[40] with respect to glycogen phosphorylase, as glycogenolytic enzyme, it showed an enhanced activity in hypoxic rats which was attributed to degradation of stored glycogen inhibition of translocase, the glucose-6-phosphate transport protein[41].

Milusheva *et al*[42] reported that ATP, ATP/ADP and AEC were significantly decreased in the absence of glucose (glucose deprivation) and ascertained that hypoxia is combined with glucose and glycogen deprivation and inhibition of the glycolysis and glycogenolytic pathways. The lack of glucose is a common factor contributing to the reduction in ATP level and AEC. This agrees with the well-known fact that during anoxia oxidative phosphorylation is impaired while ATP production proceeds *via* anaerobic glycolysis. During metabolic stress, AMPK, a highly sensitive indicator of cellular energy status, leads to an increase in ATP synthesis and inhibition of anabolic pathways to limit ATP consumption during periods of exercise or hypoxia[43]. AMPK downregulates ATP-consuming pathways, such as glycogen, cholesterol, and fatty acid synthesis[44]. However, Ou *et al*[45] declared that the evidence for increased glucose utilization under hypoxic conditions is equivocal. For example, rats acclimated to chronic hypoxia do not alter the capacity for anaerobic glycolysis in skeletal muscle, but rather increase the capacity for fatty acid metabolism, possibly sparing carbohydrate metabolism for severe hypoxic conditions. Significant amelioration was detected upon treatment of hypoxic rats with idebenone, melatonin, idebenone + melatonin, arginine and arginine + idebenone with a fluctuating percent of amelioration, where the hypoxic rats receiving combined treatment of idebenone + melatonin showed the best result followed by idebenone + arginine. In agreement with the present findings, idebenone has been reported to preserve non-protein thiols, and inhibit lipid peroxidation in rat brain during post-cardiac arrest reperfusion[46]. Idebenone also may indirectly reduce oxidative brain stress by elevating nerve growth factor[47] which acutely blocks reactive oxygen species (ROS) formation in the brain[48]. On the other hand, idebenone treatment suppressed leukocyte-enhanced cold ischemia/reperfusion injury of liver endothelium through almost complete suppression of the endothelial constitutive nitric oxide synthase mRNA expression after reperfusion[49]. Moreover, idebenone treatment reduced nitric oxide (NO) generation and apoptosis in hepatocytes treated with toxic bile salt glycochenodeoxycholate. Acute reduction in cerebral oxygen delivery is known to lead to the breakdown of neuronal energy metabolism[50]. Additionally, high concentrations of ROS, resulting from impaired oxidative phosphorylation or electron leakage, eventually leads to ATP depletion[51]; this is in contradiction with this study in which the ATP level was markedly reduced in hypoxic liver tissue.

On the basis of these data, we can hypothesise that the induction of melatonin during or post hypoxia could be a defensive mechanism by which hepatic cells contrast these alterations. To support this hypothesis, Nagaoka *et al*[38] reported that, melatonin, a lipophilic compound, acts directly or indirectly on ROS production; in fact, melatonin can directly scavenge free radicals or it can induce antioxidant enzymes *via* a specific melatonin receptor. These results could be attained in support of the presenting results that idebenone, melatonin or idebenone + melatonin produced obvious improvement in all the measured parameters in the the liver of hypoxic rats, with a greater effect being seen with combined treatment. Glycogen synthesis, which is located in cytosol, depends on the uridine triphosphate supply and hence on ATP supplies resulting from both mitochondrial oxidative phosphorylation and cytosolic glycolysis[3]. L-arginine has a protective role against ROS attack due to its direct chemical interaction with superoxide anions[52]. Data from the l-arginine treated group suggests that a pharmacologic increase in NO levels did not exacerbate the increase in free radical formation. In fact, a high level of NO/or l-arginine itself in the l-arginine treated group may be protective, probably due to their ability to scavenge free radicals as well as inhibit xanthenes oxidase (XO) enzyme[53]. Idebenone and/or l-arginine treatment ameliorated the depleting effect of nitrite-induced hypoxia on brain ATP content, suggesting that their protective effect may be mediated through improving the cerebral energy metabolism[54]. In

addition, its role in scavenging ROS and inhibiting lipid peroxidation could be an important factor in improving the mitochondrial respiratory chain. Studies have shown that under cellular low oxygen conditions idebenone prevents the free radical damaging effect and maintains relatively normal cell ATP levels[55]. The present data support these facts as the decrease in brain ATP content in hypoxic rats was accompanied by a significant increase in brain malondialdehyde (MDA) content and serum uric acid concentrations. L-arginine supplementation has been proven to significantly reduced the increased in cardiac XO activity, MDA levels, and serum uric acid caused by exhaustive exercise in rats[56].

Hepatocytes in normal rats had a normal chromatin structure with round nuclei. The quantity and the structure of the mitochondria in the cytoplasm was normal. Liver pathophysiology consists of many mechanisms that have an impact on liver damage at different levels. The morphological alterations of liver histology detected in hypoxic rats induced severe alterations in the liver, such as congestion, sinusoidal and lymphatic expansion, regional hepatocellular vacuolization and hepatocyte swelling. These results are in agreement with those of previous studies[10]. Liver injury was repaired when idebenone, melatonin, arginine, idebenone + melatonin and idebenone + arginine were administered.

In accordance with previous results[57] which revealed that melatonin exerts a beneficial role in restoring tissue alterations after subjection to hypoxia (Figure 4).

In conclusion, a combination of idebenone + melatonin and idebenone + arginine yields significantly better results than idebenone, melatonin or arginine alone. They are likely responsible for liver tissue protection in hypoxic rats through their action on NO generation, reduction as well as scavenging of ROS, maintenance of normal enzymatic and non-enzymatic antioxidant systems, and improvement of energy production.

Therefore, the current findings may have important implications in the development of therapeutic strategies aimed at manipulating either idebenone + melatonin and l-arginine + idebenone supplementation for amelioration of hypoxic liver injury.

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