INHIBITION OF ARTESUNATE-INDUCED LIVER MITOCHONDRIAL PERMEABILITY TRANSITION PORE OPENING BY VITAMIN C

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Abstract: It has been demonstrated that artesunate causes hepatoxicity by inducing opening of the mitochondrial permeability transition (MPT) pore; a condition that is exacerbated by ferrous sulphate. Experimental rats in group I, II and III received distilled water (untreated group), 2.5 mg/kg bw ART_L and 15.0 mg/kg bw ART_H respectively while group IV and V received 2.5 mg/kg bw ART_L + 200 mg/kg bwvit C and 15.0 mg/kg bw ART_H + 200 mg/kg bw vit C, respectively. All treatments were for five (5) days. Results of the study showed that ART_L and ART_H induced opening of MPT pore opening by 1.32 and 2.76 fold ($p<0.01$), respectively. However, Vit C inhibited the MPT pore opening causing 1.47 and 0.75 fold ($p<0.05$) induction in group IV and V, respectively. Vitamin C could ameliorated hepatotoxicity associated with the use of artesunate.

Keywords: Artesunate, mitochondria, vitamin C, permeability pore

Abbreviations: ART-artesunate, Vit- vitamin, MPT- mitochondrial permeability transition

Introduction

Mitochondria are the power house of the cell. Mitochondria houses important metabolic pathways such as fatty acid β-oxidation, tricarboxylic acid cycle and oxidative phosphorylation (Fromenty *et al.*, 2002). It is the main source of intrinsically generated reactive oxygen species in the cell and consequently involved in cell death resulting from mitochondrial oxidative stress (Crompton, 1999). Sadly, the mitochondria are targets of drug toxicity resulting in drug induced lesions, necrosis or apoptosis (Deschamps *et al.*, 1994; Lemasters, 1999).

Artemisinin is a sesquiterpene lactone which possesses antimalarial and cytotoxic properties derived from *Artemisia annua* (Ngokere *et al.*, 2004; Woodrow *et al.*, 2005). The compound contains an endoperoxide bridge which generates free radicals when cleaved. Consequently, the free radicals alkylate proteins in *Plasmodium falciparum*; a mechanism by which it effects antimalarial activity (Meshnick *et al.*, 1996). Unfortunately, the cells of the host organism may suffer from insult by the free radicals. There are established evidences on the hepatotoxic effect of artesunate in rats. One of the mechanisms by which artesunate elicits its hepatotoxic effect is by opening of the mitochondrial permeability pore thereby causing leakage of mitochondrial macromolecules and eventual cell death (Anyasor *et al.*, 2009; Fafowora *et al.*, 2010). Other studies supported the claims that augmentation of artesunate with ferrous sulphate exacerbates the opening of the mitochondrial pore, increased hepatotoxicity and possible cell death. The present study was designed to assess the effect of vitamin C, an antioxidant compound on mitochondrial pore opening induced by artesunate.

Materials and Methods

Drugs, chemicals and reagents

Artesunate (100 mg tablets) were products of GVS Laboratories, India. Vitamin C (100 mg tablets) were products of Mopson Pharmaceutical Limited, Nigeria. Mannitol, sucrose, HEPES, spermine, sodium succinate and rotenone were products of Sigma Aldrich, UK. All other chemicals used were of best available grade.

Animal care

Wistar strain albino male rats (180 – 200 g) were obtained from the Preclinical Animal House, of Physiology Department, of University of Ibadan, Ibadan, Nigeria. The animals were acclimatized for 15 days prior to experiments in the animal house, Biochemistry Department, University of Ibadan. Animals were fed with standard rat chow (Ladokun feed, Ibadan) and water *ad-libitum*, and were kept under standard conditions of temperature and 12 h dark/ light cycle.

Experimental design

Artesunate and vitamin C were suspended in water and administered orally to rats for five days (Utzinger *et al.*, 2007; Farombi *et al.*, 2008). Animals were allocated into groups of five (5) rats as follows:

Group I: Untreated
Group II: 2.5 mg/kg bw ART_L
Group III: 15.0 mg/kg bw ART_H
Group IV: 2.5 mg/kg bw ART_L + 200 mg/kg bwvit C
Group V: 15.0 mg/kg bw ART_L + 200 mg/kg bwvit C

On the sixth day animals were sacrificed by cervical decapitation, dissected and the livers were removed for isolation of mitochondria.

Preparation of low ionic strength liver mitochondria

Low ionic strength mitochondria were isolated according to the method described by Johnson and Lardy (1967). Briefly, the animals were sacrificed by cervical dislocation, dissected and the liver was immediately excised and trimmed to remove excess tissue. The liver was washed several times in Washing buffer (210 mM Mannitol, 70 mM Sucrose, 5 mM HEPES-KOH, pH 7.4 and 1mM EGTA) until a clear wash was obtained, then weighed and minced with a pair of scissors. A 10% suspension of the liver was prepared by homogenizing the liver in a Potter-Elvehjem glass homogenizer. The whole process was carried out on ice to preserve the integrity of the mitochondria. The homogenate was centrifuged in a refrigerated MSE centrifuge, where the nuclear fraction and cell debris was pelleted by low speed centrifugation at 2,300 rpm for 5 minutes. The supernatant was re-centrifuged at the same speed and time to remove unbroken cells. The supernatant thus obtained was centrifuged at 13,000 rpm for 10 min to sediment the mitochondria. The mitochondria pellet obtained after the supernatant was discarded was washed by re-suspending in a Suspension buffer (210 mM Mannitol, 70 mM Sucrose, 5 mM HEPES-KOH, pH 7.4, 0.5% BSA) and centrifuged at 12,000 rpm for 10 minutes. This washing stage was done twice. The mitochondria were immediately suspended in a solution of ice-cold MSH Buffer (Mannitol, Sucrose, HEPES-KOH, pH 7.4) and maintained on ice.

Mitochondria swelling assay

Mitochondria swelling was determined according to the method of Lapidus and Sokolove (1993). Mitochondria (0.4

mg/ml protein equivalent) were pre-incubated in the presence of 0.8 µM Rotenone for 3.5 min, prior to the addition 5mM Sodium succinate. When Ca^{2+} was used as a triggering agent, mitochondria were pre-incubated in the presence of 0.8 µM Rotenone for 3 minutes. Ca^{2+} was added after the 3 minutes of mitochondria pre-incubation and 30 seconds later, sodium succinate was added. Absorbance was taken at a wavelength of 540 nm in a Camspec M105 spectrophotometer every 30 seconds for 12 min. Swelling was measured as decrease in absorbance within the time space of 12 min. The temperature was maintained at 30^0C and swelling rate quantified as an A540/min/mg protein.

Protein determination

Mitochondrial protein content was estimated by the procedure of Lowry *et al.* (1951) using Bovine Serum Albumin (BSA) as standard.

Statistical analysis

Statistical analysis was carried out using Microsoft Excel and GraphadInstat. Data are reported as mean ± standard deviation of five (5) determinations. Comparison of mean values of respective groups was done by one way Analysis of Variance (ANOVA).

Results and Discussion

This study demonstrates that *in vivo* treatment with artesunate results in opening of the mitochondria permeability transition (MPT) pore and that vitamin C inhibits the effects of artesunate. This suggests that MPT is involved in hepatotoxic effect of artesunate. The results of the effect of *in vivo* treatment of rats with artesunate on MPT pore is shown in Fig. 1 and Table 1. The results show that artesunate caused a dose dependent induction of MPT pore opening. Low dose artesunate (2.5 mg/kg bw ART_L) induced 1.32 fold opening of the MPT pore though not statistically significant whereas, the high dose of artesunate (15.0 mg/kg bw ART_H) significantly induced the MPT pore ($p<0.01$) by 2.76 fold when compared to the normal untreated group. Co-administration of artesunate with vitamin C inhibited the toxic activity of artesunate. In the presence of 200 mg/kg bwvit C, low dose artesunate induced 1.47 fold MPT pore opening when compared with the untreated. Similarly, co-administration of high dose artesunate with vit C caused a significantly decreased ($p<0.05$) MPT pore opening (0.75 fold) when compared with the group treated with high dose of artesunate alone.

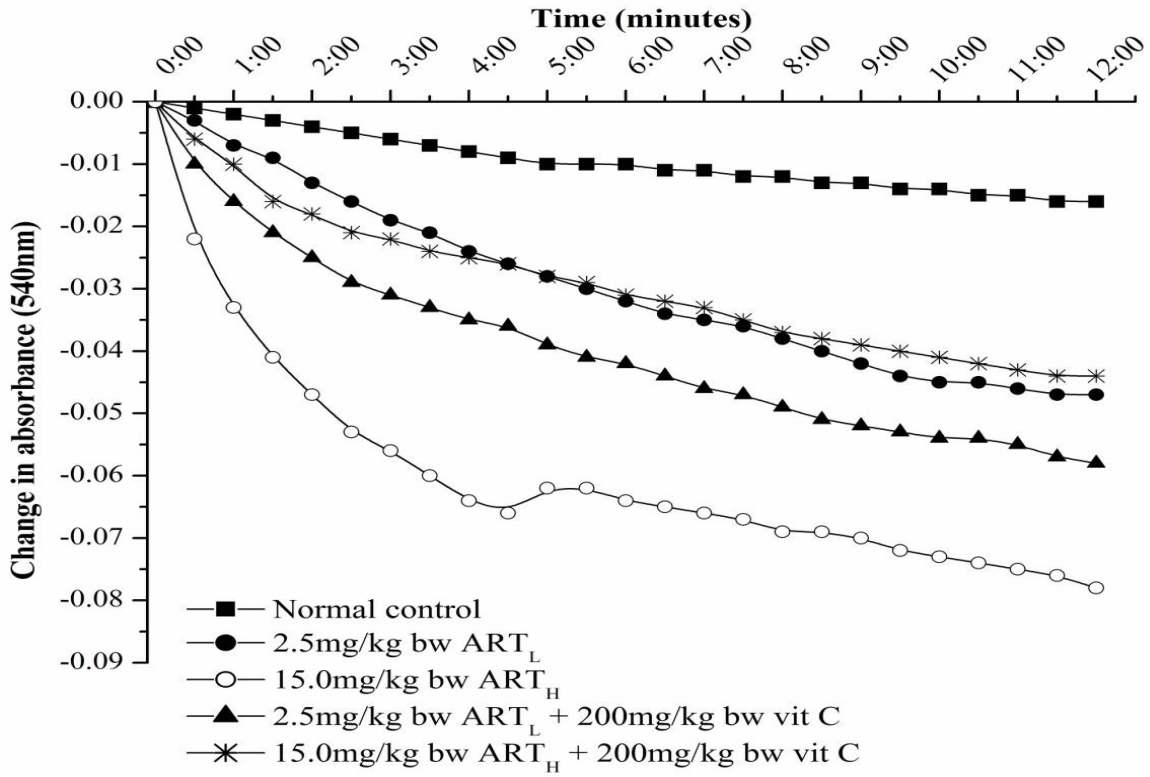

Fig. 1: Swelling of liver mitochondria of rats treated with artesunate and Vitamin C

Table 1: Varaiations in mitochondria pore opening in liver of rats treated with artesunate and vitamin C

Groups	Δ Absorbance (540 nm)	Pore induction (folds)
I: Untreated	-0.0158 ± 0.0087	--
II: 2.5 mg/kg bw ART_L	-0.0367 ± 0.0226	1.32
III: 15.0 mg/kg bw ART_H	-0.0594 ± 0.0184**	2.76
IV: 2.5 mg/kg bw ART_L + 200 mg/kg bwvit C	-0.0391 ± 0.0155	1.47
V: 15.0 mg/kg bw ART_H + 200 mg/kg bwvit C	-0.0278 ± 0.0121*	0.75

Grouping of the animals is shown in the methods section. Data are presented as mean ± SD of six (5) animals. Superscript indicate significantly different group.

**$p<0.01$ compared to control untreated rats

*$p<0.05$ compared to 15.0 mg/kg bw ART_H group

Vitamin C is a potent antioxidant and routinely co-prescribed with many drugs known to predispose people to oxidative stress (Robert *et al.*, 2000). This study has demonstrated for the first time the practice of co-prescription of artesunate and vitamin C could help in ameliorating some of the hepatotoxic effects of the former. Although, the exact mechanism that underlie such protection is not clear, previous reports shows that the MPT pore is constituted by proteins including voltage-dependent anion channel (VDAC) in the outer membrane, adenine nucleotide translocase (ANT) in the inner membrane and cyclophilin D in the matrix which are targets of free radicals (Efferth *et al.*, 2003). Compromise of the MPT pore assembly resulting from drug toxicity has been shown to cause MPT pore opening and cell death (Olliaro *et al.*, 2001).
Put together, production of free radicals by the Endoperoxide Bridge of artesunate is key to its antimalarial effect as well its adverse effects which includes liver injuries (Tailor *et al.*, 1996, WHO, 1998). Vitamin C on the other hand protects against such toxicity evident by MPT pore opening by scavenging the free radicals.
Therefore, it is advisable to co-administer artesunate with low doses of vitamin C. However, it is necessary to investigate doses of vitamin C which offer protection from 'artesunate radicals' without undermining its anti-malarial activity.

Conclusion
Vitamin C inhibits MPT pore induction by artesunate. This might offer protection from side effects of artesunate on mitochondria and artesunate-induced cell death.

Conflict of interest
The authors declare no conflict of interest.

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