Int. J. Morphol.,
38(1):48-55, 2020.

Vitamin C Administration Attenuated Artemether-Induced Hepatic Injury in Rats

La Administración de Vitamina C Atenúa la Lesión Hepática Inducida por Artemeter en Ratas

Refaat A. Eid[1]; Mohamed Samir Ahmed Zaki[2,3]; Mansour A. Alghamdi[2]; Abulqasim Mohammed Sideeg[2]; Kamal, Z. M. Ali[2]; Mohamed Andarawi[1] & Mohamed A. Haidara[4]

EID, R. A.; ZAKI, M. S. A.; ALGHAMDI, M. A.; SIDEEG, A. M.; ALI, K. Z. M.; ANDARAWI, M. & HAIDARA, M. A. Vitamin C administration attenuated Artemether-induced hepatic injury in rats. *Int. J. Morphol., 38(1)*:48-55, 2020.

SUMMARY: This research was designed to investigate the potential protective effect of vitamin C supplementation against hepatocyte ultrastructural alterations induced by artemether (antimalarial drug) administration. Twenty-four adult male albino rats were used in this study and were divided into four groups (n=6). Group I served as a control and rats in group II administrated artemether (4 mg/kg B.W) orally for three consecutive days. Group III administered artemether plus a low dose of vitamin C (2.86 mg/kg/l water) while group IV received artemether plusa high dose of vitamin C (8.56 mg/kg). At the end of the experimental period (14 days), the harvested liver tissues were examined by transmission electron microscopy (TEM), and blood samples were assayed for biomarkers of liver injury and oxidative stress. Artemether significantly ($p<0.05$) augmented biomarkers of liver injury such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), and oxidative stress such as superoxide dismutase (SOD), Glutathione Peroxidase (GPX), and caused degeneration and damage of the rough endoplasmic reticulum and disrupted mitochondria. The blood sinusoids were also damaged with distortion of their canaliculi. Administration of vitamin C showed improvement of liver biomarkers, and liver parenchyma, especially in a high dose of vitamin C.We concludes that vitamin C is a partial protective agent against artemether-induced liver injury.

KEY WORDS: Artemether; Rats; Vitamin C; Hepatocyte ultrastructure; Biomarkers liver injury; Oxidative stress.

INTRODUCTION

Malaria is an endemic public health challenge that is predominantly widespread in tropical and subtropical regions of the world (Bhatt *et al*., 2015). Artemisinin-based therapy has significantly reduced mortality, particularly for children with severe malaria (>30 %). Artemether is characterized by its novel structure which is very effective against multidrug-resistant Plasmodium falciparum malaria (Meshnick, 2002).

Oxidative stress occurs when the generation of reactive oxygen species in the body exceeds the ability of the body to neutralize and eliminate them (Li *et al*., 2018). The susceptibility of liver tissues to this stress due to exposure to drugs is a function of overall balance between the degree of oxidative stress and the antioxidant capacity (Khan *et al*., 2005).

Since malaria infection imposes tremendous oxidative stress on the host (Shichiri *et al*., 2019), the antimalarials are often prescribed with vitamin C or similar antioxidant supplements. The antioxidant effect in erythrocytes has been reported to depend upon the presence or absence of glutathione. In the presence of glutathione, ascorbic acid has synergistic antioxidant activity against haem-mediated cell toxicity (Li *et al*., 2006). In glutathione deficient red cells, as often happens in parasitized RBCs due to oxidative stress, ascorbic acid can react with iron or iron-containing compounds to generate hydrogen peroxide or hydroxyl radical and accentuate the haemolytic mechanisms in malaria (Li *et al*., 2006).

Therefore, the aim of the present work is to study the artemether induced liver toxicity on the liver and to evaluate

[1] Pathology department, College of Medicine, King Khalid University, Abha 61421, Saudi Arabia.
[2] Anatomy department, College of Medicine, King Khalid University, Abha 61421, Saudi Arabia.
[3] Histology department, College of Medicine, Zagazig University, Egypt.
[4] Physiology department, Kasr al-Aini Faculty of Medicine, Cairo University, Cairo, Egypt.

the possible protective effect of vitamin C against this toxicity in rats.

MATERIAL AND METHOD

Animals: All experimental procedures were approved by the medical research ethical committee at King Khalid University and according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. (NIH publication No. 85-23, revised 1996). Sprague–Dawley rats (n=24) weighing 150-250 g were used in this study. All rats were bred and housed in the research centre of King Khalid University, college of medicine (Abha, Saudi Arabia), at a temperature of 23 ±1 °C and a 12 h light: 12 h dark cycle. Rats had free access to tap water and fed standard laboratory chow during the acclimatization period.

Experimental design: After one-week adaptation. All rats were fed a standard laboratory diet. The rats were randomly divided into four groups (n=6 rats each). Animals in the first group (Control) were fed with standard laboratory chow for two weeks. Animals in the second group (Artemether), rats were given artemether supplementation (4 mg/kg B.W / daily by oral gavage) for three consecutive days and continue on a standard diet for two weeks. The third group, rats administered artemether, 4 mg/kg B.W /day by oral gavage for three consecutive days, plus a low dose of vitamin C, 2.86 mg/kg /l water for two weeks. Animals in the fourth rats administered artemether, 4 mg/kg B.W /day by oral gavage for three consecutive days, plus a high dose of vitamin C, 8.56 mg/kg /l water for two weeks.

Biochemical measurements

Blood samples: At the end of the experimental period, blood samples were collected by cardiac puncture under anaesthesia (sodium thiopentone at 40 mg/kg body weight) after an overnight fast of 12 hours. These blood samples were collected without anticoagulant, left for 10 min, then centrifuged for 10 min at 4000 r/min to obtain serum, which was stored at –20 °C until further biochemical analysis for determination of serum liver enzymes, oxidative stress biomarkers.

Determination of serum levels of ALT, AST: After two weeks, animals were sacrificed, and liver function was evaluated by assessing serum ALT and AST levels using an enzymatic kit (Randox Laboratories, Crumlin, UK) according to the manufacturer's instructions.

Determination of serum levels of superoxide dismutase (SOD) and Glutathione peroxidase (GPx). After two weeks, animals were sacrificed, and serum levels of (SOD) and (GPx) were measured using commercial kits supplied by SPINREACT, Spain, according to the manufacturer's instructions.

Light Microscopy (LM): The fixed liver specimens (formalin fixed tissues) were trimmed, washed, dehydrated in ascending grades of ethyl alcohol, cleared in methyl benzoate and embedded in paraffin after having completed the routine follow-up steps. Sections at 3-5 m sections were obtained from liver using rotary microtome and stained by hematoxylin and eosin (H&E) stain for light microscopically investigation to Bancroft & Gamble (2008).

Transmission Electron Microscopy (TEM): Small pieces of liver tissues were removed and immediately fixed in 2.5 % glutaraldehyde for 24 hours and washed with phosphate buffer (0.1 M, PH 7.4). Post for 1-2 hours. The samples washed in phosphate buffer to remove excess fixative, dehydrated through ascending grades of ethanol followed by clearing in propylene oxide. The specimens were embedded in Araldite 502, to form gelatin capsules. Polymerization was obtained by placing the capsules at 60 ^{o}C. Semi-thin sections (~1 mm thick) were stained with toluidine blue for orientation and observation. Ultrafiltration was made in 1 % osmium tetroxide buffered to PH 7.4 with 0.1 M phosphate buffer at 4 ^{o}C -thin sections (100 nm) were prepared using ultra-microtome and picked up on uncoated copper grids. Following double staining with uranyl acetate and lead citrate, three to five random micrographs for each section were examined and photographed using a JEM-1011 transmission electron microscope, JEOL Ltd., Musashino, Akishima, Tokyo, Japan, at 80 Kv (Haidara *et al*., 2018).

Statistical analysis. The data were expressed as mean ± standard deviation (SD). Data were processed and analyzed using the SPSS version 10.0 (SPSS, Inc., Chicago, Ill., USA). One-way ANOVA was done followed by Tukey's post hoc test. Pearson correlation statistical analysis was done for the detection of a probable significance between two different parameters. Results were considered significant if $p \leq 0.05$.

RESULTS

Vitamin C reduces biomarkers of liver injury and oxidative stress induced artemether. To determine whether vitamin C can inhibit artemether-induced up-regulation of liver injury enzymes (ALT and AST), and biomarkers of oxidative stress (SOD and GPx) and inflammation in our

animal model of artemether –induced liver injury, we measured the blood levels of ALT, AST, SOD and GPx in all rat groups. Artemether caused the augmentation of ALT (Fig. 1A), AST (Fig. 1B), which were significantly ($p<0.05$) reduced with vitamin C treatment (Artemether +Vit C). Only a high dose of vitamin C returns liver function to control levels. Artemether also caused decreased biomarkers of oxidative stress, GPx (Fig. 1C), and SOD (Fig. 1D), which were significantly ($p<0.05$) increased with vitamin C treatment (Artemether +Vit C). Low dose causes partial improvement, where high dose returns both GPx and SOD to control levels.

Vitamin C protects hepatocyte histopathological and ultrastructural damage induced by Artemether:

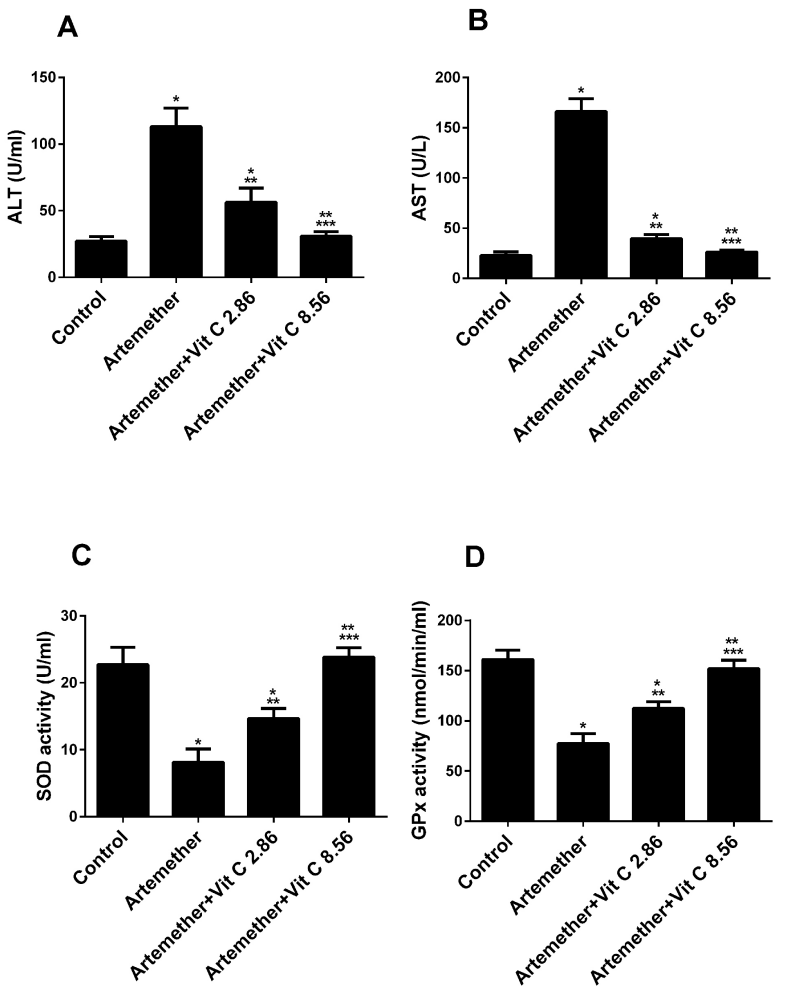

Fig. 1. Vitamin C reduces Artemether -induced ALT, AST, and increased MDA and GPx. Serum levels ofALT (A), AST (B), SOD (C), and GPx (D) were measured in 4 groups of rats; Control, Artemether, Artemether +vitamin C 2.86 and Artemether +vitamin C 8.56 groups after two weeks. Results represent the mean (±SD); n=6 for each group. Experiments were performed in triplicate. *$p<0.05$ versus control, **$p<0.05$ versus Artemether, ***$p<0.05$ versus Artemether+ vitamin C 2.86.

Histopathological findings (LM): H&E stained sections of liver of the control group revealed normal characteristic of hepatic architecture; the hepatic lobules appeared to be made up of hepatocytes arranged in cords radiating from the central veins. They were polyhedral in shape with granular acidophilic and slightly vacuolated cytoplasm and rounded vesicular, centrally located nuclei. In between the hepatic cords, the hepatic sinusoids appeared as narrow spaces lined with flattened endothelial cells and few Kupffer cells (Fig. 2A). The treated group that injected with artemether showed marked affection of hepatic architecture in rats liver sections as compared to controls. Most of hepatocytes appeared with necrotic nuclei and cytoplasmic vacuolization. Some cells had irregular-shaped nuclei. Moreover, the blood sinusoids revealed dilatation and congestion and the central vein appeared congested. Most of the blood sinusoids appeared widened as well as white blood cell infiltration and proliferation of Kupffer cells. The portal area revealed congestion of its vessels (Fig. 2B). The third group (artemether +vit C 2.86 mg/kg) revealed partial preserved hepatic architecture (Fig. 2C) were as the fourth group (artemether +vit C 8.56 mg/kg) showed normal appearance of the hepatic architecture lobules (Fig. 2D).

Ultrastructural findings (TEM): The liver of control rats revealed normal cellular architecture. The hepatocytes with rounded regular euchromatic nuclei and prominent nucleoli were seen. The cytoplasm of these cells showed numerously rounded to oval mitochondria, multiple parallel arrays of the rough endoplasmic reticulum. Bile canaliculi were seen as narrow spaces limited by short microvilli of two adjacent hepatocytes and bounded by desmosomes. Blood sinusoids with junctional complex between two adjacent hepatocytes were seen as narrow spaces limited by long microvilli (Figs. 3A, 4A and 5A).

Electron microscope investigation revealed significant structural aberrations in the liver of artemether-treated rats. Nuclei of hepatocytes appeared irregular with electron dense chromatin as compared to control. Alterations in the cytoplasmic contents of cells were observed by the presence of numerous vacuoles that vary in size and shape. Abnormally shaped mitochondria were observed with condensed opaque matrices and lacked internal organization. The

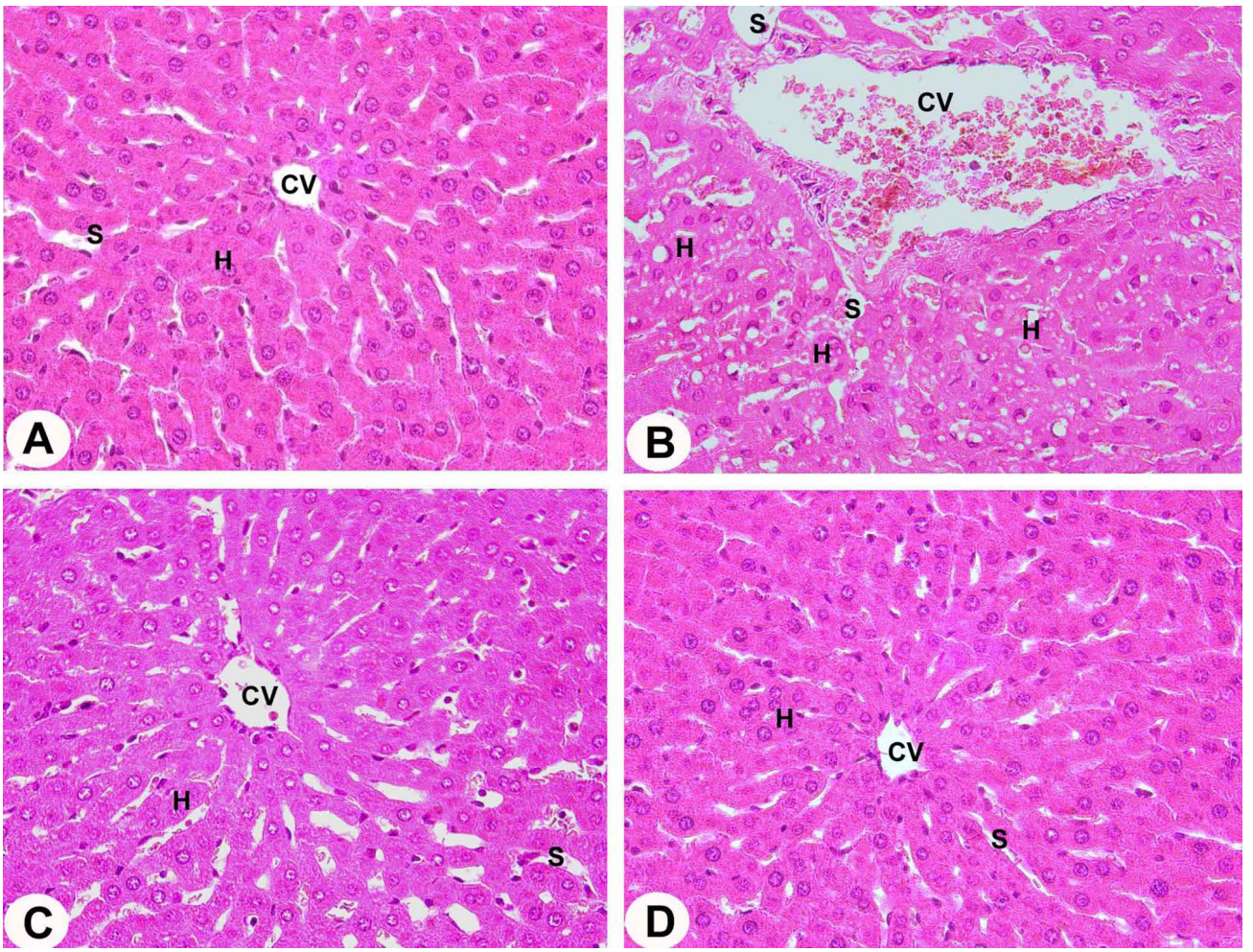

Fig. 2. LMs X400 of harvested tissues obtained from the liver of the control group (A) compared to the Artemether-treated group (B), Artemether-treated + 2.86 mg/kg Vit-C group (C), and Artemether-treated + 8.56 mg/kg Vit-C group (D) rats are visualized using light microscopy. Abbreviations: H, hepatocytes; CV, central vein; S, blood sinusoid.

cisternae of rough endoplasmic reticulum appeared dilated and fragmented. The bile canaliculi with junctional complex were distorted. (Fig. 5B). Moreover, the blood sinusoids were damaged with abnormal microvilli (Figs. 3B and 4B).

Electron microscopic examination of the third and fourth group revealed minor changes in most liver cells. The hepatocytes have normally-shaped mitochondria, the cisternae of the rough endoplasmic reticulum were normally distributed, and intact bile canaliculi with junctional complex are seen. Some vacuoles are still present. Normally distributed and intact blood sinusoids with normal microvilli were seen (Figs. 3C, 4C and 5C).

Examination of the liver sections of the fourth group by electron microscopy, the histological architecture of the hepatic lobules exhibited a more or less normal appearance (Figs. 3D, 4D and 5D).

DISCUSSION

The main objective of our study was to investigate the potential protective effect of vitamin C to the hepatocyte ultrastructure against artemether -induced liver injury in a rat model of the disease using TEM. In addition, a comparison was also made between the pathological and biochemical changes occurred in response to the disease and its potential treating drug, vitamin C. The main findings of our study were that (i) Artemether induced hepatic profound damage to hepatocyte ultrastructure; (ii) low dose vitamin C substantially but not completely slowed down the progression of the disease in rats; and (iii) high dose vitamin C significantly reduced certain biomarkers of liver injury. These conclusions are supported by the data indicating that Artemether markedly increased liver injury enzymes (ALT and AST), and reduced anti-oxidative stress

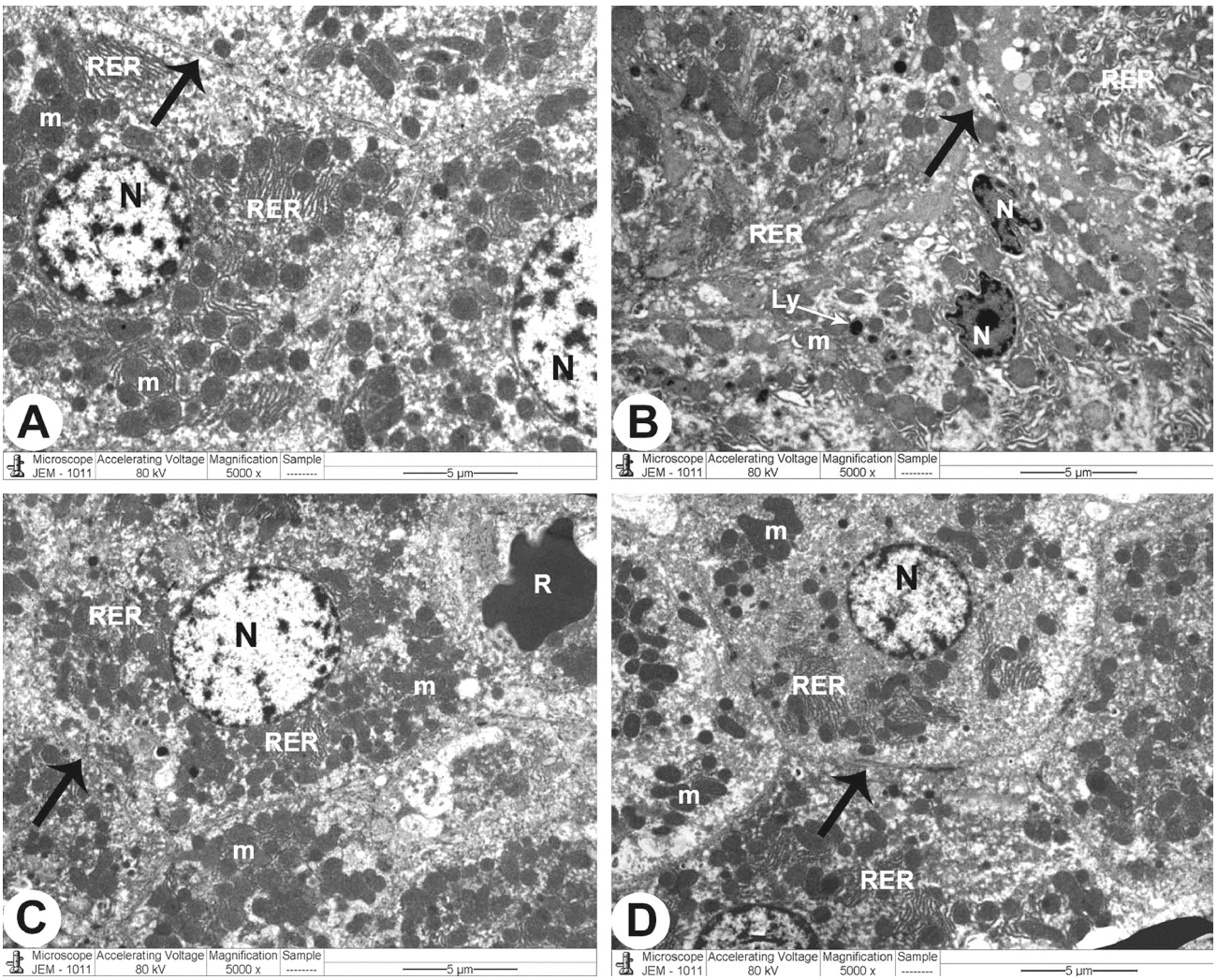

Fig. 3. TEMs X5000 (5 µm) of harvested tissues obtained from the liver of the control group (A) compared to the Artemether-treated group (B), Artemether-treated + 2.86 mg/kg Vit-C group (C), and Artemether-treated + 8.56 mg/kg Vit-C group (D) rats are visualized using transmission electron microscopy. Note that large black arrows point to intercellular spaces between hepatocytes. Abbreviations: N, nucleus; m, mitochondria; RER, rough endoplasmic reticulum; Ly, lysosomes.

biomarker (GPx and SOD), which were significantly improved with vitamin C treatment (Fig. 1). Also, vitamin C partially prevented damages occurred to liver cells after two weeks in rats fed on Artemether (Figs. 2-5).

Many xenobiotics, drugs and chemicals cause diverse forms of liver injury (Sturgill & Lambert, 1997), and this may result in distortion in liver histology. Earlier reports have also shown that artemether treatment results in congestion of hepatic sinusoids in healthy rats (Izunya *et al*., 2010).

Our results showed that treatment with artemether has a toxic effect on liver cells, associated changes in degenerative cells, which is in accordance with a previous study (Wood, 1965).

The results of the TEM examination of hepatocytes support that there was a diversity of cellular damage. Spaces of Disse were seen around hepatocytes expanded and filled with long fragmented microvilli which cause the distinct clarification of the cell's borders under the light microscopy examination. It also showed cytoplasmic vacuoles and sinusoids were filled with hypertrophy of Kupffer cells which is in accordance with Bjørndal *et al*. (2018) who reported that the neutral fat accumulates in the liver frequently, is due to inhibition of aerobic oxidation. Furthermore, artemether has been shown to induce transient

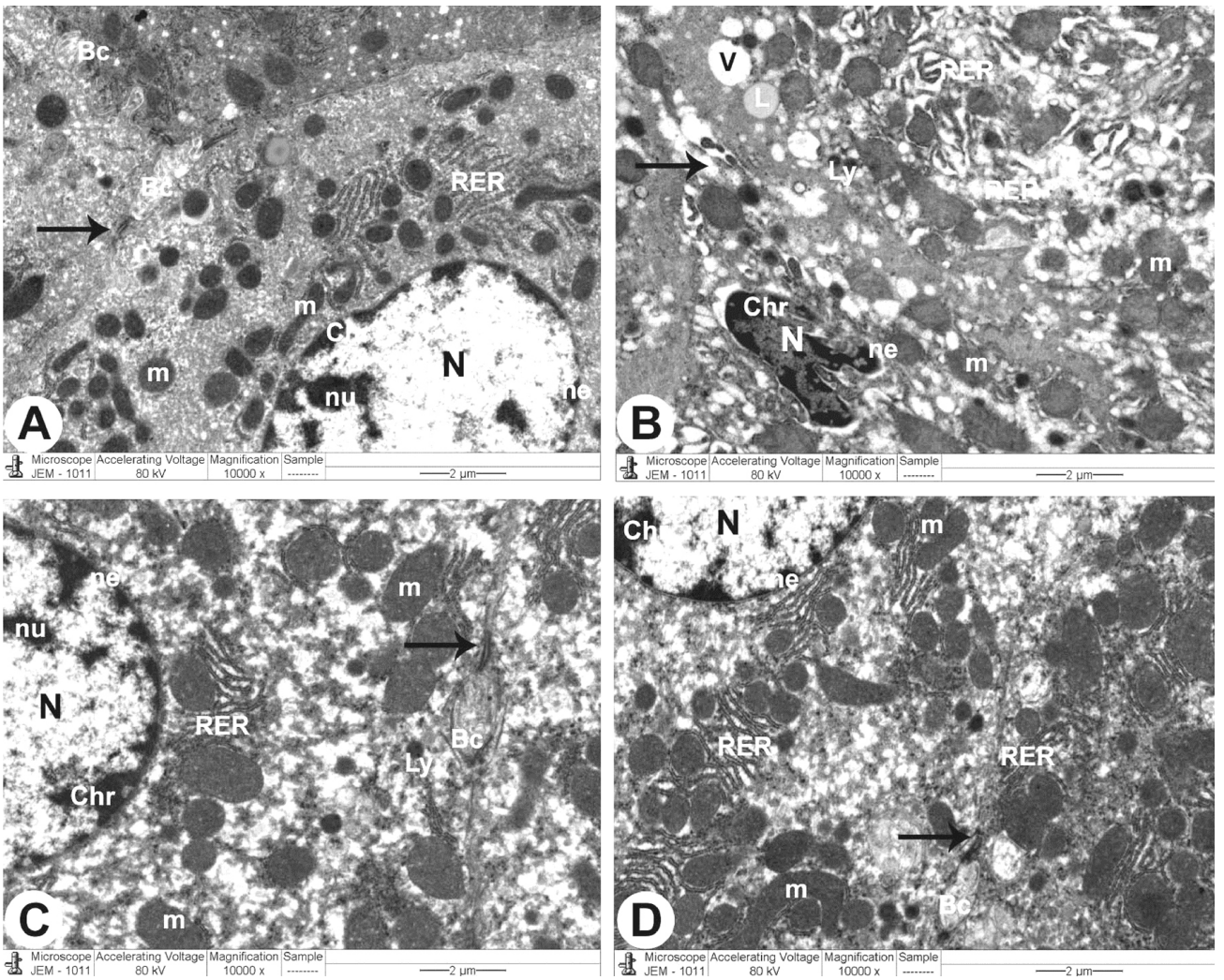

Fig. 4. Higher magnification of TEMs X10000 (2 µm) of the Vit-C protects against endoplasmic reticulum; ne, nuclear envelop; Chr, chromatin; Ly, lysosomes; Bc, bile canaliculiArtemether-induced hepatocyte ultrastructural damage in rats, at the end of the experiment, after two weeks. (A) Control group. (B), Artemether group. (C), Artemether +2.86 mg/kg Vit-C group, and Artemether +8.56 mg/kg Vit-C group (D). Note that large black arrows point to intercellular spaces between hepatocytes. Abbreviations: N, nucleus; nu, nucleolus; L, lipid droplets; V, vacuoles; m, mitochondria; RER, rough.

and moderate elevations in liver transaminases (Nwanjo *et al*., 2007).

Pro-oxidant chemicals which stimulate the oxidation effort either by synthesis or by inhibiting antioxidant may cause damage to cells and tissues (Kumar & Muralidhara, 2007) due to the formation of the superoxidation, which leads to rupture of the plasma membrane and organelles.

Vitamin C has a reducing potential that reacts with most of the important radicals and oxidants (Magdy *et al*., 2015) where it acts as a powerful hydrosoluble antioxidant in body fluids, scavenging reactive oxygen and nitrogen species (Elzoghby *et al*., 2015).

In conclusion, the overall results showed that vitamin C ameliorates the hepatotoxic effect of artemether, which was possibly mediated via free radical scavenging and inhibition of free radical generation.

ACKNOWLEDGMENTS.The authors extend their appreciation to the Deanship of Scientific Research at King Khalid University for funding this work through research groups program under grant number G.R.P.186 -39.

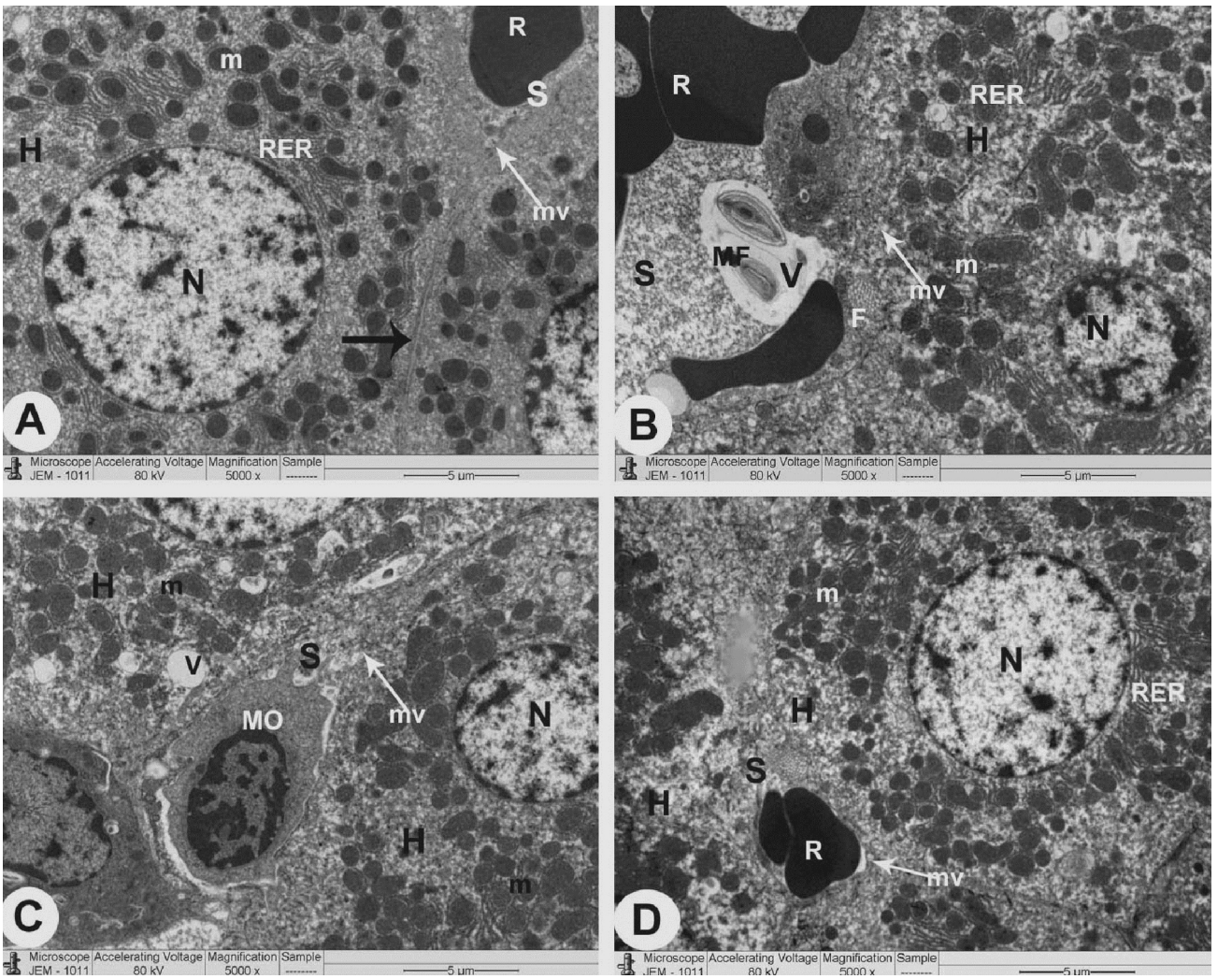

Fig. 5. TEMs X5000 (5 µm) (Blood sinusoids) of the Vit-C protects against Artemether-induced blood sinusoid and hepatocyte ultrastructural damage in rats, at the end of the experiment, after two weeks. (A) Control group. (B), Artemether group. (C), Artemether +2.86 mg/kg Vit-C group, and Artemether +8.56 mg/kg Vit-C group (D). Note that large black arrows point to intercellular spaces between hepatocytes. Abbreviations: H, hepatocytes; N, nucleus; m, mitochondria; RER, rough endoplasmic reticulum; Ly, lysosomes; MF, myelin figures; S, blood sinusoids; R, erythrocytes; V, vacuole; Mo, monocytes; mv, short microvilli.

EID, R. A.; ZAKI, M. S. A.; ALGHAMDI, M. A.; SIDEEG, A. M.; ALI, K. Z. M.; ANDARAWI, M. & HAIDARA, M. A. La administración de vitamina C atenúa la lesión hepática inducida por Artemeter en ratas. *Int. J. Morphol., 38(1)*:48-55, 2020.

RESUMEN: Esta investigación fue diseñada para investigar el posible efecto protector de la vitamina C contra las alteraciones ultraestructurales de los hepatocitos, inducidas por la administración de arteméter (medicamento antipalúdico). En el estudio se utilizaron 24 ratas albinas macho adultas y se dividieron en cuatro grupos (n = 6). El grupo I fue designado como control y las ratas en el grupo II se adminstró Arteméter (4 mg / kg de peso corporal) por vía oral durante tres días consecutivos. En el grupo III se administró arteméter, además de una dosis baja de vitamina C (2,86 mg / kg / l de agua) mientras que el grupo IV recibió arteméter más una dosis alta de vitamina C (8,56 mg / kg). Al final del período experimental (14 días), los tejidos hepáticos recolectados se examinaron por microscopía electrónica de transmisión (MET), y las muestras de sangre se analizaron en busca de biomarcadores de daño hepático y estrés oxidativo. El arteméter aumentó significativamente ($p < 0,05$) los biomarcadores de daño hepático como alanina aminotransferasa (ALT), aspartato aminotransferasa (AST) y estrés oxidativo como superóxido dismutasa (SOD), glutatión peroxidasa (GPX) y causó degeneración y daño de la retículo endoplásmico rugoso y mitocondrias alteradas. Los sinusoides sanguíneos también fueron dañados con la distorsión de sus canalículos. La administración de vitamina C mostró una mejoría de los biomarcadores hepáticos y el parénquima hepático, especialmente en una dosis alta de vitamina C. Concluimos que

la vitamina C es un agente protector parcial contra la lesión hepática inducida por arteméter.

PALABRAS CLAVE: Arteméter; Ratas; Vitamina C; Ultraestructura de hepatocitos; Lesión hepática de biomarcadores; Estrés oxidativo.

REFERENCES

Bancroft, J. D. & Gamble, M. T*heory and Practice of Histological Techniques.* 6th ed. New York, Churchill Livingstone, 2008. pp.440-50.

Bhatt, S.; Weiss, D. J.; Cameron, E.; Bisanzio, D.; Mappin, B.; Dalrymple, U.; Battle, K.; Moyes, C. L.; Henry, A.; Eckhoff, P. A.; *et al*. The effect of malaria control on Plasmodium falciparum in Africa between 2000 and 2015. *Nature, 526(7572)*:207-11, 2015.

Bjørndal, B.; Alterås, E. K.; Lindquist, C.; Svardal, A.; Skorve, J. & Berge, R. K. Associations between fatty acid oxidation, hepatic mitochondrial function, and plasma acylcarnitine levels in mice. *Nutr. Metab. (Lond.), 15*:10, 2018.

Elzoghby, R. R.; Hamoda, A. F.; Abdel-Fatah, A. & Farouk, M. Protective role of vitamin C and green tea extract on malathion-induced hepatotoxicity and nephrotoxicity in rats. *Am. J. Pharmacol. Toxicol., 9(3)*:177-88, 2015.

Haidara, M. A.; Dallak, M.; El Karib, A. O.; Abd Ellatif, M.; Eid, R. A.; Heidar, E. H. A. & Al-Ani, B. Insulin protects against hepatocyte ultrastructural damage induced by type 1 diabetes mellitus in rats. *Ultrastruct. Pathol., 42(6)*:508-15, 2018.

Izunya A. M.; Nwaopara, A. O.; Aigbiremolen, A.; Odike, M. A. C.; Oaikhena, G. A. & Bankole, J. K. Histological effects of oral administration of artesunate on the liver in Wistar rats. *Res. J. Appl. Sci. Eng. Technol., 2(4)*:314-8, 2010.

Khan, S. M.; Sobti, R. C. & Kataria, L. Pesticide-induced alteration in mice hepato-oxidative status and protective effects of black tea extract. *Clin. Chim. Acta, 358(1-2)*:131-8, 2005.

Kumar, T. R. & Muralidhara. Induction of oxidative stress by organic hydroperoxides in testis and epididymal sperm of rats in vivo. *J. Androl., 28(1)*:77-85, 2007.

Li, S. D.; Su, Y. D.; Li, M. & Zou, C. G. Hemin-mediated hemolysis in erythrocytes: effects of ascorbic acid and glutathione. *Acta. Biochim. Biophys. Sin. (Shanghai), 38(1)*:63-9, 2006.

Li, Y. F.; Ouyang, S. H.; Tu, L. F.; Wang, X.; Yuan, W. L.; Wang, G. E.; Wu, Y. P.; Duan, W. J.; Yu, H. M.; Fang, Z. Z.; *et al*. Caffeine protects skin from oxidative stress-induced senescence through the activation of autophagy. *Theranostics, 8(20)*:5713-30, 2018.

Magdy, B. W.; Mohamed, F. E.; Amin, A. S. & Rana, S. S. Ameliorative effect of antioxidants (vitamins C and E) against abamectin toxicity in liver, kidney and testis of male albino rats. *J. Basic. Appl. Zool., 77*:69-82, 2015.

Meshnick, S. R. Artemisinin: mechanisms of action, resistance and toxicity. *Int. J. Parasitol., 32(13)*:1655-60, 2002.

Nwanjo, H.; Iroagba, I.; Nnatuanya, I. & Eze, N. Antifertility activity of dihydroartemisinin in male albino rats. *Int. J. Endocrinol., 4(1)*, 2007.

Shichiri, M.; Ishida, N.; Hagihara, Y.; Yoshida, Y.; Kume, A. & Suzuki, H. Probucol induces the generation of lipid peroxidation products in erythrocytes and plasma of male cynomolgus macaques. *J. Clin. Biochem. Nutr., 64(2)*:129-42, 2019.

Sturgill, M. G. & Lambert, G. H. Xenobiotic-induced hepatotoxicity: mechanisms of liver injury and methods of monitoring hepatic function. *Clin. Chem., 438(8 Pt. 2)*:1512-26, 1997.

Wood, R. L. The fine structure of hepatic cells in chronic ethionine poisoning and during recovery. *Am. J. Pathol., 46*:307-30, 1965.

Corresponding author:
Dr. Refaat A. Eid
Department of Pathology
College of Medicine
King Khalid University
Abha
SAUDI ARABIA

Email: refaat_eid@yahoo.com

Received: 02-07-2019
Accepted: 29-07-2019