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Trihalomethanes in Liver Pathology: Mitochondrial Dysfunction and Oxidative Stress in the Mouse

Ana I. Faustino-Rocha,[1,2] D. Rodrigues,[1] R. Gil da Costa,[3] C. Diniz,[1] S. Aragão,[1] D. Talhada,[4] M. Botelho,[5] A. Colaço,[1,6] M. J. Pires,[1,2] F. Peixoto,[2,7] P. A. Oliveira[1,2]

[1]Department of Veterinary Sciences, School of Agrarian and Veterinary Sciences, University of Trás-os-Montes and Alto Douro, UTAD, 5001-911 Vila Real, Portugal

[2]Center for the Research and Technology of Agro-Environmental and Biological Sciences (CITAB), University of Trás-os-Montes and Alto Douro, UTAD, 5001-911 Vila Real, Portugal

[3]Faculty of Engineering, Laboratory for Process, Environment and Energy Engineering (LEPAE), University of Porto, UP, 4099-002 Porto, Portugal

[4]Faculty of Health Sciences, Health Sciences Research Centre, University of Beira Interior, 6201-001 Covilhã, Portugal

[5]Department of Health Promotion, National Institute of Health (INSA), 4000-055, Porto, Portugal

[6]Animal and Veterinary Research Center (CECAV), School of Agrarian and Veterinary Sciences, University of Trás-os-Montes and Alto Douro, UTAD, 5001-911 Vila Real, Portugal

[7]Department of Chemistry, School of Life Sciences and Environment, University of Trás-os-Montes and Alto Douro, UTAD, 5001-911 Vila Real, Portugal

ABSTRACT

Trihalomethanes (THMs) are disinfection byproducts found in chlorinated water, and are associated with several different kinds of cancer in human populations and experimental animal models. Metabolism of THMs proceeds through enzymes such as GSTT1 and CYP2E1 and gives rise to reactive intermediates, which form the basis for their toxic activities. The aim of this study was to assess the mitochondrial dysfunction caused by THMs at low levels, and the resulting hepatic histological and biochemical changes in the mouse. Male ICR mice were administered with two THMs: dibromochloromethane (DBCM) and bromodichloromethane (BDCM); once daily, by gavage, to a total of four administrations. Animals were sacrificed four weeks after DBCM and BDCM administrations. Blood biochemistry was per- formed for alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total bilirubin (TB), albumin (Alb), total protein (TP), creatinine, and urea. Animals exposed to DBCM and BDCM showed elevated ALT and TB levels ($p < 0.05$) as compared with controls. Histological analysis confirmed the presence of vacuolar degenerescence and a multifocal necrotizing hepatitis in 33% of animals (n 5 2). Mitochondrial analysis showed that THMs reduced mitochondrial bioenergetic activity (succinate dehydrogenase (SQR), cytochrome c

oxidase (COX), and ATP synthase) and increased oxidative stress (glutathione S-transferase (GST)) in hepatic tissues ($p < 0.05$). These results add detail to the current understanding of the mechanisms underlying THM-induced toxicity, supporting the role of mitochondrial dysfunction and oxidative stress in liver toxicity caused by DBCM and BDCM.

Keywords: chlorinated water; disinfection byproducts; liver; bioenergetics; rodents

INTRODUCTION

Trihalomethanes (THMs), including chloroform, bromoform, dibromochloromethane (DBCM), and bromodichloromethane (BDCM), have been recognized since the 1970 as some of the most common disinfection byproducts of chlorinated water (Bellar et al., 1974; Rook, 1974; Krasner et al., 2006). Brominated THMs are formed in the presence of bromide. THMs are well absorbed upon oral, respiratory, and cutaneous exposure, mainly through drinking water and while bathing, swimming, or showering (Florentin et al., 2011). Long-term exposure to THMs has been associated with increased urinary bladder (Villanueva et al., 2007; Costet et al., 2011) and colorectal (Bove et al., 2007) cancer risk (Richardson et al., 2007). For urinary bladder cancer, most of the risk seems to be associated with bathing, swimming and showering, rather than with drinking water (Villanueva et al., 2007). Genetic susceptibility studies have shown that cancer risk is associated with polymorphisms in the GSTT1, GSTZ1, and CYP2E1 genes, coding for key enzymes in THM metabolism (Cantor et al., 2010). The metabolism of THMs, and particularly that of brominated THMs, is only partially understood, although it critically underlies their mutagenic potency. Unlike chloroform, brominated THMs are activated to mutagens by glutathione S- transferase theta (GSTT-1), and originate GC to AT transitions in the RSJ100 *Salmonella* sp. strain (DeMarini et al., 1997). Using ^{14}C-radiolabelled BDCM, GSTT-1 was shown to catalyze the alkylation of DNA by BDCM to form guanine adducts (Ross and Pegram, 2004). Zhang et al. (2012) recently tested the genotoxicity of chloroform, bromophorm, DBCM, and BDCM on the HepG2 human hepatoma cell line using a single cell gel electrophoresis assay. Results indicate that the order of DNA-damaging potency is: BDCM>DBCM>bromoform >chloroform. Metabolism by cytochrome P450 seems to proceed mainly through the CYP2E1 isoform, resulting in the production of reactive intermediates and reactive oxygen species (ROS) (Porubsky et al., 2008) in laboratory rodents (Seth et al., 2013) and fish (Vega-Lopez et al., 2012; Olivares-Rubio et al., 2013). Oxidative stress induces significant cellular injury at various levels, including lipid peroxidation and oxidative DNA damage. This in turn may lead to cell death, through necrotic or apoptotic processes, or trigger carcinogenesis, if genetic mutations are allowed to accumulate. In fact, oxidative stress plays a central role in THM-induced cellular injury, including metabolic and inflammatory liver pathology (Das et al., 2013). In view of this, it becomes necessary to accurately study and describe the mechanisms through which THMs induce hepatocellular ROS accumulation and to systematically correlate this with the resulting histological hepatic lesions. The present study addresses these questions employing a mouse model of exposure to two of the most toxic THMS: DBCM and BDCM. Results concerning mitochondrial function and oxidative stress are correlated with biochemical markers of hepatocellular injury and with histological features.

MATERIALS AND METHODS

Animals

Forty-two outbred male ICR mice (*Mus musculus*) with four weeks of age were acquired from Harlan Interfauna (Spain). Animals were maintained under controlled conditions of temperature (23 6 2^{o}C), light/dark cycle (12 h/12 h) and humidity (55 6 5%). They were fed with a standard laboratory diet (Teklad Global Diet) purchased from Harlan Inter- fauna (Spain). Food and bottled water were provided *ad libitum*. All procedures were made in accordance to the European Directive 2010/63/EU.

Chemicals

DBCM and BDCM were purchased from Dr. Ehrenstorfer (Germany) and methanol was acquired from Sigma-Aldrich (USA). Methanol was used as the vehicle to administer DBCM and BDCM.

Animals' Experiments

The animals were randomly divided into four groups: DBCM-exposed (*n* 5 11), BDCM-exposed (*n* 5 11), methanol-exposed (*n* 5 11) and a negative control (*n* 5 9). Animals from DBCM, BDCM, and methanol groups received four administrations of DBCM, BDCM, and methanol by gavage in a five days period (no administration on third day). In each oral gavage, each animal from groups DBCM and BDCM received 4 mg of DBCM and BDCM, respectively. Considering a mean animal weight of 35 g, this results in a concentration of ~117 mg/kg/day (World Health Organization 1996). Methanol was used in the same volume as DBCM and BDCM. Animals from control group only drank tap water. All animals were daily monitored for signs of distress or disease. Animals' body weight and food and water intake were weekly recorded. At the end of the experimental protocol, four weeks after the end of the DBCM, BDCM, and vehicle administrations, mortality index (MI) was calculated dividing the number of deaths by the total number of animals in each group during the experimental protocol.

Necropsy and Samples Collection

Four weeks after the end of DBCM, BDCM, and vehicle administrations, all survival animals were sacrificed by pentobarbital sodium overdose administered via intraperitoneal. Complete necropsy was performed in all animals. Blood samples were collected via intracardiac puncture to evaluate biochemical parameters concerning to hepatic and renal functions: alanine aminotransferase (ALT), aspartate amino- transferase (AST), alkaline phosphatase (ALP), total bilirubin (TB), albumin (Alb), total protein (TP), creatinine, and urea. The liver, kidneys, spleen, heart, and lungs were collected, weighed, and fixed in 10% buffered formalin. The liver was sectioned into two parts: one section was fixed in 10% buffered formalin for histopathological analysis and the other one was frozen at 220^{o}C for mitochondrial analysis.

Histopathological Analysis

Formalin-fixed tissues were routinely dehydrated and paraffin-embedded. Two micrometer-thick sections were stained using hematoxylin and eosin (H&E). In addition, in the case of hepatic samples, reticulin and Masson's tri- chrome stainings were employed to study the hepatic stroma and the possible presence of fibrosis, according to the techniques described by Jones (Jones, 2002).

Liver Mitochondrial Analysis

Isolation of Liver Mitochondria

The isolation of liver mitochondria was performed as previously described by Peixoto et al. (2002). The homogenization medium contained 250 m*M* mannitol, 75 m*M* sucrose, 10 m*M* 2-[4-(2-hydroxyethyl)piperazin-1-yl] ethanesulfonic acid (Hepes), 0.5 m*M* ethylene glycol-bis(b-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) and 0.05% fatty acid-free bovine serum albumin (BSA). EGTA and BSA were omitted from the final washing medium. The final concentration of mitochondrial protein was determined by the Biuret method (Gornall et al., 1949), using BSA as standard.

Mitochondrial Respiratory Activity

Mitochondrial respiratory activity was measured according to Vilela et al. (2009). Oxygen consumption of isolated mitochondria was monitored polarographically with a Clark- type oxygen electrode, using a Hansatech Oxygraph measurement system (Hansatech, Norfolk, UK) at 25^{o}C. Mitochondria (0.5 mg) were incubated, for 2 min in 1 mL of reaction medium (250 m*M* sucrose, 20 m*M* KCl, 5 m*M* KH_2PO_4, 5 m*M* $MgCl_2$, 15 m*M* Hepes, 0.05% BSA, and 2 m*M* rotenone, pH 7.2). The mitochondria were energized with 5 m*M* succinate (complex II substrate). Oxygen consumption was measured in the absence (state 4) or presence (state 3) of 100–120 m*M* ADP. Respiratory rates were expressed in nmol O_2/citrate synthase activity and respiratory control ratio (RCR 5 state 3/state 4) was calculated.

Mitochondrial Membrane Potential Measurements

The reactions were conducted under a continuous stream of O_2 to avoid anaerobiosis. The transmembrane potential (DW) was estimated with a tetraphenylphosphonium (TPP^{1}) electrode according to the equation of Kamo et al. (1979), without correction for the "passive" binding contribution of TPP^{1} to the mitochondrial membranes (as the purpose of the experiment was to show relative changes in potential rather than absolute values). A matrix volume 1.1 mL/mg mitochondrial proteins was considered and valinomycin was used to calibrate the basal line. Mitochondrial suspension was incubated in a volume of 1 mL of reaction medium (the same medium as above described for the mitochondrial respiratory activity) supplemented with 3 m*M* TPP^{1}.

Mitochondrial Bioenergetic Activity

All assays were performed at 25^{o}C. The activity of succinate dehydrogenase (SQR) and cytochrome c oxidase (COX) was determined by polarographic methods previously described by Singer (1974) and Brautigan et al. (1978), respectively. The activity of ATP synthase was determined using the potentiometric method described by Madeira et al. (1974). The SQR activity was assessed in 1 mL of medium constituted by 250 m*M* sucrose, 5 m*M* KH_2PO_4, 15 m*M* Hepes, and 0.05% BSA, supplemented with 5 m*M* succinate, 2 m*M* rotenone, 0.1 mg antimycin A, 1 m*M* KCN, 0.03% Triton X-100 and 0.5 mg of mitochondrial fraction. The COX activity was evaluated using 1 mL of the same medium as described for mitochondrial respiratory activity, supplemented with 10 m*M* cytochrome c, 0.03% Triton X-100, and 0.5 mg of mitochondrial fraction. The activity of ATP synthase was determined using 2 mL of the same medium as described for mitochondrial respiratory activity, supplemented with 2 m*M* of PO_2^{3-}, 5 m*M* of succinate, and 1 mg of mitochondrial fraction.

Mitochondrial Oxidative Stress

Lipid peroxidation was determined measuring malondialdehyde equivalents, using the thiobarbituric acid assay, as previously described by Alves et al. (2007). Protein carbonyls were quantified through the spectrophotometric carbonyl assay, as recently described by Hawkins et al. (2009), using 2,4-dinitrophenylhydrazine. Superoxide dismutase (SOD), Glutathione-S-transferase (GST), and catalase (CAT) activity was determined as described previously Paya et al. (1992), Hatton et al. (1996) and Del R'io et al. (1977), respectively.

Statistical Analysis

All data were statistically analyzed using SPSS® (Statistical Package for the Social Sciences, version 17; USA). All variables were analyzed using independent Student's *t*-test, data are presented as mean 6 standard deviation (SD), with the results considered to be statistically significant when $p < 0.05$.

RESULTS

Animals

In the first week of the experimental protocol, when oral administrations of DBCM, BDCM, and vehicle were per- formed, the animals from these groups decreased their activity. In the remaining weeks, we did not record any alterations in animals' behavior. During the experimental protocol, 12 animals died and were excluded from the study: five animals from DBCM group (MI 5 45.5%), five animals from BDCM group (MI 5 45.5%), one animal from vehicle group (MI 5 9.1%), and one animal from control group (MI 5 11.1%). Body weight and food and water consumption were not statistically different among groups ($p > 0.05$) (data not shown).

Necropsy and Biochemical Parameters Evaluation

At animals' necropsy, no macroscopic alterations were observed and statistically significant differences were not found in organs' weight among groups ($p > 0.05$) (data not shown).

Blood biochemical analysis showed that ALT and TB levels were significantly higher in DBCM and BDCM- exposed groups than in vehicle and control groups ($p < 0.05$). AST levels were significantly increased in DBCM, BDCM, and vehicle-exposed groups compared with control group ($p < 0.05$). Regarding all other biochemical parameters (ALP, Alb, TP, creatinine, and urea), they were similar among groups ($p > 0.05$) (Table I).

Histological Analysis

The histological evaluation of liver sections did not reveal any architectural changes. However, animals from the TABLE I. Blood biochemical analysis (mean 6 SD) DBCM, BDCM, and vehicle-exposed groups showed multi- focal inflammatory cell infiltrates, multifocal hepatocellular necrosis, and cytoplasmic vacuolar degeneration, most frequently in the DBCM and BDCM-exposed groups. Hepatocellular nuclei often exhibited a disperse chromatin pattern and prominent nucleoli in DBCM, BDCM, and vehicle- exposed groups (Fig. 1 and Table II). Reticulin and Mas- son's trichrome stainings showed normal collagen expression patterns in all groups, with no differences between exposed and control animals (Fig. 1).

Liver Mitochondrial Analysis

Liver mitochondrial respiratory activity (RCR) and mem- brane potential (DW) were lower in DBCM, BDCM, and vehicle-exposed groups than in control group ($p < 0.05$) (Table III). Relatively to mitochondrial bioenergetic activity, SQR, and ATP synthase activity was lower in DBCM and BDCM-exposed groups than in other groups. However, COX activity was only lower in DBCM-exposed group, as compared with all other groups ($p < 0.05$). Regarding the analysis of mitochondrial oxidative stress, the activity of SOD and CAT was increased by DBCM, BDCM, and vehicle. GST activity was significantly increased in both DBCM and BDCM groups as compared with the other groups ($p < 0.05$) (Table III).

DISCUSSION

Human exposure to THMs correlates with increased risk for urinary bladder and colorectal cancers, while THM-exposed laboratory animals develop mainly—though not exclusively—hepatic lesions, as reviewed by Richardson et al. (2007). Recent studies indicate that THMs may also contribute to human hepatic non-neoplastic lesions by interfering with the energy metabolism and causing hepatocellular ROS accumulation (Das et al., 2013; Seth et al., 2013). Such studies show that THMs may be important environmental factors contributing to nonalcoholic steatohepatitis upon enzymatic activation (e.g., by GSTT1 or CYP2E1). The present study contributes to the current understanding of this issue by reporting the effects of two brominated THMs on hepatocellular mitochondrial function and oxidative stress. Our model relies on multiple-dose exposure to DBCM and BDCM followed by an incubation period during, which hepatocellular dysfunction and hepatic lesion are allowed to develop. Although, other authors administered higher dos- ages and longer incubation periods (Lilly et al., 1996; Stocker et al., 1997; Silva et al., 1999; Coffin et al., 2000; Torti et al., 2001), we chose to study a lower (and, in our opinion, more realistic) dose and the acute effects associated with a shorter incubation period. This approach allowed us to investigate the early mechanisms involved in trihalomethane-associated hepatotoxicity. At the end of the protocol, we observed a high MI in animals from DBCM and BDCM groups that can be explained by the method that was used to perform the administrations, the oral gavage. It was previously reported by Arantes-Rodrigues et al. (2012) that the administrations by oral gavage allow precise control of dosage but they are associated with stress and increased mortality among animals. Beyond the technique of administration, the highest MI in groups that received DBCM and BDCM can be explained by the toxicity of these compounds. It was previously described by Coffin et al. (2000) that the toxicity of the DBCM and BDCM is higher when administered by oral gavage than administered in drinking water.

Methanol, used as vehicle for THMs, shares structural, metabolic, and toxicological similarities with DBCM and BDCM. Methanol-exposed animals showed some degree of hepatocellular damage, as demonstrated by increased peripheral blood AST levels as compared with control animals. However, AST levels were even higher in DBCM and BDCM-exposed animals. The blood levels of another marker of hepatocellular damage, ALT, were only increased in DBCM and BDCM-exposed animals. This indicates that both THMs exerted their own hepatotoxicity, which exceeds the effects of methanol alone. This is further supported by results showing increased blood levels of TB in DBCM and BDCM-exposed but not in methanol-exposed animals. The fact that ALT levels were not increased in any group point to only a light intrahepatic cholestasis. Blood biochemistry results thus point to hepatocellular damage rather than cholestasis. The hepatocellular toxicity of both THMs was confirmed by histological analyses

showing that 33% of animals exposed to DBCM or BDCM developed a multifocal necrotizing hepatitis compared with only 20% in the methanol- exposed group and none in the control group. Hepatocellular damage, as reflected by cytoplasmic vacuolar degeneration, was also much more frequent in the DBMC and BDCM- exposed (100% of animals) as compared with the methanol (10%) or the control (0%) groups. Nuclear changes such as prominent nucleoli seemed to be a rather unspecific feature found in all treated groups. Vacuolar degeneration and necrosis are particularly significant phenomena in the con- text of THM-induced toxicity as both are associated with energy depletion, oxidative stress, and the consequent inability to maintain hepatocellular homeostasis. Energy depletion compromises transport at the cell membrane as well as metabolism, leading to water and/or fat accumulation in cytoplasmic vacuoles and, ultimately, to necrosis. Inflammatory responses may then perpetuate liver damage and drive stromal remodeling to the extent of impairing liver function. Seth et al. (2013) and Das et al. (2013) have recently shown that CYP2E1-mediated metabolism of BDCM induces hepatocellular oxidative stress, promoting the occurrence of non- alcoholic steatohepatitis in obese mice. In the present study, no evidence of stromal damage or remodeling was observed histologically and markers of hepatic function (albumin, TP) were at normal levels in all groups, which is in accordance with the early-stage lesions induced during the experimental procedures. Results from the mitochondrial analysis of hepatic tissues were also consistent with such findings. Those results confirmed the reduction of mitochondrial bio- energetic activity and increased oxidative stress induced by DBCM and BDCM. The changes observed on mitochondrial respiratory activity and membrane potential can be ascribed to methanol-induced toxicity (Table III). However, SQR and ATP synthase activities are significantly affected by the DBCM and BDCM, while DBCM also reduced COX activity. This is consistent with reduced available energy for cell functions, leading to increased cell stress and the resulting histological lesions. This increase in the activities of SOD, CAT, and GST certainly represents a cellular response to an increase in reactive oxygen species (ROS) due to toxicant exposure. However, this response appears not to be sufficient to prevent cellular damages as we can see by the histological analysis. One of the most discussed mechanisms behind the health effects induced by toxicant exposure is the ability to cause oxidative stress. *In vitro* studies have generally sup- ported the pathophysiological responses found in animal models, including increased generation of ROS in cells exposed to toxicants. Many *in vitro* studies have identified increased ROS generation as an initiating factor of toxicity in toxicants exposed cells. The interactions of toxicants with cell membranes result in the generation of ROS, and the generated oxidative stress may cause a breakdown of membrane lipids, an imbalance of intracellular calcium homeostasis, and DNA breakage (Botelho et al., 2013, 2014).

This is also consistent with the current knowledge concerning THM metabolism and with the associated cellular and histological lesions. THM-induced oxidative stress and reduced ATP production were suggested to cooperate with other factors, such as obesity, in the pathogenesis of nonalcoholic steatohepatitis (Das et al., 2013; Seth et al., 2013). Taken together, the present results show that BDCM and DBCM induce significant mitochondrial, cellular, and histo- logical damage in addition to that produced by methanol alone, and provide increased detail concerning the THM- induced metabolic changes.

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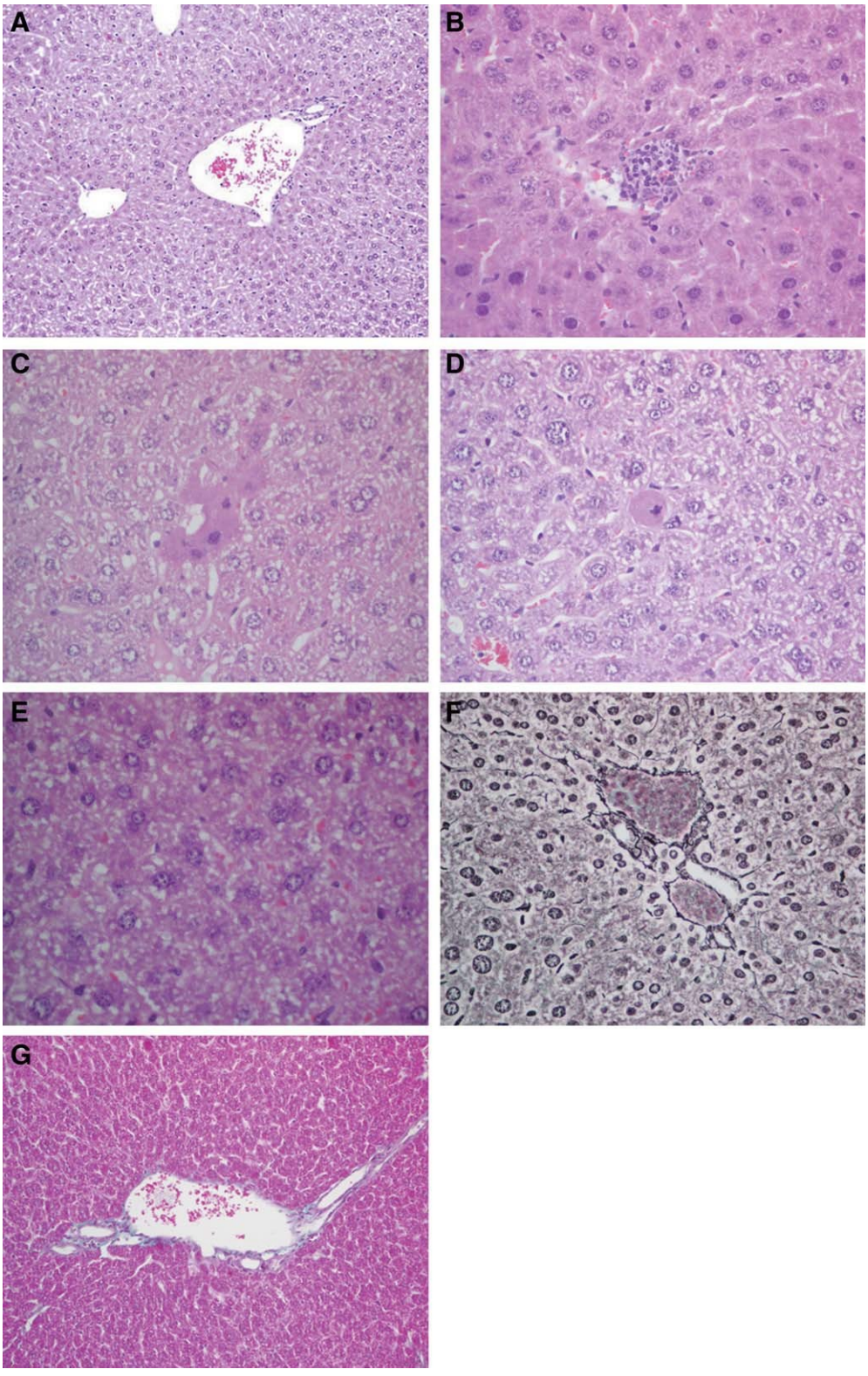

Fig. 1. Liver histological analysis: A: normal liver architecture (H&E, 203); B: liver inflammatory infiltrate (H&E, 403); C: focal hepatocellular necrosis (H&E, 403); D: hepatocellular cytoplasmic vacuolar degeneration (H&E, 403); E: hepatocyte nuclei with disperse chromatin and nucleoli (H&E, 603); F: normal collagen expression patterns (Reticulin, 403); G: normal collagen expression patterns (Masson's trichrome, 203). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

TABLE I. Blood biochemical analysis (mean 6 SD)

Group	DBCM Group ($n = 6$)	BDCM Group ($n = 6$)	Methanol Group ($n = 10$)	Control Group ($n = 8$)
ALT (UI/L)	49.2 ± 6.6^a	71.0 ± 16.0^a	40.7 ± 7.0	38.9 ± 7.5
AST (UI/L)	167.0 ± 59.0	177.0 ± 58.0	154.0 ± 33.0	99.0 ± 25.0^a
ALP (UI/L)	38.4 ± 6.8	41.0 ± 11.0	27.6 ± 7.2^a	34.0 ± 13.0
TB (mg/dL)	0.7 ± 0.2^a	0.8 ± 0.1^a	0.6 ± 0.2	0.6 ± 0.1
Alb (g/dL)	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1
TP (g/dL)	4.1 ± 0.2	4.0 ± 0.3	4.0 ± 0.3	4.0 ± 0.2
Creatinine (mg/dL)	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.1
Urea (mg/dL)	57.0 ± 14.0	53.0 ± 15.0	48.7 ± 8.8	42.9 ± 6.7

[a]For each parameter, *a* represents statistically significant differences from other groups ($p < 0.05$).

TABLE II. Histological analysis in liver sections H&E stained

	Number of Animals (%)			
Lesion	DBCM Group ($n = 6$)	BDCM Group ($n = 6$)	Methanol Group ($n = 10$)	Control Group ($n = 8$)
Inflammatory infiltrate	$n = 2$ (33.3%)	$n = 2$ (33.3%)	$n = 2$ (20%)	$n = 0$ (0%)
Hepatocellular necrosis	$n = 2$ (33.3%)	$n = 2$ (33.3%)	$n = 2$ (20%)	$n = 0$ (0%)
Hepatocellular cytoplasmic vacuolar degeneration	$n = 6$ (100%)	$n = 6$ (100%)	$n = 1$ (10%)	$n = 0$ (0%)
Hepatocyte nuclei with disperse chromatin and evidence of nucleoli	$n = 6$ (100%)	$n = 6$ (100%)	$n = 10$ (100%)	$n = 0$ (0%)

TABLE III. Liver mitochondrial analysis (mean 6 SD)

Liver Mitochondrial Analysis		DBCM Group ($n = 6$)	BDCM Group ($n = 6$)	Methanol Group ($n = 10$)	Control Group ($n = 8$)
Mitochondrial respiratory activity	RCR	1.8 ± 0.4	1.4 ± 0.3	1.6 ± 0.1	2.8 ± 0.4^a
Mitochondrial membrane potential	$\Delta\Psi$ (mV)	-165 ± 10	-165 ± 13	-170 ± 14	-193 ± 5^a
Mitochondrial bioenergetic activity	SQR ($nmolO_2$/mg)	85 ± 15^a	86 ± 13^a	101 ± 19	107 ± 12
	COX ($nmolO_2$/mg)	528 ± 77^a	677 ± 88	701 ± 127	732 ± 25
	ATP synthase ($nmolH^+$/mg)	119 ± 1^a	114 ± 1^a	146 ± 20	159 ± 38
Mitochondrial oxidative stress	SOD (U/min/mg)	2.5 ± 0.3	2.2 ± 0.1	2.4 ± 0.2	1.9 ± 0.1^a
	GST (U/min/mg)	56 ± 7^a	57 ± 12^a	46 ± 11	38 ± 2
	CAT ($nmolH_2O_2$/min/mg)	98 ± 13	103 ± 5	119 ± 41	81 ± 16^a

[a]For each parameter, *a* represents statistically significant differences from other groups ($p < 0.05$).