Fundamental Toxicological Sciences (Fundam. Toxicol. Sci.)
Vol.6, No.8, 281-286, 2019

The Japanese Society of Toxicology

Fundamental Toxicological Sciences

URL : http://www.fundtoxicolsci.org/index_e.html

Letter

Transmission electron microscopy of the benzbromarone-induced change in mitochondrial morphology in HepG2 cells

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(Received October 4, 2019; Accepted October 14, 2019)

ABSTRACT — Drug-induced mitochondrial dysfunction can lead to severe adverse effects. Accordingly, new *in vitro* assay systems for assessing mitochondrial-related toxicity are required. Current systems evaluate drug-induced mitochondrial dysfunction based on cell death. However, if mitochondria are damaged without cell death, these methods run the risk of overlooking toxic or dangerous compounds. To solve this problem, we attempted to measure morphological changes semi-quantitatively by transmission electron microscopy and to detect subtle changes in mitochondrial function. To this end, we exposed HepG2 cells cultured in galactose-containing medium to benzbromarone (BBR), which impairs mitochondrial function. After 24 hr of BBR exposure, we compared the rate of cell death between galactose and glucose cultures. Before the onset of cell death, BBR increased the ratio of damaged mitochondria to a greater extent in galactose-cultured than glucose-cultured HepG2 cells. Our results suggested that this new *in vitro* assay system could detect mitochondrial-related toxicity before the onset of cell death.

Key words: Mitochondria, Warburg effect, Benzbromarone, Transmission electron microscopy

INTRODUCTION

A drug that induces a severe adverse effect (especially cardiotoxicity or hepatotoxicity) is likely to be withdrawn from the market or discontinued as a lead compound for drug development. Drugs that impair mitochondrial function induce such severe adverse effects (Dykens and Will, 2007). Therefore, *in vitro* assay systems capable of detecting mitochondrial toxicity are required.

The mechanism underlying drug-induced mitochondrial dysfunction involves inhibition of the electron transport system (ETS) and β-oxidation, or alternatively, opening of the mitochondrial membrane permeability transition (mMPT) pore (Dykens and Will, 2007). In regard to hepatotoxicity, several studies have reported *in vitro* assay systems capable of detecting mitochondria-related toxicity (Liu *et al*., 2016; Sanuki *et al*., 2017). These systems evaluate cell death simply and quickly based on the increase in lactate dehydrogenase (LDH) level in the medium or the decrease in cellular ATP level. However, if mitochondria are damaged without cell death, these systems risk overlooking toxic and hazardous compounds.

In this study, we tried to directly evaluate mitochondrial morphology using a transmission electron microscope

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(TEM) to detect mitochondrial dysfunction following drug treatment. For this purpose, we used benzbromarone (BBR), which we selected as a model drug because (i) it can induce fatal liver injury (Arai *et al*., 2002), and (ii) it exerts severe mitochondria toxicity (Kaufmann *et al*., 2005). We administered BBR to HepG2 hepatoma cells, which are often used to predict hepatotoxicity (Garside *et al*., 2014). In general, healthy cells generate ATP via oxidative phosphorylation, whereas cancer cells rely on aerobic glycolysis to generate ATP, a phenomenon called the "Warburg effect." Circumventing Warburg effect by substituting galactose for glucose in the culture medium detects mitochondria-related toxicity (Marroquin *et al*., 2007). We exposed HepG2 cells to BBR under culture conditions that circumvent the Warburg effect and examined mitochondrial morphology by TEM.

MATERIALS AND METHODS

Materials

BBR was purchased from FUJIFILM Wako Pure Chemical Industries (Osaka, Japan). Dulbecco's Modified Eagle's Medium (DMEM), antibiotic-antimycotic (anti-anti) solution, galactose, HEPES, sodium pyruvate, Triton X-100, and dimethyl sulfoxide (DMSO) were purchased from Nacalai Tesque (Kyoto, Japan). Fetal bovine serum (FBS) was purchased from Biosera (Nuaille, France).

Cell culture condition

HepG2 cells were purchased by the RIKEN Cell Bank (Tsukuba, Japan) and cultured in glucose-containing medium (DMEM supplemented with 1.0 g/L glucose, 10% FBS, anti–anti solution, 20 mM HEPES, and 100 mM sodium pyruvate) or galactose-containing medium (identical except that the glucose was replaced with 2.0 g/L galactose). HepG2 cells were cultured at 37°C under a humidified atmosphere containing 5% CO_2. Prior to experiments, the cells were maintained in the indicated culture condition for at least 2 weeks. HepG2 cells cultured in glucose- or galactose-containing culture medium were seeded (noted as day 0) at a density of 9.26×10^4 cells/cm^2, and used for experiments on day 3.

Measurement of oxygen concentration

The oxygen concentration in the culture medium was measured using a fluorescent oxygen probe (Presens, Regensberg, Germany).

Measurement of lactate level

Lactate level in culture medium was measured as reported previously (Liu *et al*., 2016).

Measurement of cellular ATP level

Cellular ATP level was measured using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Fitchburg, Wisconsin, USA).

Measurement of cell viability following BBR treatment

HepG2 cells were exposed to BBR (0-160 μM) for 6 or 24 hr, after which the medium was collected. LDH activity released from cells into the medium was measured using the LDH Cytotoxic Detection Kit (TaKaRa Bio Inc., Shiga, Japan). Absorbance at 492 nm was detected using a Multiskan JX system (MTX Lab Systems, Vienna, VA, USA). Cell viability was assessed as shown in equation (1):

$$\text{Cell viability}(\%) = (1-(\text{experiment}-\text{control [DMSO]})/(\text{high control}-\text{control [DMSO]}))\times 100\% \quad (1)$$

'High control' represents LDH released from cells treated with 0.25% Triton X-100 for 24 hr.

Transmission electron microscopy

HepG2 cells cultured under each condition were exposed to 10 μM BBR in glucose or galactose medium for 6 hr, and then fixed and stained as reported previously (Uematsu *et al*., 2017). Forty to one hundred images were obtained for each condition described above on an H-7100 transmission electron microscope (Hitachi, Tokyo, Japan). Mitochondria with blurry outlines or ruptured outer membranes were defined as "damaged mitochondria." We calculated the ratio of damaged mitochondria number to total mitochondria in each image. Images were categorized according to this ratio into four grades: i) < 0.1, ii) ≥ 0.1 and < 0.3, iii) ≥ 0.3 and < 0.5, and iv) ≥ 0.5. Distributions were compared by chi square and Fischer's exact test using GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA).

Statistical analysis

All data are presented as means ± standard deviation (S.D.) unless otherwise noted. GraphPad Prism 7 was used to perform all statistical analyses. Data were analyzed by Student's t-tests and analysis of variance (ANOVA) followed by Tukey test to identify significant differences. In all cases, $p < 0.05$ was considered statistically significant.

RESULTS

In normal culture conditions (glucose-containing medi-

um) it was difficult to detect mitochondria-related toxicity because cancer cells produced ATP by glycolysis when glucose is present (Marroquin *et al*., 2007). Initially, we compared energy metabolism in glucose-cultured HepG2 cells (Glu-HepG2 cells) and galactose-cultured HepG2 cells (Gal-HepG2 cells) under our experimental conditions. The oxygen concentration was lower in Gal-HepG2 cells than in Glu-HepG2 cells (Fig. 1A), suggesting that oxygen consumption was higher in the former. We also compared lactate levels under each culture condition to investigate the contribution of glycolysis. Lactate level was significantly lower in Gal-HepG2 cells than in Glu-HepG2 cells (Fig. 1B), implying that the Warburg effect was circumvented in Gal-HepG2 cells under our experimental conditions. The cellular ATP level did not differ significantly between galactose and glucose (Fig. 1C). Thus, HepG2 cells were maintained in a healthy state under both culture conditions.

We next examined cell viability as a function of concentration and time in HepG2 cells treated with BBR. After 6 hr in BBR, cell viability was reduced only at 160 μM in both culture conditions (Fig. 2A). After 24 hr in BBR treatment, cell viability was reduced in a concentration-dependent manner in both culture conditions, and Gal-HepG2 cells were more sensitive (Fig. 2B).

Based on the results of our cell viability measurements (Fig. 2A and B), we exposed HepG2 cells to 10 μM BBR for 6 hr, and then observed their mitochondria by TEM.

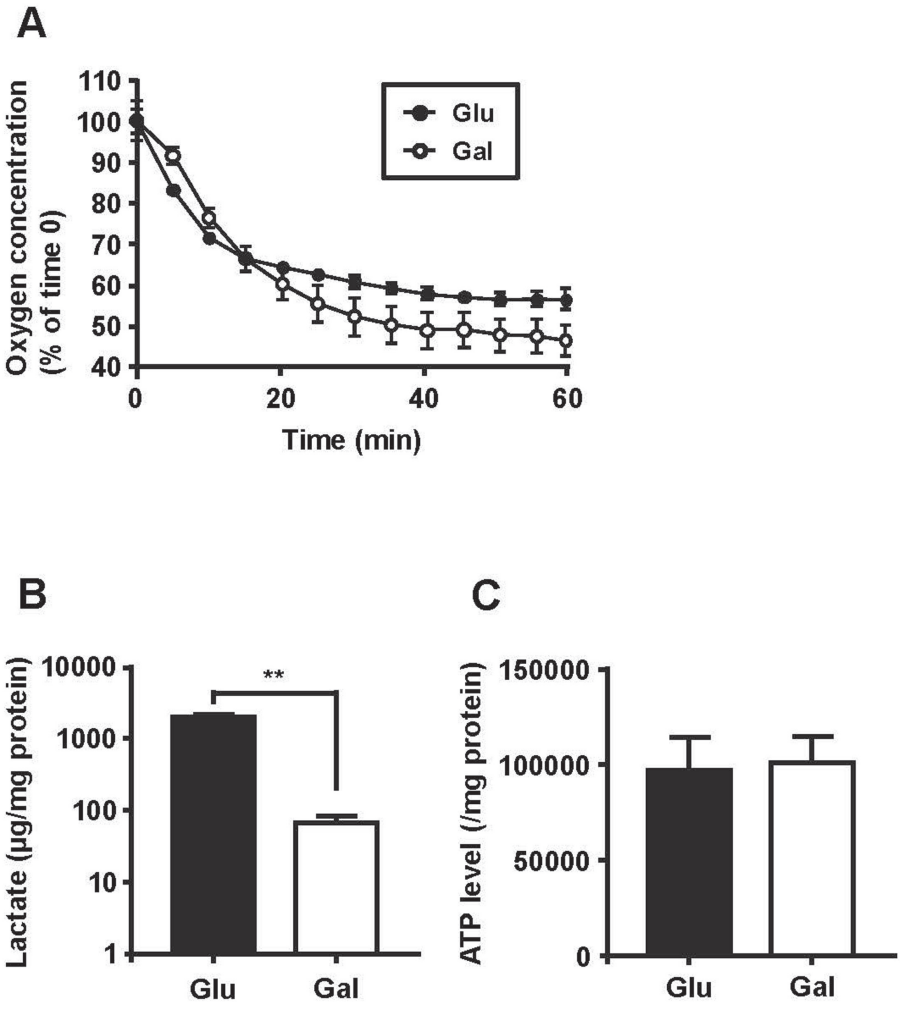

Fig. 1. Alteration of energy metabolism in glucose- or galactose-cultured HepG2 cells. Oxygen concentration (A), lactate level (B), and ATP level (C) were examined on day 3 after seeding in HepG2 cells cultured in glucose (Glu) or galactose (Gal). Data are means ± S.D. (n = 3). $^{**}p < 0.01$ vs. the glucose group.

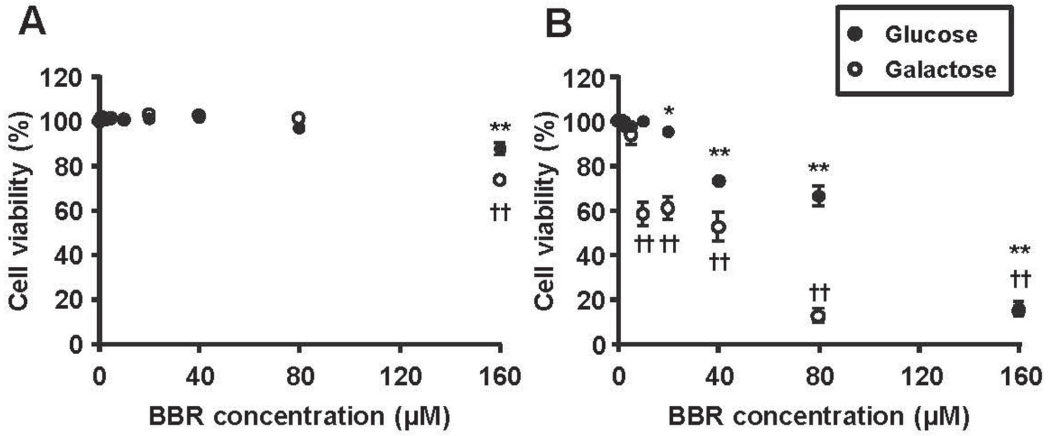

Fig. 2. Concentration- and time-dependent effects of benzbromarone (BBR) on cell viability in HepG2 cells. On day 3 after seeding, HepG2 cells were treated with BBR (0-160 μM) for 6 or 24 hr. Cell viability at 6 hr (A) and 24 hr (B) were determined based on the LDH level released into the medium. Data are means ± S.D. (n = 3). *$p < 0.05$, **$p < 0.01$ vs. HepG2 cells cultured in glucose and treated with DMSO. ††$p < 0.01$ vs. HepG2 cells cultured in galactose and treated with DMSO.

Representative TEM images from each condition are shown in Fig. 3. A-D. Glu- and Gal-HepG2 cells treated with DMSO contained many intact mitochondria (Fig. 3A and C.). Following BBR treatment, however, the number of damaged mitochondria was higher in Gal-HepG2 cells than in Glu-HepG2 cells (Fig. 3 B and D.). The distribution of the proportion of damaged mitochondria is shown in Fig. 3E. Notably, BBR increased the ratio of damaged mitochondria significantly in galactose cultures (*p*-value: 0.026) but not in glucose cultures (*p*-value:0.615).

DISCUSSION

In this study, we evaluated mitochondrial morphology using TEM with the goal of constructing a new *in vitro* assay system capable of detecting mitochondria-related toxicity following drug treatment. We observed an increase in damaged mitochondria in Gal-HepG2 cells subjected to BBR treatment (Fig. 3E). Although we did not investigate the mechanism, we suspect that the damage might be caused by mMPT, which is caused by the opening of a nonselective pore in the inner membrane, resulting in cell death (Bernardi *et al*., 2015). In addition, mMPT is associated with matrix expansion and outer membrane rupture (Feldmann *et al*., 2000; Sesso *et al*., 2004). Because BBR has the potential to induce mMPT (Kaufmann *et al*., 2005), we hypothesized that this mechanism was responsible for the increase in mitochondrial damage in Gal-HepG2 cells treated with BBR. Testing this idea will require further investigation.

We confirmed that the number of damaged mitochondria had increased 6 hr after BBR treatment in Gal-HepG2 cells (Fig. 3E), before the onset of cell death (Fig. 2A and B). These results are consistent with previous reports that alteration of mitochondrial morphology impairs mitochondrial function, activates caspase pathways, and ultimately induces cell death in a mouse model of non-alcoholic steatohepatitis (Du *et al*., 2017). Based on these findings, we thought that our TEM assay was a suitable method for detecting of mitochondria-related cell death.

One limitation of this assay is its lower throughput relative to conventional cell death assay. Hence, it may be better suited to robust safety studies or detailed toxicological analyses after the number of promising compounds has been narrowed down, e.g., in later stages of drug development. Although we only evaluated certain types of mitochondria with blurred outlines or ruptured outer membranes, other aspects, such as mitochondrial fission/fusion status, might also be important. Fused mitochondria increase aerobic respiration (Fu *et al*., 2013), whereas fragmented mitochondria are associated with cell death and mitophagy (Narendra *et al*., 2008). Considering these various aspects of mitochondrial morphological changes, semi-quantitative analysis of TEM assay data could to understand the toxic mechanisms of compounds that have the risk of drug-induced liver injury.

In conclusion, we developed a new *in vitro* assay system capable of detecting mitochondrial toxicity induced by drug treatment. Although previous studies did not focus on mitochondrial morphological alteration without

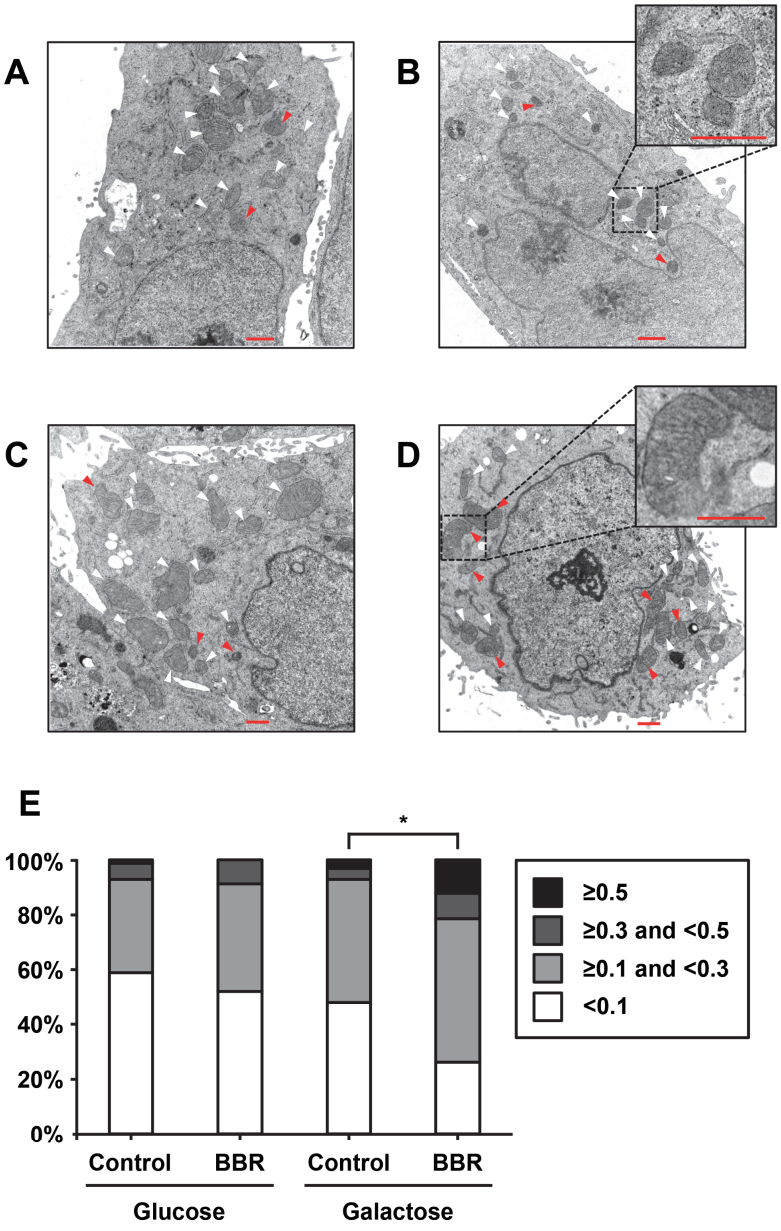

Fig. 3. Analysis of mitochondria morphology following benzbromarone (BBR) treatment by transmission electron microscopy. On day 3 after seeding, HepG2 cells were treated with DMSO or 10 µM BBR for 6 hr. (A and B) Representative images showing HepG2 cells cultured in glucose and treated with DMSO (A) or BBR (B). (C and D) Representative images showing HepG2 cells cultured in galactose and treated with DMSO (C) or BBR (D). Scale bar = 1 µm. White arrowhead represents normal mitochondria, and red arrowhead represents damaged mitochondria. (E) Distribution of the ratio of damaged mitochondria to total mitochondria. *$p < 0.05$ vs. HepG2 cells cultured in galactose and treated with DMSO.

cell death, this phenomenon may be important for accurate prediction of drug-induced mitochondrial dysfunction.

ACKNOWLEDGMENTS

This research was supported by AMED under Grant Number JP19fk0310112.

Conflict of interest---- The authors declare that there is no conflict of interest.

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