EXTENDED ABSTRACT

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Perturbation of mitochondrial dynamics in alcoholic liver disease

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Abstract

Alcoholic Liver Disease (ALD) is a global issue that causes increasing number of deaths annually. In the complex pathogenesis of ALD the involvement of mitochondria is well established and gross morphological alterations (mega-mitochondria) in the liver biopsies of patients are recognised as hallmarks of ALD. However, the impact and significance of alcohol on mitochondrial architecture, dynamics and on mitochondria-shaping proteins (MSP) remains unknown due to the lack of good experimental models. Firstly, we utilised human hepatoma VL-17A cells expressing ethanol-metabolizing enzymes to assess the kinetics (1-14 days) and the impact of increasing doses of alcohol (25-250mM) on mitochondrial morphology by confocal and electron microscopy. In order to study alcohol toxicity in normal hepatocytes and in other hepatic cell types an *ex vivo* model was also developed. Precision cut liver slices were derived from human tissue and cultured for 8-72 hours with increasing doses of ethanol (50-250mM). The morphological analysis revealed an initial mitochondrial hyper-fragmentation; while mega-mitochondria developed as a consequence of longer exposures. These structural modifications were associated with changes in the MSP regulating fragmentation but not fusion, opening new perspectives in the development of therapies for ALD aimed to modulate mitochondrial dynamics.

List of abbreviations: *MSP (Mitochondria-Shaping Protein), ALD (Alcoholic Liver Disease), EtOH (Ethanol), PCLS (Precision Cut Liver Slices), EM (Electron Microscopy).*

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Introduction

According to the recent "Global status report on alcohol and health" published by the World Health Organization, alcohol abuse results in 3.3 million deaths worldwide every year. Despite this burden, the treatment options for ALD are limited with abstinence still being the cornerstone of therapy. The lack of good experimental models has significantly hampered the elucidation of the molecular pathways associated with ALD and the discovery of diagnostic, prognostic and therapeutic targets which are urgently needed.
The involvement of mitochondria in the pathogenesis of ALD has been confirmed in several studies. Besides being the location of the main ethanol metabolising enzymes, mitochondria also play an active role in the oxidative stress caused by this insult and in the response to ethanol toxicity.

The detection of disproportionately enlarged mitochondria (mega-mitochondria) has been reported in liver biopsies from alcoholic patients since the 1970s and this feature has universally been considered a specific hallmark for alcohol-induced liver disease (Bruguera, Bertran, Bombi, & Rodes, 1977). Despite the general consensus that alcohol abuse and alcohol-related disease are associated with mega-mitochondria formation in the liver, their significance and role in the pathophysiology of ALD is unknown.

In 1986, Chedid *et al.* reported the presence of mega-mitochondria predominantly in patients with a mild or moderate form of alcoholic hepatitis (Chedid et al., 1986) and recent studies by Altamirano *et al.* and Andrade *et al.* have correlated their detection with a more favourable outcome at 90 days in patients with severe alcoholic hepatitis (Altamirano et al., 2014; Andrade et al., 2016). In contrast, Teli *et al.* interpreted the presence of mega-mitochondria in alcoholic patients "as a bad prognostic feature in the context of fatty liver" and associated them to a higher risk of progression to fibrosis/cirrhosis (Teli, Day, Burt, Bennett, & James, 1995). Therefore, the precise significance of these mitochondrial alterations in the context of ALD remains controversial and the molecular mechanisms involved in mega-mitochondria development, their function and their influence on cellular response to alcohol have not been established.
In the last decade, the concept that mitochondria have immense architectural plasticity and that their shape is strictly correlated with their function has been extensively developed and accepted. In particular, morphological studies and live cell-imaging by confocal microscopy have underlined that these organelles can go through cycles of fusion and fission in order to maintain a functional mitochondrial pool in the cell. The balance between fusion and fission determines the mitochondrial shape and is necessary for the preservation of cellular integrity and is unsurprisingly tightly regulated by the activity of multiple "mitochondria-shaping proteins" (MSP) (Lee, Jeong, Karbowski, Smith, & Youle, 2004). Alterations in MSP or in the equilibrium between fusion/fission have been associated with the pathogenesis of several disorders; however, the impact of alcohol on mitochondrial dynamics and on the expression or activity of MSP is still unknown.

This study aimed to develop *in vitro* and *ex vivo* models to allow the investigation of the changes induced by alcohol on the shape of hepatic mitochondria and on the MSP to elucidate the role of mitochondrial dynamics in the pathogenesis of ALD.

Material & Methods

Cell culture: VL-17A cells, stably expressing alcohol dehydrogenase (ADH) and cytochrome P450 2E1 (CYP2E1) (Donohue, Osna, & Clemens, 2006), were cultured in complete DMEM containing zeocin (selective for ADH) and geneticin (selective for CYP2E1) at 400µg/ml. For the short treatments (1-3 days) ethanol (25-250mM) was added to the complete medium in the multi-well plates, while in the long treatment (14 days) ethanol 100mM was added in the culture flasks and replaced at every passage (every 3-4 days); after 11 days, the cells were then seeded accordingly to the protocols for the different experiments and treated for a further 3 days. For all the experiments, treated cells were kept in an incubator saturated with ethanol to minimize evaporation (opened petri dish containing 200-500mM ethanol).

Human Precision Cut Liver Slices (PCLS): Human liver tissue was obtained from patients who underwent partial hepatectomy from The London Clinic, London. Immediately after surgical resection, the healthy portion of the liver specimen was harvested and kept on ice in sterile University of Wisconsin solution (ViaSpan; Bridge to Life Ltd) until slicing. Time from harvest to culture was kept to a minimum (3-10 hours)

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and the preparation of PCLS was performed as previously described (de Graaf et al., 2010). Each slice was placed in a 12-well plate and cultured in Williams medium E supplemented with glucose (Sigma), penicillin and streptomycin (Life technologies), with the addition of insulin-transferrin-selenium (Life technologies), epidermal growth factor 1nM (Life technologies), corticosterone 1µM (Sigma), glucagon 100nM (Sigma) and human AB serum 5% (Gemini Bio-product). The medium was saturated with carbogen (95% O_2/5% CO_2) and the plates kept in sealed chambers at 37°C in a shaking incubator. After an initial pre-incubation of 2 hours to allow the recovery after the cut, each slice was maintained in culture for 8, 24 or 72 hours with or without the addition of ethanol (50mM, 100mM or 250mM) and the medium was replaced every day. Cell viability was evaluated in 3 PCLS per patient by measuring the content of ATP with the ATP Bioluminescence Assay Kit CLS II (Roche), normalized against the total amount of proteins quantified by Pierce BCA Protein Assay Kit (Thermo Fisher Scientific), as previously described (de Graaf et al., 2010).

Confocal microscopy: To visualise mitochondrial morphology VL-17A cells were transiently transfected (Neon transfection system; Life technologies) according to manufacturer's guidelines to express a mitochondrially targeted dsRed fluorescent protein (mtRFP) with a transfection efficiency of about 40%. Transfected cells were seeded onto poly-D-lysine/laminin-coated glass coverslips (BD Bioscience) and then, after ethanol exposure, fixed with 3.7% paraformaldehyde in PBS (30 min, 4 °C) and permeabilised with 0.01% Nonidet-P40. When the cells were treated for 14 days it was not possible to use the transient transfection, thus mitochondria were visualized using immunostaining as previously described in (de Brito & Scorrano, 2008). After fixation and permeabilisation, cells were incubated with anti-Tom20 (BD Bioscience) and NorthernLights anti-mouse IgG-NL557 secondary antibodies (R&D Systems). Confocal images were collected using the PerkinElmer spinning-disk confocal microscope UltraVIEW (Perkin Elmer), a z-axis motorized stage (ProScan II; Prior Scientific), and EM-CCD camera (Hamamatsu Photonics).

Electron Microscopy (EM): The PCLS and the cells were fixed with 2% paraformaldehyde and 1.5% glutaraldehyde in 0.1M cacodylate buffer and then fixed in 1% OSO_4/ 0.1M cacodylate buffer pH7.3 at 3˚C. After the staining with 0.5% uranyl acetate, the specimens were dehydrated in a graded ethanol-water series and infiltrated with Agar 100 resin mix. Serial 1µm sections were cut and stained with 1% toluidine blue for light microscopy. Ultrathin sections were cut at 70-80 nm using a diamond knife on a Reichert ultracut microtome, collected on 300 mesh copper grids and stained with uranyl acetate and lead citrate. Then samples were viewed in a Joel 1010 transition electron microscope and images recorded using a Gatan Orius camera.

Results

In order to investigate the impact of ethanol on hepatic mitochondria we developed two different experimental models, one based on human hepatoma cells expressing the main ethanol-metabolising enzymes (ADH and CYP2E1) and another one based on the culture of human liver slices. This second system represents an *ex vivo* model that simulates the *in vivo* microenvironment and is closer to the real-life situation. The entire tissue structure is preserved in each slice and multicellular characteristics of the liver are maintained in physiological proportions. Besides hepatocytes, the other liver cell types also retain their activity including the inflammatory response induced by Kupffer cells and the development of fibrosis mediated by hepatic stellate cells and myofibroblasts. After the exposure for different time durations to increasing doses of ethanol, the toxic effect induced on the two experimental models was evaluated thorough the analysis of cellular growth, cell death rate (apoptosis and necrosis), ATP content and mitochondrial functionality in terms of respiration. The appropriate conditions were then selected for the study excluding the doses causing excessive cell death, in order to be able to perform a visual analysis of the organelles by microscopy, to quantify the morphometric parameters (mitochondrial length and width) and to detect early changes in the mitochondrial shape. After the evaluation of the general cellular toxicity caused by alcohol, a detailed study of the mitochondrial phenotypes, based on the shape and the size of the organelles, was performed both in the cells and in the slices. The blind score of the images collected by confocal and electron microscopy highlighted consistent morphological changes induced by the ethanol insult on the hepatic mitochondria,

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suggesting an unbalanced equilibrium between fusion and fission as a consequence of ethanol administration. The important finding that alcohol has a direct effect on the morphology of hepatic mitochondria could be useful to unveil unknown pathways affected in ALD and needs to be further investigated. Another striking observation that emerged from the morphometric analysis was the formation of oversized organelles in a great percentage of the cells analysed in both the cellular *in vitro* system and the *ex vivo* model based on the PCLS. These enlarged mitochondria resembled the abnormalities seen in the livers of alcoholic patients (Bruguera et al., 1977) and identified as mega-mitochondria. This observation validates the two models as suitable for the study of mega-mitochondria development, with the advantage to be able to investigate the molecular pathways, which regulate this event. The use of human *in vitro* systems opens the possibility to follow each step of the mega-mitochondria formation and to perform studies that could clarify the many controversies regarding this phenomenon. Considering the strict regulation of mitochondrial fusion and fission by the activity of many MSP and the effect of alcohol on mitochondrial shape, our study aimed also to investigate the impact of ethanol on the expression of Mitofusin-1, Mitofusin-2, Drp-1 and its receptors. In our models preliminary results on the protein and gene expression of the main MSP shown that the fragmentation pathway is the most affected by ethanol-induced hepatotoxicity and further experiments will be critical to better elucidate possible targets for ALD therapy.

Conclusion

The main achievement of this study is the development of two robust models to investigate the intracellular changes induced by alcohol. We were able to perform a careful characterisation of the morphological changes induced by ethanol toxicity on hepatic mitochondria and to elucidate the molecular pathways involved in these modifications. These preliminary findings need further investigation with an overall aim to reveal novel prognostic and therapeutic targets for ALD which are urgently needed.

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