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Ethanol-Induced Oxidative Stress in Primary Human Hepatocytes is Suppressed by VTL C3A Cell-Secreted Factors

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BACKGROUND

Hepatocellular death due to mitochondrial dysfunction is a key mechanism in alcoholic hepatitis (AH) and a leading cause of liver-related deaths. Both chronic and binge drinking can activate stress-mediated intracellular signaling pathways through induction of reactive oxygen species (ROS) and cause mitochondrial dysfunction.

The ELAD System is an investigational human hepatic cell-based liver treatment comprised of four metabolically active cartridges containing VTL C3A cells, with ancillary delivery device components and cell support circuitry, to treat severe AH.

Previous metabolome profiling studies of ELAD-treated clinical subject plasma ultrafiltrate suggested that the cellular component of ELAD treatment could provide a reducing environment[1,2]. In vitro studies further demonstrated a protective effect on primary human hepatocytes (PHH), at least in part by increasing the reduced to oxidized glutathione ratios in these cells[2].

We hypothesized that these anti-oxidant effects were produced through regulation of mitochondrial oxidative stress.

OBJECTIVES

The aim of this study was to evaluate whether VTL C3A cell-secreted factors from the ELAD System could dampen ethanol-induced oxidative stress and improve mitochondrial function using a PHH model.

MATERIALS & METHODS

ELAD Conditioned Media (ELAD CM) was prepared by aseptically infusing ELAD C3A cell cartridges with Williams' E media (WEM) containing insulin, transferrin, selenium, and glutamine, but without dexamethasone, and incubating cartridges at 37 °C for 4 h. ELAD CM was drained from the cartridges, pooled, aliquoted, and held at -80 °C, then sterile filtered prior to use.

PHH Oxidative Stress Model: Cryopreserved PHH were plated in collagen-coated plates in WEM and treated with WEM or ELAD CM diluted 1:1 in WEM, 5 h prior to exposure to 100 mM ethanol (EtOH).

Total ROS in Intact PHH and Isolated Mitochondria was measured using 2',7'-dichlorofluorescein diacetate (DCF-DA) (Sigma).

GKT137831 (50 µm), a dual inhibitor of NADPH oxidases NOX1 & NOX4, was used as a positive control for inhibition of ROS produced by NOX1 & NOX4.

Mitochondrial-specific Superoxide Anion ($O_2^{\bullet-}$) (i.e. a specific type of ROS) was measured using MitoSOX Red (Promega).

Adenosine-5'-triphosphate (ATP) was measured using CellTiter-Glo luminescent cell viability assay (Promega).

Mitochondria Oxidative Stress Model: Mitochondria were isolated from PHH[3], resuspended in WEM or ELAD CM and then exposed to 100 mM EtOH for 30 min.

Total Superoxide Dismutase (SOD) was measured using Nitro Blue Tetrazolium (NBT) (Sigma).

Manganese SOD (MnSOD) (a mitochondrial-targeted superoxide scavenging enzyme) levels were determined using 5 mM sodium cyanide (NaCN) to block mitochondrial function, then using NBT to measure SOD activity.

Hydrogen Peroxide (H_2O_2) was measured using Amplex Red (*N*-acetyl-3,7-dihydroxyphenoxazine) (Invitrogen).

Data Analyses were performed using GraphPad Prism. Unless otherwise noted, data are plotted as the average of biological replicates ± SD and deemed significant vs control if $p<0.05$ by ANOVA, with Dunnett's post-hoc test (control = WEM only, except Figure 1B where control = WEM + EtOH).

RESULTS

Since EtOH is known to induce ROS, it was first important to determine the impact of ELAD CM on total ROS levels in our PHH model using intact cells. EtOH substantially increased ROS generation in intact PHH maintained in WEM (p=0.0001), but no such effect was observed in PHH cultured in ELAD CM (Figure 1A). The NADPH oxidase (NOX) enzyme complex is a major source of ROS and plays a key role in liver injury. We evaluated the role of NOX in our model by using the NOX1- and NOX2-specific inhibitor GKT137831 and compared its effects to ELAD CM. The effect of ELAD CM attenuation of ROS was similar to GKT137831 treatment, suggesting a potential role ELAD CM on the inhibition of NOX-mediated ROS generation (Figure 1B).

RESULTS (cont.)

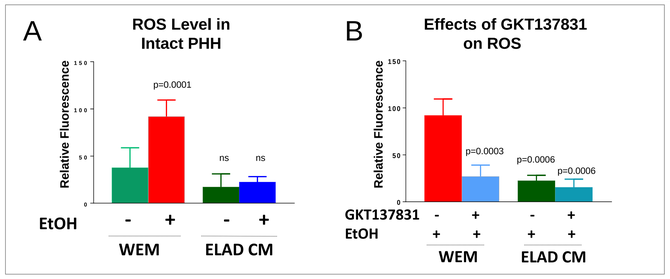

Fig 1. ELAD CM blocked ROS generation in intact PHH following EtOH treatment to a similar extent as the NOX1/4 inhibitor GKT137831 (GKT).
(A) EtOH increased ROS significantly in PHH maintained in WEM but not in ELAD CM-treated PHH. (B) GKT significantly inhibited ROS production in WEM/EtOH-treated PHH. However, GTK did not lower ROS any further in the ELAD CM/EtOH-treated group.

Since mitochondria are an important source of ROS, we next looked specifically at mitochondrial ROS. EtOH was effective at significantly increasing mitochondrial-specific ROS levels in both intact PHH (p=0.0001, Figure 2A) and isolated mitochondria (p=0.03, Figure 2B) following 5 h in culture with WEM. However, this EtOH-mediated effect was blunted by pre-treatment with ELAD CM (Figures 2A and 2B). In the intact PHH model, there was significantly less (p=0.004) ROS, specifically $O_2^{\bullet-}$ measured by MitoSox Red, in PHH cultured in ELAD CM than PHH cultured in WEM. EtOH treatment failed to induce any significant increase in mitochondrial-specific ROS in intact PHH cultured in ELAD CM (Figure 2A). Similarly, in isolated mitochondria, there was no increase in ROS in mitochondria treated with ELAD CM (Figure 2B).

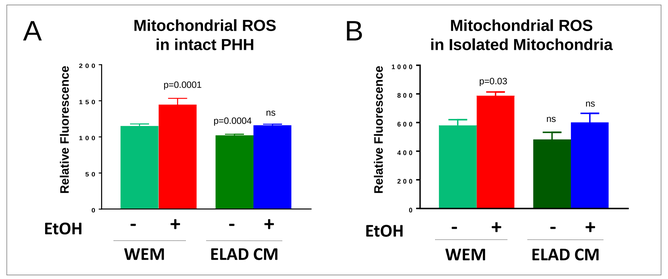

Fig 2. ELAD CM blocked mitochondrial ROS generation in PHH following ethanol treatment.
Both mitochondrial-specific ROS in intact PHH (A) and ROS from isolated mitochondria (B) were significantly increased by EtOH treatment in WEM alone. There was no increase in ROS relative to WEM following EtOH treatment in the presence of ELAD CM.

We next looked at the ability of ELAD CM to impact SOD activity in isolated mitochondria. EtOH significantly increased total SOD activity in mitochondria incubated in WEM (45% increase over WEM only, p=0.0001) (Figure 3A). Total SOD activity increased only 10% in mitochondria treated with ELAD CM + EtOH vs ELAD CM only. This EtOH-induced increase in SOD activity in ELAD CM-treated mitochondria was not different from WEM-treated mitochondria control. NaCN was used to determine MnSOD activity. As with total SOD, EtOH induced an increase in MnSOD in isolated mitochondria in WEM (72% increase vs WEM only). In contrast, MnSOD activity in EtOH-exposed mitochondria in ELAD CM was only 45% and significantly (p=0.0001) reduced compared to MnSOD activity in WEM (Figure 3B).

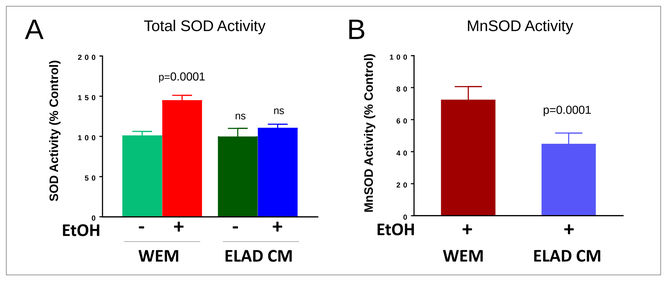

Fig 3. ELAD CM Inhibited SOD activity in isolated mitochondria following EtOH treatment
Both total (A) and (B) MnSOD activity were significantly lower in ELAD CM-treated PHH-isolated mitochondria (Student's t-test ELAD CM + EtOH vs. WEM + EtOH).

The next ROS species in the mitochondrial ROS pathway (Figure 4A) is H_2O_2. Consistent with the blunting of EtOH induction of $O_2^{\bullet-}$ and MnSOD by ELAD CM treatment, H_2O_2 production was also lower in ELAD CM-treated mitochondria exposed to EtOH. EtOH treatment induced a significant increase in H_2O_2 in PHH-isolated mitochondria in WEM (44% increase over WEM only, p=0.0001). Production of H_2O_2 in mitochondria incubated in ELAD CM + EtOH, although significantly increased relative to WEM (p=0.04), was increased less (14% over ELAD CM) than WEM + EtOH (Figure 4B).

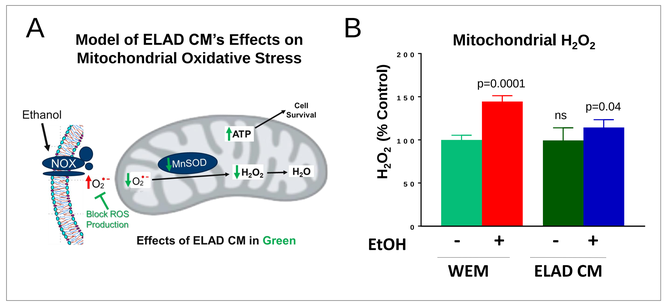

Fig 4. Model of ELAD CM's effects on oxidative stress; ELAD CM inhibited H_2O_2 production.
(A) Current model of aspects of mitochondrial oxidative stress inhibited by ELAD CM. (B) Mitochondrial H_2O_2 was significantly lower in PHH mitochondria incubated in ELAD CM.

EtOH significantly decreased ATP concentrations in intact PHH and also in isolated mitochondria maintained in WEM (44% (p=0.004) and 20% (p=0.003), respectively) (Figure 5). In contrast, cellular and mitochondrial ATP levels were not significantly decreased by EtOH in the ELAD CM-treated groups relative to WEM only control.

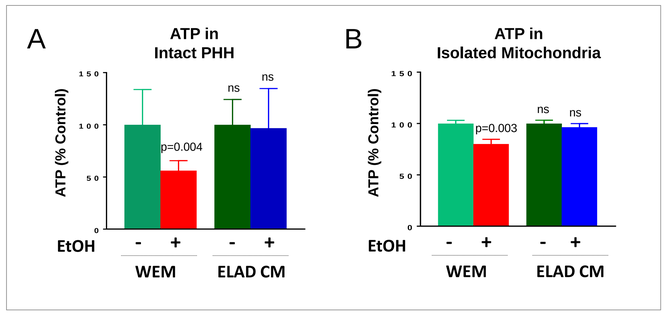

Fig 5. ATP concentrations in PHH mitochondria.
(A) ATP concentrations were significantly higher in intact PHH and (B) isolated mitochondria treated with ELAD CM than WEM.

DISCUSSION

Oxidative stress is an important driving force in alcoholic liver disease (ALD). The current study provides data in support of a protective effect of ELAD CM on hepatocyte viability by attenuating EtOH-mediated ROS generation (specifically superoxide anion ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2)) and protecting cellular ATP reserves. It also provides evidence for a potential mechanism of action of the ELAD System in the treatment of severe AH subjects. Among the multiple factors involved in the process of alcohol-induced liver injury, a crucial role is played by oxidative stress and decreased cellular antioxidant pool, including glutathione.

It is becoming increasingly apparent that the hepatic mitochondrial compartment is an important target of alcohol toxicity. Studies have linked alcohol-mediated effects to mitochondrial dysfunction, apoptosis, increase in ROS production, loss of cellular ATP, and eventually mitochondrial DNA damage[4]. Homologues of NADPH oxidase isoforms NOX1 and NOX4 are major sources of ROS, and these isoforms are widely expressed in the liver, mainly by hepatocytes, hepatic stellate cells, and endothelial cells.

DISCUSSION (cont.)

NOX-derived ROS contributes to various kinds of liver disease caused by alcohol[5]. ELAD CM reduces ROS in PHH, the effect of which is similar to GKT137831, a dual NOX inhibitor. This suggests a potential role of ELAD CM in reducing NOX-mediated ROS in response to ethanol in this PHH model.

Mitochondria are important for iron homeostasis, but are also a primary source of $O_2^{\bullet-}$ production, and, thus, are involved in the pathogenesis of ALD[6,7]. Disturbances in the redox balance within mitochondria are one of the early events in the progression of ALD. Alcohol increases ROS generation by liver mitochondria leading to oxidative stress[8]. At physiological levels, ROS function as "redox messengers" in intracellular signaling; however, excess ROS induce cell death by promoting the intrinsic apoptotic pathway. Because ALD has been linked to oxidative stress, we investigated the effect of a compromised antioxidant defense system in response to ELAD CM (which contains VTL C3A cell-secreted factors).

We have shown that ELAD CM significantly reduced ROS generation in response to EtOH exposure. EtOH did not increase mitochondrial total or MnSOD levels in the ELAD CM-treated group, consistent with both the significant reduction in measured $O_2^{\bullet-}$ generation and decreased H_2O_2 formation.

There is evidence that both mitochondrial function and glycolytic ATP synthesis declines in the liver due to EtOH exposure, promoting cell death, impaired liver function, and exacerbating the inflammation characteristic of AH[9,10] ATP levels were not perturbed in ELAD CM-exposed PHH, providing additional evidence of the protective effects of ELAD CM, and of the potential of ELAD treatment to impact disease in AH subjects.

The present studies support previous metabolome profiling of clinical samples[1] and cell-based models[2] showing the reducing effects of factors in ELAD CM.

CONCLUSIONS

Chronic liver injury results in the generation of oxidative stress, which disrupts lipids, proteins and DNA, induces necrosis/apoptosis of hepatocytes, and amplifies the inflammatory response.

ELAD CM reduced oxidative stress and mitochondrial dysfunction in PHH in each part of the SOD pathway investigated.

These data provide a potential mechanism of action for the ELAD System in the treatment of severe AH.

REFERENCES

1. Landeen LK, et al., ASAIO. 2017; Chicago, IL
2. Lapetoda J, et al., ISAD. 2016; Rostock-Warnemünde, Germany
3. Frezza C, et al., Nat Protoc. 2007; 2(2):287-95
4. Bansal S, et al., J Biol Chem. 2012; 287(19):15284-97
5. Paik YH, et al., Antiox Redox Signal. 2014; 20(17): 2854–2872
6. Mari M, et al., Redox Biol. 2014; (3), 100-108
7. Dubuquoy P, et al., Gut. 2015; 64(12):1949-60
8. Nagy LE, et al., Gastroent. 2016;150(8):1756-68
9. Arteel GE, Gastroent. 2003; 124(3):778-90
10. Osna NA. Alc Res. 2017; 38(2): 147–161

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