NITAZOXANIDE IMPROVES SYSTEMIC INFLAMMATION AND BRAIN DAMAGE IN A DISEASE MODEL OF ACUTE-ON-CHRONIC LIVER FAILURE

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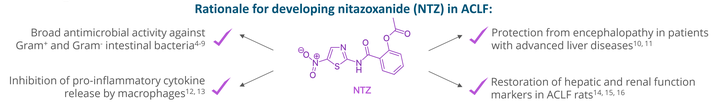
BACKGROUND & AIM

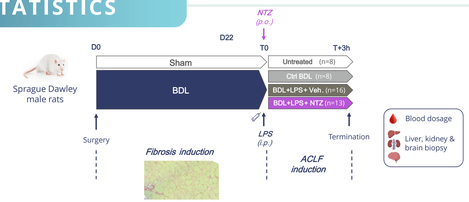
- Acute-on-chronic liver failure (ACLF) is a severe clinical syndrome in patients with cirrhosis, marked by systemic inflammation and multi-organ failure, and associated with high short-term mortality
- Overt hepatic encephalopathy (HE) represents a frequent manifestation of ACLF that can lead to cerebral edema, a critical neurological complication. HE is driven by inflammatory mediators and neutotoxic metabolites such as ammonia[1]. Bacterial translocation and infection, notably the release of pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS), play a pivotal role in the onset of systemic inflammation and neurotoxicity[2,3]
- Nitazoxanide (NTZ), demonstrated anti-inflammatory, hepatoprotective and antimicrobial properties[4-9, 12-16]. Moreover, NTZ was reported to be safe and well-tolerated in preventing recurrence of HE and improving neurocognitive outcomes[10,11]

Rationale for developing nitazoxanide (NTZ) in ACLF:



- **We previously demonstrated that NTZ mitigates systemic inflammation in rodent models of sterile endotoxemia initiated by LPS administration, and polymicrobial sepsis in mice triggered by cecal ligation and puncture[17]. In addition, we showed that the compound reduces liver and kidney injury in preclinical ACLF models[14, 15, 16]**
- **We aim to evaluate the efficacy of NTZ to counteract systemic inflammation and brain damage in a disease model of ACLF with cirrhosis induced by bile duct ligation (BDL) surgery followed by LPS injection as precipitating agent**

METHODS & STATISTICS



Evaluation of NTZ on ACLF rats with induction by BDL + LPS

Sprague Dawley male rats underwent bile duct ligation (BDL) to induce cirrhosis. Rats were stratified into treatment groups based on markers of hepatic fibrosis and functions measured 15 days after BDL surgery. ACLF was induced 22 days post-surgery by injection of LPS (*Escherichia coli* O111:B4, 1 µg/kg, i.p.). NTZ (100 mg/kg) or vehicle were orally administered concomitant to ACLF induction. Serum and liver tissue samples were collected 3 hours post LPS injection

Analysis of circulating biochemical markers

Serum levels of cytokines were measured by Luminex. Serum hepatic and renal function markers were measured by Daytona plus automate. Cystatin C and RIPK3 serum levels were measured by ELISA. To evaluate brain edema, fresh brains were weighed, dehydrated and weighed again to calculate the percentage of water

Analysis of organ samples

To evaluate brain edema, fresh brains were weighed, dehydrated and weighed again to calculate the percentage of water.
High-quality mRNA was extracted from liver and kidney tissues of ACLF rats. High-quality mRNA was extracted from liver and kidney tissues for RT-qPCR analyses (CFX96 Touch™) or Illumina RNA-sequencing. For RT-qPCR, expression data were normalized to housekeeping genes and compared across treatment groups

Transcriptomic analyses

Reads quality were analyzed using FastQC and trimmed when necessary. After mapping to the rat genome (BN7.2), DEseq2 was used to retrieve differentially expressed genes. Enrichment analyses were performed with Gene Set Enrichment Analysis (GSEA) and 181 KEGG pathways as gene sets. The Normalized Enrichment Score (NES) and adjusted p-value (FDR) were retrieved for each pathway

Statistical analysis

Experimental results are expressed as mean ± standard deviation. Box plots show the median as a line, 25th to the 75th percentile as a box and min and max as whiskers. Normality for each parameter was assessed using the Shapiro-Wilk test or Kolmogorov-Smirnov test. For variables presenting a normal distribution, comparison between groups was tested using unpaired Student's t test (*: p<0.05; **: p<0.01; ***: p<0.001). For non-normally distributed variables, a non-parametric Mann-Whitney test was applied ($: p<0.05; $$: p<0.01; $$$: p<0.001)

For some data, a log-scale was applied for better visualization. Group comparisons were performed as follows: Control (BDL) vs Healthy, Vehicle (BDL + LPS + Vehicle) vs Control and NTZ-treated (BDL + LPS + NTZ) vs Vehicle

RESULTS

NTZ RAPIDLY REVERSES SYSTEMIC, HEPATIC AND RENAL INFLAMMATORY RESPONSE IN ACLF

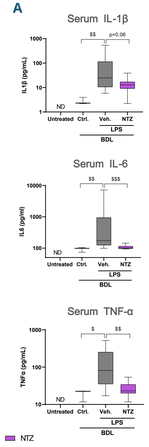
A.Serum cytokine levels

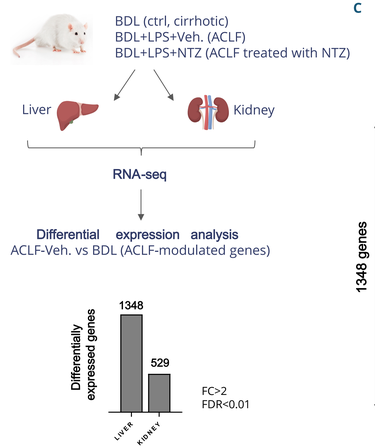
B.Transcriptomic analysis workflow. Livers and kidneys were collected in Ctrl BDL, BDL + LPS + Veh. and BDL + LPS + NTZ groups (n=5 per group)

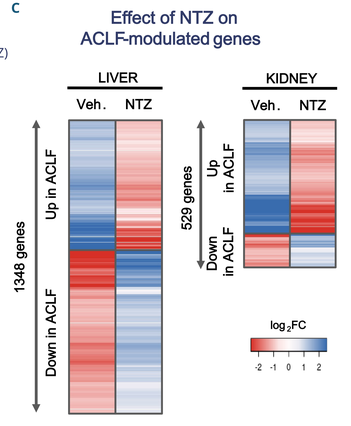
C.Number of differentially expressed genes between LPS + Veh. (ACLF) and Ctrl BDL (cirrhotic) rats (Fold-changes >2, FDR<0.01) Heatmap displaying (log2) fold-changes of ACLF-modulated genes (LPS + Veh.) and their modulation by NTZ treatment

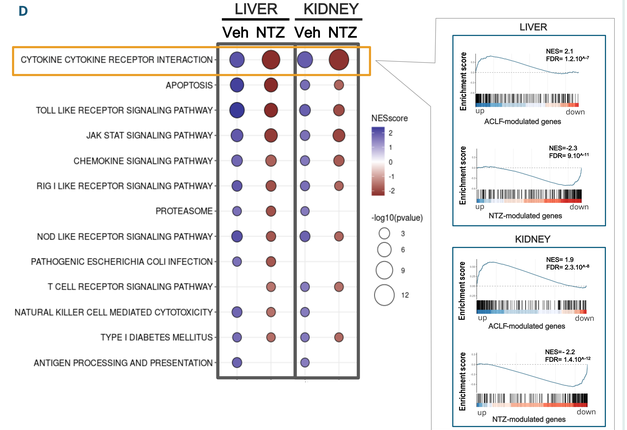
D. Bubble map displaying GSEA analysis using KEGG pathways as gene set. Colors indicate the Normalized Enrichment Score (NES) while dot areas are proportional to the false discovery rate (FDR).

E.Enrichment plots from GSEA analysis using the cytokine-cytokine receptor interaction pathway as example (265 genes)









→ **In BDL rats, LPS triggered an intense systemic inflammatory response, counteracted by NTZ administration**

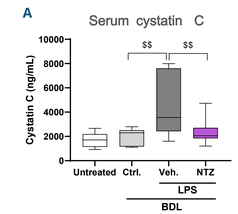
→ **NTZ reversed the ACLF-induced gene signature**

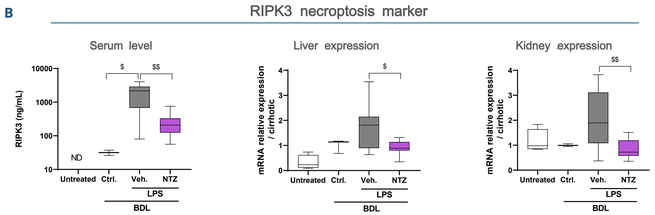
→ **ACLF pathways modulated by NTZ were mainly related to inflammatory response and immune cells pathways**

NTZ ALLEVIATES RENAL AND HEPATIC DAMAGE IN ACLF

A.Serum cystatin C

B.Serum levels of necroptosis marker RIPK3; hepatic and renal mRNA expression levels of Ripk3

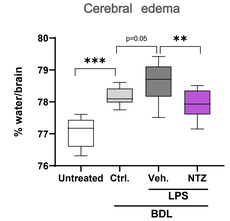




→ **NTZ improved ACLF-induced cystatin C, a renal function marker**

→ **NTZ lowered ACLF-induced circulating RIPK3 level, and tissue mRNA expression of Ripk3. This supports a protective role against necroptosis, a key pro-inflammatory cell death pathway in ACLF[18]**

NTZ RESOLVES BRAIN DAMAGE IN ACLF RATS



→ **NTZ effectively reversed LPS-induced cerebral edema in ACLF rats**

→ **This effect is potentially due to reduced inflammatory response, as ammonia levels remained unchanged 3h after treatment**

CONCLUSION

- **A single dose of NTZ rapidly attenuates systemic inflammation, associated with neuroprotective effects in an *in vivo* model of ACLF**
- **These findings further support the evaluation of NTZ as an investigational treatment in patients with acute decompensation of liver cirrhosis and ACLF**

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