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Full Length Article

Alcohol induced NLRP3 inflammasome activation in the brain of rats is attenuated by ATRA supplementation

S.H. Priyanka, A.J. Thushara, Arun A. Rauf, M. Indira *

Department of Biochemistry, University of Kerala, Kariavattom, Thiruvananthapuram, 695 581, Kerala, India

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ABSTRACT

Alcohol abuse affects several neurological pathways and causes significant alterations in the brain. Abstention from alcohol is an effective intervention against alcohol related diseases. But the recovery of the damaged cells to normal presents a major problem in those who have stopped alcohol consumption. Hence therapeutic interventions are needed. Our previous studies have shown that all trans retinoic acid (ATRA) is effective in reducing alcohol induced neuro toxicity. Chronic alcohol administration up-regulates and activates the NLRP3 inflammasome leading to caspase-1 activation and IL-1β production causing neuroinflammation. Hence, we investigated whether ATRA has any impact on NLRP3 inflammasomes activation. Rats were divided into two groups and were maintained for 90 days as control and ethanol group (4 g/kg body weight). After 90 days, ethanol administration was stopped and animals in the control group were divided into control and control + ATRA (100 μg/kg body weight per day) groups; those in the ethanol group as ethanol abstention and ATRA (100 μg/kg body weight per day) and maintained for 30 days. Administration of ATRA reduced reactive oxygen species and endotoxins which were elevated in alcoholic rats. There was also reduction in the expression of NLRP3 inflammasome and caspase 1. Our results suggested ATRA down regulated NLRP3 activation with concomitant decrease in the release of caspase −1 and production of IL1β. However, all these parameters were higher in abstention in comparison with ATRA supplemented group. In short therapeutic intervention with ATRA regressed alcohol induced inflammasome activation better than abstention.

1. Introduction

Inflammation and altered innate immune responses are observed in alcohol-induced organ damage (Szabo et al., 2011; Qin et al., 2008). Long-term alcohol intake results in neuroinflammation (Valles et al., 2004) and neurodegeneration both in humans and animal models. One of the main players triggering acute or chronic inflammation is activation of inflammasomes.

Alcohol-induced neuroinflammation is mediated by proinflammatory cytokines which is sparked both by endotoxins and reactive oxygen species (ROS). Chronic alcohol consumption leads to increased intestinal permeability and translocation of gut bacterial components into the circulation including the peripheral immune system and the central nervous system (CNS). Lowe et al. (2018) observed that chronic alcohol administration to mice induces both neuroinflammation and intestinal inflammation. Lipopolysaccharides (LPS) or endotoxins, activates proinflammatory cytokine cascades through toll-like receptor 4 (TLR4) and CD14, and leads to the production of tumor necrosis factor alpha (TNFα) and other proinflammatory cytokines. Quin et al. (2007) demonstrated that systemic LPS administration can induce TNFα production in liver, serum and brain within 1 h.

Chronic administration of alcohol generates ROS (Zhong et al., 2012). Elevated ROS can alter intracellular signaling, leading to dysregulation of the inflammatory response. Hoyt et al. (2017) reported that mitochondrial ROS production is critical for chronic ethanol-induced IL-1β hypersecretion which is a hall mark of inflammasome activation.

The NLRP3/caspase-1/IL-1 axis has emerged as a critical signaling pathway of the innate immune system in the CNS (Rosenzweig et al., 2011). The abundance of caspase-1 has been identified in neuroinflammation-related disorders (de Rivero Vaccari et al., 2016). Lippai et al. (2013) demonstrated that alcohol up-regulates and activates the NLRP3/ASC inflammasome, leading to caspase-1 activation and IL1β increase in the cerebellum.NLRP3 inflammasome complex consists of adaptor protein pycard (ASC) and pro–caspase-1, which, when activated by various endogenous and microbe-derived danger signals, directs the auto cleavage and processing of pro–caspase-1 (Martinon et al., 2002).

* Corresponding author.
E-mail address: indiramadambath@gmail.com (M. Indira).

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Activated caspase-1 cleaves both pro–IL-1β and pro–IL-18 to their mature secretory forms and causes pyroptosis, a proinflammatory form of programmed cell death (Bergsbaken et al., 2009).There are also contradictory reports on action of alcohol on inflammasome activation which may be dose dependent. Nurmi et al. (2013) reported that ethanol inhibited the NLRP3 inflammasome activation in macrophages.

One of the most important problems faced by those who have stopped chronic alcohol consumption is the recovery of damaged cells to normal. Although abstention is an effective therapy, studies have revealed that it does not bring about marked regression of alcohol induced toxicity (Stickel et al., 2017; van Eijk et al., 2013; Abhilash et al., 2012; Harikrishnan et al., 2013). Hence noninvasive economical therapeutic interventions are needed. Our recent study showed that all-trans retinoic acid (ATRA), an active form of vitamin A, ameliorated alcohol induced neuro toxicity (Priyanka et al., 2018; Nair et al., 2015) better than abstention. ATRA was also reported to negate the effects of ethanol on fetal alcohol syndrome in zebra fish (Marrs et al., 2010). Hence, we investigated whether ATRA has any impact on NLRP3 inflammasomes.

2. Materials and methods

2.1. Animals, housing and study design

Male albino rats (Sprague Dawley strain, average weight of 175 ± 25 gm) were selected and housed in polypropylene cages. The cages were kept in a room at 28°-32 °C. The light cycle was 12 h light and 12 h dark. Animals were handled as per the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) and fed on animal feed (Ashirvad Pvt. Ltd., India). The study protocol was approved by the Institutional Animal Ethics Committee [IAEC No-KU-25/2011-BC-MI (36)]. Standard diet and water were provided by ad libitum. Ethanol purchased from M/s Merck Ltd, Mumbai, India, was diluted with water (1:1) and given orally by gastric intubation. ATRA was purchased from M/s Sigma Aldrich (Bangalore, India; Product No. R2625, CAS No: 302-79-4) and dissolved in 30% ethanol and administered orally by gastric intubation. The dose of ethanol and ATRA was taken from previous report (Nair et al., 2015). A total of 36 rats were divided into two groups and were maintained for 90 days as control (C) and ethanol groups (E) (4 g/kg body weight). One cage contained 18 animals. The control group was given isocaloric glucose solution for 90 days and then discontinued. After 90 days, we sacrificed 6 animals from each group to find out whether toxicity has been induced or not. The ethanol administration was stopped after 90 days and animals in the control group were divided into two groups and then maintained for 30 days as the control (C) and ATRA (100 μg/kg body weight) and those in the ethanol group as ethanol abstention(A) and ATRA + ethanol abstention (ATRA + A) (100 μg/kg body weight). In this case each cage contained 6 animals. This is schematically presented in (Fig. No 1). Serum γ-glutamyl transferase (GGT) was assayed every week using blood collected from tail. At the end 30 days the animals were sacrificed after overnight fasting. The experimental animals were euthanized by injecting an overdose of thiopentone sodium intraperitonially. Blood was collected, and tissues were dissected out and cleaned with ice-cold saline, blotted dry and immediately transferred to ice-cold containers for various biochemical studies.

2.2. Biochemical analysis

Serum γ-glutamyl transferase (GGT) activity was assayed using the kit by Agappe Diagnostic Ltd., India (Product No. 11416001). Intracellular ROS was measured by 2, 7-dichlorofluorescein diacetate (DCDFA) method (Kengo et al., 2004).

2.3. Estimation of endotoxin

Endotoxin level was detected using ToxinSensor Chromogenic LAL endotoxin assay kit, (Cat. No. L00350, Gen Script, USA) according to manufacturer's instructions. The concentrations of endotoxin in samples were calculated from a standard curve of known amounts of *Escherichia coli* endotoxin.

2.4. Assay of intestinal permeability

The method of Wilson and Wiseman (1954) was used for the measurement of intestinal permeability towards endotoxin. A 20 cm long portion of small intestine (part of ileum, distal to colon) was isolated from experimental rats and one end of the intestine was bound to form a sac. The sac was everted so that the serosal layer was inside, and the mucosal layer was outside. The sac was filled with 10 ml of Krebs's buffer and incubated in Krebs's buffer with endotoxin standard solution at 37 °C

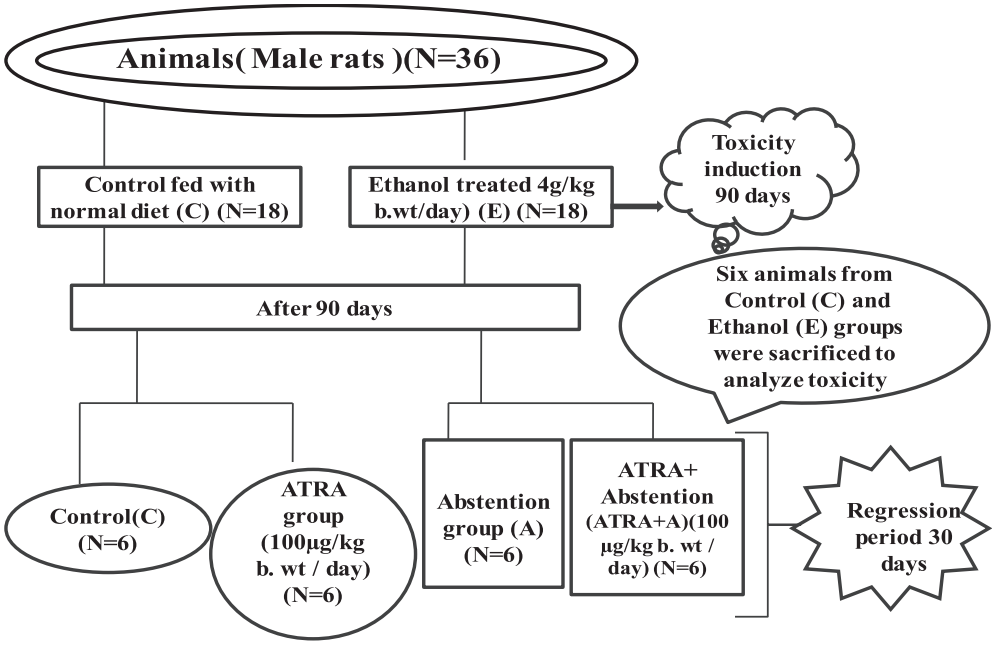

Fig. 1. Schematic representation of experimental design

Table 1
Primer sequence used.

Gene	Gene Sequence	Accession No:
β-actin	Forward 5′- ACCCGCGAGTACAACCTTCT3′ Reverse 5′- ATGGCTACGTACATGGCTGG3′	NM-031144.3
NLRP-3	Forward 5′-TGC TCT TCA CTG CTA TCA AGC CCT-3′ Reverse 5′-ACA AGC CTT TGC TCC AGA CCC TAT-3′	74446285
IL-1β	Forward 5′-TGG AAA AGC GGT TTG TCT -3′ Reverse 5′- ATA AAT AGG TAA GTG GTT GCC -3′	74446286
Caspase-1	Forward 5′-GTGTTGCAGATAATGAGGGC-3′ Reverse 5′-AAGGTCCTGAGGGCAAAGAG-3′	74446288

under O_2 supply in a sterilized conical flask. The samples were injected out from the sac and endotoxin concentrations in sample were detected by means of ToxinSensor Chromogenic LAL endotoxin assay kit (Cat. No. L00350, GenScript, USA), according to manufacturer's instructions.

2.5. Total RNA isolation

Total RNA was isolated using TRI reagent (Sigma Aldrich) extraction method described by Chomcynski and Sachi (1987).

2.6. Quantitative polymerase chain reaction (qPCR)

The isolated RNA was subjected to real time PCR (qPCR) using PCR master mix including evagreen qPCR Kit (Thermo Scientific, Bangalore, India). For RT-PCR amplification an initial amplification using primers was done with a denaturation step at 95 °C for 10 min, followed by 35 cycles of denaturation at 95 °C for 1 min, primer annealing at 55 °C for 30s, and primer extension at 72 °C for 45s. Upon completion of the cycling steps, a final extension at 72 °C for 5 min was done and then the reaction was stored at 4 °C. Real-time PCR was carried out using Eppendorf Master Cycler RealPlex. Reactions were run in triplicate in three independent experiments. The geometric mean of housekeeping gene β- actin was used as an internal control to normalize the variability in expression levels. The primer sequences are provided in Table 1. Expression data were normalized to the geometric mean of housekeeping gene β- actin to control the variability in expression levels and were analyzed using the $2^{-\Delta\Delta CT}$ method described by Livak and Schmittgen (2001).

2.7. Statistical analysis

Statistical analyses were carried out using the Statistical Package for Social Science (SPSS Inc. Chicago, IL, USA) version 17.0. Data with normal distribution and homogenous variance were analyzed using One-way analysis of variance (ANOVA). Pair fed comparisons between the groups was made by Duncan's multiple range test $p \leq 0.05$ was considered to be significant.

3. Results

3.1. GGT regression curve

Monitoring the activity of GGT in serum during treatment period showed a gradual increase in the activity upon administration of ethanol (Fig. 2). ATRA supplementation and abstention significantly decreased the activity of GGT when compared to ethanol treated group. A more significant and rapid decrease was found on ATRA supplementation compared to that of abstention.

3.2. ROS generation in whole brain

ROS were significantly increased in ethanol treated group compared with control group (Fig. 3). But it was significantly decreased in ATRA + A group compared with ethanol abstention and ethanol group.

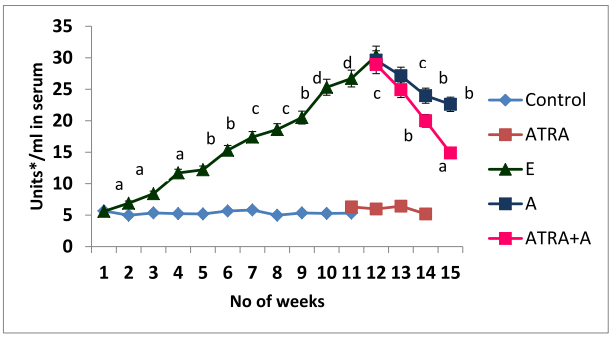

Fig. 2. Values are expressed as mean ± SEM of six rats in each group. Values not sharing a common superscript differ significantly at $p < 0.05$* The μM p-nitroaniline lib/min/mg protein.
[E = Ethanol, A = Abstention].

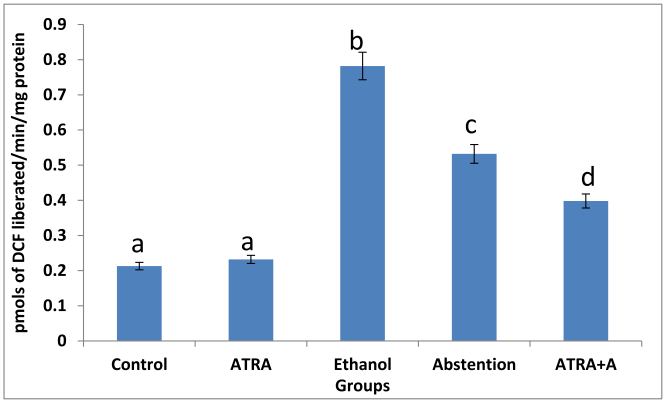

Fig. 3. Values are expressed as mean ± SEM of six rats in each group. Values not sharing a common superscript differ significantly at $p < 0.05$.

3.3. Endotoxin levels in intestinal fluid, whole brain and intestinal permeability

The level of endotoxins in the intestinal fluid and brain showed that endotoxins was significantly increased in ethanol group in comparison with control and ATRA (Fig. 4). Significant reduction was observed in the abstention and ATRA + A group when compared to ethanol group. In comparison with abstention group a more significant reduction in endotoxin level was observed in ATRA + A.

Ex vivo analysis of the intestinal permeability (Fig. 4) showed that intestine was more permeable to endotoxins on exposure to chronic alcohol. A reduction in permeability was observed on supplementation of ATRA and abstention. But more significant decrease was seen in ATRA + A group in comparison with abstension

3.4. qPCR of NLRP3, Caspase-1 and IL-1β in whole brain

The mRNA expression of NLRP3 in brain was significantly increased in ethanol group in comparison with control and ATRA groups (Fig. 5). But it was significantly reduced in ATRA + A group compared to ethanol and ethanol abstention group. More reduction in NLRP3 was observed in ATRA + A group than abstention.

4. Discussion

Alcohol is a neurotoxic compound and its administration to experimental animals can induce brain damage. Inflammatory responses are necessary steps in tissue repair (Chen et al., 2018). But uncontrolled neuroinflammation may lead to further tissue injury and neural

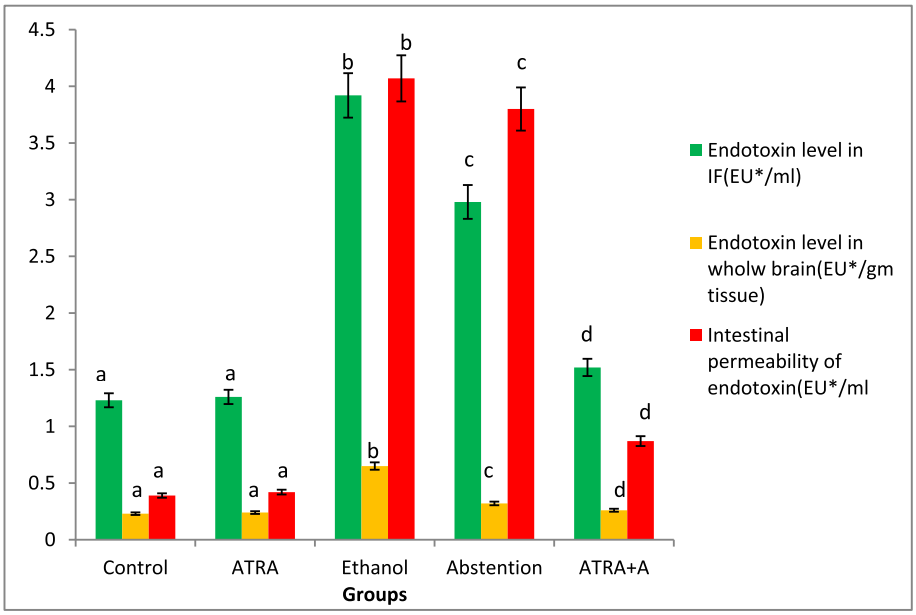

Fig. 4. *One EU is approximately equivalent to 100 pg of E. Coli LPS- the amount present in around 105 bacteria. Values are expressed as mean ± SEM of six rats in each group. Values not sharing a common superscript differ significantly at $p < 0.05$.

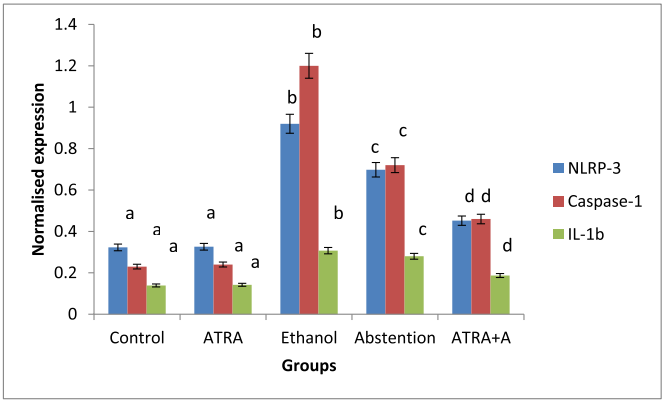

Fig. 5. NLRP3, Caspase-1 and IL-1b mRNAs quantified by qPCR and the products standardized against their respective β-actin controls. Fold change (Y-axis) represents the expression of the target gene mRNAs relative to that of the control group. Values are expressed as mean ± SEM of six rats in each group. Values not sharing a common superscript differ significantly at $p < 0.05$.

dysfunction (Choi et al., 2009; Abo-Ouf et al., 2013). Several studies are now focusing on inflammasomes especially NLRP3 due to its abundant expression in CNS (Rosenzweig et al., 2011). Our quest to find an economical and noninvasive method to regress the alcohol induced toxicity revealed the ameliorative potential of ATRA. It was able to reduce alcohol induced toxicity better than abstention as evidenced by the GGT curve. In this paper our focus was on the impact of ATRA supplementation on the NLRP3 inflammasome activation.

The major pathways for alcohol induced brain toxicity are mediated through the elevated ROS production and enhanced endotoxin levels. ROS and endotoxins can trigger NLRP3 activation (Abais et al., 2015). Consistent with our earlier reports we observed an increased production of ROS on administration of alcohol (Priyanka et al., 2018).

This study revealed that chronic alcohol exposure caused increased intestinal permeability and translocated gut bacterial components into the circulation. This is supported by the reports that impaired gut barrier function, bacterial translocation and alterations in the gut microbiome are found in animal models of chronic alcohol consumption (Keshavarzian et al., 1999; Hartmann et al., 2012). An alteration in colonic microbiome was also reported in chronic alcoholics (Mutulu et al., 2012). Several studies in animals as well as in humans have shown that alcohol consumption affects intestinal permeability (Leclercq et al., 2017). Zhong et al. (2010) demonstrated reduced distribution of tight junction proteins, claudin, occludin and ZO-1 in the distal intestine in association with increased gut permeability and plasma endotoxin level in mice chronically fed alcohol. But, ATRA administration reduced ROS, endotoxemia and decreased intestinal permeability than alcohol abstention. In addition, ATRA has been reported to have antibacterial effect (Wheelwright et al., 2014). This may be also a factor for its therapeutic potential. It has been suggested that manipulation of the gut microbiota with psychobiotics, prebiotics, or antibiotics might alleviate impaired brain functions (Dinan and Cryan, 2017).

It is well established that NLRP3 inflammasome activation triggers caspase-1 activation and IL-1β maturation. Lippai et al. (2013) reported that alcohol up-regulates and activates the NLRP3/ASC inflammasome, leading to caspase-1 activation and IL-1β increase in the cerebellum. We also observed elevated expression of caspase 1, which may be responsible for the elevated levels of IL-1β since it is involved in the processing of proIL-1β to IL-1β. These markers of NLRP3 activation were decreased on ATRA supplementation. Overall these cascades of cellular events due to ATRA supplementation would have reduced pyroptosis. NLRP3 activation was higher in abstention in comparison with ATRA supplemented group, thus pointing out the therapeutic potential of ATRA.

5. Conclusion

In short, this study points out that ATRA ameliorates alcohol toxicity in the brain better than abstention. Supplementation of ATRA to alcoholic rats reduces ROS and endotoxins in circulation leading to the down regulation of the NLRP3 inflammasome activation and concomitant release of caspase −1 and reduced production of IL-1β.

Declaration of Competing interest

The authors declare that we have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

Abais, J.M., Xia, M., Zhang, Y., Boini, K.M., Li, P.L., 2015. Redox regulation of NLRP3 inflammasomes: ROS as trigger or effector? Antioxidants Redox Signal. 22 (13), 1111–1129.

Abhilash, P.A., Harikrishnan, R., Indira, M., 2012. Ascorbic acid supplementation downregulates the alcohol induced oxidative stress, hepatic stellate cell activation, cytotoxicity and mRNA levels of selected fibrotic genes in Guinea pigs. Free Radic. Res. 46 (2), 204–213.

Abo-Ouf, H., Hooper, A.W., White, E.J., van Rensburg, H.J., Trigatti, B.L., Igdoura, S.A., 2013. Deletion of tumor necrosis factor-α ameliorates neurodegeneration in Sandhoff disease mice. Hum. Mol. Genet. 22, 3960–3975.

Bergsbaken, T., Fink, S.L., Cookson, B.T., 2009. Pyroptosis: host cell death and inflammation. Nat. Rev. Microbiol. 7 (2), 99–109.

Chen, L., Deng, H., Cui, H., et al., 2017. Inflammatory responses and inflammation-associated diseases in organs. Oncotarget 9 (6), 7204–7218.

Choi, D.Y., Liu, M., Hunter, R.L., Cass, W.A., Pandya, J.D., Sullivan, P.G., et al., 2009. Striatal neuroinflammation promotes Parkinsonism in rats. PLoS One 4, e5482.

Chomczynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidiniumthiocyanate-phenol-chloroform extraction. Anal. Biochem. 162, 156–159.

de Rivero Vaccari, J.P., Brand III., F., Adamczak, S., Lee, S.W., Perez-Barcena, J., Wang, M.Y., et al., 2016. Exosome-mediated inflammasome signaling after central nervous system injury. J. Neurochem. 136, 39–48.

Dinan, T.G., Cryan, J.F., 2017. The microbiome-gut-brain Axis in Health and disease. Gastroenterol. Clin. N. Am. 46 (1), 77–89.

Harikrishnan, R., Abhilash, P.A., Syam Das, S., Prathibha, P., Rejitha, S., John, F., Kavitha, S., Indira, M., 2013. Protective effect of ascorbic acid against ethanol-induced reproductive toxicity in male Guinea pigs. Br. J. Nutr. 110 (4), 689–698.

Hartmann, P., Chen, W.C., Schnabl, B., 2012. The intestinal microbiome and the leaky gut as therapeutic targets in alcoholic liver disease. Front. Physiol. 3, 402.

Hoyt, L.R., Randall, M.J., Ather, J.L., et al., 2017. Mitochondrial ROS induced by chronic ethanol exposure promote hyper-activation of the NLRP3 inflammasome. Redox Biol. 12, 883–896.

Kengo, T., Toshifumi, A., Naoto, K., 2004. Leptin deficiency enhances sensitivity of rats to alcoholic steatohepatitis through suppression of metallothionein. Am. J. Physiol. Gastrointest. Liver Physiol. 287, G1078–G1085.

Keshavarzian, A., Holmes, E.W., Patel, M., Iber, F., Fields, J.Z., Pethkar, S., 1999. Leaky gut in alcoholic cirrhosis: a possible mechanism for alcohol-induced liver damage. Am. J. Gastroenterol. 94, 200–207.

Leclercq, S., de, Timary, P., Delzenne, N.M., Stärkel, P., 2017. The link between inflammation, bugs, the intestine and the brain in alcohol dependence. Transl. Psychiatry 7 (2), 1048.

Lippai, D., Bala, S., Petrasek, J., Csak, T., Levin, I., Kurt-Jones, E.A., Szabo, G., 2013. Alcohol-induced IL-1β in the brain is mediated by NLRP3/ASC inflammasome activation that amplifies neuroinflammation. J. Leukoc. Biol. 94 (1), 171–182.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25, 402–408.

Lowe, P.P., Gyongyosi, B., Satishchandran, A., Iracheta-Vellve, A., Cho, Y., Ambade, A., Szabo, G., 2018. Reduced gut microbiome protects from alcohol-induced neuroinflammation and alters intestinal and brain inflammasome expression. J. Neuroinflammation 15, 298.

Marrs, J.A., Clendenon, S.G., Ratcliffe, D.R., Fielding, S.M., Liu, Q., Bosron, W.F., 2010. Zebrafish fetal alcohol syndrome model: effects of ethanol are rescued by retinoic acids supplement. Alcohol 44 (7–8), 707–715.

Martinon, F., Burns, K., Tschopp, J., 2002. The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. Mol. Cell 10 (2), 417–426.

Mutlu, E.A., Gillevet, P.M., Rangwala, H., Sikaroodi, M., Naqvi, A., et al., 2012. Colonic microbiome is altered in alcoholism. Am. J. Physiol. Gastrointest. Liver Physiol. 302, G966–G978.

Nair, S.S., Prathibha, P., Das, Syam S., Kavitha, S., Indira, M., 2015. All trans retinoic acid (ATRA) mediated modulation of N-methyl D-aspartate receptor (NMDAR) and Kruppel like factor 11 (KLF11) expressions in the mitigation of ethanol induced alterations in the brain. Neurochem. Int. 83, 41–47.

Nurmi, K., Virkanen, J., Rajamäki, K., Niemi, K., Kovanen, P.T., Eklund, K.K., 2013. Ethanol inhibits activation of NLRP3 and AIM2 inflammasomes in human macrophages–a novel anti-inflammatory action of alcohol. PLoS One 8 (11), e78537.

Priyanka, S.H., Syam, Das, Thushara, J., Arun, A.R., Indira, M., 2018. All trans retinoic acid attenuates markers of neuroinflammation in rat brain by modulation of SIRT1 and NFκB. Neurochem. Res. 43, 1791–1801.

Qin, L., He, J., Hanes, R.N., Pluzarev, O., Hong, J.S., Crews, F.T., 2008. Increased systemic and brain cytokine production and neuroinflammation by endotoxin following ethanol treatment. J. Neuroinflammation 5, 10.

Qin, L., Wu, X., Block, M.L., Liu, Y., Breese, G.R., Hong, J.S., Knapp, D.J., Crews, F.T., 2007. Systemic LPS causes chronic neuroinflammation and progressive neurodegeneration. Glia 55, 453–462.

Rosenzweig, H.L., Planck, S.R., Rosenbaum, J.T., 2011. NLRs in immune privileged sites. Curr. Opin. Pharmacol. 11, 423–428.

Stickel, F., Datz, C., Hampe, J., Bataller, R., 2017. Pathophysiology and management of alcoholic liver disease. Gut Liver 11 (2), 173–188.

Szabo, G., Mandrekar, P., Petrasek, J., Catalano, D., 2011. The unfolding web of innate immune dysregulation in alcoholic liver injury. Alcohol Clin. Exp. Res. 35, 782–786.

Valles, S.L., Blanco, A.M., Pascual, M., Guerri, C., 2004. Chronic ethanol treatment enhances inflammatory mediators and cell death in the brain and in astrocytes. Brain Pathol. 14, 365–371.

van Eijk, J., Demirakca, T., Frischknecht, U., Hermann, D., Mann, K., Ende, G., 2013. Rapid partial regeneration of brain volume during the first 14 days of abstinence from alcohol. Alcohol Clin. Exp. Res. 37 (1), 67–74.

Wheelwright, M., Kim, E.W., Inkeles, M.S., De Leon, A., Pellegrini, M., Krutzik, S.R., Liu, P.T., 2014. All-trans retinoic acid-triggered antimicrobial activity against Mycobacterium tuberculosis is dependent on NPC2. J. Immunol. 92 (5), 2280–2290.

Wilson, T.H., Wiseman, G., 1954. The use of sacs of everted small intestine for the study of the transference of substances from the mucosal to the serosal surface. J. Physiol. 193, 116–125.

Zhong, Y., Dong, G., Luo, H., Cao, J., Wang, C., Wu, J., Feng, Y.Q., Yue, J., 2012. Induction of brain CYP2E1 by chronic ethanol treatment and related oxidative stress in hippocampus, cerebellum, and brainstem. Toxicology 302, 275–284.

Zhong, W., McClain, C.J., Cave, M., Kang, Y.J., Zhou, Z., 2010. The role of zinc deficiency in alcohol-induced intestinal barrier dysfunction. Am. J. Physiol. Gastrointest. Liver Physiol. 298, G625–G633. https://doi.org/10.1152/ajpgi.00350.2009.