Original Article

Effects of retinoic acid on hepatocyte morphology and sinusoidal fenestrations in NAFLD rats

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ABSTRACT

Objective

This study aimed to evaluate the effects of retinoic acid (RA) treatment on hepatocytes and hepatic sinusoidal endothelial cells fenestrae and porosity in a non-alcoholic fatty liver disease (NAFLD) rat model.

Materials and Methods

Fifteen male Sprague Dawley rats were randomly divided into five groups (n=3 each): Group 1,control diet (CD); Group 2, CD with retinoic acid (RA); Group 3, high cholesterol diet (HCD); Group 4, HCD with vehicle; and Group 5, HCD with RA. The experiment duration was eight weeks. RA and vehicle were administered subcutaneously twice weekly during the last four weeks. Liver tissues were harvested and processed for scanning electron microscopy. Hepatocyte morphology was assessed descriptively. Fenestrae frequency and porosity of liver sinusoidal endothelial cells were quantified using ImageJ. Data were analysed with one-way ANOVA with Scheffé post hoc test.

Results and Discussion

Hepatocyte morphology was preserved in Groups 1 and 2, with polygonal cells and distinct borders. Groups 3, 4 and 5 showed swollen hepatocytes with irregular borders. RA treatment in Group 5 partially preserved hepatocyte morphology compared with untreated HCD groups. Fenestra frequency was reduced in HCD-fed groups but did not differ significantly with CD groups. However, porosity was significantly lower in Groups 3, 4 and 5 compared with Groups 1 and 2 ($p < 0.05$). Although RA treatment partially improved hepatocyte morphology, it did not effectively reverse sinusoidal endothelial alterations. These findings indicate that RA alone may be insufficient to restore fenestration and porosity once endothelial capillarisation is established in NAFLD.

Conclusion

Retinoic acid showed some morphological preservation of hepatocytes but had no significant effect on restoring LSEC fenestrations or porosity. These findings highlight the limited structural impact of RA on hepatic microvasculature in NAFLD.

Keywords

non-alcoholic fatty liver disease; rat model; retinoic acid; sinusoidal endothelial cell; fenestration

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD), recently redefined as metabolic dysfunction-associated steatotic liver disease (MASLD), is among the most prevalent chronic liver disorder globally. It is closely linked to metabolic abnormalities, particularly dyslipidaemia and hyperglycaemia.[1] The liver parenchyma mainly comprises hepatocytes arranged in lobules that are separated from hepatic sinusoidal endothelial cells (LSECs) by the space of Disse.[2] LSECs are distinctive endothelial cells lining the hepatic sinusoids, representing roughly 15–20% of the hepatocyte population.[3] They possess transcellular pore called fenestrae, which are organised into sieve plates that regulate the selective passage of lipids and macromolecules between the bloodstream and hepatocytes.[4-5] In addition, LSECs exhibit scavenging and clearing functions, facilitating the removal of circulating biomolecules.[6]

The preservation of these fenestrae is essential for maintaining normal hepatic microcirculation. Their reduction or loss, termed defenestration, is an early marker of sinusoidal endothelial dysfunction and is commonly associated with capillarisation, which involves endothelial thickening, basement membrane deposition

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and diminished porosity.[7,8] Such structural alterations are observed in various chronic liver diseases, including MASLD. Ageing further contributes to pseudocapillarisation, characterised by a decline in fenestrations and accumulation of extracellular matrix components.[9]

Evidence increasingly supports the pivotal role LSECs in regulating lipid homeostasis and liver disease progression. When fenestrae are lost, lipid transfer between blood and hepatocytes becomes impaired, potentially promoting hepatic steatosis and inflammation.[3] Although the precise mechanisms that trigger defenestration are still being investigated, factors such as excessive lipid intake, high carbohydrate consumption, and microbial metabolites from the gut are implicated. Endothelial nitric oxide (NO), produced by nitric oxide synthase (eNOS), maintains sinusoidal permeability; its depletion contributes to capillarisation and microvascular injury.[10-11]

Fat soluble vitamins and their derivatives, including vitamins A (retinoids), D (calciferols), E (tocopherols) and K (quinones), have been increasingly recognised for their therapeutic values in treating metabolic, inflammatory and degenerative conditions due to their capacity to modulate multiple cellular pathways.[12-15] These effects highlight the expanding role of vitamin-based compounds as potential treatment options beyond their traditional nutritional functions.

Retinoic acid (RA), the bioactive derivative of vitamin A, exerts multiple biological effects, including lipid-modulating and hepatoprotective effects by influencing lipid metabolism and gene expression.[16-17] Beyond hepatic tissue, RA also regulates cell adhesion, differentiation, and extracellular matrix remodelling. In trophoblastic cells, all-trans retinoic acid (ATRA) has been shown to downregulate matrix metalloproteinase-2 and upregulate E-cadherin, reflecting its potential to preserve cellular integrity and suppress tissue invasion.[18] Similarly, studies in other tissues have reported that ATRA reduces matrix degradation and improves structural stability through transcriptional control of retinoid-responsive genes.[13] In hepatic models, RA alleviates hepatic steatosis by activating the SIRT1/AMPK pathway, indicating its role in metabolic regulation.[19] Vitamin A signalling has been shown to influence endothelial homeostasis via nitric oxide synthase activation and modulation of vascular permeability.[20] Given that LSECs play a pivotal role in the development of NAFLD and non-alcoholic steatohepatitis[21], the effect of RA on LSEC fenestration in non-alcoholic fatty liver disease remains poorly understood.

The present study aimed to investigate the morphological effects of RA treatment on hepatocytes and quantify its impact on LSEC fenestrae and porosity as structural indicators of sinusoidal endothelial integrity in a rat model of NAFLD.

MATERIALS AND METHODS

Experimental Design

A total of fifteen male Sprague Dawley rats (*Rattus norvegicus*), each weighing 200-250 g, were randomly divided into five experimental groups of three animals each. A pilot study had previously determined that four weeks of high cholesterol diet (HCD) was sufficient to induce hepatic steatosis, and this duration was adopted in the present experiment. Groups 1 and 2 received a normal control diet for 8 weeks, with Group 2 additionally treated with retinoic acid (RA) during the last 4 weeks. Groups 3, 4 and 5 received a high cholesterol diet (HCD) for 8 weeks; Group 4 was treated with vehicle (dimethyl sulfoxide, DMSO) and Group 5 with RA during the last 4 weeks.

Control and High Cholesterol Diets Preparation

During the first two weeks, all animals were acclimatised with standard laboratory rat pellets (Gold Coin Feedmills (Malaysia) Sdn. Bhd). Subsequently, animals in Groups 1 and 2 received a similar diet prepared by grinding commercial pellets into a powdered form, without any added chemicals. The HCD was freshly prepared daily by mixing 120 g of analytical-grade cholesterol and 2 g of cholic acid (Nacalai-Tesque, Japan) into 1 kg of ground commercial rat pellets, resulting in a 12 % cholesterol diet.[22]

Retinoic acid preparation

Retinoic acid was obtained from Tokyo Chemical Industry, Japan (CAS RN: 302-79-4). Retinoic acid powder was dissolved in dimethyl sulfoxide (DMSO, CAS RN: 67-68-5) from R&M Chemicals, Malaysia at a

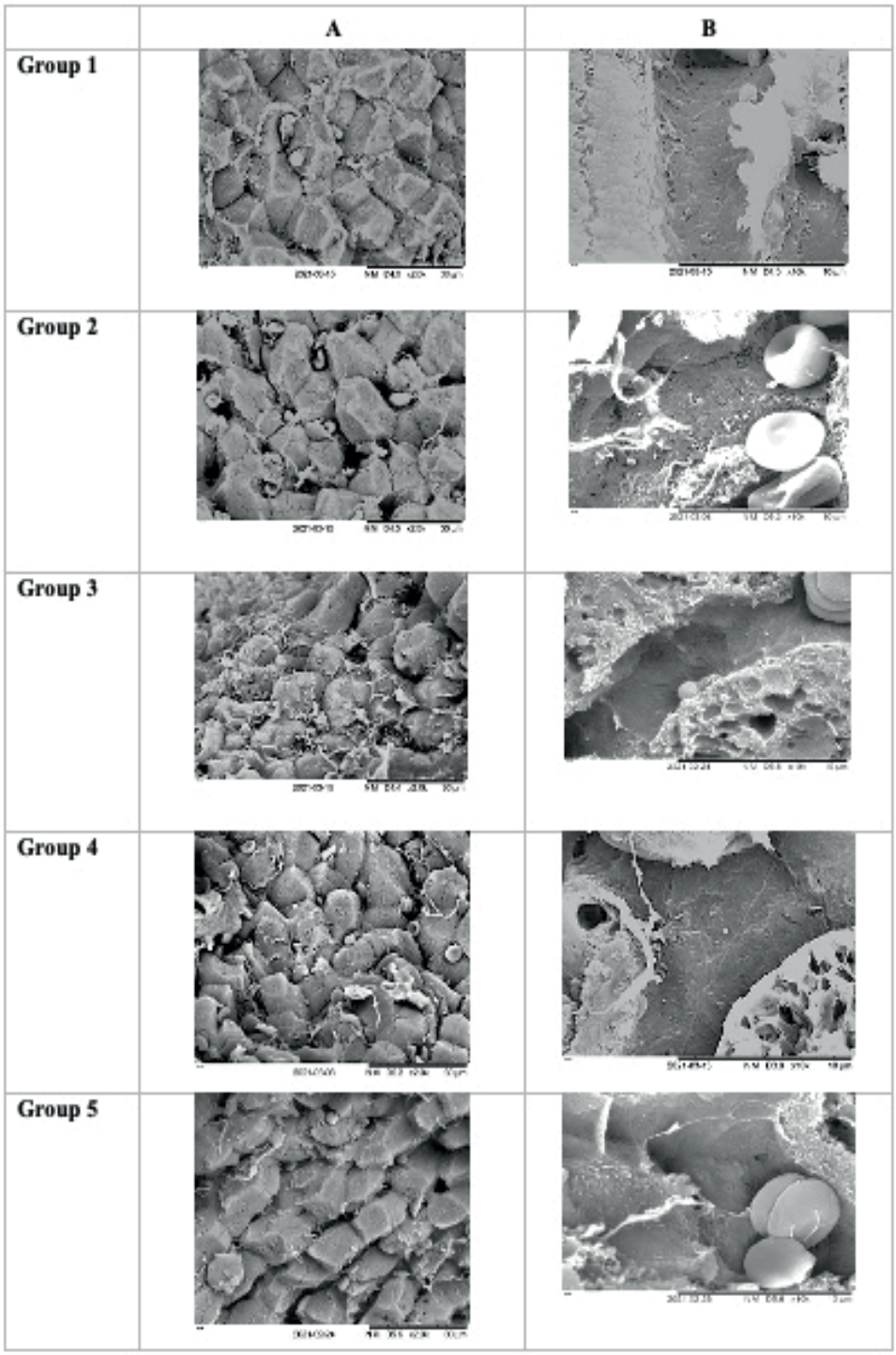

Figure 1: (A) Low magnification SEM micrographs (2,000X), showing parenchymal cells and sinusoids opening with scattered erythrocytes. Hepatocytes exhibit preserved hexagonal shape and distinct cell borders in Groups 1, 2, and 5, while Groups 3 and 4 show loss of characteristic architecture, with swollen and crowded hepatocytes. Scale bar = 30 µm (B) Higher magnification SEM micrographs (10,000X) demonstrating sinusoidal endothelial fenestrations, which are clearly visible in Groups 1 and 2 but reduced in Groups 3, 4 and 5. Scale bar = 10 µm.

concentration of 2 mg/mL of DMSO.[13,19] Rats in Group 2 and Group 5 received between 1.25 mL to 1.75 mL of the prepared retinoic acid solution via subcutaneous injection using 26 G needles, twice weekly for four weeks. Rats in Group 4 received 1.25 - 1.75 mL of DMSO subcutaneously twice weekly for four weeks.

Sample Preparation for Scanning Electron Microscopy

Liver samples were washed with cold phosphate buffered solution, followed by wedge fixation of a portion of the middle lobe measuring 1x1x1 cm^3. The tissue was injected with McDowell's fixative, comprising 4 % formalin and 1 % glutaraldehyde in 0.1 M phosphate buffer (pH 7.2), and kept at 4 ˚C until further processing. Subsequently, the tissue was post-fixed in 2 % osmium tetroxide (Agar Scientific, UK) in phosphate buffer for 2 hours, rinsed and dehydrated in upgraded concentration of ethanol. The samples were then dried at the critical point of CO_2 (CPD 030, Bal-Tec AG, Liechtenstein). After drying, the tissue was fractured into two parts and mounted on metallic stubs with fractured surface facing upward by carbon adhesive tape. Finally, the specimens were sputter-coated with gold (Leica EM SCD005, Leica Microsystems, Germany).

Quantitative Analysis

Specimens were examined using a scanning electron microscope (TM3030Plus, Hitachi High-Tech Corporation, Japan) and five images per sample were captured at a magnification of 10,000X. Fenestrations were quantified following the protocol described by Cogger et.al[23] using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Porosity was defined as the percentage of the LSEC cell membrane surface occupied by fenestrae, while fenestra frequency was defined as the number of fenestrae per μm^2.[24]

Statistical Analysis

Data are presented as mean ± standard deviation (SD). The Shapiro-Wilk and Levene's tests were used to assess normality and homogeneity of variance, respectively. Statistical comparisons between groups were performed using one-way ANOVA followed by Scheffe post hoc tests. Analyses were conducted using SPSS Statistics version 24.0 (IBM Corp., Armonk, NY, USA). A p-value of < 0.05 was considered statistically significant.

Ethical Clearence:

All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the International Islamic University Malaysia (IIUM), under approval number IIUM/IACUC/2019(14), and conducted in accordance with relevant ethical guidelines and regulations.

RESULTS

Scanning electron microscopy (SEM) examination revealed differences in hepatocytes and sinusoidal endothelial structure across experimental groups. In the control diet group (Group 1), hepatocytes maintained a polygonal shape with distinct cellular boundaries, and LSECs exhibited abundant fenestrae organized into sieve plates (Fig. 1). The fenestra frequency was 3.6 ± 1.0 per μm^2 and porosity was 2.3 ± 0.3 % (Table 1).

The control diet + RA group (Group 2) showed comparable hepatocyte ultrastructural features. Fenestra frequency (3.7 ± 0.7 per μm^2) and porosity (2.1 ± 0.4 %) were not statistically different from Group 1 ($p > 0.05$).

In contrast, the high cholesterol diet groups (Groups 3 and 4) exhibited structural changes. Hepatocytes appeared swollen with irregular cell borders, and the sinusoidal walls were flattened and covered with thread-like collagen fibrils. Fenestrae frequency decreased to 0.6 ± 0.2 (Group 3) and 0.5 ± 0.1 per μm^2 (Group 4), while porosity reduced to 0.3 ± 0.2 % in Group 3 and 0.3 ± 0.1 % in Group 4.

The HCD treated with RA group (Group 5) showed partial preservation of hepatocyte morphology, with slightly more regular cell outlines. Nevertheless, RA treatment did not restore sinusoidal fenestrations. Fenestra frequency (0.4 ± 0.1 per μm^2) remained significantly lower compared to Group1 ($p < 0.05$) and was slightly lower than Group 3, though not significant ($p > 0.05$). Porosity (0.2 ± 0.1 %) was also significantly lower than Group 1 ($p < 0.05$) and not significantly different from Group 3 and 4 ($p > 0.05$).

Although RA appeared to preserve hepatocyte morphology on descriptive assessment, it was not sufficient to reverse sinusoidal capillarization or restore endothelial fenestrae once defenestration had occurred.

Table 1. Fenestra frequency and porosity percentage in liver sinusoidal endothelial cells across experimental groups.

Parameter	Group 1	Group 2	Group 3	Group 4	Group 5
Fenestra frequency (number/1μm²)	3.6 ± 1.0	3.7 ± 0.7	0.6 ± 0.2	0.5 ± 0.1	0.4 ± 0.1
Porosity % (total area of fenestrae/ total area of sinusoidal endothelial cell) X 100	2.3 ± 0.3	2.1 ± 0.4	0.3 ± 0.2*	0.3 ± 0.1*	0.2 ± 0.1*

Data are presented as mean ± standard deviation (SD). Statistical comparisons were performed using Scheffe post hoc test. *$p < 0.05$ indicates a significant difference compared to Group 1 (n=3); Group 1: control diet group; Group 2 : control diet +RA; Group 3: high cholesterol diet only; Group 4: high cholesterol diet +DMSO; Group 5: high cholesterol diet +RA.

DISCUSSION

The present study explored the structural effects of retinoic acid (RA) on hepatocytes and liver sinusoidal endothelial cells (LSECs) in a non-alcoholic fatty liver disease (NAFLD) model. Increasing attention has been directed toward LSEC pathology in NAFLD, as alterations in their morphology are believed to occur early in disease progression.[3,25-26] Previous investigations have reported that RA improves hepatic steatosis primarily through modulation of lipid metabolism and suppression of inflammation.[13,16] However, its ability to influence sinusoidal fenestration remains uncertain.

Our findings demonstrated that the control diet group displayed normal sinusoidal microarchitecture, with LSECs containing numerous fenestrae arranged in sieve-like plates. The fenestra frequency and porosity observed in this group (3.6 ± 1.0 per μm² and 2.3 ± 0.3 %, respectively) were consistent with values previously documented in rodents.[24,27] In contrast, rats fed a high cholesterol diet (HCD) exhibited mark structural alterations, including hepatocyte swelling, irregular borders, and reduced sinusoidal porosity. These findings align with earlier reports linking defenestration and sinusoidal capillarisation to hepatic microvascular dysfunction in NAFLD.[5,7]

Quantitatively, HCD-fed groups showed reduced fenestra frequency, although the differences were not statistically significant, whereas porosity decreased significantly in comparison with controls. The descriptive assessment suggested that RA treatment preserved hepatocyte morphology to some extent; nonetheless, it failed to restore the fenestrae or improve sinusoidal porosity. This outcome suggests that once endothelial alterations are established, RA may have limited efficacy in reversing them.

Discrepancies between this study and previous research might relate to variations in species, diet composition, RA dosage, or treatment duration. Regional heterogeneity of fenestrae across the hepatic lobule, as described in previous report,[5] could also account for differences in quantitative measurements.

This study has several limitations that warrant consideration. The small sample size may have limited statistical power to detect subtle changes in fenestral dimensions. Furthermore, this model represented an early steatosis stage and might not fully mimic the spectrum of advanced NAFLD. Only a single RA dose and duration of retinoic acid (RA) treatment were tested, which may not capture the pharmacological potential of RA.

CONCLUSION

Our study indicates that RA exhibited minimal capacity to reverse sinusoidal endothelial alterations, despite mild improvement in hepatocyte morphology. Future investigations should include molecular analyses and varied dosing protocols to clarify whether RA could exert preventive rather than restorative effects on hepatic microvasculature in NAFLD.

Authors' Contributions

Nawal Ahmed Mohamed: overall responsibility, including concept, research questions, study design, data collection, analysis and draft manuscript.

Zunariah Buyong: overall responsibility, including concept, research questions, study design, and draft manuscript.

Nor Zamzila Abdullah, Asmah Hanim Hamdan, KNS Sirajudeen and Noraihan Mat Harun: Overall concept, research questions and study design.

All authors revised manuscript and approved the final version for submission.

Declarations of Interest

The authors declare no conflict of interest.

Compliance with Ethical Standards

All applicable institutional and national guidelines for the care and use of animals were followed. Ethical approval was obtained from the Institutional Animal Care and Use Committee (IIUM/IACUC/2019(14)).

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