List of Publications

1. **Malladi N**, Alam MJ, Maulik SK, Banerjee SK. The role of platelets in non-alcoholic fatty liver disease: From pathophysiology to therapeutics. Prostaglandins Other Lipid Mediators. 2023 Jul 20;169:106766. doi: 10.1016/j.prostaglandins.2023.106766. Epub ahead of print. IF: 2.5
2. **Malladi N**, Johny E, Uppulapu SK, Tiwari V, Alam MJ, Adela R, Banerjee SK. Understanding the Activation of Platelets in Diabetes and Its Modulation by Allyl Methyl Sulfide, an Active Metabolite of Garlic. Journal of Diabetes Research. 2021 Oct 19;2021:6404438. doi: 10.1155/2021/6404438. IF: 3.6
3. Tiwari V, Alam MJ, Bhatia M, **Navya M**, Banerjee SK. The structure and function of lamin A/C: Special focus on cardiomyopathy and therapeutic interventions. Life Sci. 2024 Mar 15;341:122489. doi: 10.1016/j.lfs.2024.122489. Epub 2024 Feb 8. PMID: 38340979. IF-5.2
4. **Malladi N**, Lahamge D, Somwanshi BS, Tiwari V, Deshmukh K, Balani JK, Chakraborty S, Alam MJ, Banerjee SK. Paricalcitol attenuates oxidative stress and inflammatory response in the liver of NAFLD rats by regulating FOXO3a and NFκB acetylation. Cell Signal. 2024 Sep;121:111299. doi: 10.1016/j.cellsig.2024.111299. Epub 2024 Jul 14. PMID: 39004324. IF-4.3
5. Tiwari V, Gupta P**, Malladi N**, Salgar S, Banerjee SK. Doxorubicin induces phosphorylation of lamin A/C and loss of nuclear membrane integrity: A novel mechanism of cardiotoxicity. Free Radic Biol Med. 2024 Jun;218:94-104. doi: 10.1016/j.freeradbiomed.2024.04.212. Epub 2024 Apr 5. PMID: 38582228. IF-7.1
6. Tariq, U., Sarkar, S., **Malladi, N**. *et al.* Correction: Knockdown of *SCN5A* alters metabolic-associated genes and aggravates hypertrophy in the cardiomyoblast. *Mol Biol Rep* **51**, 852 (2024). https://doi.org/10.1007/s11033-024-09742-9. IF-2.6

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Paricalcitol attenuates oxidative stress and inflammatory response in the liver of NAFLD rats by regulating FOXO3a and NFκB acetylation

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ABSTRACT

The lack of therapeutics along with complex pathophysiology made non-alcoholic fatty liver disease (NAFLD) a research hotspot. Studies showed that the deficiency of Vitamin D plays a vital role in NAFLD pathogenesis. While several research studies focused on vitamin D supplementation in NAFLD, there is still a need to understand the regulatory mechanism of direct vitamin D receptor activation in NAFLD. In the present study, we explored the role of direct Vitamin D receptor activation using paricalcitol in choline-deficient high-fat diet-induced NAFLD rat liver and its modulation on protein acetylation. Our results showed that paricalcitol administration significantly reduced the fat accumulation in HepG2 cells and the liver of NAFLD rats. Paricalcitol attenuated the elevated serum level of alanine transaminase, aspartate transaminase, insulin, low-density lipoprotein, triglyceride, and increased high-density lipoprotein in NAFLD rats. Paricalcitol significantly decreased the increased total protein acetylation by enhancing the SIRT1 and SIRT3 expression in NAFLD liver. Further, the study revealed that paricalcitol reduced the acetylation of NFκB and FOXO3a in NAFLD liver along with a decrease in the mRNA expression of IL1β, NFκB, TNFα, and increased catalase and MnSOD. Moreover, total antioxidant activity, glutathione, and catalase were also elevated, whereas lipid peroxidation, myeloperoxidase, and reactive oxygen species levels were significantly decreased in the liver of NAFLD after paricalcitol administration. The study concludes that the downregulation of SIRT1 and SIRT3 in NAFLD liver was associated with an increased acetylated NFκB and FOXO3a. Paricalcitol effectively reversed hepatic inflammation and oxidative stress in NAFLD rats through transcriptional regulation of NFκB and FOXO3a, respectively, by inhibiting their acetylation.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is a major global health challenge of chronic liver diseases [1]. NAFLD is a series of disorders characterized by fat deposition in the liver, leading to more advanced steatosis, fibrosis, cirrhosis, and, in some cases, hepatocellular carcinoma (HCC) [2]. Further, the global prevalence of NAFLD is 25.2% to 29.8% between the years 2016 to 2019 [3,4]. As a result of the increased global prevalence of NAFLD and its risk factors, such as obesity, metabolic syndrome, and type 2 diabetes, it is projected that chronic liver disease (CLD) will become more common [5]. Non-alcoholic fatty liver (NAFL) and non-alcoholic steatohepatitis (NASH) are the two phases of NAFLD. Liver fat of >5% with little or no inflammation of the liver is the hallmark of NAFL. Normal NAFL progression does not result in liver damage or complications, but NAFL can cause pain due to liver enlargement. NASH is distinguished by an inflammatory process in which the liver cells are damaged in the presence of fat in the liver [2]. Moreover, the metabolism of the NAFLD liver is entirely different from

Abbreviations: ALT, Alanine Transaminase; AST, Aspartate Transaminase; CDHF, Choline Deficient High; CLD, Chronic Liver Disease; ER, Endoplasmic Reticulum; HCC, Hepatocellular Carcinoma; HDL, High-Density Lipoprotein; HFD, High Fat Diet; HDACS, Histone Deacetylases; LDL, Low-Density Lipoprotein; MPO, Myeloperoxidase; NAFL, Non-Alcoholic Fatty Liver; NAFLD, Non-Alcoholic Fatty Liver Disease; NASH, Non-Alcoholic Steatohepatitis; PTM, Post-Translational Modification; ROS, Reactive Oxygen Species; SIR 2, Silent Information Regulator 2; VDR, Vitamin D Receptor.

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the healthy liver, and several transcription factors, enzymes, and signaling molecules may initiate the disease progression by altering their function through post-translational modification (PTM) [6,7]. PTM regulates the functional diversity of the protein by covalently linking functional groups. PTM alters the protein localization, interaction with other biomolecules, and protein's function [8]. These modifications are brought about by the addition of acetylation, phosphorylation, glycosylation, ubiquitination, and other chemical groups that affect the normal cell [9]. Among all PTMs, acetylation plays a crucial role in regulating the protein function in different ways in normal physiology as well as in different pathogenesis [10]. Also, much evidence proves that acetylation plays an essential role in chronic liver disease [11]. Further, the acetylation status in proteins is mostly regulated by two groups of enzymes, namely acetyltransferase and sirtuins (SIRT) [12]. SIRT comes under the category of histone deacetylases (HDACs) and has the function of removing the acetyl group from both histone and non-histone proteins. The seven distinct mammalian SIRT types (SIRT 1–7) that make up the silent information regulator 2 (SIR 2) families each carry out a distinct subcellular function. SIRT1 and SIRT3 serve as crucial energy and metabolic sensors that directly link the metabolic end product [13]. Changes in SIRT expression are observed in several chronic diseases, such as metabolic syndrome, cardiac hypertrophy, diabetes, and allergic disorders [14–18]. Hence, in the present study, we focused on understanding the acetylation status as well as the expression of its regulator enzyme SIRT in NAFLD liver, along with its role in developing disease.

Despite having enough data on NAFLD pathophysiology, the treatment is still limited [19]. Previous studies showed that vitamin D (fat-soluble vitamin) can affect the liver through Vitamin D receptor (VDR) [20,21]. VDR is present in hepatic cells, and its expression can reduce inflammation in chronic hepatic diseases [22]. Studies showed that VDR could be a draggable target in NAFLD [23]. A vitamin D analog, paricalcitol, has shown a beneficial role in diseases like chronic kidney injury, cholestatic liver injury, ischemia/reperfusion liver injury, CCL4 induced NASH and hepatitis B [24–28]. However, the role of paricalcitol in NAFLD is not known. Hence, in the present study, we have evaluated the effect of paricalcitol in NAFLD and the molecular mechanism thereof. Our study focused on the acetylation modification of crucial hepatic proteins in NAFLD progression and the effect of paricalcitol in reversing the same.

2. Material and methods

2.1. Reagents

CDHF diet purchased from Rodent Diets Inc., USA. Lipid profile and uric acid analytical kits were obtained from Randox Laboratories, Crumlin, United Kingdom; ALT and AST biochemical assay kits were obtained from Accurex Biomedical, India; Insulin ELISA kit was purchased from KRISHGEN Biosystems, India; immunoprecipitation kit was purchased from Abcam, Cambridge, United Kingdom; GAPDH (AC033), NFκB (A2547), and FOXO3a (A0102) antibodies were purchased from ABclonal, USA; SIRT1(9475), SIRT3 (5490S), and acetylated lysin (9441) antibodies were purchased from Cell Signaling Technology, USA; G-SNAP in gel visualizing reagent was obtained from GCC BIOTECH, India. Emerald GT PCR Master mix was purchased from Takara, USA.

2.2. Cell culture treatment

HepG2 (ATCC, USA) was grown in modified eagle high glucose medium (MEM) (Gibgo) containing 10% fetal bovine serum (FBS) (Gibgo) and 1% penicillin-streptomycin (HiMedia) under a humid atmosphere with 5% CO2 in an incubator at 37 °C.

2.3. Confocal imaging of oleic acid-induced fat accumulation in HepG2 cells

We tried to observe the effect of paricalcitol on the *in-vitro* fat accumulation in HepG2 cells. According to the protocol described [29], the cells were seeded on coverslips and starved for 12 h in 0.5% FBS-containing MEM. The cells were pretreated with paricalcitol (100 nM) for 12 h in the presence of 0.5% FBS and exposed to 400 μM oleic acid for a further 24 h. The cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% triton X. Later the cells were washed and stained with Nile red (5 μg/mL) and Hoechst stain (2 μg/mL) for 30 min. The stained cells were washed with PBS and mounted on a clean glass slide. Further, the cells were observed under a confocal microscope (Leica, DMi8, Germany) at 63×.

2.4. Immunocytochemistry to observe the expression of proteins in oleic acid-treated HepG2 cells

We did immunocytochemistry to observe the effect of paricalcitol on the *in-vitro* expression of NFκB and FOXO3a in HepG2 cells. The cells were seeded on coverslips and starved for 12 h in 0.5% FBS-containing MEM. The cells were pretreated with paricalcitol (100 nM) for 12 h in the presence of 0.5% FBS and exposed to 400 μM oleic acid for a further 24 h. The cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% triton X. The cells were then washed and incubated with the primary antibody (NFκB /FOXO3a) overnight. Later the cells were added with secondary antibody Alexa flour 488 and incubated for 2 h followed by Hoechst stain (2 μg/mL) for 10 min. The stained cells were washed with PBS and mounted on a clean glass slide. Further, the cells were observed under a confocal microscope (Leica, DMi8, Germany) at 63×.

2.5. Animal experiment design

We have received approval from the Institutional Animal Ethical Committee Approval (IAEC), NIPER-Guwahati, to conduct the animal experiment (NIPER/BT/2020/01). NAFLD was induced by feeding a choline-deficient high-fat (CDHF) diet in male Sprague-Dawley rats (weight: 200-250 g) (NAFLD group) for 20 weeks. Animals were monitored every week till 20 weeks for their body weight change and food intake. After 12 weeks of CDHF diet feeding, paricalcitol 0.08μg /kg/day according to their body weight was administered to the NAFLD rats (NAFLD + PCAL group) for up to 8 weeks (from 12 to 20 weeks). Paricalcitol was dissolved in propylene glycol: ethanol (95:5) and diluted further with PBS to administer in rats *via* the intraperitoneal (IP) route at a dose of 0.08μg/kg/day. A corresponding number of weight-matched rats (Control group) were maintained as controls by feeding them with the chow diet and administrating IP dose of vehicle (propylene glycol: ethanol diluted in PBS).

2.6. Histopathology examination

After 20 weeks of the study, the liver tissue was subjected to histopathology to observe the morphological changes. Briefly, whole liver tissue was excised and cleaned with ice-cold, then fixed in the 10% formalin, routinely processed, and embedded into paraffin. Paraffin sections were cut into 5 μM thick sections and mounted in a glass slide then stained with hematoxylin and eosin (H& E) stain and examined under a light microscope (EVOS, ThermoFisher Scientific, USA).

2.7. Biochemical analysis

Animals were numbed using isoflurane, and blood samples were drawn through the retro-orbital puncture. Serum was isolated and used to analyze various serum parameters such as total cholesterol, triglycerides (TG), low-density lipoprotein (LDL), high-density lipoprotein

(HDL), and uric acid using Randox biochemical analyzer. Serum alanine transaminase (ALT) and aspartate transaminase (AST) were measured using biochemical assay kits. While insulin level in serum was measured using an ELISA kit.

2.8. Immunoprecipitation and western blotting

Immunoprecipitation was performed by using an immunoprecipitation kit. Five mg of liver tissue incubated with 2.0 μL of anti-acetylated lysin antibody. Following antibody (anti-acetylated lysine antibody) binding, the acetylated protein precipitated by adding 25 μL of Protein A/G Sepharose beads slurry. Further immunoblotting was performed with precipitated proteins against FOXO3a and NFκB to look the individual protein acetylation. Similarly, whole protein lysates were used to perform immunoblotting against acetylated lysin, SIRT1, SIRT3, FOXO3a, and NFκB [30,31]. Similarly, we tried to look the effect of paricalcitol (100 nM) pretreatment for 12 h on protein expression of acetylated lysin, SIRT1, and SIRT3 in the HepG2 cells treated with either oleic acid (400 μM) or oleic acid (400 μM) pretreated with paricalcitol at 100 nM concentration for 12 h. After the treatment cell lysate was collected by lysing the cells with RIPA lysis buffer. The lysate was centrifuged at 14000 rpm for 15 min at 4 °C. In all the immunoblot experiments, stain-free gel loading was used to represent equal loading of proteins in each well. The protein levels were represented as fold change among groups. IgG control was used in immunoprecipitation studies and evaluate and confirm the band specific to the antibody.

2.9. Gene expression profiling

RNA was isolated from liver tissues of all groups using TRIzol reagent. Quantification and quality assessment of RNA was performed using a Spectrophotometer (Epoch Biotek microplate reader). cDNA was synthesized using 1 mg of RNA and performed polymerase chain reaction (PCR) to know the expression of inflammatory genes *i.e.*, TNF-α, IL1-β, and NFkB, and antioxidant genes *i.e.*, catalase, MnSOD using Emerald GT PCR Master mix and respective primers (Table S1). The data were normalized to the expression of the housekeeping gene *ribosomal protein L32* RPL32 and fold change between groups was calculated. The PCR image density was quantified using Image J software.

2.10. Lipid peroxidation assay

As per the protocol mentioned by Ohkawa et.al [32], lipid peroxidation was performed by homogenizing liver tissue in 10% (*w/v*) of ice-cold 0.05 M potassium phosphate buffer (pH 7.4). Homogenate of 30 μL was combined with 0.8% thiobarbituric acid (TBA), 0.2 mL of 8.1% SDS, and 1.5 mL of 20% acetic acid. The volume was made with distilled water to 4.0 mL, and the solution was held in a water bath with a temperature of 95 °C for one hour. After centrifuging the supernatant was isolated and an equivalent volume of butanol: pyridine (15:1) was added to the supernatant. The optical density of the organic layer was assessed at 532 nm. Then the mM of MDA formed per mg of protein using a standard curve of 1,1,3,3-tetraethoxypropane at a different concentration.

2.11. Glutathione assay

The assay of glutathione was carried out using Ellman's technique [33]. In short, 10% (*w/v*) of ice-cold 0.05 M potassium phosphate buffer (pH 7.4) was used to homogenize the liver tissues. The resulting homogenate was centrifuged at 14000 rpm for 20 min at 4 °C and supernatant was collected. A hundred μL of supernatant was mixed with 500 μL of 5% trichloroacetic acid (TCA) and the mixture was centrifuged at 2300 *g* for 10 min to deproteinize the sample. Further, 250 μL of dithiol-nitro-benzoic acid (DTNB) and 1.5 mL of 0.3 M disodium hydrogen phosphate were added to 100 μL of the deproteinized sample. At last, the optical density of the prepared sample was measured at 412 nm and the results were calculated as a micromole of glutathione (GSH) present in mg of protein.

2.12. Catalase activity assay

Catalase assay was carried out using Aebi's method [34]. In brief, 0.5 μL of the tissue supernatant prepared by homogenizing the liver tissue in 10% of 0.05 M phosphate buffer was taken and added to 0.5 mL of 50 mM phosphate buffer (pH 7.0). Finally, the prepared sample mixture is added with 250 μL of 30 mM H_2O_2. The change in absorbance at 240 nm was monitored with a 15-s gap for 1.5 min. The catalase activity in the liver was measured as the rate of H_2O_2 oxidation per minute per milligram of protein.

2.13. DPPH (total antioxidant) assay

Percentage antioxidant activity was measured using a 2,2-diphenyl-picrylhydrazyl (DPPH) assay [35]. Briefly, liver tissue was homogenized in 10% (*w/v*) of ice-cold 0.05 M potassium phosphate buffer (pH 7.4). DPPH solution was prepared at the concentration of 0.195 mg/mL in methanol. To 5 μL of tissue homogenate, 100 μL DPPH solution and 100 μL tris buffer were added. Further incubated for 30 min and absorbance was taken at 517 nm by a microplate reader using methanol as blank. Free radical scavenging activity of the liver was measured through the obtained optical density readings. Lower optical density represents the higher antioxidant status in the liver.

2.14. DCFDA assay

Liver reactive oxygen species (ROS) was measured using 2, 7-dichlorofluorescein diacetate (DCF-DA) method [36], where 100 mM of DCF-DA was added to 5 μL of liver tissue homogenate and incubated for 30 min at room temperature in the dark. After incubation, phosphate buffer saline (PBS, 0.1 M, pH -7.4) was used to regulate the reaction's volume, and the fluorescence was measured at 488 nm excitation and 525 nm emission wavelengths using a multimode reader.

2.15. Myeloperoxidase assay

Myeloperoxidase (MPO) assay was performed using the Bradley protocol [37]. In brief, the liver tissue homogenate was added to 5% hexadecyl trimethyl ammonium bromide (HTAB) and 10 mM EDTA. Then the homogenates were freeze-thawed followed by centrifuged at 13000 *g* for 20 min. The supernatant obtained was used for the estimation of MPO activity using 0.167 mg/mL of o-dianisidine hydrochloride and 0.005% hydrogen peroxide at 460 nm. The MPO activity was expressed as U/g of tissue.

2.16. Statistical analysis

Statistical analysis was performed using Graph pad Prism (Graph Pad Software, San Diego, CA, USA). All statistical analyses were made by either means of one-way ANOVA or two-way ANOVA followed by Turkeys' multiple comparison test. The data were represented as mean ± SEM. $P < 0.05$ is considered significant.

3. Results

3.1. Body weight and food intake changes in rats

The body weight of rats was monitored every week till the end of the study (20 weeks). The percentage changes in body weight of rats were calculated and the graph was plotted (Fig. 1A). There was a significant increase in the percentage body weight change of NAFLD rats starting from the11th week to the 20th week (except week 12) of the study period

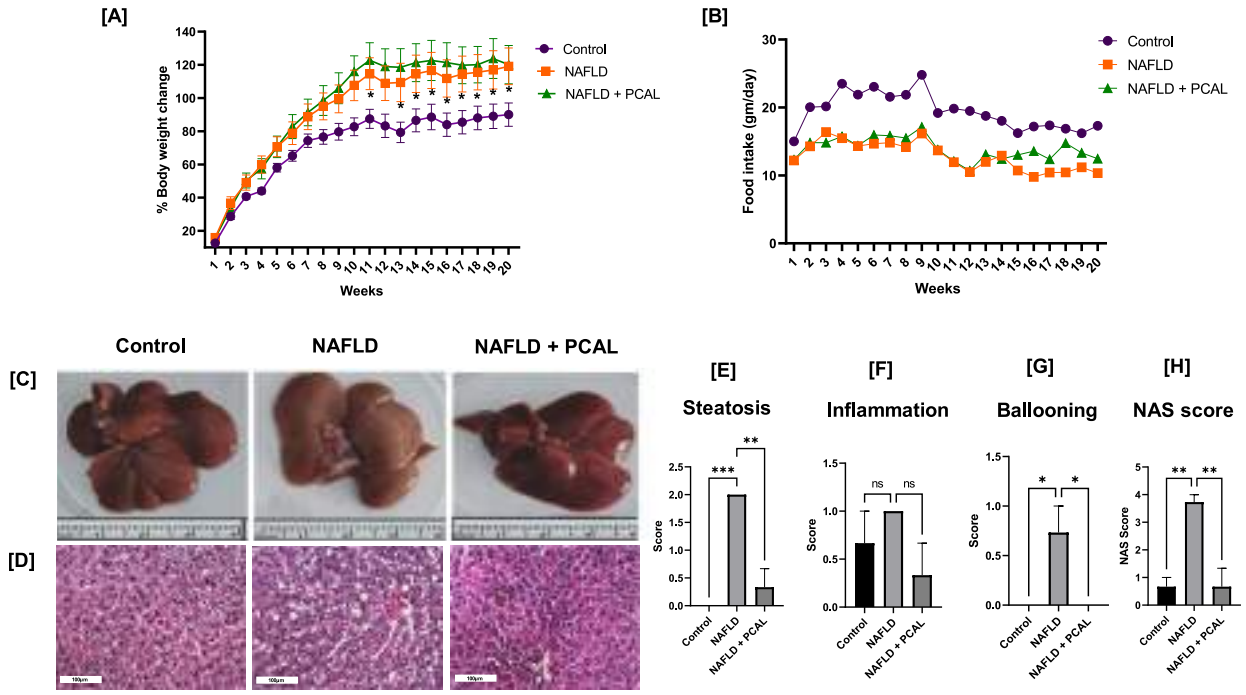

Fig. 1. Effect of paricalcitol on [A] Percentage (%) body weight changes ($n = 7$), [B] changes in food intake (n = 7), and [C] liver morphological picture and [D] images of liver histopathology (H&*E*-stained sections) of rats where scale is 100 μm ($n = 3$). Bar graph showing the histopathological score of [E] steatosis, [F] lobular inflammation, [G] ballooning of hepatocytes and [H] NAS scoring. The data were analyzed using a two-way ANOVA followed by Tukey's test and represented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns = non-significant.

compared to control. However, NAFLD rats treated with paricalcitol showed no changes in percentage body weight change compared to NAFLD rats. Similarly, rats were also monitored for food intake where both NAFLD and NAFLD treated with paricalcitol (NAFLD + PCAL) rats showed less food intake compared to control (Fig. 1B).

3.2. *Paricalcitol reduced the fat accumulation in NAFLD rat liver and oleic acid-treated HepG2 cells*

The liver of rats was collected after euthanizing the animals during the 20th week of the study. Liver morphology of NAFLD is slightly varied by color compared to control rats whereas treatment with paricalcitol improved the morphology in NAFLD liver to normal (Fig. 1C). Further, histopathological evaluation of rat liver by H&*E*-stained sections showed a significant increase in steatosis (fat accumulation) in NAFLD rats compared to the control (Fig. 1D, E). After paricalcitol treatment (NAFLD + PCAL group), a decrease in steatosis was observed when compared to NAFLD liver. Although there was no significant change in lobular inflammation was observed, paricalcitol decreased the ballooning significantly in the NAFLD group (Fig. 1D, F, G). The total NAS score was found to be higher in NAFLD liver in comparison to the control. Further paricalcitol has normalized the NAS score in NAFLD rats (Fig. 1H). The detailed scoring of each animal and a detailed illustration of histopathology changes were supplemented in Table S2 and Fig. S1 respectively. Further, we tried to elucidate the effect of paricalcitol on fat accumulation *in-vitro* in HepG2 cells by feeding the cells with excess fat (oleic acid). In untreated cells, there was very minimal fat droplet observed by Nile red stain. Upon oleic acid-treated HepG2 showed an

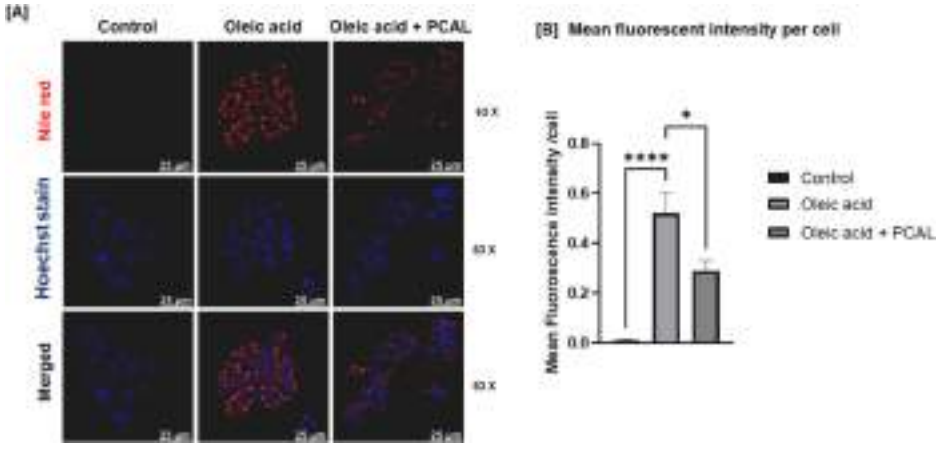

Fig. 2. Paricalcitol effect on oleic acid-induced fat accumulation in HepG2 cells where [A] confocal images and [B] Bar graph showing the changes in mean fluorescence intensity of the Nile red per cell in control, oleic acid and oleic acid + paricalcitol treated HepG2 cells. The data were analyzed using a two-way ANOVA followed by Tukey's test and represented as mean ± SEM. *$p < 0.05$, ****$p < 0.0001$ ($n = 200 \pm 5$ cells). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

elevated fat accumulation (mean fluorescent intensity of Nile red stain per cell) in hepatocytes significantly (Fig. 2). Moreover, pretreatment of paricalcitol effectively reduced the fat accumulation in HepG2 cells.

3.3. Effect of paricalcitol on serum biochemical parameters

The effect of paricalcitol on serum biochemical parameters was measured and plotted the changes as bar graphs (Fig. 3). Serum levels of uric acid, AST, ALT, and insulin were significantly increased in NAFLD compared to the control. Paricalcitol treatment effectively reduced the levels of AST, ALT, and insulin but not uric acid in the NAFLD + PCAL group. Moreover, serum lipid parameters such as LDL and triglyceride were significantly elevated in serum of NAFLD compared to control. Paricalcitol significantly reduced LDL and triglyceride in the NAFLD + PCAL group. Further, HDL levels were reduced in NAFLD and elevated significantly after paricalcitol treatment. There was no significant change observed in cholesterol levels among all the groups.

3.4. Effect of paricalcitol on the reversal of protein acetylation in the liver of NAFLD rats and oleic acid-treated HepG2 cells

After confirming the effectiveness of paricalcitol in reducing the fat accumulation in NAFLD rat liver and oleic acid-treated HepG2 cells, we tried to explore the effect of paricalcitol on the protein acetylation in the liver by western blot (Fig. 4A, D). There was an increase in acetylated lysin protein in the liver of NAFLD rats compared to the control. Interestingly, paricalcitol reversed the acetylation status in NAFLD + PCAL liver. Further, the protein expression of deacetylating enzymes such as SIRT3 (Fig. 4B, E) and SIRT1 was measured (Fig. 4C, F) where NAFLD liver showed a decrease in expression of SIRT1(significantly) and SIRT3 (non-significantly) in NAFLD compared to control. Paricalcitol treatment has significantly improved the SIRT1 and SIRT3 levels in the NAFLD + PCAL group. In support of the animal data, we have noticed there was an increase in protein acetylation in HepG2 cells when exposed to a high amount of oleic acid (Fig. S2). Pre-treatment with paricalcitol (12h) decreased the protein acetylated status as compared to high oleic acid treated cells but the results are non-significant. Further, we tried to look at the SIRT1 and SIRT3 expression, the regulators of acetylation of proteins in oleic acid-treated HepG2 cells (Fig. S3). A decrease in SIRT 1 and SIRT 3 protein expression was observed similar to the *in-vivo* data. Although, 12 h of paricalcitol pretreatment increased the expression of SIRT 3 but it was not significant. Similarly, the expression of SIRT1 has not increased significantly when compared to oleic acid group.

3.5. Paricalcitol regulates the inflammation in the liver of NAFLD rats by attenuating the acetylation of NFκB and modulating gene expression of inflammatory makers

We measured the levels of the transcription factor NFκB in the liver of control, NAFLD, and paricalcitol (NAFLD + PCAL) groups by western blot (Fig. 5A-C). Data showed a significant increase in the expression of the NFκB in the NAFLD compared to the control. Similarly, we checked the expression of the acetylated NFκB. Data showed a significant increase in the levels of the acetylated NFκB in NAFLD compared to the control. After paricalcitol treatment (NAFLD + PCAL group), a significant decrease was seen in both total and acetylated NFκB expression. In support to the animal data, our *in-vitro* immunofluorescence data showed an increased NFκB protein expression in HepG2 cells treated with oleic acid which has been reduced with paricalcitol treatment (Fig. S4). Further, mRNA expression of inflammatory genes such as TNFα, IL 1ß, and NFκB was measured by PCR (Fig. 5E-H). Data showed a significant increase in expression of TNFα, IL 1ß, and NFκB in rat liver of NAFLD when compared to control. Further paricalcitol treatment has

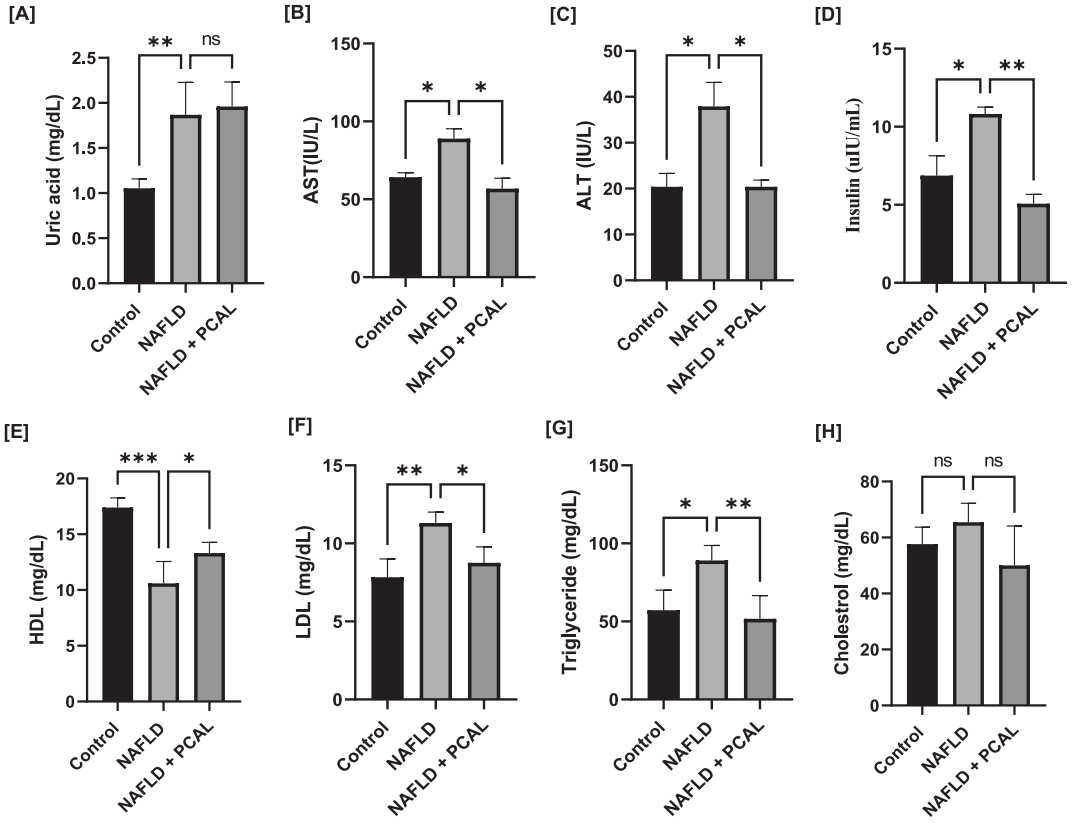

Fig. 3. Bar graphs showing changes in various serum biochemical parameters of control, NAFLD and NAFLD + PCAL groups. [A] Uric acid, [B] AST, [C] ALT, [D] insulin, [E] HDL, [F] LDL, [G] triglyceride and [H] cholesterol. The data were analyzed using a One-way ANOVA followed by Tukey's test and represented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns = non-significant ($n = 3$–4).

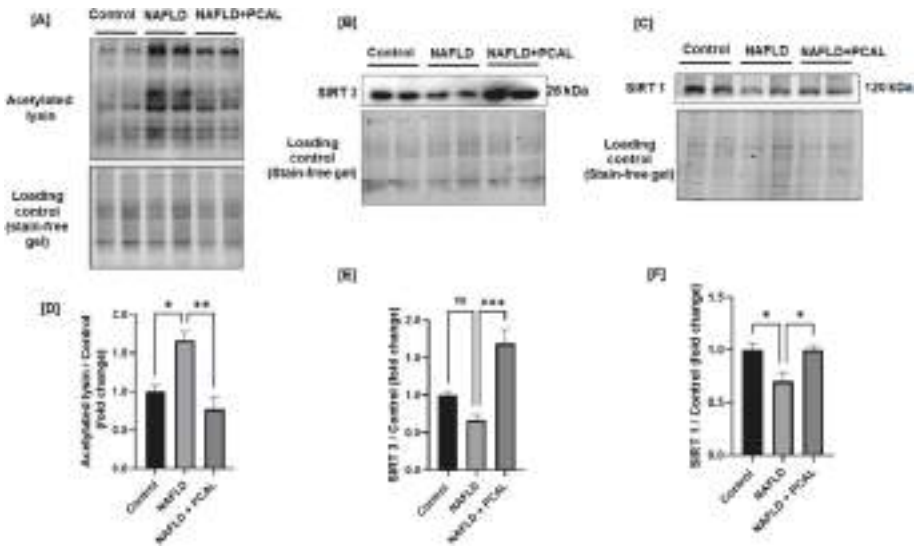

Fig. 4. The expression of the total acetylated protein and its regulatory enzymes SIRT 1 and SIRT 3 in rat liver of control, NAFLD, NAFLD + PCAL groups. [A, D] Total acetylated lysin expression, [B, E] protein expression of SIRT3 and [C, F] protein expression of SIRT1. The data were analyzed using a One-way ANOVA followed by Tukey's test and represented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns = non-significant ($n = 4$ from two independent blots).

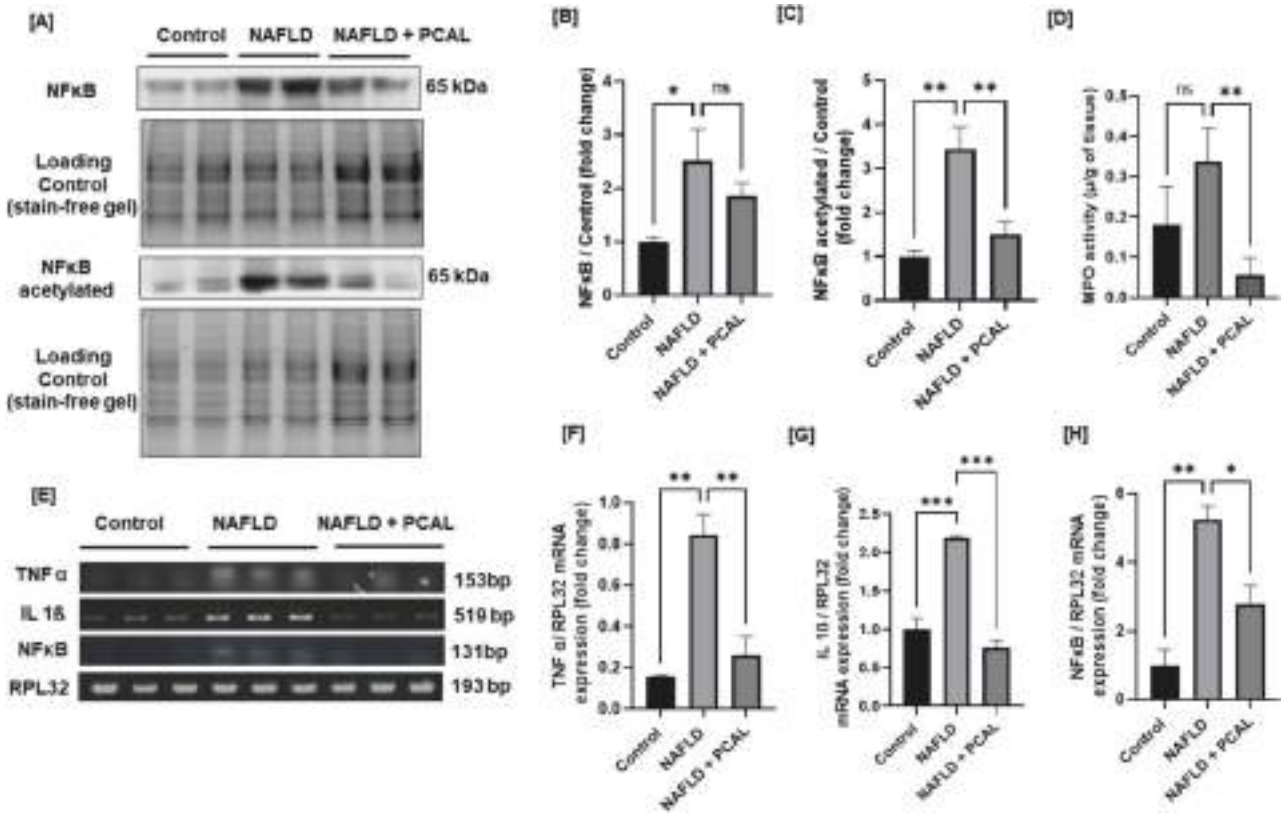

Fig. 5. Expression of inflammatory markers in the liver of control, NAFLD, NAFLD + PCAL group. [A-C] Protein expression of NFκB and acetylated NFκB expression in the rat liver ($n = 4$ from two independent blots), [D] MPO activity, [E] Blots showing the mRNA expression of TNFα, IL 1ß, and NFκB. Bar graphs showing the changes in expression of [F] TNFα, [G] IL 1ß, and [H] NFκB respectively where $n = 3$. The data were analyzed using an ordinary one-way ANOVA and represented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns = non-significant.

significantly reduced this gene expression in the NAFLD + PCAL group. Additionally, the inflammation in the liver was measured by MPO assay, where the data showed an increase (but not significant) in MPO activity in NAFLD the rat liver compared to control (Fig. 5D). But paricalcitol treatment showed a significant reduction in MPO activity in NAFLD + PCAL liver.

3.6. Paricalcitol attenuates the acetylation of FOXO3a and gene expression of oxidative stress markers in the liver of NAFLD rats

We measured the protein levels of the transcription factor FOXO3a in the liver of control, NAFLD, and paricalcitol (NAFLD + PCAL) groups by western blot (Fig. 6A-C). Data showed a significant increase in the

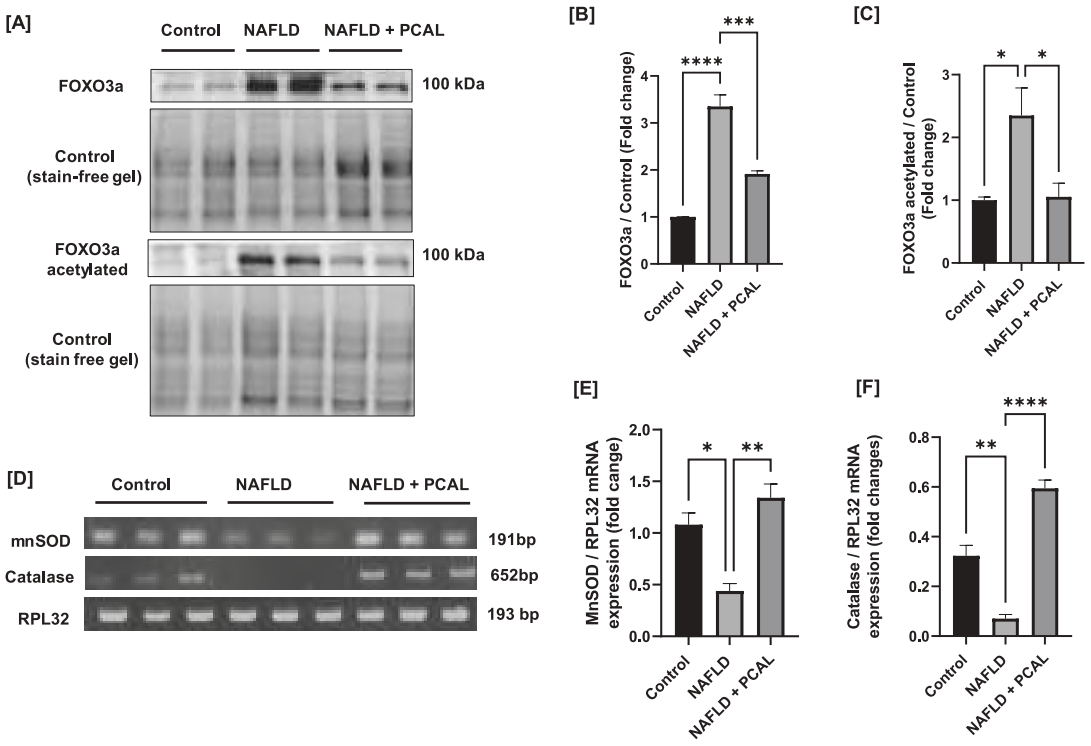

Fig. 6. Expression of oxidative stress markers in the liver of control, NAFLD, NAFLD + PCAL groups. [A-C] FOXO3a and its acetylated protein status ($n = 4$ from two independent blots); [D-F] mRNA expression of MnSOD and catalase ($n = 3$). The data were analyzed using a One-way ANOVA followed by Tukey's test and represented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

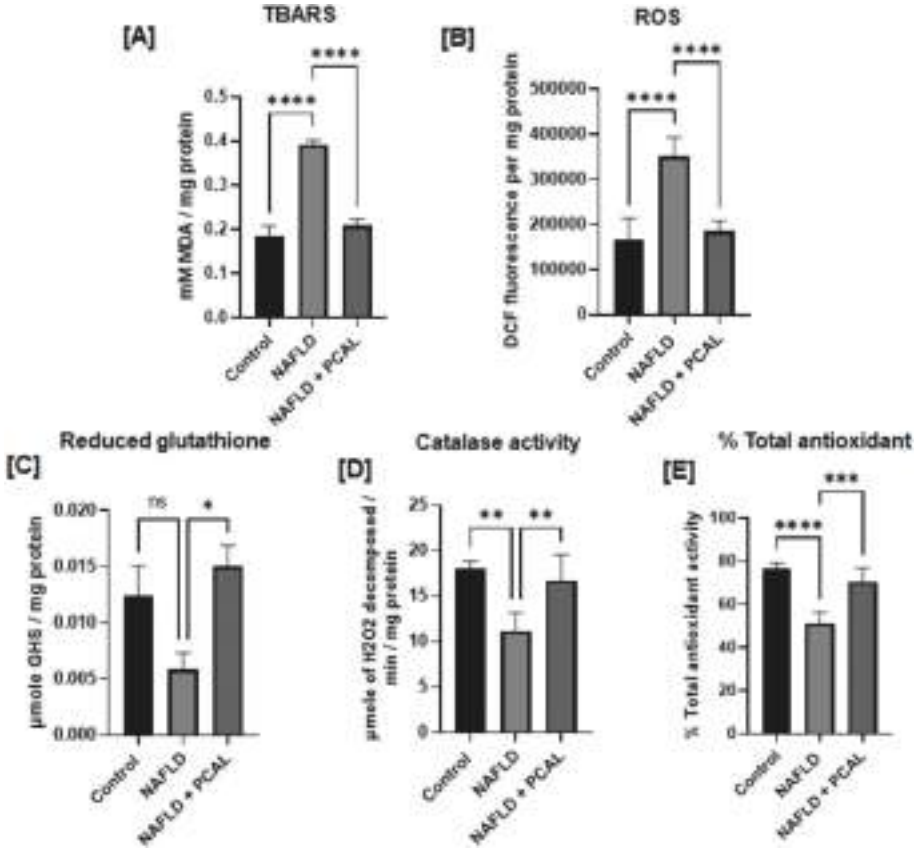

Fig. 7. Oxidative stress and antioxidant level/activity were measured in the liver of control, NAFLD, NAFLD + PCAL groups. [A] Lipid peroxidase activity, [B] ROS level, [C] GSH concentration, [D] catalase activity, [E] % total antioxidant activity. The data were analyzed using a One-way ANOVA followed by Tukey's test and represented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, ns = non-significant ($n = 3$–4).

expression of FOXO3a in the NAFLD compared to the control. Similarly, the liver after paricalcitol treatment (NAFLD + PCAL group) showed a significant decrease in the expression of FOXO3a. Further, we check the levels of the acetylated FOXO3a. Data showed a significant increase in the levels of the acetylated FOXO3a in NAFLD compared to the control. However, the paricalcitol treatment showed a significant decrease in the levels of acetylated FOXO3a. In support to the animal data, our *in-vitro* immunofluorescence data showed an increased FOXO3a protein expression in HepG2 cells treated with oleic acid which has been reduced with paricalcitol treatment (Fig. S5). Then we measured oxidative stress parameters in the rat liver by evaluating mRNA gene expression levels of two antioxidant genes, MnSOD and catalase (Fig. 6D-F). Data showed a significant decrease in the expression of the MnSOD and catalase genes in the NAFLD compared to the control. The paricalcitol treatment showed a significant increase in the expression of both MnSOD and catalase genes in the NAFLD + PCAL group when compared to the NAFLD group.

3.7. Paricalcitol attenuates oxidative stress and enhances the antioxidant activity in the liver of NAFLD rats

We measured the oxidative stress in the liver of control, NAFLD, and paricalcitol (NAFLD + PCAL) groups using a lipid peroxidation assay. Our data showed that lipid peroxidation was significantly increased in the liver of NAFLD rats compared to the control rats. Paricalcitol treatment significantly decreased lipid peroxidation levels in NAFLD + PCAL rats. (Fig. 7A). Additionally, we measured ROS levels by using a fluorescence dye, DCFDA in the liver homogenate of control, NAFLD, and paricalcitol (NAFLD + PCAL) groups. Our data showed that the ROS level increased significantly in the NAFLD group compared to the control. The paricalcitol treatment showed a significant decrease in the ROS levels in the liver of NAFLD + PCAL rats (Fig. 7B).

Furthermore, to evaluate the antioxidant properties we measured GSH levels, an antioxidant in the liver of control, NAFLD, and paricalcitol (NAFLD + PCAL) groups. Data showed that the GSH levels in the liver were decreased in NAFLD rats compared to the control. However, paricalcitol treatment significantly increased GSH levels in the NAFLD + PACL group (Fig. 7C). Further, catalase, an antioxidant enzyme, activity in the liver of control, NAFLD, and paricalcitol (NAFLD + PCAL) groups were measured. Our data showed that the catalase enzyme activity was significantly decreased in the NAFLD liver compared to the control. However, paricalcitol treatment showed a significant increase in catalase enzyme activity in the liver of NAFLD + PCAL rats (Fig. 7D). We have also measured the percentage of total antioxidant levels in the liver of control, NAFLD, and paricalcitol (NAFLD + PCAL) groups by using DPPH assay. Our data showed that the total antioxidant activity was significantly decreased in the NAFLD liver compared to the control. Paricalcitol treatment significantly increased the percentage of total antioxidant activity in the liver of NAFLD + PCAL rats (Fig. 7E).

4. Discussion

NAFLD is a condition of the liver with excessive accumulation of fats in the hepatocytes [38]. The pathophysiology of NAFLD involves several pathways that are interlinked in a complex way and, therefore, make the disease difficult to understand [39,40]. PTMs of proteins such as acetylation play a crucial role in regulating various protein functions and maintaining cellular homeostasis [41]. In the liver, protein acetylation regulates several metabolic pathways such as glucose and lipid metabolism [42,43]. Previous studies showed that the alteration in the acetylation of proteins may lead to fatty liver disease [44,45]. Further, recent findings highlighted the significance of lysine acetylation on subcellular distribution, transcriptional activity, DNA affinity, and stability of transcription factors [46]. Therefore, understanding the acetylation of transcription factors in NAFLD may add strength to existing pathophysiology.

Along with the increasing heterogenicity of NAFLD pathophysiology, the lack of effective treatment in NAFLD highlights the need for exploring more therapeutic strategies in NAFLD [19]. Although studies showed that lifestyle interventions such as dietary control and exercise are the effective methods available for reducing NAFLD at an early stage by improving insulin resistance, and liver enzymes and improving glucose and fatty acid metabolism [47–49]. Further numerous investigations have shown that vitamin D insufficiency is associated with both a higher frequency of NAFLD and a higher grade of NAFLD severity [50]. The expression of VDR in hepatic cells generally helps to reduce inflammation in chronic hepatitis [22]. Paricalcitol, a vitamin D analog and drug to treat hyperparathyroidism in patients with chronic kidney disease [51], could be a promising therapeutic agent to treat NAFLD by preventing the accumulation of fat in liver cells [52]. Further, paricalcitol prevented fibrosis in carbon tetrachloride (CCL4) induced chronic liver disease model in rats [27]. Previously, paricalcitol showed its beneficial effect by reducing oxidative stress and inflammation in diabetes, kidney diseases, allergic disorders, and cardiovascular diseases [53–56]. However, the role of paricalcitol in regulating inflammation and oxidative stress in NAFLD is still not evaluated. Further, it is not proven if paricalcitol shows any effect by modulating the acetylation of crucial proteins. Therefore, the present study aims to look at the effect of paricalcitol in attenuating fat accumulation in the hepatocytes of both *in vitro* and *in vivo* models of NAFLD. Thus, the present study tried to explore the effect of paricalcitol on oxidative stress and inflammation of the liver by modulating the acetylation of proteins in an *in-vivo* rat model of NAFLD.

We developed a NAFLD rat model by administration of a choline-deficient high-fat (CDHF) diet [57], and tried to find the efficacy of paricalcitol by administering the drug at a dose of 0.08 μg/kg/day by intraperitoneal route for a period of 8 weeks. We observed the beneficial effect of paricalcitol on reducing hepatocyte and/or liver fat accumulation both *in-vitro* and *in-vivo* studies. Moreover, in our *in-vitro* data, we observed a very minimal fat droplet in untreated cells which reflects the normal physiology of liver cells as per the previous literature [58,59]. Further *in-vivo* data showed the role of paricalcitol in decreasing ballooning and total NAS score in NAFLD liver. Paricalcitol reduced the liver injury markers such as AST and ALT in NAFLD rat serum. Previous studies showed that elevated ALT and AST are surrogate markers of the disease NAFLD [60]. Another study showed that calcitriol, a vitamin D analog, reduced the ALT and AST in NAFLD patients [61]. Moreover, in the present study, the elevated ALT and AST in NAFLD have been effectively reduced by paricalcitol, suggesting paricalcitol's role in decreasing liver injury in NAFLD. Apart from transaminases, uric acid is also known to be associated with the occurrence and progression of NAFLD. According to a study, elevated uric acid was found in patients with NAFLD and can be used as a predictor of disease [62]. Similarly, recent research showed a beneficial effect of paricalcitol in reducing uric acid levels in diabetic nephropathy [63]. Although increased uric acid levels were found in NAFLD rats, paricalcitol did not show any effect on uric acid levels. NAFLD is also known to associate with insulin resistance and hyperinsulinemia which stimulates lipogenesis by SREBP-1c in NAFLD liver [64,65]. Paricalcitol effectively reduced the insulin in serum suggesting a beneficial role of paricalcitol in NAFLD. Further, a previous study showed that paricalcitol enhances insulin sensitivity in Type 2 diabetes [66]. A large proportion of NAFLD patients are known to be accompanied by dyslipidemia [67]. Increased serum lipids such as TG, LDL, and cholesterol and decreased HDL were linked with increasing degree of fatty liver [68]. As expected, vitamin D deficiency is also linked with dyslipidemia in NAFLD [50]. In the present study, paricalcitol reduced the elevated TG, LDL, and decreased HDL in NAFLD rats.

According to a study, exposure to a high-fat diet (HFD) increased the acetylation of proteins involved in gluconeogenesis, methionine metabolism, mitochondrial oxidative metabolism, endoplasmic reticulum

(ER) stress response, and liver damage [69]. Similarly, the present study increased lysine acetylation of hepatic proteins in NAFLD rats which effectively reversed by paricalcitol treatment. It is well-known that the increased acetylation is associated with a decrease in sirtuins activity or protein levels. Among all sirtuins, SIRT1 and SIRT3 are the major players in regulating protein acetylation in the nucleus, cytoplasm, and mitochondria [70]. Also, studies showed a strong relationship between sirtuins and the development of NAFLD [71,72].

In the present study, our *in-vivo* data showed that the SIRT1 and SIRT3 protein expression is significantly downregulated in NAFLD disease condition suggesting that the increased acetylation is due to a decreased deacetylases SIRT1 and SIRT3 in the liver of NAFLD rats. Further, NAFLD treated with the paricalcitol showed an upregulation in both SIRT1 and SIRT3 expression *in-vivo* indicating an increase in deacetylation activity. Further, we tried to see the effect of paricalcitol *in-vitro* on oleic acid induced HepG2 cells. In support with our *in-vivo* results, an increase in acetylation and decrease deacetylase enzyme expression in oleic acid treated HepG2 cells suggests that the excess fat in hepatocytes has a role in elevating protein acetylation by down regulating SIRT1 and SIRT3 which has been not reduced effectively with 12 h pretreatment of paricalcitol. Our *in-vitro* data suggests that the longer exposure (>12 h) of paricalcitol might effective in reducing fat and its associated protein acetylation in *in-vitro*. A study proved that the downregulation of SIRT1 and SIRT3 caused by excessive fat intake increases lipotoxicity, ROS production, mitochondrial damage, apoptosis, and inflammatory responses in the liver leading to cell injury [73,74]. Further, a study showed that paricalcitol reduces cholestatic liver damage by activating the SIRT1 pathway [24]. Vitamin D supplementation has effectively increased the level of SIRT1 in the serum of diabetic patients [75]. Further vitamin D supplements have also elevated SIRT3 in HFD-fed rats [76]. All the above studies suggest that paricalcitol has the property to attenuate elevated acetylation of proteins in NAFLD by activating the SIRT1 and SIRT3 pathways. Moreover, in our study, the upregulation of SIRT3 expression was observed in NAFLD rat liver after paricalcitol treatment. This could be due to the selective activation of VDR receptors by paricalcitol [77]. As previous literature suggests VDR activation by calcitriol, a non-selective VDR agonist enhances the transcriptional activation of SIRT3, thereby increases the expression of SIRT3 in cardiomyocytes [76]. Similarly, in our study, paricalcitol, a selective Vitamin D analogue has more selectivity to bind VDR and therefore enhances higher SIRT3 expressions compared to control in liver tissue.

Moreover, sirtuins are well known to affect the function of several transcription factors and regulate cell function [78]. One of the important downstream targets of SIRT1 is NFκB which regulates inflammation in hepatocytes of fatty liver [79–81]. In the present study, paricalcitol reduced the protein expression of NFκB in the liver of NAFLD rats suggesting that the beneficial role in reducing the NFkB mediated inflammation. Additionally, in our study, an increased NFκB acetylation has been observed along with increased proinflammatory cytokines like IL-1ß and TNFα in the liver of NAFLD rats. TNFα is a pleiotropic cytokine that regulates pathways involved in the regulation of inflammation, cell metabolism, and tissue homeostasis [82]. IL-1ß is a pro-inflammatory cytokine that can disrupt lipid signaling pathways in lipid metabolism [83]. Besides that, these both genes are responsible for causing liver fibrosis, steatosis, and inflammation [84,85]. It is known that the site-specific acetylation of NFκB (K310) increases its binding to the DNA promoter regions and enhances the expression of several proinflammatory cytokines [86,87]. Our previous *in-vitro* work showed that loss of function by knocking down of SIRT1 using siRNA or gain of function by activating the SIRT1 altered the acetylation status of NFκB in cardiomyoblast [88]. Similarly, our previous study on SIRT3 knockdown resulted in acetylation of various mitochondrial proteins [89,90]. Besides this, an *in-vivo* studies showed that SIRT1 deletion increases NFκB acetylation and induces NFκB nuclear translocation in HFD-fed mice [91]. As a primary regulator in the inflammatory response, NFκB can be targeted to reduce inflammation in NAFLD. [92]. Our results suggested that the paricalcitol is beneficial in reducing the NFκB acetylation thereby reducing the proinflammatory gene expression in NAFLD rat liver. Similar to our study, a previous study showed that paricalcitol treatment reduced hepatic ischemia/reperfusion injury in rats by down-regulating the TLR4 signaling pathway [28]. Further, it is well known that TLR 4 activation is involved in NFκB mediated inflammatory responses in the liver [93]. Therefore, the decreased inflammatory response of paricalcitol showed in the present study is due to a decrease in both NFκB and its acetylation, thereby inhibiting the expression of inflammatory genes. Additionally, a recent study showed that the inflammatory process in NAFLD is also mediated by an elevated myeloperoxidase (MPO) by Kuffer cells which leads to an increased CXC chemokine and neutrophile infiltration, and causes MPO-mediated liver damage [94]. In the present study, we observed an elevated MPO in the liver was effectively reduced by paricalcitol. Further, increased hepatic ballooning and inflammation in the liver of NAFLD rats were also attenuated by paricalcitol. Together the study proved the anti-inflammatory properties of paricalcitol in NAFLD.

Further, in our study, paricalcitol reduced the ROS level and enhanced the antioxidant levels in the rat liver of NAFLD suggesting that the paricalcitol not only reduces oxidative stress, it also maintains an optimum level of the ROS in the liver which is essential for cellular signaling processes. It is well known that an increased ROS may lead to liver damage in NAFLD by triggering the lipid peroxidation followed by activation of the inflammatory response [95,96]. Previously, it was proved that increased lipid peroxidation, an indicator of oxidative stress, is associated with NAFLD progression [97]. This study showed that paricalcitol reduced the lipid peroxidation of the liver. The present study also evaluated FOXO3a, one important transcription factor that activates several antioxidant genes and is modulated by both SIRT1 and SIRT3 for its regulation [98,99]. In current research, we observed an elevated FOXO3a expression in NAFLD rat liver. In support to present results, a previous study also showed an increased FOXO3a expression is associated with fatty liver disease [100]. In contrast, another study proved that deficiency of FOXO3a has increased the inflammation and fibrosis in high fat diet fed rats [101]. Therefore, the above two literatures together make it difficult to draw the exact FOXO3a role in NAFLD. However, it is known that FOXO3a transcriptionally binds to promotors of antioxidant genes such as mitochondrial manganese superoxide dismutase (MnSOD) and catalase and increases their expression [102,103]. Hence, observing the post transcriptional regulation of FOXO3a in NAFLD is important. One of the PTMs of FOXO3a is acetylation where acetylated FOXO3a was known to be highly instable and inactive compared to deacetylated FOXO3a [103,104]. Overexpression studies showed that SIRT3-mediated deacetylation of FOXO3 at Lys271 and Lys290 reduces FOXO3 phosphorylation, ubiquitination and degradation, thereby stabilizing FOXO3 in cells [104]. Further, another study showed that the knockdown of FOXO3a prevented the activation of the antioxidant gene even though the SIRT1 is over-expressed [98]. Both of the studies suggest that the SIRT1/3 expression enhances the transcriptional activation of FOXO3a, thereby enhancing antioxidant genes. In contrast, the knockdown of SIRT3 demonstrated an increase in FOXO3a acetylation and an increase in ROS levels in hypoxia conditions. Further, they found that dominant-negative SIRT3 decreased the level of MnSOD, Prx3, Prx5 and Trx2. [104]. In the present study, the increased expression of acetylated FOXO3a along with decreased MnSOD, catalase and GSH levels suggests that the acetylated FOXO3a is transcriptionally inactive towards antioxidant gene expression in NAFLD rats. Although there was an increase of FOXO3a protein in our study, its transcriptional activity might have been reduced due to the acetylation of this protein. Previously, it was reported that deacetylation of FOXO3a by resveratrol effectively reduces the oxidative stress by enhancing the antioxidant genes [105]. Another study showed that the SIRT1 mediated deacetylation of FOXO3a might be protective against alcoholic fatty liver disease [106]. In the current research, the paricalcitol shows its effect by

SIRT1 mediated deacetylation of FOXO3a and thereby enhancing the antioxidant gene expression, its activity and its level. Our data as well as previous studies suggesting that the paricalcitol is effectively reversed the oxidative stress condition in NAFLD by transcriptional regulation of FOXO3a by SIRT1/SIRT3.

Overall, we have established the role of paricalcitol, an FDA-approved drug for renal failure, in NAFLD and looked at the molecular changes focusing on the acetylation of two important proteins especially NFκB and FOXO3a that regulate inflammation and oxidative stress, respectively. The present study has shown the potential to repurpose the drug paricalcitol, well-known for its use in renal failure, in NAFLD.

5. Conclusion

Increased acetylation of total proteins along with downregulation of the SIRT1 and SIRT3 were observed in the liver of NAFLD rats. The decreased expression of SIRTs further enhances the acetylation of two main transcription factors namely FOXO3a and NFκB. Increased acetylation of the above two transcription factors resulted in the activation of hepatic inflammatory and oxidative stress. We have explored the role of paricalcitol, well-known for its use in renal failure, in NAFLD. Paricalcitol treatment has reduced the acetylation of protein by enhancing the SIRT1 and SIRT3 expression. Moreover, the increased oxidative stress and inflammation in NAFLD were attenuated by paricalcitol administration in rats. Further, the increase in hepatic fat accumulation, ballooning, and inflammation was reversed by the paricalcitol treatment. In short, we have shown the possibility of repurposing paricalcitol, an FDA-approved drug for renal failure, in NAFLD and established its efficacy in a rat model of NAFLD by looking at its ability to reverse protein acetylation, especially NFκB and FOXO3a.

Ethical approval

We received approval of Institutional Animal Ethical Committee (IAEC) from the National Institute of Pharmaceutical Education and Research, Guwahati, to conduct the animal experiment (NIPER/BT/2020/01).

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Authors' contributions

Navya Malladi: Methodology, data curation, formal analysis, writing-original draft, validation. **Devidas:** Methodology, formal analysis., **Balaji Sanjay Somwanshi:** Methodology, data curation. **Vikas Tiwari:** Methodology, data curation. **Kajal Deshmukh:** Methodology. **Jagdish Kumar Balani:** Methodology, data curation. **Samhita Chakraborty:** Methodology, data curation. **Md Jahangir Alam:** Validation, investigation. **Sanjay Kumar Banerjee:** Conceptualization, methodology, writing-review and editing of the original manuscript, validation and supervision.

Declaration of competing interest

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

Data availability

The data of this study will be available with the corresponding author and will be provided upon request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellsig.2024.111299.

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Research Article

Understanding the Activation of Platelets in Diabetes and Its Modulation by Allyl Methyl Sulfide, an Active Metabolite of Garlic

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Background. Diabetes mellitus (DM) is a chronic metabolic disorder associated with higher risk of having cardiovascular disease. Platelets play a promising role in the pathogenesis of cardiovascular complications in diabetes. Since last several decades, garlic and its bioactive components are extensively studied in diabetes and its complications. Our aim was to explore the antiplatelet property of allyl methyl sulfide (AMS) focusing on ameliorating platelet activation in diabetes. *Method.* We used streptozotocin- (STZ-) induced diabetic rats as model for type 1 diabetes. We have evaluated the effect of allyl methyl sulfide on platelet activation by administrating AMS to diabetic rats for 10 weeks. Flow cytometry-based analysis was used to evaluate the platelet activation, platelet aggregation, platelet macrophage interaction, and endogenous ROS generation in the platelets obtained from control, diabetes, and AMS- and aspirin-treated diabetic rats. *Results.* AMS treatment for 10 weeks effectively reduced the blood glucose levels in diabetic rats. Three weeks of AMS (50 mg/kg/day) treatment did not reduce the activation of platelets but a significant ($p < 0.05$) decrease was observed after 10 weeks of treatment. Oral administration of AMS significantly ($p < 0.05$) reduced the baseline and also reduced ADP-induced aggregation of platelets after 3 and 10 weeks of treatment. Furthermore, 10 weeks of AMS treatment in diabetic rats attenuated the endogenous ROS content ($p < 0.05$) of platelets and platelet macrophage interactions. The inhibition of platelet activation in diabetic rats after AMS treatment was comparable with aspirin treatment (30 mg/kg/day). *Conclusion.* We observed an inhibitory effect of allyl methyl sulfide on platelet aggregation, platelet activation, platelet macrophage interaction, and increased ROS levels in type 1 diabetes. Our data suggests that AMS can be useful to control cardiovascular complication in diabetes via inhibition of platelet activation.

1. Introduction

Diabetes mellitus (DM) is one of the chronic and complex disorders among all metabolic diseases. "Diabetes mellitus" is characterized by persistent hyperglycemia with disturbances of carbohydrate, fat, and protein metabolism resulting from defects in insulin secretion, insulin action, or both. Changes in lifestyle and rapid urbanization have increased the incidence of diabetes and its prevalence day by day. According to the International Diabetes Federation (IDF), 463 million adults (20–79-year age) are diabetic in the year 2019 with a record of 4.2 million deaths. Further, IDF interprets that there could be still more diabetic patients by the years 2030 and 2045 with an estimation of 578.4 and 700.2 million cases, respectively [1].

Type 1 diabetes is a condition that resulted from insufficient production of insulin by the pancreas while type 2 is due to improper utilization of produced insulin and ultimately leads to higher glucose levels in the circulatory system [2]. Both type 1 and type 2 diabetes require careful monitoring and control of blood glucose levels, if not this uncontrolled condition over time may root to several complications including cardiovascular disease (CVD). Cardiovascular complications in diabetes occurring as the disease progresses and leads to premature mortality [3–5]. IDF has suggested that diabetic patients are having 2-3 times more

probability of getting CVDs than nondiabetic patients [6]. The central mechanism for these cardiovascular complications in diabetes includes an imbalance in the systems which maintain the homeostasis of blood coagulation and fibrinolysis [7]. This imbalance results in diabetic thrombocytopathy [8], a condition that majorly affects platelet function and ultimately results in heart attacks or stroke in diabetes [9–12]. Scientific research has shown that antiplatelet therapy can reduce cardiovascular complications in diabetes and premature death [13, 14].

Despite having many drugs and therapies for the management of diabetes, it is still under the category of life-threatening diseases because of its complications. Reducing these cardiovascular complications in diabetes has become more challenging than controlling the disease itself. This has made an immense impact on scientific researchers for exploring new strategies for diabetic treatment. Dietary therapy in diabetes is one among them showing a tremendous effect on preventing as well as controlling diabetes [15, 16].

Among large numbers of nutritional diets, garlic has been showing a very promising effect on diabetic as well as diabetic complications [17–21]. An antiplatelet property has been observed in raw garlic, preventing cardiovascular complications in diabetes [22–24]. Scientific literature strongly supports that garlic is showing these beneficial effects mainly due to its sulfur-containing compounds [25–27]. Allyl methyl sulfide (AMS), an active metabolite observed inside the body after oral administration of raw garlic, has been shown to effect diabetes by reducing glucose levels, increasing insulin levels, and reducing hepatic oxidative stress caused by glucotoxicity in diabetes [28, 29]. However, the effect of AMS on diabetic complications in thrombosis especially on platelet activation and aggregation is yet to be reported.

Therefore, in this present study, we aim to understand the various altered parameters of platelets isolated from diabetic rats and the effect of garlic metabolite, allyl methyl sulfide, on altered parameters of diabetic platelet.

2. Materials and Methods

2.1. Reagents. Allyl methyl sulfide (cat no. A34201-25G) and phorbol 12-myristate 13-acetate (PMA) (cat no. P8139-1MG) were obtained from Sigma (St. Louis, Missouri, USA). Flow cytometry antibodies such as CD61 FITC antibody (cat no. 104305) and APC anti-mouse/rat CD62P (P-selectin) antibody (cat no. 148303) are from Bio Legend (San Diego, California, USA), and CD14 PE antibody (cat.no: 561707) is from BD Bioscience (Franklin Lakes, NJ, USA). Adenosine diphosphate (ADP) was obtained from Hi-Media (cat no. RM437-1G). H_2DCFDA dye (cat no. C6827) was from Invitrogen. Cell culture reagents such as RPMI-1640 (cat no. 31800-014), antibiotic-antimycotic (100X) (cat no. 15240062), and fetal bovine serum (cat no.10270106) are from Gibco.

2.2. Animals and Study Design. Wistar rats 200-250 g were used to develop diabetes and evaluate the effect of AMS on altered platelet function in diabetes. Animals were procured from Jeeva Life Sciences (Hyderabad, India). The study was approved by the Institutional Animal Ethics Committee (IAEC) of National Institute of Pharmaceutical Education and Research (NIPER), Guwahati, India (NIPER/BT/2020/37). The animals were housed in individually ventilated cages (IVC) at an animal house facility of NIPER, Guwahati, under standard conditions (temperature 23 ± 1 °C, 50 ± 15% relative humidity, and 12 h light/dark cycle). Wistar rats were allowed free access to food and water ad libitum during the study. Post seven days of acclimatization, animals were randomly allocated into four groups ($n = 6$): group 1: control; group 2: diabetes (STZ 35 mg/kg); group 3: diabetes+AMS (50 mg/kg); and group 4: diabetes+aspirin (30 mg/kg). All these rats were maintained for 10 weeks. Every week, the body weight of all animals was recorded to understand the body weight gain or loss during the experimental period and glucose levels were monitored by using a glucometer (Accu Chek Active, Roche). The doses of AMS and aspirin were chosen from our previous work and scientific literature [30, 31].

2.3. Induction of Diabetes. Diabetes was chemically induced in Wistar rats (weight 200-250 g) with streptozotocin. After 6 hours of fasting, adult Wistar rats were administered with a single intraperitoneal (I.P) injection of a freshly prepared solution of streptozotocin (STZ) in ice-cold citrate buffer (0.01 M, pH 4.5) at a dose of 35 mg/kg body weight. Animals were then monitored for the next seven days for their blood glucose levels by using a glucometer (Accu Chek Active, Roche). The induction of diabetes was confirmed by monitoring fasting blood glucose. The rats with >250 mg/dl of blood glucose levels were considered diabetic.

2.4. Oral Dosing. Allyl methyl sulfide was administered orally at a dose of 50 mg/kg in corn seed oil for 10 weeks in diabetes Wister rats and referred to as the 'diabetes +AMS' group. Aspirin was given at a dose of 30 mg/kg in 0.5% carboxy methyl cellulose (CMC) orally to another group of diabetes Wistar rats and referred to as the 'diabetes+aspirin' group. The control group was referred to the rats feed with corn seed oil.

2.5. Cell Culture. Human macrophages (THP-1 cells; an acute monocytic leukemia cell line) were a kind gift from Translational Health Science and Technology Institute (THSTI, Faridabad, India). The cells were cultured in the RPMI 1640 medium enriched with fetal bovine serum (10% *v*/*v*) and antimicrobial agents (antibiotic and antimycotic) (100 U/ml) and cultured under standard conditions that are 37°C and 5% CO_2 incubator (eppendorf CellXpert C170).

2.6. Blood Collection and Isolation of Platelets. The platelets were isolated from blood by dual centrifugation. It has been carried out at room temperature. Preventive measures have been taken to avoid platelet activation during the process. Initially, the animals were anesthetized with the help of isoflurane. Through retroorbital plexus using capillary tube, blood was collected into a tube containing 3.8% sodium citrate (9 : 1 ratio) and centrifuged at 500 rpm for 15 minutes at

20°C temperature. The centrifugation results in isolation of platelet-rich plasma (PRP) as the upper layer in a tube. Almost 3/4th of this layer was taken into a fresh tube using a wide-bore pipette tip. Further, platelets were pelleted by centrifuging the PRP at 400 g for 10 minutes at 20°C. The platelets pellet was washed, resuspended in HEPES-Tyrode buffer.

2.7. Measuring the Number of Activated Platelets by Flow Cytometer. The *in vivo* activated status of isolated platelets was measured by flow cytometry using fluorochrome-tagged antibodies. All these works were carried out within 2 hours of blood collection and took all measures to avoid platelet activation during handling of the sample. 7 μl of PRP was taken in a tube and diluted by adding 50 μl of HEPES-Tyrode's buffer having 1% BSA. Samples were added with antibody mix having FITC anti-mouse/rat CD61 antibody (platelet surface marker) and APC anti-mouse/rat CD62P antibody (platelet activation marker) diluted with HEPES-Tyrode's buffer (with 1% BSA). Stained platelets were incubated in dark for 30 minutes at room temperature. Samples were immediately analyzed by using Attune™ NxT Flow Cytometer, where analysis was done by taking 50,000 events for each sample. The compensation was done using individual antibody-stained cells and unstained cells in order to avoid spillover from one channel to other. Platelets were gated on forward light scatter (FSC) vs. side light scatter (SSC) plot, and the percentage of CD62P positive cells is enumerated among platelets positive for CD61 cells which give % activated platelets.

2.8. Measuring Platelet Aggregation in Presence of ADP, a Platelet Agonist, by Flow cytometry. With a few modifications to previously mentioned procedure, the hypersensitivity of platelets in presence of platelet-aggregating agents (ADP) has been observed by measuring the percentage aggregation of platelets by flow cytometry [32]. 50 μl of freshly isolated platelet-rich plasma (PRP) was resuspended in 450 μl of HEPES-Tyrode buffer. Platelets were activated by adding 20 μM ADP as a final concentration and incubated for 10 minutes at 37°C under shaking conditions at 1000 rpm using a thermoshaker. Diluted PRP without agonist was used as a negative control. Cells were analyzed by flow cytometry for the presence of platelet aggregation. Platelets were differentiated from platelet aggregated by gating cells on forward light scatter (FSC) versus side light scatter (SSC) plot where the platelet aggregates are having increased size and density.

2.9. Analysis of Platelet and Macrophage Interaction by flow cytometry. The heterogeneous interactions of platelets with macrophage were analyzed by flow cytometry method using phorbol 12-myristate 13-acetate- (PMA-) differentiated THP-1 macrophages [33, 34].

For monocyte differentiation to macrophage, THP-1 monocytes (1.5×10^6 cells) were seeded in 6-well plate having 1 ml of Roswell Park Memorial Institute medium (RPMI-1640) which was supplemented with 100 μg/ml streptomycin, 100 U/ml penicillin, and 10% fetal bovine serum. The monocytes were differentiated to macrophages by adding 50 ng phorbol 12-myristate 13-acetate (PMA) and incubated for 48 hours in an incubator maintaining 5% CO_2 at 37°C under humidified conditions. After 48 hours of incubation, media was changed to fresh RPMI-1640 complete media without the addition of PMA and incubated for 48 hours in an incubator.

For macrophage and platelet coculture study, isolated PRP of 7 μl was added in a culture plate having macrophages derived from THP1 and incubated for 30 minutes in a CO_2 incubator. After successive coculturing of platelets, each well has been washed with PBS to remove floating platelets. Cells were trypsinized by adding 0.25% trypsin EDTA and incubated in a CO_2 incubator for 2 minutes. The detached cells were collected in an individual tube and centrifuged at 400 g for 10 minutes at 20°C. The supernatant was discarded; the pellet was washed with PBS and fixed by incubating with 4% paraformaldehyde for 10 minutes. The cells further were washed and resuspended in PBS for labelling with fluorescently conjugated antibodies of macrophage surface marker (CD14 PE) and platelet-specific marker (CD61 FITC). The cells were incubated in dark for 30 minutes at room temperature and analyzed by Attune™ NxT Flow Cytometer. Analysis was done by gating the macrophage population under FSC vs. SSC plot. To compensate for the overlapping spectra single-stained cells were used. Finally, we measured the mean fluorescence intensity of CD61-FITC in macrophage (CD14+) population inorder to quantify platelet macrophage interaction.

2.10. Measurement of Endogenous ROS in Platelets. Intracellular ROS was measured by using 2′,7′-dihydrodichlorofluorescein diacetate, H_2DCFDA, dye as per the procedures mentioned in articles [35, 36]. The freshly isolated PRP (7.0 μl) was diluted in 100 μl of HEPES-Tyrode's buffer and incubated with 10 μM of H_2DCFDA dye under dark conditions for 30 min at 37°C. Later, samples were further diluted with 200 μl HEPES-Tyrode's buffer and mixed gently with a pipette. The tubes were centrifuged at 400 g for 10 minutes at 20°C for pelleting the cells and discarding the supernatant. Further, the pellet was resuspended by adding 100 μl of HEPES-Tyrode's buffer. The unstained samples were used as a negative control. All of these samples were analyzed using Attune™ NxT Flow Cytometer by taking 10,000 events where the mean fluorescent intensity of DCFDA in platelet population was determined for each sample.

2.11. Statistical Analysis. All the statistical analysis was performed using GraphPad Prism version 8.0.2(263) (GraphPad Software, San Diego, CA, USA), and its comparisons were made utilizing ANOVA test followed by Tukey's test post hoc analysis. Results were expressed as mean ± standard error, and a p value < 0.05 was considered significant.

3. Results

3.1. Body Weight and Blood Glucose Level Changes in Rats. We monitored body weights and blood glucose levels of

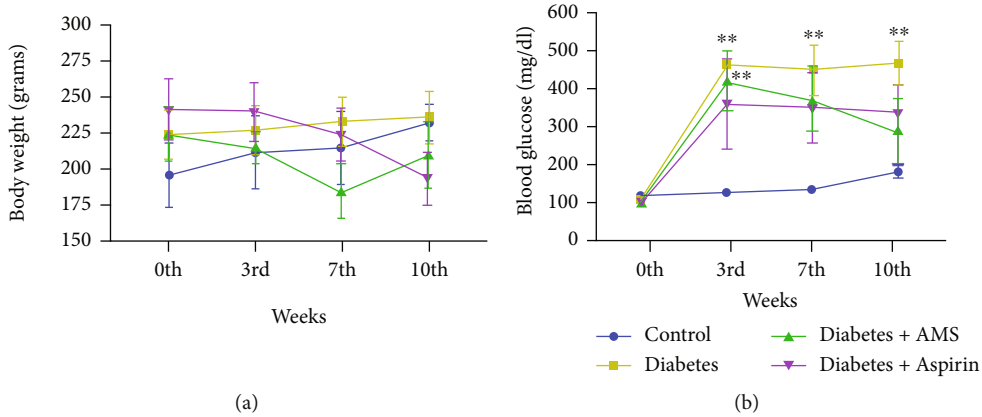

FIGURE 1: (a) Body weights and (b) blood glucose levels of the control, diabetes, AMS-treated, and aspirin-treated rats at the end of the 0^{th}, 3^{rd}, 7^{th}, and 10^{th} weeks. All values are represented as mean ± SEM ($N = 4$-6). $^{**}p < 0.01$ vs. the control.

experimental rats until 10 weeks. The mean values of body weight in grams of all study groups were compared in 0^{th}, 3^{rd}, 7^{th}, and 10^{th} week and plotted as a graph. As shown in Figure 1(a), we did not observe any significant body weight changes among all four groups. The blood glucose levels of the diabetes group significantly increased at all (3^{rd}, 7^{th}, 10^{th} weeks) the time points when compared to the control. However, AMS treatment decreased the glucose levels at 7 and 10 weeks when compared to the 0^{th} and 3^{rd} week of treatment (Figure 1(b)).

3.2. Effect of AMS on Platelet Activation in Diabetes. The *in vivo* activated status of platelets in the control, diabetes, and treatment groups after 3^{rd} and 10^{th} weeks of study duration has been analyzed by flow cytometry. The graph represents percent CD61 positive on the *X*-axis and CD62P-positive platelets on the *Y*-axis (Figure 2(a)). The dual positive cells are considered activated platelets, and percent of the activated platelets were represented as bar graphs. At the end of 3^{rd} week, the percentage of activated platelets was significantly increased in the diabetes group (~32.4%) when compared to the control (~19.11%). We did not observe any significant changes in the AMS and aspirin-treated groups (Figure 2(b)). Similarly, at the end of 10^{th} week, the percentage of activated platelets was increased significantly in the diabetes group (~47.19%) when compared to the control group (~35.24%). Moreover, both aspirin and AMS treatments significantly decreased this activation of platelets (~30.15 and 25.22%, respectively) induced in diabetes (Figure 2(c)).

3.3. Effect of AMS on ADP-Induced Platelet Aggregation. To assess the effect of AMS on platelet aggregation, flow cytometry analysis was performed on platelets derived from all the experiment groups in the absence or presence of ADP at the 3^{rd} week and 10^{th} week. Figure 3(a) represents FSC vs. SSC density plots showing the 3^{rd} week platelet aggregation with and without ADP where aggregates are gated as R2. In the absence of ADP, we did not observe any significant change in the percentage of platelet aggregation in the diabetes group compared to the control. However, a significant decrease (3-fold) in percent aggregation was observed in the AMS- and aspirin-treated diabetes groups compared to diabetes (Figures 3(a) and 3(b)). Similarly, as shown in Figures 3(a) and 3(c), we observed a significant induction (~1.6-fold) in platelet aggregation by ADP in the diabetes group when compared to the control. With AMS and aspirin treatment, we observed a significant decrease (~3.8- and 4.2-fold, respectively) in platelet aggregation when compared to the diabetes group.

Similar flow cytometry analysis in the presence and absence of ADP was performed at the end of the 10^{th} week, and results were represented as FSC vs. SSC density plots (Figure 4(a)). In the absence of ADP, we observed that the percentage of platelet aggregation increased 1.2-fold in diabetic rats when compared to the control (Figures 4(a) and 4(b)). A significant ($p < 0.05$) decrease in the percentage of platelet aggregation was observed after AMS and aspirin treatment (~2.3- and 3.3-fold, respectively) when compared to diabetes. Similarly, ADP-induced aggregation was increased by 1.1-fold in diabetes while it was decreased by 1.1- and 1.5-fold in the AMS and aspirin treatment groups, respectively (Figures 4(a) and 4(c)).

3.4. Effect of AMS on Diabetes-Induced Reactive Oxygen Species Generation in Platelets. After platelet activation and aggregation study, we measured the reactive oxygen species (ROS) levels in the platelets isolated from the control, diabetic, and treated diabetic rats at 10^{th} week of the study duration. ROS levels in platelets were measured by using 2′,7′-dihydrodichlorofluorescein diacetate (H_2DCFDA) dye and analyzed it by flow cytometry. H_2DCFDA dye is a direct measure of amount of ROS present in cells, and the results are expressed as the mean fluorescent intensity (MFI) of DCFDA (Figure 5). Platelet ROS levels was significantly ($p < 0.05$) increased in the diabetic group when compared to the control. However, the ROS levels was decreased significantly ($p < 0.05$) in platelets isolated from the AMS-treated diabetic rats when compared to diabetes rats. We did not observe any significant change in ROS levels in platelets isolated from the aspirin-treated diabetic rats.

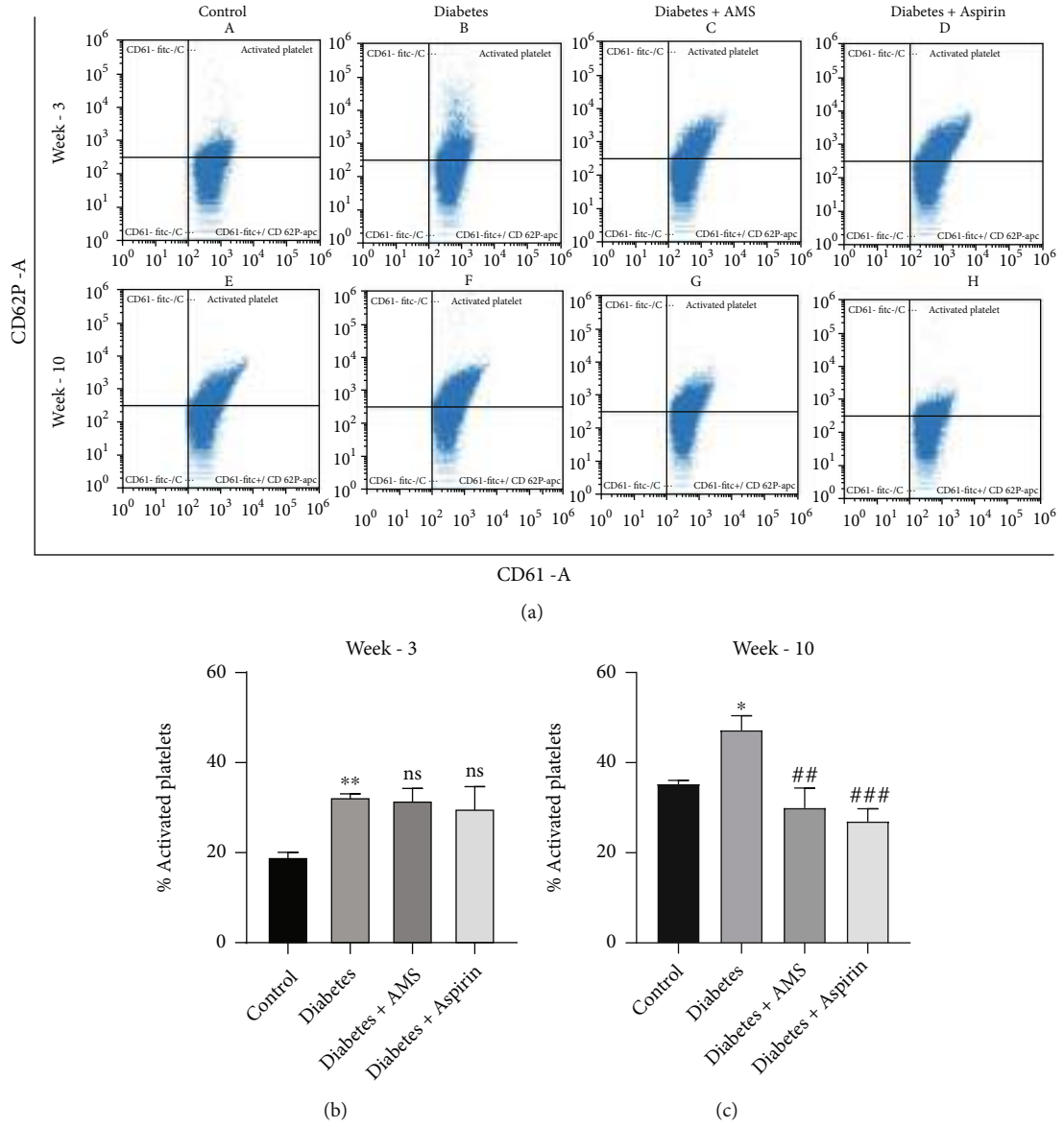

Figure 2: Flow cytometry analysis of platelet activation showing percentage dual positive cells (CD61+, CD62P+). (a) Representation of the scattered plots of percentage platelet activation in the control (A, E), diabetes (B, F), AMS-treated diabetic (C, G), and aspirin-treated diabetic (D, H) groups after 3 and 10 weeks of study. Bar graph showing percentage platelet activation at the end of the 3rd week (b) and 10th week (c). All values are represented as mean ± SEM ($N = 4$-5). $^{*}p < 0.05$ vs. the control; $^{**}p < 0.01$ vs. the control; $^{\#\#}p < 0.01$ vs. diabetes; and $^{\#\#\#}p < 0.001$ vs diabetes; ns: nonsignificant.

3.5. Effect of AMS on Diabetes-Induced Macrophage and Platelet Interaction. Platelet macrophage interaction has been considered an important phenomenon for platelet activation in diabetes [37, 38]. To look at this interaction, macrophage and platelet were incubated together and FACS analysis was performed. THP 1, a well-known monocyte cell, were differentiated with phorbol 12-myristate 13-acetate (PMA) and later cocultured with platelets collected from the control, diabetes, and AMS-/aspirin-treated rats at 10^{th} week of study duration. The results are shown in mean fluorescent intensity of CD61-FITC (marker of platelets) among CD14-positive THP1 macrophages. The data was represented as a bar graph (Figure 6). The increased mean fluorescent intensity was considered an aggregate cell of macrophages and platelets. In our data, the significant ($p < 0.05$) increase (~2.4-fold) in mean florescent intensity of CD61-FITC was observed in macrophages cocultured with diabetes platelets when compared to the control. Although we observed a decrease in macrophage platelet interactions in the AMS and aspirin treatment groups (~1.2- and 1.5-fold) when compared to the diabetic group, these changes were not significant.

4. Discussion

Diabetes mellitus is not a single clinical entity but a spectrum of diseases with various diabetic complications [39–41]. Several reports have shown a strong relation

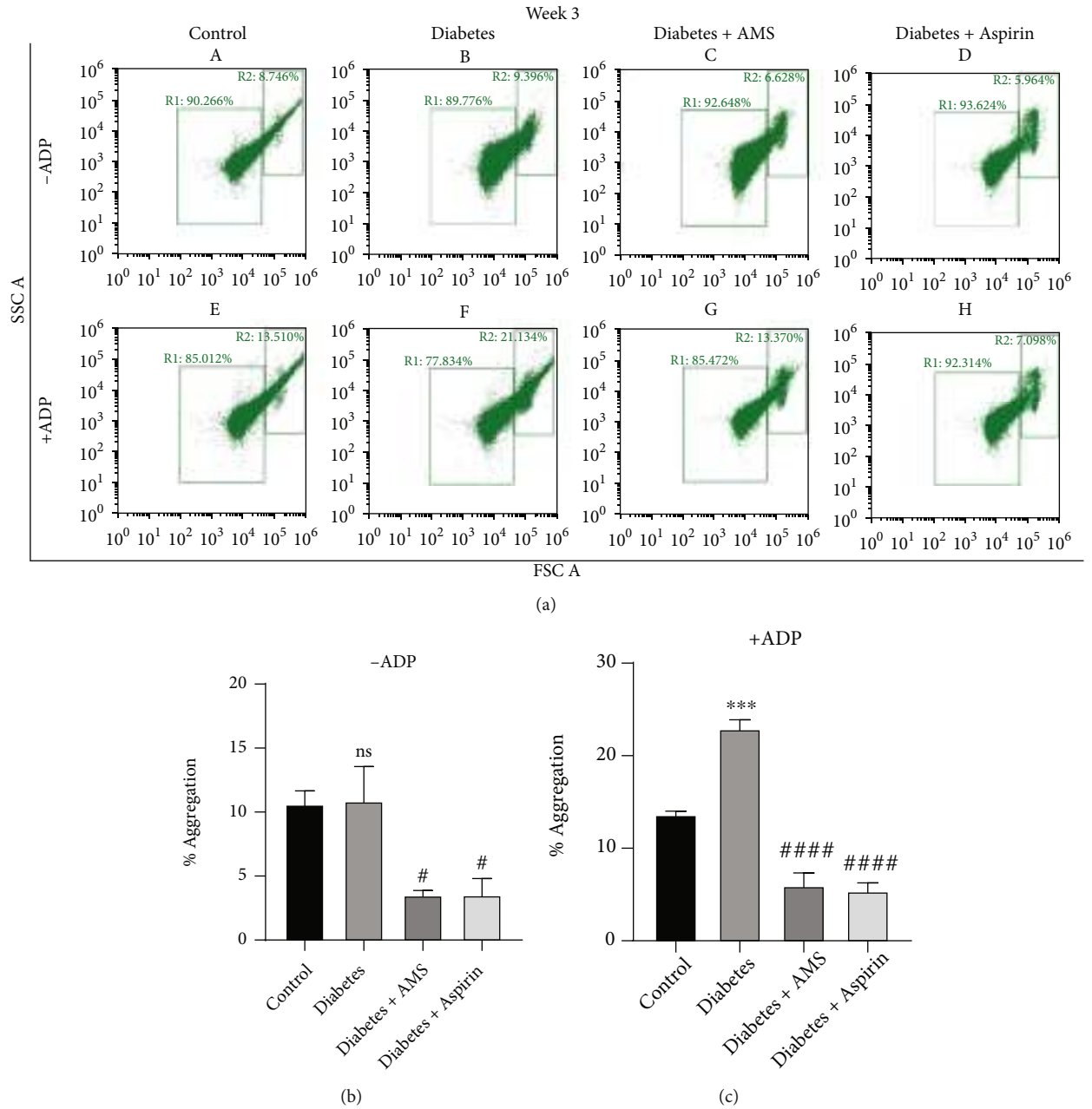

Figure 3: Flow cytometry analysis of platelet aggregation after 3-week treatment. (a) Scatter plot of forward vs. side scatter of platelets from the control (A, E), diabetic (B, F), AMS-treated diabetic (C, G), and aspirin-treated diabetic (D, H) groups, representing percentage of platelet aggregation in the absence and presence of ADP. Bar graph represents percentage of platelet aggregation observed in absence (b) and presence (c) of ADP. All values are represented as mean ± SEM ($N = 3$). $^{***}p < 0.001$ vs. the control; $^{\#}p < 0.05$, $^{\#\#\#\#}p < 0.0001$ vs. diabetes; ns: nonsignificant.

between diabetes mellitus and premature cardiovascular events [4, 5]. The underlining mechanisms of cardiovascular complication in diabetes include several physiological and pathological changes in the heart, blood vessels, blood cells, and kidneys [42]. Previous scientific literature identified numerous risk factors i.e., hyperglycemia [43–45], dyslipidemia [46, 47], inflammation [48, 49], endothelial dysfunction, and oxidative stress [50, 51], which together can induce several complexities including cardiovascular complication in diabetes. Studies have also identified that alteration of normal platelet function as one of the major risk factors of diabetic complication and characterized by increased thromboxane synthesis [52], reduced membrane fluidity [53], and increased expression of activation-dependent adhesion molecules (e.g., GpIIb-IIIa and P-selectin) [54]. All of these changes make platelets more reactive and create a prothrombic environment in a diabetes patient [55, 56]. Nevertheless, the studies focusing on platelet dysfunction in type 1 diabetes (T1DM) and its modulation by pharmacological agents are limited. Recent literature says that among all the popular natural remedies, organosulfur compounds from garlic have shown a potential antidiabetic

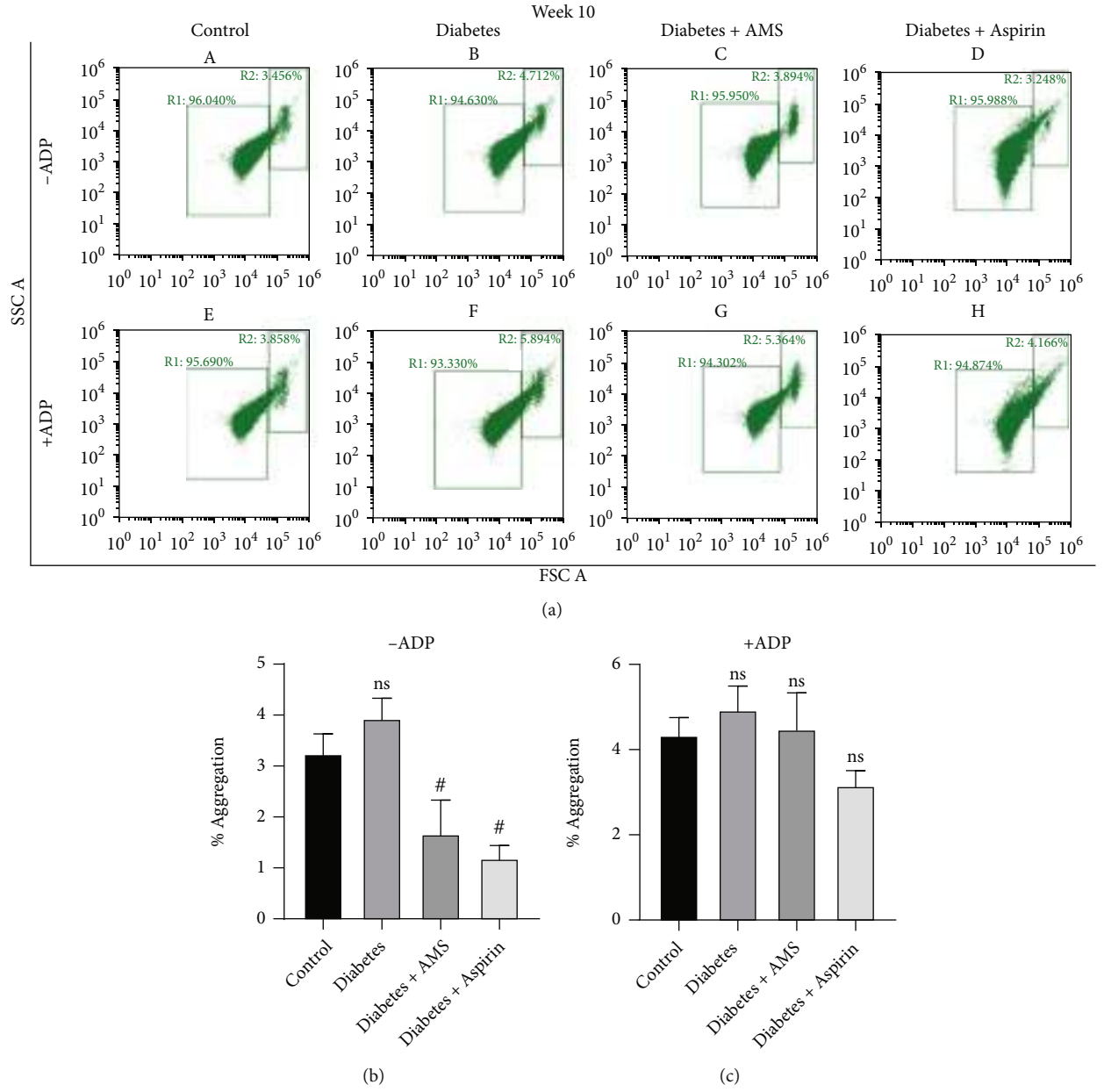

Figure 4: Flow cytometry analysis of platelet aggregation after 10-week treatment. (a) Scatter plot of forward vs. side scatter of platelets from the control (A, E), diabetic (B, F), AMS-treated diabetic (C, G), and aspirin-treated diabetic (D, H) groups, representing percentage of platelet aggregation in the absence and presence of ADP. Bar graph represents percentage of platelet aggregation observed in absence (b) and presence (c) of ADP. All values are represented as mean ± SEM ($N = 3$). $^{\#}p < 0.05$ vs. diabetes; ns: nonsignificant.

and as well as antithrombotic effect in diabetic individual [17, 18, 22, 23, 57]. Previous research work also supported the role of garlic to attenuate cardiovascular complications in diabetes [18]. Allyl methyl sulfide (AMS) is one of the important sulfur compounds obtained from garlic, and studies showed that AMS is a major metabolite that is detected in the human breath and plasma [28]. Our previous study on AMS suggested that chronic administration of AMS is safe in control rats, where the body weight, food, and water intake along with the histopathology of major organs and serum biomarkers remained normal [30]. Further, the same study showed a beneficial effect of AMS on isoproterenol-induced cardiac fibrosis and dysregulated extracellular matrix (ECM) deposition in the myocardium [30]. Also, our recent finding showed a cardioprotective effect of AMS in pressure overload-induced cardiac hypertrophy and heart failure by ameliorating endogenous antioxidants and mitochondrial function [31]. Further research identified the therapeutic role of AMS on type 1 diabetes where different parameters like blood glucose, HbA1c, oxidative stress, inflammation, and insulinotropic activity were normalised after AMS treatment. All the above parameters remained normal in control rats after AMS administration [29, 58]. However, there is no study to find the effect of AMS on platelet activation. Therefore, in the current study, we determined the major platelet alterations in STZ-induced diabetic

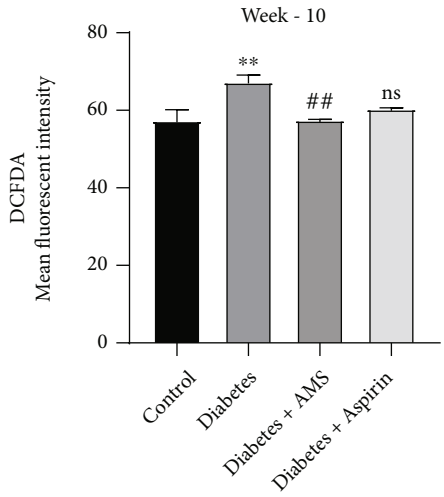

Figure 5: Comparison of ROS levels among the control, diabetes, and treatment groups. All values are represented as mean ± SEM ($N = 3$-4). $^{**}p < 0.01$ vs. the control; $^{\#\#}p < 0.01$ vs. diabetes; ns: nonsignificant.

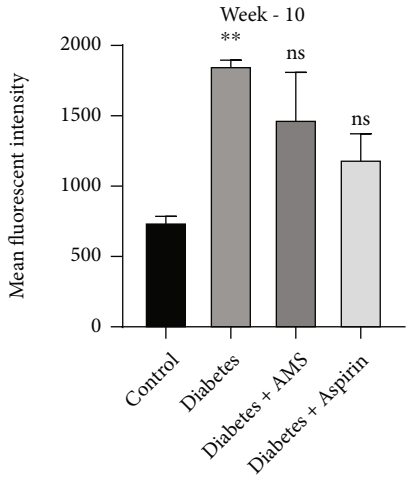

Figure 6: Macrophage platelet interaction in the control, diabetes, and treatment groups. All values are represented as mean ± SEM ($N = 3$-5). $^{**}p < 0.01$ vs. the control; ns: nonsignificant.

rats and explored the antithrombotic effect of AMS, a bioactive derivative of garlic, mainly focusing on platelet activation.

In diabetes, controlling hyperglycemia is the primary goal to reduce complications of diabetes. Poor glycemic control affects platelet activation and vascular dysfunction in diabetes. A previous study reported that a low dose of aspirin reduces blood glucose levels in diabetic rats [59]. In our present study, AMS administration to rats reduced blood glucose levels. The result showed that AMS is more effective to reduce blood glucose level in diabetic rats than aspirin. Our data is supporting the recent studies on AMS, where AMS administered to STZ-induced diabetic rats showed a significant decrease in blood glucose level [58, 60].

Hyperglycemia along with other factors helps to aggregate the platelets in the presence of a small stimulus. Such platelets are referred to as hyperactivated platelets [61]. This hyperactivated platelet has distinct morphology and expresses P-selectin (CD69P) and GP IIb/IIIa receptor on the surface [62–64]. Supporting the previous data, our present study also observed an increase in platelet activation (CD62P levels) in type 1 diabetic rats when compared to nondiabetic rats. This activation of platelets was higher at a later stage (10 weeks) when compared to the initial stage (3 weeks) of diabetes. Moreover, we also observed the effect of AMS on platelet activation. Interestingly, we found a little reduction in platelet activation after initial treatment for 3 weeks with AMS but a significant decrease was found after 10 weeks of treatment. Here, our data suggest that long-term administration of AMS has a superior effect on reduction of platelet activation in diabetes.

Increased platelet activation results in increased platelet aggregation and has been detected in diabetes mellitus [37, 38]. This increased platelet aggregation is a result of increased systemic production of TxA 2 by platelet [65], increased sensitivity of platelets agonist like ADP [66], and impaired production of platelet aggregation inhibitors PGI 2 and NO [67, 68]. The previous finding has shown that the platelet of the diabetes patient was found to be 1.6-fold more sensitive to the ADP-induced aggregation than that of nondiabetes persons [69]. In the present study, we observed the aggregation of platelets (basal level) and its sensitivity to ADP stimuli in both the early (3^{rd} week) and late (10^{th} week) stages of diabetes. At early stages of the disease, there was no difference in the baseline aggregation property of platelets between the diabetes and control groups. However, in the presence of ADP, the diabetic platelets showed an increase in aggregation than the control group. The data suggests that the platelets from diabetic rats were highly sensitive and prone to thrombus formation. A decrease in baseline aggregation was observed in diabetic rats after AMS and aspirin treatment. The percentage of aggregation was also reduced when the platelets were activated by the addition of ADP in treatment groups. The study suggests that AMS has a beneficial effect in reducing the platelet sensitivity and aggregatory properties. Similar to platelet aggregation data in the 3^{rd} week, we observed a decrease in baseline platelet aggregation in the 10^{th} week after AMS and aspirin treatment. Interestingly, in the 10^{th} week of diabetes, we did not observe any significant increase in ADP-induced platelet aggregation when compared to the control. Although we cannot explain the reason for not showing the sensitivity of platelets after ADP addition at week 10, it may be due to desensitization of P2Y1 and P2Y12 ADP receptors of platelets after the long-term interaction with endogenous ADP in chronic diabetes [70]. Further, the control group at 10^{th} week showed higher basal platelet aggregation than the control group at 3^{rd} week. This can be explained by the fact that age itself may enhance the platelet aggregation in the absence of diabetes [71]. After treatment with AMS for 10 weeks, we observed a decrease in ADP induced aggregation. The data overall indicated that inhibition was less at later stages of diabetes and may be due to the alteration of platelet structure and expression of major protein levels that resist the AMS's beneficial effect.

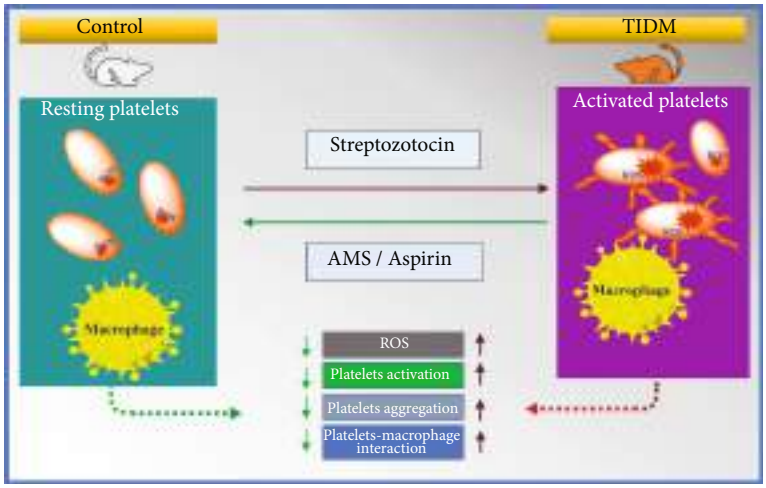

Figure 7: Attenuation of the platelet activation and platelet-macrophage interaction in type 1 diabetes by AMS. AMS: allyl methyl sulfide; ROS: reactive oxygen species; T1DM: type1 diabetes mellitus.

Next, we correlated the platelet aggregation phenotype with intracellular ROS production. Increased ROS has been observed in activated platelets and activates the PKC pathway which led to platelet hyperactivity and aggregation [72]. Supporting the previous literature, our study also found increased ROS content in diabetic platelet when compared to the control. A recent study indicates that AMS administration improved the oxidative stress in STZ-induced hyperglycemic rats [58]. Based on their observation, we evaluated the endogenous ROS generation in platelets after AMS treatment and found an inhibitory effect of AMS on ROS production in platelets.

Increased ROS production can activate the platelets and help to participate in the signaling event of atherosclerosis in diabetes by forming aggregates with monocytes through P-selectin–PSGL-1 interactions [73]. Therefore, platelets play an important role in promoting inflammation in diabetes. The inflammatory condition created by PSGL-1-mediated monocyte activation leads to the synthesis and release of various chemokines, cytokines, and reactive oxygen species. Monocyte platelet interactions also have a role in the coagulation system by a surface expression of phosphatidylserine [74]. Previous literature suggested that increased platelet-monocyte aggregates are the indicator of *in vivo* platelet activation. These aggregates are responsible for the prothrombic stage and play a major role in the development of atherosclerosis in type 1 diabetes [75, 76]. Additionally, macrophage accumulation also plays a vital role in causing diabetic complications [77]. It was reported that a similar interaction between macrophages and platelets causes releases of chemokines and phagocytosis of platelets which further involve in atherothrombosis formation [78, 79].

In the present study, we observed platelet macrophage interactions to evaluate the platelet activation status as well as proinflammatory condition in type 1 diabetes. Here, in the present study, we observed an increase in macrophage and platelet interaction at late stages of diabetes compared to control, whereas after AMS treatment, diabetic rats showed less macrophage and platelet interactions compared to diabetes. The study suggests that AMS can reduce the macrophage and platelet interaction and can further inhibit pro-inflammatory condition which leads to vascular complication in diabetes.

5. Conclusion

In this study, we found that allyl methyl sulfide inhibits platelet activation and their aggregation in type 1 diabetes. It has also shown an inhibitory effect on increased platelet-macrophage interaction in type 1 diabetes. The increased platelet activation was associated with elevated levels of ROS. Further, our results showed a decrease in ROS levels in platelets after AMS treatment. The summary of the present study has been represented in Figure 7. Overall, this study provides a piece of strong evidence that treatment with AMS could prevent the phenotypic platelet changes seen in type 1 diabetes and may act as a potential therapeutic molecule for cardiovascular complications especially thrombosis in diabetes.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Ethical Approval

Institutional Animal Ethics Committee (IAEC) Approval (NIPER/BT/2020/37) from National Institute of Pharmaceutical Education and Research (NIPER)-Guwahati to conduct the animal experiment was obtained.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Authors' Contributions

S.K.B., R.A, and N.M have designed the study. Animal handling and dosing were done by N.M. and S.K.U. Blood collection, platelet isolation, and sample preparation for flow analysis were performed by N.M., S.K.U., and V.T. Flow cytometry experiment design and analysis of platelet samples were done by both N.M and E.J. Statistical analysis and writing of the manuscript were done by S.K.B., R.A, M.J.A, and N.M.

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