Title: Repurposing of paricalcitol in Non-alcoholic fatty liver disease: By targeting the acetylation of FOXO3a and NFκB

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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

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 more than 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 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 12hrs in 0.5% FBS-containing MEM. The cells were pretreated with paricalcitol (100nM) for 12 hours in the presence of 0.5% FBS and exposed to 400µM oleic acid for a further 24 hr. The cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% triton X. Later the cells were washed and stained with Nile red (5µg/mL) and Hoechst stain (2µg/mL) for 30 minutes. 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 63X.

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 12hrs in 0.5% FBS-containing MEM. The cells were pretreated with paricalcitol (100nM) for 12 hours in the presence of 0.5% FBS and exposed to 400µM oleic acid for a further 24 hr. 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 2hrs followed by Hoechst stain (2µg/mL) for 10 minutes. 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 63X.

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: 200-250g) (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 0.08ug /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 0.08ug/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 4 % 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 (100nM) pretreatment for 12hrs on protein expression of acetylated lysin, SIRT1, and SIRT3 in the HepG2 cells treated with either oleic acid (400μM) or oleic acid (400μM) pretreated with paricalcitol at 100nM concentration for 12 hrs. After the treatment cell lysate was collected by lysing the cells with RIPA lysis buffer. The lysate was centrifuged at 14000 rpm for 15 minutes at 4ºC. 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 1mg 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 minutes 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 2,300 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 0.05M 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-second gap for 1.5 minutes. 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-diphenylpicrylhydrazyl (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 minutes and absorbance was taken at 517nm 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. Measuring the number of activated platelets by using antibodies by flow cytometer

Flowcytometry is performed to detect platelet activation, immunophenotyping and platelet immune cell interaction. Blood was collected in sodium citrate tubes. Isolated the PRP by centrifuging at 500rpm for 10 minutes at 18-22 °C. All these works were carried out within 2 hours of blood collection to avoid platelet activation during handling of sample. 7 µl of PRP is incubated with platelet surface markers such as FITC anti-mouse/rat CD61 Antibody (Bio legend, cat.no: 104305) and also with platelet activation marker APC anti-mouse/rat CD62P (P-selectin) antibody (bio legend, cat.no: 148303) for 20 minutes at room temperature. Wash with HEPES Tyrode buffer saline and analyze in flow-cytometer.

2.17. Platelet macrophage interaction measured by confocal

THP 1 is a human monocytic cell line. We seeded the THP-1 monocytes (2.0×10^6 cells) in 6 well plate having 1ml RPMI 1640 media and differentiated them by using 50ng phorbol 12-myristate 13-acetate (PMA), incubate for 48 hours in CO2 incubator. Differentiated THP 1 monocytes were grown in RPMI-1640 medium and supplemented with 100 µg/mL streptomycin, 100 U/mL penicillin, 2 mM L-glutamine, and 10% fetal calf serum in an incubator maintaining 5% CO2 at 37 °C under humidified condition. We have isolate the PRP (20ul) and stained with cell tracker green (20um) for 10 min. Centrifuged the platelets for 10 min at 400g. After collection of the platelet pellets, add 1ml of complete RPMI media was added. Now, the stained platelet were added in cells (6 well plate) and kept it for 1 hour incubation at room temperature. After incubation, we have removed the media and washed the cells with PBS. After washing, cells were fixed with 4% formaldehyde solution, DAPI and Rhodamine were added to stain microphase and incubated for 15 min followed by washing the cover slip mounted on slides with the help of

mounting solution. Cell-platelet interaction was observed under confocal microscope. Similarly, monocytes-platelets interaction was also analyzed by flow cytometry**.**

2.18. 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 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)**.

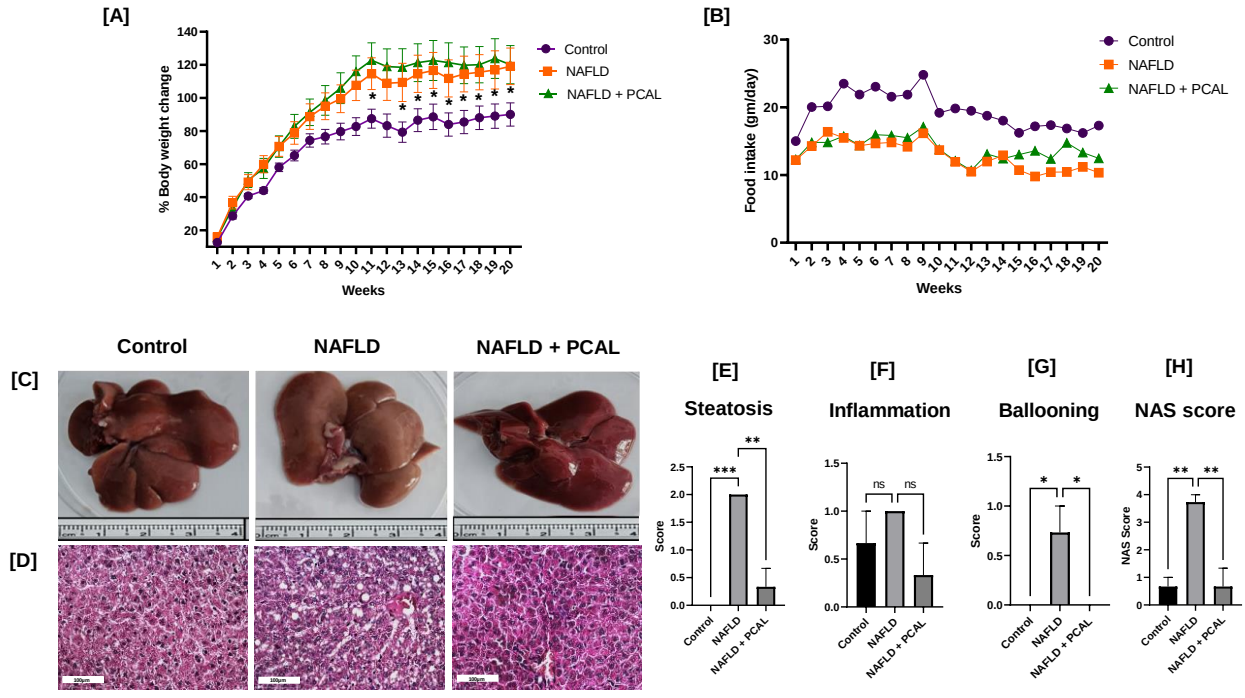

Figure 1: Effect of paricalcitol on **[A]** Percentage (%) body weight changes (n=7), **[B]** changes in food intake (n=7), and **[C]** liver morphological picture and [D] images of liver histopathology (H&E-stained sections) of rats where scale is 100µm (n=3). Bar graph showing the histopathological 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.

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, 1E)**. 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, 1F, 1G)**. The total NAS score was found to be higher in NAFLD liver in comparison to the control. Further paricalcitol has normalised 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 Figure 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 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.

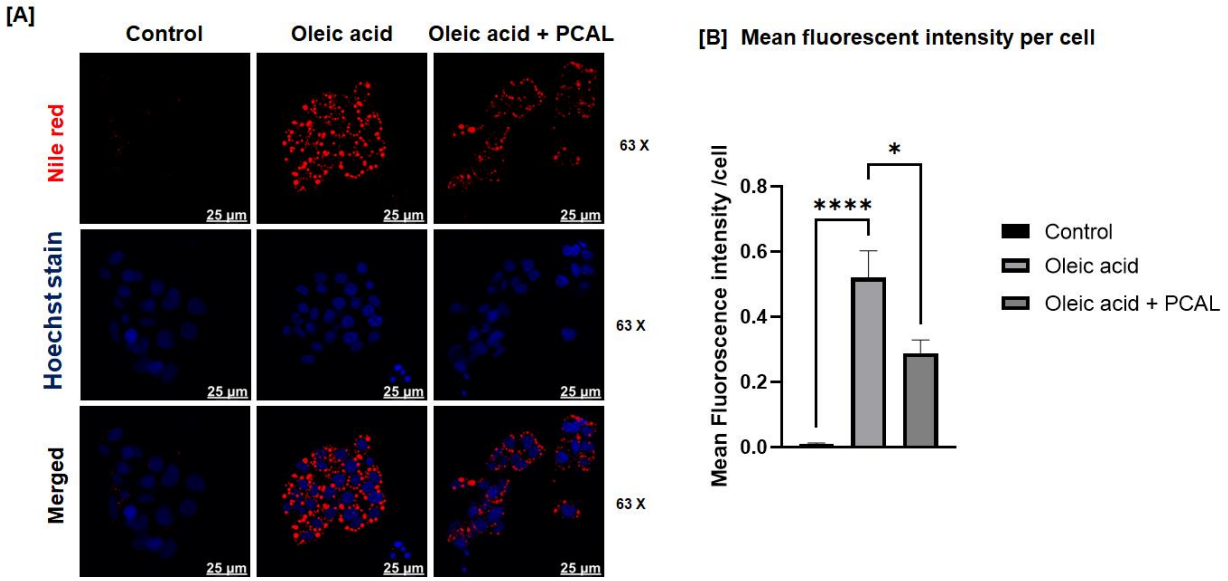

Figure 2: Paricalcitol effect on oleic acid-induced fat accumulation in HepG2 cells where [A] confocal images and **[B]** Bar graph showing the changes in mean fluorescence intensity of the Nile red per cell in control, oleic acid and oleic acid + paricalcitol treated HepG2 cells. The data were analyzed using a two-way ANOVA followed by Tukey's test and represented as mean ± SEM. *$p< 0.05$, ****$p<0.0001$ (n= 200±5 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.

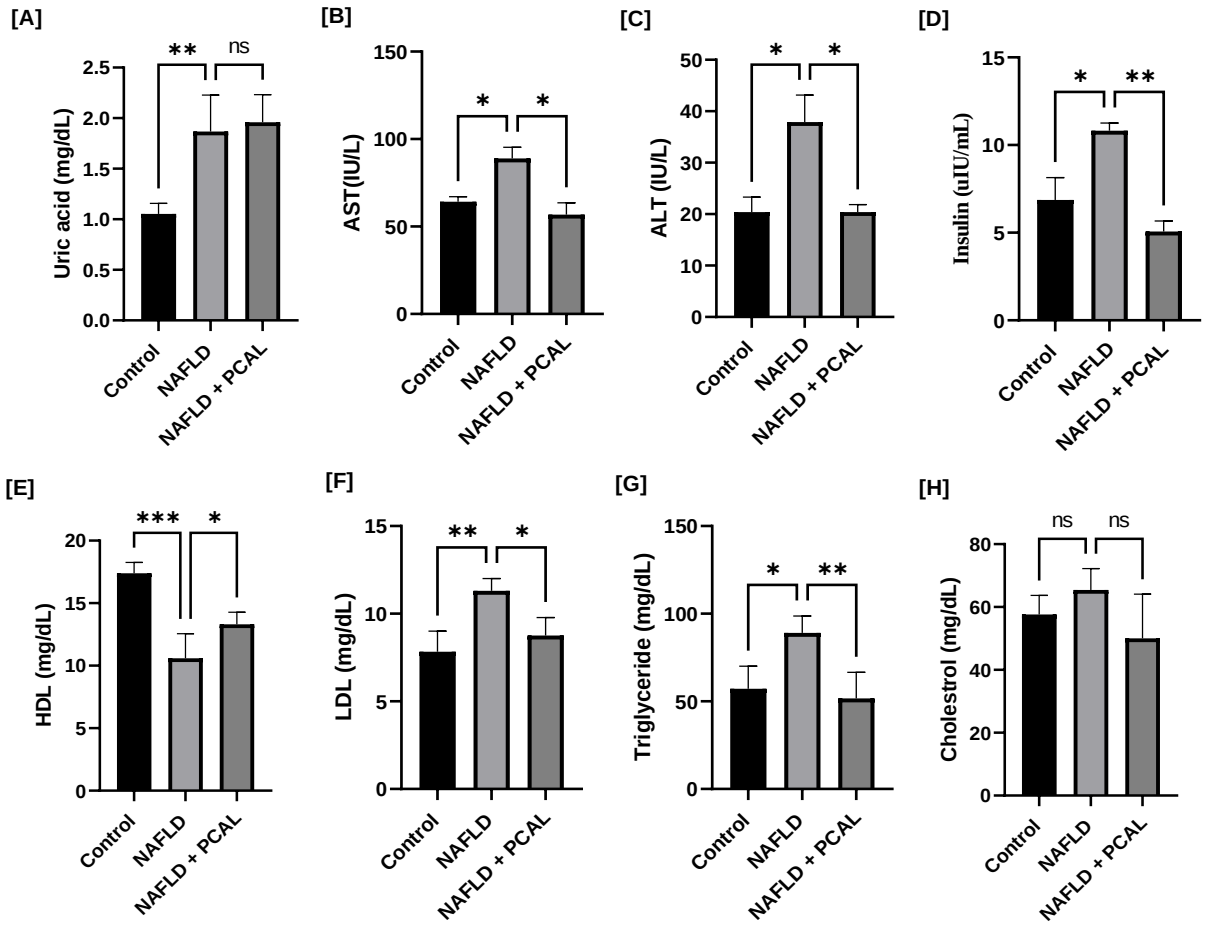

Figure 3: Bar graphs showing changes in various serum biochemical parameters of control, NAFLD and NAFLD + PCAL groups. [**A]** Uric acid**, [B]** AST, **[C]** ALT, **[D]** insulin, **[E]** HDL, **[F]** LDL, **[G]** triglyceride and **[H]** cholesterol. The data were analyzed using a One-way ANOVA followed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

3.4. Effect of paricalcitol on the reversal of protein acetylation in the liver of NAFLD rats

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, 4D**). 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, 4E)** and SIRT1 was measured **(Fig. 4C, 4F**) 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

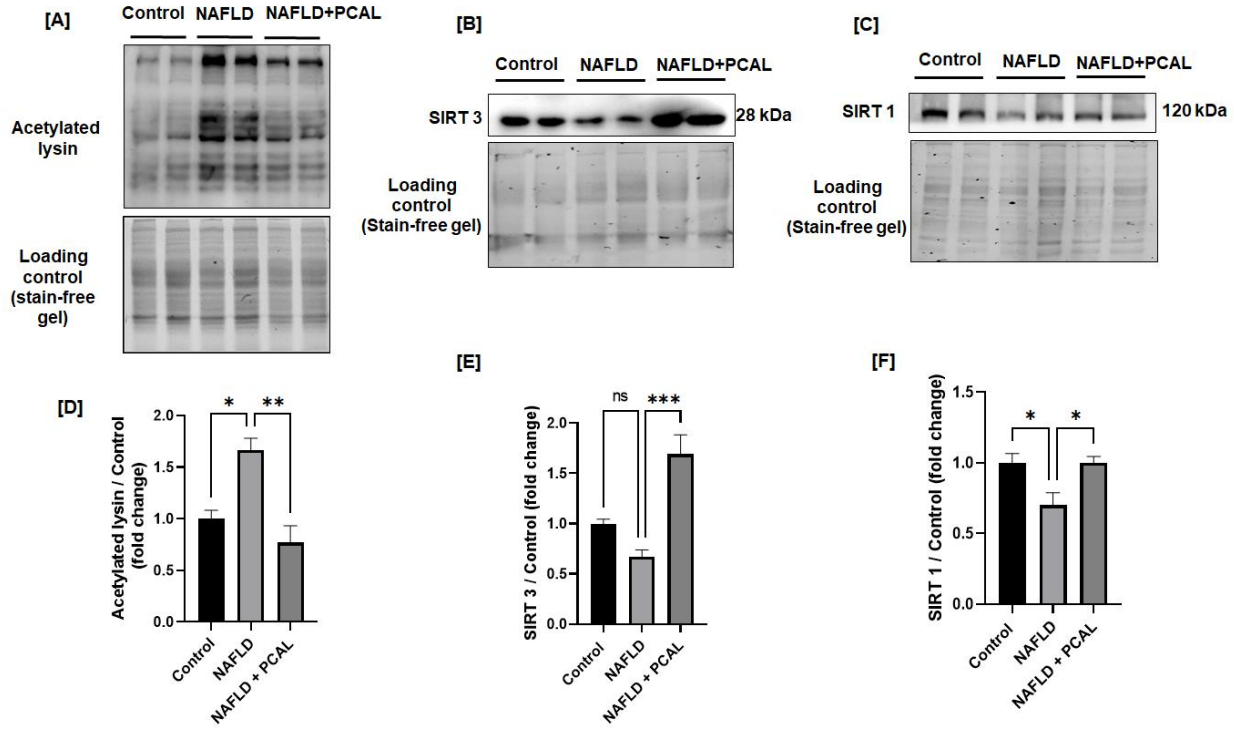

Figure 4: The expression of the total acetylated protein and its regulatory enzymes SIRT 1 and SIRT 3 in rat liver of control, NAFLD, NAFLD + PCAL groups. [**A, D]** Total acetylated lysin expression, **[B, E]** protein expression of SIRT3 and **[C, F]** protein expression of SIRT1. The data were analyzed 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).

3.5. Effect of paricalcitol on the reversal of protein acetylation in the oleic acid-treated HepG2 cells

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 (12hrs) 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, 12hrs 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.

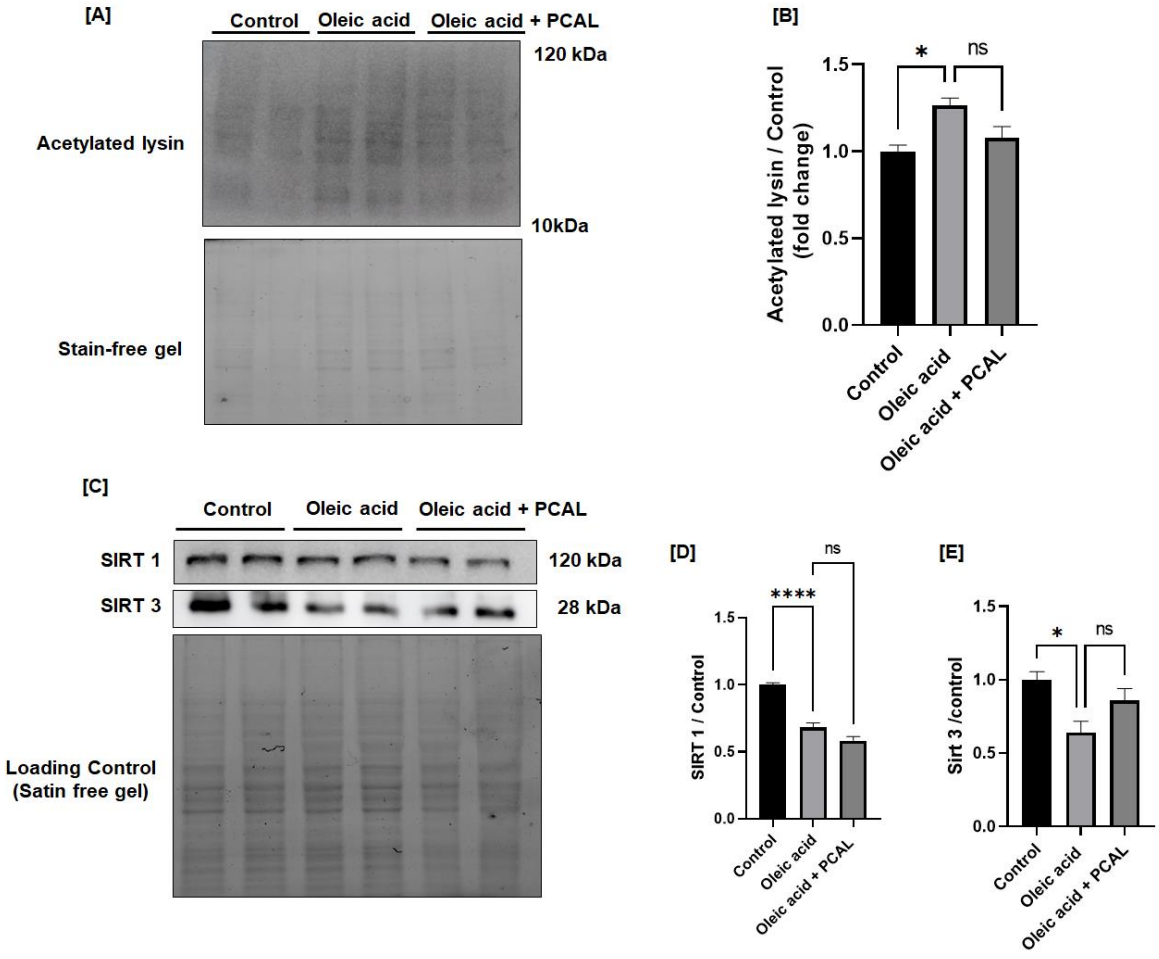

Figure 5: The expression of the total acetylated protein and its regulatory enzymes SIRT 1 and SIRT 3 in control, oleic acid, oleic acid + PCAL treated HepG2 cells. [**A, B]** Total acetylated lysin expression, **[C-E]** protein expression of SIRT1 and SIRT3. The data were analyzed using a One-way ANOVA followed by Tukey's test and represented as mean ± SEM. *$p< 0.05$, ****$p< 0.0001$, ns= non-significant (n=4 from two independent blots).

3.6. 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. 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 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.

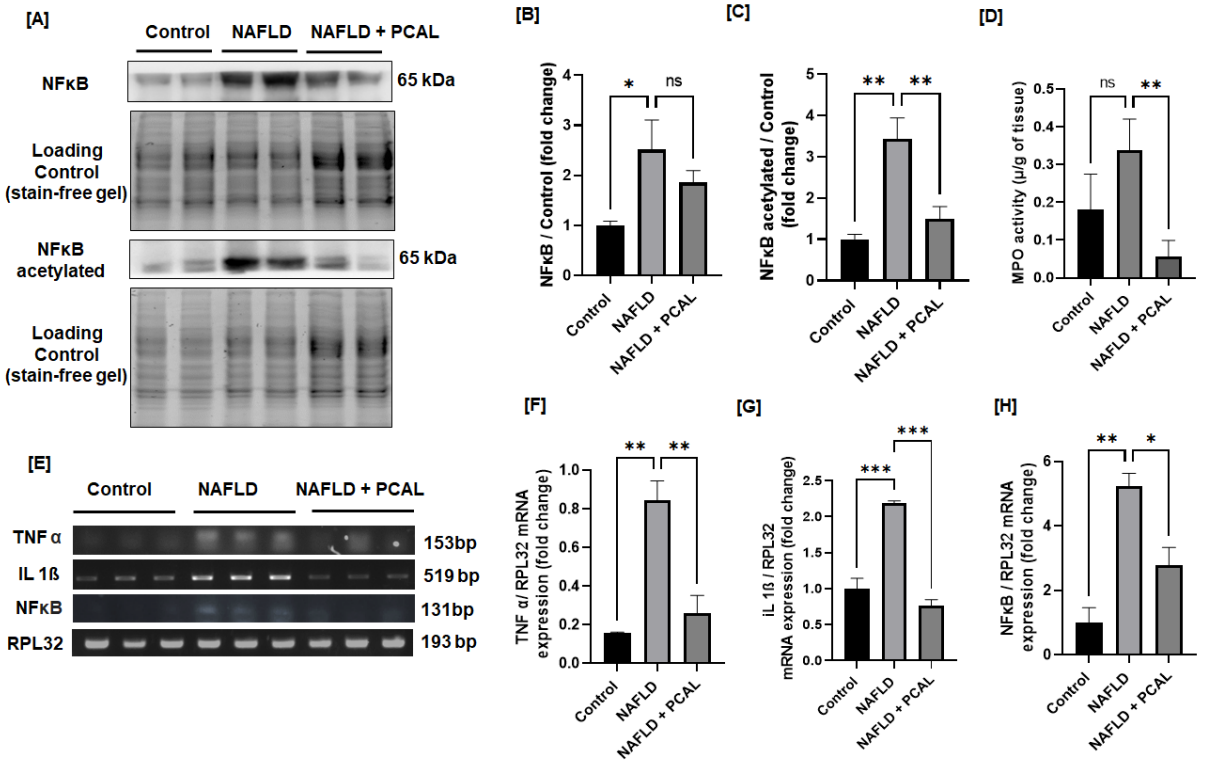

Figure 6: Expression of inflammatory markers in the liver of control, NAFLD, NAFLD + PCAL group. [**A-C**] Protein expression of NFκB and acetylated NFκB expression in the rat liver (n=4 from two

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.

3.7. 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 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**.** 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.

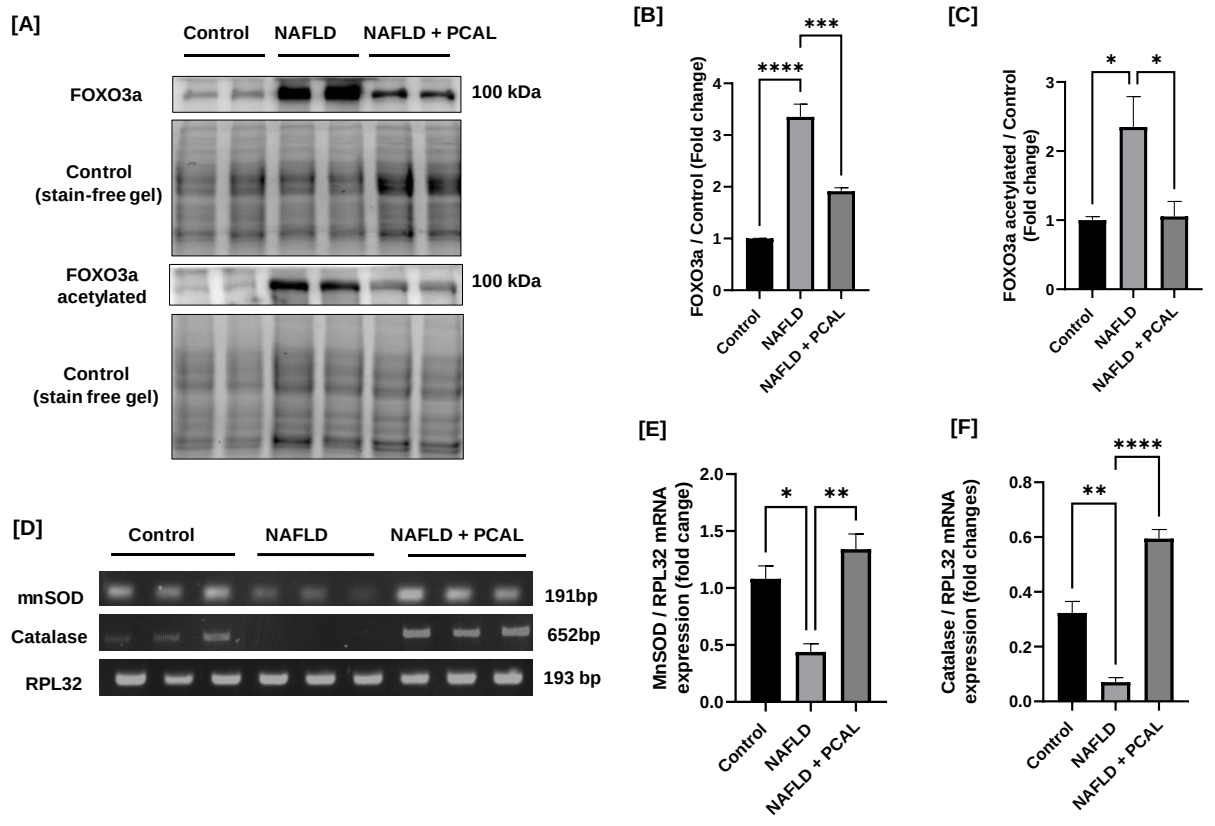

Figure 7: **Expression of oxidative stress markers in the liver of control, NAFLD, NAFLD + PCAL groups.** [**A-C]** FOXO3a and its acetylated protein status (n=4 from two independent blots); [**D-F]** mRNA expression of MnSOD and catalase (n=3). The data were analyzed using a One-way ANOVA followed by Tukey's test and represented as mean ± SEM. **p< 0.05, **p< 0.01, ***p<0.001, ****p<0.0001*.

3.8. Paricalcitol decreases the NFκB and FOXO3a protein expression in oleic acid-treated HepG2 cells

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. 8A, 8C).** Similarly, 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. 8B, 8D)**.

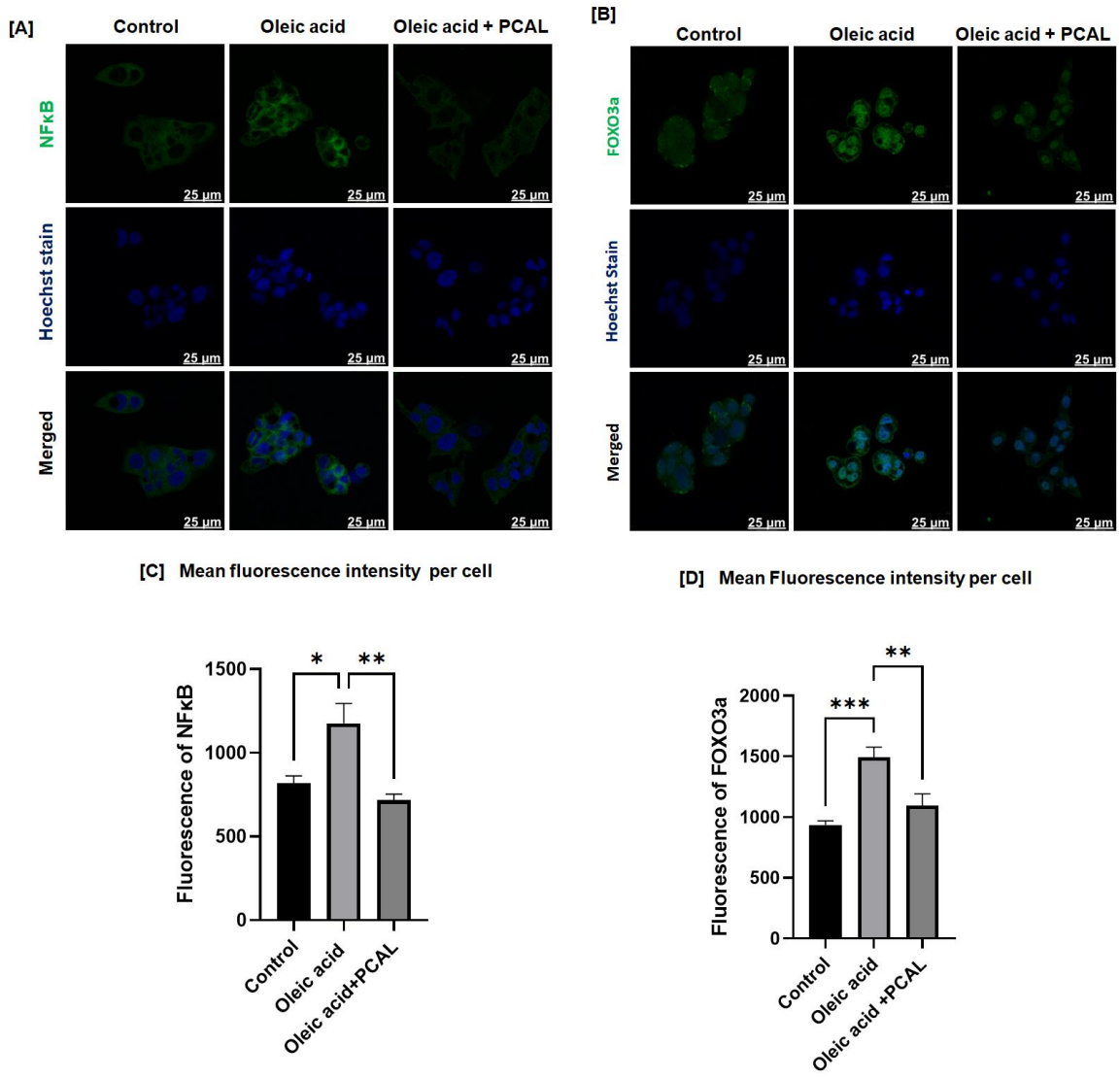

Figure 8: Paricalcitol effect on expression of NFκB and FOXO3a in oleic acid treated HepG2 where [A, B] confocal images showing the expression of **NFκB and FOXO3a** and **[B]** Bar graph showing the changes in mean fluorescence intensity of NFκB and FOXO3a per cell in control, oleic acid and oleic acid + PCAL treated HepG2 cells respectively. The data were analyzed using a two-way ANOVA followed by Tukey's test and represented as mean ± SEM. *$p< 0.05$, **$p<0.01$, ***$p<0.001$ (n= 200±5 cells).

3.9. 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**).

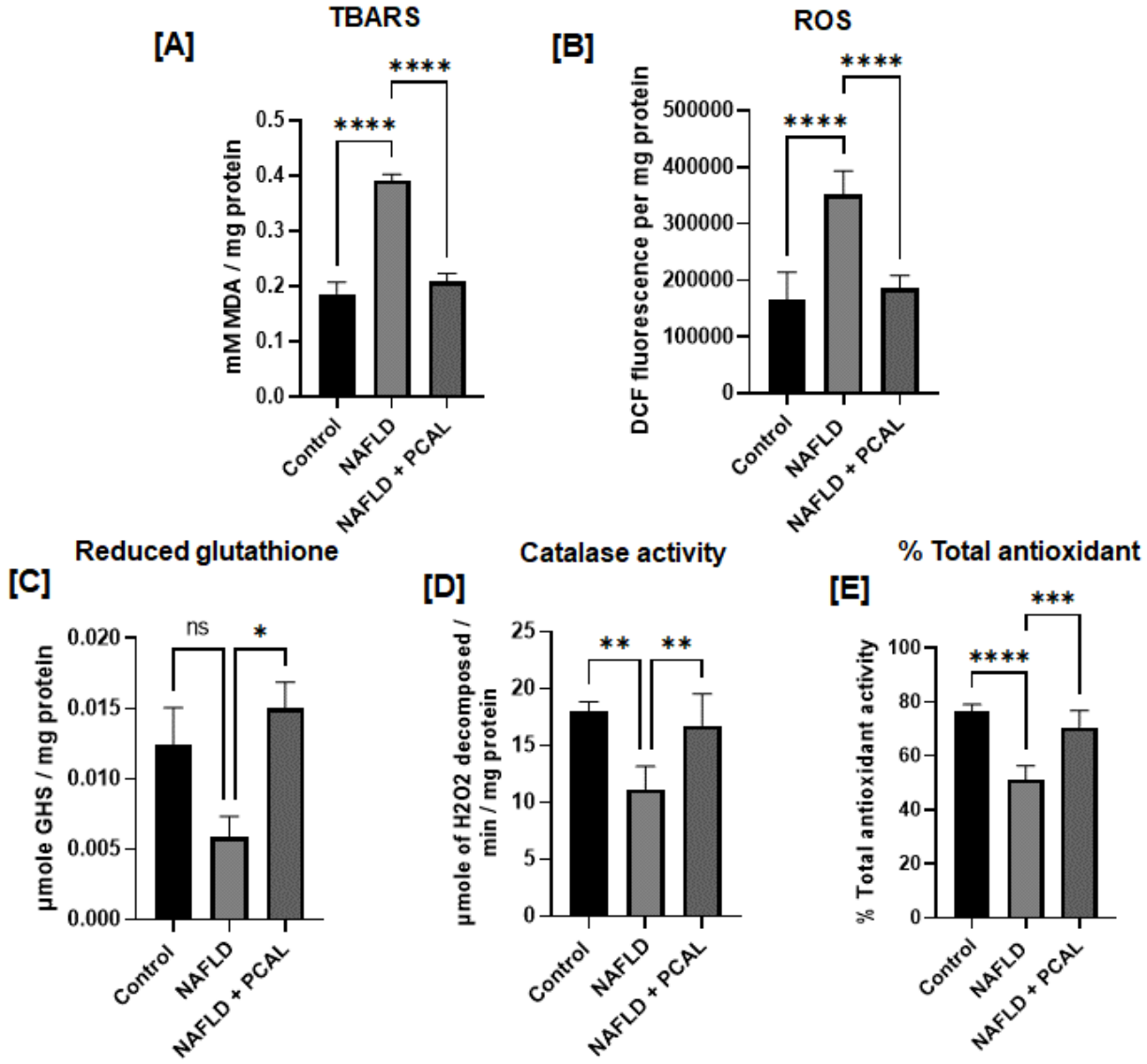

Figure 9: Oxidative stress and antioxidant level/activity were measured in the liver of control, NAFLD, NAFLD + PCAL groups. **[A]** Lipid peroxidase activity, **[B]** ROS level, **[C]** GSH concentration, **[D]** catalase activity, **[E]** % total antioxidant activity. The data were analyzed using a 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).

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 0.08µg/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 12hrs pretreatment of paricalcitol. Our *in-vitro* data suggests that the longer exposure (>12hrs) 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 knock-down 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 (87). 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.

6. Impact of research in the advancement of knowledge and benefit to mankind

NAFLD is a recent global burden among chronic liver disease which is known to be associated with cardiometabolic dysfunction. Because of the disease's complexity, there is a lack of clear

views on NAFLD pathogenesis. In our study, we showed the role of post-translational modification (acetylation) of proteins in NAFLD pathogenesis by increasing the acetylation of NFkB and FOXO3A. Our research on the role of protein acetylation in NAFLD has strengthened the existing pathophysiology of NAFLD. Further paricalcitol, a vitamin D analog has effectively reversed the acetylation of protein in NAFLD. This helps to understand the importance of acetylation of proteins in NAFLD disease development as well as the beneficial role of paricalcitol in reversing the increased acetylation in the liver. This makes paricalcitol a potential drug to treat NAFLD. As paricalcitol has already been approved for chronic kidney disease, repurposing of paricalcitol in NAFLD can be translated to humans easily.

Therefore, my study has a great potential to translate in humans without any regulatory hurdles. I am looking forward to translating the preclinical data in clinical settings.

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).

Acknowledgments

The authors are grateful to the National Institute of Pharmaceutical Education and Research, Guwahati for providing the funds, support, and facility for this work.

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