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Original Article

Whole-grain millets attenuate atherosclerosis by modulating cholesterol metabolism and the FGF-2/PI3K/Akt and Wnt-1/β-catenin pathways in high-cholesterol-fed rats

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Abstract

Millets, rich in nutrients, have the potential to enhance immunity and combat diseases, including atherosclerosis. As a chronic inflammatory disease contributing to high global mortality, atherosclerosis involves key signaling pathways such as Reverse Cholesterol Transport, FGF-2, and Wnt-1/β-catenin. Hence, this study investigated the anti-atherogenic effects of wholegrain millets on these pathways in high-cholesterol-fed rats, highlighting their potential as plant-based therapeutic alternatives to current treatments with adverse effects. Serum lipid profile, atherogenic index, tissue cholesterol levels, activity of lipogenic enzymes, hepatic 3-hydroxy-3-methylglutaryl-CoA reductase, plasma lecithin cholesterol acyl transferase, cardiac and inflammatory markers, gene expression of key lipid metabolism and FGF-2-Wnt-1 pathway genes by RT PCR and qPCR. Protein levels of the FGF-2-Wnt-1 pathway by ELISA and histopathological and Oil-red-O analysis were evaluated. 10% millet intervention significantly improved lipid metabolism by normalizing lipid profiles, reducing atherogenic index, lowering tissue cholesterol and lipogenic enzyme activities, enhancing LCAT activity, upregulating ABCA1 and Apo A1, and downregulating Apo B in HCD-fed rats. Among the millets, Little Millet (LM) showed the most potent effect, significantly reducing cardiac markers (CK-MB, LDH, CRP), downregulating FGF-2/PI3K/Akt and Wnt-1/β-catenin signaling by upregulating GSK3β, and improving aortic structure with no lipid accumulation as shown by Oil-red O staining. Our study suggests that whole-grain millet consumption can effectively reduce atherosclerosis progression. Specifically, LM shows strong potential as a natural intervention for managing atherosclerosis through its regulatory effects on key signaling pathways.

Keywords: Atherosclerosis, Reverse Cholesterol Transport, FGF-2, Wnt-1, GSK3β, Little millet

1. Introduction

Over 5,000 years ago, millets were apparently consumed in India. But the use of millets has greatly decreased as a result of the adoption of modern diets. Based on their grain size, millets are classified into major and minor millets. Sorghum, Pearl millet, Finger millet are the major millets and Proso millet, Kodo millet, Little millet, Foxtail millet, Barnyard millet, etc are considered as minor millets. Compared to rice and wheat, millets have significantly higher nutrient content. Millets are naturally gluten-free and provide an excellent source of dietary fiber, essential minerals, B-complex vitamins, beneficial polyphenols, healthy fatty acids, and proteins [1].

Changes in dietary habits in current society are linked to an increased risk of metabolic syndrome, which includes diabetes, obesity, hypertension, atherosclerosis, stroke, and cancer. Hypercholesterolemia (HC) is a leading cause of atherosclerosis risk, characterised by high lipoprotein levels in the serum, especially LDL-C [2]. Apolipoprotein A1 (Apo A1) interacts with ATP-binding cassette A1 (ABCA1) and facilitates High-density lipoprotein (HDL) to carry excess cholesterol from peripheral tissues and cells to the liver, where it is excreted as faeces, a process known as reverse cholesterol transport (RCT) [3]. Consequently, since ABCA1, Apo A1, Apolipoprotein B (Apo B), 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) and Lecithin-cholesterol acyltransferase (LCAT) are the key regulators responsible for maintaining cholesterol equilibrium, it is critical to determine changes in serum and tissue lipids as well as the fate of these molecules.

Wnt signaling is a developmental process that is very active during embryogenesis and also persists in a small subset of proliferating adult cells [4]. Adult-onset Wnt signaling pathway dysfunction has been linked to a variety of cardiac disorders, including arrhythmias, heart failure, hypertrophy, and illnesses, including atherosclerosis and myocardial infarction repair [5]. It has been observed that, in the various stages of atherosclerosis, from endothelial dysfunction to vascular calcification, the Wnt/β-catenin pathway is activated [6]. Similarly, FGF-2 or bFGF is

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responsible for the development of microvessels in the plaque, which can result in thrombosis, bleeding, and neoplasia [7]. Moreover, more research is needed to determine how FGF-2- Wnt-1/β-catenin crosstalk contributes to atherosclerosis.

Although pharmacological agents are widely used for managing atherosclerosis, concerns regarding their adverse effects have intensified the search for safer alternatives [8]. In this regard, dietary strategies have gained prominence, as fiber-rich foods such as whole grains and legumes are consistently associated with reduced cardiovascular risk. Millets, being nutrient-dense cereals rich in dietary fiber, polyphenols, unsaturated fatty acids, and essential micronutrients, possess remarkable nutraceutical potential. Their regular incorporation into the diet may offer a safe and sustainable means of delaying the onset and progression of cardiovascular diseases. Therefore, one of the main goals of our research is to highlight the nutraceutical potential of different fiber-rich millets available in the market and to explore their role in mitigating cardiovascular risk without side effects.

There is no scientific evidence to support millet's anti-atherogenic properties. Therefore, the first part of the study was designed to find out which of the six millets— Foxtail millet, Proso millet, Pearl millet, Finger millet, Kodo millet, and Little millet—had a high anti-atherogenic effect on atherosclerosis by modifying the expression of lipid metabolism-related genes and enzymes in rats given a high-cholesterol diet (HCD). The second part of the study was set out to determine whether Little Millet (*Panicum sumatrense*) alleviates the progression of atherosclerosis through the FGF-2 and Wnt-1/β-catenin pathway.

2. Materials and Methods

2.1. Collection and Preparation of the millet

Foxtail millet (*Setaria italica*), Proso millet (*Panicum miliaceum*), Pearl millet (*Pennisetum glaucum*), Finger millet (*Eleusine coracana*), Kodo millet (*Paspalum scrobiculatum)*, and Little millet (*Panicum sumatrense*) were collected from the Indian Council of Agricultural Research, Hyderabad. The millets were cleaned and soaked overnight. After that, each millet was cooked separately for 40 minutes at 100°C by mixing with distilled water at a 1:20 ratio [9]. Collected millet grains by using sieve filtration of millet porridge. For the investigation, pulverised millet was combined with rat chow.

2.2. Animal Experiment

Male Sprague Dawley rats of 100-150g body weight were bred and reared in the department animal house and were used for this study. They were provided laboratory chow (Amrut AF-1000M Lab diet) and water *ad libitum* throughout the experimental period. The rats were housed in polypropylene cages in a room with temperature maintained at 26 ± 1°C and a 12-hour light and dark cycle. All experiments were conducted according to the guidelines of the Animal Ethics Committee for Control and Supervision of Experiments on Animals (CPCSEA) (IAEC 1-KU-07/21-BCH-AH (39)) according to the Government of India accepted principles for laboratory animal use and care.

2.3. Experimental design and treatment protocol

The normal control diet was made up entirely of rat chow (100%). 1.5% cholesterol and 0.5% cholic acid [10] were added to the rat chow to create an HCD. The treatment diets were made by mixing HCD, rat chow, and 10% millet. An effective dose of millets, i.e., 10% (w/w), was selected from previous studies [11,12].

In the first part of the study, experimental animals were divided into eight groups of 3 rats each as follows: Group I was treated as normal and received standard commercial laboratory rat chow. Group II received HCD. Group III: HCD + 10% Little millet (LM), Group IV: HCD + 10% Proso millet (PrM), Group V: HCD + 10% Pearl millet (PeM), Group VI: HCD + 10% Kodo millet (KM), Group VII: HCD + 10% Finger millet (FiM), Group VIII: HCD + 10% Foxtail millet (FoM).

In the second part of the study, experimental animals were divided into three groups of 3 rats each as follows: Group I was treated as normal and received standard commercial laboratory rat chow. Group II received a high-cholesterol diet (HCD). Group III: HCD + 10% Little millet (LM). After 60 days of the experimental period, the animals were sacrificed after an overnight fast. Body weights were recorded weekly during the experimental period. Blood was collected in tubes without anticoagulants for serum separation. Tissues were removed, washed with ice-cold saline, and stored in ice containers for evaluation of different parameters.

2.4. Rat aortic endothelial cell (EC) isolation

As previously described, the rat aortic EC was isolated and cultured by enzymatic dissociation [13]. In brief, the full length of the thoracic aorta was removed from Sprague-Dawley rats under sterile conditions, rinsed 3 times with phosphate-buffered saline solution (PBS), and placed into a 100-mm culture dish (Corning, New York, USA) filled with serum-free Dulbecco Modified Eagle medium (DMEM, Himedia) on ice. The vessel was gently cleaned of periadventitial fat and connective tissue. The rat thoracic aorta was opened and rinsed with serum-free DMEM and then placed intimal side down on a sterile plate containing 0.2% collagenase type I (Sigma, St. Louis, MO) and incubated at 37ºC for 30 min. The cells were gathered by gentle abrasion with a cell scraper (Costar, Pleasanton, California) from the edges to the center of the specimen, and rinsed with 5 ml of DMEM plus serum to arrest the digestion process. The medium containing detached EC was collected and the cell suspension was centrifuged at 2000 rpm for 10 min. The cell pellet was washed twice, suspended in DMEM supplemented with 10% fetal bovine serum (FBS, Sigma), 100U/ ml penicillin, 100mg/ ml streptomycin, 75mg/ml endothelial cell growth supplement (Sigma) and placed in a 60-mm collagen type I coated culture dish (Corning). The dish was then placed in a humidified incubator at 37ºC with 5% CO_2. Thereafter, the medium was changed every other day. After the cells formed a confluent monolayer, they were harvested.

2.5. Estimation of serum lipid profile

Total Cholesterol (TC), triglycerides (TG), LDL-C, and HDL-C concentrations in the serum were estimated using Agappe diagnostics kit [14,15].

The atherogenic index (AI) was calculated by the following formula: AI = LDL-C/ HDL-C.

2.6. Extraction and estimation of tissue lipids

Total lipids from the heart, liver and aorta were extrac-

ted using chloroform/methanol according to the method described by Folch *et al* [16].

2.7.Measurement of hepatic 3-hydroxy-3-methylglutaryl-CoA reductase, plasma lecithin cholesterol acyl transferase

The activity of HMG-CoA reductase was measured, as described by Rao *et al* [17], by determining the ratio of HMG-CoA: mevalonate. Plasma lecithin cholesterol acyl transferase (LCAT) activity was assayed as described by Schoenheimer *et al* [18].

2.8. Measurement of the activity of hepatic lipogenic enzymes

The rat liver tissue was finely minced and homogenized in glycyl-glycine buffer under ice-cold conditions. Homogenates were centrifuged at 9000 g at 40ºC for 20 min and the supernatant fraction was used for the measurement of various lipogenic enzyme activities. The activities of glucose-6-phosphate dehydrogenase (G6PDH) and isocitrate dehydrogenase (IDH) were measured by previous method [19]. The activity of malic enzyme (ME) was measured by the method described by Ochoa [20].

2.9. Biochemical estimations

Creatine Kinase in serum samples was assayed by using a kit from BioSystems. Lactate dehydrogenase (LDH) was estimated by using a kit from Enzopark Pvt. Ltd. C-reactive protein (CRP) in plasma was determined by an Immunoturbidimetric CRP turbilatex kit (Spinreact, Spain).

2.10. Enzyme linked immunosorbent assay (ELISA)

ABCA1, Apo A1, APO B, FGF-2, PI3K, Akt, GSK3β, Wnt-1, β-catenin, MMP-7, and LEF-1 concentrations were measured using enzyme-linked immunosorbent assay [21]. Tissue lysate precoated onto ELISA plates served as the antigen. O-dianisidine was used as substrate and the absorbance of the coloured horse radish peroxidase (HRP) product was measured spectrophotometrically at 405 nm by an automated microplate reader (Thermo Multiskan Sprectrum).

2.11. Histopathological Analysis and Oil-red-O

The aorta of the sacrificed animals was removed and preserved in 10% formalin, and then the tissue was fixed with paraffin. Following that, sections were taken and stained with hematoxylin and eosin (H&E). A microscopic analysis was carried out to study pathological variations. To evaluate lipid deposition, aorta tissue was embedded in an OCT embedding medium, and cross sections were stained with oil-red-O.

2.12. RT PCR Analysis

The RT-PCR kit from Thermo Scientific was used to perform PCR amplification and RT-PCR of significant Wnt pathway genes. Three cycle stages are conducted after the initial 15-minute 95°C PCR activation step. Every cycle consists of 40 repetitions of denaturation for 1 minute at 94 °C, annealing for 1 minute at 65 °C, extension for 1 minute at 72 °C, and final extension for 10 minutes at 72 °C. A UV transilluminator was used to view the PCR results after they had been run on 1% agarose gels and stained with ethidium bromide. Primer sequences used in the study are listed in Table 1.

2.13. Real-time polymerase chain reaction (qPCR)

Following the manufacturer's instructions, total RNA from the cells was extracted using Trizol reagent (cat. No. 93289; Sigma Aldrich). First-strand cDNA was produced with an RT kit (Thermo Scientific, Cat. No. AB1453A). The SYBR green Real-time PCR kit technique was used for qPCR using an Eppendorf thermocycler, according to the manufacturer's instructions. The real-time analysis started after a 7-minute incubation period at 95°C and 40 cycles of 10 s at 95°C and 30 s at 60°C. An incubation phase at 4°C ended the process. $2^{-\Delta\Delta Ct}$ was used to perform the relative quantification; ΔC_t is the difference between the endogenous GAPDH control and the sample's triplicates' mean C_t value. Primer sequences used in the study are listed in Table 1.

2.14. Protein assay

The protein concentration of the samples was determined by a method by Lowry *et al* [22].

2.15. Statistical analysis

Values were analysed using GraphPad Prism version 5.0 and provided as Mean ± SEM (standard error of the mean). Comparisons were made using one-way ANOVA and Tukey's multiple comparison test. Statistical significance was determined with $P<0.05$.

3. Results

3.1. Effect of millets on lipid profile and tissue cholesterol levels

In phase 1 of the experiment, consumption of HCD resulted in a significant reduction ($p < 0.05$) in HDL-C and an increase in serum TC, TG, and LDL-C when compared to rats on a regular diet. As opposed to HCD-feeding rats, the administration of 10% millets significantly increased

Table 1. Primers sequences.

Gene	Forward	Reverse
ABCA 1	AACAGTTTGTGGCCCTTTTG	AGTTCCAGGCTGGGGTACTT
APO A	CCTGGATGAATTCCAGGAGA	TCGCTGTAGAGCCCAAACTT
APO B	AATGGAGCACTTTTCAAG	GGAACAGCAGCAGTAGCG
Akt	GCCACGGATACCATGAACGA	TTGAGGAGGAAGTAGCGTGG
GSK3β	AGACCAATAACGCCGCTTCTGC	AACGTGACCAGTGTTGCTGAGTG
β-catenin	GGAAAGCAAGCTCATCATTCT	AGTGCCTGCATCCCACCA
MMP-7	CAGGAAGCCGGAGAAGTGAC	TCTCCGGCAAACCGAAGAAC
GAPDH	CAACTCCCTCAAGATTGTCAGCAA	GGCATGGACTGTGGTCATGA

the level of HDL-C and significantly decreased ($p < 0.05$) the serum TC, TG, and LDL-C (**Table 2**). In addition, liver, heart, and aortic lipid levels were analysed and results showed rats administered with 10% millets had significantly lower ($p < 0.05$) liver, heart, and aortic lipid levels in all millets-supplemented groups than rats fed the HCD alone (Table 3). According to the findings, millet supplementation reduces the lipid content and thereby maintains normal cardiac function.

3.2. Effect of millets on activities of lecithin cholesterol acyl transferase, 3-hydroxy-3-methylglutaryl-CoA reductase and lipogenic enzymes

To further understand the effect of millets on liver lipid metabolism, the activities of lipogenic enzymes HMG CoA, LCAT and lipid transporters were analysed. The administration of 10% millets to hypercholesterolemic rats resulted in a significant increase in LCAT activity, whereas rats fed cholesterol showed a decrease in LCAT activity (Fig. 1A). These findings indicate that millets can aid in reducing elevated lipid levels by preventing the synthesis of cholesterol and boosting the clearance of excess cholesterol from peripheral tissues by HDL. In Fig. 1B, HMG-CoA reductase activity was seen to be elevated in the HCD-fed group, but it dropped following administration with 10% millets, particularly in the little millet-fed group.

Hence, the activity of the aforementioned lipogenic enzymes was investigated. In the current study, after 10%

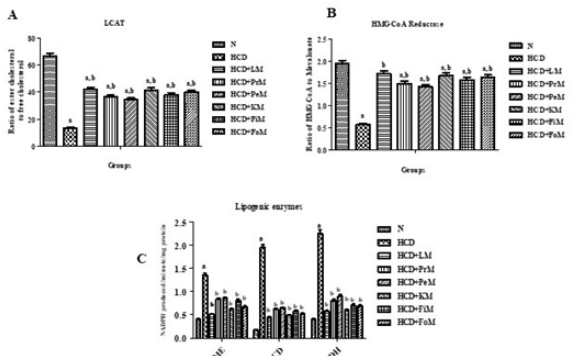

Fig. 1. (A) Plasma LCAT activity; (B) Hepatic HMG-CoA levels; (C) Hepatic lipogenic enzyme activities. Values are expressed as mean ± SEM (n = 6 per group). *a* – significantly different from Group I ($p < 0.05$); *b* – significantly different from Group II ($p < 0.05$). Group I: Normal control (standard laboratory diet); Group II: HCD (standard diet containing 1.5% cholesterol and 0.5% cholic acid); Group III: HCD + Little millet (HCD + LM); Group IV: HCD + Proso millet (HCD + PrM); Group V: HCD + Pearl millet (HCD + PeM); Group VI: HCD + Kodo millet (HCD + KM); Group VII: HCD + Finger millet (HCD + FiM); Group VIII: HCD + Foxtail millet (HCD + FoM). *Note:* A lower ratio indicates higher enzyme activity.

Table 2. Effect of millets on lipid profile.

	TC (mg/dL)	TG (mg/dL)	HDL-C (mg/dL)	LDL-C (mg/dL)	AtherogenicIndex
N	54.15± 2.02	69.48±2.59	34.63± 1.33	20.75±0.77	0.599
HCD	170.43±6.51[a]	156.35±5.82[a]	16.32±0.623[a]	91.88±3.42[a]	5.63
HCD+LM	66.62±2.48 [a,b]	97.28±3.62 [a,b]	29.78±1.14 [a,b]	23.71±0.88 [a,b]	0.796
HCD+PrM	85.41±3.18 [a,b]	121.61±4.53 [a,b]	26.59± 1.02 [a,b]	35.56±1.32 [a,b]	1.337
HCD+PeM	92.62±3.45 [a,b]	125.08±4.66[a, b]	22.43± 0.86 [a,b]	38.53±1.44 [a,b]	1.717
HCD+KM	74.14±2.76 [a,b]	107.67±4.01 [a,b]	29.19± 1.11 [a,b]	24.69±0.92 [a,b]	0.84
HCD+FiM	81.31±3.03 [a,b]	114.66±4.27 [a,b]	27.13± 1.04 [a,b]	32.59±1.21 [a,b]	1.20
HCD+FoM	79.06±2.95 [a,b]	108.06±4.03 [a,b]	28.44± 1.09 [a,b]	30.61±1.14 [a,b]	1.076

Lipid marker concentrations were examined in order to assess the prevalence of atherosclerosis during cholesterol supplementation. Values expressed as average of 6 values± SEM in each group. A significantly different from group I ($p< 0.05$), b- significantly different from group II ($p< 0.05$). Group I – Normal (standard laboratory diet), Group II - HCD (standard diet containing 1.5% cholesterol and 0.5% cholic acid), Group III – HCD with Little millet (HCD+LM), Group IV – HCD with Proso millet (HCD+PrM), Group V – HCD with Pearl millet (HCD+PeM), Group VI – HCD with Kodo millet (HCD+KM), Group VII – HCD with Finger millet (HCD+FiM), Group VIII – HCD with Foxtail millet (HCD+FoM).

Table 3. Effect of millets on the concentration of tissue cholesterol.

GROUPS	LIVER	HEART	AORTA
N	388.25±14.46	188.30±7.02	166.95±6.22
HCD	543.56±20.25 [a]	310.61±11.57 [a]	359.14±13.38 [a]
HCD+LM	399.90±14.89 [a,b]	194.14±7.23 [a,b]	221.29±8.24 [a,b]
HCD+PrM	456.19±16.99 [a,b]	211.59±7.88 [a,b]	254.31±9.47 [a,b]
HCD+PeM	462.02±17.21 [a,b]	216.84±8.08 [a,b]	258.19±9.62 [a,b]
HCD+KM	414.51±15.44 [a,b]	198.01±7.38 [a,b]	225.18±8.39 [a,b]
HCD+FiM	427.08±15.91 [a,b]	205.77±7.67 [a,b]	244.59±9.11 [a,b]
HCD+FoM	421.25±15.69 [a,b]	201.88±7.52 [a,b]	242.66±9.04 [a,b]

Values expressed as average of 6 values± SEM in each group. A significantly different from group I ($p< 0.05$), b- significantly different from group II ($p< 0.05$). Group I – Normal (standard laboratory diet), Group II - HCD (standard diet containing 1.5% cholesterol and 0.5% cholic acid), Group III – HCD with Little millet (HCD+LM), Group IV – HCD with Proso millet (HCD+PrM), Group V – HCD with Pearl millet (HCD+PeM), Group VI – HCD with Kodo millet (HCD+KM), Group VII – HCD with Finger millet (HCD+FiM), Group VIII – HCD with Foxtail millet (HCD+FoM).

of millets were fed along with cholesterol diet in rats, the activity of lipogenic enzymes (ME, IDH and G6PDH) were found to be decreased, but the liver of rats with high cholesterol diet showed significantly increased activities of lipogenic enzymes (Fig. 1C). According to these findings, 10% of millets may have played a role in regulating the metabolism of cholesterol by reducing the synthesis of cholesterol.

3.3. Effect of millets on mRNA expression and protein level of RCT transporters

Hence, our investigation focused on the protective role of millets in the control of a group of genes crucial to RCT. In our findings, the millets group showed significantly higher expression levels of ABCA1 and apo A1 compared to the HCD-fed group. However, Apo B expression was higher in the HCD-fed group and lower in the millets-fed group (Fig. 2A). The protein levels were also assessed using ELISA (Fig. 2B), confirming the findings of mRNA expression analysis and showing consistent results. Taken together, millets enhanced the efflux of cholesterol, thereby enhancing the cardioprotective effect.

3.4. Effect of LM on cardiac and inflammatory markers

In the current investigation, the high cholesterol diet showed elevation in the activity of enzymes such as CK-MB and LDH, suggesting that the heart muscle has been damaged, leading to heart failure. On the other hand, 10% LM supplementation resulted in decreased activities of these enzymes, which indicates the cardioprotective property of Little Millet (Fig. 3A and B). The HCD rats exhibited a significant increase in CRP level as compared to the normal group. Whereas millet supplementation significantly decreased serum CRP level (Fig. 3C). These findings indicate that including Little Millet in the diet could exert an anti-atherosclerotic impact by decreasing the level of pro-inflammatory marker CRP.

3.5. Effect of LM on FGF-2/PI3K/Akt signaling pathway

The findings demonstrated that FGF-2 activity is higher in atherosclerosis-prone rats than in control rats. While LM supplementation decreased FGF-2 level in aortic endothelial cells. Due to high cholesterol supplementation, the protein level of GSK3β in aortic endothelial cells of HCD rats was found to be decreased. On the other hand, rats supplemented with LM showed significant increase in the concentration of PI3K/Akt signal transduction pathway, which controls the activity of GSK3β (Fig. 4A and 4B).

3.6. Effect of LM on Wnt-1/β-catenin signaling pathway

To assess the anti-atherogenic impact of LM in aortic endothelial cells, the level of Wnt-1 was examined. When compared to normal rats, HCD rats had considerably ($P<0.05$) higher Wnt-1 activity in aortic endothelial cells. When compared to HCD rats, the Wnt-1 activity significantly decreased upon administration of LM. As expected, the protein levels of β-catenin, LEF-1, and MMP-7 in aortic endothelial cells were significantly lowered in the hypercholesterolemic rats treated with LM. Moreover, upregulated expression of β-catenin and MMP-7 was also determined in the aortic endothelial cells of rats treated with HCD. However, LM significantly reduced the expression of β-catenin & MMP-7 (Fig. 5A and 5B).

3.7. Effect of LM on atherosclerotic lesions in the aorta

The impact of little millet supplementation on struc-

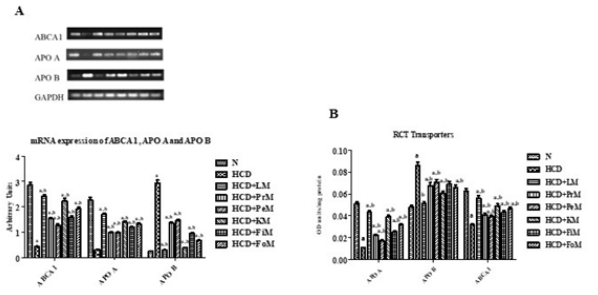

Fig. 2. (A) mRNA expression levels of **ABCA1**, **ApoA**, and **ApoB**; (B) Hepatic protein levels of **ABCA1**, **ApoA**, and **ApoB** determined by ELISA. Values are expressed as mean ± SEM (n = 6 per group). *a* – significantly different from Group I ($p < 0.05$); *b* – significantly different from Group II ($p < 0.05$). Group I: Normal control (standard laboratory diet); Group II: HCD (standard diet containing 1.5% cholesterol and 0.5% cholic acid); Group III: HCD + Little millet (HCD + LM); Group IV: HCD + Proso millet (HCD + PrM); Group V: HCD + Pearl millet (HCD + PeM); Group VI: HCD + Kodo millet (HCD + KM); Group VII: HCD + Finger millet (HCD + FiM); Group VIII: HCD + Foxtail millet (HCD + FoM).

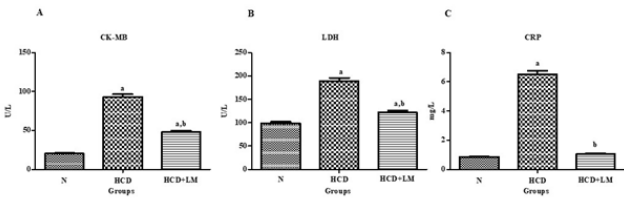

Fig. 3. Effect of Little millet (LM) on (A) CK-MB and (B) LDH activities, and (C) CRP concentration in serum. Values are expressed as mean ± SEM (n = 6 per group). *a* – significantly different from Group I ($p < 0.05$); *b* – significantly different from Group II ($p < 0.05$). Group I: Normal control (standard laboratory diet); Group II: HCD (standard diet containing 1.5% cholesterol and 0.5% cholic acid); Group III: HCD + Little millet (HCD + LM). U/L = units per litre; mg/L = milligrams of CRP per litre.

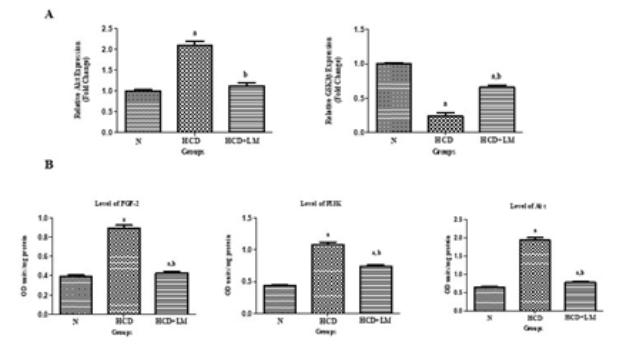

Fig. 4. (A) Gene expression of **Akt** and **GSK3β** determined by qPCR; (B) Levels of **FGF2**, **PI3K**, and **Akt** in aortic endothelial cells. Values are expressed as mean ± SEM (n = 6 per group). *a* – significantly different from Group I ($p < 0.05$); *b* – significantly different from Group II ($p < 0.05$). Group I: Normal control (standard laboratory diet); Group II: HCD (standard diet containing 1.5% cholesterol and 0.5% cholic acid); Group III: HCD + Little millet (HCD + LM).

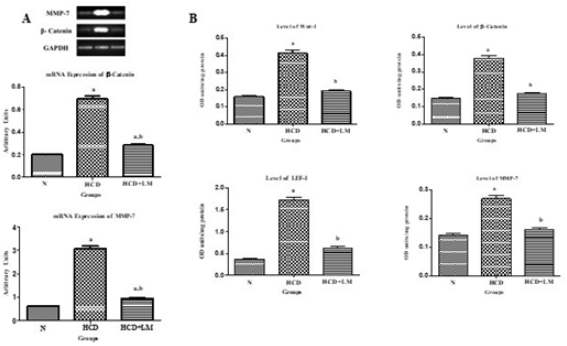

Fig. 5. (A) Gene expression of **β-catenin** and **MMP-7** determined by RT-PCR; (B) Levels of **Wnt-1**, **β-catenin**, **MMP-7**, and **LEF-1** in aortic endothelial cells. Values are expressed as mean ± SEM (n = 6 per group). *a* – significantly different from Group I ($p < 0.05$); *b* – significantly different from Group II ($p < 0.05$). Group I: Normal control (standard laboratory diet); Group II: HCD (standard diet containing 1.5% cholesterol and 0.5% cholic acid); Group III: HCD + Little millet (HCD + LM).

tural changes in the aorta brought on by atherosclerosis has not been investigated. The histopathological study using Hematoxylin and eosin staining of the aorta showed distorted elastic lamellae and exhibited intimal hyperplasia along with thickening of the intimal layer; in contrast, histological examination of aortic tissue in the normal and LM groups showed normal cells without any abnormalities (Fig. 6A). The cross-section of the aorta of the experimental rats in each group were stained with Oil red O. The control group was shown to have no lesions since they were not stained red. Lesions were found to be significantly distributed in the aortic cross-section of the HCD group, as evidenced by the intense red stain. Similarly, the Oil red O area in the LM group was significantly less than in the HCD group (Fig.6B).

4. Discussion

This study aims to evaluate the effects of cooked wholegrain millets on the progression of atherosclerosis in Sprague Dawley rats. Each of the six millets demonstrated the ability to reduce progression of atherosclerosis. However, Little Millet was the most effective among them in providing cardiovascular protection. In light of this, cooked whole-grain millets have the potential to regulate cholesterol metabolism and support RCT. Additionally, in the second phase of the study, Little Millet specifically regulated the Wnt/β-catenin, FGF-2/PI3K/Akt pathways. It played a key role in normalizing these signaling mechanisms associated with atherosclerosis progression. Histopathological analysis and Oil Red O staining of the aorta further confirmed its protective effects. These findings highlight the potential of millets, particularly LM, in preventing atherosclerosis progression when included as part of a regular diet. The protective role of millets can be largely due to their rich phytochemical profile, including polyphenols (ferulic acid, caffeic acid, catechins), flavonoids, tannins, dietary fibers, and essential micronutrients such as magnesium and potassium [1]. These compounds act synergistically to regulate lipid metabolism and modulate key signaling pathways, thereby reducing atherosclerotic burden.

Increased levels of TG, TC, and LDL-C, along with reduced HDL-C, serve as key biomarkers of dyslipidemia [23]. In the 60-day study, dietary supplementation with 10% millets effectively mitigated progression of atherosclerosis by enhancing HDL-C levels while significantly lowering TC, TG, and LDL-C, highlighting its potential role in lipid regulation and cardiovascular health. The reduced lipid accumulation in the liver, heart, and aorta of 10% millet-fed groups highlights its protective role against lipid overload. Overall, these findings reinforce the importance of whole-grain millet consumption in maintaining lipid homeostasis and promoting heart health.

LCAT is a key component of the RCT pathway that stimulates HDL-mediated efflux by converting free cholesterol into cholesteryl esters on HDL through a transesterification reaction [24]. The enhanced LCAT activity in millet-fed rats suggests that millet consumption supports cholesterol homeostasis by improving HDL function and promoting reverse cholesterol transport. The rate-limiting enzyme HMG-CoA reductase is responsible for catalyzing the conversion of HMG-CoA to mevalonate in the cholesterol biosynthesis pathway, as lower ratio indicates higher enzyme activity [25]. Reduced HMG-CoA reductase activity in the millet-fed groups suggests that millet consumption may play a role in regulating lipid metabolism and reducing endogenous cholesterol production. A reduction in lipogenic enzyme activity was observed in millet-fed groups, with the most significant decrease in the HCD+LM group, while the HCD+PeM group exhibited higher enzyme activity compared to other millet-supplemented groups, indicating variations in the effectiveness of different millet types in regulating lipid metabolism. Since de novo lipid synthesis in vertebrate tissues requires cytoplasmic dehydrogenases like ME, IDH, and G6PDH to generate NADPH and acetyl-CoA for converting glucose into triacylglycerol, the decreased enzyme activity in millet-fed groups suggests a potential inhibitory effect on lipid biosynthesis, contributing to improved lipid regulation [26].

The PCR and ELISA results showed that millet-fed groups had higher ABCA1 and ApoA-1 expression, indicating improved cholesterol efflux and HDL function. In contrast, ApoB expression, linked to LDL-C and athe-

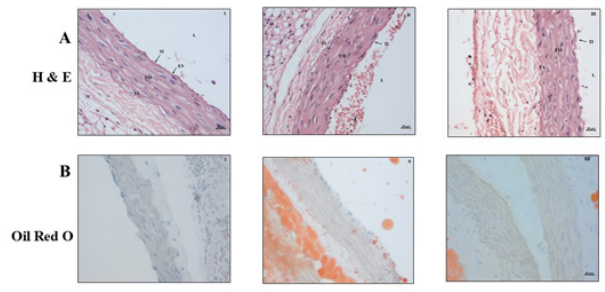

Fig. 6. (A) Histopathological image of rat aorta; (B) Oil Red O staining of rat aorta (photomicrograph at 40× magnification; scale bar = 20.0 μm). (TI) Tunica intima; (TM) Tunica media; (TA) Tunica adventitia; (EN) Endothelium. Values are expressed as mean ± SEM (n = 6 per group). *a* – significantly different from Group I ($p < 0.05$); *b* – significantly different from Group II ($p < 0.05$). Group I: Normal control (standard laboratory diet); Group II: HCD (standard diet containing 1.5% cholesterol and 0.5% cholic acid); Group III: HCD + Little millet (HCD + LM).

rosclerotic risk, was elevated in the HCD-fed group but lower in millet-fed groups. Among the millets, HCD+LM group showed the best regulation of cholesterol transporters, while HCD+PeM group had the least effect. The cardioprotective effects of HDL are primarily attributed to its role in reverse cholesterol transport, where HDL particles facilitate the removal of excess cholesterol from peripheral cells [27]. ApoA-1, the major protein component of HDL, plays a crucial role in this process by interacting with ABCA1, a macrophage cholesterol exporter, thereby reducing atherosclerotic risk. Conversely, elevated ApoB-100 levels, the key protein of LDL-C, are strongly associated with the progression of atherosclerotic coronary artery disease [28]. These findings suggest that millet consumption supports cardiovascular health by enhancing reverse cholesterol transport, improving HDL function, and reducing atherogenic lipoproteins.

Studies have revealed that an increased atherosclerotic plaque burden is correlated with elevated CK-MB and LDH levels [29,30]. Oxidative stress-induced cellular damage, resulting from an inadequate oxygen or glucose supply, compromises membrane integrity, leading to enzyme leakage into the bloodstream and subsequently increasing their serum concentrations. Also, an accelerated atherosclerosis process can be diagnosed with the help of the CRP level [31]. The HCD+LM supplemented rats exhibited significantly lower levels of CK-MB, LDH, and CRP compared to the HCD group, indicating reduced oxidative stress and myocardial damage. This suggests that LM may play a protective role in maintaining cardiac health by mitigating oxidative stress, preserving cellular integrity, and reducing inflammation.

The second phase of our study focused on evaluating the effects of LM on the FGF-2/PI3K/Akt and Wnt/β-catenin pathways. According to reports, the instability of atherosclerotic plaque and FGF-2 are strongly associated [32]. FGF-2 and its receptors stimulate intraplaque angiogenesis, intimal thickening, and inflammatory processes in atherosclerotic lesions. Since our result showed that the rats in the HCD+LM group reduced FGF-2 levels in aortic endothelial cells, this highlighting the potential of LM in mitigating these pathological processes. Activation of the PI3K/Akt signaling pathway contributes to plaque development by promoting monocyte chemotaxis, macrophage migration, intracellular lipid accumulation, neovascularization, and smooth muscle cell proliferation, ultimately leading to lesion dysfunction [33]. This pathway regulates GSK3β activity through phosphorylated Akt, which influences plaque stability and endothelial function. Our study demonstrated that LM supplementation significantly reduced PI3K and Akt protein levels, suggesting an inhibitory effect on their phosphorylation in aortic endothelial cells. This inhibition, in turn, activated GSK3β, indicating that LM plays a protective role in modulating the PI3K/Akt/GSK3β axis, potentially mitigating atherosclerotic progression.

Wnt signaling plays a crucial role in atherogenesis, in which Wnt-1 becomes proatherogenic in advanced stages of atherosclerosis by contributing to plaque instability, smooth muscle cell proliferation, and vascular inflammation [34]. Under pathological conditions, β-catenin translocates into the nucleus, where it acts as a transcriptional activator by binding to T cell factors (TCFs) or lymphoid enhancer factor (LEF) family members, leading to the induction of target genes such as MMP-7. Our study demonstrated that LM supplementation significantly reduced Wnt-1 activity, resulting in lower protein levels of β-catenin, LEF-1, and MMP-7 in aortic endothelial cells. The ability of LM to downregulate these proteins suggests its protective role in counteracting Wnt-1/β-catenin-driven atherosclerotic changes.

Many polyphenolic components contained in millet shells have been shown in a prior study to suppress the development of atherosclerotic plaques in the aorta [35]. Large concentrations of lipids and cholesterol build up in the aorta and result in fibrous plaques and the fundamental morphometric technique used to assess atherosclerosis burden is Oil red O-staining. As evidenced by our results, histological examination showed LM treatment significantly reduced lesion formation, indicating its protective effects against atherosclerosis. The decrease in lesion severity suggests that LM may aid in modulating lipid metabolism and inflammation, thereby preventing plaque buildup and vascular complications. Also, the reduced Oil Red O staining intensity in the aorta of LM-supplemented rats indicates a significant decrease in lipid accumulation, highlighting the potential of LM in preventing excessive lipid deposition and atherosclerotic plaque formation. These findings support the potential of LM as a dietary intervention for preventing or managing atherosclerosis.

5. Conclusion

The preliminary study shows that 10% millet, along with HCD supplementation, reduces atherosclerosis progression by regulating genes of reverse cholesterol transport, maintaining a normal lipid profile, decreasing lipid biosynthesis in tissues, and reducing activities of lipogenic enzymes. Among the six millets, little millet showed more anti-atherogenic properties than other millets and is ranked from highest to lowest: Little Millet> Kodo Millet> Foxtail Millet> Finger Millet> Proso Millet> Pearl Millet. From the second part of the study, it is revealed that little millet maintained an equilibrium of FGF-2 and Wnt-1/β catenin pathways, decreased serum markers and preserved the integrity of the aortic structure, hence the inhibition of atherosclerosis progression. In conclusion, our study highlights the nutraceutical value of millets as safe, fiber- and polyphenol-rich grains suitable for regular dietary inclusion. The presence of nutraceutical bioactive compounds in millets may contribute to maintaining lipid balance and reducing oxidative as well as inflammatory stress, thereby limiting the progression of atherosclerosis. Overall, these findings reinforce the importance of adopting a nutraceutical approach to cardiovascular health through millet-based diets.

Abbreviations

FGF2, Fibroblast growth factor-2; bFGF, Basic fibroblast growth factor; PI3K, Phosphoinositide 3-kinase; Akt, Protein kinase B; GSK3β, Glycogen Synthase Kinase 3 Beta; Wnt, Wingless-related integration site; β-Catenin, Beta-catenin; LEF-1, Lymphoid enhancer-binding factor 1; MMP-7, Matrix metalloproteinase 7; HC, Hypercholesterolemia; Apo A, Apolipoprotein A; Apo B, Apolipoprotein B; ABCA1, ATP binding cassette A1; RCT, Reverse Cholesterol Transport; HMG CoA, Hydroxy-3-Methylglutaryl Coenzyme A; LCAT, Lecithin-cholesterol acyltransferase; HCD, High Cholesterol Diet; LM, Little millet; PrM,

Proso millet; PeM, Pearl millet; KM, Kodo millet; FiM, Finger millet; FoM, Foxtail millet; EC, Endothelial cell; DMEM, Dulbecco modified eagle medium; FBS, Fetal bovine serum; TC, Total cholesterol; TG, Triglyceride; LDL-C, Low density lipoprotein-cholesterol; HDL-C, High density lipoprotein-cholesterol; AI, Atherogenic Index; G6PDH, Glucose-6-phosphate dehydrogenase; IDH, Isocitrate dehydrogenase; ME, Malic enzyme; CK-MB, Creatine kinase MB; LDH, Lactate dehydrogenase; CRP, C-reactive protein; ELISA, Enzyme linked immunosorbent assay; HRP, Horseradish peroxidase; H&E, Hematoxylin and eosin; RT PCR, Reverse transcription polymerase chain reaction; qPCR, Real-time polymerase chain reaction; RNA, Ribonucleic acid; cDNA, Complementary deoxyribonucleic acid; TI, Tunica intima; TM, Tunica media; TA, Tunica adventitia; EN, Endothelium; NADPH, Nicotinamide adenine dinucleotide phosphate; TCFs, T cell factors.

Conflict of Interests
The author has no conflicts with any step of the article preparation.

Consent for publications
The author read and approved the final manuscript for publication.

Ethics approval and consent to participate
The animal use protocol was approved by the Committee for Control and Supervision of Experiments on Animals (CPCSEA) (Registration Number: 218/GO/ReBi/2000/CPCSEA), IAEC 1-KU-07/21-BCH-AH (39), according to the Government of India accepted principles for laboratory animal use and care.

Declaration of competing interest
The authors declare that there are no competing interests to declare

Availability of data and material
All data generated during this study are included in this published article.

Authors' contributions
Haritha R- Methodology, Resources, Investigation, Data curation, Writing-original draft, Writing—review and editing. **Abhirami S**- Investigation, Writing—review and editing. **Mani Sebastian**- Investigation, Writing-original draft. **Amrutha D S**- Data curation, Investigation. **Salu V S**- Data curation, Investigation, Writing—review and editing. **A Helen**- Conceptualization, Resources, Methodology, Supervision, Validation, Writing—review and editing. All authors read and approved the final manuscript.

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