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Original Research Article

Combination of melatonin and chondroitin sulphate provides better chondroprotection in osteoarthritis in male Wistar rats

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Abstract

Purpose: *To investigate the effect of melatonin (MEL) and chondroitin sulphate (CHS) in osteoarthritis (OA) in male Wistar rats.*
Methods: *A total of 42 Wistar rats (175 - 200 g) were divided randomly into 7 groups (n = 6) including control (received 0.5 mL normal saline orally), sham (received 0.5 mL of normal saline intra-articularly, osteoarthritis-induced (OA; received 2 mg monoiodoacetate intra-articularly) and OA treated groups (CHS: received 300 mg/kg CHS orally, MEL: received 100 mg/kg MEL orally, CHS and MEL: received 300 mg/kg CHS and 100 mg/kg MEL orally), and positive control (diclofenac: received 2 mg/kg diclofenac orally). After 21 days of daily oral treatment, blood samples were obtained via retro-orbital collection, and the synovial fluid was collected and used for biochemical analysis. The matrix metalloproteinases-13 (MMP-13) gene expression and immunohistochemical analysis of collagen type-2 (COL-2) were also carried out on the cartilage.*
Results: *Osteoarthritis-induced group showed significantly lower levels of antioxidant enzymes and Bcl-2, and significantly higher levels of pro-inflammatory markers, caspase-3, ADAMTS-5 levels, and up-regulation in mRNA expression of MMP-13 compared to control and treated groups ($p < 0.05$). Also, treatment with MEL plus CHS significantly reversed down-regulation of COL-2 induced in OA group ($p < 0.05$).*
Conclusion: *Combination of CHS and MEL significantly improves outcomes, lowers levels of pro-inflammatory markers, interleukins, caspase-3, and downregulates MMP-13 gene expression compared to either CHS, MEL, or diclofenac alone in osteoarthritis-induced Wistar rats.*

Keywords: *Osteoarthritis, Melatonin, Chondroitin Sulphate, Antioxidant, Wistar Rats*

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INTRODUCTION

One of the most common types of arthritis is osteoarthritis (OA). Complex problems affecting the entire synovial joint are the hallmarks of this degenerative, long-term, and incapacitating disorder. In 2021, more than 22 % of adults over 40 years old had knee OA, and in the year 2022, it was estimated that over 500 million individuals worldwide were OA patients [1]. Degradation of cartilage and sclerosis of subchondral bone are the primary pathologic characteristics. Wang *et al*

[2] showed that cytokines increase the inflammatory reaction by causing cartilage destruction and consequently production of synovial fluids. This triggers the production of enzymes that destroy the extracellular matrix, such as disintegrin and metalloproteinase with thrombospondin motifs-5 (ADAMTS-5) and matrix metalloproteinases-13 (MMP-13).

Many connective tissues' extracellular matrix contains a significant amount of chondroitin sulphate (CHS). CHS's anti-inflammatory and immunomodulatory properties contribute to its positive effects in OA [3]. In aging and several inflammatory autoimmune disorders, melatonin (MEL) has anti-inflammatory and antioxidant properties [4]. There is no treatment to stop the advancement of OA. Current OA treatments are expensive, harmful, and only effective at reducing pain and unpleasant symptoms. However, they have little effect on the underlying pathologies. The best treatment for OA, according to some studies, should involve a combination of pharmaceutical approaches [5].

There is a dearth of information on the effect of the combination of MEL and CHS on OA. Therefore, this study investigated the effect of melatonin (MEL) and chondroitin sulphate (CHS) in experimental osteoarthritis (OA) in male Wistar rats.

EXPERIMENTAL

Animals

A total of 42 Wistar rats weighing between 175 – 200 g were purchased from the University of Ilorin, Nigeria. The rats were housed and acclimatised for a week under standard conditions (12 h light/dark cycle, humidity, 23 ± 2 ^{o}C) with unrestricted access to feed and water at the Animal House of Bioresources Hub, Ilorin. Ethical approval was obtained from the Ethical Review Committee of the University of Ilorin (approval no. UERC/ASN/2024/2907).

Drugs and chemicals

Melatonin, monoiodoacetate (MIA), and Chondroitin sulphate were procured from Sigma-Aldrich, St. Louis, USA. Moreover, pentobarbital sodium and diclofenac sodium were purchased from Nicholas Piramal Ltd., India. Analytical grade of other materials and chemicals were produced by Elabscience Biotechnology Inc., Texas, USA, and obtained from Bridge Biotechnology Ltd., Nigeria.

Treatment

The 42 rats were randomly divided into seven groups (n = 6; Table 1). Osteoarthritis was induced by anaesthetizing the rats with ketamine/xylazine (50/10 mg/kg body weight) [6]. Thereafter, the left knee was shaved, and 75 % ethanol was used to swab the surface. A 27 G needle was then used to inject 2 mg of MIA intra-articularly into the left knee joint space after it had been dissolved in 50 µL of sterile saline. Treatment lasted for 21 consecutive days.

Body weight determination

Body weights were measured weekly during the study.

Measurement of knee joint swelling

The area of knee joint swelling after MIA injection was accurately measured using a Vernier calliper.

Knee bending test

This was carried out within the physiological range of knee extension/flexion by counting the struggle or squeak reactions to five alternative anatomical extensions and flexions of the knee joint. The knee bend values were graded as follows: 0 corresponds to 180 ^{o}, 0.5 corresponds to 120 – 150 ^{o}, 1 corresponds to 40 – 75 ^{o}, and 2 corresponds to 30 ^{o}.

Table 1: Animal groups and doses of drugs administered

Group (n=6)	Dosage that was administered
Control	0.5 mL oral administration of normal saline
Sham	0.5 mL normal saline intra-articularly
OA	2 mg MIA intra-articularly [7]
OA+CHS	2 mg MIA intra-articularly + 300 mg/kg of CHS per oral once daily [8]
OA+MEL	2 mg MIA intra-articularly + 100 mg/kg of melatonin per oral once daily [9]
OA+CHS+MEL	2 mg MIA intra-articularly + 300 mg/kg of CHS + 100 mg/kg of melatonin per oral, once daily
OA+Diclofenac Sodium (DS)	2 mg MIA intra-articularly + 2 mg/kg body weight diclofenac sodium per oral once daily [10]

Determination of paw withdrawal threshold

Pain perception was determined by evaluating the paw withdrawal threshold (PWT) in response to reflex stimulation by the von Frey filament. Assessment of the secondary tactile allodynia was performed by placing the animals on a Perspex chamber with small holes, which enabled the von Frey hairs to be applied to the hind paws' plantar surface without distracting the rat. The PWT was defined as the lowest value (in grams) necessary to elicit a positive reaction.

Sample preparation

After treatment for 21 days, all the rats were anesthetised and blood samples were obtained through retro-orbital collection. The samples were centrifuged, and serum collected and stored at -20 °C for enzyme-linked immunosorbent assay (ELISA).

Collection of synovial fluid was done before euthanizing the Wistar rats. Prior to collection, a sterile syringe was used to inject 500 µL of phosphate-buffered saline (PBS) into the articular joint cavity of the rat knee. After the PBS was injected and withdrawn five times, the synovial fluid was aspirated out, centrifuged and kept at a cold temperature.

Determination of biochemical parameters

Serum levels of malondialdehyde (MDA), superoxide dismutase (SOD) and catalase were determined based on the methods of Siddique *et al* [11], Magnani *et al* [12], and Aebi [13] respectively. Furthermore, interleukin-1β (IL-1β), tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), vascular endothelial growth factor (VEGF), Bcl-2, caspase-3, and ADAMST-5 were determined using ELISA.

MMP-13 gene expression

For each group, the cartilages were promptly harvested into a TRIzol Reagent (ThermoFisher Scientific) and used for total RNA isolation following the manufacturer's kit protocol (Elabscience Biotechnology Inc., Texas, USA).

The primer name for the gene expression is metallopeptidases (MMP-13) mRNA, accession number is NM_133530.1, Forward Primer sequence (5'-3') - GGGAACCACGTGTGGAGTTAT, Reverse Primer sequence (5'-3') is GACAGCATCTACTTTGTCGCC, optimum temperature is 59 ^{o}C, and the amplicon size is 108 bp.

Immunohistochemical analysis of COL-2

Immunohistochemical analysis was carried out on the cartilage according to the procedure stated by the manufacturer (Elabscience Biotechnology Inc., Texas, USA). Immunohistochemistry-stained slides were examined using an 800 × magnification microscope (Olympus, Germany).

Statistical analysis

Data was analyzed using GraphPad Prism 9.0 (GraphPad Software, San Diego, CA, USA). Data was expressed in mean ± standard error of mean (SEM) and compared using one-way ANOVA. Two-way ANOVA was used for analysing knee circumference and knee bending data. $P < 0.05$ was considered statistically significant.

RESULTS

Effect of MEL and CHS on body weight

Combination regimen with CHS, MEL, and diclofenac alone significantly lowers body weight compared to osteoarthritis-induced group (OA; $p < 0.05$; Table 2).

Effect of MEL and CHS on weekly knee circumference

Knee circumference was significantly higher in OA-induced group compared to control and treatment groups on days 7, 14, 21, and 28 ($p < 0.05$; Table 3).

Effect of MEL and CHS on knee bending test

Treatment with CHS in combination with MEL significantly reduced knee bending score on the last day of the study ($p < 0.05$; Figure 1).

Effect of MEL and CHS on paw withdrawal threshold (PWT)

There was significant decrease in PWT in OA group compared to control and sham groups from day 7 to day 28 ($p < 0.05$).

Following treatment, PWT significantly increased compared to OA group from day 7 to day 28 ($p < 0.05$). Combination regimen resulted in significantly higher PWT on day 28 ($p < 0.05$; Figure 2).

Table 2: Effects of MEL and CHS on the weekly weights

Group	Initial (g)	Week 1 (g)	Week 2 (g)	Week 3 (g)	Week 4 (g)	Week 5 (g)
Control	182.80±7.17	220.8±8.35	222.00±12.98	227.00±15.80	252.3±12.49	257.50±17.86
Sham	191.00±7.72	193.30±8.85	209.00±7.72	222.00±8.28	239.8±8.82	245.00±18.01
OA	175.50±13.52	200.30±11.31	208.80±10.75	228.5±12.55	248.3±15.70	270.50±14.51[a]
OA+CHS	187.80±4.39	216.50±3.80	220.00±5.12	222.00±6.84	238.00±10.45	246.30±11.24[b]
OA+MEL	175.00±5.89	201.80±6.51	210.30±6.91	222.3±8.08	240.30±9.95	248.80±11.55[b]
OA+CHS+MEL	181.5±6.40	181.30±6.21	206.5±6.01	230.00±10.52	232.50±6.51	241.00±6.75[b]
OA+DIC	188.80±9.64	213.50±11.43	206.00±17.07	212.00±17.28	226.30±10.73	230.50±14.55[b]

Mean ± SEM (n = 6). [a]$P < 0.05$ compared to sham, [b]$p < 0.05$ compared to OA. OA. Osteoarthritis, CHS: Chondroitin sulphate, MEL: Melatonin, DIC: Diclofenac

Table 3: Effects of MEL and CHS on knee circumference

Group	Initial (cm)	Day 1 (cm)	Day 4 (cm)	Day 7 (cm)	Day 14 (cm)	Day 21 (cm)	Day 28 (cm)
Control	3.65±0.15	3.65±0.15	3.65±0.15	4.13±0.05	4.65±0.10	4.95±0.13	5.25±0.13
Sham	3.70±0.10	4.05±0.17	4.65±0.21	4.70±0.31	4.85±0.30	5.15±0.36	5.45±0.17
OA	3.25±0.26	4.55±0.22	5.60±0.42	5.70±0.45[a]	5.75±0.10[a]	5.85±0.10[a]	6.08±0.11[a]
OA+CHS	3.85±0.15	5.25±0.15	6.00±0.14	4.75±0.45[b]	5.20±0.16[b]	5.45±0.17[b]	5.65±0.10[b]
OA+MEL	3.70±0.06	4.55±0.10	6.20±0.38	4.40±0.22[b]	4.80±0.14[b]	5.23±0.17[b]	5.45±0.13[b]
OA+CHS+MEL	4.05±0.17	4.65±0.17	5.50±0.21	5.05±0.19[b]	5.10±0.17[b]	5.30±0.21[b]	5.30±0.13[b]
OA+DIC	4.00±0.26	4.70±0.26	5.90±0.06	4.50±0.13[b]	5.05±0.33[b]	5.20±0.29[b]	5.15±0.10[c]

Mean ± SEM (n = 6). [a]$P < 0.05$ compared to sham. [a]$P < 0.05$ compared to control, [b]$p < 0.05$ compared to osteoarthritis-induced group (OA). OA: Osteoarthritis, CHS: Chondroitin sulphate, MEL: Melatonin, DIC: Diclofenac

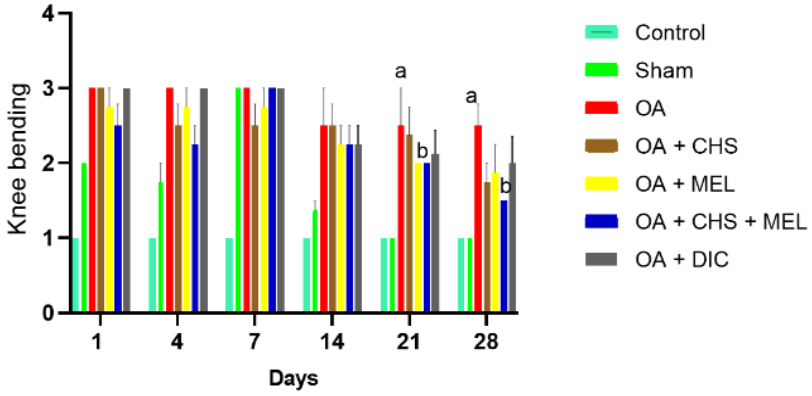

Figure 1: The Effects of MEL and CHS on knee bending score. Mean ± SEM (n=6). [a]$P < 0.05$ compared to control, [b]$p < 0.05$ compared to osteoarthritis-induced group (OA). (OA). OA: Osteoarthritis, CHS: Chondroitin sulphate, MEL: Melatonin, DIC: Diclofenac

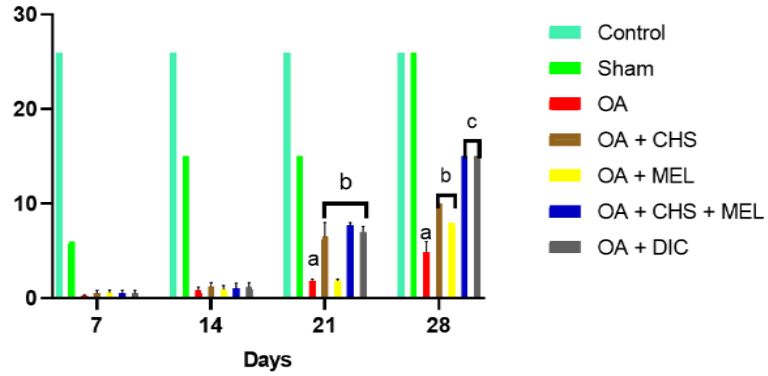

Figure 2: Effects of MEL and CHS on paw withdrawal. Mean ± SEM (n = 6). [a]$P < 0.05$ compared to control, [b]$p < 0.05$ compared to osteoarthritis-induced group (OA). OA: Osteoarthritis, CHS: Chondroitin sulphate, MEL: Melatonin, DIC: Diclofenac

Effect of MEL and CHS on synovial MDA levels

There was a significant increase ($p < 0.05$) in MDA levels of OA group compared to control and sham groups ($p < 0.05$). However, treatment with CHS + MEL resulted in significantly lower MDA levels compared to other groups ($p < 0.05$; Figure 3).

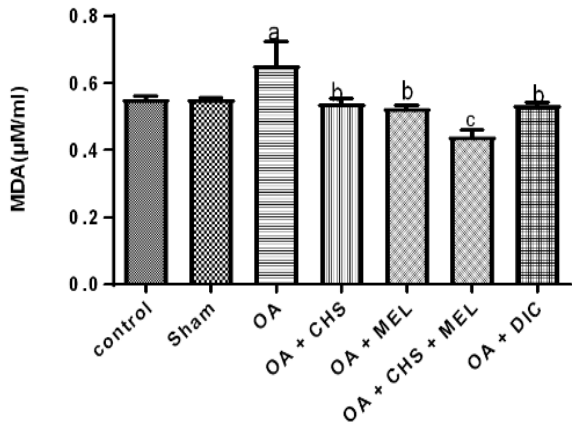

Figure 3: Effects of MEL and CHS on synovial malondialdehyde (MDA) levels. Mean ± SEM (n = 6). [a]$P < 0.05$ compared to control, [b,c]$p < 0.05$ compared to osteoarthritis-induced group (OA). OA: Osteoarthritis, CHS: Chondroitin sulphate, MEL: Melatonin, DIC: Diclofenac

Effect of MEL and CHS on catalase and SOD levels

Levels of catalase (Figure 4 A) and SOD (Figure 4 B) significantly reduced in OA group compared to control and sham groups ($p < 0.05$). Furthermore, catalase and SOD levels were significantly increased in study groups (CHS, MEL, CHS + MEL, diclofenac) compared to OA group ($p < 0.05$). Also, combination group (CHS + MEL) demonstrated significantly higher levels ($p < 0.05$).

Effect of MEL and CHS on synovial IL-1β, IL-6, TNF-α, and serum VEGF levels

Levels of pro-inflammatory markers were significantly increased in OA group ($p < 0.05$). However, IL-1β (Figure 5 A), IL-6 (Figure 5 B), TNF-α (Figure 5 C), and serum VEGF levels (Figure 5 D) were significantly reduced following treatment with CHS, MEL, and CHS + MEL compared to OA group. Furthermore, levels of interleukins were significantly reduced following treatment with CHS + MEL compared to the other groups.

Effect of MEL and CHS on the serum caspase

Level of caspase-3 significantly increase in OA group ($p < 0.05$). Furthermore, treatment with CHS, MEL, CHS + MEL, and DIC resulted in significantly lower levels of caspase-3 ($p < 0.05$; Figure 6).

Effect of MEL and CHS on serum Bcl-2 levels

Level of serum Bcl-2 was significantly reduced in OA group ($p < 0.05$). However, treatment with CHS, MEL, CHS + MEL, and DIC resulted in significantly higher Bcl-2 levels compared to OA group ($p < 0.05$; Figure 7).

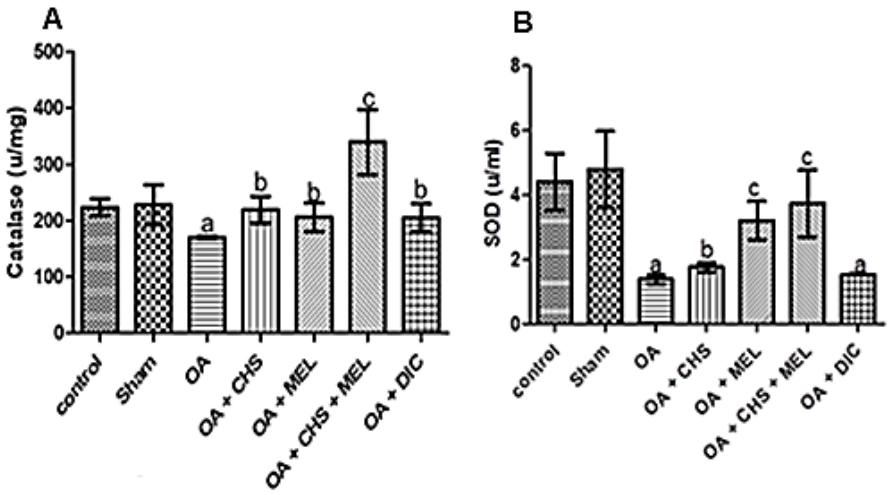

Figure 4: Effects of MEL and CHS catalase and SOD levels. Mean ± SEM (n= 6). [a]$P < 0.05$ compared to control, [b,c]$p < 0.05$ compared to osteoarthritis-induced group (OA). OA: Osteoarthritis, CHS: Chondroitin sulphate, MEL: Melatonin, DIC: Diclofenac

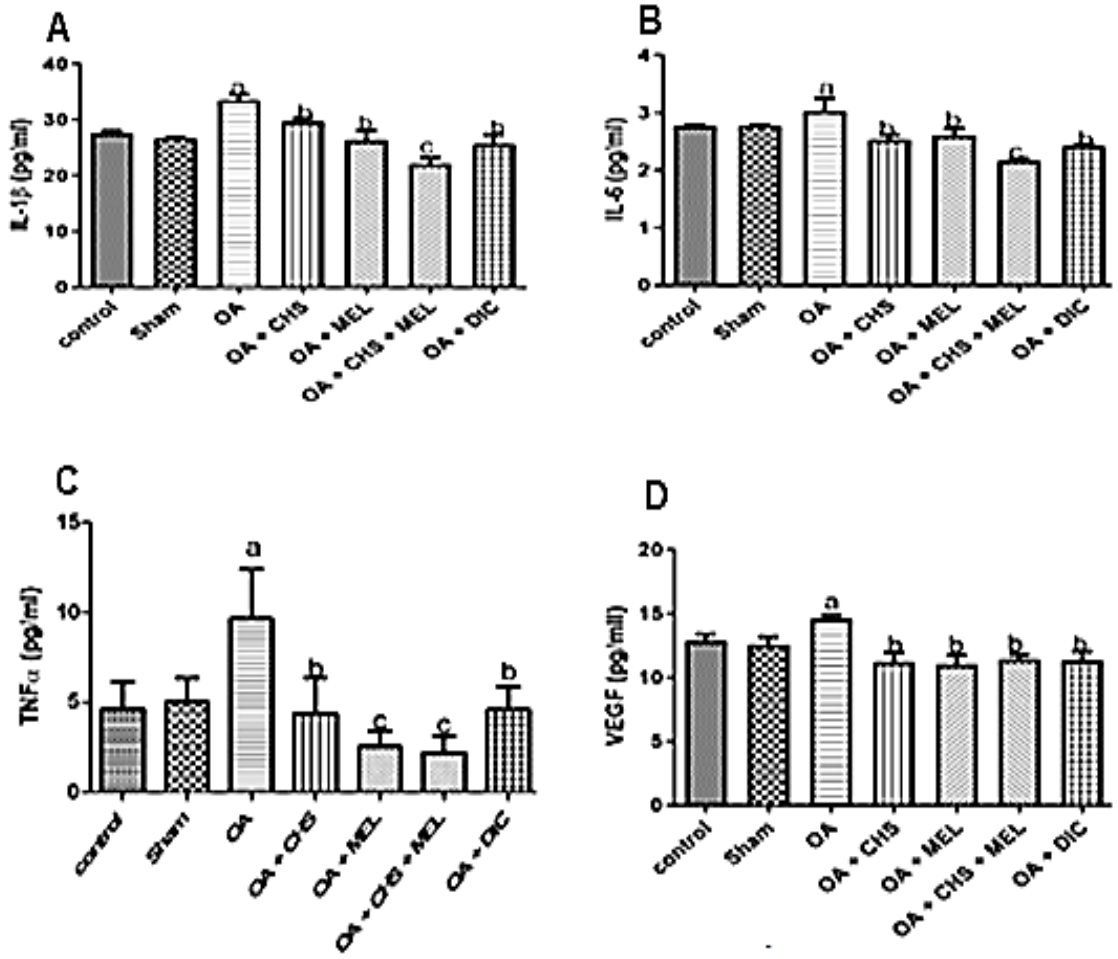

Figure 5: Effects of MEL and CHS on levels of pro-inflammatory markers. Mean ± SEM (n= 6). $^{a}P < 0.05$ compared to control, $^{b,c}p < 0.05$ compared to osteoarthritis-induced group (OA). OA: Osteoarthritis, CHS: Chondroitin sulphate, MEL: Melatonin, DIC: Diclofenac

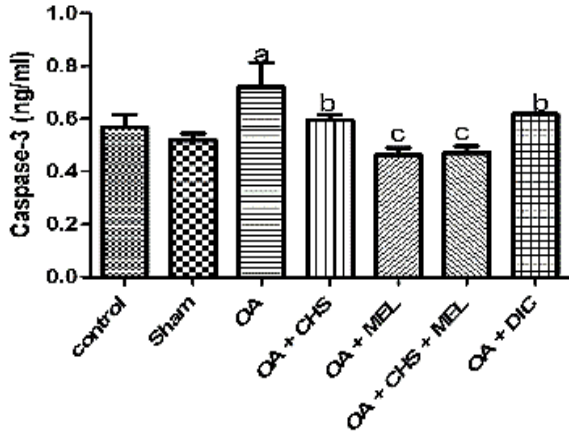

Figure 6: Effects of MEL and CHS on levels of serum caspase-3. Mean ± SEM (n = 6). $^{a}P < 0.05$ compared to control, $^{b,c}p < 0.05$ compared to osteoarthritis-induced group (OA). OA: Osteoarthritis, CHS: Chondroitin sulphate, MEL: Melatonin, DIC: Diclofenac

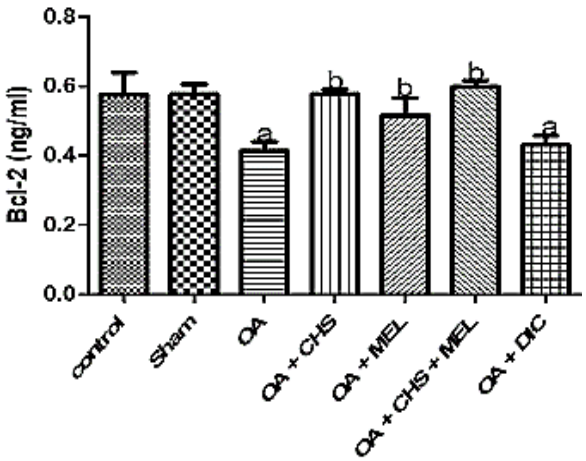

Figure 7: Effects of MEL and CHS on serum Bcl-2 level. Mean ± SEM (n= 6). $^{a}P < 0.05$ compared to control, $^{b}p < 0.05$ compared to osteoarthritis-induced group (OA). OA: Osteoarthritis, CHS: Chondroitin sulphate, MEL: Melatonin, DIC: Diclofenac

Effect of MEL and CHS on serum ADAMTS-5 levels

Level of ADAMTS-5 significantly increases in OA group ($p < 0.05$). Treatment with CHS, MEL, CHS + MEL, and DIC resulted in significantly lower levels of ADAMTS-5 compared to OA group ($p < 0.05$). Furthermore, treatment with CHS + MEL resulted in significantly lower levels of ADAMTS-5 compared to other groups ($p < 0.05$).

Effect of MEL and CHS on MMP-13 gene expression

Gene expression of MMP-13 was significantly upregulated in OA group ($p < 0.05$). Furthermore, MMP-13 gene expression was significantly down-regulated following treatment with CHS, MEL, CHS + MEL, DIC compared to OA group ($p < 0.05$). Also, MMP-13 gene expression was significantly down-regulated following treatment with CHS + MEL compared to other groups ($p < 0.05$).

Immunohistochemical analysis of COL-2 in articular cartilage of Wistar rats

The control group (A) revealed up-regulation of COL-2, Sham (B) showed moderate expression, OA (C) revealed little expression, OA + CHS treated group (D) revealed moderate expression, OA + MEL treated group (E) revealed moderate expression of COL-2 in territorial and inter-territorial spaces, OA + CHS + MEL treated group (F) revealed up regulation of COL-2 in the territorial and less expression in the inter-territorial spaces, while OA + diclofenac treated group (G) revealed little expression of COL-2 in the territorial and less expression in the inter-territorial spaces (Figure 10).

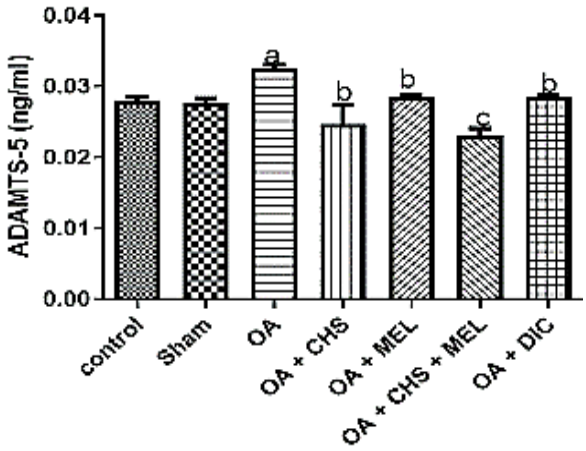

Figure 8: Effects of MEL and CHS on serum ADAMTS-5 level. Mean ± SEM (n= 6). $^{a}P < 0.05$ compared to control, $^{b,c}p < 0.05$ compared to osteoarthritis-induced group (OA). OA: Osteoarthritis, CHS: Chondroitin sulphate, MEL: Melatonin, DIC: Diclofenac

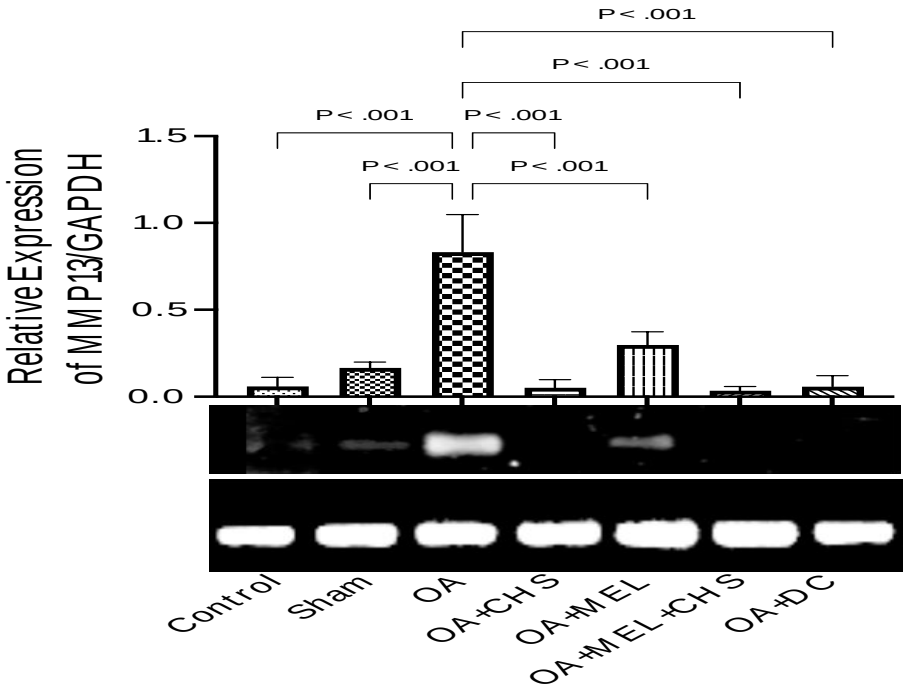

Figure 9: Effects of MEL and CHS on mRNA expression of MMP-13. OA, Osteoarthritis; CHS, Chondroitin sulphate; MEL, Melatonin; DIC, Diclofenac. OA: Osteoarthritis, CHS: Chondroitin sulphate, MEL: Melatonin, DIC: Diclofenac

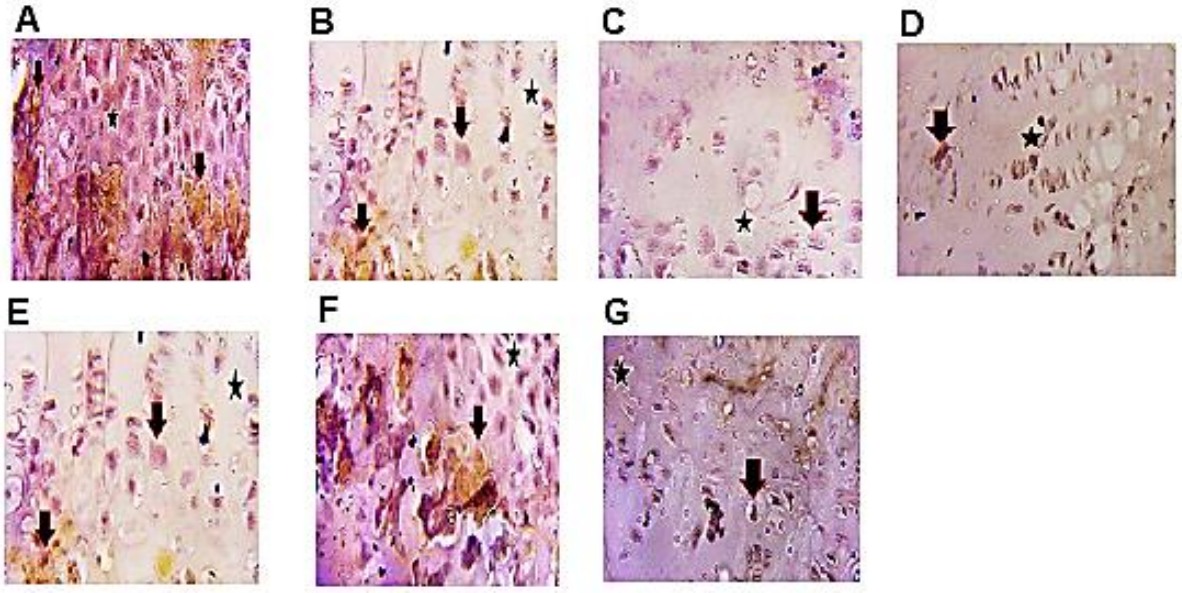

Figure 10: Effect of MEL and CHS on immunohistochemical expression of COL-2. ***Key:*** A: Control, B: Sham, C: OA, D: OA + CHS, E: OA + MEL, F: OA + CHS + MEL, G: OA + DIC. OA is Osteoarthritis, CHS is Chondroitin sulphate, MEL is Melatonin, DIC. is Diclofenac. Black star: inter-territorial area, black arrow: territorial area, brown precipitates indicate immunochemical detection of COL-2. (Mag. x800; Scale bar: 51 µm)

DISCUSSION

Osteoarthritis (OA) has been reported to be associated with behavioural alterations. Those behavioural alterations, such as decreased knee bending score, decreased paw withdrawal threshold, and increased knee circumference, observed in OA-induced rats may be the direct result of inflammation. However, improvement in behavioural tests in intervention groups in this study may be as a result of the anti-inflammatory properties of MEL and CHS. The fact that MEL and CHS improved behavioural deficits comparable with diclofenac (DIC) suggests that they share similar anti-inflammatory properties. The current study showed an increase in lipid peroxidation, as evident by MDA level elevation, in OA-induced rats. It has been documented that overproduction of ROS causes DNA destruction and chondrocyte loss [14]. Results from this study indicated that levels of catalase and SOD in OA-induced rats were significantly reduced compared to normal rats, demonstrating that inadequate or low level of antioxidants may lead to cartilage destruction. In this study, the fact that MEL, CHS, and both combinations reversed the elevated MDA and the reduced antioxidant enzymes might be as a result of their antioxidant properties [3,15].

Increased levels of pro-inflammatory factors, including TNF-alpha, IL-1β, IL-6, and VEGF in OA-induced rats have been reported to be responsible for the development of proteolytic enzymes like MMP-13, which causes damage to the cartilage [16]. Collectively, pro-inflammatory cytokines are potent catabolic substances in synovial fluid, which stimulate OA process via up-regulation of inflammatory and catabolic responses and cause cartilage matrix degradation [17]. In this study, treatment with MEL and CHS resulted in lower levels of pro-inflammatory factors, possibly due to their anti-inflammatory properties, anabolic and anti-catabolic effects [14,3] respectively. It has been reported that excessive ROS production induced apoptosis of chondrocytes and irreversible damage to articular cartilage [18]. This may be due to the ability of ROS to open the transition pore of mitochondria and cause apoptogenic substances, like cytochrome c, to be released in order to stimulate caspase reactions [19]. Significant increase in the activity of antioxidant enzymes and Bcl-2, followed by a reduction of caspase-dependent apoptosis, demonstrated the effectiveness of MEL, CHS, and their combination in reducing apoptosis caused by oxidative stress in OA rats. This might be due to their anti-apoptotic activities.

Earlier studies have reported that lower COL-2 is a predisposing or contributing factor to OA progression [14]. Also, MMP-13 and ADAMST-5 are specifically linked to articular cartilage destruction in OA by degrading COL-2. This is evident in this study as there was a significant increase in ADAMST-5 activity and a significant reduction in COL-2. However, the significant increase in COL-2 level may be due to MEL and CHS's ability to inhibit the activities of MMP-13

and ADAMTS-T5 and stop OA progression. The breakdown of bone and cartilage in osteoarthritis is mostly caused by MMPs, especially MMP-13. This is in agreement with the findings of this study, as MMP-13 gene expression was significantly up-regulated in OA untreated group compared to others. However, the down-regulation of MMP-13 mRNA expression by MEL, CHS, and their combination may be due to their anti-inflammatory properties. Furthermore, MEL, CHS, and their combination significantly reversed increased interleukin levels, which are known to elevate MMP-13 levels and reduce newly synthesized matrix protein levels, thereby causing an enhanced degradation of tissues in the pathogenesis of OA [20].

CONCLUSION

Combination of CHS and MEL significantly improves outcomes, lowers levels of pro-inflammatory markers, interleukins, caspase-3, and downregulates MMP-13 gene expression compared to either CHS, MEL, or diclofenac alone in OA-induced animals. Thus, this regimen may be considered as a treatment option for osteoarthritis due to the multi-factorial nature of its etiopathogenesis.

DECLARATIONS

Acknowledgement/Funding

We duly acknowledged the Staff of the Physiology Department, University of Ilorin, Ilorin, Nigeria, for their support during this study.

Ethical approval

As stated in the 'Experimental' section.

Use of Artificial intelligence/Large language models

We also declare that we did not use Generative artificial intelligence (AI) and AI-assisted technologies in writing the manuscript.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Salam Babatunde, Maryam Tayo Ayinla, and Luqman Aribidesi Olayaki participated in conceiving and designing of the study, and data collection. Abraham Olufemi Asuku analysed the data, and Salam Babatunde Saliu wrote the manuscript. All authors read and approved the manuscript for publication.

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