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Original Research Article

S-ALLYL-L-CYSTEINE-INDUCED ANTI-INFLAMMATORY AND ANTI-APOPTOTIC EFFECTS IN CHONDROCYTES IS ASSOCIATED WITH SUPPRESSION OF THE MITOCHONDRIAL INFLAMMATION PATHWAY

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Abstract: *One major aspect to consider while dealing with osteoarthritis is oxidative stress. This deleterious oxidative stress is responsible for the increased production of Reactive Oxygen Species and triggers several inflammatory pathways, including Mitochondrial Inflammation Pathway (MIP), which leads the cell to apoptosis. Chondrocytes, under oxidative stress, are unable to synthesize cartilage efficiently. S-Allyl-L-Cysteine (SAC) is known to be a potent natural, water-soluble antioxidant derived from garlic whose antioxidant properties have been evaluated in several diseases at the molecular level; other than osteoarthritis. Herein, we investigated the potential of S-Allyl-L-Cysteine (SAC) preconditioning of chondrocytes against oxidative stress-mediated mitochondrial inflammation. SAC priming alleviated oxidative stress-induced injuries by significantly improved cell viability, morphology and activated cell migration. In addition, decreased lactate dehydrogenease, increased superoxide dismutase release and retention of glycosaminoglycans were observed. SAC preconditioning ameliorated the injurious effects of oxidative stress as revealed by significant downregulation in gene expression of hypoxia-inducible factor 1α (Hif-1α), Xanthine Oxidase (XO), Caspase-9 (Casp-9), Caspase-3(Casp-3), Interleukin 1 beta (IL-1β) and inducible nitric oxide synthase (iNOS). These findings suggest that SAC preconditioning might enhance the antioxidant and anti-inflammatory efficacy of chondrocytes by regulating the MIP pathway and improving cellular responses.*

Keywords: Osteoarthritis, Chondrocytes, S-Allyl-L-Cysteine (SAC), Antioxidant, Mitochondrial Inflammation, Anti-apoptotic, Anti-inflammation

Introduction

Osteoarthritis (OA) is an inflammatory disease characterized by the degeneration of cartilage tissue, especially in the knee and hip joints. In severe OA, damaged cartilage tissue results in swollen joints and cartilage dysfunction (Fosang and Beier, 2011). The damage caused is due to the disruption of Collagen and Aggrecan meshwork in the cartilage's deep zone, and the chondrocyte alignment is compromised. During OA, the chondrocytes experience oxidative stress, leading to the production of reactive oxygen species (ROS), inflammatory cytokines, pro-oxidant enzymes and triggers several inflammatory pathways such as ER-STRESS pathway and Mitochondrial Inflammation pathway (MIP), which ultimately leads to cells apoptosis. Consequently, the chondrocytes lose their cartilage regeneration potential and die out. Of several inflammation pathways being activated during OA, MIP is critical and is triggered when the chondrocytes experience oxidative stress microenvironment. During MIP, the electron transport chain of mitochondria is inhibited. The main regulator of oxidative stress in MIP is hypoxia-inducible factor-1α (Hif-1α) which can trigger apoptosis by up-regulation of the pro-apoptotic proteins like Caspase-9. The Caspase-9 further cleaves the downstream Caspase-3 and 6, finally lead the cell to death (Boyd et al., 1994; Fulda et al., 2000; Li et al., 1997; Nagata and Golstein, 1995; Nicholson and Thornberry, 1997; Wei et al., 2001). $CoCl_2$ has been reported to impersonate various aspects of oxidative stress *in vitro*, such as up-regulation of Hif1α, Caspase-3 and Caspase-9 and the production of Reactive Oxygen Species (ROS) like superoxide anion (O_2^-). It also initiates DNA fragmentation and loss of mitochondria membrane potential, and the cells acquire apoptotic morphology. Different strategies are being used to treat the OA. Still, they are not successful as cartilage is not subjected to systemic regulation because of its avascular, neural and alymphatic nature. Due to prolonged oxidative stress microenvironment, the

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chondrocytes continuously face oxidative stress, and their cartilage regeneration potential is compromised. Introducing and reinforcing the chondrocytes' anti-oxidant capabilities seems a promising approach in making chondrocytes combat persistent oxidative stress and continue their function without being affected. Different plant extracts such as those of *Tridaxprocumbens* (Bahule et al., 2018), Psidiumguajava L. cv. Red Suprema (Alvarez-Suarez et al., 2018) and Glycyrrhizaglabra L. (Hejazi et al., 2017) have been observed to possess antioxidant and radical scavenging potential (Lan et al., 2012). S-Allyl-L-Cysteine (SAC), a sulfur-containing compound **(Figure 1)** present in garlic, is formed by the catabolism of γ-glutamyl-S-allylcysteine. Compared to other garlic compounds, SAC is stable, less toxic and water soluble. Its amount is highest in garlic aged up to 22 months (Colín-González et al., 2012). SAC is reported to prevent apoptosis via the downregulation of many apoptotic proteins like Caspase-3, Caspase-9, and Hif-1α and XO and scavenges ROS like superoxide anion (Colín-González et al., 2015; Maldonado et al., 2003; Medina-Campos et al., 2007; Orozco-Ibarra et al., 2016).

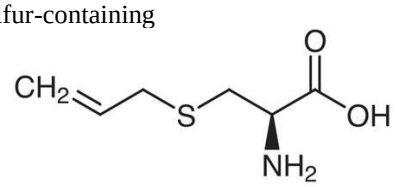

Figure 1: Chemical structure of S-Allyl-L-cysteine. Source: Tokyo Chemical Industry. Catalog # A-1468. Source link: https://www.tcichemicals.com/AU/en/p/A1468

Previously, SAC has been evaluated for its protective properties against diseases like cardiovascular diseases, neural(Colín-González et al., 2015), and renal(Maldonado et al., 2003) disorders etc. However, its role in inducing nti-apoptotic properties in chondrocytes has not been evaluated at the molecular level.

Based on the antioxidant properties possessed by the SAC, it was proposed that preconditioning the chondrocytes with SAC will reduce apoptosis via attenuation of MIP and make them survive oxidative stress better. It was further anticipated that SAC preconditioning would enable chondrocytes to perform their characteristic metabolic processes under oxidative stress. Therefore, the present study assessed the antioxidant and anti-apoptotic potential of S-Allyl-L-Cysteine (SAC) against $CoCl_2$ damaged chondrocytes.

Materials and Methods

Isolation of Chondrocyte from knee cartilage

The animals used in this study were treated as per *Instructions of the care*, and *use of laboratory animals* published by the U.S. National Institute of Health (NIH publication No. 85-23, revised 1985) and the study was approved by the institutional review committee at the National Centre of Excellence in Molecular Biology, Lahore. Healthy male Wister rats 4-5 months old were anaesthetized using chloroform. The isolated knee joints were washed with 1X PBS supplemented with penicillin and streptomycin and first incubated in pronase (2% w/v in 0.15 M NaCl) and then overnight in 1.5% w/v collagenase-B prepared using DMEM. The debris was removed, and the filtrate was centrifuged at 1200 rpm for 13min. The resulting pellet was re-suspended in a complete DMEM (10% FBS) medium and incubated (37°C, 5% CO_2) for cell attachment (Gouze et al., 2002). The cells were sub cultured up to Passage 3 before preconditioning.

Dose optimization of SAC and $CoCl_2$

Time and concentration-dependent dose optimization for SAC and $CoCl_2$ were evaluated through MTT (Sigma Cat No. M2128) viability assay by seeding cells (5 x 10^3 cells/well) in triplicates in 96 well plates. Different concentrations of SAC and $CoCl_2$ were selected by reviewing the previously published data. Optical density was measured at 570 nm wavelength and 650nm (reference wavelength) using Spectrostar Nano (BMG LABTECH) microplate reader.

Experimental design

The following experimental groups were designed based on the data generated during dose optimization.

A. *Control:* Cells grown in 10% FBS-supplemented DMEM medium and neither treated with SAC nor with $CoCl_2$.
B. *$CoCl_2$-400-24h (Stress Control):* Cells to which only stress of 400µM of $CoCl_2$ was given for 24 hours.
C. *SAC-5mM-24h & $CoCl_2$-400-24h:* Cells preconditioned with 5mM SAC for 24 hours and then stress of 400µM of $CoCl_2$ was given for 24 hours
D. *SAC-5mM-48h & $CoCl_2$-400-24h:* Cells preconditioned with 5mM SAC for 48 hours and then stress of 400µM of $CoCl_2$ was given for 24 hours

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E. *SAC-7.5mM-24h & $CoCl_2$-400-24h:* Cells preconditioned with 7.5mM SAC for 24 hours and then stress of 400µM of $CoCl_2$ was given for 24 hours

F. *SAC-7.5mM-48h & $CoCl_2$-400-24h:* Cells preconditioned with 7.5mM SAC for 48 hours and then stress of 400µM of $CoCl_2$ was given for 24-hour

Biochemical analysis

Cell cytotoxicity assessment

The culture medium of each group was used to perform a Lactate Dehydrogenase (LDH) assay using (Roche Diagnostics, Cat No. 04744926001) according to the manufacturer's protocol. Briefly, 50µl culture medium of each experimental group was mixed with 100µl reaction mixture (Catalyst + Dye Solution) and incubated at room temperature for 30 minutes. Next, 50µl of LDH Stop Solution was added, and optical density was measured at 490nm and 650nm (reference wavelength) using Spectrostar Nano (BMG LABTECH) microplate reader.

Sulfated Glycosaminoglycan (sGAG) evaluation

The sulfated glycosaminoglycan assay kit (Blyscan, Biocolor, UK) was used to conduct the assay. The assay was performed according to the manufacturer's protocol, and absorbance was taken at 656nm using Spectrostar Nano (BMG LABTECH) microplate reader.

Superoxide Dismutase (SOD) activity assessment

Superoxide dismutase activity was determined by following manufacturer protocol (SOD Determination Kit, Sigma Cat No. 19160). The experiment was run in triplicate, and absorbance was taken at 656nm using Spectrostar Nano (BMG LABTECH) microplate reader.

Gene expression analysis

Chondrocytes were plated into 6-well plates. The seeding density was 7 X 10^4 cells/well. The cells were left to attain 60-80% confluency; a pre-treatment strategy was applied corresponding to the experimental design. The experiment was run in triplicate.

RNA and cDNA preparation

RNA was isolated soon after the completion of the experiment following the protocol described in TRIzol Reagent (Sigma Cat No. T9424). The RNA pellets were dissolved in 20ul of DEPC-treated water, and their concentration was determined using NanoDrop-1000. cDNA synthesis was performed using the Revert Aid H-Minus First Strand cDNA Synthesis Kit (Invitrogen Cat# K1612). cDNA of all the experimental groups were made soon after the RNA extraction.

Analysis of relative quantification of gene expression via Real-Time Polymerase Chain Reaction

The relative quantification in gene expression of apoptotic and inflammatory molecular markers (Table 1) among different experimental groups was analyzed by executing Real-time PCR. Maxima SYBR Green/ROX qPCR Master Mix (2X) (Thermofisher cat # K0221) was used and the remaining components were added according to the manufacturer's protocol in N11471 ver 2.2 PikoReal Real-time PCR System. The expression of each gene was normalized by using β-actin (internal control). Each real-time experiment was executed in triplicate. Data acquisition was performed during the extension step.

Table 1: Primers sequences along with product size

Primer	Sequence (5'-3')	Annealing temperatures (ºC)	Product size (bp)
Hif-1a(F)	CCCATCCATGTGACCATGAG	58	263
Hif-1a(R)	AATCAGCACCAAGCACGTCA		
Caspase-3 (F)	ACCGATGTCGATGCAGCTAAC	59	203
Caspase-3 (R)	ACAGGTCCGTTCGTTCCAAA		
Caspase-9 (F)	AGAAGCTTCAGCACCGCTTC	60	220
Caspase-9 R)	TCGATGGACACAGAGCATCC		
XO (F)	AAGCTTGGCTGTGGAGAAGGT	59	240
XO (R)	AATGCCAGGAGTGCAGAACC		
IL-1β(F)	TCTGACCCATGTGAGCTGAA	58	170
IL-1β(R)	GTGCCGTCTTTCATCACACA		
iNOS (F)	GCTACACTTCCAACGCAACA	58	116
iNOS (R)	CATGGTGAACACGTTCTTGG		
β-actin (F)	GCTGTGTTGTCCCTGTATGC	57	200
β-actin (R)	GAGCGCGTAACCCTCATAGA		

Results

SAC and $CoCl_2$ affect Chondrocytes' viability in a time and dose-dependent manner

Sixteen concentrations of $CoCl_2$ were chosen, and their effect on cell viability was assessed via MTT assay. Cell viability was observed to be decreased with the increase in $CoCl_2$ concentration. The selected SAC concentrations were 5mM and 7.5mM versus control each for 24 hours (5mM: 0.941 ± 0.088, 7.5mM: 0.953 ± 0.053 vs Control: 0.899 ± 0.049 respectively) and 48 hours (5mM: 0.975 ± 0.032, 7.5mM: 1.007 ± 0.022 vs Control: 0.944 ± 0.162 respectively)No significant increase in cell viability was observed with the increase in SAC

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concentration as compared to control (**Figure 2-a, b)**. 400µM $CoCl_2$ for 24h was chosen as the cell viability at this concentration ranged between 50 to 60% versus control (400µM $CoCl_2$: 0.281 ± 0.073 vs Control: 0.485 ± 0.071) **(Figure 2-c)**.

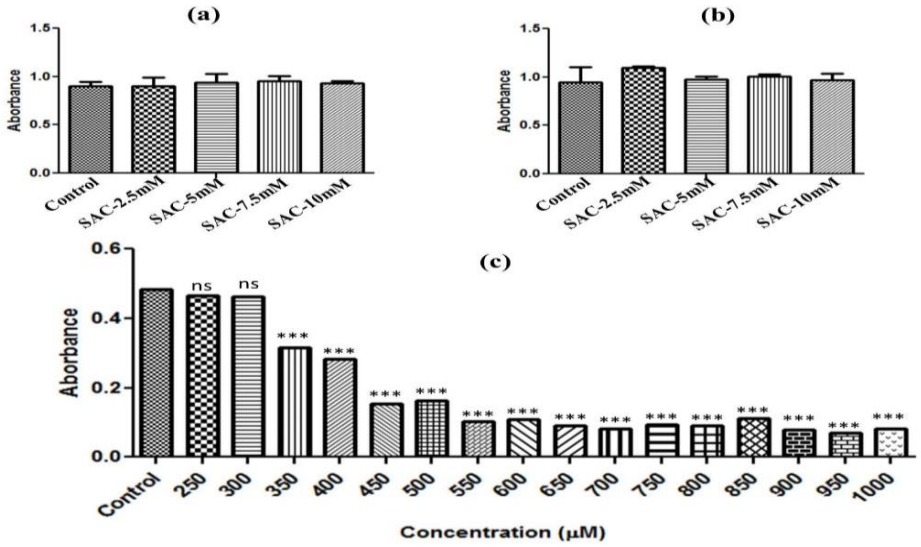

Figure 2: ***(a & b) Graphical representation of MTT absorbance at different concentrations of SAC at 24h and 48h, respectively. (c) Graphical representation of $CoCl_2$ dose optimization*** *and its analysis using One-way Anova.*p = 0.05, **p= 0.01 and **p<0.001*

SAC preconditioning triggers cell migration

The effect of selected SAC doses (i.e., 5 mM and 7.5 mM) on cell migration was assessed by scratch wound healing assay. The cells were seeded in a 6-well plate and were left to form a monolayer. We found that the wound closure was easily observable at 12 hrs (SAC-5mM: 38.760 ± 0.978mm, SAC-7.5mM: 22.290 ± 2.098mm **vs** Control: 50.535 ± 8.846mm) and significant cell migration was evident at 24 hrs (SAC-5mM: 29.567 ± 2.683mm, SAC-7.5mM: 19.579 ± 2.061mm **vs** Control: 36.578 ± 1.960mm). The wounds were almost closed at 48 hrs (SAC-5mM: 10.981 ± 3.849mm, SAC-7.5mM: 6.483 ± 1.391mm **vs** Control: 22.092 ± 2.560mm) as compared to control cells **(Figure 3-a, b)**.

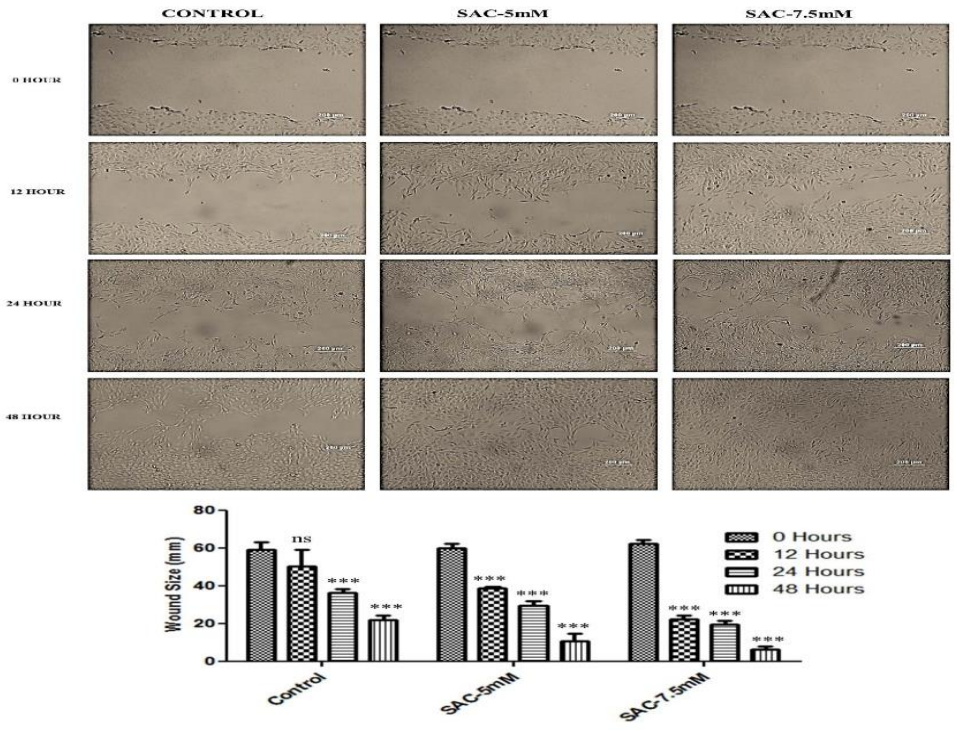

*Figure 3:Pictorial and Graphical representation of Scratch wound healing assay to assess the effect of SAC preconditioning of chondrocytes in time and dose-dependent manner. The wound closure was evaluated at 0, 12, 24 and 48 hours.*p = 0.05, **p= 0.01 and **p<0.001*

SAC preconditioned Chondrocytes tend to retain better morphological features in oxidative stress microenvironment

Phase-contrast microscopy showed that the 24-hour **(Figure 4-c, d)** and 48-hour **(Figure 4-e, f)** SAC preconditioned cells had fibroblastic morphology and possessed prolonged membrane extensions, less detachment, less change in their morphology and were confluent as compared to stress control **(Figure 4-c, d)**. The stress control showed a visible change in the morphology; the cells were dilated. Their cytoplasmic membrane underwent disintegration **(Figure 4-b)**. The cells also showed a prominent decrease in cell confluency as compared to the control **(Figure 4-a, b)**.

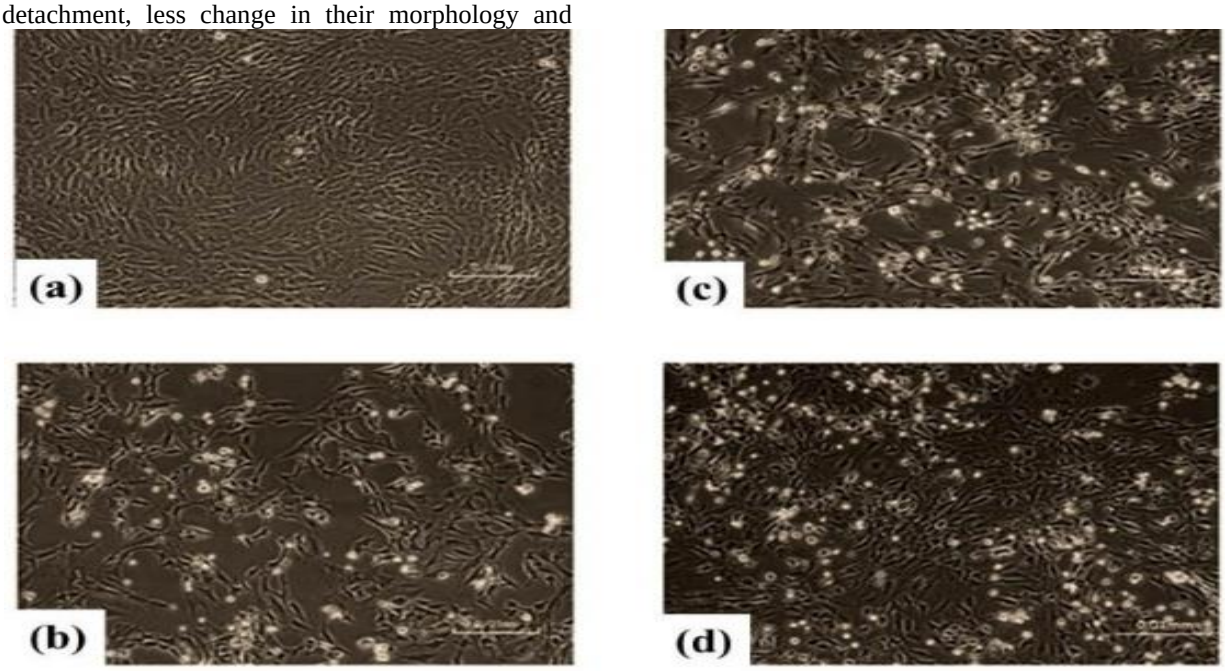

Figure 4: Phase contrast microscopy (10X magnification) of different experimental groups preconditioned with SAC followed by $CoCl_2$-500µM oxidative stress induction. (a) Normal control, (b) Chondrocytes exposed to $CoCl_2$-500µM mediated oxidative stress, (c) Chondrocytes preconditioned with SAC-5mM for 24 hours, (d) Chondrocytes preconditioned with SAC-5mM for 48 hours, (e) Chondrocytes preconditioned with SAC-7.5mM for 24 hours, (f) Chondrocytes preconditioned with SAC-7.5mM for 48 hours.

SAC preconditioned Chondrocytes showed enhanced viability in oxidative stress conditions

Cell viability of all the groups was accessed by performing MTT assay. It does so by analyzing cellular metabolic activities. Cells that are metabolically active and viable actively reduce the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) salt into its insoluble Formazan. The absorbance values of MTT assay revealed that preconditioning of chondrocytes with 5mM and 7.5mM SAC for 48 hours (SAC-5mM: 0.826 ± 0.044, SAC-7.5mM: 0.752 ± 0.015) were capable of significantly withstanding the oxidative stress as compared to the $CoCl_2$-treated group (0.555 ± 0.019)**(Figure 5-a).**

SAC preconditioning reduces cell cytotoxicity via a reduction in LDH release

Lactate Dehydrogenase (LDH) Assay was performed to determine cellular toxicity. LDH is an enzyme released into the culturing medium by the cells when their cell membrane is damaged, and consequently, cellular death occurs. The LDH release is directly proportional to cell death. The absorbance values revealed significant reduction in LDH release in 5mM and 7.5mM-SAC preconditioned chondrocytes for 24 hours (0.185 ± 0.005 & 0.199 ± 0.004 respectively) and 48 hours (0.199 ± 0.001 & 0.167 ± 0.004 respectively) in response to CoCl2-400µM mediated oxidative stress for 24 hours (0.672 ± 0.011) **(Figure 5-b).**

SAC preconditioned Chondrocytes have better retention of Glycosaminoglycan

The sulfated Glycosaminoglycans (sGAG) are in higher amounts in cartilage but during osteoarthritis, sGAG translocate, and their level in the synovial fluid increases. The absorbance data of the present study revealed that the amount of sGAG released in the medium by SAC preconditioned chondrocytes of 24 hours (SAC-5mM: 0.044 ± 0.002, SAC-7.5mM:

0.039± 0.005) and 48 hours (SAC-5mM: 0.042 ± 0.007, SAC-7.5mM: 0.048 ± 0.0007) was significantly lower as compared to the $CoCl_2$-treated chondrocytes (0.515 ± 0.0007), and the SAC retained more GAG content preconditioned chondrocytes **(Figure 5-c).**

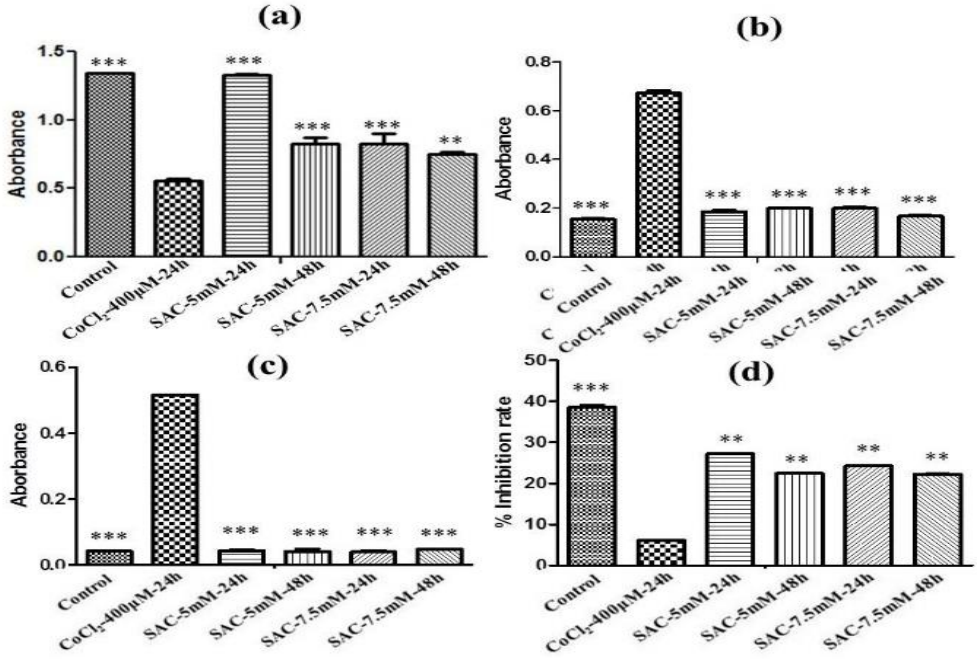

Figure 5:Graphical representation of MTT (a), LDH (b), sGAG (c), and SOD (d) of chondrocytes preconditioned with SAC followed by $CoCl_2$ mediated oxidative stress and its analysis using One-way Anova. High metabolic activity was observed in sac-pretreated chondrocytes as compared to stress. Additionally, SAC-pretreated chondrocytes revealed less LDH, sGAG and SOD activity than the stress group.***p*= 0.05, ****p*= 0.01 **and** *****p* <0.001

SAC preconditioning boosts the anti-oxidant activity of Chondrocytes

Superoxide Dismutase (SOD) is an important anti-oxidative enzyme in the cell capable of reducing superoxide ions into less harmful products. Contrary to SOD, Xanthine Oxidase is a pro-oxidant enzyme involved in producing reactive oxygen species, especially superoxide anion. SOD assay was performed on the culture media. At the same time, XO activity was assessed quantitatively through qPCR technology, and it was observed that the 24 hours SAC preconditioned chondrocytes showed a significant increase in SOD activity (SAC-5mM: 27.25 ± 0.030%, SAC-7.5mM: 24.3 ± 0.051%). SOD activity in 48 hours of SAC preconditioned chondrocytes (SAC-5mM: 22.54 ± 0.029%, SAC-7.5mM: 22.27 ± 0.052%) was also increased as compared to $CoCl_2$-treated cells (6.13 ± 0.025%) **(Figure 5-d)** and showed a simultaneous reduction in fold change of XO gene expression in 24 hours SAC preconditioned chondrocytes (SAC-5mM: 5.926 ± 2.353, SAC-7.5mM: 2.333 ± 3.018) the XO activity in 48 hours SAC preconditioned chondrocytes was also reduced to a significant level (SAC-5mM: 2.575 ± 2.238, SAC-7.5mM: 0.044 ± 0.029) **(Figure 6-d)** in comparison to $CoCl_2$-treated chondrocytes (26.726 ± 9.918).

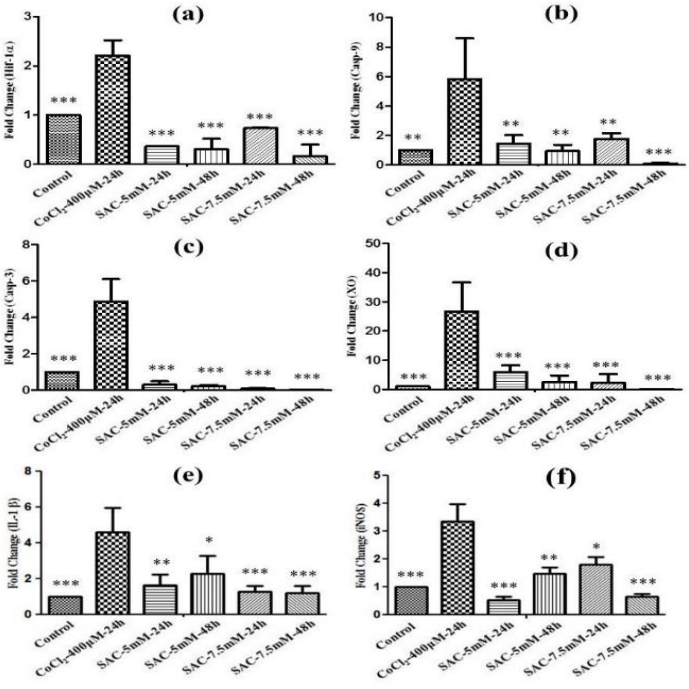

Figure 6: Expression of Hif-1α (a), Caspase-9 (b), Caspase-3 (c), Xanthine Oxidase-XO (d), IL-1β (e) and iNOS (f) in chondrocytes preconditioned with SAC followed by $CoCl_2$ mediated oxidative stress and its analysis using One-way Anova. Significant

*downregulation of all the inflammatory markers was observed in SAC pretreated groups compared to the stress group.*p = 0.05, **p = 0.01 and **p< 0.001*

SAC preconditioning reduces apoptosis via inhibition of the Mitochondrial Inflammation Pathway

Hif-1α is the main regulator of MIP, which up-regulates during oxidative stress. Downstream the MIP, two other very important pro-apoptotic proteins are called Caspase-3 and Caspase-9. The increased gene expression of all of these molecular markers of MIP leads the cell towards apoptosis. The present study revealed that24 48 hours SAC-preconditioned chondrocytes showed a significant reduction in gene expression of Hif-1α (SAC-5mM: 0.362 ± 0.011, SAC-7.5mM: 0.737 ± 0.008) and (SAC-5mM: 0.306 ± 0.214, SAC-7.5mM: 0.161 ± 0.240) respectively, Caspase 3 (SAC-5mM: 0.311 ± 0.321, SAC-7.5mM: 0.085 ± 0.046) and (SAC-5mM: 0.220 ± 0.123, SAC-7.5mM: 0.020 ± 0.014) respectively and Caspase 9 (SAC-5mM: 1.452 ± 0.568, SAC-7.5mM: 1.759 ± 0.392) and (SAC-5mM: 0.943 ± 0.430, SAC-7.5mM: 0.099 ± 0.050) respectively **(Figure 6-a,c,b)** as compared to $CoCl_2$-treated cells (Hif-1α: 2.207 ± 0.311, Caspase3: 4.879 ± 2.129, Caspase9: 5.836 ± 2.757). The 24 and 48-hours SAC-preconditioned chondrocytes also showed a reduction in gene expression of iNOS (SAC-5mM: 0.521 ± 0.216, SAC-7.5mM: 1.810 ± 0.471) and (SAC-5mM: 1.475 ± 0.402, SAC-7.5mM: 0.655 ± 0.192) respectively versus $CoCl_2$-treated chondrocytes (3.352 ± 1.072) **(Figure 6-f)** which impairs mitochondrial respiration and produces ROS.

SAC preconditioning reduces inflammation in Chondrocytes

Besides the antioxidant and anti-apoptotic potential of SAC preconditioning, anti-inflammatory perspective was also determined by evaluating the gene expression of one of the very important inflammatory cytokines, IL-1β. The qPCR results showed that SAC preconditioning of chondrocytes for 24 and 48 hours showed a significant decrease in the expression of IL-1β (SAC-5mM: 1.634 ± 0.618, SAC-7.5mM: 1.288 ± 0.310) and (SAC-5mM: 2.280 ± 1.006, SAC-7.5mM: 1.213 ± 0.380) respectively as compared to the $CoCl_2$-treated cells (4.610 ± 1.351)**(Figure 6-e).**

Discussion

Osteoarthritis is a joint disease where chondrocytes face mild to severe oxidative stresses, also known as oxidative stress. The induction of oxidative stress in cartilage tissue triggers the formation of reactive oxygen species (ROS). It also affects mitochondrial membrane potential and triggers apoptosis (Guo et al., 2009; Jung and Kim, 2004; Wang et al., 2000; Zou et al., 2002). The activation of Hif-1α and regulated catabolic processes up-regulates the many pro-inflammatory cytokines and cytoplasmic proteins (Lan et al., 2012; Lan et al., 2011; Pfander and Gelse, 2007). Various natural antioxidant compounds have been used to treat oxidative stress so far. S-Allyl L-Cysteine (SAC) is a sulfur sulfur-containing phytochemical formed by the catabolism of γ-glutamyl-S-allyl cysteine, present in garlic. Compared to other compounds isolated from garlic, SAC is relatively much more stable, less toxic and can retain its antioxidant properties even after 2 years of isolation without undergoing any decomposition (Colín-González et al., 2012; Lawson, 1998). SAC has also been known for its neuroprotective, anti-oxidant, anti-apoptotic and anti-inflammatory properties against various pathological conditions like Cerebral Ischemia and renal damage (Colín-González et al., 2015; Maldonado et al., 2003).

Due to the protective nature of SAC and its anti-inflammatory properties against various pathological conditions, it was hypothesized that SAC would be able to revert OA by making the chondrocytes resistant to oxidative stress and helping them to perform better and retain their distinguishing cartilage synthesizing properties under oxidative stress. To provide an oxidative stress environment *in-vitro*, $CoCl_2$ was used, which has already been reported as the inducer of chemical oxidative stress (Yudoh et al., 2005). The results revealed that exposure of chondrocytes to 400µM $CoCl_2$ for 24 hours up-regulated the Hif-1α gene expression, which indicated that $CoCl_2$ was able to mimic oxidative stress in chondrocytes (Lan et al., 2011; Wang et al., 2000).

The cell viability of SAC-preconditioned cells was significantly increased as compared to CoCl2 treated cells, affirming that SAC helps restore the cell viability in the dosage and time-dependent manner. The positive effect of SAC on cell viability was in correspondence with the previous research where SAC had been reported to increase the cell viability of PC-12 cells against chemically induced oxidative stress(Orozco-Ibarra et al., 2016). Lactate dehydrogenase (LDH) is released into the synovial fluid by the chondrocytes and ECM when the cartilage experiences any damage and collagen fibers start disintegrating (Roughley and Mort, 2014). The LDH activity has already been reported to be higher in H_2O_2-treated human mesenchymal stem cells (Ali et al., 2016). A decrease in LDH release was observed in the SAC preconditioned chondrocytes as compared to the untreated cells. As the LDH release is directly proportional to cytotoxicity, our findings suggested that SAC was able to protect the chondrocytes against oxidative stress-mediated cytotoxicity. Reduction in LDH release strongly suggests SAC's anti-inflammatory and anti-oxidant potential as it has already been reported that the increase in LDH activity is associated with the

progression of OA and Rheumatoid arthritis (Najeeb and Aziz, 2015).

The glycosaminoglycans are proteoglycans primarily located on the surface of chondrocytes and in the ECM. The sGAGs are highly polar molecules and hence play an important part in water retention and cartilage lubrication. During OA, chondrocytes' ability to retain GAG on their cell surfaces and in the ECM is reduced, and the level of GAGs increases in the synovial fluid(Khan et al., 2008; King and Rosenthal, 2015). In the present study, the significantly less release of GAGs in the medium by the SAC-preconditioned cells, as compared to the stressed cells, strongly suggested that SAC enabled the chondrocytes to retain the GAG content. Our findings also corresponded with the research work in which GAG release by chondrocyte treated with Morindaelliptica leaf extract was less, and the cell showed resistance to OA (Osman et al., 2017).

Superoxide dismutase is an antioxidant enzyme in the cells that catalyze the conversion of superoxide anion into less toxic hydrogen peroxide and molecular oxygen (Weydert and Cullen, 2010). Our results revealed that the SAC-preconditioned cells possessed significantly high SOD activity as compared to stressed cells. The elevated SOD activity in the SAC-preconditioned cells further reinforced the antioxidant potential of SAC against oxidative stress in chondrocytes. SAC has also been reported to increase the expression of superoxide dismutase in cerebral ischemia-induced damage and exhibited antioxidant potential(Javed et al., 2011; Tsai et al., 2011). The increase in SOD activity is also related to the decrease in oxidative stress to the cells (Li et al., 2017), which implies that SAC can also induce antioxidant potential in chondrocytes against oxidative stress.

It has been reported the $CoCl_2$-mediated oxidative stress leads to up-regulation and stabilization of hypoxia-inducible factor 1α (Hif-1α) which marks the starting point for activation of mitochondrial inflammation pathway and activates many other pro-apoptotic proteins (Greijer and Van der Wall, 2004). Hence, the downregulation of Hif-1α can prevent the cells from undergoing apoptosis. We observed a significant decrease in the Hif-1α gene expression in the SAC preconditioned cells compared to stressed cells. The role of Hif-1α has previously been suggested in leading the cells toward apoptosis by up-regulating the Hif-1α dependent genes in the cells (Orozco-Ibarra et al., 2016). Hence the downregulation of Hif-1α suggested that SAC pretreatment made the chondrocytes resistant to the $CoCl_2$-mediated oxidative stress and oxidative stress-mediated apoptosis.

Caspase-9 is a pro-apoptotic protein from the Caspase family and plays a central role by initiating the formation of apoptotic bodies in the cells. The activation of Caspase-9 downstream of the mitochondrial pathway further up-regulates other Caspase-3 and Caspase-7(Würstle et al., 2012). Similar to caspase-9, Caspase-3 is also an important pro-apoptotic protein that has been seen to be up-regulated in the cells undergoing apoptosis (Musumeci et al., 2011). In the present study, we observed a significant reduction in the Caspase-9 and Caspase-3 gene expression in the SAC preconditioned cells compared to stressed cells. The findings strongly suggested that SAC was able to alleviate mitochondrial inflammation and protect the chondrocytes from undergoing apoptosis. The induction of $CoCl_2$ mediated oxidative stress has been reported to up regulate the Caspase-9 expression in different inflammatory pathways including the mitochondrial pathway, and ultimately leads to apoptosis (Orozco-Ibarra et al., 2016). It has been seen that the decrease in Caspase-9 gene expression is associated with the cell's capability to resist oxidative stress (Li et al., 2017). Similar to the reduction in Caspase-9 expression, the downregulation of Caspase-3 further strengthened the hypothesis by confirming the anti-apoptotic potential of SAC against oxidative stress. We observed a significant decrease in the Caspase-3 expression in SAC-preconditioned cells compared to stressed cells. Our findings corresponded to another study in which the bellidifolin, a Gentiana-derived xanthone compound, treated PC12 cells showed decreased expression of Caspase-3 in response to oxidative stress(Zhao et al., 2017). As the decrease in Caspase-3 gene expression is seen to be related to the increase in the cellular potential to resist oxidative damage (Li et al., 2017).

Xanthine oxidase (XO), a pro-oxidant enzyme, is upregulated during oxidative stress and is involved in the conversion of hypoxanthine into xanthine together with the production of superoxide anion, which is very toxic for the cells as it damages proteins, lipids and DNA in the cells and hence promotes apoptosis (Colín-González et al., 2012). The XO and SOD are antagonistic to each other. The former promotes the production of superoxide anion while the latter suppresses it. We observed that the SAC-preconditioned cells showed a significant reduction in XO expression as compared to the stressed cell. The less expression of XO and up-regulation of SOD activity in the SAC-preconditioned cells further confirmed the induction of antioxidant potential by SAC into chondrocytes against $CoCl_2$-mediated oxidative stress. It has also been reported in previous studies that the formation of Reactive Oxygen Species (ROS) in response to oxidative stress induces the up-regulation XO activity in the cells which later on produces superoxide anion (Ramalingam et al., 2017). Hence the reduction in XO activity by SAC strongly

suggested its antioxidant potential against oxidative stress.

Inflammatory cytokines, especially Interleukin-1β plays an important role in the progression of OA. IL-1β triggers the production of matrix metalloproteinases (MMP's) and reactive oxygen species (ROS), leading to matrix proteolysis (Rousset et al., 2015). Interleukin-1β is responsible for promoting apoptosis in bovine chondrocytes, glioblastoma cells and human articular chondrocytes (Mathy-Hartert et al., 2008; Rousset et al., 2015; Sun et al., 2014). Consistent with the previous studies (Lee et al., 2012), the present study also reveals the downregulation of IL-1β in SAC-preconditioned chondrocytes. Nitric oxide synthases (NOS) represent a group of isomorphic enzymes which have been found responsible for the production of nitric oxide (NO), an intoxicating dilator. Inflammatory conditions trigger the upregulation of inducible NOS (iNOS), leading to the production of a large amount of NO (Lind et al., 2017). The presence of NO· free radical in large amounts impedes mitochondrial respiration by hindering the electron transfer chain. This, in turn, leads to the production of surplus superoxide anion (O2·–), which combines with NO·to produce highly toxic peroxynitrite ($ONOO^-$) radicals. $ONOO^-$ together with NO·(Prime et al., 2009)not only impedes ATP synthesis by compromising oxidative phosphorylation (Escames et al., 2007; Esteban et al., 1997), but also triggers tyrosine nitration and produces different reactive species deeply disturbing mitochondrial functions (Brown, 1999, 2001; Schild et al., 2003). Also numerous tissues have been observed to have varying levels of iNOS in their mitochondria during inflammation (Gitto et al., 2001; Takemura et al., 2000). Consistent with the previous studies, the present study reports the downregulation of iNOS in SAC-Preconditioned chondrocytes when subjected to oxidative stress.

Conclusion

In conclusion, SAC protects chondrocytes against oxidative stress *in vitro.* We observed that SAC preconditioning of rat chondrocytes diminished the harmful effects induced by cobalt chloride-mediated oxidative stress by down-regulating the MIP pathway and stimulation of various cellular defensive mechanisms.Therefore, our study provides an effective alternative cellular therapy for damaged articular cartilage and proposes the potential use of SAC as a supplement agent for transplanting chondrocytes into injured cartilage. However, *in vivo* osteoarthritis animal model studies should be performed to elucidate SAC's further role.

Declaration

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Ethics approval: This research has been approved by the Institutional review board of Centre of Excellence in Molecular Biology.

Consent to participate: This research does not involve any human samples involvement.

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