Stichopus chloronotus aqueous extract as a chondroprotective agent for human chondrocytes isolated from osteoarthritis articular cartilage in vitro

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Abstract The proinflammatory cytokines, metalloproteinases family (MMPs), inflammatory mediators PGE₂, COX-2 and NO are the most important group of compounds responsible for the loss of metabolic homeostasis of articular cartilage by promoting catabolic and destructive processes in the pathogenesis of osteoarthritis (OA). *Stichopus chloronotus*, a marine sea cucumber which is rich in n-3 PUFAs and phenolic compound, may exert a favorable influence on the course of the disease. The objective of this study was to investigate the regeneration and anti-inflammatory potential of *S. chloronotus* aqueous extract (SCAE) on human OA articular chondrocytes (HOC). Methods: The HOC isolated from knee joint cartilage

removed during surgery were cultured with SCAE for 7 days. The effect of SCAE on anabolic and catabolic gene expression was verified by real-time PCR. Monolayer chondrocytes were stained with toluidine blue whereas sGAG, NO and PGE₂ production in medium were analyzed by ELISA. Results: The HOC cultured in various SCAE have polygonal morphology maintaining their chondrocytes characteristic. SAE supplementation tested was found to be effective pro-chondrogenic, anti-inflammatory and anti-oxidative agents, as evidenced by upregulation of cartilage specific markers collagen type II, aggrecan core protein and sox-9 expression and downregulation of collagen type I, IL-1, IL-6, IL-8, MMP-1, MMP-3, MMP-13, COX-2, iNOS and PAR-2 expression. The presence of SCAE in the culture was able to increase sGAG and reduce NO and PGE₂ production significantly. Conclusions: These results suggested that SCAE demonstrated chondroprotective ability by suppressing catabolic activities, oxidative damage and effectively promoting chondrocytes growth.

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Introduction

The pathogenesis of osteoarthritis (OA) is complex and involves the interaction of multiple factors ranging from genetic predisposition to altered mechanical loading and changes in the gene expression repertoire of the articular chondrocytes (Akhtar et al. 2011). The metabolic and structural changes in articular cartilage are thought to play a leading role in the initiation and the progression of the disease process. The varieties of stress are believed to stimulate chondrocyte metabolism, providing a mechanism for the cartilage to adapt to the demands imposed by the body. However, the balance between cartilage matrix synthesis and degradation is disturbed in focal and degenerative lesions caused by trauma or disorders, resulting in tissue breakdown and the risk of OA progression.

Normal articular cartilage extracellular matrix (ECM) composed primarily of collagen type II for tensile strength and aggrecan which contains sulfated glycosaminoglycans (GAGs), for stiffness in compression (Cucchiari et al. 2016). The chondrocytes are the sole, differentiated cellular resident of articular cartilage that are responsible for the generation and maintenance of the ECM. Phenotypically, articular chondrocytes are characterized by their ability to synthesize a specific matrix consisting of collagen type II and aggrecan (Liu et al. 2016). However, during expansion of the chondrocyte in vitro, the loss of phenotype due to cell dedifferentiation causes chondrocytes to lose their round shape and become flattened fibroblast-like cells with an increased proliferative capacity (Jiménez et al. 2015). This is accompanied by a decrease in expression of chondrocyte markers such as collagen type II, aggrecan, and the transcription factor *sox9*, and increased in expression of fibroblastic markers such as collagen type I and collagen type X (Jiménez et al. 2015). The differentiated chondrocytes are important for cartilage tissue engineering since dedifferentiated chondrocytes become fibroblastic and do not produce hyaline cartilage, which is necessary for the proper function of articulating joints. The proinflammatory cytokines such as IL-1, IL-6, and chemokine, IL-8, are known to be upregulated during OA progression (Kapoor et al. 2011). The action of these inflammatory mediators within the cartilage is predominantly to drive catabolic pathways, inhibit matrix synthesis and inhibit

autophagy, leading to an increase in chondrocyte apoptosis. IL-1 is synthesized by chondrocytes at concentrations that are capable of inducing the synthesis of matrix metalloproteinases (MMP 1, MMP 3 and MMP 13) and other inflammatory cytokines such as TNF, IL-6 and chemokine IL-8. These substances may induce additive or synergistic effects that drives the cartilage matrix breakdown in the catabolic cascade to further enhance articular chondrocytes destruction (Houard et al. 2013). IL-1 could also contribute to the degradation of the cartilage matrix by decreasing the synthesis of cartilage-specific collagens and proteoglycans (Goldring and Otero 2014). In addition, the effects are observed in a number of other secreted enzymes and mediators involved in the pathophysiology of OA. The proinflammatory cytokines, IL-1 is known to induce the synthesis of prostaglandin E2 (PGE₂) by stimulating the gene expression of COX-2, and upregulates the production of nitric oxide (NO) via iNOS (Shimpo et al. 2009). NO can combine with superoxide anions (O₂[−]) to generate peroxynitrite, which has also been implicated in proinflammatory effects on cartilage. Peroxynitrite also has proapoptotic effects on cartilage where it can induce mitochondrial dysfunction in cells (Abramson 2008). NO and COX-2 has been shown to upregulate the synthesis of matrix metalloproteinases in a cGMP-dependent manner to induce inhibition of both proteoglycans and collagen synthesis (Lee et al. 2013). Besides mediating pain and inflammation, the PGE₂ produced by COX-2 in OA cartilage may play a role in the bone and cartilage degeneration that results in the formation of osteophytes (Hardy et al. 2002). There is a possibility that the proteinase-activated receptor 2 (PAR-2) system is involved in the inflammatory response-mediated degradation of ECM in OA. Studies have shown that secreted IL-1 up-regulated the expression of PAR-2, stimulating more secretion of proinflammatory cytokines (IL-6, IL-8), metalloproteinases and PGE₂ to enhance inflammatory responses (Boileau et al. 2007).

The use of sea cucumbers due to their potential health benefits to humans, are gaining much recognition among consumers, medical and biomedical researchers. *Stichopus chloronotus*, a species of sea cucumber, which is found on the sea floor worldwide, is mainly consumed in Asian countries as both food and traditional medicine. Many Asians consume sea cucumbers to cure disorders which includes asthma,

hypertension, cancer and arthritis, as well as intestinal and urinary dysfunctions. Fredalina et al. (1999) demonstrated that fatty acids profile which were arachidonic, eicosapentaenoic (EPA) and docosahexaenoic acid (DHA) extracted from *Stichopus chloronotus* have a potential role in tissue repair and wound healing. Several in vitro and in vivo studies on eicosapentaenoic (EPA) and docosahexaenoic acid (DHA) (*n*-3 PUFAs), have demonstrated modulation of several pathophysiologic pathways involved in the pathogenesis of osteoarthritis (Wann et al. 2010; Knott et al. 2011). Several studies showed a decreased in GAG loss and collagenase cleavage of collagen type II, and decreased in expression of enzymes and inflammatory mediators involved in joint destruction, when the OA articular cartilage were cultured with *n*-3 PUFAs (Curtis et al. 2004; Knott et al. 2011). This indicated that *n*-3 PUFAs may have a beneficial effect on articular OA. Athunibat et al. (2009) reported that aqueous extract derived from sea cucumber *Stichopus chloronotus* contained significantly higher amounts of total phenolics which were known to have antioxidant properties since the cartilage was an avascular tissue and chondrocytes were in an environment with high oxidative stress due to repeated ischemia and reperfusion. Although there was a lack of information about the *Stichopus chloronotus*, it has been suggested that this sea cucumber species may provide some beneficial effects in modulating the pathophysiological processes of arthritis. The aim of study was to investigate the effect of *S. chloronotus* aqueous extract on anabolic and catabolic activity of primary human chondrocytes from OA cartilage in vitro.

Materials and methodology

Human articular chondrocytes isolation and culture

Prior ethical approval was obtained from the Research and Ethical Committee of Faculty of Medicine, Universiti Kebangsaan Malaysia (FF-2015-235). All the human study subjects provided informed consent.

Human articular cartilage was obtained from six consented patient aged between 55 and 70 years old that underwent total knee arthroplasty (TKR). All patients were diagnosed with knee osteoarthritis with lesion scored grade 4 according to International

Cartilage Repair Society (ICRS) https://www.secot.es/uploads/descargas/.../ICRS._TRAUMA_CARTaILAGO.pdf. The cartilage was harvested from medial and lateral condyle of the distal femur, weighing approximately 300 mg. All samples were processed within 24 h following surgery.

Specimens were washed with Phosphate-Buffered Saline (PBS; pH 7.2, Gibco, Grand Island, NY) containing 100 U/ml penicillin. Each sample was minced into 1 mm³ fragments and digested with 0.6% Collagenase II (Gibco, Grand Island, NY, USA) in an orbital incubator (Stuart Scientific, Redhill, UK) at 37 °C for 6–8 h. After digestion the cell suspension was centrifuged and the cell pellet were then cultured in chondrocytes growth medium consisting of Ham's F-12:Dulbecco's Modified Eagles's Medium (Gibco, Grand Island, NY), 10% foetal bovine serum (FBS, Gibco, Grand Island, NY) 1% of antibiotic and antimycotic (Gibco, Grand Island, NY), 1% of 50 µg/ml ascorbic acid (Sigma-Aldrich, Missouri, USA), 1% 200 mM L-glutamine (Gibco, Grand Island, NY) and 15 mM herpes buffer (Gibco, Grand Island, NY) in T25 culture flasks (Nunc, Denmark). Primary cultured chondrocytes were maintained in a humidified incubator at 37 °C with 5% CO₂. The culture media were changed every other day. Once the cultured cells reached 80% confluency, the chondrocytes were trypsinized using 0.05% Trypsin-EDTA (Gibco, Grand Island, NY) and subcultured at a seeding density of 5000 cells/cm² in T75 culture flasks. The cultured chondrocytes were incubated overnight in chondrocytes growth medium to allow cell attachment before the medium was changed to Ham's F-12:Dulbecco's Modified Eagles's Medium (Gibco, Grand Island, NY, USA) supplemented with various concentrations of *S. chloronotus* aqueous extract (SCAE) ranging from 0.0 to 1.0% for another 7 days. The new SCAE was prepared from the same dried extract for culture medium supplementation during culture medium changing, which was every 48 h and the culture supernatant was pooled in a 50 ml tube and stored at – 80 °C until further use.

The morphological features of chondrocytes were assessed daily under an inverted light microscope (Olympus, Tokyo). Cell viability and proliferation rate for each sample was evaluated after 7 days of culture.

Preparation of *S. chloronotus* aqueous extract

S. chloronotus (SC) were freshly harvested from Pulau Bidong, Terengganu, Malaysia. SC species identification was confirmed by a marine expert, Dr. Zulfigar Yasin from University Malaysia Terengganu, Ridzwan (2011), Forbes et al. (1999) and also through the information given by local residents. The SC was identified according by its morphological characteristic which emphasized on body shape, body colour, the existence and shape of papillae on both dorsal and ventral parts. Fresh samples of 20 SC were collected. The extract used was pooled from one batch to avoid variations in the result. There were abundant of SC at coastal areas of Terengganu, Malaysia. The extracts were prepared according to Fredalina et al. (1999) with slight modification. The dried product from the extraction process was converted into water extract for cell culturing purposes using sterile Phosphate-Buffered Saline (PBS; pH 7.2, Gibco Grand Island, NY, USA). The extract was filtered into a 50 ml sterile tube using a 0.2 µm syringe filter and stored for further use.

Determination of fatty acids composition of SC was not done in this recent study, we based on previous studies on *S. chloronotus* that have been done in Malaysia by our local marine scientists Fredalina et al. (1999) and Mazliadiyana et al. (2017). It shown that *S. chloronotus* contained bioactive substances which is fatty acids composition, especially arachidonic (C20:0) and PUFA: eicosapentaenoic (C20:5, EPA) and docosahexaenoic acid (C22:6, DHA) which was described in introduction.

MTT assay

The quantitative evaluation of cell viability and proliferation of each cultured human osteoarthritis chondrocytes in 0.0–1.0% concentration of SCAE was determined by MTT Assay using commercial kit ((3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; Life technologies, California, USA). The chondrocytes were subcultured in 96-well microtiter plate at a density of $1 \times 10^4/100 \mu\text{l}$ of chondrocytes. The assay was started following 7 days of culture by adding 100 µl of fresh medium, followed by 10 µl of MTT solution into each well and incubated for 4 h at 37 °C in a CO₂ incubator. Consequently, the formed formazan crystals by living cells were solubilized by mixing 100 µl of Sodium dodecyl sulfate (SDS) in

0.01 M HCl and incubated for another 4 h. The concentration of the formazan solution was measured at 570 nm by ELISA microplate reader.

Total RNA extraction

Approximately 1.0×10^6 chondrocytes cultured with or without supplementation of SCAE were harvested via trypsinization and counted using haemocytometer. The isolated chondrocytes were then centrifuged at 5000 rpm for 5 min at 37 °C. The chondrocyte pellet was suspended and the total RNA purified using the RNeasy kit (Qiagen Inc., Valencia, CA, USA). The yield and purity of isolated RNA was determined by using the Infinite 200 PRO NanoQuant spectrophotometer (Tecan, Austria GmbH) measured at 260 nm. Total RNA was stored at – 80 °C immediately after extraction.

cDNA synthesis

Complementary DNA was synthesized from standardized total RNA from each treatment group (cultured chondrocytes with or without SCAE supplementation) using iScriptTM reverse transcription (Biorad, California, USA). The reaction was carried out according to the protocol recommended by the manufacturer.

Real time reverse transcriptase-polymerase chain reaction (qRT-PCR)

The expression of cartilage anabolic markers; Collagen I, Collagen II, Sox9 and Aggrecan core protein and catabolic markers; MMP 1, 3 and 13, IL-1, IL-6 and IL-8, COX-2, iNOS and PAR-2 were evaluated by quantitative real-time PCR protocol (SsoAdvancedTM Universal SYBR[®] Green Supermix, Biorad, California, USA). The sequences of the both forward and reverse primers were designed based on the sequences published in GenBank using primer-3 software (as shown in Table 1). Human GAPDH gene was used as housekeeping gene. The quantitative RT-PCR protocol was performed in Bio-Rad iCycler set for 40 cycles for each run. The data were analyzed using Bio-Rad iCycler software. For gene expression quantitation, the comparative Ct method was used. The Ct values of the gene of interest were normalized to GAPDH. The Ct value was calculated when the fluorescence of the sample exceeded the threshold level. The relative

Table 1 List of primers used in qPCR for chondrogenic genes (www.ncbi.nlm.nih.gov/nucleotide)

Gene name	Accession number	Primer sequence 5'–3'
GAPDH	NM_002046	F: 5'-tcc ctg agc tga acg gga ag-3' R: 5'-gga gga gtg ggt gtc gct gt-3'
COL I	NM_000088	F: 5'-agg gct cca acg aga tcg aga-3' R: 5'-tac agg aag cag aca ggg cca-3'
COL II	NM_001844	F: 5'-cta tct gga cga agc agc tgg ca-3' R: 5'-atg ggt gca atg tca atg atgg-3'
SOX-9	NM_000346	F: 5'-cac tgt tac cgc cac ttc cc-3' R: 5'-acc agc gga agt ccc ctt cg-3'
ACP	NM_001135	F: 5'-gcg gag gaa gtc ggt gaa ga-3' R: 5'-ccc tct cgc ttc agg tca gc-3'
IL-1	NM_000576	F: 5'-gga caa gct gag gaa gat gc-3' R: 5'-tcg tta tcc cat gtg tcg aa-3'
IL-6	NM_000600	F: 5'-tac ccc cag gag aag att cc-3' R: 5'-ttt tct gcc agt gcc tct tt-3'
IL-8	NM_000584	F: 5'-gtg cag ttt tgc caa gga gt-3' R: 5'-ctc tgc acc cag ttt tcc tt-3'
MMP1	NM_002421	F: 5'-agg tct ctg aag gtc aag ca-3' R: 5'-ctg gtt gaa aag cat gag ca-3'
MMP3	NM_002422	F: 5'-tgc ttt gtc ctt tga tgc tg-3' R: 5'-gga aga gat ggc caa aat ga-3'
MMP13	NM_002427	F: 5'-ggt ctt gac cac tcc aag gac-3' R: 5'-ctc ctc gga gac tgg taa tgg-3'
iNOS	NM_000625	F: 5'-aca agc cta ccc ctc cag at-3' R: 5'-tcc cgt cag ttg gta ggt tc-3'
COX-2	NM_000963	F: 5'-tga gca tct acg gtt tgc tg-3' R: 5'-tgc ttg tct gga aca act gc-3'
PAR2	NM_005242	F: 5'-tcc agg aag aag gca aac att-3' R: 5'-cac ata ggc aga ggc tgt gag-3'

mRNA expression of the target gene was calculated with the following equation: $2^{(Ct\text{ GAPDH}-Ct\text{ target gene})}$.

Nitric oxide (NO) assay

The measurement of NO in a system was measured by the determination of total nitrite and nitrate concentrations in the samples. The total nitrite and nitrate concentration in pooled culture supernatants from the cultured chondrocytes with or without SCAE supplementation was measured using Colometric In Vitro Nitric Oxide Assay Kit (OxiSelect™, Cell Biolabs, Inc, USA). The pooled culture supernatants from the cultured chondrocytes with or without SCAE supplementation was used to measure total nitrite and nitrate using Colometric In Vitro Nitric Oxide Assay Kit

(OxiSelect™, Cell Biolabs, Inc, USA). Sample preparation and reaction protocol was performed according to the manufacturer's instruction. A standard curve was drawn using Nitrate standard solution and was used to determine the amount of NO released in the culture supernatant. Briefly, the kit uses the enzyme nitrate reductase to convert the nitrate to nitrite. Total nitrite is then detected with Griess Reagents as a colored azo dye product. The optical density of each well was determined using ELISA microplate reader at 540 nm. The total nitrite and nitrate concentration were calculated according to the mean absorbance from the standard curve.

Prostaglandin E2 (PGE₂) assay

The Prostaglandin E2 in the pooled culture supernatant from the cultured chondrocytes with or without SCAE supplementation was measured using PGE2 Immunoassay Kit (Parameter™, R&D Systems Inc, USA). Sample preparation and reaction were performed according to the manufacturer's instruction. A standard curve was drawn using PGE2 standard solution and was used to determine the amount of PGE2 released in the culture supernatant. The reading of PGE2 concentration for each sample was then multiplied by the dilution factor (DF); DF = 3.

Sulphated glycosaminoglycan (sGAG) assay

The amount of sGAG released into the medium of all culture supernatant with or without SCAE supplementation was measured spectrophotometrically by using Blyscan assay kit (Biocolor, Carrickfergus, Northern Ireland), according to the manufacturer's protocol. The GAG content in each type of culture medium was determined by a standard curve drawn using standard solutions containing bovine tracheal chondroitin 4-sulfate. The sGAG absorbance was determined using ELISA microplate reader at 620 nm wavelength.

Toluidine blue staining

The monolayer cultured chondrocytes was stained using 0.04% toluidine blue. The cultured cells were washed twice with Phosphate-Buffered Saline (PBS; pH 7.2, Gibco, Grand Island, NY) and fixed in 4% buffered formaldehyde for at least 30 min. After fixation, the cultured cell were washed again twice and stained with toluidine blue for 30 min. Then cell cultured was washed with running distilled water for 5 min for three times before viewing under the microscope.

Statistical analysis

The quantitative data were presented as mean $\pm$ standard error of mean (SEM). Data comparisons between each control and treated group were conducted using one-way ANOVA with significant value at $p < 0.05$. All data has been analyzed using

GraphPad Prism version 7.0 device (GraphPad Software, Inc., USA).

Results

Cell proliferation (MTT assay)

The proliferation of human osteoarthritis chondrocytes was evaluated by MTT assay at 7 days post treatment with different concentrations of SCAE. The result showed that SCAE supplementation at lower concentration (0.05–0.5%) promoted the proliferation of chondrocytes (Fig. 1). This result suggested that SCAE at a concentration of $\leq 0.5\%$ has no adverse effect on the viability and proliferation ability of chondrocytes. However, the chondrocytes proliferation was slower than the control group with increasing SCAE concentration $> 0.5\%$. The IC₅₀ which was the inhibitory concentration of SCAE that reduced 50% of

Fig. 1 MTT assay analysis. Human osteoarthritic articular chondrocytes cultured in various concentration of SCAE in the culture medium. The values were expressed as mean $\pm$ SEM, $n = 6$. * $p < 0.05$; compared values of control medium and Ham's F-12:Dulbecco's Modified Eagles's Medium (FD) with 0.05–1% SCAE. The IC₅₀ which was the inhibitory concentration of SCAE that reduced 50% of cells viability as compared to untreated cells was estimated at 0.93%

cells viability as compared to untreated cells was estimated to be at 0.93%. This showed that SCAE exhibited anti-proliferative effect against chondrocytes at higher concentration. Similar anti-proliferative effect of SCAE towards in vitro human non-small lung carcinoma and cervical cancer cell has been reported by Althunibat et al. (2009). These effects may be due to the presence of flavonoids and phenols, the main active compounds found in SCAE, which are known to have anti-oxidant properties. The concentrations of 0.1% and 0.2% of SCAE were chosen for further experiments.

Cell morphology

The osteoarthritic chondrocytes that were subcultured in F12:DMEM medium, F12:DMEM with SCAE 0.1% and 0.2% supplementation showed different morphological characteristic after 7 days. The chondrocytes cultured in F12:DMEM with SCAE supplementation retained an almost polygonal morphology (Fig. 2e, f) while chondrocytes cultured in F12:DMEM only, appeared to have lost their spherical shape and adopted a spindle-like fibroblastic morphology (Fig. 2d). Besides, chondrocytes cultured in F12:DMEM with SCAE supplementations were found to be actively proliferating evidenced by their shining borders which could be more clearly seen under higher magnification and were relatively larger in size as compared to chondrocytes cultured in F12:DMEM only.

Gene expression analysis

The effect of SCAE supplementation on chondrogenic differentiation genes expression

The OA chondrocytes cultured in medium with SCAE supplementation demonstrated a higher expression of chondrogenic genes (collagen type II, aggrecan core protein and sox9) as compared to OA chondrocytes cultured in medium without SCAE supplementation. However, no significant differences were observed. Collagen type II was expressed higher in the OA chondrocytes cultured in medium with 0.1% and 0.2% SCAE supplementation represented by 1.4-fold and 1.8-fold respectively. The expression of genes encoding the aggrecan core protein was expressed higher in 0.1% and 0.2% SCAE supplementation, differed by

1.6-fold and 2-fold respectively as compared to culture medium without SCAE supplementation. The sox9 expression was detected at low level in all monolayer culture, however, the expression was found higher in 0.1% SCAE and 0.2% SCAE supplementation as compared to culture medium without supplementation. The expression level of chondrogenic dedifferentiation gene, collagen type I is comparable in culture medium with 0.1% and 0.2% SCAE supplementation as compared to culture medium without supplementation (Fig. 3). The chondrocytes phenotype index (ratio of collagen II/collagen I) which represented the differentiation status of chondrocytes at monolayer culture, is increased by 1.7-fold in 0.1% SCAE supplementation and 2.4-fold in 0.2% SCAE supplementation.

The effect of SCAE supplementation on proinflammatory cytokines (IL-1, IL-6) and chemokine IL-8 genes expression

The OA chondrocytes cultured in medium with 0.1% and 0.2% SCAE supplementation resulted in significant reduction in IL-1 mRNA expression level by 86% and 96% respectively ($p = 0.01, 0.009$) as compared to OA chondrocytes cultured without SCAE supplementation. Similarly, the expression level of IL-8 mRNA decreased by 62% and 81% in response to 0.1% and 0.2% SCAE supplementation respectively compared to OA chondrocytes cultured in medium without SCAE, with significantly difference $p = 0.04$ in 0.2% SCAE supplementation. Whereas OA chondrocytes cultured in medium with 0.1% and 0.2% SCAE supplementation showed a decrease in the level of IL-6 mRNA expression. However, this decrement was not significantly different. The effect of the SCAE on IL-1, IL-6 and IL-8 are shown in Fig. 4.

The effect of SCAE supplementation on proteolytic enzymes: matrix metalloproteinase genes expression

The expression of genes encoding the MMP-1, MMP-3 and MMP-13 were decreased in OA chondrocytes cultured in medium with 0.1% and 0.2% SCAE supplementation as compared to OA chondrocytes cultured in medium without SCAE. Supplementation of 0.1% and 0.2% SCAE showed a decreased in the level of MMP-13 mRNA expression by 40% and 60% respectively as compared to OA chondrocytes cultured

Fig. 2 Human OA articular chondrocytes in monolayer culture. Photomicrographs of human OA articular chondrocytes cultured in Ham's F-12:Dulbecco's Modified Eagles's Medium (FD) (**a**, **d**), FD supplemented with 0.1% SCAE (**b**, **e**) and 0.2% SCAE (**c**, **f**) were taken after 7 days. The red arrow in **e** and **f** showed chondrocytes cultured in F12:DMEM with SCAE

supplementation were retained an almost polygonal morphology while red arrow in **D** showed chondrocytes had lost their original morphology and changed to more fibroblast-like cells. **a–c** Magnification $\times 4$, **d–f** magnification $\times 10$. Bar = 100 μm (**a–c**), bar = 50 μm (**d–f**)

in medium without SCAE, with a significant difference ($p = 0.03$, $p = 0.004$). The expression of MMP-1 and MMP-3 mRNA was decreased in response to both 0.1% and 0.2% SCAE supplementation, however, the decrement was not significantly different. The result showed that the SCAE supplementation had a significant effect on MMP13 down-regulation. The effect of the SCAE on MMPs are shown in Fig. 5.

The effect of SCAE supplementation on inflammatory mediators genes expression

In order to obtain a broad view of the catabolic activities, expression of other inflammatory mediators such as iNOS, COX2 and PAR2 was also studied. The expression of iNOS decreased significantly by 90% in response to both 0.1% SCAE ($p = 0.03$) and 0.2% SCAE ($p = 0.03$) supplementation compared to OA chondrocytes cultured in medium without SCAE. Both COX2 and PAR2 mRNA expression decreased

Fig. 3 The expression of cartilage anabolic markers. Collagen I (COL I), collagen II (COL II), sox9 (SOX 9) and aggrecan core protein (ACP) gene expression of human osteoarthritic articular chondrocytes cultured for 7 days in a DMEM with 0.1% and 0.2% SCAE medium compared with FD only. A higher expression of the following genes COL II, SOX 9 and ACP were observed in the Ham's F-12:Dulbecco's Modified Eagles's Medium (FD) with 0.1% and 0.2% SCAE medium compared

with the FD only after 7 days of culture however there were no significant difference whereas the expression level of collagen type I (COL I) was comparable: Collagen I (COL I), collagen II (COL II), sox9 (SOX 9) and aggrecan core protein (ACP) values were expressed as the mean $\pm$ SEM ($n = 6$). The relative mRNA expression of the target gene was the Ct values of the gene of interest, normalized to GAPDH

significantly by 80% ($p = 0.04$) and 90% ($p = 0.04$) respectively in response to 0.2% SCAE supplementation compared to OA chondrocytes cultured in medium without SCAE. A significant decreased for PAR2 mRNA expression level for chondrocytes cultured in medium with 0.1% SCAE supplementation ($p = 0.02$) was detected, however no significant difference in COX2 mRNA was observed although the expression decreased dramatically. The effect of the SCAE on iNOS, COX2 and PAR2 are shown in Fig. 6.

The effect of SCAE supplementation on NO production

The release of NO in the culture medium was determined indirectly through spectrophotometric analysis of total nitrite and nitrate. The supplementation of 0.2% SCAE in culture medium for 7 days, significantly down-regulated the production of NO in monolayer human OA chondrocytes (70%, $p = 0.02$) compared to control. The decreased in the level of NO production was also observed in monolayer OA human chondrocytes with 0.1% SCAE

supplementation however there was no significant differences. The effect of the SCAE on NO production is shown in Fig. 7.

Fig. 4 Proinflammatory cytokines and chemokine genes expression. Proinflammatory cytokines and chemokine gene expression of human osteoarthritic articular chondrocytes cultured for 7 days in a Ham's F-12:Dulbecco's Modified Eagles's Medium (FD) with 0.1% and 0.2% SCAE medium compared with a control medium. A significantly lower expression of the following genes was observed in the FD with 0.1% and 0.2% SCAE medium compared with the control medium after 7 days of culture: IL-1, IL-6 and IL-8 values were expressed as the mean $\pm$ SEM, ($n = 6$), # $p < 0.05$; compared values of control medium and FD with 0.1% SCAE and * $p < 0.05$; compared values of control medium and FD with 0.2% SCAE medium. The relative mRNA expression of the target gene was the Ct values of the gene of interest, normalized to GAPDH

The effect of SCAE supplementation on PGE₂ production

The monolayer human OA chondrocytes produced low levels of PGE₂ and this was inhibited by the supplementation of 0.1 and 0.2% SCAE. The supplementation of SCAE at 0.1% and 0.2% concentration in the medium was able to reduce the PGE₂ productions as compared to untreated control, however the decrement was comparable and has no significant difference. In the absence of SCAE, the production of PGE₂ was increased most probably due to increased COX2 enzyme activity by human OA chondrocytes. The effect of the SCAE on PGE₂ production is shown in Fig. 8.

SGAG analysis

The total concentration of sGAG, an index of cartilage damage, in the medium of human cartilage cultures, was measured through quantitative spectrophotometric analysis. The concentration of sGAG in the medium was substantially greater in the monolayer OA chondrocytes supplemented with 0.1% SCAE and 0.2% SCAE which showed a 3-fold and 4-fold increase in the amount of sGAG released compared with untreated controls at day 7. The differences in monolayer OA chondrocytes supplemented with 0.2% SCAE compared with untreated controls was statistically significant with a $p = 0.02$ (Fig. 9). Therefore, in this study, SCAE supplementation could enhance proteoglycan production of cartilage.

Fig. 5 Matrix metalloproteinase genes expression. Matrix metalloproteinase gene expression of human osteoarthritic articular chondrocytes cultured for 7 days in a Ham's F-12:Dulbecco's Modified Eagles's Medium (FD) with 0.1% and 0.2% SCAE medium compared with a control medium. A lower expression of the following genes, MMP1, MMP3 and MMP13 was observed in the FD with 0.1% and 0.2% SCAE medium compared with the control medium after 7 days of culture: Supplementation of 0.1% and 0.2% SCAE showed a significant decreased in the level of MMP-13 mRNA expression as compared to OA chondrocytes cultured in medium without SCAE ($p < 0.05$). MMP1, MMP3 and MMP13 values were expressed as the mean $\pm$ SEM, ($n = 6$), # $p < 0.05$; compared values of control medium and FD with 0.1% SCAE and * $p < 0.05$. The relative mRNA expression of the target gene was the Ct values of the gene of interest, normalized to GAPDH

medium supplemented with SCAE showed significant staining for clusters of chondrocytes and their extracellular matrix as compared with control culture medium. This result indicates accumulation of large amounts of GAG in the matrix surrounding chondrocytes. In particular, the chondrocytes in medium conditions with 0.1% and 0.2% SCAE had structures where cells were surrounded by a violet coloured shell of the matrix (Fig. 10).

Discussion

The stability of chondrocytes phenotype is critically depending on the cell shape and density (Otero et al. 2012). Instead, is well accepted that high cell density in cultures favor the maintenance of the chondrocyte phenotype and support redifferentiation of dedifferentiated articular chondrocytes. The arthritic chondrocytes are not able to fully recover to the normal tissue phenotype in vitro culture condition as evident by low cellularity and decreased ECM production as compared to chondrocytes from healthy cartilage (Maldonado and Nam 2013). Supplementation of SCAE in culture was able to maintain chondrocytes characteristic morphology, keeping up their phenotype with growth acceleration. The chondrocytes appeared typical polygonal shape rather than adopting spindle-like fibroblastic shape. Hsieh-Bonassera et al. (2009) reported that chondrocytes in monolayer culture underwent dedifferentiation, stopped expressing aggrecan core protein and collagen type II but expressed collagen type I, which represented a

Histological analysis

Toluidine blue staining is proportional to proteoglycans and GAG content in cartilage. The culture

Fig. 6 Inflammatory mediators genes expression. COX2, iNOS and PAR2 gene expression of human osteoarthritic articular chondrocytes cultured for 7 days in a Ham's F-12:Dulbecco's Modified Eagles's Medium (FD) with 0.1% and 0.2% SCAE medium compared with a control medium. A significantly lower expression of the following genes was observed in the FD with 0.1% and 0.2% SCAE medium compared with the control medium after 7 days of culture: COX2, iNOS and PAR2, values were expressed as the mean $\pm$ SEM, ($n = 6$), # $p < 0.05$; compared values of control medium and FD with 0.1% SCAE and * $p < 0.05$; compared values of control medium and FD with 0.2% SCAE medium. The relative mRNA expression of the target gene was the Ct values of the gene of interest, normalized to GAPDH

Fig. 7 Nitric oxide production in human osteoarthritic articular chondrocytes. Nitric Oxide production (total nitrite and nitrate concentrations) of human osteoarthritic articular chondrocytes cultured for 7 days in a Ham's F-12:Dulbecco's Modified Eagles's Medium (FD) with 0.1% and 0.2% SCAE medium compared with a control medium. A lower expression of NO production was observed in the FD with 0.1% and 0.2% SCAE medium compared with the control medium after 7 days of culture. NO values were expressed as the mean $\pm$ SEM, ($n = 6$), * $p < 0.05$; compared values of control medium and FD with 0.2% SCAE medium

potential reason for anabolic failure of chondrocytes in osteoarthritic cartilage.

In this study showed the high expression of collagen type I mRNA in monolayer culture with or without SCAE supplementation. Yin et al. (2011) reported that expression of the fibroblastic marker,

collagen type I, increased with severity of OA degeneration, indicating a loss of cartilage phenotype. Although the high expression of collagen type 1 was detected in OA chondrocytes in monolayer culture, SCAE supplementation was able to down-regulate the expression of collagen type I compared to those cultured in medium without SCAE supplementation. In addition, the increased chondrocytes phenotypes index indicated that SCAE supplementation was able to reduce dedifferentiation and maintained the chondrocytes phenotype. A decreased in chondrocytes phenotypes index was a disadvantage for monolayer

Fig. 8 PGE₂ production in human osteoarthritic articular chondrocytes. PGE₂ production of human osteoarthritic articular chondrocytes cultured for 7 days in a Ham's F-12:Dulbecco's Modified Eagle's Medium (FD) with 0.1% and 0.2% SCAE medium compared with a control medium. A lower expression of PGE₂ production was observed in the FD with 0.1% and 0.2% SCAE medium compared with the control medium after 7 days of culture: PGE₂ values was expressed as the mean ± SEM, ($n = 6$)

Fig. 9 Sulphated glycosaminoglycan (sGAG) assay. sGAG production of human osteoarthritic articular chondrocytes cultured for 7 days in a Ham's F-12:Dulbecco's Modified Eagle's Medium (FD) with 0.1% and 0.2% SCAE medium compared with a control medium (FD). A higher sGAG production was observed in the FD with 0.1% and 0.2% SCAE medium compared with the control medium after 7 days of culture. sGAG values was expressed as the mean ± SEM, ($n = 6$), * $p < 0.05$; compared values of control medium and FD with 0.2% SCAE medium

culture as it results in the production of extracellular matrix typical of fibrotic tissue that might compromise cartilage regeneration (Jiménez et al. 2015). The presence of inflammatory cytokine IL-1 has been shown to play a role in suppressing ECM synthesis

through down-regulation of *sox9* (Dai et al. 2012). This was the reason why the *sox9* mRNA was detected at low level in this study. However, the presence of this gene was important since it was associated with chondrogenesis that promoted the spherical differentiated chondrocyte phenotype and ECM synthesis. Supplementation of SCAE in monolayer culture was able to up-regulate the expression of collagen type II, aggrecan core protein and *sox9* mRNA, which indicated the presence of cartilage-specific phenotype. It was suggested that this up-regulation may be attributed by polyunsaturated fatty acids (PUFA): eicosapentaenoic acid, docosahexaenoic acid that suppressed the IL-1-induced aggrecanase and collagenase activity and decreased the inflammation mediator PGE₂ (Jerosch 2011).

During the progression of OA, the compositional change of cartilage matrix from collagen type II to I compromise the integrity of ECM networks formed by collagen and proteoglycan (Munirah et al. 2010; Maldonado and Nam 2013). Glycosaminoglycan (GAG) content gives a measure of the development of a proteoglycan known to be a feature of hyaline cartilage. Dodge and Jimenez (2003) demonstrated that there was an increase of GAG content in cultures in parallel with an increase aggrecan core protein mRNA expression. In this study, the production of sGAG in monolayer OA chondrocytes culture supernatants supplemented with SCAE was increased along with the aggrecan core protein mRNA expression suggesting enhanced chondrogenic ability. Furthermore, cartilage specific proteoglycans were also clearly more apparent in monolayer OA chondrocytes supplemented with SCAE than in the control as assessed by toluidine blue staining demonstrating an increase sGAG synthesis by the OA chondrocytes. The action exhibited by SCAE under our experimental conditions could be explained by its active phenols. Cardile et al. (2003) reported that the flavonoid, an active phenolic constituent, was able to promote sGAG synthesis in chondrocytes. Hurst et al. (2010) demonstrated that EPA and DHA found in SCAE was able to suppress ADAMTS-4 and -5 mRNA levels, which are known to cause the initial proteolysis of cartilage in osteoarthritis.

Proinflammatory cytokines, such as IL-1, IL-6 and chemokine IL-8 were known to be up-regulated during OA progression. These inflammatory cytokines inducing the aging and apoptosis of chondrocytes, and

Fig. 10 Toluidine blue staining of monolayer human OA chondrocytes. Toluidine blue staining of monolayer human OA chondrocytes cultured for 7 days in a Ham's F-12:Dulbecco's Modified Eagle's Medium (FD) (a), FD with 0.1% SCAE (b) and 0.2% SCAE (c) medium. The red arrows in b, c showed the production of glycosaminoglycans (GAGs) by human OA chondrocytes cultured under above media by toluidine blue staining where the chondrocytes had structures where the cells were surrounded by a violet coloured shell of the matrix. Magnification $\times 4$. Bars = 100 μm

IL-8 mRNA significantly. The IL-1 mRNA expression was detected at low level in this study. However, the presence of this gene was important since it was associated with the synthesis of matrix metalloproteinases (MMPs), and suppression of aggrecan and collagen type II genes. Moreover, it was shown that IL-1 can induce the production of IL-6 and IL-8 by synovial cells and chondrocytes (Dozin et al. 2002). In normal adult cartilage, chondrocytes synthesize matrix components very slowly. Differences in age of donors with OA may also have contributed to differences in cytokines levels. In this study, both IL-6 and IL-8 were expressed higher than IL-1 are because these cytokines also secreted by senescent cells sometimes is known as the senescence associated secretory phenotype (Tsuchida et al. 2014).

The inflammatory cytokines IL-1 and $\text{TNF-}\alpha$ synthesized by OA chondrocytes were capable to increasing the synthesis of MMPs and decreasing the synthesis of MMP enzyme inhibitors. We demonstrated that supplementation of SCAE to OA chondrocytes in monolayer culture was able to down-regulate the collagenase encoding genes including MMP 1, 3 and 13. This result suggested that SCAE constituted a potential agent in reducing MMP-mediated degradation of native collagen and proteoglycans during the degenerative progression of OA. The degradation of type II collagen has been studied by many researchers. They showed that MMP-13, which was highly expressed in OA cartilage, was the enzyme responsible for most of the collagen degradation (Houard et al. 2013). The role of NO in OA was not really understood, but it can inhibit collagen and proteoglycan synthesis and activate metalloproteinases. Several studies have implicated nitric oxide as an important mediator in chondrocyte apoptosis, a feature that is common in progressive OA (Sun et al. 2011). In this our study, the results showed that SCAE

decreasing the synthesis of ECM key components such as proteoglycans, aggrecan and collagen type II (Lee et al. 2013). In this study, the supplementation of SCAE in monolayer culture of OA chondrocytes was able to down-regulate the expression of IL-1, IL-6 and

significantly inhibited the iNOS mRNA expression and NO production in monolayer OA chondrocytes. The ability of SCAE to suppress the expression of iNOS mRNA indicated that the extract could help to retain the chondrogenic properties in monolayer cultured chondrocytes. Sun et al. (2011) reported that the increased expression of iNOS mRNA showed an association with NO production and hypertrophy of human chondrocytes, which suggested that NO played a role in the modulation of chondrocytes phenotype.

Several studies indicated that NO enhances cyclooxygenase activity and PGE₂ production in various cell types including normal human chondrocytes. Amin et al. (1997) reported that OA cartilage spontaneously releases high levels PGE₂ compared to normal cartilage, and this effect may be due to transcriptional up-regulation of COX-2. Increased PGE₂ production caused cartilage resorption by suppressing the production of proteoglycans and enhancing the degradation of both aggrecan and collagen type II (Wang et al. 2011). In this present study, we showed that supplementation of SCAE on monolayer OA chondrocytes resulted significant down-regulation of COX-2 mRNA expression and PGE₂ production. Hajjaji et al. (2003) reported that COX inhibitor had an effect on cartilage metabolism, therefore, SCAE could produce similar beneficial effects in treating osteoarthritis.

The OA chondrocytes were found to express PAR-2 at a level seven times higher than normal chondrocytes and this could be due to the effects of IL-1 or TNF- α produced by chondrocytes, in response to inflammatory stimuli. The activated PAR-2 might further stimulate the production of proteinases, such as MMP-1 and MMP-13 as well as COX-2 in chondrocytes leading to the degradation of cartilage (Boileau et al. 2007). In the present study, SCAE supplementation was found to down-regulated the PAR-2 mRNA expression in monolayer OA chondrocytes culture. The low PAR-2 mRNA expression was expected as its level was modulated by the proinflammatory cytokines, IL-1 in which the earlier result showed that SCAE supplementation also down-regulated IL-1 mRNA expression.

In this present study, all of the disease markers present in the OA articular cartilage could be reduced by culture with SCAE supplemented media. This showed the capability of SCAE as a potential substance to reduce the cartilage degeneration process

during pathogenesis of OA. Curtis et al. (2004) and Janakiram et al. (2015) suggested that eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) were involved in a metabolic coupling mechanism causing the suppression of expression of the degradative enzymes, MMP3 and MMP13 as well as the cytokines IL-1 and inflammation factors COX2 that induce and propagate their expression in cartilage metabolism. The capability exhibited by SCAE as an antioxidant in the present study could be explained by phenolics to reduce iNOS mRNA and NO levels, thus slowing various catabolic processes in the cartilage, such as the loss of chondrocyte phenotype, chondrocyte apoptosis, and ECM degradation.

Conclusion

This study showed that SCAE could effectively promote proliferation of chondrocytes, enhance secretion and synthesis of cartilage ECM. Meanwhile, it could prevent chondrocyte dedifferentiation by up-regulating the expression levels of the aggrecan, collagen II, and sox9 genes while down-regulating the expression of collagen type I gene. SCAE was able to down regulate the expression of proinflammatory cytokines, IL-1, IL-6 and IL-8, proteolytic enzymes, MMP-1, MMP-3 and MMP-13, inflammatory mediators, iNOS, COX2 and reduced the productions of NO, PGE₂, PAR-2 in human osteoarthritis articular chondrocytes in vitro. *S. chloronotus* aqueous extract may attenuate the severity of articular cartilage degradation in OA through an increase in anabolic and a decrease in catabolic activity. Thus, SCAE may constitute a promising therapeutic option for the management of OA.

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Authors' contribution We declare that all authors listed contributed to the acquisition of data, drafting, critical revision and final approval of this manuscripts. Prof. Dr. Ahmad Nazrun Shuid and Dr. Chua Kien Hui conceived of the study and designed research. Dr. Mohd Heikal Mohd Yunus and Dr. Norzana Abd Gafar analyzed data. Dr. Mohd. Heikal Mohd Yunus performed research. Prof. Dr. Ahmad Nazrun Shuid and Dr. Chua Kien Hui helped coordinate the study. Dr. Mohd Heikal bin Mohd Yunus wrote the paper. All authors read and

approved the final manuscript. Dr. Mohd Heikal bin Mohd Yunus takes the integrity of this work.

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Compliance with ethical standards

Competing interest The authors declare that they have no competing interest.

Ethics approval Prior ethical approval was obtained from the Research and Ethical Committee of Faculty of Medicine, Universiti Kebangsaan Malaysia (FF-2015-235).

Informed consent All the human study subjects provided informed consent.

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