Chronic Diseases Prevention Review 9 (2019) 28-33

Available at http:// www.cancercellresearch.org ISSN 2158-0820

Protective effect of Alginate oligosaccharide on senescent cardiomyocytes

Jie Mou, Yongjun Mao*

Department of Geriatrics, the Affiliated Hospital of Qingdao University, Qingdao, 266003, China

Abstract: **To investigate the effect of Alginate oligosaccharide (AOS) on senescent cardiomyocytes. In this study, we established a senescence model of H9c2 cardiomyocytes by hydrogen peroxide (H_2O_2). Subsequently, senescent H9c2 cardiomyocytes was treated with AOS for 48h. Cell viability was determined with the methyl thiazolyl tetrazolium (MTT) assay. The protein expression of p21 was detected by Western blot. The mRNA expressions levels of p16 and p53 were detected by RT-PCR. We found that compared with the control group, cells viability was decreased, the expression of p21 protein, the expressions of p16 and p53 mRNA were significantly increased in the H_2O_2-induced senescent H9c2 cells ($p<0.05$). After treated with AOS, AOS significantly increased cell viability, inhibited the expression of p21 protein ($p<0.05$), meanwhile decreased the expressions of p16 and p53 mRNA ($p<0.05$). In conclusion, these results suggest that AOS was able to delay H_2O_2-induced cardiomyocytes senescence.**

Keywords: Alginate oligosaccharide; Senescence; H9c2 cells; Hydrogen peroxide

Received 15 January 2019, Revised 7 March 2019, Accepted 8 March 2019

*Corresponding Author: Yongjun Mao, mmc168@126.com

1. Introduction

Advanced age is an important risk factor for cardiovascular disease (CVD)[1]. Age-related CVD is a disease with high mortality. Cardiac aging result in a series of pathological changes, such as cardiac hypertrophy, myocardial fibrosis, ventricular diastolic dysfunction[2]. With the population ages, cardiovascular disease is seriously damaging the health of the elderly. However, there have no effective treatments for Age-related CVD.

Nowadays, aging has been widely concerned in many medical researches. Aging is characterized by degenerative changes in the body, accompanied by dysfunction of cells, tissues and organs[3]. Studies have shown that aging is associated with increased activation of pro-inflammatory and oxidative stress, and decreased DNA damage repair and mitochondrial function[4,5].

With the recognition and exploitation of marine resources, marine polysaccharides, a marine natural products, have been widely used in pharmaceutical, biomedical and cosmetics fields[6,7]. Alginate is an acidic marine polysaccharides, due to its large molecular weight and gelation, its application has been limited in medicine. Alginate oligosaccharide (AOS) is a kind of oligosaccharide formed by alginate degradation, it possesses good gel properties and a variety of biological activities, such as anti-oxidant, anti-inflammatory, growth-promoting[8-10]. Many studies have shown that it has high medical application value[11-13]. The effect of AOS on senescent cardiomyocytes remains unclear. In this study, we used H_2O_2-induced cardiomyocytes senescence model to explore whether AOS could delay the senescence of cardiomyocytes.

2. Materials and Methods

2.1. Cell and Reagents

H9c2 cell was obtained from the Shanghai Cell Bank, Chinese Academy of Sciences. Dulbecco's modified eagle medium (DMEM) was purchased from Hyclone (USA). Fetal bovine serum (FBS) was purchased from Gibco (USA). Penicillin and streptomycin were purchased from Solarbio (China). Cell culture flasks and 6-well plates were purchased from Corning (USA). AOS was obtained from School of Medicine and Pharmacy, Ocean University of China. Methyl thiazolyl tetrazolium (MTT) Assay Kit was purchased from Beyotime (China). p21 antibody was purchased from Cell Signaling Technology (USA), β-actin antibody was purchased from abcam (England). Goat anti-rabbit secondary antibody was purchased from Elabscience (China). Trizol was purchased from Invitrogen company (USA). Reverse transcription kits and Faststart Essential DNA Green Master mixes were purchased from Roche (Switzerland). The PCR primer were purchased from the Shanghai Sangon Biotech (China).

2.2. Cell culture

H9c2 cardiomyocyte line were cultured in DMEM medium with 10% FBS and 1% penicillin and 1% streptomycin at 37°C in a humidified incubator with 5% CO_2. Replaced the fresh complete medium every two days. At approximately 90% of confluence, cells were subcultured for the following experiments. The H9c2 cells used were between passages 3 and 8. All experiments were repeated 3 times.

2.3. H2O2-induced senescence model and drug treatments

When the confluency of cells was approximately 50%, the H_2O_2 group and AOS treatment group cells were added to 100μM H_2O_2 for 4h to establish cardiomyocytes senescence model. The induction conditions of H_2O_2 were determined by preliminary experiments. After 4h, replaced the fresh complete medium, and the H_2O_2+AOS group was treated with AOS (100μg/mL) for 48 h. The control group was only given synchronous replaced fresh medium.

2.4. MTT assay

The methyl thiazolyl tetrazolium (MTT) assay was used to determine cell viability. H9c2 cells (2000 cells/well) were seeded in 96-well plate, and added 100μM H_2O_2 for 4h to establish the senescence model, and then replaced the fresh complete medium, H9c2 cells were treated with AOS (100μg/mL) for 48h. According to the manufacturer's instructions, 10μL MTT solution was added to 96-well plate and incubated for 4h at 37°C. After adding 100μL of Formazan solution to each well, the 96-cell plate was shocked several times and incubated for 4 h at 37°C until the crystals was completely dissolved. Absorbance was measured at 570nm wavelength by enzyme immunoassay analyzer to calculate the relative ratio of cell viability.

2.5. Western blot analysis

Cells were lysed by RIPA buffer with 1% PMSF for 30min on ice, and then cell lysates were centrifuged at 12,000g for 20min at 4°C. Protein concentration was measured using BCA protein assay kit (Beyotime, China). Equal protein extracts (20μg) were re-suspended in SDS loading buffer and separated by 10% SDS-PAGE, and then transferred to PVDF membranes, which were blocked with 5% skimmed milk at room temperature for 2h. Next the membranes were washed with Tris-buffered Saline with Tween 20 (TBS-T) for 3 times, 5min each time. Then the PVDF membranes were incubated with p21 antibody (1:1000) and β-actin antibody (1:2000) overnight at 4°C. After that, the PVDF membranes were washed with TBS-T for 6 times, 10 min each time and incubated with goat anti-rabbit secondary antibody (1: 5,000) at room temperature for 2h. the membranes were treated with ECL reagent and exposed to the ECL imaging system. The blots were analyzed by Quantity One.

2.6. Quantitative Real Time-PCR (qRT-PCR) analysis

Total RNA was extracted from the cells using Trizol. According to the manufacturer's instructions, RNA was reversely transcribed into cDNA used reverse transcriptase kit. The relative expressions of mRNA were determined by RT-qPCR and normalized to β-actin. The total volume of PCR was 20μL, consisting of 10μL Faststart Essential DNA Green Master Mix, 1μL cDNA, 0.5μL forward primer and reverse primer, and 8μL RNase-free water. The qRT-PCR was performed using Light Cycler® 96 System (Roche, Switzerland). The primer sequences are listed in table 1. The relative mRNA expressions of p53 and p16 were calculated by the formula $2^{-\Delta\Delta Ct}$.

Table 1. The sequence of primers

Gene name		Sequence (5′-3′)
P53	Forward	GTCACCTCCACACCTCCACCTG
	Reverse	TGCCTGTCGTCCAGATACTCAGC
P16	Forward	CTCACCATGGATGATGATATCGC
	Reverse	CGTACCCAGTCTTCCTAAGGA
β-actin	Forward	ACCCAGAAGACTGTGGATGG
	Reverse	CACATTGGGTAGGAACAC

2.7. Statistical analysis

Statistical analysis was performed by SPSS 21.0 software. The data was expressed as means ± SD. Differences between two groups were compared using Student's t-test, differences between multiple groups were compared using one-way ANOVA. $P<0.05$ was considered to be statistically significant difference.

3. Results

3.1. Effect of AOS on cell viability

We measured the effect of AOS on cell viability by MTT assay. As shown in Figure 1, after the cardiomyocytes were exposed to H_2O_2, the cell viability was significantly decreased compared with the control group. However, we used 100 μg/mL AOS treated the H_2O_2-induced senescent cardiomyocytes, the result shown that AOS significantly increased cell viability compared with the H_2O_2 group.

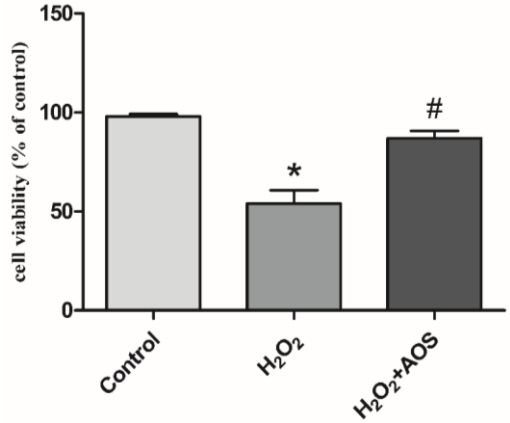

Figure 1. Effect of H_2O_2 and H_2O_2+AOS treatment on H9c2 cells viability. Cell viability was detected by MTT assay. *p <0.05 versus control group; #p <0.05 versus H_2O_2 group.

3.2. The expression of aging marker protein p21 in H9c2 cells

Senescent cells exhibit cell cycle arrest. p21 plays a key role in regulating the cell cycle. We investigated the effect of AOS on the expression of p21 protein by Western blot. As shown in Figure 2, compared with the control group, the protein expression of p21 was significantly increased in the H_2O_2-induced senescent cardiomyocytes. Compared with the H_2O_2 group, the expression of p21 was inhibited when treated by AOS. The result suggested that AOS treatment attenuated cell cycle arrest in the H_2O_2-induced senescent cardiomyocytes.

3.3. The mRNA expressions of aging-associated gene p53 and p16 in H9c2 cells

Tumor suppressor p53 and p16 are considered as biomarker for cellular senescence[14]. The effect of AOS on the mRNA expressions of p53 and p16 were determined by qRT-PCR. As shown in Figure 3, compared with the control group, the mRNA expression levels of p53 and p16 were obviously increased in the H_2O_2 group. Compared with the H_2O_2 group, AOS significantly decreased the mRNA expression levels of p53 and p16. Therefore, AOS alleviated H_2O_2-induced cardiomyocytes senescence.

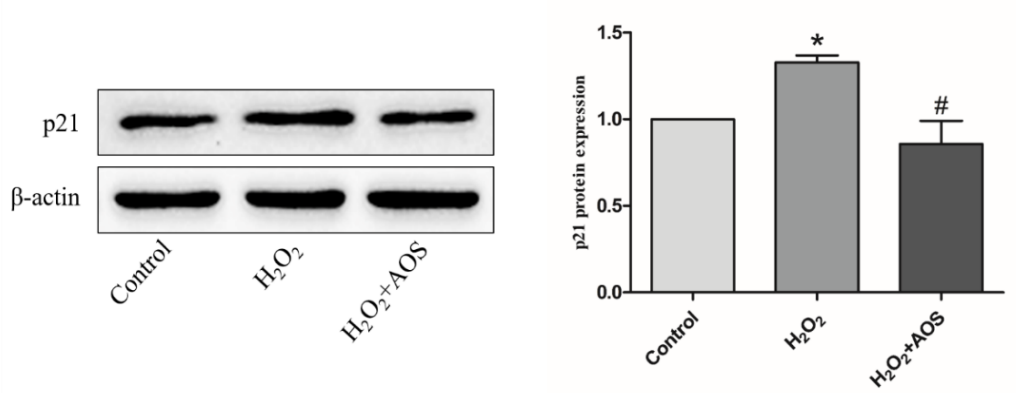

Figure 2. Effects of AOS on the expression of p21 protein in H_2O_2-induced senescent H9c2 cells. p21 protein expression was determined by Western blot. *p <0.05 versus control group; #p <0.05 versus H_2O_2 group.

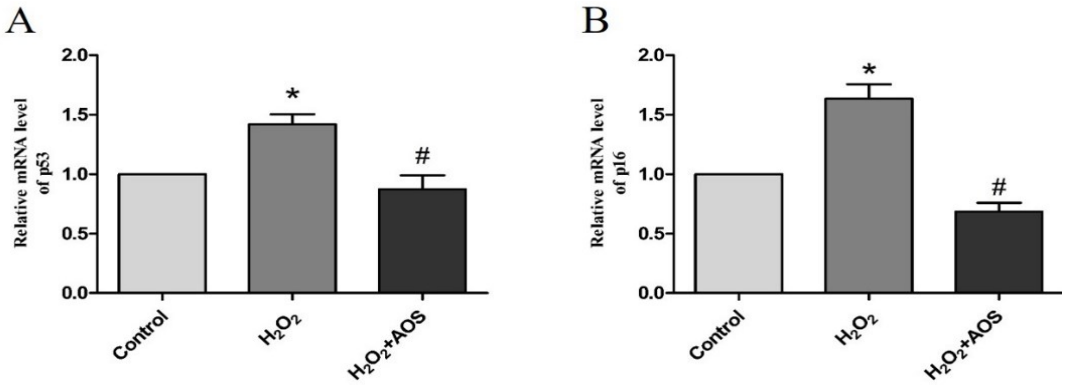

Figure 3. Effects of AOS on the expressions of p53 and p16 mRNA in H_2O_2-induced senescent H9c2 cells. The mRNA expressions were determined by qRT-PCR. A: the expression level of p53 mRNA; B: the expression level of p16 mRNA. *p <0.05 versus control group; #p <0.05 versus H_2O_2 group.

4. Discussion

Cardiovascular disease (CVD) is one of the major causes of mortality and morbidity in the world, Risk factors of CVD include high blood pressure, diabetes, smoking, and aging[15,16]. In particular, aging is an independent risk factor of CVD[17,18]. The cellular senescence is characterized by cell cycle arrest and decreased viability of cell[19]. Oxidative stress also play an important role in aging. Cardiac aging increases reactive oxygen species (ROS) levels in cardiac myocytes[1]. H_2O_2 can enhance intracellular oxidative stress and induce cell senescence, and it has been widely used to establish cell senescence models[20]. In this study, we used 100 μM H_2O_2 to induce H9c2 cardiomyocytes senescence model. We found that H_2O_2 significantly decreased cell viability and inhibited cell proliferation. AOS is an algal oligosaccharide derived from marine brown algae, and has been proved to have a variety of biological activities[21, 22]. When the H_2O_2-induced senescent cardiomyocytes were treated using AOS, AOS obviously enhanced cell viability. The result indicated that AOS had a protective effect on cell viability and proliferation of senescent cardiomyocytes.

Cellular senescence is characterized by cell cycle arrest and distinct epigenetic changes. As cyclin-dependent kinase inhibitors, p21, p53 and p16 play the critical role in senescence. Cell cycle arrest in senescent cells is associated with activation of two key pathways that include the p53/p21 and p16/retinoblastoma (Rb) proteins[23-25]. When senescence occur, the expression level of p53 increases, resulting in activation of its downstream factor p21, which induce senescence by blocking the activity of cyclin-dependent kinase[26,27]. Increased expression levels of p21 and p53 proteins have been observed in senescence[28,29]. Meanwhile, premature aging can occur in mice with overexpression of p16 gene[27]. Studies have shown that activation of Rb can drive the cell cycle via releasing E2F transcription factors. p16 inhibits the activation of Rb, which consequently induces senescence[29, 30]. Activation of p53 is also associated with DNA damage response in senescent cells. In normal cells, p53 maintain the low expression level in unstressed conditions, however, the expression levels of p53 is increased in senescent cells[31,32]. These previous studies have shown that the expressions of p21, p16 and p53 were increased after aging and could be used as biomarkers to determine aging. Therefore, we detected the protein expression of p21 by western blot, and determined the mRNA expressions of p53 and p16 by qRT-PCR. As expected, the expression levels of p21 protein, p53 and p16 mRNA were significantly increased in the H_2O_2-induced senescent cardiomyocytes. The results suggest that the model of cardiomyocytes senescence was well established. Subsequently, we attempt to demonstrate the hypothesis that AOS delayed the cardiomyocytes senescence. In this study, the H_2O_2-induced senescent H9c2 cells were treated with 100 μg/mL AOS, we found that AOS significantly inhibited the expression levels of p21 protein, p53 and p16 mRNA. Our results indicated that AOS not only increased cell activity, promoted cell proliferation, but also inhibited the expressions of senescence related markers in senescent cardiomyocytes. Thus we believe that AOS may delay H_2O_2-induced cardiomyocytes senescence.

In recent years, senescence-related CVD have seriously damaged people's health. With the intensive studies in the fields of cytology, molecular biology and genetics, delaying senescence and treating senescence-related CVD have become the research hotspot. Therefore, it is important to find new effective drugs for senescence-related CVD. Our study demonstrated the protective effect of AOS on H_2O_2-induced cardiomyocytes senescence. With the development of marine drugs, AOS will be possible application in clinic.

5. Conclusion

We demonstrated the protective effect of AOS on H_2O_2-induced H9c2 cells senescence, and this finding suggests that AOS may make a significant contribution to the treatment of senescence-related CVD.

Acknowledgements

This research was supported by National Natural Science Foundation of China (NSFC) (31571829 and 31640050), Natural Science Foundation of Shandong Province (ZR2016HQ23) and China Postdoctoral Science Foundation (2018M630757).

References

[1] Dai DF, Chen T, Johnson SC, et al. Cardiac aging: from molecular mechanisms to significance in human health and disease[J]. Antioxid Redox Signal, 2012, 16(12):1492-1526.

[2] Horn MA, Trafford AW. Aging and the cardiac collagen matrix: Novel mediators of fibrotic remodelling[J]. J Mol Cell Cardiol, 2016, 93:175-185.

[3] Booth LN, Brunet A. The Aging Epigenome[J]. Mol Cell, 2016, 62(5):728-744.

[4] Levine ME, Lu AT, Quach A, et al. An epigenetic biomarker of aging for lifespan and healthspan[J]. Aging, 2018, 10(4):573-591.

[5] Mikhed Y, Daiber A, Steven S. Mitochondrial Oxidative Stress, Mitochondrial DNA Damage and Their Role in Age-Related Vascular Dysfunction[J]. Int J Mol Sci, 2015, 16(7):15918-15953.

[6] de Jesus Raposo MF, de Morais AM, de

Morais RM. Marine polysaccharides from algae with potential biomedical applications[J]. Mar Drugs, 2015, 13(5):2967-3028.
[7] Fedorov SN, Ermakova SP, Zvyagintseva TN, et al. Anticancer and cancer preventive properties of marine polysaccharides: some results and prospects[J]. Mar Drugs, 2013, 11(12):4876-4901.
[8] Zhu B, Chen M, Yin H, et al. Enzymatic Hydrolysis of Alginate to Produce Oligosaccharides by a New Purified Endo-Type Alginate Lyase[J]. Marine Drugs, 2016, 14(6):108-119.
[9] Sellimi S, Younes I, Ayed HB, et al. Structural, physicochemical and antioxidant properties of sodium alginate isolated from a Tunisian brown seaweed[J]. Int J Biol Macromol, 2015, 72:1358-1267.
[10] Murat S. Effects of molecular weight and ratio of guluronic acid to mannuronic acid on the antioxidant properties of sodium alginate fractions prepared by radiation-induced degradation[J]. Appl Radiat Isot, 2011, 69(1):126-129.
[11] Khan S, Tondervik A, Sletta H, et al. Overcoming drug resistance with alginate oligosaccharides able to potentiate the action of selected antibiotics[J]. Antimicrob Agents Chemother, 2012, 56(10):5134-5141.
[12] Tøndervik A, Sletta H, Klinkenberg G, et al. Alginate Oligosaccharides Inhibit Fungal Cell Growth and Potentiate the Activity of Antifungals against Candida and Aspergillus spp[J]. Plos One, 2014, 9(11):e112518.
[13] Yang Y, Ma Z, Yang G, et al. Alginate oligosaccharide indirectly affects toll-like receptor signaling via the inhibition of microRNA-29b in aneurysm patients after endovascular aortic repair[J]. Drug Des Devel Ther, 2017, 11(9):2565-2579.
[14] Wang Y, Sharpless N, Chang S. p16(INK4a) protects against dysfunctional telomere-induced ATR-dependent DNA damage responses[J]. J Clin Invest, 2013, 123(10):4489-4501.
[15] Lagerweij GR, de Wit GA, Moons KG, et al. A new selection method to increase the health benefits of CVD prevention strategies[J]. Eur J Prev Cardiol, 2018, 25(6):642-650.
[16] Muntner P, Whelton PK. Using Predicted Cardiovascular Disease Risk in Conjunction With Blood Pressure to Guide Antihypertensive Medication Treatment[J]. J Am Coll Cardiol, 2017, 69(19):2446-2456.
[17] Foote K, Reinhold J, Yu EPK, et al. Restoring mitochondrial DNA copy number preserves mitochondrial function and delays vascular aging in mice[J]. Aging Cell, 2018:e12773.
[18] Marques FZ, Chu PY, Ziemann M, et al. Age-Related Differential Structural and Transcriptomic Responses in the Hypertensive Heart[J]. Frontiers in Physiology, 2018, 9:817-830.
[19] Zhang M, Du Y, Lu R, et al. Cholesterol Retards Senescence in Bone Marrow Mesenchymal Stem Cells by Modulating Autophagy and ROS/p53/p21(Cip1/Waf1) Pathway[J]. Oxid Med Cell Longev, 2016, 2016:7524308.
[20] Lin XL, Liu Y, Liu M, et al. Inhibition of Hydrogen Peroxide-Induced Human Umbilical Vein Endothelial Cells Aging by Allicin Depends on Sirtuin1 Activation[J]. Medical Science Monitor, 2017, 23:563-570.
[21] Powell LC, Pritchard MF, Ferguson EL, et al. Targeted disruption of the extracellular polymeric network of Pseudomonas aeruginosa biofilms by alginate oligosaccharides[J]. NPJ Biofilms Microbiomes, 2018, 4(1):13-23.
[22] Guo JJ, Xu FQ, Li YH, et al. Alginate oligosaccharide alleviates myocardial reperfusion injury by inhibiting nitrative and oxidative stress and endoplasmic reticulum stress-mediated apoptosis[J]. Drug Des Devel Ther, 2017, 11:2387-2397.
[23] Jin HJ, Kwon JH, Kim M, et al. Downregulation of Melanoma Cell Adhesion Molecule (MCAM/CD146) Accelerates Cellular Senescence in Human Umbilical Cord Blood-Derived Mesenchymal Stem Cells[J]. Stem Cells Transl Med, 2016, 5(4):427-439.
[24] Jiang C, Liu G, Luckhardt T, et al. Serpine 1 induces alveolar type II cell senescence through activating p53 - p21 - Rb pathway in fibrotic lung disease[J]. Aging Cell, 2017, 16(5):1114-1124.
[25] Banerjee K, Mandal M. Oxidative stress triggered by naturally occurring flavone apigenin results in senescence and chemotherapeutic effect in human colorectal cancer cells[J]. Redox Biol, 2015, 5:153-162.
[26] Chen Y, Pan K, Wang P, et al. HBP1-mediated Regulation of p21 Protein through the Mdm2/p53 and TCF4/EZH2 Pathways and Its Impact on Cell Senescence and Tumorigenesis[J]. J Biol Chem, 2016, 291(24):12688-12705.
[27] Boquoi A, Arora S, Chen T, et al. Reversible cell cycle inhibition and premature aging features imposed by conditional expression of p16Ink4a[J]. Aging Cell, 2015, 14(1):139-147.
[28] Ji M, Su X, Liu J, et al. Comparison of naturally aging and D-galactose induced aging model in beagle dogs[J]. Exp Ther Med, 2017, 14(6):5881-5888.
[29] Bourgeois B, Madl T. Regulation of cellular senescence via the FOXO4-p53 axis[J]. FEBS

Lett, 2018, 592(12):2083-2097.
[30] Ben-Porath I, Weinberg RA. The signals and pathways activating cellular senescence[J]. Int J Biochem Cell Biol, 2005, 37(5):961-976.
[31] Kim YY, Jee HJ, Um JH, et al. Cooperation between p21 and Akt is required for p53-dependent cellular senescence[J]. Aging Cell, 2017, 16(5):1094-1103.
[32] Qian Y, Chen X. Senescence regulation by the p53 protein family[J]. Methods Mol Biol, 2013, 965:37-61.

