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Effects of pine pollen polysaccharide and its sulfate on the production of ROS in cardiomyocytes

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By using the fluorescent probe 2′,7′-di-chlorofluorescein diacetate (DCHF-DA), 2-chloro- 1,3-dibenzothiazoline-cyclohexene (DBZTC) and 2,2,6,6-tetramethyl-1-piperidinoxyl-boron dipyrromethene (TEMPO-BDP) to mark reactive oxygen species (ROS) in cardiomyocytes (H9C2(2-1), the effect of PPM60 and SPPM60 on the production of ROS with laser scanning confocal microscope (LSCM) was detected. The results showed that PPM60 could increase the concentration of ROS, which indicated that PPM60 had an effect of inducing the production of ROS in cardiomyocytes. However, SPPM60 could reduce the concentration of ROS, so SPPM60 had an effect of eliminating ROS in cardiomyocytes.

Key words: Pine pollen, polysaccharide, sulfated polysaccharide, cardiomyocytes, reactive oxygen species (ROS).

INTRODUCTION

Oxidation is an essential process in all living organisms for the production of energy necessary for biological processes (Duan et al., 2006). In addition, oxygen-centered free radicals are involved in development of a variety of diseases, including cellular aging, mutagenesis, carcinogenesis, coronary heart disease, diabetes and neurodegeneration (Halliwell et al., 1999; Moskovitz et al., 2002). As reported recently, polysaccharides isolated from fungal, bacterial and plant sources were found to exhibit antioxidant activity and were proposed as useful therapeutic agents (Kodali et al., 2008; Li et al., 2006; Liu et al., 1997; Wang et al., 2008). Sulphated polysaccharides were found to possess wider pharmacological actions, especially potent free radical scavenging (Parket al., 2005) and antioxidant (Xue et al., 2001) effects. In the present study, the antioxidant activity of a polysaccharide fraction isolated from pine pollen and its sulfated derivative were investigated by molecule fluorescent probe in order to understand the potential usefulness of bioactive compound in pine pollen.

MATERIALS AND METHODS

Pinus massoniana pollen was obtained from Yantai New Era Health Industry Co., Ltd. The polysaccharide content of 60% alcohol precipitated pollen polysaccharide (PPM60) was 83.24%; SPPM60, sulfated derivative of PPM60 was derivated by chlorosulfonic acid-pyridine method and the degree of substitution was 1.762. They were mainly composed by mannose, galactose, fucose and an unknown monosaccharide. They were prepared in our laboratory. Rat cardiac muscle cell strain H9C2(2-1) was bought from the cell bank in Shanghai of Chinese Academy of Science.

Reagent

DMEM powder (GIBCO); DMSO (Shanghai Sangon Biological Engineering Technology & Services Co., Ltd, China); Trypsin (Sigma); FBS (Hangzhou Sijiqing Biological Engineering Material Co., Ltd, China); Fluorescent probe 2′,7′-di-chlorofluorescein diacetate (DCHF-DA Sigma); Probe 2-chloro-1,3-dibenzothiazoline-cyclohexene (DBZTC) and 2,2,6,6-tetramethyl-1- piperidinoxyl-boron dipyrromethene (TEMPO-BDP) were presented by key laboratory of molecular and nano probes, Shandong Normal

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University, China. The concentrations of PPM60 and SPPM60's water solution were 200 μg/ml.

Apparatus

Laser Scanning Confocal Microscope (LSCM; Leica TCS SPE, Germany); carbon dioxide cell incubator (NUAIRE, USA); 35 mm glass bottom cell culture dish (Shanghai Baisai Biological Technology Co., Ltd, China); inverted microscope (OLYMPUS, Japan).

Experimental

H9C2(2-1) cells in good condition were digested by pancreatin and made into cell suspension with DMEM. Cell suspension (0.5 ml) plated on 35 mm glass bottom cell culture dish was added another 1.5 ml DMEM, incubated in 37°C, 5% CO_2 incubator. After cells adhered to the glass bottom, DMEM medium was discarded and washed with D-Hanks 3 times. The DMSO solution of each fluorescent probe (10 μM, 0.5 ml) was added to each dish loaded with serum-free DMEM medium, incubated for 30 min. Before taking photos, each dish was washed with D-Hanks 3 times and added 0.5 ml D-Hanks for detection.

Confocal fluorescence imaging was performed with high resolution spectral confocal microscope with an oil immersion lens (100×). Excitation of probe-loaded cells at 488 nm was carried out with an argon ion laser, and emission was collected at 530 nm using detection system. Photos of cells in the same visual field were taken before and after adding PPM60 and SPPM60 (200 μg/ml, 0.5 ml) continuously (time interval was 30 s). Quantitative analysis was carried out on the images that were stored and the average fluorescence intensity of each image was calculated.

RESULTS

Effects of PPM60 and SPPM60 on the production of ROS in cardiomyocytes were detected by DCHF-DA

From Figures 1 to 4, it was observed that PPM60 could increase the fluorescence intensity of DCHF-DA in cardiomyoctyes and reached at the maximum at about 120 s after adding PPM60. The increasing rate was 115.02% (vs. control). Although the fluorescence intensity declined gradually after 2 min, it was still higher than resting state. However, SPPM60 could decrease the fluorescence intensity of DCHF-DA. Although they increased at 210 and 240 s, the increasing extent was very low. After that, it declined further and arrived at the minimum after 360 s, the decreasing rate was 30.55% (vs. control). The same trends were also observed in fluorescence images.

Effects of PPM60 and SPPM60 on the production of superoxide anion radicals ($O_2^{\cdot-}$) in cardiomyocytes detected by DBZTC

From Figures 5 to 8, it was observed that both PPM60 and SPPM60 could increase the concentration of $O^{\cdot-}_2$ in cardiomyoctyes. The extents of increasing were 98.34 and 36.46%, respectively.

Effects of PPM60 and SPPM60 on the production of hydroxyl radicals (·OH) in cardiomyocytes detected by TEMPO-BDP

From Figures 9 to 12, it was observed that both PPM60 and SPPM60 could decrease the concentration of ·OH in cardiomyoctyes. The extents of declining were 14.82 and 66.87%, respectively.

DISCUSSION

DCHF-DA is a lightless fluorochrome. After entering cell, DCHF-DA can be deacetylated to the oxidant-sensitive compound 2′,7′-dichlorofluorescein (DCHF) by cellular esterase. For DCHF to be oxidized to fluorescent DCF by ROS, the concentration of ROS has a direct proportion to the fluorescence intensity of DCF. So, by detecting the fluorescence intensity of DCF, we can observe the changes of ROS qualitatively and quantitatively (Hempel et al., 1999). DBZTC as a cell-permeable probe can selectively and dose dependently detect $O^{\cdot-}_2$ in cellular system and respond to micromole changes of $O^{\cdot-}_2$ concentrations. This probe features good stability, quick-reaction, high sensitivity and exhibits a better selectivity for $O^{\cdot-}_2$ over other ROS and biological compounds (no interference encountered from 500-fold molar H_2O_2) (Gao et al., 2007). TEMPO-BDP is a novel fluorescent probe that can rapidly and high selectively detect changes of ·OH, which sensitivity is very high. The principle of detection is that ·OH could react with DMSO to produce methyl free radicals. TEMPO-BDP will emit hyperfluorescence when combined with methyl free radicals. It has stable chemical and optic property and low level cytotoxic which is available for visualizing ·OH in living cells (Li et al., 2010).

It was discovered from our laboratory in previous researches that SPPM60 could enhance the contraction of isolated toad cardiac muscle significantly and the probable mechanism may work through L-type Ca^{2+} channel in order to increase the concentration of Ca^{2+} (Xia et al., 2009). Earlier investigation indicated that abnormal increase of $[Ca^{2+}]i$ could damage the structure of cell and disturb metabolism in cells called Ca^{2+} overload. When Ca^{2+} is overloading, the $[Ca^{2+}]i$ could be 8 to 10 times more than that in normal conditions. Ca^{2+} overload is an important factor in cardiomyoctyes injury. It always happen in ischemic-reperfusion injury, viral myocarditis, left ventricular remodeling, heart failure, etc (Park et al., 2005). In addition, Ca^{2+} overload can increase the combination of Ca^{2+} and CaM, it will affect the signal transduction within the cells. Besides activating Ca^{2+}-dependent protease, Ca^{2+} overloading also activates

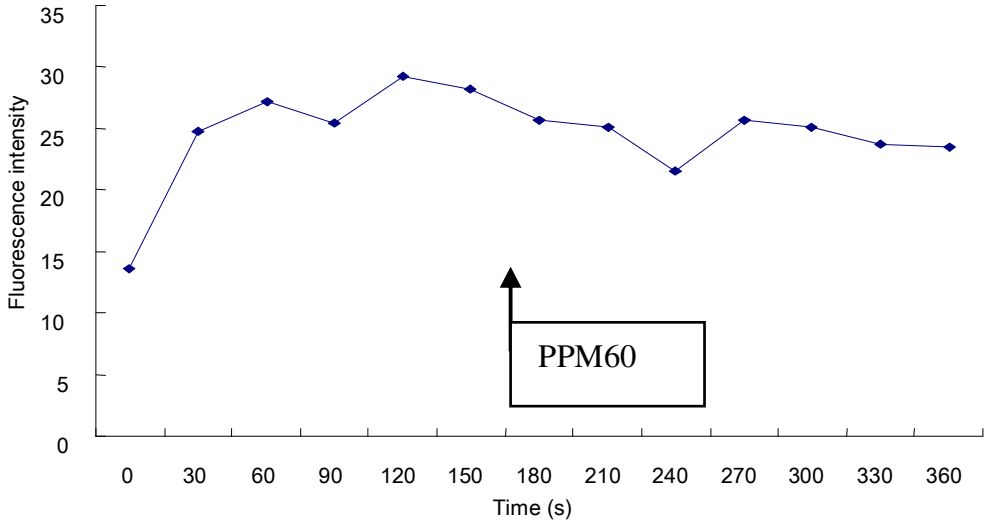

Figure 1. Kinetic changes of ROS in murine cardiomyocytes induced by pine pollen polysaccharide PPM60 (200 μg/ml). The vertical marker line indicates the time when PPM60 was added.

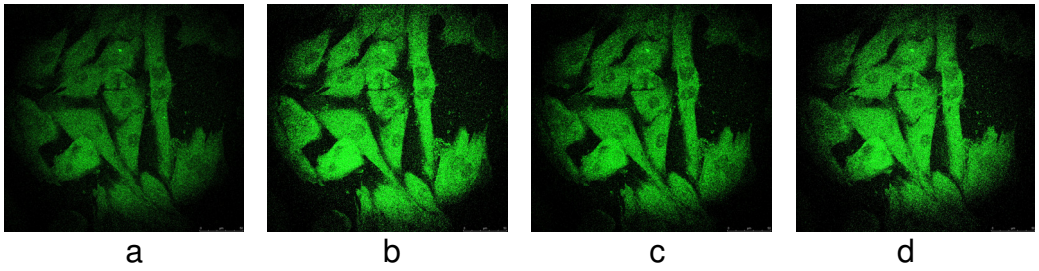

Figure 2. Confocal fluorescence images of murine cardiomyocytes. Cardiomyocytes were incubated with 10 μmol/L DCHF-DA for 30 min and then stimulated with 200 μg/ml PPM60 for 0 min (a), 2 min (b), 4 min (c), and 6 min (d).

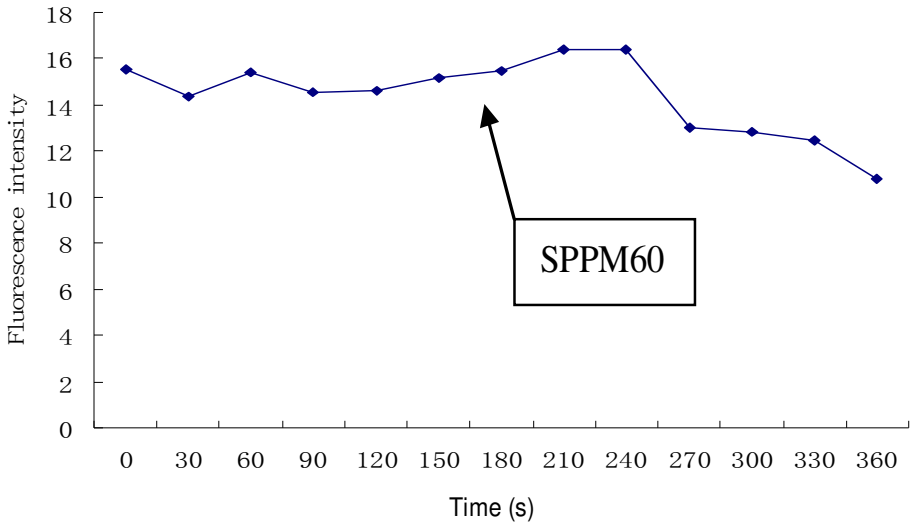

Figure 3. Kinetic changes of ROS in murine cardiomyocytes induced by sulfated pine pollen polysaccharide SPPM60 (200 μg/ml). The vertical marker line indicates the time when PPM60 was added.

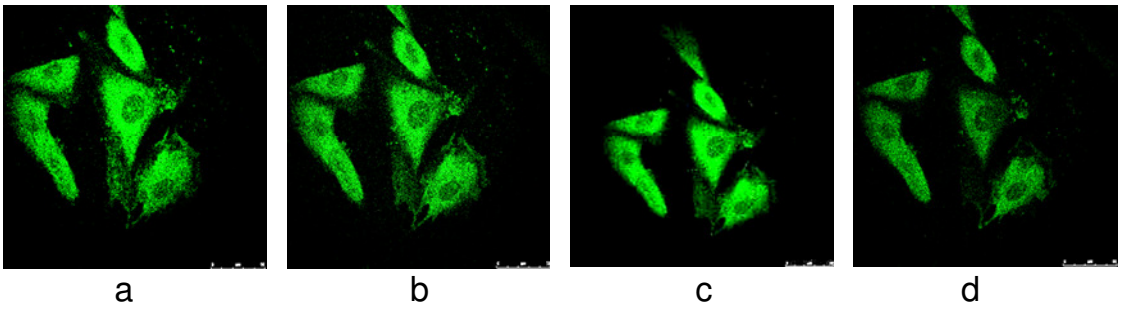

Figure 4. Confocal fluorescence images of murine cardiomyocytes. Cardiomyocytes were incubated with 10 µmol/L DCHF-DA for 30 min and then stimulated with 200 µg/ml SPPM60 for 0 min (a), 2 min (b), 4 min (c), and 6 min (d).

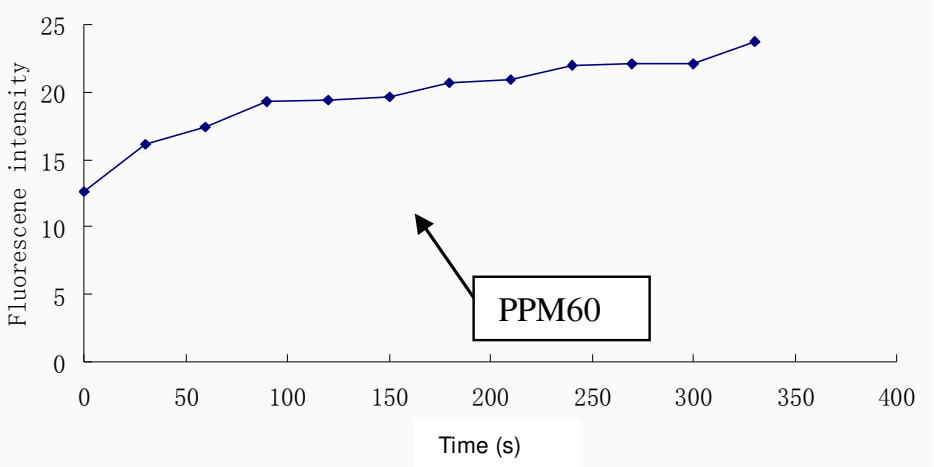

Figure 5. Kinetic changes of $O_2^{\cdot-}$ in murine cardiomyocytes induced by Pine pollen polysaccharide PPM60 (200 µg/ml). The vertical marker line indicates the time when PPM60 was added.

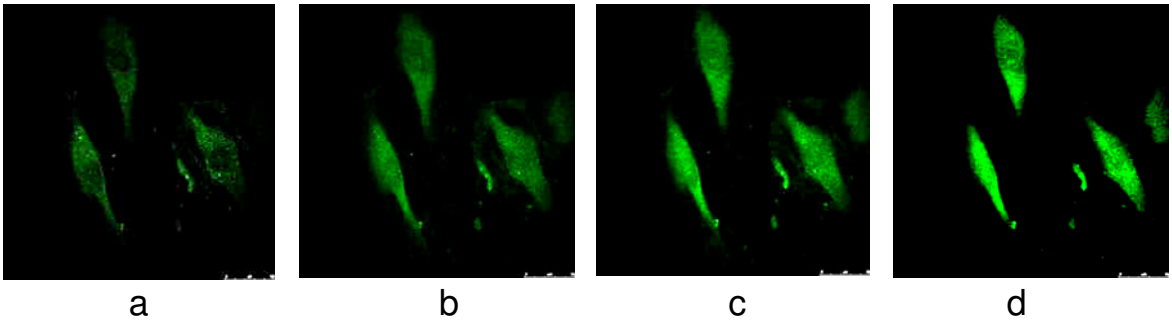

Figure 6. Confocal fluorescence images of murine cardiomyocytes. Cardiomyocytes were incubated with 10 µmol/L DBZTC for 30 min and then stimulated with 200 µg/ml PPM60 for 0 min (a), 2 min (b), 4 min (c), and 6 min (d).

many Ca^{2+}-dependent degradation enzyme. The activated phospholipase can hydrolysis biomembrane phospholipid, which will induce membrane and organelle injury; moreover, the activation of protein hydrolase and endonuclease could cause the decomposition of cytoskeleton and nucleic acid (Ray et al., 2000). Increasing $[Ca^{2+}]i$ could make muscle fiber contracture and break, injure (or damage) biomembrane and cytoskeleton, eventually result in irreversible damage of cardiomyocytes (Nayler, 1992).

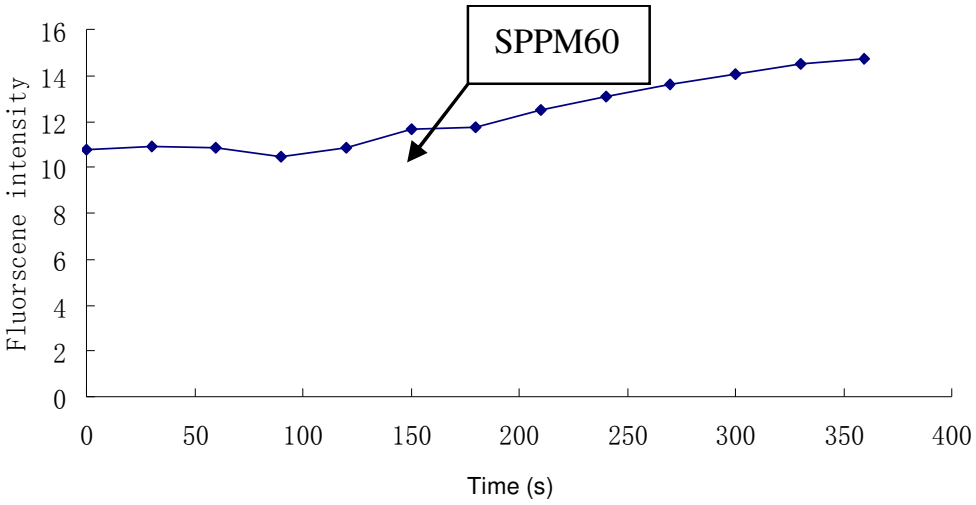

Figure 7. Kinetic changes of $O_2^{-}\cdot$ in murine cardiomyocytes induced by Sulfated Pine pollen polysaccharide SPPM60 (200 µg/ml). The vertical marker line indicates the time when PPM60 was added.

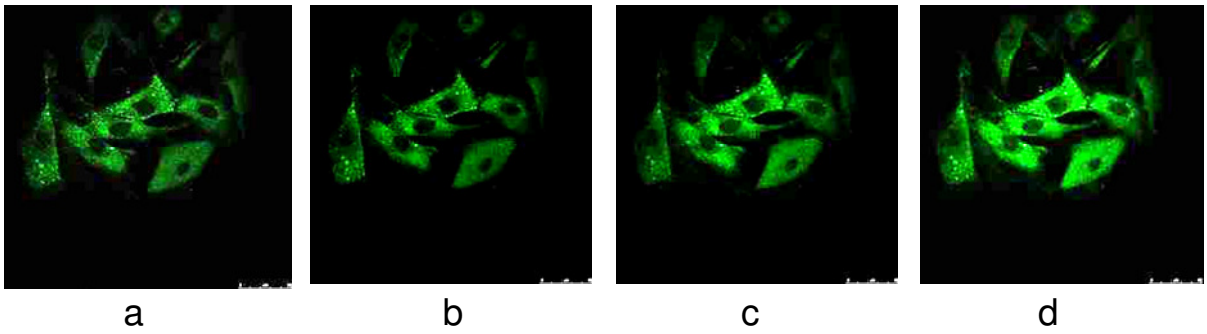

Figure 8. Confocal fluorescence images of murine cardiomyocytes. Cardiomyocytes were incubated with 10 µmol/L DBZTC for 30 min and then stimulated with 200 µg/ml SPPM60 for 0 min (a), 2 min (b), 4 min (c), and 6 min (d).

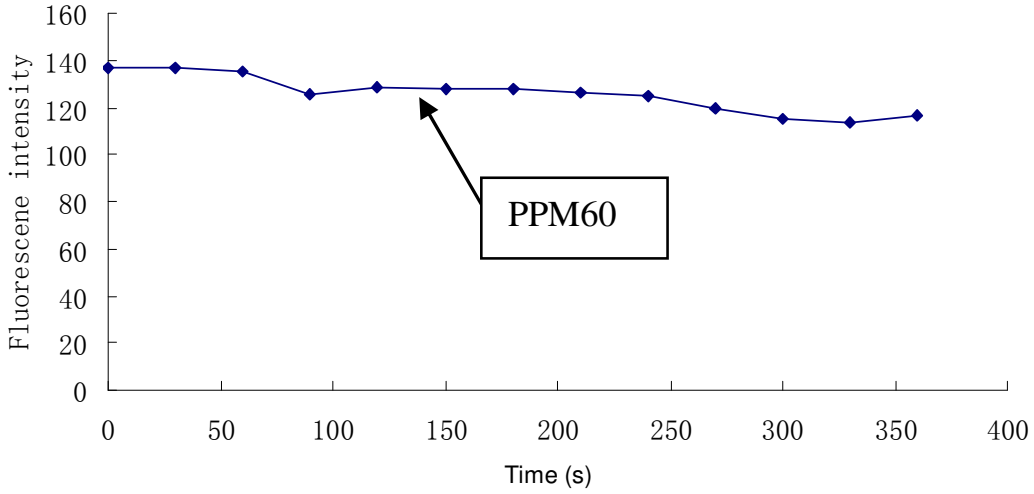

Figure 9. Kinetic changes of ·OH in H9C2(2-1) cells induced by PPM60 (200 µg/ml). The vertical marker line indicates the time when PPM60 was added.

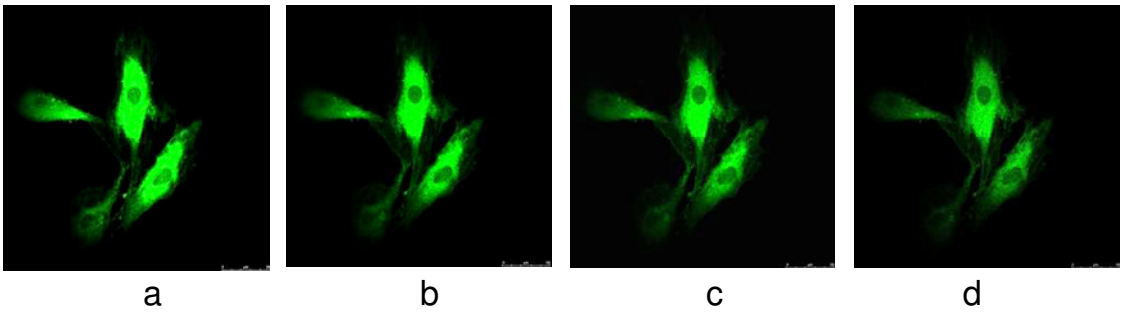

Figure 10. Confocal fluorescence images of H9C2(2-1) cells. Cardiomyocytes were incubated with 10 µmol/L TEMPO-BDP for 30 min and then stimulated with 200 µg/ml PPM60 for 0 min (a), 2 min (b), 4 min (c), and 6 min (d).

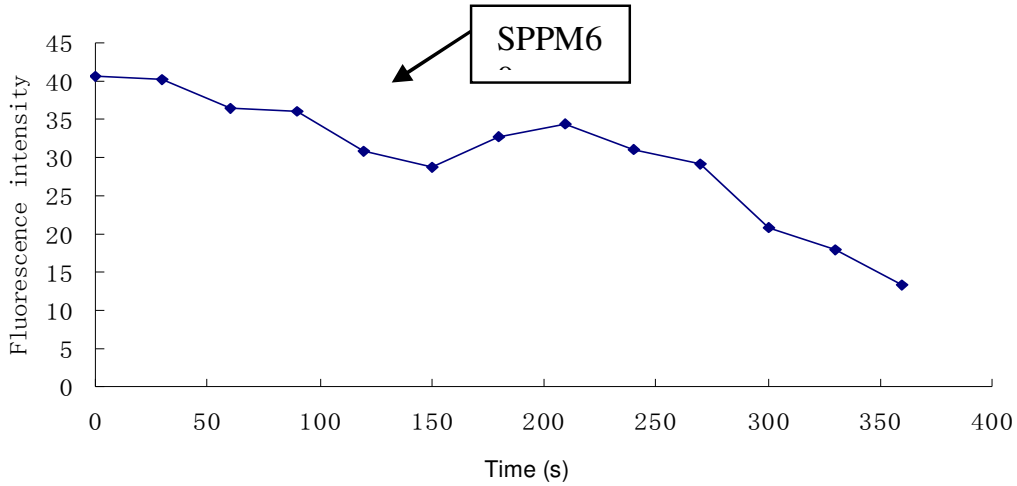

Figure 11. Kinetic changes of ·OH in H9C2(2-1) cells induced by SPPM60 (200 µg/ml). The vertical marker line indicates the time when SPPM60 was added.

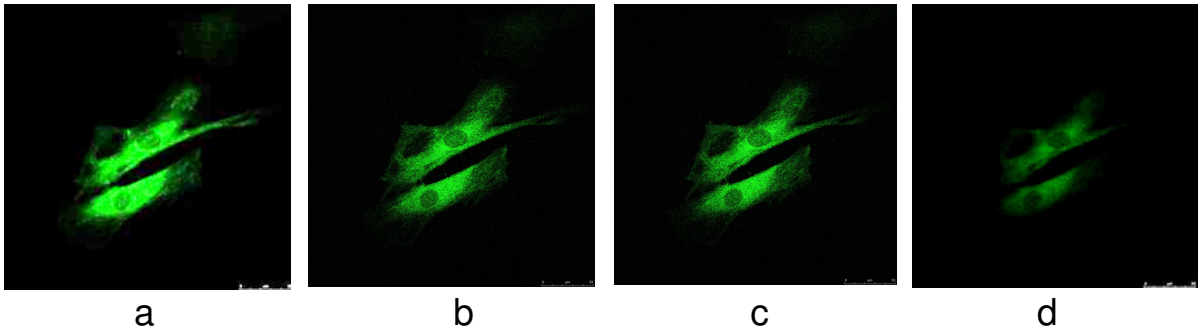

Figure 12. Confocal fluorescence images of H9C2(2-1) cells. Cardiomyocytes were incubated with 10umol/L TEMPO-BDP for 30 min and then stimulated with 200 µg/ml SPPM60 for 0 min (a), 2 min (b), 4 min (c), and 6 min (d).

It has been approved that Ca^{2+} overload has a close relation with ROS (Dixon et al., 1990). On one hand, high concentration of $[Ca^{2+}]i$ can activate phospholipase and Ca^{2+} sensitive protease easily. The activation of phospholipase A2 and protein kinase C could induce the production of arachidonic acid, which will produce lots of H_2O_2 and ·OH by cyclooxygenase. On the other hand, lipid peroxidation can occur between ROS and plasmalemmatic unsaturated fatty acid, and then alter the membrane enzymes, receptor and ionic lipid micro-

environment, finally damage the regular function of these protease and lead to Ca^{2+} overload. Besides, ROS could also act on the Ca^{2+} pump on sarcoplasmic reticulum and Na^{+}-Ca^{2+} exchanger and caused both functions destroyed and promoted Ca^{2+} overload (Otani et al., 1986).

Therefore, in order to certify whether the increasing of $[Ca^{2+}]i$ caused by SPPM60 induced Ca^{2+} overload and further promote the production of ROS, we used specific probe DCHF-DA, DBZTC and TEMPO-BDP to observe the effects of PPM60 and SPPM60 on the production of ROS in cardiomyocytes.

The results showed that SPPM60 did not induce the production of ROS, however, it had a certain effect of lowering of ROS production. Although the increasing effect of $O^{\cdot-}_2$ was low (26.72%), but the effect of decreasing ·OH was relatively strong (66.8%). This suggested that SPPM60 did not cause Ca^{2+} overload when it increase the concentration of $[Ca^{2+}]i$. So it will not induce the production of ROS and then avoid the probable cell injury. In addition, the lowering function of SPPM60 may even prevent in cellular Ca^{2+} overload, accordingly protect cardiac muscle in another side. At the same time, it was observed that PPM60 could decrease the concentration of ·OH, but the effect was very low, only 14.82%. However, its effect of increasing ROS and $O^{\cdot-}_2$ were 115.02 and 98.34%, so PPM60 could induce the production of ROS. This result opposed to the conclusion of PPM60's ROS scavenging qualities *in vitro* (Liu et al., 2008). We guess the probable mechanism is that the research *in vitro* carried out in a chemical system, PPM60, could react with the substance directly. But polysaccharide could not get into cell to take effect as macromolecule. So, the mechanism of it effect may through acting on certain membrane receptor, then affect the cellular signal transduction. There are more researches needed to be done to explore the exact mechanism.

Conclusions

The polysaccharide (PPM60) from pine pollen had an effect of inducing the production of ROS in cardiomyocytes. But its sulfated derivative (SPPM60) could eliminate ROS in cardiomyocytes. It is suggested that SPPM60 could be considered as a source of antioxidant agent which might be applied in pharmaceutical and cosmetic products.

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