

Background

Ageing of the vasculature has been associated with endothelial dysfunction, impaired angiogenesis and enhanced occurrence of atherosclerosis. The onset of senescence in vascular endothelial cells has been implicated in accelerating vascular aging and thereby increasing the risk of atherosclerosis. This is supported by the detection of senescent cells in atherosclerotic plaques of human autopsy material. Senescence in endothelial cells can be induced by cell cycle associated factors such as telomere attrition, a process referred to as replicative senescence, or by stress factors such as enhanced levels of reactive oxygen species (ROS), a process referred to as stress induced senescence (SIPS). Interestingly, DNA damage responses are activated in both pathways of senescence.

Oligomeric proanthocyanidins (OPCs) are complex, readily bio-available phytonutrients, composed of oligomers of 2 to 5 flavan-3-ol (catechin) units, whose presence in the diet is compromised by the fact that OPCs are mostly found in discarded food parts such as skins and seeds. The specific OPCs-rich compound used in the present study has been previously shown in human intervention trials to localize to vascular tissue and exert significant beneficial effects on vascular function. The OPCs-rich compound was also found to exhibit strong ROS-scavenging activity and protect vascular endothelial cells from oxidative damage. Based on the above observations, we tested the hypothesis that this OPCs-rich compound might delay vascular endothelial cell senescence and consequently have potential application in reducing atherosclerosis risk.

Material and Methods

Umbilical cords were obtained from the Department of Obstetrics and Gynecology, Diaconessen Hospital, Utrecht, The Netherlands, with the informed consent of the parents. Human Umbilical Vein Endothelial Cells (HUVECs) were isolated and cultured towards replicative senescence as described [1]. The cells were counted at the moment of seeding and passaging to allow the calculation of population doublings (PD, = $(\ln[\text{number of cells seeded}] - \ln[\text{number of cells harvested}]) / \ln 2$) after each passage. Senescence-associated- β -galactosidase (SA- β -gal) activity (Cell Signalling Technology, Beverly, MA, USA) was detected to determine the presence of senescent cells. For immunofluorescence the phosphorylated form of H2AX (γ H2AX) was detected by a monoclonal antibody from Upstate (clone JBW301). To induce SIPS cells were incubated with a range of rotenone (Sigma) concentrations and ROS production was determined by dihydro-ethidium (Invitrogen), followed by examination by fluorescence microscopy and quantification of the signal by image analysis software. Subsequently the effect OPCs on rotenone-induced SIPS was determined, using cell number and SA- β -gal as parameters.

Results

Analysis of the OPCs-Compound by HPLC (Figure 1):

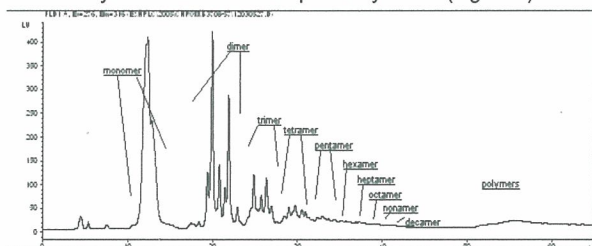
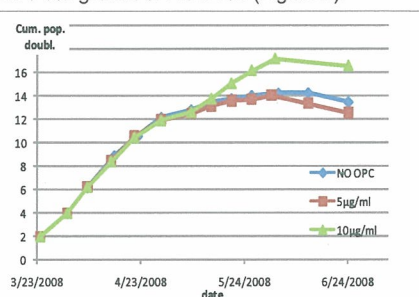


Figure 1: The OPCs-Compound was analyzed by HPLC. As the chromatogram in Fig.1 indicates, the product comprises of catechins and oligomers of 2-5 flavan-3-ol units. Catechins and dimeric forms of flavan-3-ols accounted for over 40% of the total product. The remaining flavan-3-ols were trimers, tetramers and pentamers. The extraction process ensured that clusters of 6 or more flavan-3-ol units formed less than 1% of the product.

OPCs delay the halt in replicative cell growth of HUVECs (Figure 2):

Figure 2: Human umbilical vein endothelial cells were cultured in the absence or continuous presence (5 μ g/ml or 10 μ g/ml) of OPCs, and the onset of replicative senescence was determined by calculating cumulative population doubling at each passage. By passage 11, cells cultured in the absence of OPCs exhibited a halt in cell growth, suggesting replicative senescence, and had a cumulative population doubling of 14. In contrast, at passage 11, cells grown in the presence of 10 μ g/ml of OPCs continued to show cell growth and had a cumulative population doubling of 17.



OPCs Attenuate Replicative Senescence (Figure 3):

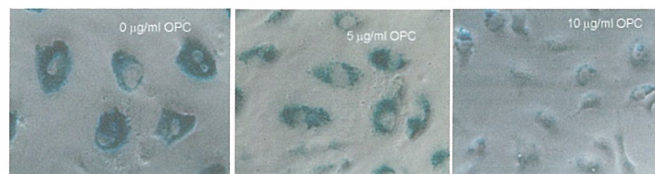


Figure 3: HUVECs grown in the absence (0mg/ml) or continuous presence (5mg/ml or 10mg/ml) of OPCs at passage 11 from the above experiment (Fig 2) were stained for S.A. β -galactosidase activity, a marker of senescence. Cells grown in the absence of OPCs exhibited characteristic morphology and strong β -gal staining, indicating onset of senescence. Culturing the HUVECs in the presence of OPCs caused a marked decrease in β -gal staining in a dose-dependent manner, with cells exhibiting morphology closer to early passage cells.

OPCs Attenuate γ -H2AX foci (Figure 4):

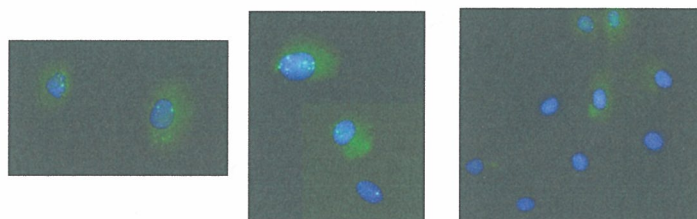


Figure 4: HUVECs grown in the absence (0 μ g/ml, left) or continuous presence (5 μ g/ml, middle, or 10 μ g/ml, right) of OPCs at passage 11 from the above experiment (Fig 2) were stained for γ -H2AX protein. Cells grown in the absence of OPCs or low concentrations of OPCs had several γ -H2AX foci, indicating DNA strand breaks or bare ends. Culturing the HUVECs in the presence of 10 μ g/ml of OPCs caused a marked decrease in the number of γ -H2AX foci.

OPCs Protect Cells from Stress Induced Premature Senescence (Figure 5):

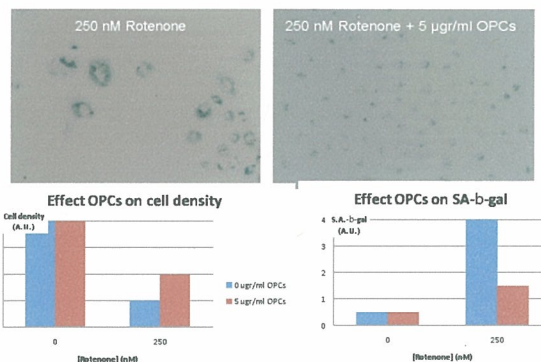


Figure 5: HUVECs were either pre-treated with 5 μ g/ml of OPCs or left untreated, and subsequently exposed to the superoxide-generating agent rotenone. Onset of stress-induced senescence was assessed by performing cell count at the start of the experiment and on day 6 after rotenone addition. Rotenone exposure caused marked a decrease in cell count by day 6 suggesting onset of senescence. However, pre-treatment with OPCs resulted in protection from rotenone-induced senescence as indicated by a smaller decrease in cell count by day 6 relative to cells not pre-treated with OPCs and by a lower levels of SA- β -gal stain by day 6 relative to cells not pre-treated with OPCs.

Summary and conclusion

-) The specific OPCs-compound used in this study significantly delays replicative senescence in human umbilical vein endothelial cells (HUVECs).
-) The OPCs-induced delay in replicative senescence is associated with decrease in levels of γ -H2AX foci, a marker of DNA damage and terminal DNA ends.
-) The OPCs-compound also protects HUVECs from stress-induced senescence caused by exposure to the oxidative stress-inducing agent rotenone.

In conclusion, the results suggest that the specific OPCs-compound used in this study might delay senescence in human vascular endothelial cells, possibly by reducing DNA damage and ROS stress. The results therefore provide a basis for further investigating the potential effects of this specific OPCs-compound in reducing risk of atherosclerosis and the molecular mechanisms involved.