Figure 2: Concentration of caspase-3 in the livers and brains of normal and aging mice. Data expressed as mean ± SD (n = 10);a,b,c Means in the same column with common superscript are not significantly different whereas values with different superscript are significantly different at P < 0.05

Figure 3: Activities of Na+-K+-adenosine triphosphatase (ATPase) and Ca2+-Mg2+-ATPase in the liver mitochondria of normal and aging mice. Data expressed as mean ± SD (n = 10);a,b,c Means in the same column with common superscript are not significantly different whereas values with different superscript are significantly different at P < 0.05

Table 5: Membrane potential ($\Delta\Psi$m) and fluidity in liver mitochondria of normal and aging mice

Groups	$\Delta\Psi$m	Fluorescence polarization (P)	Microviscosity (η)
NCG	44.46±0.48[a]	0.16±0.02[a]	1.05±0.15[a]
ACG	24.94±1.54[b]	0.32±0.02[b]	4.49±0.69[b]
SMO	41.19±1.03[c]	0.23±0.01[c]	1.97±0.24[c]
PCG	47.04±1.08[d]	0.24±0.02[c]	1.96±0.27[c]

Data expressed as mean±SD (*n*=10);[a, b, c] Means in the same column with common superscript are not significantly different, whereas values with different superscript are significantly different at *P*<0.05. NCG: Normal control group; ACG: Aging control group; SMO: *Silybum marianum* oil; PCG: Positive control group
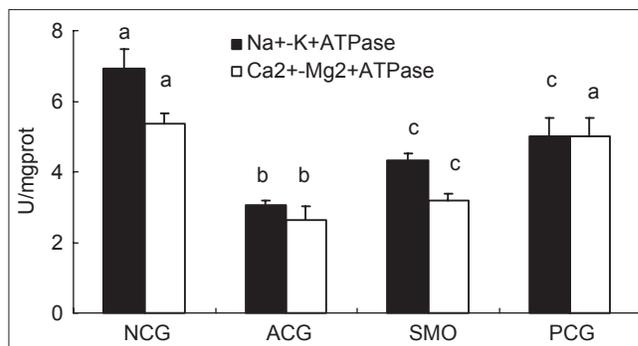
fluorescence polarization (*P*) in the liver mitochondrial membrane were significantly higher in ACG. SMO or olive oil significantly decreased average microviscosity and fluorescence polarization (*P*) of the liver mitochondrial membrane in D-gal-induced aging mice. Compared with NCG, the level of membrane potential ($\Delta\Psi$m) in the liver mitochondrial membrane was significantly lower in ACG. Supplementation with SMO or olive oil significantly increased the level of membrane potential ($\Delta\Psi$m).

Activities of Na+-K+-ATPase and Ca2+-Mg2+-ATPase in liver mitochondria

The effect of SMO on the activities of Na+-K+-ATPase and Ca2+-Mg2+-ATPase are presented in Figure 3. The results show that the activities of Na+-K+-ATPase and Ca2+-Mg2+-ATPase were significantly decreased in ACG mice as compared to NCG mice. Mice treated with SMO or olive oil had significantly higher levels of Na+-K+-ATPase and Ca2+-Mg2+-ATPase than ACG mice.

DISCUSSION

Aging is a biological process characterized by a general and progressive decline in physiological functions, especially of

the brain and liver.[34] D-gal has caused age-related changes in many animal models. It has been reported that the level of free radicals increases in animals that receive D-gal. The free radical theory of aging suggests that increased free radicals might contribute to the incidence of age-related degenerative diseases.[35-37]

The free radical damage hypothesis states that the generation of ROS or free radicals can lead to cell and tissue damage as well as alterations in the function of the genetic apparatus, resulting in aging and untimely cell death. SOD, GSH-Px, and Catalase (CAT) are the most important antioxidant enzymes that inhibit free radical formation. These enzymes also act as the primary defense system against ROS that are generated *in vivo* during oxidative stress.[38] Both SOD and GSH-Px are principally localized in the cytosolic and mitochondrial compartments of cells, whereas CAT is localized in the peroxisomes of cells. Under normal physiological conditions, these enzymes can efficiently counteract oxidative damages induced by free radicals in normal cells. In contrast, there are not enough endogenous antioxidant enzymes against excessive free radicals under abnormal physiological conditions.[39] In the present study, the mean activities of SOD and GSH-Px were found to be significantly lower in the liver and brain of D-gal-treated mice than in normal mice. Similar low levels of SOD and GSH-Px have been noted in tissues of D-gal-treated mice.[12] However, SMO significantly increased the activities of SOD and GSH-Px in the liver and the activity of GSH-Px in the brain. T-AOC reflects the capacity of the non-enzymatic antioxidant defense system. In the present study, FRAP (ferric-reducing antioxidant power) assay was used to evaluate the T-AOC of antioxidants. As shown in Table 2, SMO treatment increased the T-AOC in the liver indicating that the non-enzymatic antioxidant defense system of aging mice had been enhanced.

Lipid peroxidation is the process that involves chain reactions of free radicals with polyunsaturated fatty acids. These reactions lead to hydroperoxide generation and lipid breakdown into lower molecular weight fragments. Therefore, the inhibition of lipid peroxidation is of great importance in disease processes that involves free radicals.[40] MDA is a major reactive aldehyde resulting from the peroxidation of biological membrane-polyunsaturated fatty acids. It is therefore, used as an indicator of tissue damage involving a series of chain reactions. The levels of MDA were significantly higher in the liver and brain of D-gal-induced aging mice than in normal mice [Tables 2 and 3]. Similar findings have been reported in tissues of D-gal-treated mice.[41] Treatment with SMO or olive oil significantly reduced levels of MDA in both the liver and the brain of mice. These results indicate that the administration of SMO was effective in inhibiting the effects on lipid peroxidation in aging mice.

MAO is the major enzyme that catalyzes the degradation of neuroactive and vasoactive amines. Products of MAO-catalyzed reaction are the compelling inductors of lipid peroxidation. The activation of MAO is associated with age-related disturbances, homeostasis, and the generation of free radicals that involves the nervous tissue.[42] Brain tissues from mice injected with D-gal showed an increase in the activity of MAO. However, the administration of SMO or olive oil could significantly prevent this increase. The inhibitory effect of SMO or olive oil may correct the maladjusted metabolism of monoamine transmitter.

Apoptosis, also known as programmed cell death, is a biological process that plays a crucial role in the normal development and tissue homeostasis.[43] In this study, we found that chronic treatment with D-gal induced a significant increase in apoptosis in the liver and brain. The results also indicated that SMO or olive oil treatment significantly reduced apoptosis in the liver and brain of D-gal-treated mice [Figures 1 and 2].

The proto-oncogene Bcl-2 is one of the major regulators of the mitochondrial apoptotic pathway and plays a significant role in the regulation of apoptosis. The stability and expression levels of Bcl-2 protein can be regulated by ROS and NO through various mechanisms.[44] In our study, chronic administration of D-gal led to low levels of Bcl-2 in the liver and brain. But SMO treatment prevented the decline in Bcl-2 levels [Figure 1]. This may be due to the fact that SMO may reduce the level of oxidative stress.

Caspases, a family of cysteine proteases, are integral parts of the apoptotic pathway. Caspase-3, a key executor of apoptosis playing a vital role in programmed cell death, could be activated by ROS, leading to neuronal

dysfunction.[45] Studies have shown that an increase in Bcl-2 levels limits the effects of Bax and prevents the mitochondrial release of cytochrome c, thereby inhibiting the activation of caspase cascade and apoptosis.[46] In the present study, we found that SMO reversed the elevation of caspase-3 activity in the liver and brain of D-gal-treated mice [Figure 2]. Similarly, low levels of Bcl-2 and high levels of caspase-3 have been reported in the hippocampus of D-gal-treated mice.[47] These results suggest that SMO probably regulates the expression of apoptosis-related proteins by mitigating oxidative stress to attenuate apoptosis in the mitochondrial pathway. However, the detailed mechanism of action of SMO is unknown and requires further research.

Oxidant-induced mitochondrial damage, resulting in progressive loss of cellular energy (ATP) resources, degeneration, and eventually cell death, is believed to play a key role in the aging process.[48] Mitochondrial oxidative stress should be considered a hallmark of cellular aging and the identification of mitochondria as the major source of ROS led to the mitochondrial theory of aging. ROS can oxidize membrane lipids in close proximity to the respiratory chain; thus, decreasing the fluidity and increasing the permeability of the mitochondrial membrane.[49] Research has shown that mitochondrial dysfunction is a key factor in the mechanism of D-gal-caused aging acceleration.[15] In this study, we found that chronic treatment with D-gal induced mitochondrial dysfunction in the liver of mice. Treatment of mice with SMO was effective in ameliorating D-gal-induced mitochondrial dysfunction [Table 4 and Figure 3].

The mitochondrial membrane is a target for attack by ROS. Membrane fluidity and membrane potential reflect the biophysical and biochemical characteristics of the mitochondrial membrane and are the markers of membrane function, especially the production of energy. In the present study, membrane fluidity and membrane potential of the liver mitochondria in ICR mice were assessed. A significant reduction in the membrane potential and fluidity was observed when mice were treated with D-gal. However, SMO or olive oil significantly increased levels of membrane fluidity and membrane potential of liver mitochondria. This could be due to reduced oxidative damage thereby preventing mitochondrial membrane damage.

Mitochondrial oxidative phosphorylation is the primary source of energy for metabolism. Ion transport across the membranes regulates a number of biochemical reactions in the cell. About 40% of the total ATP is consumed by Na^+-K^+-ATPase and Ca^{2+}-Mg^{2+}-ATPase, which maintain the plasma membrane potential and intracellular Ca^{2+} sequestration. ATPases are very sensitive to peroxidation reactions since they are intimately associated

with the plasma membrane and participate in the energy requiring translocation of Na+, K+, and Ca^{2+} ions.[50] In the present study, the levels of Na^+-K^+-ATPase and Ca^{2+}-Mg^{2+}-ATPase were lower in the liver mitochondria of D-gal-treated mice compared to normal mice (NCG). Treatment with SMO or olive oil significantly increased levels of ATPases probably by preventing free radical induced membrane damage.

CONCLUSIONS

The results of this study indicate that SMO has a protective effect in D-gal-induced aging mice. SMO can attenuate lipid peroxidation, renew the activities of antioxidant enzymes, modulate the expression of apoptosis-related factors, and alleviate mitochondrial damage. The data suggested that the effect of SMO is similar to that of olive oil. Therefore, SMO has the potential to be further explored as a new vegetable oil.

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