Prevention of Oxidative Stress-Induced Apoptosis of C2C12 Myoblasts by a *Cichorium intybus* Root Extract

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**Note**

Cell injury associated with reactive oxygen species (ROS) has been reported in various muscular disorders. We found that a *Cichorium intybus* (Cii) extract reduced H$_2$O$_2$-induced viability loss in C2C12 myoblasts, inhibited oxidative stress-induced apoptosis and increased intracellular heat shock protein 70 (Hsp 70) expression. Cii also inhibited the level of intracellular ceramide. These results indicate that Cii may prevent skeletal muscle atrophy by inducing the expression of Hsp 70 and inhibiting the level of ceramide.

**Key words:** *Cichorium intybus*; heat shock protein 70; muscle atrophy; ceramide

Atrophy of skeletal muscles occurs when these muscles are not used due to immobilization, bed rest, or damage to related nerves. In special cases such as spaceflight, osteoporotic changes and muscle atrophy occur due to lack of consistent stimulation because of the low gravity in space. To regenerate dystrophic muscles, myoblasts can be transplanted and dystrophin can be delivered transiently to improve muscle strength.

Reactive oxygen species (ROS) are associated with various dystrophic muscular disorders, including Duchenne muscular dystrophy. Furthermore, extreme exercise increasing the production of ROS can lead to muscle injury. Oxygen influx and electron transport happens rapidly when skeletal muscles contract, making skeletal muscle cells highly susceptible to ROS-induced damage. Antioxidants may therefore help to prevent ROS-induced muscle damage in skeletal muscle cells.

Exposure of a cell to various stressors increases the expression of heat shock protein 70 (Hsp 70). One study, however, has reported that the expression of Hsp 70 was reduced in skeletal muscle atrophy. Hsp 70 is thought to protect cells from stress-induced apoptosis, including that induced by oxidative stress. The increased expression of Hsp 70 can therefore be expected to have a positive effect on reducing ROS-induced cell injury in skeletal muscle cells.

*Cichorium intybus* (Cii) is an edible plant commonly used as a salad ingredient that is also used to manage various chronic diseases. Components of this plant have been reported to have antimicrobial, anti-inflammatory, and hepatoprotective effects based on several studies. Moreover, this plant may be a potential source of antioxidants.

We assessed in this study the effects of stem and root extracts from this plant on cell viability and apoptosis in H$_2$O$_2$-treated C2C12 myoblasts. We also investigated the effects of these extracts on the ceramide amount and Hsp 70 expression.

C2C12 myoblasts were treated with H$_2$O$_2$ to determine the effects of the Cii extracts on oxidative stress. The cell viability after the H$_2$O$_2$ treatment was 27.80 ± 1.65%. However, the cells treated with the Cii extract showed increased cell viability in a concentration-dependent manner. In particular, when the cells were treated with 5, 10, 25 and 50 μg/mL of the Cii stem extract, the respective cell viability was 29.26 ± 8.38, 35.44 ± 7.46, 49.48 ± 8.01, 50.57 ± 1.93%, whereas the cells treated with these concentrations of the root extract had a respective cell viability of 33.18 ± 4.07, 51.84 ± 6.70, 109.80 ± 19.86, and 145.29 ± 9.75%. N-Acetyl cysteine (NAC) was included as a reference (positive control) to evaluate the strength of the Cii extract effect on the cellular response to stress. In comparison with the antioxidant, NAC, the Cii root extract showed a reduction in the H$_2$O$_2$-induced decrease in cell viability of the C2C12 myoblasts (Fig. 1A).

We determined the cell toxicity of these extracts by treating C2C12 cells with 5, 10, 25, and 50 μg/mL of each extract without H$_2$O$_2$. Neither of the Cii extracts were cytotoxic at any of the concentrations tested (data not shown).

We next investigated whether the root extract directly affected apoptosis. When the cells were treated with H$_2$O$_2$ alone, the percentage of apoptotic cells was 19.04 ± 6.17% of the total cells, but when the cells were treated with 25 μg/mL or 50 μg/mL of the Cii root extract, the percentage of apoptotic cells respectively decreased to 12.96 ± 2.52 and 5.56 ± 0.62% of the total number of cells, indicating that the Cii root extract significantly and concentration-dependently reduced

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**Abbreviations:** Cii, *Cichorium intybus*; Hsp 70, heat shock protein 70; ROS, reactive oxygen species
apoptosis (Fig. 1B).

Ceramide is involved in the regulation of cell death and acts as a lipid mediator of cellular stress responses.\textsuperscript{23,24} The ceramide level in cells is up-regulated by various types of stress conditions.\textsuperscript{25} Hsp 70 has been reported to block caspase-3 and SAPK/JNK activation and to inhibit the SAPK/JNK signaling cascade in ceramide-induced apoptosis, thus inhibiting apoptosis.\textsuperscript{26–28} We evaluated the effects of the Cii root extract on the endogenous ceramide levels involving the C2C12 cells damage induced by oxidative stress. There was an increase in the ceramide content when we examined endogenous ceramide by using an HPLC assay after treating with $H_2O_2$. Conversely, pre-treating with the Cii root extract maintained the ceramide content at the control level (Fig. 2).

The protective effect of Hsp 70 on cells is related to the inhibition of apoptosis.\textsuperscript{12,13} Based on several studies that have shown that Hsp 70 protected cells from stress-induced apoptosis, we hypothesized that the Cii root extract reduced C2C12 apoptosis by increasing Hsp 70 production. We performed a western blot analysis of Hsp 70 to test this hypothesis, and found that the expression of Hsp 70 increased in the group treated with the Cii root extract (Fig. 3). Given that apoptosis was significantly reduced by the Cii root extract and that treating with the Cii root extract increased the expression of Hsp 70, it is likely that Hsp 70 played a role in preventing the $H_2O_2$-induced apoptosis of C2C12 myoblasts.

Cii is a plant in the family Asteraceae that is used as a coffee supplement or in salads, but it has recently been shown to also have pharmacological activity.\textsuperscript{17–22} For example, this plant contains components that can reduce cholesterol and that have an anti-hyperglycemic effect.\textsuperscript{17} Furthermore, extracts of this plant have been reported to reduce hepatocellular damage, possibly by acting as potent scavengers of highly reactive radicals.\textsuperscript{19,22} A previous study has reported that components of the Cii root were alkaloids and/or nitrogenous bases, carbohydrates and/or glycosides, tannins, flavonoides, saponins and unsaturated sterols and/or triterpenes in an EtOH MeOH extracts.\textsuperscript{19} In addition, components in the EtOH extracts were dihydrolactucin, lactucin, (4-OH-phenyl) acetate ethyl-ester, 8-deoxylactucin, (epi) jacquinelin, and dihydrolactu-copicrin.\textsuperscript{29} Such muscular disease as muscle dystrophy is closely related to oxidative stress.\textsuperscript{5–7} Furthermore, as mentioned in the introduction, normal muscle cells and myoblasts are both sensitive to oxidative stress, making antioxidative
of oxidative stress. We also showed for the first time that a Cii root extract protected myoblasts against oxidative stress. We focused this study only on Hsp 70 production. However, a cell activates several complex mechanisms to protect itself from oxidative stress such as increasing the production of antioxidative enzymes. Various cell signaling pathways are also activated; therefore, further studies are required to gain further insight into the protective mechanisms of Cii.

In conclusion, the Cii root extract may inhibit or slow the progress of skeletal muscle atrophy by inducing the expression of Hsp 70 and inhibiting the level of ceramide.

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