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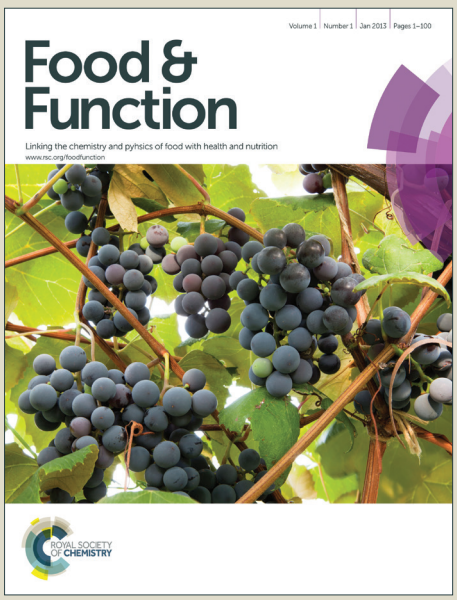

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Hydroxytyrosol induces apoptosis in human colon cancer cells through ROS generation

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Running title: HT-induced colon cancer cell death

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Abstract

Cancer cells are usually under higher levels of oxidative stress compared to normal cells. We hypothesized that the introduction of additional reactive oxygen species (ROS) or the suppression of antioxidant activity may selectively enhance cancer cell killing by generating oxidative agents through stress overload or stress sensitization. The aim of this work was to test whether hydroxytyrosol (HT), one of the major polyphenolic constituents of extra virgin olive oil, could exert anti-cancer effects on human colon adenocarcinoma cells via its ability to induce apoptosis through ROS generation. HT exhibits preferential anti-proliferative effects on human colon cancer cells (DLD1 cells) but not normal colon epithelial 1807 cells. HT causes oxidative stress, activates the phosphoinositide 3-kinase/Akt pathway, phosphorylates FOXO3a and then downregulates FOXO3a's target genes. Combined with SOD or catalase treatment, there are different responses in HT treated DLD1 cells. The results support the hypothesis that the two main species of ROS, superoxide and H_2O_2, play different roles in cancer survival. The present work shows that HT induces apoptotic cell death and mitochondrial dysfunction by generating ROS in colon cancer cells. This research presents important evidence on the *in vitro* chemopreventive effect of HT and shows that the disruption of the intrinsic redox status promotes cancer cell death.

Introduction

The regular consumption of extra virgin olive oil is believed to protect against a variety of pathological processes, including the development of cancer, particularly colon cancer[1]. Although extra virgin olive oil is rich in a variety of phenolic compounds, hydroxytyrosol (HT) has been the subject of most investigations primarily because it is the most bioavailable. In contrast to its benefits to normal cell

function, the anticancer properties of HT have been previously demonstrated in HL60 leukemia cells [2, 3], prostate cancer cells [4] and colon cancer cell lines [3, 5].

Cancer cells often have increased ROS generation from the mitochondria [6] or NADPH oxidase [7] and decreased levels of antioxidant enzymes, such as manganese superoxide dismutase (MnSOD, SOD2), CuZnSOD (SOD1), and catalase [8]. A small shift toward an oxidizing condition in cells may lead to elevated proliferation and the induction of an adaptive response. However, a high oxidizing condition often results in cell injury and cell death. Persistently high ROS levels in cancer cells often lead to increased cell proliferation and adaptive responses that may contribute to tumorigenesis, metastasis, and treatment resistance. Further exposure to exogenous ROS is hypothesized to push tumor cells, which already have high constitutive oxidative stress levels, towards cell death, whereas normal cells may still maintain redox homeostasis through adaptive responses. Therefore, regulating the intracellular redox state may represent an ideal strategy to selectively sensitize cancer cells to oxidative stress-inducing therapy.

Our previous studies have shown that HT induces phase II enzyme activation, which activates the endogenous antioxidant system and glutathione production and is capable of stimulating mitochondrial biogenesis in ARPE-19 and 3T3-L1 cells [9-12]. These effects both eliminate the production of reactive oxygen species (ROS) and prevent oxidative damage.

However, the effects of HT on colon cancer cells have not been well studied. Therefore, the aim of the present work was to ascertain the contribution of HT and its anti-proliferative effects on epithelial colorectal adenocarcinoma cells and to investigate its cellular mechanism of action.

Here, we report that HT is more effective in reducing the viability of colon cancer DLD1 cells than that of non-malignant colon epithelial (CRL1807) cells. HT induced mitochondrial dysfunction, resulting from superoxide-mediated defects in apoptosis.

Similar to other reports about the pro-oxidation activity of HT, *in vitro* hydrogen peroxide generation may be the main reason for its chemopreventive activity.

Materials and Methods

Materials

Hydroxytyrosol was purchased from Cayman Chemical Company; 5-(and-6)-chloromethyl-2′, 7′-dichlorodihydrofluorescein diacetate acetyl ester (DCFDA) were purchased from Molecular Probe (Eugene, OR). Antibodies against Akt, phospho-Akt Ser 473, phospho-FOXO3a Ser 253, cleaved PARP1, cleaved caspase-3 and cleaved caspase-7 were purchased from Cell Signaling (Beverly, MA). Antibodies against PI3K, SOD1 and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-catalase antibody was purchased from Novus Biologicals (Littleton, CO). The anti-SOD2 antibody was purchased from Millipore (Billerica, MA). Annexin V and PI were purchased from Sigma-Aldrich (St. Louis, MO). Trypsin and 5,5',6,6'-tetrachloro-1,1',3,3'-tetra ethylbenzimidazolylcarbocyanine iodide [JC-1; CBIC2(3)], Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), gentamicin, and L-glutamine were purchased from Invitrogen (Carlsbad, CA).

Cell culture and treatments

Colorectal adenocarcinoma DLD1 cells were cultured in a monolayer at 37 ℃ with 5% CO_2. The cells were grown in DMEM supplemented with 10% FBS, 2 mmol/L L-glutamine, and 25 μg/ml gentamicin. For the cell viability assay, the mitochondrial membrane potential assay, the H_2O_2 generation assay and the apoptosis assay, DLD1 cells were pretreated with different concentrations (50, 100, or 200 μmol/L) of HT for 24 or 48 h. For the SOD and catalase pretreatment assays, cells were incubated with 200 μmol/L HT for 24 h. Immortalized non-malignant human colon epithelial CRL1807 cells were cultivated in McCoy's Modified 5A media supplemented with

10% FBS, 0.1 mmol/L nonessential amino acids, 50 units/ml streptomycin, and 50 units /ml penicillin. The cells were maintained in a 5% CO_2 incubator at 32 ℃.

Cell viability assays

Cell viability was measured by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, which is based on the conversion of MTT to formazan by mitochondrial and cytosol dehydrogenases 13, 14. After cells (about 1 × 103 cells/well) were treated with the indicated concentrations for 24 or 48 h after seeding. At the indicated time, they were incubated with 50 μL MTT solution (5mg/ml) for 4 hr at 37 ℃, 5% CO2. The MTT solution was removed, and 100 μL DMSO was added to each well to dissolve the formazan. The absorbance of the cultures was measured using a multi-well spectrophotometer at a wavelength of 560 nm (Specter Max 190; Molecular Devices, Sunnyvale, CA). Cell viability was expressed as O.D. values. The results were calculated as the percentage of absorbance in the control cultures.

Mitochondrial membrane potential assay

Changes in mitochondrial membrane potential were measured using JC-1 staining. When JC-1 enters the mitochondrial matrix and accumulates, it forms red fluorescent J-aggregates. However, the amount of monomeric, green fluorescent JC-1 present in the cytoplasm increases as the mitochondrial membrane potential decreases. The ratio of red to green fluorescence was used to assess the mitochondrial membrane potential. $1*10^6$ cells were resuspended in 0.5 ml medium and incubated with 10 μg/ml JC-1 for 20 min at room temperature. The cells were then washed twice with ice-cold PBS and analyzed by flow cytometry (FACSAria; Becton Dickinson, San Jose, CA). The fluorescence intensity at emission wavelengths of 530 and 590 nm was measured by ratiometric analysis [15].

Apoptosis analysis

Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide [2] double staining was used to measure the percentage of apoptotic cells according to the manufacturer's protocol (BD Pharmingen). Briefly, DLD1 cells were grown in 12-well plates in DMEM. When the cells reached 80–90% confluence, the cells were pretreated with HT, SOD or catalase. After 24 or 48 h, the cells were washed with PBS, digested with 0.25% trypsin/EDTA, and washed with PBS. The cells were re-suspended in 500 μl of 1× binding buffer, and Annexin V-FITC/PI was added. After incubation at room temperature for 10 min, the cells were measured using flow cytometry.

Immunoblotting analysis

DLD1 cells were lysed in RIPA buffer. The protein concentration was measured using the Bradford Protein Assay Reagent (Bio-Rad), and 30 μg of sample was separated using SDS-PAGE. The proteins were then transferred to membranes and incubated with antibodies. The blots were then incubated with secondary antibodies conjugated to horseradish peroxidase. The immunoreactive bands were detected with the enhanced chemiluminescence reagent (Amersham).

ROS assay with dichlorofluorescein (DCF)

The dichlorofluorescein (DCF) assay was performed using carboxy-H2DCFDA (sensitive to oxidation; Invitrogen) and oxidized carboxy-DCFDA (insensitive to oxidation; Invitrogen) as optimized by Wan and colleagues [16]. The fluorescence in cells preloaded with carboxy-H_2DCFDA was normalized to the fluorescence of cells preloaded with carboxy-DCFDA (ratio of H_2DCFDA/DCFDA) to control for cell number, dye uptake, and ester cleavage differences between the different treatment groups.

Statistical analysis

Statistical analysis was performed using either Student's t test (for two-group comparison) or one-way ANOVA (for multiple-group comparison). The data were reported as the mean ± SEM.

Results

Effects of HT on cell viability

Human colon cancer DLD1 cells and non-malignant human colon epithelial CRL1807 cells were treated with 0, 50, 100 or 200 μmol/L HT for 24 or 48 h. After treatment, an MTT assay was performed to investigate the influence of HT on cell viability. Fig. 1a shows that HT inhibited the growth of DLD1 cells in a concentration and time-dependent manner. However, the growth inhibitory effect of HT on CRL-1807 cells was less pronounced (Fig. 1b) at the same concentrations.

SOD (100 units) and catalase (1000 units) alone had no effect on DLD1 colon cancer cells. Treating DLD1 cells with 200 μM of HT for 48 h caused a marked increase in cell death, which was increased further when SOD (100 units) was added to the cell culture medium (Fig. 1c). However, catalase (1000 units) addition reduced the amount of cell death (Fig. 1c).

These results clearly indicate that HT induces the production of H_2O_2, which is responsible for its induction of cell death in DLD1 cells.

Effects of HT on mitochondrial membrane potential

HT decreased the mitochondrial membrane potential in a dose-dependent manner, as shown by the red–green JC-1 fluorescence ratio (Fig. 2a). Similar to the cell viability results, the addition of SOD (100 units) enhanced the decrease in mitochondrial membrane potential induced by HT. In contrast, the addition of catalase (1000 units) resulted in the recovery of the mitochondrial membrane potential (Fig. 2b).

HT induced the generation of reactive oxygen species (ROS) in DLD1 cells

Oxidative stress is the major cause of HT-induced cell death [4]. We determined the effect of HT on cellular ROS levels in DLD1 cells using the DCF assay. As shown in Fig. 3a, HT significantly increased the normalized carboxy-H_2DCFDA fluorescence, a general indicator of cellular ROS levels, in a dose-dependent manner after 24 and 48 h of treatment. When combined with SOD or catalase treatment, HT decreased the cellular ROS levels in DLD1 cells (Fig. 3b).

Similar to other polyphenols, HT is unstable and generates hydrogen peroxide in the cell culture media. Superoxide dismutase [17] catalyzes the superoxide conversion into oxygen and hydrogen peroxide, whereas catalase (CAT) catalyzes the breakdown of hydrogen peroxide into water and oxygen.

Effects of HT on apoptosis

We next determined whether the HT-induced growth inhibition was caused by the induction of apoptosis. The results show that HT induced apoptosis in DLD1 cells in a dose-dependent manner after 48 h of treatment (Fig. 4a). The amount of apoptosis was further increased by SOD treatment, but decreased following catalase treatment. Moreover, the addition of SOD alone increased the amount of apoptosis observed in the DLD1 cells (Fig. 4b).

To confirm the involvement of apoptotic proteins, the expression levels of three key proteins, cleaved PARP1, cleaved caspase-3 and cleaved caspase-7, were examined in DLD1 cells. The results showed that HT treatment resulted in a dose-dependent increase in cleaved PARP1, cleaved caspase-3 and cleaved caspase-7 expression in DLD1 cells (Fig. 4c).

Effects of HT on the Akt/FOXO3a pathway

FOXO3a is a major target of activated Akt. There are three conserved Akt phosphorylation sites on FOXO3a: Thr 32, Ser 253, and Ser 315. HT treatment resulted in an increase in phosphorylation on Ser 253 of FOXO3a in a dose-dependent

manner. HT treatment also resulted in an increase in the phosphorylation of Ser 473 on Akt (Fig. 5a).

FOXO3a regulates a wide range of target genes. Because HT kills cells, in part through the generation of ROS, we examined the expression of two FOXO3a targets, catalase and SOD2. The expression of SOD2 protein was increased in a dose-dependent manner following HT treatment. HT treatment resulted in a decrease in catalase levels in DLD1 cells (Fig. 5b).

The phosphorylation of Akt and FOXO3a were further increased by SOD treatment, but decreased following catalase treatment (Fig. 5c).

Discussion

Extra virgin olive oil in a major component of the Mediterranean diet, and its regular ingestion has been proposed to provide protection against the development of numerous disease, most notably cancer[1]. The *in vitro* anticancer properties of HT, a minor component of olives, were investigated in different cell lines [3-5, 17-19]. Extra virgin olive oil polyphenols have been suggested to possess anticancer effects against colorectal carcinogenesis by inhibiting different stages of the colon carcinogenesis process [20] or by inducing apoptosis [21]. The cellular mechanisms by which olive oil polyphenols exert these anticancer effects are still poorly understood. In this study, we showed that HT had an anti-proliferative effect (Fig. 1) and induced apoptotic cell death (Fig. 4) through ROS generation (Fig. 3) in human colon cancer cells. HT cytotoxicity has also been reported in human prostate cancer cells [4]. Our current study confirmed these finding in human colon cancer cells, and we further examined the effects that HT-induced ROS on colon cancer cells. The current findings confirmed this observation from human colon cancer DLD1 cell line, which demonstrated the formation of intracellular ROS when incubated with HT in a time-dependent manner (Fig.3).

Over the years, redox regulated cell fate signaling during carcinogenesis has been studied [22-24]. Despite the dogmatic view of ROS as a detrimental agent, emerging

evidence has shown that nontoxic concentrations ROS can act as proliferative and survival signals [23]. To that end, a slight increase in intracellular superoxide levels has been shown to promote cancer cell survival by inhibiting apoptotic execution, irrespective of the trigger [23, 25-28]. Other research has shown that the regulation of cell fate by intracellular redox status was the result of a tight balance in the ratio of superoxide to H_2O_2, and this ratio could be affected by the constitutive expression of antioxidant enzymes, in particular the SODs [24]. This balance is tightly regulated by a plethora of antioxidant defenses, and the deregulation of these mechanisms could potentially alter the cell fate decision, as is the case in cancer cells that are deficient in the antioxidant enzyme superoxide dismutase [17]; depletion of this enzyme leads to the accumulation of superoxide, which stimulates cell survival and proliferation. On the contrary, slight accumulation of H_2O_2 generates an intracellular environment that permits cell death signaling.

Polyphenols produce hydrogen peroxide in the auto-oxidation process, which is a common event in cell culture media[29]. It has been reported that the chemopreventive effect of hydroxytyrosol is due to extracelluar hydrogen peroxide[30]. In our previous studies, we found that NAC, catalase and pyruvate can scavenge hydrogen peroxide generation in media induce by hydroxytyrosol, which prevented prostate cancer cell viability loss[4]. However, superoxide generates in polyphenol (such as EGCG) auto-oxidation process, which works as a catalyst to hydrogen peroxide. SOD can suppress hydrogen peroxide generation via inhibition of the catalytic effect of superoxide on polyphenol auto-oxidation[31].

In our study, we found that combining HT treatment with the addition of SOD significantly increased the amount of apoptosis in H_2O_2 sensitized cells. In contrast, the addition of catalase, which catalyzes the conversion of H_2O_2 to water and oxygen, prevented the loss of viability induced by HT.

ROS plays an important role in redox signaling due to its highly regulated activation [32]. The PI3K/Akt/FOXO3a cascade leads to the down regulation of the antioxidant

enzymes MnSOD and catalase, which are also involved in the oxidative stress response induced by HT.

Many studies suggest that antioxidant systems are critical in protecting against tumor-promoting agents, and that one or more components of these systems are deficient in many forms of cancer[33]. Both catalase and manganese SOD (Mn-SOD) appear to be particular important in this regard.

Akt is perhaps the most frequently activated oncoprotein in human cancers. Akt is activated by multiple mechanisms, including Pten mutations, p110-activating mutations, Ras activation, and receptor tyrosine kinase activation [34, 35]. Thus, Akt is an attractive target for cancer therapy, and a strategy that allows for the selective eradication of cancer cells with hyperactivated Akt is highly desirable. It was reported that cancer cells expressing activated Akt are selectively killed by oxidative stress [36]. Furthermore, our results show that HT treatment increases Akt activity, which further sensitizes the cells to oxidative stress-induced apoptosis.

In summary, our study shows that HT induced ROS generation in DLD1 cells, and ROS acted as a second messenger to activate the PI3K/Akt pathway, leading to a decrease in the antioxidant defense capacity through FOXO3a suppression. Our results imply that modulating the intracellular redox state might be an ideal strategy for cancer therapy.

Acknowledgments

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The author's responsibilities were as follows: LJ.S, C.L and JK.L designed and conceived the study; LJ.S conducted the cell culture and other experiments assays; C.L undertook statistical analyses; LJ.S interpreted the findings and wrote the

manuscript; Jk.L reviewed and edited the paper. All authors read and approved the final manuscript.

LJ.S, C.L and JK. L have no conflicts of interest to declare.

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Figure legends

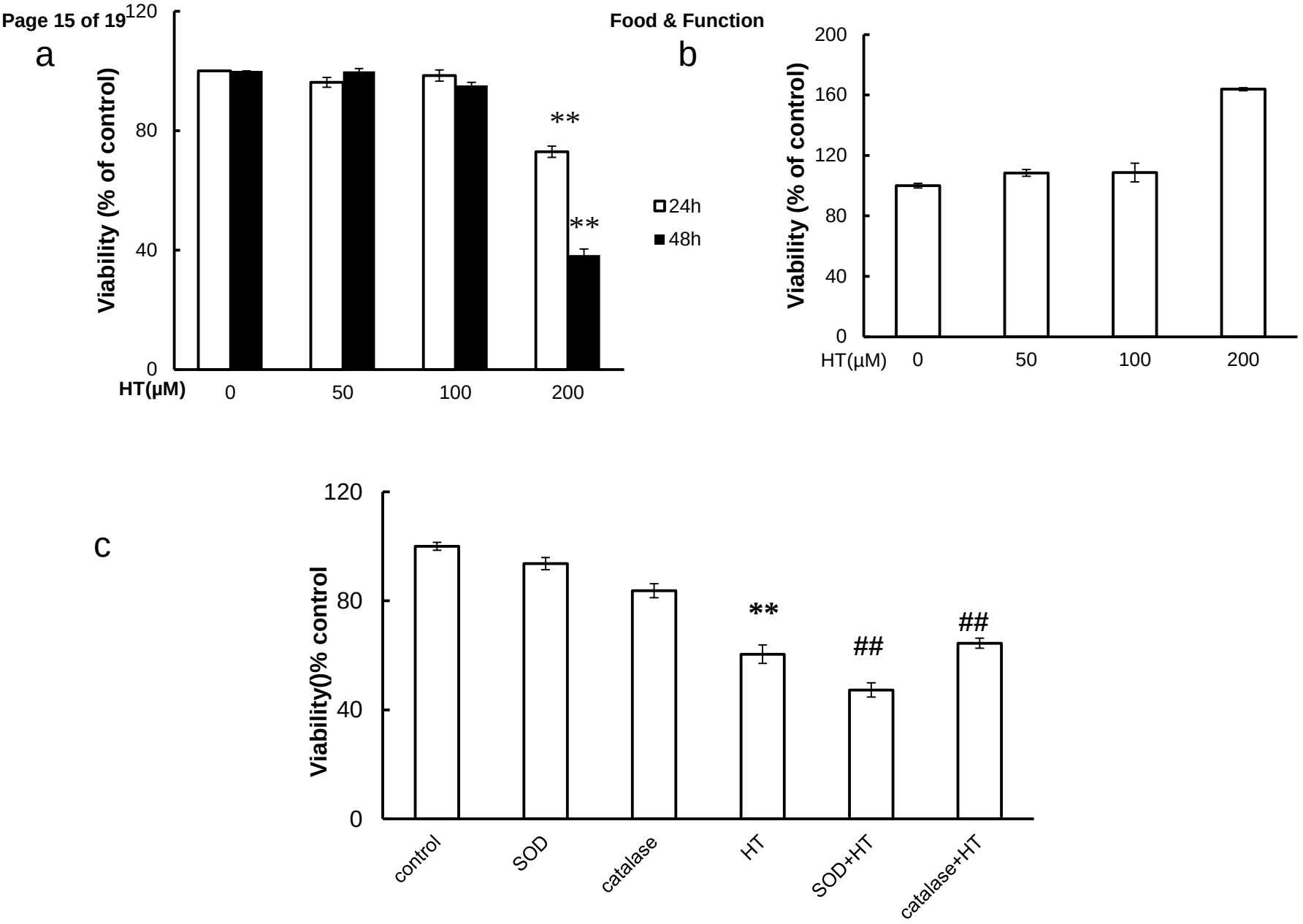
Fig. 1 Inhibition of cell viability by HT treatment in colon cancer cells. (a) Colon cancer DLD1 cells (24 or 48 h). (b) Normal colon epithelial CRL1807 cells (24 h). (c) Combination effects of HT and SOD, or catalase on the DLD1 cells (48 h). The values represent the mean ± SEM. from three independent experiments. ** $p<0.01$ versus the control group. ## $p<0.01$ versus the HT group.

Fig. 2 Changes of mitochondrial membrane potential in colon DLD1 cancer cells. (a) treatments with HT alone (24 or 48 h). (b) Combination effects of HT and SOD, or catalase (48 h). The values represent the mean ± SEM. from three independent experiments. **p<0.01 versus the control group. ##p<0.01 versus the HT group.

Fig. 3 Changes of H_2O_2 in colon DLD1 cancer cells. (a) treatments with HT alone (24 or 48 h). (b) Combination effects of HT and SOD, or catalase (48 h). The values represent the mean ± SEM. from three independent experiments. **p<0.01 versus the control group. ##p<0.01 versus the HT group.

Fig. 4 Apoptosis induction in colon DLD1 cancer cells. Annex V/PI assay for (a) treatment with HT alone (48 h), and (b) combination treatment with HTand SOD, or catalase (48 h). (c) Western blot analysis. The values represent the mean ± S.E.M from three independent experiments. **p<0.01 versus the control group. ##p<0.01 versus the HT group.

Fig. 5 Activation of Akt and FOXO3a, expression of SOD and catalase in colon DLD1 cancer cells with HT treatment alone or combination with SOD or catalase.



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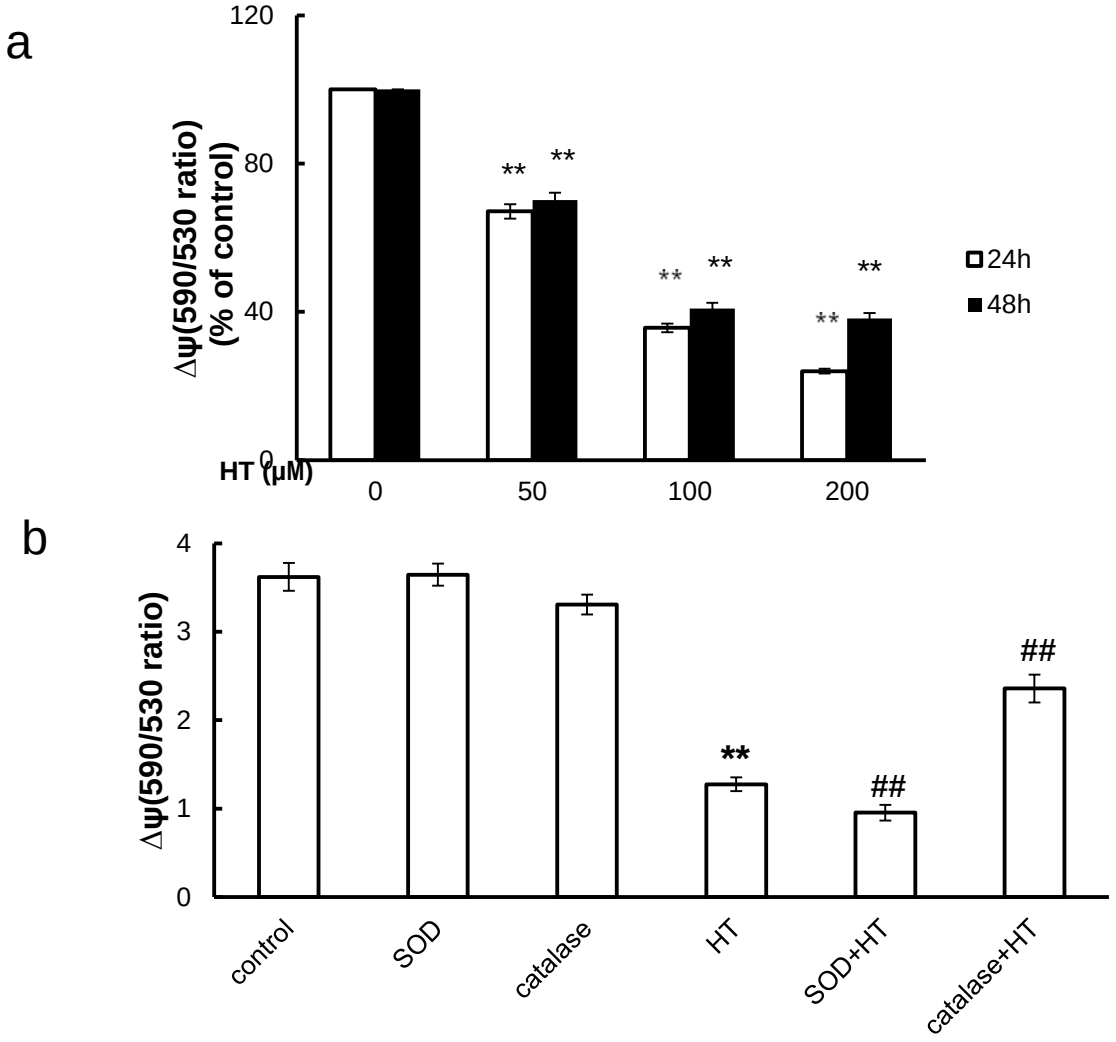

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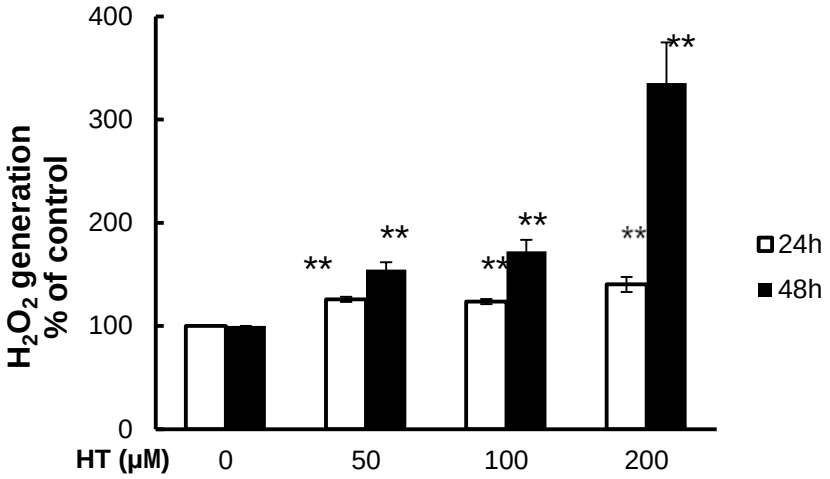

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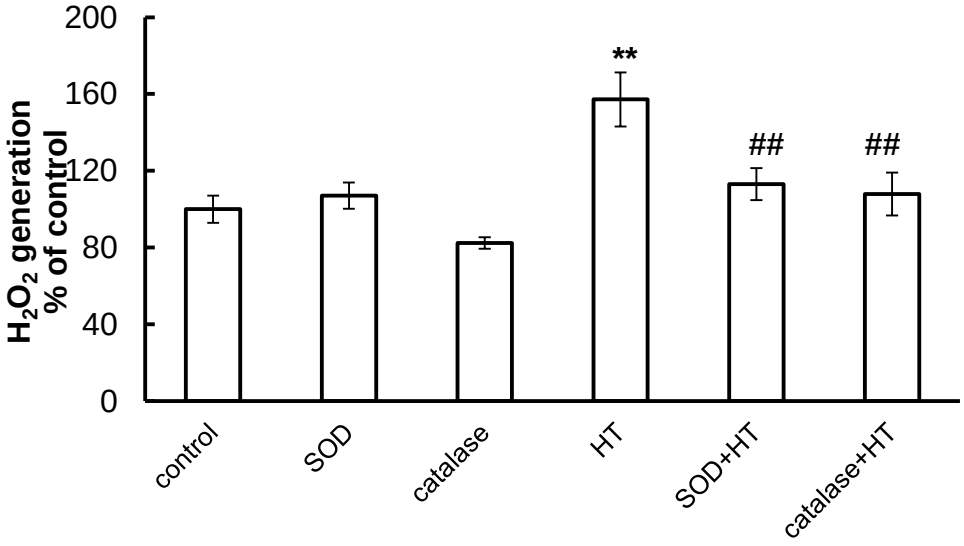

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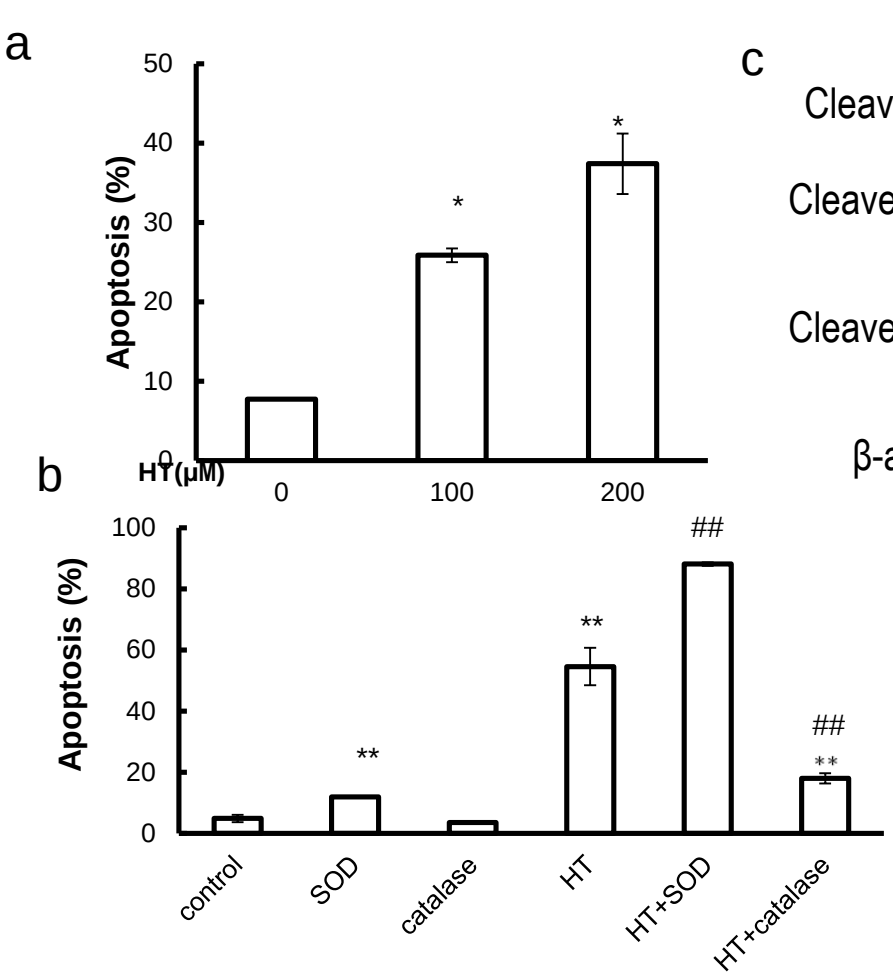

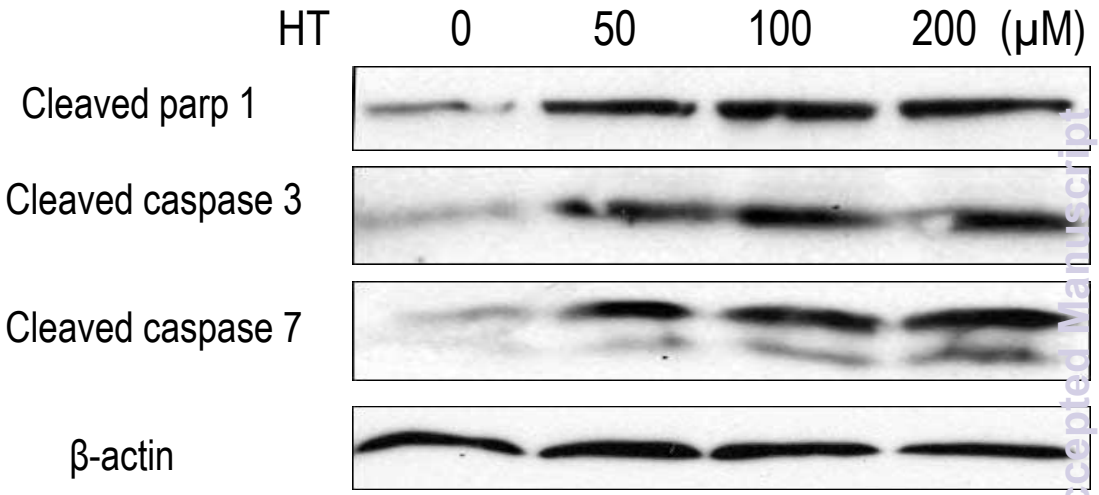

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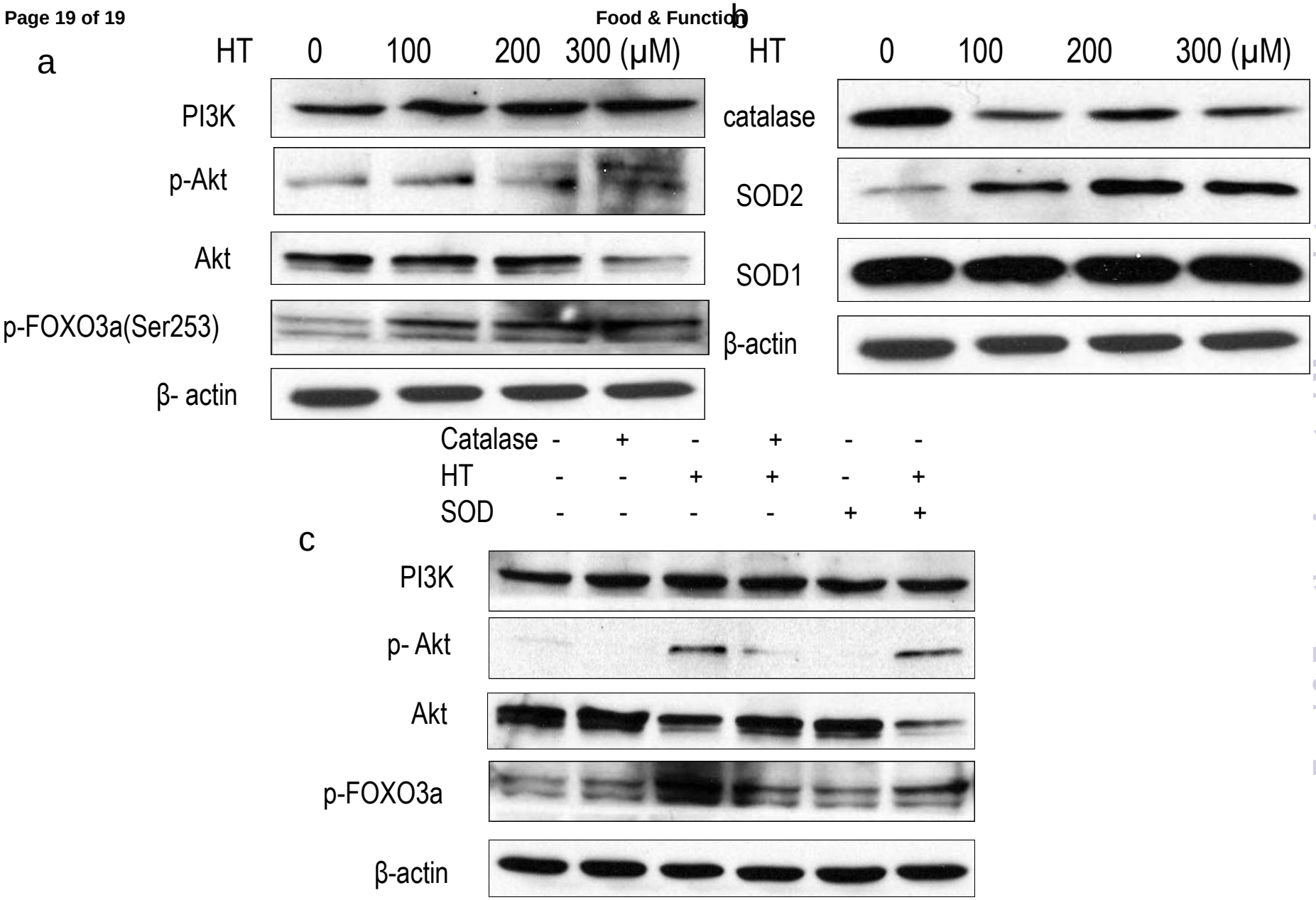

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