Myricetin Prevents Cataract Formation by Inhibiting the Apoptotic Cell Death Mediated Cataractogenesis

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Background: The current research work aimed to explore the protective role of myricetin against cataractogenesis in humans, in terms of its anti-apoptotic potential.

Material/Methods: Human eye lens epithelial cells were exposed to oxidative stress by treating with hydrogen peroxide (H$_2$O$_2$). The levels of superoxide dismutase (SOD), catalase (CAT), and glutathione (GSH) were determined using standard detection kits. DAPI (4',6-diamidino-2-phenylindole), AO/EB (acridine orange/ethidium bromide) and Annexin V/propidium iodide (PI) staining assays were used for the assessment of cell apoptosis. Western blotting was used to examine the protein concentrations.

Results: The exposure of human epithelial eye lens cells to H$_2$O$_2$ led to significant accumulation of reactive oxygen species molecules. Treatment of the H$_2$O$_2$-stressed epithelial cells with myricetin caused significant (P<0.05) increased levels of SOD, CAT, and GSH. Western blot analysis also showed a significant (P<0.05) increase in the expression of SOD, CAT, and GSH levels in human epithelial eye lens cells. Additionally, myricetin administration to H$_2$O$_2$-treated epithelial eye lens cells caused a significant decline in cell apoptosis ratio. The induction of apoptosis was associated with upregulation of Bax and downregulation of Bcl-2.

Conclusions: The results of this study showed the potential of myricetin in protecting the apoptosis driven cataract formation in humans.

MeSH Keywords: Apoptosis • Cataract • Superoxide Dismutase

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Background

Blindness results mainly from age-related cataract forma-
tion [1]. The cataract formation is further influenced by envi-
ronmental factors and genetic alterations [2]. The presently
employed strategy against cataract disease involves the sur-
gical removal of the layer of cataract from the anterior part
of the eye [3]. Molecular etiology of cataractogenesis has not
been fully elucidated. However, a recent study has shown that
apoptosis of epithelial cells of the human lens is responsible
for the development of an opaque layer on the eye lens (cat-
aract) [4]. The accumulation of reactive oxygen species (ROS)
in the epithelial cells of human lens has been shown to act as
a trigger for apoptotic cell death of epithelial cells [5]. Hence,
studies are being directed to identify the agents with anti-oxi-
dant properties which may prove helpful in preventing the in-
duction of apoptotic cell death and thus reduce the chances
of cataract formation. The present study was therefore undertak-
en with the purpose to explore the effects of a natural flavo-
neglycoside, myricetin, in preventing the apoptosis of human
eye lens epithelial cells. The rationale for using myricetin in
this study was that the flavonoid bear tremendous anti-oxi-
dant potential and have been shown to act as a scavenger of
ROS molecules [6]. The anti-oxidant and anti-apoptotic role of
myricetin has already been proven in previous studies [7,8]. In
the present study, the administration different concentrations
of myricetin to human lens epithelial cells resulted in a signif-
icant level of decline of ROS entities and inhibited the apop-
tosis of epithelial cells. Myricetin treatment was shown to en-
hance the anti-oxidant power of epithelial cells by increasing
the levels of superoxide dismutase (SOD), catalase (CAT), and
 glutathione (GSH). The inhibition of apoptosis was mediat-
ed through the Bax/Bcl-2 signaling pathway. Taken together,
the study highlights the protective role of myricetin to inhib-
it the apoptosis of human eye lens epithelial cells and thus
implicates its effectiveness in preventing the cataract forma-
tion on the eye lens.

Material and Methods

Cell culture

The human eye lens epithelial cells (HLEB-3) were bought from
the American Type Collection Center (ATCC, USA). The culturing
of HLEB-3 cells was performed using the Dulbecco’s modified
Eagle’s medium (DMEM, Thermo scientific). For maintaining
the HLEB-3 cells, the cells were incubated using 5% CO₂
concentration at 37°C in a humidified CO₂ incubator. The DMEM
medium was supplemented with 10% fetal bovine serum (FBS,
Thermo Scientific).

Induction of oxidative stress

For the induction of oxidative stress in HLEB-3 cells, the cells
were administered with H₂O₂. The H₂O₂ was added at the fi-
nal concentration of 425 µM in the liquid DMEM medium and
the cell suspension was incubated at 37°C for 24 hours with
5% CO₂.

Assessment of ROS levels and anti-oxidant power of
epithelial lens cells

To monitor the production of intracellular level of ROS mol-
ecules, the microplate-based ROS estimation was performed
using the 525 nm as emission wavelength and 495 nm as ex-
citation wavelength. Prior to determination of intracellular
levels of SOD and CAT activities and GSH level, the cells were
harvested with centrifugation, washed with phosphate-buff-
ered saline (PBS) thrice and lysed by ultrasonication. The ac-
tivities of SOD, CAT, and GSH levels were then estimated with
the help of respective detection kits.

Analysis of apoptosis

To investigate the apoptosis of HLEB-3 cells, the cell cultures
were centrifuged, and the pellets were washed thoroughly
with PBS buffer. Afterwards, the fixation of cells was done
using 70% ethyl alcohol. Subsequently, the HLEB-3 cells were
stained using DAPI solution (Thermo Scientific) or dual AO/EB.
Lastly, the DAPI or AO/EB stained cells were visualized under
the fluorescent microscope to examine the level of apoptosis
of HLEB-3 epithelial lens cells. Annexin V/propidium iodide
(PI) assay was used as described previously [7] to determine
the levels of apoptosis.

Western blotting

For extracting the total proteins from the HLEB-3 epithelial eye
lens cells, the radioimmunoprecipitation assay (RIPA) buffer
was used for cell fractionation. The lysed cells were analyzed
by Bradford method for examining the total protein concen-
tration. Exactly, 40 µg of proteins were separated by running
on 10% sodium dodecyl sulfate-polyacrylamide gel electro-
phoresis (SDS-PAGE). The separated proteins were blotted to
PVDF (polyvinylidene fluoride) nylon membrane. Using the ex-
posure of primary and secondary antibodies, the protein bands
of proteins of interest were visualized through chemilumines-
cence method. Human β-actin was used as internal control.

Statistical analysis

To validate the experimental findings, the values were depict-
ed as mean±standard deviation (SD). The Student’s t-test and
ANOVA were performed with the help of GraphPad Prism 7.0
statistical analysis software, offline, and the $P<0.05$ were used as the measure of statistically significant difference.

**Results**

**Myricetin inhibited the accretion of ROS levels in lens epithelial cells**

To produce the oxidative stress in the HLEB-3 epithelial eye lens cells, the cell cultures were inoculated with $H_2O_2$ at a final concentration of 425 µM. The cell cultures were then administered with myricetin, whose molecular structure is presented in Figure 1, to infer whether myricetin treatment is effective in reducing the ROS levels by enhancing the intracellular antioxidant parameters like SOD, CAT, and GSH. Myricetin was used in the concentrations of 0, 5, 10, and 20 µM. The results showed that the myricetin administration was effective in reducing the intracellular ROS levels (Figure 2A). The administration further
Figure 3. Western blot analysis showing the expression of SOD, CAT, and APX in H$_2$O$_2$ treated HLEB-2 cells. The experiments were performed in triplicate. SOD – superoxide dismutase; CAT – catalase; GSH – glutathione; APX – ascorbate peroxidase; H$_2$O$_2$ – hydrogen peroxide; HLEB-3 – human eye lens epithelial cells.

Figure 4. Inhibition of apoptosis of HLEB-3 cells by myricetin. DAPI staining for the assessment of apoptosis of H$_2$O$_2$ stressed HLEB-2 cells treated with 0, 5, 10, or 20 µM myricetin. The experiments were performed in triplicate. DAPI – 4',6-diamidino-2-phenylindole; H$_2$O$_2$ – hydrogen peroxide.
Figure 5. Myricetin inhibits apoptosis of HLEB-3 cells. AO/EB dual staining for the assessment of apoptosis of H₂O₂ stressed HLEB-2 cells treated with 0, 5, 10, or 20 µM myricetin. The experiments were performed in triplicate. AO/EB – acridine orange/ethidium bromide; H₂O₂ – hydrogen peroxide.

Figure 6. Annexin V/PI staining showing the percentage of apoptosis at different concentration of myricetin in H₂O₂ treated HLEB-2 cells. The experiments were performed in triplicate. Annexin V/PI – Annexin V/propidium iodide (PI); H₂O₂ – hydrogen peroxide.
enhanced the intracellular level of SOD enzyme (Figure 2B). Similarly, the intracellular counts of CAT and GSH were also seen to be enhanced when the HLEB-3 cells were treated with myricetin (Figure 2C, 2D). The effects of myricetin in reducing the intracellular ROS levels and enhancing the antioxidant parameters of HLEB-3 cells were more prominent at the higher concentrations used. The western blot analysis also showed that the expression of SOD, CAT, and GSH increased dose dependently upon myricetin treatment (Figure 3).

**Myricetin inhibited the apoptosis of H₂O₂ stressed lens epithelial cells**

To investigate the effects of myricetin on the apoptosis of HLEB-3 epithelial cells stressed with H₂O₂, the oxidative stress induced cells were treated with 0, 5, 10, or 20 µM for 24 hours of myricetin and the cells were processed for DAPI or AO/EB staining. The results showed that myricetin treatment reduced the apoptosis of HLEB-3 cells in a dose-dependent manner as evident from the DAPI (Figure 4) and AO/EB staining assays (Figure 5). It can be then stated that myricetin, through its ant-apoptotic potential, is effective in prevention of apoptosis driven cataractogenesis of the human eye lens. The Annexin V/PI assay showed that the percent apoptosis decreased from 19.8% in untreated HLEB-3 cells to 7.5% in HLEB-3 cells treated with 20 µM dosage of myricetin (Figure 6).

**Myricetin decreased the Bax/Bcl-2 ratio in HLEB-3 cells**

The western blotting of Bax and Bcl-2 proteins from the HLEB-3 cells stressed with H₂O₂ and administered with 0, 5, 10, or 20 µM myricetin was performed to determine the concentrations of Bax and Bcl-2 proteins. The results showed that Bax protein concentration decreased while that of Bcl-2 increased in myricetin concentration dependent fashion (Figure 7). The results suggest that the myricetin prevents the apoptosis of human epithelial lens cells by modulating the Bax/Bcl-2 protein concentration ratio.

**Discussion**

The formation of cataract on the human eye lens is the dominant cause of human blindness [9]. The chances of cataract formation particularly increases in the elder age group [10]. Environmental factors together with genetic alterations have been shown to play a role in the cataractogenesis of the human eye [11,12]. Furthermore, the apoptosis of human epithelial lens cells has been implicated as responsible for the development of their opaqueness which is ultimately manifested as the layer of cataract [13]. Oxidative stress is very detrimental to normal cell functioning and reactive oxygen species (ROS), when accumulated, leads to the induction apoptosis of human cells [14]. The natural occurring compounds have the potential to enhance the oxidant potential of human cells and enhance their survival by inhibiting the cell apoptosis [15]. Myricetin, a flavonoglycoside, is isolated from the stem, bark, branches, and fruits of Myrica rubra or other plant sources. Research studies have shown that myricetin has the potential to inhibit the induction of apoptosis of human cells [16]. In this study, we investigated the effects of myricetin on the apoptosis of human lens epithelial cells, HLEB-3. In confirmation with previous reports, the myricetin treatment resulted into the inhibition of apoptosis of HLEB-3 epithelial cells. The reduction of...
apoptosis of HLEB-3 epithelial cells was attributed to the potential of myricetin to decline the intracellular ROS levels and enhancement of CAT, SOD, and GSH activities. Researchers have reported similar types of effect of myricetin on the human cells in previous reports [16]. Moreover, the myricetin administration led to the inhibition of Bax/Bcl-2 apoptotic signal by increasing the concentration of Bcl-2 and decreasing the Bax protein. This leads to decrease of Bax/Bcl-2 protein ratio which inhibits cell apoptosis [17]. Summing up, myricetin was shown to inhibit the accumulation of ROS molecules in HLEB-3 cells. The inhibition of HLEB-3 cell apoptosis provides insights into the potential of myricetin to prevent the cataract formation through the reduction of the apoptosis driven cataractogenesis of epithelial eye lens in humans.

Conclusions

The results of the current study reveal the potential of myricetin to inhibit the apoptosis of human eye lens epithelial cells and thus highlights its protective role in inhibiting the apoptosis driven cataractogenesis of human eye.

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