Effect of Lycopene Administration on Necrotic Gene Expression in Renal Epithelial Cell Line (NRK-52E) Exposed to Sodium Fluoride

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ABSTRACT

Objective: The aim of this study was to determine the effects of lycopene administration as a protective agent against necrotic damage of NaF, a fluoride compound found to have high cytotoxic effects in the renal epithelial cell.

Material- Method: The renal epithelial cell was cultured in DMEM high glucose medium, containing 10%FBS, 1%L-Glutamine (2mM) and 1% penicillin/streptomycin. With the MTT viability test, the non-toxic dose of lycopene (1 µM) and the IC₅₀ value of NaF at the 24th hour was determined to be 3200 µM. The study groups were divided into four as control, NaF, lycopene and NaF+lycopene (the combination of NaF and lycopene). After the total mRNA obtained from these groups were converted to cDNA, expression levels of the identified necrotic genes were determined by real-time PCR method.

Results: While the Ripk1 gene did not change in the group given lycopene at the 24th hour, it was found that it increased 2.6 times in the group that received only fluoride, while it increased 7 times in the group treated with NaF+lycopene. A significant difference was detected between the groups in terms of gene expression pattern. While the Ripk3 gene increased slightly in the 24th hour applied lycopene group, it was observed that only NaF applied group increased 8 times and NaF+lycopene applied group increased in the 9 times.

Conclusion: Based on the results obtained from this study, it was seen that activation of necrotic genes is important in explaining the molecular basis of cell death from NaF, which is applied as fluoride source, in revealing the molecular basis of the necrotic pathway. It was found that the decrease in cell viability due to NaF increased with lycopene, but the use of lycopene with fluoride also increased necrotic gene expression.

Keywords: NaF, in vitro, Lycopene, Necrotic Genes

INTRODUCTION

Fluoride (F) is a highly electronegative element that can be found naturally in water and various nutrients. Prolonged exposure and high concentrations cause damage to teeth, bones and various tissues (Agalakova and Gusev, 2012; Perumal et al., 2013; Çetin et al., 2020).

Fluoride has a high penetrative ability and can easily penetrate the cell membrane. It may enter deeper soft tissues such as the liver, brain, and kidney, and therefore, nephrotoxicity could occur due to the accumulation and retention of inorganic fluoride in the renal tubules (Quadri et al., 2016). In a study in the northern region of Sri Lanka, where the disease of fluorosis is intense, Dharmaratne
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(2015) found that the concentration of fluoride directly correlates with renal diseases in the settlements where the drinking water has high levels of fluoride.

Necrosis or necroptosis is an irregular process that develops randomly and cannot be controlled by genes, and it’s most common cause is hypoxia. Toxic substances such as arsenic, cyanide, insecticides and heavy metals cause necrosis. During necrosis, mitochondrial ROS production increases, nonapoptotic proteases are activated, ATP production decreases and Ca^{++} channels are opened (Nicotera et al., 2004; Golstein and Kroemer, 2007).

Lycopene, (LYC) has an acyclic structure with 11 conjugated double bonds; the double bonds are in an all-trans form and have antioxidant properties. It has been reported that lycopene has many uses due to its anti-inflammatory, anticancer and antioxidant effects. In addition to protecting cells from free radical damage, LYC strengthens the bonds between cells and improves cell metabolism. It is reported that lycopene is protective against prostate, uterus, liver cancer, aging, Alzheimer’s and cardiovascular diseases (Bramley, 2000; Mashima et al., 2001; Pruthi et al., 2003; Cetin et al., 2017).

This study aimed to determine the effects of NaF-induced necrotic damage, a fluoride compound, which is found to be highly cytotoxic in the renal epithelial cell, and the application of lycopene as a preservative.

MATERIALS and METHODS

Cell Culture

The study material comprised rat renal epithelial NRK-52E (ATCC® CRL-1571™) cells. NRK-52E cells were cultured in vitro with cultured in a medium containing, 10% fetal bovin serum (FBS), 1% penicillin/streptomycin, 1% L-glutamine and DMEM high glucose at 37°C, 95% humidity, 5%CO₂.

Preparation of Analysis Groups

Stock solutions of NaF and LYC used in the study were prepared by referring to the concentrations in our previous study. NaF and lycopene was dissolved in the medium. The dose that increased lycopene cell proliferation was determined as 1 µM (Cetin et al., 2017). Cell viability was measured by MTT assay to measure the cytotoxic effect of NaF IC₅₀. NRK-52E cells were treated with various NaF concentrations and lycopene in a 24-hour incubation (Figure 1).

Cell Viability Assay (MTT)

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell viability tests were performed to determine IC₅₀ values of compounds. The proliferative doses of lycopene and lycopene were determined. For this purpose, NRK-52E cells were seeded at 2×10⁶ cells/well in 96-well plates and incubated overnight at 37°C. After exposure the described doses of compounds for 24 h, medium of wells was discarded and MTT (0.5mg/ml in sterile PBS) solution (10% of completed medium) was added to each well and incubated for 3 h at 37 °C. At the end of the incubation time, MTT medium was discarded and added to lysis solution (1% Triton-X, 10% 0.1mol/l HCl, 89% Isopropanol) to each well for solubilization of the formazan crystals. The absorbance of each well was measured at 570 nm by using a microplate reader. Inhibition and increasing of growth in cells were analyzed Graphad Prism 8 software (San Diego CA). Each experiment in MTT assay was repeated at least four times.

Obtaining RNA

The cells of the experimental groups were collected after 24 hours. RNAs of these cells were isolated by using TRizol® Reagent (Chomczynski and Mackey, 1995).

cDNA synthesis and real-time PCR analysis (RT-qPCR)

cDNA was obtained using the isolated mRNA and a commercial cDNA synthesis kit (WizScript, Cat. No: w2211). SYBR green master mix (WizPure, Cat. No: w1711) was used in the study. Ct (cycle threshold) was determined at the start of the logarithmic amplification phase. The differences between the Ct values of the control group and the replicates were used for determining the appropriate expression. The reaction content is presented in Table 1

Table 1. Reaction content for real-time-PCR

<table>
<thead>
<tr>
<th>Reaction content</th>
<th>For a example</th>
</tr>
</thead>
<tbody>
<tr>
<td>Master mix (2X)</td>
<td>10 µl</td>
</tr>
<tr>
<td>Primer (F/R)</td>
<td>F: 1 µl, R: 1 µl</td>
</tr>
<tr>
<td>dH₂O</td>
<td>7 µl</td>
</tr>
<tr>
<td>cDNA</td>
<td>1 µl</td>
</tr>
<tr>
<td>Total</td>
<td>20 µl</td>
</tr>
</tbody>
</table>
Amplification protocol was applied as preliminary denaturation at 95°C for 5 minutes, and denaturation at 95°C for 15 seconds, annealing at 60°C for 60 seconds, in total of 40 cycles, Melting Curve Ramp: 50-99 (1 degree increase).

The difference between the groups was normalized using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the control. Gene products were determined by using ΔΔCt and 2^-ΔΔCt values (Livak and Schmittgen, 2001). The expression levels of the target genes were compared with the fold change number and evaluated statistically.

**RESULTS**

It was revealed that the cell viability of the cells, which were applied for 24 hours in different concentrations of NaF, decreased gradually. It was found that the application of lycopene in association with NaF has significantly reduced the cytotoxication (Figure 1).

**DISCUSSION**

High levels of fluoride exposure, causes damage starting from cell, to tissue and organ damage. Many studies, both cellular and experimental, has been made to investigate the damage especially on molecular basis. Parameters seen in apoptotic, autophagic and necrotic pathways have significant role on fluoride dependent cell deaths (Yüksek et al, 2017; Kuang et al, 2018; Tu et al., 2018).

Fluoride reveals various cellular effects depending on time, mixture and cell type. The main toxic effect of fluoride occurs in cells that interact with its enzymes. In most cases, fluoride acts as an enzyme inhibitor, but fluoride ions can occasionally stimulate enzyme activity. Mechanisms depend on the type of enzyme affected (Adamek et al., 2005).

Fluoride at micromole levels is considered to be an effective structural agent because it increases cell reproduction and with the millimolar mixtures stops enzymes such as both living and inanimate phosphates (Mendoza-Schulz et al., 2009). Metabolic, functional and structural damage has been reported due to chronic fluoride poisoning in many tissues. Research data strongly suggests that fluoride inhibits protein synthesis and / or secretion, and effects many pathways such as cell reproduction and apoptosis, mitogen activated protein kinase (MAPK), p53, activator protein-1 (AP-1) and nuclear factor kappa B (NF-B) (Zhang et al., 2007; Zhang et al., 2008; Karube et al., 2009).

Intensive studies are underway to clarify fluoride related toxicity mechanisms. DNA damage due to oxidative stress and activation of apoptotic and necrotic pathways have an important place among these mechanisms (Cao et al., 2015; Yüksek et al., 2017; Tan et al, 2018; Cetin et al., 2019).

Xiong et al. (2007) reports in their study that fluoride levels higher than 2.0 mg/l in drinking water may cause liver and renal damage and tooth fluorosis is independent of liver damage but not independent of renal damage.

Ripk1 and Ripk3 (Receptor interacting protein kinase) are activated as a result of cellular stress or by activation of TNF and Fas receptors. Ripk1 and Ripk3 either directly activate the mitochondria or indirectly affect NADPH oxidase-induced oxygen species (ROS) and induce necrosis (Hengartner et al., 1992). The effects of excess fluoride on the health
of many organisms have been investigated extensively and free radicals have been shown as the mechanism causing fluorosis (Wang et al., 1997). There are literatures that fluoride increases the formation of reactive oxygen species (ROS) and free radicals in vivo and in vitro, causes excessive oxidative stress and lipid peroxidation, and reduces antioxidant enzyme activities (Lu et al., 2010; Varol et al., 2013). Recently, reactive oxygen species (ROS) induced by excess fluoride have been shown to play an important role in DNA damage (Rzeusk et al., 1998). Fluoride can also cause endoplasmic reticulum (ER) stress and suppress protein synthesis and secretion (Kubota et al., 2005).

According to our findings this situation depending on the time of application of the lycopene, it can be said that it caused the continuation of the already activated path with NaF and that the expected inhibition phase has not yet started.

Fluoride has high penetration ability and can easily pass through the cell membrane. It can enter deeper soft tissues, such as the liver, brain, and kidney, and therefore nephrotoxicity may occur due to the retention and accumulation of inorganic fluoride in the renal tubules (Quadri et al., 2016).

LYC shows a strong antioxidant property in vitro, while it is protective against oxidation of DNA, protein and lipids in vivo environments (Matos et al., 2011; Karahan et al. 2018). Li et al. (2017) reported that lycopene significantly affected NaF-induced ameloblast and dental fluorosis by reducing oxidative stress and caspase pathway. They also demonstrated that lycopene administration in rats given Sodium Fluoride (NaF) can minimize the toxic effects of fluoride indicating free radical and strong antioxidant activities (Mansour and Tawfik, 2012).

CONCLUSION
As a result, in this study; it was understood that administration of NaF at cytotoxic concentrations accelerated cell deaths by making necrotic genes more active in nephrons. It was understood that the administration of lycopene alone did not affect the necrotic pathway. However, when lycopene was administered together with NaF, considering the dose used in this study and the time of sample collection, the positive effect detected on cell viability was not found positive on the necrotic pathway. In order to confirm this situation, it was concluded that new studies should be planned to apply lycopene at different hours and to follow the necrotic pathway in the samples to be taken at 36, 48, and 72 hours after the application.

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REFERENCES


