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Lycopene improves methotrexate-induced functional alterations of the Madin-Darby kidney cells in a concentration-dependent manner

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Abstract

Lycopene is one of the most potent antioxidants among carotenoids due to its ability to quench singlet oxygen and react with free radicals to reduce DNA damage. Methotrexate is widely used in the treatment of several types of cancers and autoimmune diseases. One of the most common side effects of high-dose of methotrexate is kidney injury. In this study we evaluated effects of lycopene on the Madin-Darby canine kidney cells (MDCK) treated with methotrexate through the estimation of their mitochondrial and lysosomal functions (MTT reduction assay and NR uptake assay) and changes in cell oxidative status (determination of advanced oxidized proteins concentrations and reduced glutathione levels) and lysosomal enzymes activity (β -N acetyl glucosaminidase activity). Results of our study showed that lycopene applied in high concentration caused significant impairment of the MDCK function leading to cell death. Contrary, in relatively low concentrations lycopene moderately ameliorated methotrexate-induced MDCK cells' death estimated by both biochemical and microscopic analyses. It also prevented a significant decline in the MDCK cells' lysosomal function estimated by neutral red accumulation ability and activity of lysosomal enzyme β -N acetyl glucosaminidase.

Key words: lycopene, methotrexate, kidney injury, Madin-Darby canine kidney cells, oxidative stress.

Introduction

Lycopene (Lyc), a fat-soluble red pigment, is the major carotenoid (not related to vitamin A) found mainly in tomatoes but also in pink grapefruit, watermelon and papaya (Chandrika et al., 2009). Evidence exists that Lyc has wide spectrum beneficial properties including hypocholesterolemic and cardioprotective effects, and anti-inflammatory, anti-mutagenic and especially anti-cancerogenic potentials (Agarwal and Rao, 2000). In recent years, Lyc has received particular attention as one of the most potent antioxidants among carotenoids due to its ability to quench singlet oxygen, react with free radicals and reduce DNA damage (Rao and Agarwal, 2000). However, due to a low concentration of Lyc in body fluids (plasma) it has been questioned whether its antioxidant activity resulted from the direct action on reactive oxygen species (ROS) and/or reactive nitrogen species (RNS), or is a consequence of Lyc metabolites property to change the expression of different genes involved in cellular oxidative stress (Erdman et al., 2009).

Methotrexate (MTX) is a folic acid antagonist, widely used in the treatment of several types of malignant tumors and leukemia. It is also used as a therapy of certain autoimmune diseases, including rheumatoid arthritis, psoriasis, and lupus erythematosus (Caglar et al., 2013). One of the most common side effects that follow the administration of high-dose of MTX is kidney injury. This adverse effect is likely induced by two MTX derivatives, 7-hydroxy-MTX, and 2,4-diamino-*N*10-methylpteroic acid, that can form intraluminal precipitates in renal tubules (Dalaklioglu et al., 2012). Since more than 90% of MTX is cleared by the kidney, this effect directly leads to a delayed MTX elimination and therefore sustained elevation of its plasma levels and further tissue damage (Widemann and Adamson, 2006). Another possible effect contributing to MTX induced tissue damage might arise from its ability to induce cellular oxidative stress, possibly by increasing generation of ROS and RNS (Ali et al., 2014). In line with this, several studies shown that

supplementation with different antioxidants can ameliorate MTX-induced nephrotoxicity (Abraham et al., 2010; Howard et al., 2016); however, cellular mechanisms of this beneficial effect have not been fully explored. In our previous study we showed that Lyc has nephroprotective effect in kidney tissue damage induced by systemic MTX administration in rats (Stojiljkovic et al. 2018).

Here, we evaluated effect of Lyc at cellular level using the Madin-Darby canine kidney (MDCK) cells, a well-known model of collective tubular epithelial cells (Valentich, 1981), through the estimation of their mitochondrial and lysosomal functions. In addition, we studied the effects of Lyc on the MDCK cells treated with MTX, and traced the changes in cell oxidative status and lysosomal enzymes activity.

Materials and Methods

Reagents and Chemicals

Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), stable glutamine, antibiotic-antimycotic solution, trypsin-EDTA and Hank's Balanced Salt Solution (HBSS) were purchased from Capricorn (Germany). Lycopene (Lyc) and Neutral red (NR) were purchased from Sigma-Aldrich, while (4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) was obtained from Carl Roth (Germany). Methotrexate (MTX), (Metoject, 10 mg/ml) injection solution was purchased from Medac GmbH (Germany).

Preparation of substances for *in vitro* testing

Lycopene was dissolved in chloroform (5 mg/ml) according to the manufacturer's instructions, to obtain a stock solution, which was used to make the final concentrations by dilution in complete cell culture medium (DMEM). The final concentrations used for testing were ranged from 10^{-4} to 10^{-8} M. Methotrexate injection solution was diluted in complete DMEM and the final concentration used in the MDCK cell assay was 250 μ M (Zhu et al., 2016).

***In vitro* cell culture experiments**

The MDCK epithelial cells, obtained from American Type Culture Collection (ATCC), were used as a model of kidney collective tubular cells. Cells were cultivated in DMEM supplemented with 10% FBS, 2 mM stable glutamine and 1% antibiotic-antimycotic solution (complete DMEM), at the 37 °C in a humidified atmosphere containing 5% CO₂.

For the experiments, the MDCK cells were detached using trypsin-EDTA solution and appropriate cell density was adjusted according to Trypan Blue Dye Exclusion method. Cells were seeded in 96 well plates (Greiner Bio-One, Germany) at density of 2×10^4 cells per well. Twenty four hours after cultivation, cells were treated with Lyc, in different concentrations from 10^{-4} to 10^{-8} M. In a separate set of experiments the combination of Lyc with MTX was incubated with cells in order to analyze the potential of *in vitro* cytoprotective effects of Lyc in MTX-induced MDCK cells' damage. Cells incubated only with complete DMEM medium, and with omission of tested substances was used as a control. Each concentration of Lyc alone or combination of Lyc and MTX, or MTX alone and complete DMEM, were tested in four to eight replicates. Cells were incubated with Lyc and its combination with MTX for 24 h under standard cell culture conditions.

MTT reduction assay

Following 24 h-incubation with tested substances, cells were washed with phosphate buffer saline (PBS) and then 100 μ l of MTT solution (1 mg/ml) was added to all wells. Plates were further incubated with MTT for 3 h, and afterward, the formed formazan crystals were dissolved with isopropanol. The absorbance of dissolved formazans was measured using Multiscan Ascent Photometric plate reader (Thermo Labsystems) at 540 nm with correction of absorbance at 650 nm. The results are expressed as the percentage of viable cells calculated according to the formula: % cell viability = (absorbance value of treated cells/absorbance value of control cells) * 100. The value of control culture cell viability was considered to be 100%.

NR uptake assay

After 24 h of incubation with tested substances, cells were washed with PBS before 100 μ l of NR solution at 1 mg/ml concentration was added to the cells. Then, the cells were incubated with NR solution for the next 60 minutes, washed again with PBS, and the remaining intracellular dye was dissolved with 1% acetic acid in 50% ethanol. The absorbance of dissolved dye was measured at 540 nm. The results are expressed as the percentage of NR uptake according to the control, calculated with the formula: % NR uptake = (absorbance value of treated cells/absorbance value of control cells) * 100.

Determination of the MDCK cell enzyme activity and cell oxidative damage

For this experiment, the MDCK cells were seeded in 12 well plates (Greiner Bio-One) at density of 2×10^5 cells/well/ml. After 24 h, the medium was replaced with either Lyc (10^{-4} , 5×10^{-7} and 10^{-8} M) or the combination of Lyc (10^{-5} , 5×10^{-7} and 10^{-8} M) and MTX, and incubation continued for additional 24 h. After that, cells were washed with PBS and frozen at -80°C until further

analysis. Protein concentration in kidney cell homogenates was measured according to Lowry's method (1951).

β-N acetyl glucosaminidase (β-NAG) activity determination

The activity of β-N acetyl glucosaminidase (β-NAG, EC 3.2.1.52) was determined using a colorimetric assay kit (ab204705). A colorimetric reaction is based on the formation of product generated after *p*-nitrophenol degradation under the influence of the enzyme. The lowest enzyme activity that could be determined was 50 μU. Obtained results are presented as U of β-NAG per mg of proteins.

Determination of advanced oxidized proteins (AOPP) concentrations

The concentration of advanced oxidized proteins products (AOPP) in kidney cells was determined by a spectrophotometric method based on the reaction of AOPP with KI in an acidic medium (Witko-Sarsat et al., 1996). The concentrations of AOPP were expressed as mmol/mg of proteins.

Measurement of reduced glutathione (GSH) levels

The concentrations of GSH in the MDCK cells homogenates was determined using a standard protocol previously described by Ellman (1959). After short incubation of deproteinized cell homogenate with DTNB reagent, the absorbance of the reaction mixture was measured at 412 nm. The amount of GSH was determined based on the standard curve and expressed as nmol of GSH/mg of proteins.

Statistical analysis

Statistical analysis was performed with ANOVA followed by Tukey's post-hoc test using GraphPad Prism (GraphPad Software, San Diego, USA). Analyzed values were obtained from at least two independent experiments. Statistical significance was considered for $p \leq 0.05$. Effective concentration (EC_{50}) was calculated using GraphPad Prism following Probits analysis. The lowest observed adverse effect level (LOAEL) was defined as the lowest dose tested with statistically significant effect, while the NOAEL (no observed adverse effect level) was defined as the highest non-statistically significant dose tested (Moffett et al., 2007).

Results

High concentrations of Lyc decrease the MDCK cells ability to reduce MTT and to accumulate NR, while lower concentrations do not show these effects

Incubation of the MDCK cells with Lyc at concentrations 10^{-4} and 5×10^{-5} almost completely abolished the ability of these cells to reduce MTT and accumulate NR (Fig. 1A and B). On the other hand, seven lower Lyc concentrations tested ($10^{-5} - 10^{-8}$ M), did not significantly affect these cellular functions (Fig. 1). The EC_{50} for Lyc on the MDCK ability to reduce MTT and accumulate NR was calculated to be 12.2×10^{-5} and 11.7×10^{-5} M, respectively. The values of NOAEL and LOAEL, same in both MTT and NR assay, were 10^{-5} and 5×10^{-5} M, respectively.

Functional changes of the MDCK cells under the influence of Lyc were followed by the reduction in cell density, with no apparent changes in their morphology (Fig. 2).

MTX-induced damage of the MDCK cells' ability to reduce MTT or to accumulate NR is not significantly improved by Lyc

Co-incubation of Lyc with MTX did not significantly improve the ability of the MDCK cells to metabolize MTT or to accumulate NR compared to DMEM treated cells (Fig. 3A and B). At highest concentrations, Lyc and MTX even caused damage to cells, with almost complete deprivation of their functions studied here. Interestingly the activity of Lyc and MTX was more pronounced on the MDCK cells' ability to accumulate NR, than on their ability to metabolize MTT (Fig. 3B).

Co-incubation of Lyc (10^{-4} M) and MTX reduced the number of the MDCK, while in case of single Lyc (10^{-5} and 10^{-8} M) and MTX treatments cell density remained identical. Application of just MTX had a barely visible impact on cell density (Fig. 4C and D). When cells were incubated with NR for 1h, changes in NR accumulation were clearly visible as some of the cells appeared empty (white) suggesting the impairment of lysosomal function.

Application of Lyc affects AOPP and GSH concentrations and β -NAG activity in MDCK cells

Application of Lyc at a concentration of 10^{-5} M had no detectable effects on AOPP and GSH concentrations, nor on the β -NAG activity (Fig. 5A-C), while lower tested concentrations of Lyc (5×10^{-7} and 10^{-8} M) significantly increased all three measured parameters (Fig. 5A-C). When just MTX was applied, a significant decrease in AOPP concentrations and β -NAG activity was observed, but without significant reduction of GSH concentrations. Co-incubation of Lyc (in nontoxic concentrations 5×10^{-7} and 10^{-8} M) with MTX prevented the drop in β -NAG activity, increased GSH cell content, but also caused a more pronounced increase in AOPP concentrations (Fig. 5).

Discussion

The results of this study revealed for the first time that Lyc at low concentrations ($<10^{-5}$ M) causes a significant decrease in MDCK cells ability to metabolize MTT and to accumulate NR. Cytotoxic potential of Lyc has been shown recently on HEP-2 cells (Ramanathan and Ramalakshmi, 2017), in which concentration of $\approx 1\text{mM}$ caused almost complete depletion of cells, while lower concentrations exhibited less cytotoxic potential. In line with that, it was suggested that tomato extract containing a high concentration of Lyc (90% of total carotenoids), does not affect CCD-18 cells' ability to metabolize MTT (Guil-Guerrero et al., 2011). These discrepancies in results could be possibly explained by the different concentrations and different source of Lyc tested (high grade of purity or extracted from tomato). Also, the difference might account for the property of cell type studied, since the cancerous cells (HEP-2) are more resistant to the effect of Lyc than the normal (non-transformed) cells (CCD-18 and MDCK).

Previous studies have shown that Lyc is able to decrease pro-inflammatory cytokine production in human keratinocytes (TNF- α and IL-1 β), which were found to be curtailed for gap junctional intercellular communication (GJIC) (Zefferino et al., 2005). The GJIC is responsible for numerous cellular functions such as cell adhesion and cell-cell communication. Also, it participates in the regulation of signal transduction pathways involved in cell proliferation and growth control by enabling direct exchange of signaling molecules between cells (Mally et al., 2006). Although Lyc prevented the GJIC disruption, at significantly lower doses than used in the present study (Zefferino et al., 2005), the same could not be claimed here given the direct microscopic and MTT test analyses which revealed significant decrease in cell density after exposure to Lyc (Fig 1, 2C and 2D).

Previous studies found that the MDCK cells represent a good substitution model for human collecting tubule cells (Valentich, 1981), a well-known target for the adverse action of numerous

chemotherapeutics (Widemann and Adamson, 2006). Since MTX is extensively excreted via kidneys and concentrated in the urine, these collecting tubule cells, which are located in relatively hypoxic surroundings and have high metabolic demands (Shirali and Perazella, 2014), are particularly sensitive to the action of this drug. In our previous study, we found that MTX causes proximal tubule cell vacuolation and desquamation, with areas of focal tubular necrosis (Stojiljkovic et al. 2018). Application of Lyc in combination with MTX prevented a decline in the MDCK cells' ability to metabolize MTT and to a certain extent to accumulate NR. These results align with previously reported beneficial properties of Lyc in MTX-induced rat kidney damage *in vivo* (Stojiljkovic et al., 2018, Oguz et al., 2015).

The effect of MTX on kidney tubule cells morphology is not clear, although several studies suggested that MTX causes cell swelling (in concentrations from 1-100 μM) which depends on Na^+/H^+ ionic pump function and/or to the intracellular Ca^{2+} accumulation (Grönroos et al., 2006). In the present study, we did not detect any significant changes in the MDCK cellular morphology (Fig. 4C), which might be due to a significantly higher concentration of MTX used (250 μM) that caused significant cell death after 24 h (Fig. 3 and 4C). Similar findings related to tubule cell damage was observed in animals exposed to MTX (Stojiljkovic et al., 2018), where clear tubule cell necrosis was observed. Although MTX does not accumulate in lysosomes nor it affects its intravacuolar pH (Zhitomirsky and Assaraf, 2017), our data showed that NR accumulation in the MDCK cells decreased after MTX application (Fig. 3B and 4D). These results are probably the consequence of a relatively high concentration of MTX that potentially caused rapid cell death, which in turn reflects the MDCK cells' ability to accumulate NR.

An increase in intracellular GSH content under the effect of Lyc (lower concentrations, Fig. 5B) is not an unexpected finding since the application of Lyc to healthy animals also caused an increase

in this non-enzymatic antioxidant levels in kidney (Khan et al., 2016). When the MTX was applied to cells, an increase in GSH concentration could be connected to its involvement in ROS detoxication (Dhanesha et al., 2015), as well as to its usage for MTX transport inside the cell (Leszczyńska and Pfaff, 1982). Such an increase in GSH concentrations probably reflects a cellular adaptive mechanism to MTX-induced toxicity (Dhanesha et al., 2015). Therefore, it is not surprising that Lyc could not completely protect cells from MTX damage, even though its application significantly increases GSH levels.

The β -NAG is one of the constitutive enzymes of the MDCK cells, (Gstraunthaler et al., 1985), and its activity represents a sensitive urinary marker of tubular cell damage (Vaidya et al., 2008). Due to a significant decrease in cell viability under the influence of MTX, the activity of this enzyme in the MDCK cell lysate was significantly decreased (Fig. 5C). An increase in the activity of β -NAG after incubation of Lyc is detected here for the first time. There might be several explanations for this kind of activity. Since Lyc induces gene expression and thus protein synthesis (Erdman et al., 2009), it is reasonable to expect an increase in activity of β -NAG. Recently this enzyme was found to be responsible for the removal (degradation) of oligosaccharides generated during posttranslational modification in the endoplasmic reticulum suggesting its role in the basic biological processes of eukaryotic cells (Suzuki et al., 2002). Given the fact that MTX is an anti-metabolic drug (Shirali and Perazella, 2014), and that Lyc increases cell protein synthesis (Erdman et al., 2009) such changes in the MDCK cells' β -NAG activity might be expected.

Similar explanations related to the action of MTX and Lyc on protein synthesis (Erdman et al., 2009; Shirali and Perazella, 2014) might be applied to the levels of AOPP determined in the MDCK cells after treatment. An increase in protein synthesis under the influence of Lyc might be followed by its potentially damaging effect in the cellular microenvironment when present in

higher concentration. It is well known that ROS particularly targets constitutive cellular proteins (their damage accounts for about 70% of all molecules impacted by ROS). Also, the proteins that are newly synthesized and/or still in the process of post-translational modification are sensitive to oxidative damage (Dahl et al., 2014). Given the effect of MTX and Lyc on cellular functions, it is not unexpected for Lyc to cause an increase in AOPP levels.

The partially inconsistent results between *in vivo* and *in vitro* studies might be explained through different exposure/treatment time, metabolism of Lyc, as well as its interactions with different cellular molecules. In the present *in vitro* study, we applied Lyc at fixed concentrations during 24 h on a single cell type, while in *in vivo* studies the Lyc is mostly administered in various concentrations during several days and it most certainly affects different cell types (Stojiljkovic et al., 2018, Ramanathan and Ramalakshmi, 2017, Guil-Guerrero et al., 2011, Oguz et al., 2015). Additionally, in *in vivo* conditions, Lyc passes numerous metabolic “barriers” (i.e. enterocytes, liver, etc.), where this rather specific carotenoid is subjected to both enzymatic (mainly through β -carotene oxygenase 1) and non-enzymatic degradations (Rao et al., 2006; Story et al., 2010). Interestingly, the MDCK cells do possess β -carotene oxygenase 1 which would enable them to metabolize Lyc (see: <http://amp.pharm.mssm.edu/Harmonizome/>); however, it is debatable how these rather high concentrations of either Lyc or MTX affect activity of this enzyme.

Conclusions

Results of our study showed that Lyc applied in high concentration causes significant impairment of the MDCK cells' function that leads to cell death. Contrary, in relatively low concentrations Lyc shows beneficial effects, since it moderately ameliorates MTX-induced MDCK cells' toxicity estimated by both biochemical and microscopic analyses. It also prevented a

decrease in the MDCK lysosomal function, estimated through their ability to accumulate neutral red and to preserve the activity of lysosomal enzyme β -N acetyl glucosaminidase.

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Figure captions:

Figure 1. Effect of different concentrations of Lyc on the MDCK cells ability to reduce MTT (A) and to accumulate NR (B). Data are given as mean $\pm$ SD; * $p < 0.001$ vs. DMEM treated cells (by ANOVA and Tukey's post hoc test).

Figure 2. The MDCK cells appearance when cultured with DMEM only (A and B), Lyc 10^{-4} (C and D), Lyc 10^{-8} (E and F). Images A, C, and E were obtained under the invert objective lens, while B, D and F were from cells incubated with NR. Magnification was $\times 100$ for all.

Figure 3. Effect of different concentrations of Lyc and MTX (250 μ M) on the MDCK cells ability to reduce MTT (A) and accumulate NR (B). Data are given as mean $\pm$ SD; * $p < 0.001$ vs. Lyc and MTX treated cells; # $p < 0.001$ vs. MTX only treated cells (by ANOVA and Tukey's post hoc test).

Figure 4. The MDCK cells' appearance when cultured with DMEM only (A and B), MTX (C and D), Lyc 10^{-5} + MTX (E and F), Lyc 10^{-8} + MTX (G and H). Images A, C, E, and G were obtained under the invert objective lens, while B, D, F, and H are from cells incubated with NR. Magnification was $\times 100$ for all.

Figure 5. Effect of different concentrations of Lyc and MTX (250 μ M) on the MDCK cells AOPP (A) and GSH (B) concentrations, as well as on the β -NAG activity (C). Data are given as mean $\pm$ SD; * $p < 0.001$, ** $p < 0.01$ vs DMEM treated cells; # $p < 0.001$ vs. MTX only treated cells (by ANOVA and Tukey's post hoc test).

218x270mm (300 x 300 DPI)

1066x531mm (96 x 96 DPI)

185x266mm (300 x 300 DPI)

1422x531mm (96 x 96 DPI)

182x275mm (300 x 300 DPI)