Original Article

In vitro effect of 1-methyltryptophan isomers on epithelial-mesenchymal transition transcription factors in tubular epithelial cells after ischemia-reperfusion injury

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How to cite this article: Ali NejadKasbakhi N, Vavrincová D, Čepcová D. *In vitro* effect of 1-methyltryptophan isomers on epithelial-mesenchymal transition transcription factors in tubular epithelial cells after ischemia-reperfusion injury. Archives of Razi Institute Journal. 2024;79(2):307-314. DOI: 10.32592/ARI.2024.79.2.307

ABSTRACT

The compound 1-methyltryptophan (1-MT) has been shown to act protectively in renal ischemia-reperfusion injury. Toll-like receptor 4 signaling is also a regular process of epithelial-mesenchymal transition (EMT) that can after ischemia-reperfusion injury (IRI) result in an increase in renal fibrosis. EMT is associated with specific transcription factors: Snai1, Snai2, Zeb1, and Twist. 1-MT could regulate EMT and act as an antifibrotic agent. This study aimed to investigate the effect of 1-MT on EMT transcription factors in tubular epithelial cells that underwent 30 min. Renal tubular epithelial cells (TECs) were isolated from Lewis rats using a standard protocol with Fe_2O_3 magnetic separation and selective media as previously mentioned. Cells were cultivated and divided into 4 groups, namely C-TECs: control cells, IRI-TECs: IRI-induced TECs, D-IRI-TECs: IRI-induced TECs treated with 1-methyl-D-tryptophan, and L-IRI-TECs: IRI-induced TECs treated with 1-methyl-L-tryptophan. IRI was induced in all groups for 30 min by mineral oil (except for C-TECs) followed by 48-hour reperfusion. RNA and proteins were isolated from harvested cells. Using a semi-quantitative polymerase chain reaction, we assessed the relative mRNA expression of EMT transcription factors Snai1, Snai2, Zeb1, and Twist. Hereby, we showed that the treatment of ischemia-induced TECs with both 1-MT isomers lowered the expression of EMT transcription factors Snai1 and Zeb1 which were increased by ischemia and reperfusion of TECs. This could act favorably in renal IRI decreasing EMT and renal fibrosis, therefore showing the potential of 1-MT as a part of therapy in renal transplantation aimed at renal ischemia-reperfusion injury.

Keywords: Gene expression, Ischemia, Kidney, mRNA, Renal fibrosis

Article Info:
Received: 13 August 2023
Accepted: 18 September 2023
Published: 30 April 2023

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1. Introduction

To date, chronic kidney disease has affected 10% of the world's population (1). Owing to the rise in the elderly population and the growing occurrence of diabetes and hypertension, the prevalence of the disease would most likely increase over the next decade. Kidney transplant is the treatment of choice in end-stage renal disease (ESRD) patients. Kidney transplantation, peritoneal dialysis, and hemodialysis are options for renal replacement therapy for ESRD patients (2). Variations of the mentioned renal replacement therapies are being tried to enhance the results, such as morbidity reduction, mortality, and days of hospitalization, in conjunction with existing ongoing demonstration projects (2). Renal transplantation still faces some critical problems, one of which is ischemia-reperfusion injury (IRI) (3). Ischemia-reperfusion injury is triggered via a sudden temporary impairment of the blood flow to the organ. In general, IRI is correlated with a robust response of inflammatory and oxidative stress to hypoxia and reperfusion that disturbs the role of the organ (4). Acute kidney injury caused by renal IRI leads to high morbidity and mortality rates in a wide range of injuries (5). Renal IRI remains a significant kidney transplant problem. Renal-IRI-related therapy is currently not available (3, 4). For IRI treatment, it is important to decrease the hypoperfusion time to keep the organ functioning. Furthermore, indirect tissue injuries can be serious due to shock/resuscitation. Concerning sepsis, primary treatment with antibiotics and suitable recovery with satisfactory vasopressors and fluids are advised to lessen hypoperfusion of peripheral tissue (4). Epithelial-mesenchymal transition (EMT) is primarily regulated by a central group of EMT-activating transcription factors (EMT-TFs), including Snail (also known as SNAI1), Slug (also known as SNAI2), Twist-related protein 1 (TWIST1), zinc finger E-box-binding homeobox 1 (ZEB1), and ZEB2 (6). These factors trigger the classic EMT process which results in the breakdown of cellular adhesions, the loss of epithelial cell polarity, and the manifestation of a mesenchymal, motile phenotype 1 (7). Moreover, to mediate the classical EMT functions, EMT-TFs are related to the induction of several other characteristics, particularly stemming, survival, and changes in cell metabolism (7). It has been well established for many years that tubular epithelial cells, far from being innocent perpetrators of renal injury, are actively involved in interstitial events occurring during both immunologically and non-immunologically mediated renal disease (8). Moreover, a growing body of evidence has documented their ability to produce a variety of chemokines, cytokines, and growth factors that further contribute to interstitial inflammatory and reparative events (9). 1-methyltryptophan (1-MT) was suggested as a molecule with potential in IRI treatment (10). 1-methyltryptophan is a chemical substance that is an inhibitor of the tryptophan catabolic enzyme indoleamine 2,3-dioxygenase (IDO). This chiral compound can exist as both D- and L-isomers (enantiomers) (10). 1-methyl-D-tryptophan is also recognized as indoximod and is currently used in cancer treatment clinical trials; for instance, for advanced melanoma, IDO expression was observed in glomerular and tubular cells in a model of nephrotoxic serum nephritis, and its inhibition with 1-methyltryptophan intensified the renal damage (11). This study aimed to investigate the effect of 1-MT on EMT transcription factors in tubular epithelial cells that underwent 30-minute ischemia and 48-hour reperfusion.

2. Materials and Methods

2.1. Animals

A local Ethical Committee and the State Veterinary and Food Administration of the Slovak Republic approved this experimental procedure involving the use of laboratory animals. We used 16 Lewis rats (male, 12-week-old, RT1l, 180-220 g) in the experiment. Standard housing conditions (12h light/dark cycle, well-ventilated room, 23°C temperature, humidity-controlled environment) were maintained, and rats had access to water and food ad libitum.

2.2. Renal tubular epithelial cell isolation

Renal tubular epithelial cells (TECs) were isolated from Lewis rats using a standard protocol with Iron oxide (Fe_2O_3) magnetic separation and selective media based on a study described by Čepcová et al. (12) with minor modifications. Rats were anesthetized with 3% isoflurane, shaved, cannulated the aorta, and collected the blood sample. The rats were lysed with a solution of 2 × 20 ml sterile saline and then with a solution of 5 × 10 ml Fe_2O_3 (0.9 g Fe_2O_3/50 ml/rat). The kidneys were collected and transferred to cold Dulbecco's phosphate-buffered saline (DPBS) (Gibco, Germany). In a sterile bacteriological Petri dish, the renal capsule was cut, and the kidney cortex was minced with a surgical blade and incubated with 20 ml of collagenase IV. At 37°C (40 mg Dulbecco's Modified Eagle Medium [DMEM]/F12 [Gibco] medium/rat collagenase intravenous/20 ml) for 1 h and resuspended every 10 min. The suspension was moved to 50 ml falcon and 3 ml DPBS was added to avoid the reaction. Then, the suspension was filtered into a new 50 ml falcon via a 100 µm cell strainer. The pellet was washed in 50 ml falcon with 20 ml of DMEM/F12 media, and the cell suspension was divided by flipping the magnet device that attracted glomeruli trapped by Fe_2O_3. With the magnet held in place, the suspension was moved

to a new falcon (separation was repeated several times until all glomeruli attached to iron beads were removed) and filled with DMEM/F12 medium supplemented with epidermal growth factor (Peprotech, 10 ng/ml) and hydrocortisone (Hydrocortisone Medochemie, 200 ng/ml) and then divided into 6-well plates. The suspension consisted of tubules that bind to the culture flask at the bottom. The tubular epithelial cells developed out of tubules after 5-6 days of culture. Tubules were then washed out with PBS and inserted with fresh content. Cells were ready for the experiments in 75% confluence over the next 2-3 days. The epithelial nature of these cells was confirmed by their typical polygonal shape and cobblestone-like arrangement. No spindle-shaped cells that could resemble renal fibroblasts were identified in each period using this technique.

2.3. IRI model (hypoxia-reoxygenation) on TECs

TECs induced by IRI were cultured in standard conditions in DMEM/F12 medium, and renal TECs were grown in six-well plates (n=3), supplemented as mentioned above with regular medium exchange. Cells were subjected to ischemic (hypoxic) conditions after 80% confluence: medium was extracted and TECs washed twice with DPBS, then 350 µl of sterile mineral oil (Sigma-Aldrich, Germany) was applied at 37°C for 30 min. Mineral oil was washed out with PBS after ischemia, and added medium (reoxygenation). Cells were treated with either D-MT or L-MT (750 µmol/l dissolved in 0,1N NaOH) 24 h and 1 h before ischemia induction and 24 h after reperfusion for a total of 48 h. TECs were collected 48 h after reperfusion for further analysis. The groups (n=3) involved:

- C-TECs: control group
- IRI-TECs: TECs with induced IRI for 30 min and 48 h reperfusion
- D-IRI-TECs: TECs with induced IRI for 30 min and 48 h reperfusion treated with 1-D-MT
- L-IRI-TECs: TECs with induced IRI for 30 min and 48 h reperfusion treated with 1-L-MT

2.4. RNA isolation and RNA concentration assessment

For the RNA isolation, we used a commercially available kit NucleoSpin RNA/protein (Macherey-Nagel, Germany). We added 350 µl of lysis buffer (RP1) and 3.5 µl β-mercaptoethanol (β-ME) to the harvested cells and then mixed the sample by shaking it. We filtered the lysate with NucleoSpin® Filter and centrifuged for 1 min at 11,000 rpm. We added 350 µl 70% ethanol to the solution and we homogenized it by pipetting up and down. We transferred the lysate into the NucleoSpin® RNA/protein colon and centrifuged it for 1 minute at 11,000 rpm. The RNA got separated (in the top filter). We placed the top filter/membrane in a new tube. We desalted the membrane with 350 µl membrane desalting buffer and centrifuged it for 1 min at 11,000 rpm. We prepared rDN-ase reaction mixture: 10 µl rDN-ase with 90 µl rDN-ase buffer. We incubated the sample with 95 µl of the rDN-ase reaction mixture at room temperature for 15 min. We washed the membrane with 200 µl washing buffer (RA2) and centrifuged it for 30 s at 11,000 rpm. We placed the membrane in a new tube and added 600 µl washing buffer (RA3) and centrifuged it for 30 s at 11,000 rpm. We discarded the collection tube, added another 250 µl of washing buffer (RA3), and centrifuged it for 2 min at 11,000 rpm to dry the membrane with RNA. We dissolved the RNA with 60 µl of water without RN-ases and centrifuged it for 1 more min to obtain the RNA solution. We measured its concentration using wavelength 280/260 nm.

2.5. Reverse transcription and semi-quantitative reverse transcription-polymerase chain reaction

The concentration of total RNA isolated from TECs was adjusted to 500 ng and used in reverse transcription (LunaScript® RT SuperMixKit in Mastercyclerpersonal-Eppendorf®, Germany) to obtain cDNA. The reverse transcription-polymerase chain reaction (PCR) was operated using 5xHot FIREPol EvaGreen qPCR MixPlus ROX kit (Solis Biodyne, Estonia) in QuantStudioTM 3, Real-Time PCR Systems (ThermoFisher, USA). The optimized PCR program was 95°C for 15 min, then 40 cycles at 95°C for 30 s, 60°C for 40 s, and 72°C for 30 s, followed by a final elongation step at 72°C for 10 min. The reaction was terminated by a step of dissociation. Designed using Primer-BLAST, specific primers were acquired from Sigma-Aldrich, Germany (Figure 1). Results were standardized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and analyzed using the standard method of calibration curve.

2.6. Statistical analysis

Values of relative mRNA expression (standardized to GAPDH as a housekeeper gene) were analyzed by ANOVA in SPSS software (version 26.0) with post hoc test LSD. Differences between groups were considered significant at $P < 0.05$.

3. Results

3.1 Effects of 1-MT isomers on Snai1 mRNA expression

The IRI induction in TECs caused an increase in relative mRNA expression of transcription factor Snai1, compared to C-TECs. Treatment with both D-MT and L-MT decreased the relative mRNA expression of Snai1 compared to IRI-TECs (Figure 2).

Gene	Primer sequences Fwd(5′-3′)/Rev (3′-5′)
Snai1	Fwd GACGCGTGTGTGGAGTTCA
	Rev GAGAGAGTCCCAGATGAGGGT
Snai2/Slug	Fwd CACATTAGAACACACACTGGGGA
	Rev TGCCCTCAGGTTTGATCTGTC
Zeb1	Fwd TGGGATGTACGCATGTGACC
	Rev GGGGCCTCTTACCTGTATGC
Twist	Fwd CCGGAGACCTAGATGTCATTGT
	Rev CACGCCCTGATTCTTGTGAAA
Gapdh	Fwd TCTCTGCTCCTCCCTGTTCT
	Rev TACGGCCAAATCCGTTCACA

Figure 1. Specific primers used in sq-PCR.

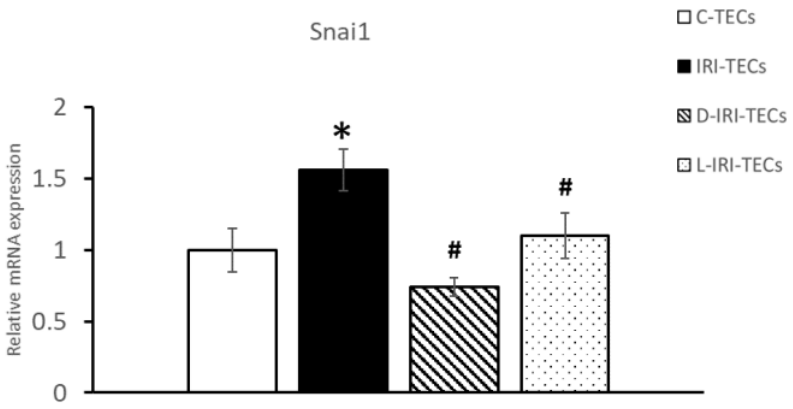

Figure 2: Effect of 1-MT isomers on Snai1 relative mRNA expression in IRI-induced TECs * IRI, IRID vs. C $p < 0,05$; # IRI vs. IRIL, IRID $p < 0,05$; C - control cells; IRI-TECs – IRI-induced TECs; D-IRI-TECs – IRI-induced TECs treated with D-MT; L-IRI-TECs – IRI-induced TECs treated with L-MT.

3.2 Effects of L-MT treatment on Snai2 mRNA expression

The IRI in TECs did not cause a significant change in relative mRNA expression of the second investigated EMT transcription factor Snai2 (also referred to as Slug). The D-MT treatment did not have any effect on IRI-induced TECs compared to C-TECs and IRI-TECs. On the other hand, we observed increasing trends in the L-MT-treated group of TECs, compared to C-TECs and IRI-TECs (Figure 3).

3.3. Effects of 1-MT isomers on Zeb1 mRNA expression

In the relative mRNA expression of Zeb1, we found a significant increase in the IRI-TECs group, compared to C-TECs. Treatment with both D-MT and L-MT significantly decreased mRNA expression of Zeb1, compared to IRI-TECs, although D-MT had an even stronger effect on Zeb1 mRNA expression than L-MT (Figure 4).

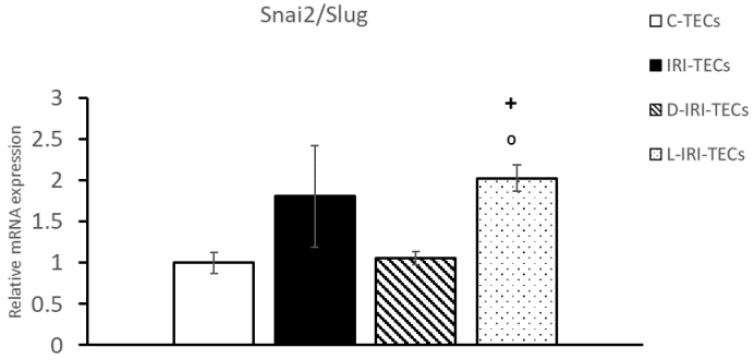

Figure 3. Effect of 1-MT isomers on Snai2 (Slug) relative mRNA expression in IRI-induced TECs. +RIL vs. IRID $p = 0,07$; ^{0} IRIL vs. C $p=0,06$; C - control cells; IRI-TECs – IRI-induced TECs; D-IRITECs – IRI-induced TECs treated with D-MT; L-IRI-TECs – IRI-induced TECs treated with L-MT.

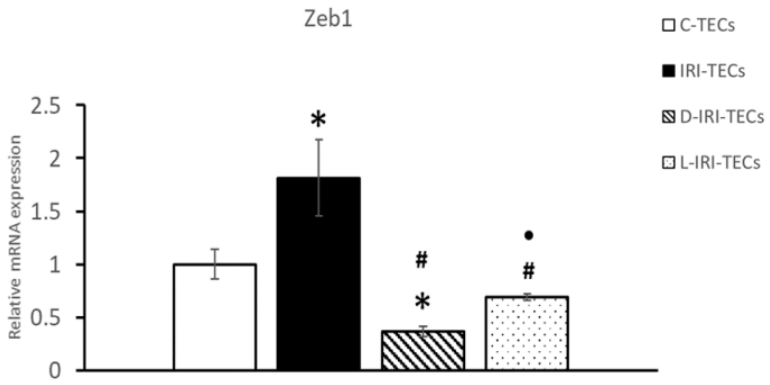

Figure 4. Effect of 1-MT isomers on Zeb1 relative mRNA expression in IRI-induced TECs. *IRI, IRID, IRIL vs. C $p < 0,05$; # IRI vs. IRIL, IRID $p < 0,05$; • IRIL vs. IRID $p < 0,05$; C - control cells; IRI-TECs – IRI-induced TECs; D-IRI-TECs – IRI-induced TECs treated with D-MT; L-IRITECs – IRI-induced TECs treated with L-M.

3.4 Effects of 1-MT isomers on Twist mRNA expression

The IRI alone did not change the mRNA expression of Twist in TECs and neither did treatment with D-MT. In contrast, L-MT increased the mRNA expression of Twist, compared to C-TECs (Figure 5).

4. Discussion

1-MT isomers in rats have been previously demonstrated to reduce renal IRI - kidney function and pre-fibrosis. In this study, we investigated whether they could potentially reduce pre-fibrosis through EMT - one of the processes caused by IRI that contributes to renal fibrosis (12). Our previous study suggested that 1-MT might interfere with the regulation of EMT signaling because it interfered with two pathways crucial to EMT regulation - transforming growth factor-β (TGF-β) and toll-like receptor 4 (12). Thus, in this study, we investigated the effect of 1-MT isomers on epithelial-to-mesenchymal following IRI in the TEC, particularly EMT transcription factors. We also evaluated the effect of 1-MT isomers on the relative mRNA expression of Snai1, Snai2/Slug, Zeb1, and Twist in the TEC. At first, we investigated the effect of 1-MT isomers on Snai1 relative expression in IRI-induced TECs. Snail1 is the key member of the Zinc-finger transcription factors of the Snail superfamily, involved in cell differentiation and survival, and usually acts as a transcriptional repressor (13). Snail1 performs a crucial role in regulating EMT, a mechanism by which epithelial cells acquire a migratory, mesenchymal phenotype as a result of its repression of E-cadherin. In our experiment, the induction of IRI in TECs triggered an increase in the relative expression of Snai1 mRNA, compared to C-TECs (14). Treatment with both D-MT and L-MT decreased Snai1 relative mRNA expression compared to IRI-TECs suggesting at least partly inhibitory effect of both isomers on EMT signaling pathway (15). Previous research suggests that 1-MT isomer interference with TGF-β signaling (documented in our prior research) is a possible cause of these changes. For example, Peinado et al. have

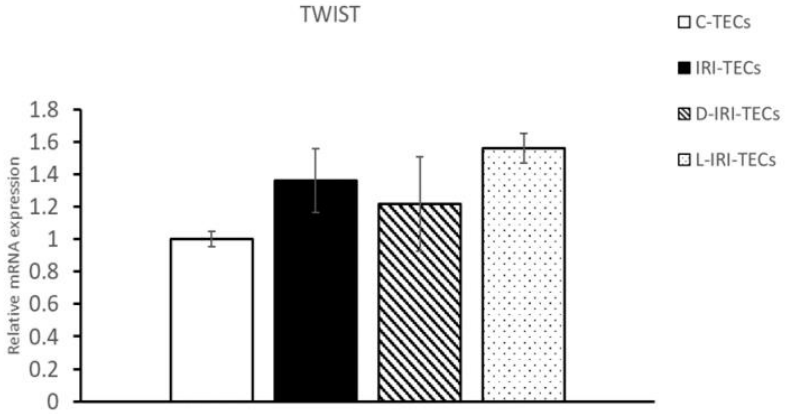

Figure 5. Effect of 1-MT isomers on Twist relative mRNA expression in IRI-induced TECs. IRIL vs. C $p < 0,05$; C - control cells; IRI-TECs – IRI-induced TECs; D-IRI-TECs – IRI-induced TECs treated with D-MT; L-IRI-TECs – IRI-induced TECs treated with L-MT.

shown that TGFβ1 stimulates Snail expression in canine kidney cells of Madin-Darby and causes epithelial-mesenchymal transitions through a mitogen-activated protein kinase-dependent signaling mechanism (16). Liu et al. found that TGFβ1-induced SMAD3 phosphorylation promoted Snail transcriptional activation, through Stat3 signaling. The pSMAD3 can bind to PIAS3, leading to the dissociation of PIAS3 from Stat3, allowing Stat3 to be phosphorylated and then the target Snail promoter to activate it. Significant increases in Snail and phosphorylated Stat3, an activated Stat3 form, were observed in the kidney following the induction of unilateral ureteral obstruction (UUO) in mice (17). Regardless of the mechanism of 1-MT, we can suggest that decreased Snai1 expression is a sign of the protective antifibrotic effect of 1-MT isomers in IRI-induced cells. It was demonstrated that Snai1 knockout animals had considerably diminished inflammation, reduced a-SMA+ myofibroblasts, and fibrosis following UUO (another model in which fibrosis and EMT are considered pathological features) (18). Furthermore, benefits (inhibited collagen deposition and cardiac fibrosis) of lowered SNAI1 expression were documented in heart IRI in mouse models (19). Subsequently, we analyzed the role of 1-MT isomers on the relative expression of SNAI2 (also called Slug) in IRI-induced TECs. SNAI2 (Slug) also has properties like Snail, including E-cadherin transcriptional repression and anti-apoptotic activity. The ischemia induction did not induce any significant change in the relative mRNA expression of Snai2 in TECs nor did treatment with D-MT. On the other hand, as compared to C-TECs and IRI-TECs, we observed an increasing trend in L-MT-treated TECs culture. Our results were surprising because the overexpression of Snai2 like Snai1 in cultured epithelial cells was necessary to induce EMT (20) and we did not register any significant change. On the other hand, it might change with more than 3 biological replicates of TECs in each group - an increased sample size could result in statistical significance. Interestingly, the Snai transcription factors are also described as key transcription factors in EMT since their ectopic over-expression promotes EMT also when TGFβ1 is absent (20). Afterward, we studied the effect of 1-MT isomers on Zeb1 mRNA expression. Zinc finger E-box binding homeobox 1 (ZEB1) is a member of the family of transcription factor zinc finger-homeodomain modulating cell differentiation and unique cellular functions in multiple tissues (13). It can suppress E-cadherin in solid tumors, induce EMT, stimulate chemo- and radio-resistant phenotypes, and promote the development of stem cells from cancer. ZEB1 alone was shown to initiate renal tubular dedifferentiation and the development of renal fibrosis (21). Yao et al. demonstrated that ATII cells ZEB1-dependent EMT led to fibrosis through epithelial-fibroblast crosstalk and that ZEB1-regulated paracrine signaling contributes to the production of a profibrogenic microenvironment contributing to interstitial fibrosis (22). We observed a significant increase in IRI-induced TECs, compared to control cells in relative mRNA expression of ZEB1. Treatment with both D-MT and L-MT significantly reduced ZEB1 mRNA expression, while D-MT had an even greater effect on ZEB1 mRNA expression than L-MT. Finally, we investigated the mRNA expression of Twist. Twist is a member of the transcription-factor basic helix-loop-helix family. Upregulated during growth of mesoderms, neural tube formation, tissue fibrosis, and tumor metastases. The TWIST gene has been identified as an EMT participant in all three types of EMT and is considered an independent pathway to repressing E-cadherin from SNAI1 and SLUG and upregulating fibronectin and N-cadherin (23). Our results concerning Twist's mRNA expression showed that TECs and the IRI alone did not alter Twist's mRNA expression and neither did D-MT therapy. Contrary to this, L-MT increased Twist's mRNA expression compared to control cells. Sun et al. demonstrated the role of hypoxia-inducible factor-1α (HIF-1 α) Twist activation in EMT-induced hypoxia. Their results demonstrated the substantial upregulation of HIF-1α and Twist in tubular epithelial cells undergoing EMT. Although they do not provide clear evidence that Twist activation in tubular epithelial cells induces EMT and *in vivo* fibrosis (as seen for Snail), it is highly probable that Twist activation contributes to renal fibrogenesis (24). In our rat IRI model, we did not find changes in HIF-1α expression that would correlate with an increase of Twist in L-IRI-TECs. To investigate this pathway further, it would be desirable to assess the HIF-1α expression in our model - RI-induced TECs treated with 1-MT isomers. Taken together, both isomers of 1-MT decreased mRNA expression of EMT transcription factors SNAI1 and ZEB1 in IRI-induced TECs compared to IRI-induced untreated cells. These results led to the conclusion that 1-MT isomers inhibited EMT and acted as antifibrotic agents in renal IRI. The data available regarding the topic of this thesis are scarce. Furthermore, the findings of only two articles we are aware of containing information close enough to our model are contradictory to our data - both concerning only D-MT. D-MT was used in the model of UUO through IDO inhibition (25) and it potentiated the developing EMT. According to Matheus et al., D-MT could even induce this process. Even though both models (IRI and UUO) have renal fibrosis as a pathological consequence, their mechanisms are not completely identical, and various signaling cascades could be altered (by injury or D-MT) in one and not the other that we were not aware of

and that could lead to different results (25). The same group investigated the effect of D-MT in T24 cells stimulated by TGF (26). They have concluded that the effect was IDO-independent. Added TGF-β1 as profibrotic stimulus in cell culture caused increased EMT and according to their data it was potentiated by D-MT. In contrast, in our model, the only profibrotic stimulus was IRI and the amount of TGF-β1 in cell culture was unknown (yet to be determined). Since we have in our previous research established that 1-MT isomers inhibit TGF-β1 signaling, the observed effect could be simply a result of decreased levels of TGF-β1 in our TECs culture treated with 1-MT (26). Although both 1-MT isomers decreased the relative expression of transcription factors Snai1 and Zeb1 after IRI, D-MT was clearly more potent than the L isoform. Additionally, in the case of Snai2 and Twist L-MT has even increased their expression. L-MT was not previously investigated in this context; therefore, there is no additional information available. However, the biological and pharmacological differences between the 1-MT isomers are documented and described in this study. We demonstrated that both 1-MT isomers had antifibrotic properties in the TECs IRI model with some differences. They both alter the EMT signaling pathway probably through the TGF-β pathway. These properties of D-MT and L-MT may be partially responsible for their renoprotective effect in renal IRI. Thus, our data support 1-MT isomers as potential drugs in IRI therapy. In this study, we investigated the effect of 1-MT isomers (D and L) in IRI-induced TECs. Our focus was on the expression of transcription factors of EMT. We have demonstrated that both 1-MT isomers acted as inhibitors in the EMT pathway; that is, they lowered the expression of Snai1 and Zeb1 transcription factors. We showed that 1-MT isomers had antifibrotic properties by interfering with EMT in the IRI model in TECs. Furthermore, for the first time, we investigated the link between the L isomer of 1-MT and EMT. Our data support both 1-MT isomers as potential novel drugs that could be used in renal IRI therapy while such therapy is currently not available even though it is highly needed. As the main limitation of the present study, we can mention the lack of efficacy of 1-MT in animal models of IRI.

Acknowledgment
We would like to thank

Authors' Contribution
NANK, and DV designed the experiments; NANK, and DC performed experiments and collected data; DV Supervised, directed, and managed the study; all authors approved the final version to be published.

Ethics
The study protocol was approved by the Ethic Committee of Comenius University in Bratislava, Slovak Republic (No.2018-22-10)

Conflict of Interest
The authors declare no conflicts of interest.

Grant Support
No funding/support.

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