รายงานวิจัยฉบับสมบูรณ์

โครงการ “ฤทธิ์ของสารแมงจิเฟอรินที่พบในมะม่วงต่อการดูดกลับน้ำตาลกลูโคสและการป้องกันการเกิดพังผืดในเซลล์ท่อไตส่วนต้นของคน”

“Effect of Mangiferin, a constituent found in mango, on glucose reabsorption and its protective effect on fibrosis of human renal proximal tubular epithelial cells”

โดย ดร. พร้อมสุข ชุตาภา

พฤศจิกายน พ.ศ. 2565

สัญญาเลขที่ RDG6220034

รายงานวิจัยฉบับสมบูรณ์

โครงการ “ฤทธิ์ของสารแมงจิเฟอรินที่พบในมะม่วงต่อการดูดกลับน้ำตาลกลูโคสและการป้องกันการเกิดพังผืดในเซลล์ท่อไตส่วนต้นของคน”

“Effect of Mangiferin, a constituent found in mango, on glucose reabsorption and its protective effect on fibrosis of human renal proximal tubular epithelial cells”

คณะผู้วิจัย	สังกัด
1. ดร. พร้อมสุข ชุตาภา	โรงเรียนแพทย์รามาธิบดี สถาบันการแพทย์จักรีนฤบดินทร์ คณะแพทยศาสตร์โรงพยาบาลรามาธิบดี มหาวิทยาลัยมหิดล
2. นางสาววันอังคาร พูลศรี	หลักสูตรเวชศาสตร์ปริวรรต คณะแพทยศาสตร์โรงพยาบาลรามาธิบดี มหาวิทยาลัยมหิดล

สนับสนุนโดยสำนักงานคณะกรรมการส่งเสริมวิทยาศาสตร์ วิจัยและนวัตกรรม (สกสว.)
(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกสว.ไม่จำเป็นต้องเห็นด้วยเสมอไป)

กิตติกรรมประกาศ (Acknowledgement)

งานวิจัยนี้ได้รับทุนอุดหนุนการวิจัยจากสำนักงานคณะกรรมการส่งเสริมวิทยาศาสตร์ วิจัยและนวัตกรรม (สกสว.) ประจำปีงบประมาณ 2562 และได้รับการสนับสนุนด้านเครื่องมือและสถานที่ห้องปฏิบัติการจากสถาบันการแพทย์จักรีนฤบดินทร์ คณะแพทยศาสตร์โรงพยาบาลรามาธิบดี มหาวิทยาลัยมหิดล หัวหน้าโครงการวิจัยขอขอบคุณนางสาววันอังคาร พูลศรี นักศึกษาปริญญาเอกสาขาเวชศาสตร์ปริวรรต ที่ช่วยดำเนินการทดลองส่วนใหญ่ในโครงการวิจัยจนสำเร็จลุล่วง และขอขอบคุณ นางสาวระพีวรรณ เศรษฐคมกุล นายกานต์ ช้างศร และนางสาวกรกมล อักษร ผู้ช่วยวิจัย ที่ช่วยเก็บข้อมูลบางส่วนในโครงการ

นอกจากนี้ผู้วิจัยขอขอบคุณ ศาสตราจารย์เกียรติคุณ ดร.ภญ.นันทวัน บุณยะประภัศร ที่ให้คำปรึกษาชี้แนะเรื่องการบริหารจัดการและดำเนินการวิจัยนี้โดยตลอด

พร้อมสุข ชุตาภา

21 พฤศจิกายน 2565

สารบัญเรื่อง (Table of Contents)

หน้า

กิตติกรรมประกาศ i

สารบัญเรื่อง (Table of Contents) ii

สารบัญตาราง (List of Tables) iii

สารบัญภาพ (List of Illustrations) iv

คำอธิบายสัญลักษณ์และคำย่อที่ใช้ในการวิจัย (List of Abbreviations) v

สรุปโครงการวิจัย (Executive Summary 1

บทคัดย่อ 5

Abstract 6

บทนำ (Introduction) 8

วรรณกรรมที่เกี่ยวข้อง (Literature Review) 10

วิธีดำเนินการวิจัย (Methods) 15

ผลการวิจัยและอภิปรายผลการทดลอง (Results and Discussion) 21

สรุปผลการวิจัยและข้อเสนอแนะ (Conclusion and Suggestion) 47

เอกสารอ้างอิง (References) 49

ภาคผนวก 54

สารบัญตาราง (List of Tables)

หน้า

Table 1. Primer sequences for polymerase chain reaction of transporter genes 16
Table 2. Primer sequences for quantitative polymerase chain reaction of genes 19

สารบัญภาพ (List of Illustration)

หน้า

Figure 1 Structure of mangiferin..... 10
Figure 2 Renal proximal epithelial cell lines 21
Figure 3 Image of RT-PCR of RPTEC/TERT1 and SA7K cells 22
Figure 4 The RPTEC staining with proximal tubule marker..... 23
Figure 5 The RPTEC staining with LTL, Na/K ATPase, ZO-1..... 23
Figure 6 RPTEC cells cultured in regular 2D staining with transporters 24
Figure 7 RPTEC cells cultured in transwell-inserts staining with transporters..... 25
Figure 8 RPTEC cells cultured in 3D platform (proximal tubule-on-a-chip) 25
Figure 9 Effect of mangiferin on the cell viability of RPTEC cells..... 26
Figure 10 The functional test of OCTs in RPTEC cells 27
Figure 11 The uptake of 4-Di-1-ASP (ASP^+) by RPTEC cells 28
Figure 12 The uptake by OAT1-transfected MDCK cells (MDCK-OAT1) 29
Figure 13 The fluorescein uptake by OAT3-transfected MDCK cells (MDCK-OAT3) 30
Figure 14 The [^{3}H] glucose uptake in RPTEC cells 31
Figure 15 The mRNA expression of glucose transporters in RPTEC cells with high glucose..... 32
Figure 16 The mRNA levels of inflammatory cytokines and profibrotic markers 33
Figure 17 The mRNA levels of kidney injury and fibrogenesis markers..... 34
Figure 18 The levels of inflammatory cytokines detected in cell culture supernatant 35
Figure 19 The effect of mangiferin on IL-6 release in RPTEC cells..... 36
Figure 20 The IL-6 and LDH release in RPTEC cells in 3D culture platform 36
Figure 21 The RPTEC cell survival test in 3D culture platform of proximal tubule-on-a-chip..... 37
Figure 22 The induction of fibrogenesis with IL-1□ in RPTEC cell..... 39
Figure 23 The induction of fibrogenesis with TGF-β1 for 48h in RPTEC cell..... 40
Figure 24 The induction of fibrogenesis with TGF-β1 for 24h in RPTEC cell..... 41
Figure 25 The effect of mangiferin on the reduction of TGF-β1-induced fibrosis..... 42
Figure 26 The effect of mangiferin on the reduction of TGF-β1-induced the alteration..... 43
Figure 27 The effect of mangiferin on the mechanism of TGF-β1-induced fibrosis. 44
Figure 28 The effect of mangiferin on the expression of fibronectin in 3D platform..... 45

คำอธิบายสัญลักษณ์และคำย่อที่ใช้ในการวิจัย (List of Abbreviations)

2D	Two dimensional
3D	Three dimensional
α-SMA	Alpha smooth muscle actin
Col I	Collagen type I
DM	Diabetes mellitus
ESRD	End-stage renal disease
FN	Fibronectin
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFR	Glomerular filtration rate
hEGF	Human epidermal growth factor
IL-1□	Interleukin 1 beta
IL-6	Interleukin 6
MRP	Multidrug resistance-associated protein
MTT	3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium
NF-κB	Nuclear factor kappa B
OAT	Organic anion transporter
OCT	Organic cation transporter
PCR	Polymerase chain reaction
P-gp	P-glycoprotein
RPTEC	Renal proximal tubule epithelial cells
ROS	Reactive oxygen species
SGLT	Sodium-coupled glucose transporter
TGF-□1	Transforming growth factor beta 1
TNF-α	Tumor necrotic factor alpha
ZO-1	Zona occludens 1 or tight junction protein 1

สรุปโครงการวิจัย
Executive Summary

1. ชื่อโครงการ

(ภาษาไทย) **:** ฤทธิ์ของสารแมงจิเฟอรินที่พบในมะม่วงต่อการดูดกลับน้ำตาลกลูโคสและการป้องกันการเกิดพังผืดในเซลล์ท่อไตส่วนต้นของคน

(ภาษาอังกฤษ) **:** Effect of mangiferin, a constituent found in mango, on glucose reabsorption and its protective effect on fibrosis of human renal proximal tubular epithelial cell

2. ชื่อคณะผู้วิจัย/หน่วยงานที่สังกัด/หมายเลขโทรศัพท์/โทรสาร/e-mail

หัวหน้าโครงการ

ชื่อ นามสกุล: นางสาวพร้อมสุข ชุตาภา

หน่วยงานที่สังกัด: สาขาวิชาพรีคลินิก โรงเรียนแพทย์รามาธิบดี สถาบันการแพทย์จักรีนฤบดินทร์ คณะแพทยศาสตร์โรงพยาบาลรามาธิบดี มหาวิทยาลัยมหิดล

สถานที่ตั้ง: 111 หมู่ 14 ต.บางปลา อ.บางพลี จ.สมุทรปราการ 10540

โทรศัพท์: 02-839-5216

e-mail: promsuk.jut@mahidol.ac.th

ผู้ร่วมวิจัย

ชื่อ นามสกุล: นางสาววันอังคาร พูลศรี

หน่วยงานที่สังกัด: หลักสูตรเวชศาสตร์ปริวรรต โรงเรียนแพทย์รามาธิบดี สถาบันการแพทย์จักรีนฤบดินทร์ คณะแพทยศาสตร์โรงพยาบาลรามาธิบดี มหาวิทยาลัยมหิดล

สถานที่ตั้ง: 111 หมู่ 14 ต.บางปลา อ.บางพลี จ.สมุทรปราการ 10540

โทรศัพท์: 02-839-5216

e-mail: wanangkan.poo@student.mahidol.edu

3. สาขาที่ทำการวิจัย

วิทยาศาสตร์สุขภาพ

4. Keywords

mangiferin, mango extract, diabetes mellitus, renal fibrosis, renal proximal tubule, chronic kidney disease

5. งบประมาณทั้งโครงการ

2,040,500.00 บาท

6. ระยะเวลาดำเนินการ

3 ปี (ตั้งแต่ เดือนกันยายน ปี พ.ศ. 2562 ถึง เดือนสิงหาคม ปี พ.ศ. 2565)

7. ความสำคัญและที่มาของปัญหาการวิจัย

ในปัจจุบันอุบัติการณ์ของโรคเบาหวานเพิ่มมากขึ้นในประชากรทั่วโลก ผู้ป่วยโรคเบาหวานมักจะมีภาวะแทรกซ้อนทางไตเกิดขึ้น (Diabetic nephropathy) ซึ่งจะตรวจพบการมีโปรตีนขับทิ้งในปัสสาวะมากขึ้น มีอัตราการกรองของไตลดลง เมื่อมีการดำเนินโรคไประยะหนึ่งประมาณ 10-15 ปี อาการของภาวะไตเสื่อมจะเพิ่มมากขึ้น ผู้ป่วยมักจะมีการเกิดพังผืดในไต จนกระทั่งเกิดภาวะไตวายในระยะสุดท้าย (end-stage renal failure, ESRD) การรักษาจะต้องรอการเปลี่ยนไต (Renal transplantation) ซึ่งจะทำได้ไม่มาก จำเป็นต้องรอไตจากการบริจาค ส่วนใหญ่จึงทำได้เพียงการล้างไตผ่านทางหน้าท้อง (peritoneal dialysis) หรือ การฟอกเลือด (hemodialysis) เพื่อช่วยขับของเสียที่เกิดขึ้นออกไปเท่านั้น ยังไม่มียารักษาที่มีประสิทธิภาพที่จะชะลออาการไตเสื่อมหรือการเกิดพังผืดในไต

ความสนใจการใช้ประโยชน์จากสารธรรมชาติ โดยเฉพาะอย่างยิ่งสารสกัดหรือสารบริสุทธิ์ที่ได้จากพืชอาหารและสมุนไพรต่างๆ เพื่อใช้เป็นอาหารเสริมหรือยาเพื่อป้องกันและรักษาโรคมากขึ้น ด้วยความเชื่อที่ว่าจะมีความปลอดภัยมากกว่ายาหรือสารเคมีสังเคราะห์เริ่มมีมากขึ้น อย่างไรก็ตามสารสำคัญจากพืชหรือตำรับยาโบราณต่างๆที่มีการใช้ในตำรับยาแผนโบราณ ยังขาดหลักฐานทางวิทยาศาสตร์สนับสนุนอีกมาก และงานวิจัยส่วนใหญ่ทำในสัตว์ทดลอง มีข้อมูลสนับสนุนในคนน้อยมาก เนื่องจากขาดโมเดลที่จะเลียนแบบการทำงานในร่างกายคน โดยเฉพาะอย่างยิ่งโมเดลของการเลียนแบบภาวะเบาหวานในไตที่จะนำไปสู่การเกิดการบาดเจ็บของไต การเกิดพังผืด และท้ายที่สุดอาจดำเนินไปสู่ไตวายได้ ซึ่งการศึกษาภาวะเบาหวานที่จะทำให้เกิดการบาดเจ็บที่ไตโดยตรงในหลอดทดลองนั้น ยังขาดโมเดลเซลล์ที่จะเป็นตัวแทนที่เหมาะสมในการนำมาใช้เพื่อลดการใช้สัตว์ทดลองลง โมเดลเซลล์ท่อไตส่วนต้นของคนที่นำมาศึกษากันนั้น เป็นการเลี้ยงเซลล์ในรูปแบบสองมิติ โดย Primary cells แม้จะมีคุณลักษณะที่คงคุณสมบัติหลายๆอย่างของเซลล์ในร่างกายไว้ แต่มีอายุการใช้งานสั้นมาก และผลการศึกษามีความแปรปรวนค่อนข้างสูง ยากต่อการควบคุมให้ได้ผลคงที่ จึงเริ่มมีการพัฒนา immortalized cells ขึ้นมาเพื่อให้สามารถคงสภาวะการที่จะได้ผลการทดลองที่คงที่ และสามารถเลี้ยงเซลล์ต่อๆไปได้หลายรุ่น แต่ cell line ที่ได้จากไต ไม่เฉพาะแต่ส่วนท่อไตส่วนต้นเท่านั้น ท่อไตในส่วนอื่นๆก็ยังไม่พบการแสดงออกของโปรตีนขนส่ง (Transporter) ที่เหมาะสมเหมือนที่พบในร่างกายได้ จึงเป็นข้อจำกัดในการแปลผลการทดลอง การเลี้ยงเซลล์ในแบบสามมิติทั้งในแบบ organoid และ Tubuloid เริ่มเป็นที่สนใจในทุกๆอวัยวะ เพื่อเลียนแบบสภาวะที่คล้ายในร่างกายมากยิ่งขึ้น ในระยะต่อมาการใช้ microfluidic model มีบทบาทสำคัญสำหรับอวัยวะที่มีการไหลเวียนของสารน้ำ เช่น หลอดเลือด และ ท่อไตส่วนต่างๆ (renal tubules) ซึ่งมีข้อมูลสนับสนุนว่าจะช่วยให้เซลล์มีคุณสมบัติที่เจริญเต็มที่ (cell maturation and differentiation) ใกล้เคียงในร่างกายมากที่สุด ผู้วิจัยจึงมีความสนใจที่จะเพาะเลี้ยงเซลล์ท่อไตส่วนต้นของคนในรูปแบบ proximal tubule-on-a-chip เพื่อที่จะเริ่มให้มีการพัฒนาโมเดลนี้ในประเทศไทยต่อไป

จากข้อมูลเกี่ยวกับสารที่สกัดได้จากเปลือกและใบของมะม่วงที่มีชื่อว่าแมงจิเฟอริน (mangiferin) ซึ่งมีคุณสมบัติในการลดน้ำตาล และลดการเกิดพยาธิสภาพที่ไตในหนูทดลองที่ถูกเหนี่ยวนำให้เป็นเบาหวาน และยังมีคุณสมบัติทางเภสัชวิทยาในการลดการเจริญของเชื้อโรค ต้านการอักเสบ ลด oxidative stress และการตายของเซลล์ที่ได้รับสารพิษได้ ด้วยคุณสมบัติดังกล่าวที่พบในสัตว์ทดลอง ซึ่งอาจไม่เหมือนกับสภาพในร่างกายมนุษย์ จึงจำเป็นที่จะต้องทำการศึกษาในโมเดลที่ใช้เซลล์จากร่างกายมนุษย์ เพื่อลดความแตกต่างระหว่าง Species และเป็น

การทดสอบผลโดยตรงในเซลล์นั้นๆด้วย ซึ่งอาจจะสามารถนำแมงจิเฟอรินมาพัฒนาเป็นยาเพื่อป้องกันหรือลดภาวะแทรกซ้อนของไตจากเบาหวานต่อไป

8. วัตถุประสงค์การวิจัย

โครงการวิจัยนี้มุ่งเน้นการศึกษาฤทธิ์ทางเภสัชวิทยาของสารแมงจิเฟอรินโดยมีวัตถุประสงค์ย่อยดังนี้

8.1. เพื่อศึกษาฤทธิ์ของสารแมงจิเฟอริน ในการลดระดับน้ำตาล โดยใช้เซลล์เยื่อบุท่อไตส่วนต้นของคน

8.2. เพื่อศึกษาและให้ข้อมูลที่มีหลักฐานทางวิทยาศาสตร์รองรับเกี่ยวกับฤทธิ์ของสารแมงจิเฟอรินในการลดการเกิดพังผืดในเซลล์ท่อไตส่วนต้นของคน

9. ระเบียบวิธีวิจัย

เป็นการศึกษาในเซลล์เยื่อบุท่อไตส่วนต้นของคน โดยเริ่มจาก

9.1. การตรวจสอบการแสดงออกของโปรตีนขนส่ง (Transporter) ที่สำคัญ เช่น โปรตีนขนส่งน้ำตาล โปรตีนขนส่งสารอินทรีย์ประจุลบ (OATs) และบวก (OCT2) ทั้งในรูปแบบการเลี้ยงเซลล์สองมิติทั่วไปและแบบสามมิติที่มีการไหลของของเหลวผ่านท่อไตจำลอง

9.2. ประเมินความเป็นพิษของสารแมงจิเฟอรินโดยใช้ MTT assay

9.3. ตรวจสอบการทำงานของโปรตีนขนส่ง และอันตรกิริยาของสารแมงจิเฟอรินกับ OAT1, OAT3 และ OCT2 transporters

9.4. ตรวจสอบฤทธิ์การลดการดูดซึมน้ำตาลของสารแมงจิเฟอรินในเซลล์

9.5. ตรวจสอบการเปลี่ยนแปลงการแสดงออกของ glucose transporters ในเซลล์เยื่อบุท่อไตส่วนต้นในภาวะน้ำตาลสูง โดย Quantitative RT-PCR

9.6. ตรวจสอบการเปลี่ยนแปลงการแสดงออกของ injury inflammatory และ profibrotic markers. ในเซลล์ที่อยู่ในภาวะน้ำตาลสูงหรือภาวะเลียนแบบเบาหวาน

9.7. การสร้างโมเดลเลียนแบบภาวะเบาหวานโดยการเหนี่ยวนำให้เกิดพังผืดในเซลล์เยื่อบุท่อไตส่วนต้นโดยการให้ TGF-□1 และตรวจสอบความสามารถและกลไกของสารแมงจิเฟอรินในการลดการพังผืดในเซลล์เยื่อบุท่อไตส่วนต้น

10. สรุปผลการวิจัยที่ได้

10.1. การตรวจสอบการแสดงออกของโปรตีนขนส่ง พบว่ามีโปรตีนขนส่งสารอินทรีย์ประจุบวกชนิดที่ 2 (OCT2) แสดงออกที่เยื่อหุ้มเซลล์ของเซลล์เยื่อบุท่อไตส่วนต้น และสามารถทำหน้าที่ขนส่งสารประจุบวก ASP^+ ได้ แต่ไม่พบการแสดงออกของโปรตีนขนส่งน้ำตาล (SGLTs, GLUTs) หรือสารอินทรีย์ประจุลบ (OATs) ที่เยื่อหุ้มเซลล์ พบเพียงในรูปแบบ mRNA เท่านั้น การเลี้ยงเซลล์แบบสามมิติที่มีการไหลของสารน้ำในท่อไตจำลองไม่ได้เพิ่มการแสดงออกของ SGLTs หรือ OATs ที่เยื่อหุ้มเซลล์ ยังคงพบเพียง OCT2 เช่นเดียวกับการเลี้ยงเซลล์แบบสองมิติเท่านั้น

10.2. การตรวจสอบความเป็นพิษของสารแมงจิเฟอรินที่ความเข้มข้นต่างๆ พบว่าในการที่เซลล์เยื่อบุท่อไตส่วนต้นได้รับสารแมงจิเฟอรินที่ความเข้มข้น 1-500 μM เป็นระยะเวลา 1-5 วัน ไม่มีผลทำให้เซลล์ตาย แต่การให้สารดังกล่าวเป็นระยะเวลา 7 วัน ตรวจพบการตายของเซลล์ในกลุ่มที่ได้รับสารความเข้มข้นสูง 100-500 μM

อย่างมีนัยสำคัญทางสถิติ ดังนั้นในการใช้สารแมงจิเฟอรินที่ความเข้มข้นสูง สามารถใช้ได้ในระยะเวลาไม่เกิน 5 วัน ในการทดลองในเซลล์นี้

10.3. การตรวจสอบอันตรกิริยาของสารแมงจิเฟอรินต่อโปรตีนขนส่ง พบว่ามีการยับยั้งการขนส่งของโปรตีน OCT2 โดยมีค่า IC_{50} ที่สูงกว่า 1000 μM ส่วนการยับยั้งการขนส่งของ OAT1 และ OAT3 มีค่า IC_{50} ที่ 188.7 และ 62.67 μM ตามลำดับ จะเห็นได้ว่าอันตรกิริยากับโปรตีนขนส่ง OAT3 มีมากที่สุด ดังนั้นอาจต้องระวังในการใช้สารนี้ร่วมกับยาที่ขับออกทางไตผ่านโปรตีนขนส่ง OAT1 และ OAT3 ซึ่งอาจมีผลให้ยาขับออกจากร่างกายช้าลง

10.4. ในการเลี้ยงเซลล์ในภาวะที่มีน้ำตาลสูง ไม่มีผลต่อการเปลี่ยนแปลงระดับการแสดงออกของ mRNA ของ glucose transporters ในเซลล์เยื่อบุท่อไตส่วนต้นของคน (RPTEC)

10.5. การเหนี่ยวนำให้เกิดการบาดเจ็บของเซลล์เยื่อบุท่อไตส่วนต้น โดยให้เซลล์อยู่ในภาวะที่มีน้ำตาลสูงอย่างต่อเนื่องที่ความเข้มข้น 30 mM หรือให้ร่วมกับการมีอินซูลิน และไซโตไคน์สูงขึ้นเลียนแบบภาวะเบาหวานที่เกิดขึ้น พบว่าในการมีภาวะน้ำตาลสูงอย่างเดียว หรือร่วมกับการเลียนแบบภาวะเบาหวาน สามารถพบการหลั่งของไซโตไคน์ที่เหนี่ยวนำการอักเสบ IL-1□ และ IL6 สูงขึ้น มีการเพิ่มการหลั่งของ TGF-□1 ซึ่งเป็นสารที่เหนี่ยวนำให้เกิดพังผืดที่ไตได้ และตรวจพบการเพิ่มขึ้นของ mRNA ในกลุ่มที่แสดงถึงการเกิดการบาดเจ็บที่ไต ได้แก่ KIM-1 นอกจากนี้ยังพบการเพิ่มขึ้นของการแสดงออกของ mRNA ที่นำไปสู่การสะสมของ collagen I, fibronectin และ alpha-smooth muscle actin (α-SMA) อย่างไรก็ตามในภาวะเลียนแบบเบาหวานนี้ไม่ได้เหนี่ยวนำให้เกิดเซลล์ตายหรือการเกิดการหลั่ง LDH ที่สูงขึ้นในกลุ่มเซลล์ที่เลี้ยงแบบสามมิติ

10.5. สารแมงจิเฟอรินความเข้มข้น 40 μM สามารถลดการหลั่งไซโตไคน์ที่เหนี่ยวนำการอักเสบ IL-6 ในเซลล์ท่อไตส่วนต้นที่อยู่ในสภาวะน้ำตาลสูงหรือเลียนแบบโรคเบาหวานได้

10.6. ในภาวะเหนี่ยวนำให้เกิด fibrosis ด้วย TGF-□1 พบว่ามีผลให้เกิดการเพิ่มขึ้นของ Fibronectin ซึ่งเป็นตัวบ่งชี้การแสดงออกของภาวะ fibrosis และพบว่ามีการแสดงออกของโปรตีน E-cadherin ซึ่งเป็น adhesion protein ลดลง การเปลี่ยนแปลงดังกล่าวสามารถถูกยับยั้งได้ด้วยการให้สารแมงจิเฟอรินความเข้มข้น 50-100 μM เป็นเวลา 3 วัน ก่อนการเหนี่ยวนำด้วย TGF-□1

11. การเผยแพร่ผลงานวิจัยสู่สาธารณะ

ส่วนหนึ่งของงานวิจัยนี้ ได้นำเสนอในรูปแบบโปสเตอร์ ในการประชุมวิชาการวิทยาศาสตร์การแพทย์ ครั้งที่ 30 ระหว่างวันที่ 22-24 มิถุนายน 2565 ณ โรงแรมริชมอนด์ นนทบุรี

บทคัดย่อ

ภาวะไตเสื่อมจากเบาหวานเป็นปัญหาทางสุขภาพที่มีทำให้เกิดไตวายได้ การป้องกันการดำเนินของโรคไปสู่ไตวายระยะสุดท้ายเป็นสิ่งจำเป็น แมงจิเฟอรินเป็นสารที่สกัดได้จากใบมะม่วง มีรายงานวิจัยพบว่าสารนี้มีฤทธิ์ต้านอนุมูลอิสระ ต้านการอักเสบ ลดระดับน้ำตาลในเลือด และลดการบาดเจ็บของไตในสัตว์ทดลองที่ถูกเหนี่ยวนำให้เป็นโรคเบาหวานได้ จากงานวิจัยที่ผ่านมาพบว่าการศึกษาฤทธิ์ของสารแมงจิเฟอรินจะทำการทดลองโดยใช้สัตว์ทดลองซึ่งยังมีข้อจำกัดในการนำข้อมูลมาใช้จริงกับมนุษย์ ดังนั้นในงานวิจัยนี้ได้ใช้เซลล์เยื่อบุท่อไตส่วนต้นของมนุษย์ (RPTEC) เพื่อทดสอบฤทธิ์ทางเภสัชวิทยาของแมงจิเฟอรินที่มีต่อการขนส่งกลูโคส และฤทธิ์ในการลดการเกิดพังผืด ผลการศึกษาพบว่าในสภาวะที่มีระดับน้ำตาลกลูโคสสูง และสภาวะเลียนแบบในผู้ป่วยเบาหวาน มีผลให้มีการเพิ่มการแสดงออก mRNA ของไซโตไคน์ที่เหนี่ยวนำให้เกิดการอักเสบ (IL-1□ และ IL-6), ตัวบ่งชี้การเกิดพังผืด (TGF-□1) และ การสะสมของสารที่บ่งชี้ว่าเริ่มมีการเปลี่ยนสภาววะจากเซลล์เยื่อบุเป็นพังผืด (collagen I, fibronectin และ α-SMA) ซึ่งเป็นกระบวนการที่เกิดภาวะไตเสื่อมจากโรคเบาหวาน โรคไตเรื้อรัง และนำไปสู่ภาวะไตวายระยะสุดท้าย ในการศึกษานี้ยังพบว่าแมงจิเฟอรินที่ความเข้มข้น 40 ไมโครโมลาร์ สามารถลดการหลั่ง IL-6 จากเซลล์เยื่อบุผิวที่ท่อไตส่วนต้นในสภาวะที่ถูกเหนี่ยวนำให้เป็นเบาหวานได้ ต่อมาผู้วิจัยได้ใช้ recombinant TGF-□1 เพื่อเหนี่ยวนำให้เกิดพังผืด เป็นการสร้างโมเดลของโรค พบว่าแมงจิเฟอรินที่ความเข้มข้น 50-100 ไมโครโมลาร์ ยับยั้งการลดลงของโปรตีน e-cadherin และการเพิ่มขึ้นของโปรตีน fibronectin ที่เกิดจากการเหนี่ยวนำให้เกิดพังผืดในเซลล์เยื่อบุท่อไตส่วนต้นของคนได้ ซึ่งฤทธิ์ของแมงจิเฟอรินในการป้องกันการเกิดพังผืดนี้ผ่านทางการยับยั้งการทำงานของ Smad3 ในการศึกษาอันตรกิริยากับตัวขนส่งยา พบว่าแมงจิเฟอรินสามารถยับยั้งการขนส่งสาร ASP^+ผ่านทางตัวขนส่งสารอินทรีย์ประจุบวก OCT2 ในเซลล์ RPTEC ได้ที่ค่า IC_{50} สูงกว่า 1000 ไมโครโมลาร์ ส่วนการศึกษาอันตรกิริยากับตัวขนส่งสารอินทรีย์ประจุลบ OAT1 และ OAT3 ได้ทดสอบในเซลล์ท่อไตส่วนปลายที่เพิ่มการแสดงออกของโปรตีน OAT1 และ OAT3 (MDCK-OAT1, MDCK-OAT3) แทนการใช้ RPTEC เนื่องจากไม่พบว่ามี OAT1 และ OAT3 แสดงออกที่เยื่อหุ้มเซลล์ RPTEC ค่า IC_{50} ของแมงจิเฟอรินต่อ OAT1 และ OAT3 มีค่า188.7 และ 62.87 ไมโครโมลาร์ ตามลำดับ สำหรับการศึกษาอันตรกิริยาของแมงจิเฟอรินต่อการขนส่งกลูโคสนั้น ไม่พบว่าแมงจิเฟอรินมีผลยับยั้งการขนส่งกลูโคสในเซลล์เยื่อบุท่อไตนี้ อย่างไรก็ตามการศึกษาวิจัยต่อไปควรใช้เซลล์ที่มีการแสดงออกของตัวขนส่งกลูโคส GLUTs และ SGLTs ถ้ามีเซลล์ที่มีคุณสมบัติดังกล่าวในอนาคต

จากฤทธิ์ของแมงจิเฟอรินที่พบในการศึกษานี้ต่อการยับยั้งการอักเสบและการเกิดพังผืดในเซลล์เยื่อบุท่อไตส่วนต้นของคนอาจจะเป็นประโยชน์ในการนำไปพัฒนาเป็นยาเพื่อป้องกันหรือชะลอการดำเนินโรคไตจากเบาหวานไปสู่ไตวายระยะสุดท้ายต่อไปได้

คำสำคัญ: แมงจิเฟอริน, สารสกัดจากมะม่วง, เบาหวาน, การเกิดพังผืดที่ไต, เซลล์เยื่อบุท่อไตส่วนต้น, โรคไตเรื้อรัง

Abstract

Diabetic nephropathy is one of the health problems that increased the incidence of renal failure. The prevention of the progression to end-stage renal failure is needed. Mangiferin, a constituent found in mango leaves, has been reported for anti-oxidant and anti-inflammatory activities, reduced blood glucose and renal injury in diabetic animal models. Most of research studies of mangiferin activities were performed in animal models. The extrapolation of information from animal to human are still limited. In the present study, we aim to study the pharmacological effect of mangiferin on glucose uptake and its activity on the reduction of renal fibrosis in human renal proximal tubule epithelial cells (RPTEC). The results showed that high glucose and diabetic mimicking conditions induced the mRNA expression of the inflammatory cytokines IL-1□ and IL-6, profibrotic marker TGF-□1 and epithelial to mesenchymal transition (EMT) markers collagen I, fibronectin and α-smooth muscle actin (α-SMA) which are the alterations similar to those found in patients with diabetic nephropathy progression to chronic kidney disease and end-stage renal failure. Mangiferin at 40 μM reduced the inflammatory marker IL-6 release in diabetic-induced human renal proximal tubule epithelial cells. We used recombinant human TGF-□1 to induce fibrosis in RPTEC cells in order to obtain the disease model. We found that mangiferin at concentrations of 50-100 μM inhibited the reduction of e-cadherin expression and the elevated expression of fibronectin observed in the TGF-□1-induced fibrosis model of human RPTEC cells. The anti-fibrotic activity of mangiferin was demonstrated via the inhibition of TGF-□1-induced Smad3 activation. For the interaction of mangiferin with a renal organic cation transporter OCT2 in RPTEC cells using the uptake of a fluorescent OCT substrate, ASP^+, the inhibition of OCT2 was detected with an IC_{50} value higher than 1000 μM. Since no plasma membrane expression of organic anion transporter proteins, OAT1 and OAT3 in this RPTEC cell, we tested the interaction of mangiferin in OAT1 and OAT3 over-expressed MDCK cells instead of RPTEC cells. The IC_{50} values of mangiferin on OAT1 and OAT3 were 188.7 and 62.87 μM, respectively. The glucose transport in the RPTEC cells was not inhibited by mangiferin in this study. However, further experiments should be performed in the human kidney cell that expresses glucose transporters, GLUTs and SGLTs in order to finalize the direct action of mangiferin on glucose reabsorption in kidney if the cells with the mentioned properties are available in the future.

The information about anti-inflammatory and anti-fibrotic activities of mangiferin obtained from this study may be useful for further drug development to reduce the progression of diabetic nephropathy to end-stage renal failure.

Keywords: mangiferin, mango extract, diabetes mellitus, renal fibrosis, renal proximal tubule, chronic kidney disease

Introduction

The rapid increasing global prevalence of diabetes mellitus has been reported. There was about 285 million people worldwide diagnosed diabetes in 2010 and was estimated to be 439 million persons by 2030 [1]. Diabetic patients usually develop to diabetic nephropathy (DN), which is diagnosed by the presence of abnormal quantity of albumin excreted in urine, glomerular lesions and reduced glomerular filtration rate (GFR) [2], within 10-15 years, in both type 1 (T1DM; approximately 30%) and type 2 (T2DM; approximately 40%) diabetes mellitus. DN is the most common cause of the increased morbidity and mortality in diabetic patients and about 20-40% of them may develop to end-stage renal disease (ESRD) which required treatment by hemodialysis or renal transplantation, causing increased expense for health care and disturbing quality of life [2]. In addition, the mortality rate of patient on dialysis was increasing every year and transplantation was limited by the shortage of donated kidney and matching with recipient. Hyperglycemia is a risk factor of chronic complication in diabetes mellitus. It is associated with the enhanced reactive oxygen species (ROS) production, induction of oxidative stress and inflammatory mediator such as NF-□B which is involved in progression of renal failure [3]. Therefore, specific and effective prevention or therapy of DM and diabetic kidney disease is really needed to delay the progression of DN to renal fibrosis and ESRD.

Diabetes mellitus (DM) is one of the metabolic disorders fundamentally characterized by elevated level of blood glucose and insufficient of endogenous insulin secretion or action. Hyperglycemia is known to be a pathogenic factor of chronic complication in DM, generating reactive oxygen species (ROS), known as oxidative stress which is being mediator of kidney injury [3]. DM has been known to be a major cause of end-stage renal disease (ESRD). Diabetic nephropathy (DN) has been characterized by glomerular hypertrophy and hyperfiltration, increased urinary albumin secretion, thickness of glomerular basement membrane and expansion of mesangial cells. Diabetic glomerulopathy is found in early stages of DN while tubulointerstitial fibrosis occurs in the advanced stage of the disease [4-5]. *In vitro* study demonstrated that exposure of renal epithelial cells to high glucose concentration, the cells could produce type I and type IV collagen leading to fibrosis [6]. Therefore, this method can be used as model for developing early fibrosis of renal tubule epithelial cells. TGF-β 1 has been reported as mediator for making *in vitro* model of fibrosis in various cell culture types [7].

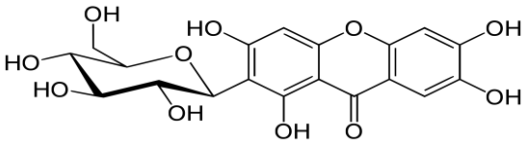
Natural compounds are in trend for drug discovery since they have various pharmacological activities and believed that they might have benefits for prevention or health

promoting when use them as functional foods or nutraceuticals. Mangiferin, a constituent extracted from leaves or stem bark of mango (*Mangifera Indica*), has been reported to possess numerous potential effects to protect kidney and delay progression of renal failure as observed in the studies with diabetic animals [8-9]. These studies demonstrated its anti-oxidative and anti-diabetic including restoration from diabetic nephropathy. The mechanisms underline its effects on DN usually performed in rat kidney and focused on glomeruli, podocytes and mesangial cells, but lack of data on proximal tubule, which is important part of kidney to involve in renal function alteration and ESRD progression [10]. Translation information from rodents to human sometimes had limitation because of different species and physiological roles of some proteins, especially in kidney. To study protective effect of mangiferin and its safety for human kidney, it should include the studies in diabetic model of human renal proximal tubular epithelial cells. Proximal tubule is the major site of nephron for reabsorption and secretion of endogenous compounds such as glucose, amino acids, uric acid and exogenous compounds for example xenobiotic drugs. In the past, human renal proximal tubular cell lines could not represent the real characteristics of renal proximal tubule, its expression of various enzyme and endogenous proteins were limited. The culturing method and type of renal proximal tubule cell line have influenced the expression of protein [11-14]. Therefore, in this project I used newly developed cell line with adjustment of culturing method and try to establish disease model in more physiological microfluidic platform, proximal tubule–on-a-chip [15-16].

This project is aimed to investigate potential effect of mangiferin, which is an important bioactive compound found in mango leaves, on the reduction of glucose reabsorption in renal proximal tubular epithelial cells and to provide scientific evidence to support the protective effect of this compound on fibrosis of renal proximal tubular epithelial cell. We hypothesized that mangiferin exert significant protective activities on renal function under hyperglycemic condition by inhibiting renal glucose reabsorption and inhibiting inflammatory mediator which can delay progression of renal fibrosis.

Literature Review

Mangiferin



Mangiferin, MW = 422.34

Figure 1. Structure of Mangiferin

Mangiferin is an important constituent extracted from leaves or stem bark of Mango (*Mangifera Indica*). Many research studies reported numerous pharmacological benefits of mangiferin for example anti-inflammatory, anti-oxidative, anti-aging, anti-tumor, antibacterial, antiviral, anti-diabetic, hepatoprotective, neuroprotective, cardiovascular protective and analgesic effects [17, 18]. The pharmacokinetic study of mangiferin in human showed that its absorption after oral administration was not good. Mangiferin level in plasma after oral administration of 0.9 grams was detected about 38.64±6.75ng/mL at 1 hour and the elimination half-life was 7.85±1.72h [19]. Mangiferin was distributed to various organs and it was the highest detection in heart after 1.5 hours of oral administration (30 mg/kg) in Wistar rats. It was also detected in liver and kidney but the heist dection was in small intestine after 3 hours of oral administration [20].

There were only 2 studies about mangiferin reported in Thailand. Yoosook *et al.* (2000) showed that water extract of mango leaves could obtain mangiferin and had anti-HSV1 and anti-HSV2 activities [21]. From report of TRF grant #RDG5220049, Chancharunee S and coworkers claimed that the ethanol extraction and purification processes from mango dried leaves gave a yield of mangiferin 6.89%. Since the limited solubility of mangiferin in water and organic solvents affecting its addition into cosmetics or food development, they tried to modify mangiferin's structure to improve its solubility by 3 methods, (1) metal salt formation, (2) esterification of hydroxyl group on sugar ring, and (3) conversion of hydroxyl group on xanthone ring to alkoxy and acyl derivatives. All new 14 derivatives have better solubility than mangiferin and some of them still showed anti-herpes simplex activity [22].

Many studies about anti-diabetic effect of mangiferin had been reported. Most of studies were performed in STZ-induced diabetic rats (Type 1 DM), some had been done with type 2 DM model rats. The effect of mangiferin on improving blood glucose level in diabetic rats or mice has been controversial [8-9, 23-25], however the recovery of renal function had been

observed. It attenuated diabetic nephropathy by inhibiting oxidative stress [9]. Mangiferin also improved glomerulosclerosis and podocyte function in diabetic rats [25]. Rat with diabetic nephropathy had a decrease in nephrin, a podocyte marker protein, expression level and was restored with long term oral gavage treatment (12 weeks) of 12.5-50 mg/kg mangiferin. It protected podocytes via enhancing autophagy, up-regulation of AMPK phosphorylation, down-regulation of mTOR phosphorylation [25] and osteopontin, a pro-fibrotic adhesion molecule which increased in diabetic kidney and involved in glomerular damage [26]. Those studies concerned the alteration in glomeruli but lack of information about renal proximal tubule which is an important part of nephron having roles for reabsorption and excretion of various endogenous and exogenous compounds and involves in the renal function alteration in both normal and diabetic nephropathy including progression of renal fibrosis. Therefore, how mangiferin acts on renal proximal tubule should be explored to provide scientific information about protective effect of mangiferin on renal proximal tubule in order to finally develop its benefit to be new nutraceuticals.

Renal proximal tubule

The kidney is an important organ for homeostasis of our body**.** It does not only regulate blood pH and volume homeostasis, but it also functions for nutrient re**-**absorption and the excretion of waste products and xenobiotics**.** It has been known that renal proximal tubule is the major site of nephron for reabsorption and secretion of endogenous compounds such as glucose, amino acids, uric acid and exogenous compounds for example xenobiotic drugs. In the past, human renal proximal tubular cell lines could not represent the real characteristics of renal proximal tubule, its expression of various enzymes and endogenous proteins were limited. The culturing method and type of renal proximal tubule cell line have influence to the expression of protein [11-13, 27]. The idea of new culturing platform of renal proximal tubule has been created and are developing to obtain more physiological imitation and hopefully to obtain an appropriate testing platform for renal toxicity that can reduce animal usage and extrapolate to human. In 2013, Jang and co-workers [28] had developed kidney tubule-on-a-chip as 3D platform for drug toxicity testing and after that the microfluidic platforms of renal proximal tubule were developed and use to study more physiological renal function and renal toxicity [15-16, 29]. Weber et al. [16] and Vriend et al. [30] showed that microfluidic flow had effects on cell maturation and cilia development of the proximal epithelial cells. The flow rate increased the length of cilia but not the number of cilia on the cells [30]. The use of proximal tubule-on-a-chip might help to obtain more physiological condition of testing platform for *in vitro* drug development.

Proximal tubule injury in diabetic condition

Hyperglycemia activates renal mechanisms, including tubular growth and sodium-glucose cotransporters, SGLT2 and SGLT1 activities to increase glucose reabsorption in the proximal tubule. These modifications also result in the reabsorption of sodium. Then, the glomerular filtration rate (GFR) is increased to restore sodium excretion. Moreover, tubular growth, hyper-reabsorption, and glomerular hyperfiltration are an important feature that can develop DN. The higher GFR enhances oxygen requirements, inflammation, and tubulointerstitial fibrosis. The molecular signaling of tubular growth in diabetes is associated with the 'salt paradox' of the diabetic kidney, whereby renal vasodilation unexpectedly occurs in response to a low dietary NaCl intake. Proinflammatory and profibrotic factors also release from tubular growth in diabetes condition and can promote kidney failure [31]. Furthermore, diabetic conditions can promote proximal tubule injury leading to podocyte detachment and causing albuminuria. The expression of sirtuin-1 (Sirt1) in the proximal tubular epithelial cell was decreased, leading to the reduction of nicotinamide mononucleotide (NMN) concentrations that promote Claudin-1 expression in podocytes, which causes podocyte detachment and increases urinary albumin [32-33].

Mechanisms of proximal tubule fibrosis in DN

In DN, multiple cell types are involved in interstitial fibrosis and responsible for extracellular matrix (ECM) accumulation [34]. Activation of myofibroblasts is mainly promoted the renal fibrogenesis by the synthesis of matrix proteins, such as collagen types I, III, and IV and fibronectin. The activation of myofibroblasts for tubulointerstitial fibrosis is stimulated by diabetes factors, which are myofibroblasts progenitors, including interstitial fibroblasts, bone marrow-derived fibrocytes, endothelial cells, vascular pericytes and tubular cells. It has been contributed to the activation of myofibroblasts via different processes or mechanisms such as proliferation, recruitment, endothelial-to-mesenchymal transition (EndMT), epithelial-to-mesenchymal transition (EMT), and differentiation [35].

For the proximal tubule fibrosis in DN, the early phenotype usually related to hyperglycemic condition-induces growth factors such as insulin-like growth factor 1 (IGF-1), phosphoinositide 3-kinase (PI3K) pathway. An initial phase of cell proliferation and an early transition to hypertrophy are also associated with G1 cell cycle arrest via TGF-β1-induced p21, the cyclin dependent kinase (CDK) inhibitor. The molecular pathways in the tubule involved the activation of AGE and Ang II, the activation of protein kinase C β1 (PKCβ1) and Janus kinase/signal transducers (JAK/STAT), which is the activator molecules of TGF-β1 transcription, leading to the increase of proinflammatory and profibrotic factors and resulting in the inflammation,

tubulointerstitial fibrosis, and renal failure [36]. An important cellular mechanism of renal fibrosis is the stimulation of myofibroblasts, which results in ECM components and inflammatory cell infiltration [37]. Several cellular mechanisms important to renal fibrosis have been identified. Some anti-fibrotic agents have shown better promise in slowing and preventing fibrosis. However, the translation of this knowledge to effective anti-fibrotic therapies in humans has been limited due to a lack of sufficient effects, off-target effects, or both [38].

Pathological changes in DN lead to irreversible loss of tissue and kidney functions and eventually end-stage renal failure that requires renal replacement therapy. Furthermore, the adverse outcomes related to cardiovascular complications have also been reported [39]. Therefore, treatment strategies for DN are aimed at preventing and delaying the destructive effects and progression of ESRD. At the key of the current treatment of chronic kidney disease (CKD) is blocking renin-angiotensin-aldosterone system (RAAS) by angiotensin-converting enzyme (ACE) inhibitors and angiotensin receptor blocker (ARBs), which have a renal protective effect, significantly slowing the progression of CKD [40-41]. However, the incidence of CKD continues to increase worldwide and eventually the number of CKD patients developing ESRD remains high. Therefore, it is imperative to find novel effective drugs. The fibrogenic pathways has been an interesting therapeutic target for inhibiting or slowing the loss of renal function in patients with DN. A growing number of studies show that TGF-β1 plays an important role in the pathogenesis of renal fibrosis associated with progressive kidney diseases [42]. The new compounds, for example, fresolimumab could neutralize the activity of TGF-β1 [8], pirfenidone, a small synthetic molecule, had shown the anti-fibrotic effect in preclinical study by blocking the TGF-β1 promotor [43]. Molecular targets other than blockade of the RAAS by ACE inhibitors, ARBs and the inhibition of SGLT2 function, such as connective tissue growth factor (CTGF) inhibitor, and BMP-7 agonists are very promising targets for developing anti-diabetic drugs. Inhibition of CTGF, a profibrotic factor acting downstream of TGF-β1, can prevent the ECM accumulation in kidney disease models [44]. BMP-7, a natural antagonist of TGF-β1, had a potent renal protective effect by suppression of renal fibrosis *in vivo* models [45]. Although great progress has been made in many preclinical studies on the mechanisms and therapeutic targets of renal fibrosis, there are still no agents beyond ACE-Is and ARBs have been approved to treat renal fibrosis in clinical practice. Therefore, the finding a novel effective anti-fibrotic therapy in patients with DN to prevent or slow the progression of ESRD.

As mentioned above, mangiferin demonstrated anti-hyperglycemic, anti-oxidant and anti-inflammatory activities. Mangiferin can be detected in various organs after oral administration

[20]. It's possible that mangiferin may enter renal proximal tubular cells via some xenobiotic transporters, i.e., OATs or OCTs, before secretion into tubular lumen and mangiferin may also reduce glucose uptake via SGLT2 transporter at apical side of renal proximal tubule. This project also aimed to provides scientific evidence to support how mangiferin protects renal proximal tubular epithelial cell injury and fibrosis. We hypothesized that mangiferin exerts significant protective activities on hyperglycemic induced renal injury by inhibiting inflammatory mediator which can delay progression of renal tubule injury and fibrosis. Therefore, in this project we used renal proximal tubular epithelial cells (RPTEC) with diabetic model by adjustment of culturing method and also tried to establish the fibrosis model both in 2D platform and in more physiological microfluidic model, a proximal tubule–on-a-chip [15-16, 28-30] with hope to obtain the model that close to physiological function in human kidney.

Methods

1. Study the characteristics of human renal proximal tubular epithelial cell lines (RPTEC)

- Renal proximal tubular epithelial cell culture

Before started the experiment in this project, we firstly characterized 2 types of RPTEC cells (SA7K clone and RPTEC/TERT1) that used recently in many publications. Human renal proximal tubular epithelial cells (RPTEC control cells SA7K clone) purchased from Sigma-Aldrich (St. Louis, MO, USA) were cultured according to the manufacturer's instruction in MEMα supplemented with RPTEC complete supplement (Sigma-Aldrich, St. Louis, MO, USA), 2.5 mM L-glutamine, 30 mg/ml gentamicin and 13 ng/ml amphotericin B. Human renal proximal tubular epithelial cells (RPTEC/TERT1) were from Evercytes, Inc. (San Diego, CA, USA). The cells were grown in DMEM/F-12 supplemented with 10 ng/ml hEGF, 3.5 µg/ml L-ascorbic acid, 25 ng/ml hydrocortisone, 1x ITS (Sigma, St. Louis, MO, USA) and 100 µg/ml G418. Both types of RPTEC cells were maintained culturing in humidified incubator with 5% CO_2 at 37 C. The cells were grown and sub-cultured once they reached 85-90% confluency. Sub-culture was done using 0.25% trypsin-EDTA and spit cells about 1:5. Culture medium was changed every 2-3 days.

- 3D culture of renal proximal tubule-on-a-chip

Cultrex rat collagen I (R&D systems, Minneapolis, MN, USA) was used as extracellular matrix (ECM) for 3D platform. 4 mg/ml collagen I was load into gel loading inlet to prepare ECM for RPTEC/TERT1 cells and kept at 37 C for more than 3 hours until the gel became solid. Then RPTEC/TERT1 cells at the density of 20,000 cells in 2 µl medium were loaded to the cell inlet channel of the 3D platforms. After cells attached to collagen gel about 3 hours after seeding, culture medium was added into the cell inlet and outlet parts of 3D-platform. The flow inside the tube is controlled by the movement of specific stage on rocker. The cells on chip were incubated in 37 C humidified CO_2 incubator. About 7-9 days the tube of cells was formed. The culture medium was changed every 2 days. When the cells on tube are completely confluence, this 3D-platform can be used for testing in each experiment.

- Transporter expression in renal proximal tubular epithelial cell lines

RPTEC-SA7K and RPTEC/TERT1 were seeding into 6-well plate and continued culturing in humidified incubator with 5% CO_2 at 37 C for 7 days. The cells were washed with PBS and

harvested into 1.5-ml tubes and lysed with lysis buffer. Total RNA from each cell type was extracted according to the protocol of total RNA extraction and purification kit (New England BioLabs Inc., Ipswich, MA, USA). 1 µg of total RNA was used to synthesized cDNA using iScript cDNA synthesis kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Classical PCR was performed to detect the expression of transporters (OAT1, OAT3, OCT2, SGLT1, SGLT2, p-gp and MRP4) expression of GAPDH was used as internal control. The primer sequences are shown in Table 1.

Table 1. Primer sequences for polymerase chain reaction of transporter genes

Transporter	Forward primer (5'->3')	Reverse primer (5'->3')
OAT1	GGCTTCCTTGTCATCAACTCCC	CACAGCAAGAGAGGTTCGGACA
OAT3	CAACAGCACCAAGGACTCCATTG	CTGTCAGACAGGTCTCCAAGCA
OCT2	GAGATAGTCTGCCTGGTCAATGC	GTAGACCAGGAATGGCGTGATG
SGLT1	CGCCTATCCAACCTTAGTGGTG	CGCTGTTGAAGATGGAGGTCAG
SGLT2	GCTGGAACATCTATGCCTCCGT	TGACGAAGGTCTGTACCGTGTC
p-gp	GCTGTCAAGGAAGCCAATGCCT	TGCAATGGCGATCCTCTGCTTC
MRP4	CTGTTGGAGGATGGTGATCTGAC	CTGCTAACTTCCGCATCTACTGC
GAPDH	GTCTCCTCTGACTTCAACAGCG	ACCACCCTGTTGCTGTAGCCAA

- **Immunostaining of transporter proteins in classical 2D culture, transwell and 3D-platform of renal proximal tubule**

RPTEC cells cultured in 2D, 12-well transwell 0.4 µm PET insert and 3D-platforms were fixed with 4% paraformaldehyde for 15 min at room temperature. After washing with phosphate-buffered saline (PBS), the cells were permeabilized with 0.3% triton X-100 for 15 min and blocked with 2% BSA, 0.3% triton X-100 for 30 min at room temperature. The fixed cells were incubated with primary antibody at 4 C overnight. After washing with PBS, the cells were incubated with secondary antibody (Alexa 488) plus DAPI or Hoechst 33342 for 1 hour. The images were visualized under fluorescence microscope or confocal laser scanning microscope (Zeiss, Germany).

2. Evaluating the appropriate non-toxic concentrations of mangiferin

- **Cell viability assay**

The concentration of mangiferin that will be used for other studies should be the non-toxic concentrations. Therefore, MTT assay was used to screen effect of various concentrations of mangiferin on cell viability.

MTT assay is a colorimetric assay for detection metabolic activity of the cells, the mitochondria enzyme activity of NADPH-oxidoreductase can reflect number of viable cells. This enzyme can convert tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to its insoluble formazan, which has a purple color. This assay can also be used to measure cytotoxicity (loss of viable cells) or cytostatic activity (shift from proliferation to quiescence) of potential pharmacological activity compound and toxic compound. MTT assays are usually performed in the dark because of light sensitive reagent.

RPTEC cells were seeded into 96-well plate with a density of 10,000 cells/well. The cells were incubated with various concentrations of mangiferin (1, 3, 10, 30, 100, 300 and 500 μM) for 1, 3, 5 and 7 days. At the end of treatment period, the cells were washed once with PBS and replaced with 0.1 ml of fresh MTT reagent (0.4 mg/ml)-supplemented medium and incubated at 37 C for 3 h, then the reagent-supplemented medium was aspirated and 0.1 ml DMSO was added to each well. The plate was covered with foil and shaken on plate shaker for 10 min. The absorbance at OD 570 nm were determined using microplate reader. The absorbance of each well was subtracted with background control (the average absorbance of 3 wells that have no cells but were incubated with MTT reagent-supplemented medium). The cell viability was calculated and expressed as percentage of control treatment (solvent treatment without mangiferin) in which the control is 100%.

3. Transport function assay and the interaction of mangiferin with transporters

Human OAT1 and OAT3 transfected MDCK (MDCK-OAT1 and MDCK-OAT3) and RPTEC cells were cultured in 48-well tissue culture plate at a density of 3×10^4 cells/well at 37°C for 2 days. On the day of transport function assay, the cells were washed 2 times with pre-warmed D-PBS (2.7 mM KCl, 1.5 mM KH_2PO_4, 136.9 mM NaCl, 8.9 mM Na_2HPO_4, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 5 mM D-glucose) and pre-equilibrate for 15 min. In case of glucose transport assay, glucose was omitted from pre-warmed D-PBS during 15-min incubation. The cells were incubated with D-PBS containing a substrate with or without inhibitor at 37°C for 10 min (for OAT1, OAT3 and OCT2) or 40 min (for GLUTs/SGLTs). At the end of incubation periods, the cells were washed 3 times with ice-cold D-PBS to stop transport assay. The cells were measured fluorescent intensity at Ex/Em 485/535 nm using microplate reader (Synergy Neo2, Biotek, VT, USA). For ^{3}H-glucose

transport, the cells were lysed with 0.1N NaOH and counted radioactivity with beta counter (Microbeta-2, PerkinElmer, USA). Substrates used for OATs are fluorescein, and 6-carboxyfluorescein (6-CF). The fluorescent dye 4-Di-1-ASP (4-(4-(Dimethylamino) styryl) -1-Methylpyridinium Iodide, ASP^+) is a substrate for OCT2 and ^{3}H-D-glucose (PerkinElmer, Boston, MA, USA) is GLUTs/SGLTs' substrate. All fluorescent substrates used are from Sigma (St. Louis, MO, USA). The positive control of inhibitors used in this study are probenecid for OAT1, estrone-3-sulfate for OAT3, tetrapentylammonium (TPeA) for OCT2 and phloretin for GLUTs and phlorizin and dapagliflozin for SGLTs and SGLT2, respectively. The IC_{50} values of mangiferin on OAT1, OAT3 and OCT2 were calculated from Graphpad prism software.

For the transporter function in 3D-platform, the ASP^+ substrate (10 μM) in D-PBS was loaded in to the basolateral side of the tubule and incubated for 20 min at 37 °C. The ASP^+ transport into the cells was observed under fluorescence microscope and determined fluorescent intensity with ImageJ (NIH. software).

4. The alteration of glucose transporters' expression in high glucose or diabetes mimicking conditions

RPTEC cells were cultured in 12-well plate. After 1-day of seeding in the culture plate, the cells were treated with high glucose condition (30 mM D-glucose into culture media) for 3, 7 and 14 days. To imitate insulin resistance condition in diabetes, condition media was used instead of high glucose medium. The compositions of condition medium were 30mM D-glucose, 2ng/ml TNF-α, 1ng/ml IL6 and 100nM Insulin. The mRNA expression level of glucose transporters (SGLT1, SGLT2 and GLUT2) were determined by real-time quantitative-PCR. Total RNA was isolated from the cell pellet using the Monarch Total RNA Miniprep Kit (New England BioLabs Inc., Ipswich, MA, USA) according to the manufacturer's protocols. RNA yields were measured by NanoDrop Spectrophotometers. The synthesis of double-stranded cDNA from total RNA was using iScript Reverse Transcription Supermix for RT-qPCR (Bio-rad, Hercules, CA, USA) according to the manufacturer's instruction. Real-Time qPCR was performed using the SensiFAST SYBR No-ROX Kit (Meridian Bioscience, Memphis, TN, USA). The expression level was determined using a CFX96 Touch Real-Time PCR Detection System (Bio-rad, Hercules, CA, USA). The activation was performed with 95°C for 2 min and then 40 cycles of denaturation at 95°C for 5 seconds and annealing/extension at 60°C for 30 seconds. Quantitative analysis was performed using the comparative ΔΔCt method by detecting of interesting mRNA level and GAPDH as reference genes.

5. The alteration of kidney injury marker, proinflammatory and profibrotic markers in high glucose treated RPTEC cells

RPTEC cells treated with treated with high glucose condition (30 mM D-glucose into culture media) or condition media imitated DM condition for 3, 7 and 14 days, in some case 5 mM D-glucose plus 25 mM mannitol was used as osmotic control. To imitate insulin resistance condition in diabetes, condition media (30mM D-glucose, 2ng/ml TNF-α, and 100nM Insulin) was used instead of high glucose medium. IL-1, IL-6 and TGF β1 released into culture media were analyzed by ELISA assay (Thermo Fisher Scientific, Waltham, MA USA). The cells pellet was also used to extract total RNA and used for real-time qPCR. The primer sequences used for qPCR are shown in Table 2. The appropriate duration of high glucose or condition media treatment was also used to examine the cell injury in 3D-platform.

The treatment of mangiferin was given to the cells together with high glucose or condition media. The concentrations of mangiferin used in the experiment were indicated in the data.

Table 2. Primer sequences for quantitative polymerase chain reaction of genes

Gene name	Forward primer (5'->3')	Reverse primer (5'->3')
IL-1β	ACCTGTGTCTTTCCCGTGG	TCATCTCGGAGCCTGTAGTG
TGF-β1	TACCTGAACCCGTGTTGCTCTC	GTTGCTGAGGTATCGCCAGGAA
KIM-1	CTTCACCTCAGCCAGCAGAAAC	GCCATCTGAAGACTCTGTCACG
α-SMA	AATCCTGTGAAGCAGCTCCAG	TTACAGAGCCCAGAGCCATTG
Fibronectin	ACAACACCGAGGTGACTGAGAC	GGACACAACGATGCTTCCTGAG
Collagen 1A1	GATTCCCTGGACCTAAAGGTGC	AGCCTCTCCATCTTTGCCAGCA
GAPDH	GTCTCCTCTGACTTCAACAGCG	ACCACCCTGTTGCTGTAGCCAA

6. The induction of fibrogenesis using IL-1β and TGF-β1 and the anti-fibrotic activity test of mangiferin

Since the increased IL-1β and TGF-β1 have been found in diabetes-induced kidney injury and possible to induce the severe progression of renal fibrosis. The fibrosis model was set up in RPTEC cells.

RPTEC cells were seeded into 96-well plate at the density of 15,000 cells/well. One day after seeding the cells were treated with IL-1β or TGF-β1 at various concentrations for 1 or 2 days. In some cases, mangiferin at varying concentrations were pre-incubate for 2 days before TGF-β1 treatment, then it was also co-treated together with TGF-β1. The ALK5II inhibitor (CAS. 446859-33-2, Calbiochem, CA, USA), an inhibitor of TGF-β1 receptor type 1 kinase, has been used as a positive control for inhibition of fibrosis forming. At the end of treatment period, the cells in each well were fixed with 100 µl of 4% paraformaldehyde in PBS for 20 min at room temperature. After rinsing the cells with cold PBS twice, the cells were stained with 50 µl picro-sirius red (PSR, ab246832, abcam, MA, USA.) for 1h at room temperature to detect collagen deposition, a marker of fibrosis. The cells in each well were washed three times with 100 µl of 0.5% acetic acid to remove unbound dye and air-dried the plates in a fume hood overnight. Next day, the cells were photograph for a qualitative record under a microscope. After that, the PSR dye that bound to collagen was eluted with 50 µl of 0.5 N NaOH by mixing on an orbital shaker for 30 min and measured the absorbance at 540 nm by spectrophotometry.

For the treatment with mangiferin, the cells were pre-treated with varying concentration of mangiferin for 3 days before induced fibrosis with TGF-β1. Fibronectin and e-cadherin proteins, markers of fibrosis and normal cell adhesion, were detected by western blot analysis in the separate treatment of the cells in 6-well plate. The mechanism of anti-fibrotic activity of mangiferin was also studied via phospho-NF-κB and Smad3 and detected by immune-blotting.

7. Statistical analysis

The statistical difference was determined using Mann-Whitney test for the comparison between 2 groups. In case of more than 2 experimental groups, ANOVA with Tukey's multiple comparisons test was used to determine the statistical difference. Data were considered statistically significant when $P<0.05$.

Note: *Mangiferin used in this study was kindly provided by Dr. Uthai Wichai, Department of Chemistry, Faculty of Science, Naresuan University, Phitsanulok, Thailand.*

Results and Discussion

1. **Characteristics of renal proximal tubular epithelial cell lines**

The characteristic of human renal proximal tubular epithelial cell line (RPTEC-SA7K and RPTEC/TERT1) looks similar when start culturing at low density, the RPTEC-SA7K cells showed loosely contact each other and once they reached complete confluence the cell will change their appearance to long spindle shape whereas RPTEC/TERT1, when reach confluent, the cells were closely contact each other and tighter than SA7K cells. The appearance of these cells is shown in Figure 2.

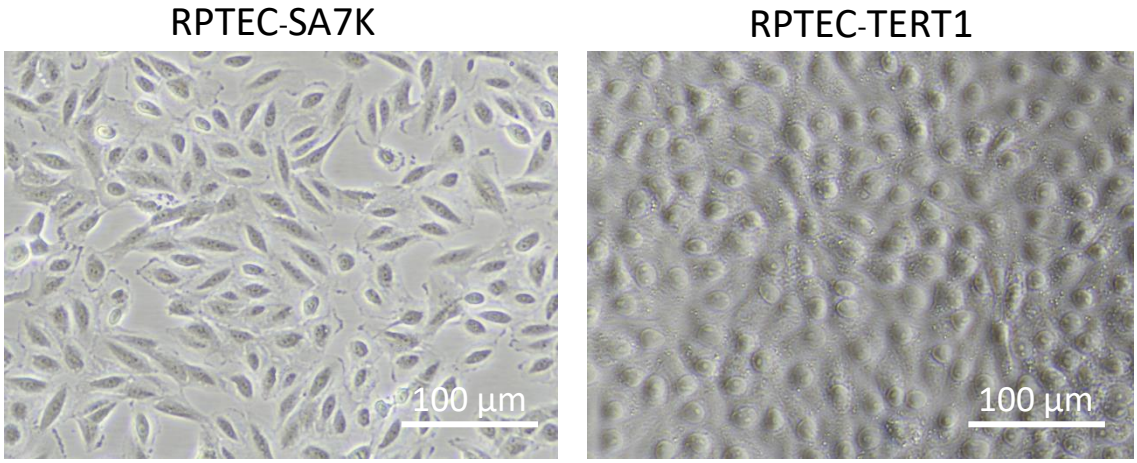


Figure 2. Renal proximal epithelial cell lines. Left, RPTEC-SA7K (from Sigma) and right, RPTEC/TERT1 (from Evercyte).

- **mRNA expression of transporters in renal proximal tubular epithelial cell lines**

Using classical PCR, mRNA expression of 2D-cultured RPTEC-SA7K and RPTEC/TERT1 cells showed dominant expression of two ABC-transporters, P-gp and MRP4, whereas expression of SLC transporters, OAT1 was undetectable in both cell types within our PCR cycles tested. The expression of OAT3 was detected in RPTEC/TERT1 better than in SA7K. OCT2 was detected in both cell types and seemed that it was higher in RPTEC-SA7K cells. Very low expression of SGLT1 mRNA was seen in the PCR products. SGLT2 was moderately detected in both types of the cells, as shown in Figure 3. With the higher expression of OAT3 and SGLT2, RPTEC/TERT1 cells has been used in further experiments.

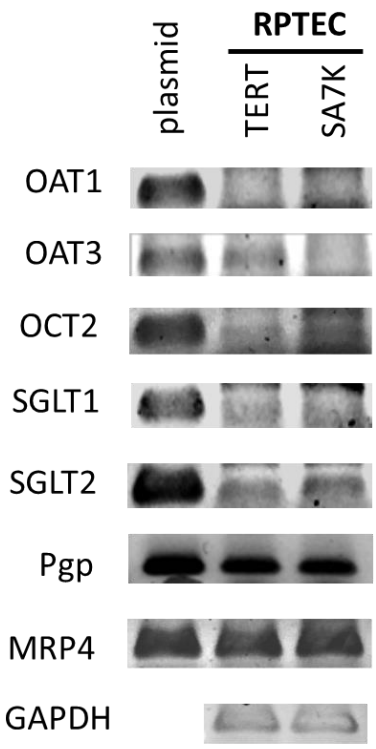


Figure 3. Image of RT-PCR of RPTEC/TERT1 and SA7K cells on 2% agarose gel electrophoresis. The bands of DNA from plasmid were used as control PCR product size.

- **Immunostaining of marker proteins in renal proximal tubular epithelial cells**

RPTEC cells were characterized using proximal tubule marker by fixing the cells on 96-well plate and staining with lotus tetragonolobus lectin (LTL). LTL has been used to identify renal proximal tubule because it can bind with fucose-containing oligosaccharide that express on brush border membrane of renal proximal tubule, not in other nephron segments. In the cultured epithelial cells, microvilli on the brush border were not well developed, however, the staining of lotus tetragonolobus lectin can be used to identify renal proximal epithelial cells [46-48]. RPTEC cells showed the fluorescent staining, it means that the RPTEC cells are from exact renal proximal tubular epithelial cells. The human colon T84 cells were used as negative control (Figure 4).

To confirm the normal characteristic of the cells, staining of sodium/potassium pump (Na^+/K^+ ATPase) and tight junction protein ZO-1 were investigated. The cells showed clear staining of sodium/potassium pump and ZO-1 at the plasma membrane as shown in Figure 5.

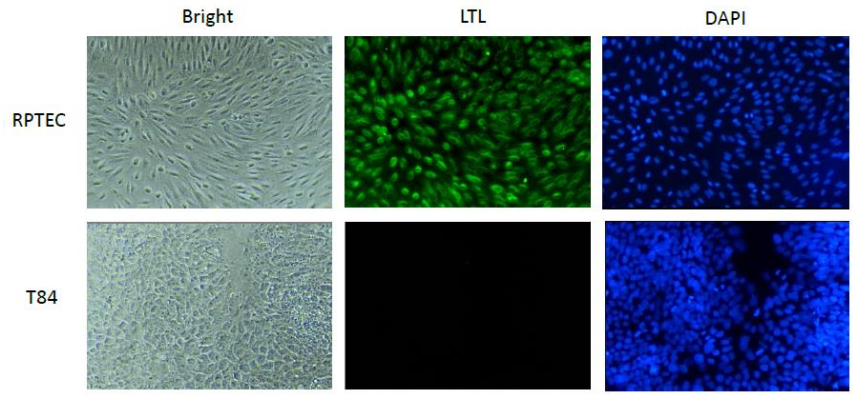


Figure 4. RPTEC cells staining with proximal tubule marker, lotus tetragonolobus lectin (LTL; green) in upper middle. Nuclei are stained with DAPI (blue) in upper right. Human colon T84 cells (lower panel) does not show the staining with proximal tubule marker, LTL (lower middle).

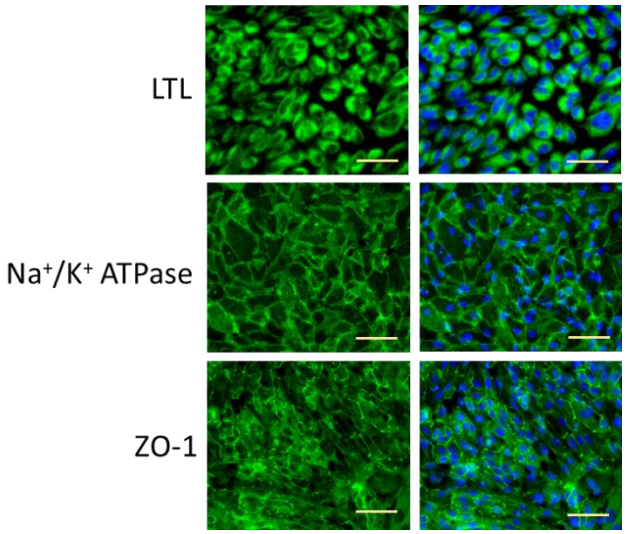


Figure 5. RPTEC cells staining with proximal tubule marker, lotus tetragonolobus lectin (LTL) in upper. Na^+/K^+ ATPase (middle) and ZO-1 (bottom). Nuclei are stained with DAPI (blue), in right panel. The scale bar: 50 μm

- **Immunostaining of transporter proteins in renal proximal tubular epithelial cells cultured on different platforms**

In RPTEC cells, OAT1 and OAT3 proteins had been very weak detected by western blot as reported by Aschauer et al. in 2015 [11]. In that article, they did not show protein staining or localization even though they showed the protein expression but the functional transport of OAT1 were undetectable in that article [11]. It might be due to low membrane expression of OAT1 in that cell line. The immunostaining of various transporters expressed in RPTEC cells was performed in the present study. As shown above in Figure 5, the basolateral marker protein, Na^+/K^+-ATPase and tight junction protein, ZO-1, could be detected, whereas Organic anion transporters (OATs) expression as well as the expression of sodium-glucose cotransporter SGLT2

and glucose transporter GLUT2 did not appear on the plasma membrane (Figure 6). Only organic cation transporter OCT2 could be detected on the plasma membrane, as seen in Figure 6.

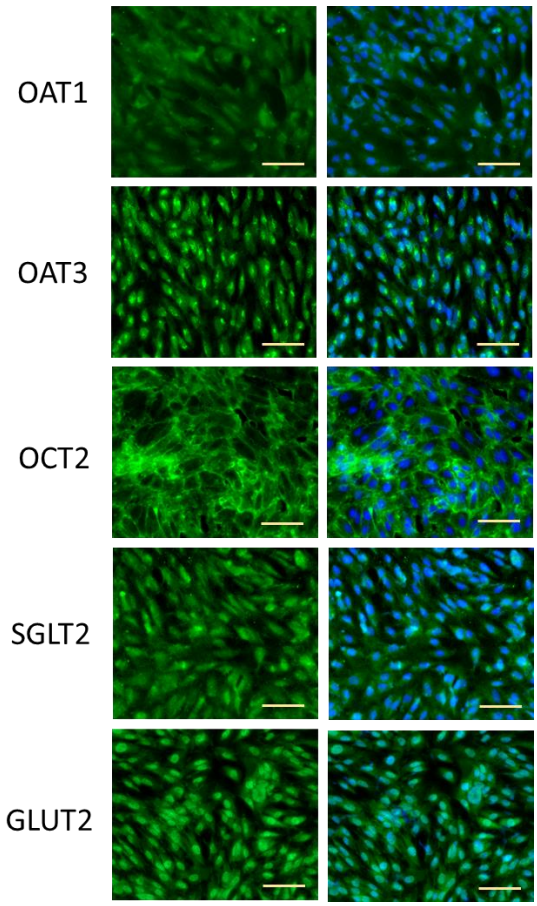


Figure 6. RPTEC cells cultured in regular 2D plate staining with antibodies of proximal tubule transporters, OAT1, OAT3, OCT2, SGLT2 and GLUT2 (green). The merge figures with nucleus staining (blue, DAPI) are in right panel. The scale bar: 50 μm

When the RPTEC cells were cultured on the membrane insert in transwell format (Figure 7), plasma membrane expressions of Na^+/K^+-ATPase and OCT2 were clearly detected similar to the regular 2D culture platform (Figure 6) but the sub-membranous expression of OAT1, OAT3, SGLT1 and SGLT2 was observed in the cells. However, GLUT2 was still localized inside the cells similar to that detected in regular 2D culture plate (data not shown). Na^+/K^+-ATPase and OCT2 expression on the plasma membrane were also revealed in the cells cultured in 3D microfluidic platform (proximal tubule-on-a-chip), as shown in Figure 8. Other transporters were still disappeared from the plasma membrane localization (data not shown).

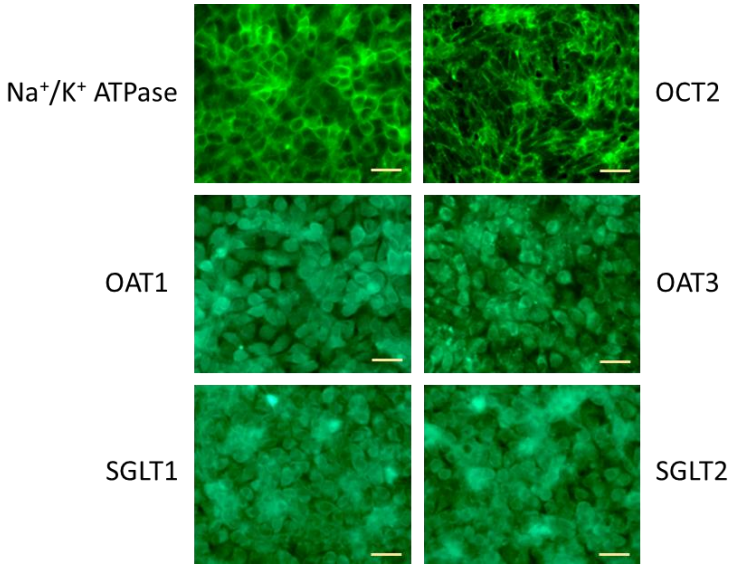


Figure 7. RPTEC cells cultured in transwell-inserts with 0.4 µm pore size clear polyester membrane staining with antibodies of Na^+/K^+-ATPase and proximal tubule transporters, OCT2, OAT1, OAT3, SGLT1 and SGLT2 (all are in green color). The scale bar: 50 µm

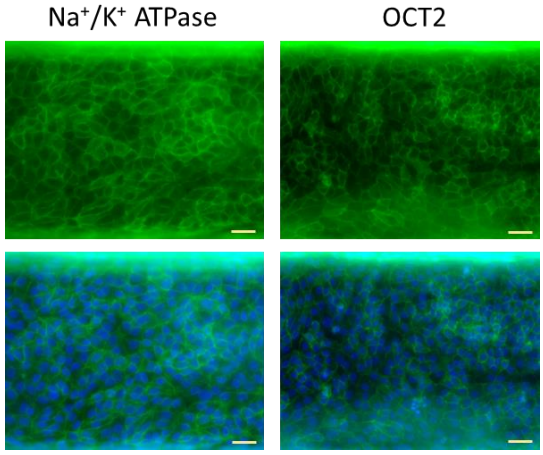


Figure 8. RPTEC cells cultured in 3D microfluidic platform (proximal tubule-on-a-chip) staining with antibodies of Na^+/K^+-ATPase (left, in green) and OCT2 (right, in green). The merge figures with nucleus staining (blue, Hoechst 33342) are in lower panel. The scale bar: 50 µm

From the above results, RPTEC cells are still lack the expression of renal proximal tubule transporters, especially OATs and SGLTs. There may be other factors beyond the physical flow rate and extracellular matrix, since the culture in 3D platform did not help the expression of the necessary transporters in renal tubule The combination of other cells might be necessary; for example, renal tubular capillary. Some factors or cytokine release from the capillaries may send a signal to activate other factors to promote movement of transporter to locate on the cell membrane. This is our prediction and need to be proven in other challenging projects of the grants.

2. Evaluating the appropriate non-toxic concentrations of mangiferin

- **Cell viability assay**

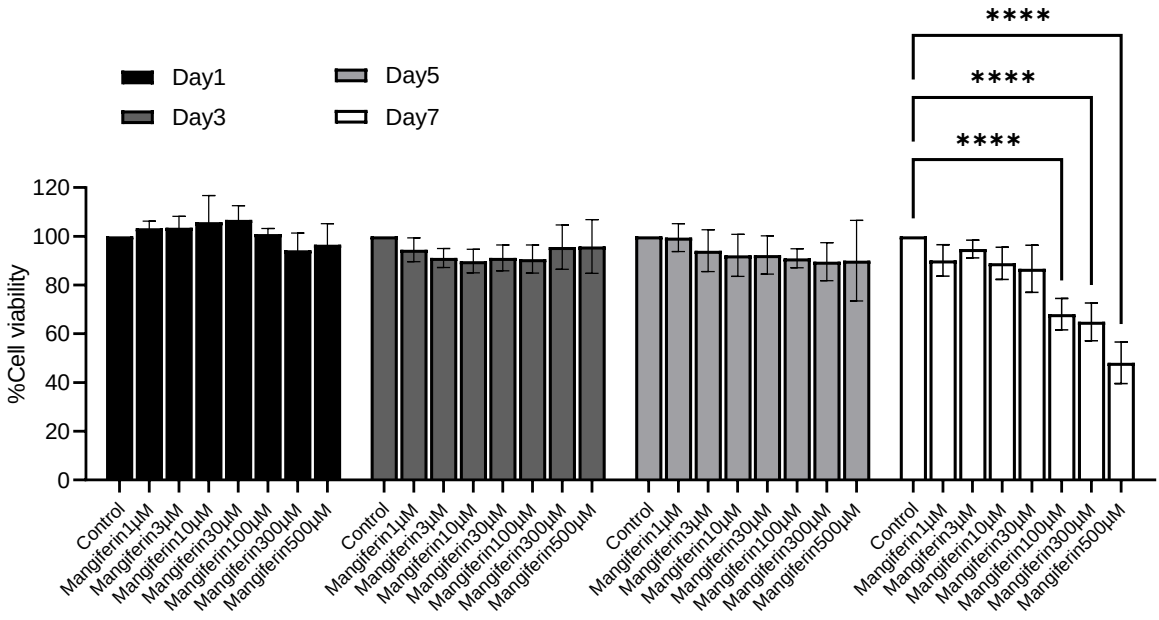


Figure 9. Effect of mangiferin on the cell viability of RPTEC cells. The cells were incubated with mangiferin at concentrations of 0, 3, 10, 30, 100, 300 and 500 μM for 1, 3, 5 and 7 days. Cell viability was determined by MTT assay. Data are expressed as % of control, mean ± S.D., n=3, ****p<0.0001 vs. control at the same incubation period, two-way ANOVA with Tukey's multiple comparisons test.

In order to determine the non-toxic concentrations of mangiferin for further experiments, MTT assay was used to examine cell viability of RPTEC cells treated with varying concentrations of mangiferin (0, 1, 3, 10, 30, 100, 300 and 500 μM) for 1, 3, 5 and 7 days. The concentrations that showed no toxicity were used for further experiments. In this study the concentrations of mangiferin up to 500 μM can be used without toxicity when duration of treatment is increasing up to 5 days. The higher concentrations (100 and 500 μM) of mangiferin showed toxicity when the cells were longer exposed to these concentrations for 7 days (Figure 9). Therefore, the concentrations of mangiferin for the experiments should bring the exposure time of treatment to be considered, too.

3. Transport function assay and the interaction of mangiferin with transporters

- **The functional test of transporter proteins in renal proximal tubule cells**

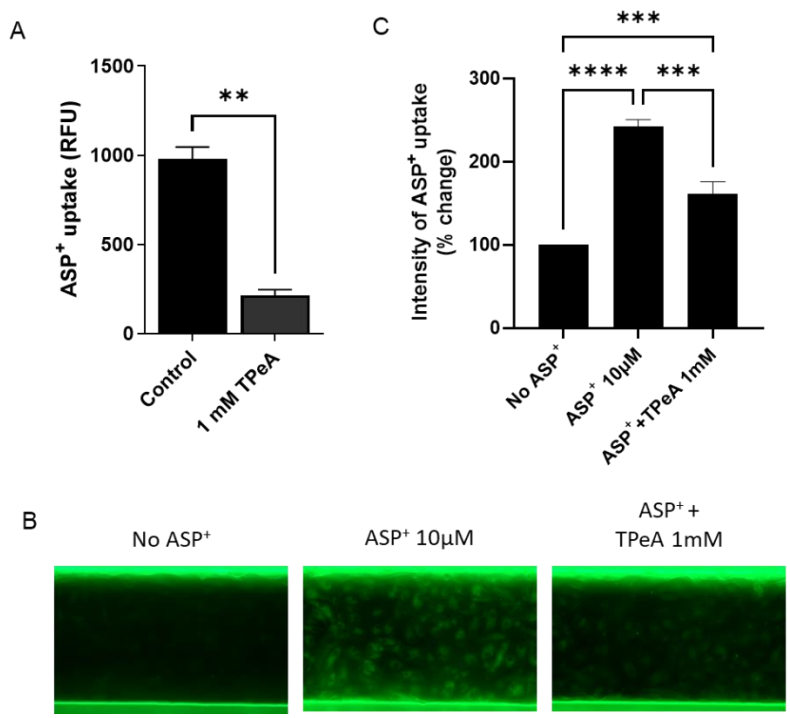


Figure 10. The functional test for OCTs transporters in RPTEC cells. Transport of 4-Di-1-ASP (ASP^+) were performed at 37°C for 10 min in the cells cultured in classical 2D platform (A), and 20 min in 3D microfluidic platform (B and C). Control is the uptake without an inhibitor The fluorescent uptake into the cells was observed under fluorescent microscope (B) and the calculated intensity of the fluorescent dye (C).: ASP^+: 4-Di-1-ASP; TPeA: Tetrapentyl ammonium. **p<0.01, ***p<0.001, ****p<0.0001. Data are mean ± SD, n=3.

The other method to confirm the membranous localization of transporter expressed in RPTEC cells is functional test of that transporter. The functional study using known substrates and inhibitors for OCT2 transporters were examined. The uptake of fluorescent substrate of OCTs, 4-Di-1-ASP (ASP^+), was performed both in 2D (Figure 10A) and in 3D (Figure 10B) platforms. The result showed that OCT2 was existing on the plasma membrane of the cells since ASP^+ could be transported into RPTEC cells and was inhibited by tetrapentylammonium (TPeA), an inhibitor of OCTs (Figure 10). In 3D platform the substrate and inhibitor were added to the basolateral side which is the position that OCT2 localized on the membrane. After 20 min of incubation the cells had changed to be green under fluorescent microscopy, as shown in Figure 10B. The % change of substrate uptake in the presence of inhibitor (TPeA) was higher in 2D than 3D platform. It may explain by having the ECM space between the loading site and the cells in 3D structure platform, whereas the direct exposure of the substrate and inhibitor were applied to the cells in 2D culture system.

The inhibition test of OCT2 by mangiferin was also performed in RPTEC cells cultured on conventional 2D platform since the cells were proven to express OCT2 on the membrane.

The dose-dependent inhibition was observed and the inhibition was significant difference from control when the concentrations were 500 µM or more. The IC_{50} value of mangiferin on the inhibition of OCT2 in RPTEC cells was higher than 1000 µM (Figure 11). This indicate that the interaction of mangiferin with OCT2 was not strong.

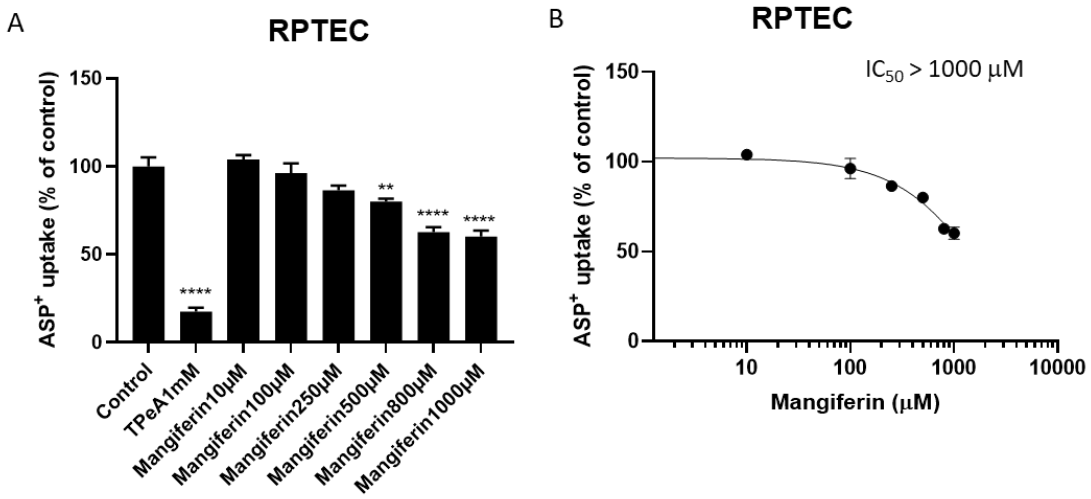


Figure 11. The uptake of 4-Di-1-ASP (ASP^+), a fluorescent substrate of OCTs by RPTEC cells. The cells were incubated with 10 µM ASP^+ in the absence (control) and presence of 1 mM tetrapentylammonium (TPeA), an inhibitor of OCTs, and various concentrations of mangiferin for 10 min at 37°C. The fluorescent intensity in the cell was measured to determine the accumulation of fluorescein inside the cells (A). The IC_{50} of mangiferin for ASP^+ uptake by OCTs was calculated (B). **p<0.01, ****p<0.0001 vs. control, one-way ANOVA with Bonferroni's multiple comparisons test. Data are mean ± S.D., n=3.

The studies with OAT1 and OAT3 substrates and inhibitors uptake on the RPTEC cells showed no transport function of OATs (Data was not shown.). The results were correlated with the immunofluorescence staining mentioned above. Therefore, the interaction of mangiferin with OATs could not be performed in RPTEC cells. The over-expressing OAT1 and OAT3 were constructed, instead, in MDCK cells. As shown in Figure 12C and 13C, OAT1 and OAT3 were existed on the membrane of MDCK cells after transfection. 6-carboxyl fluorescein (6-CF) and fluorescein (FC) were used as fluorescent substrates of OAT1 and OAT3, respectively. The function of these two transporters was confirmed by the inhibition test using their known inhibitors, probenecid (PB) for OAT1 and estrone-3-sulfate for OAT3, respectively. The mangiferin inhibition of OAT1 was dose-dependent with an IC_{50} of 188.7 µM (Figure 12). The OAT3 inhibition by mangiferin was with an IC_{50} value of 62.76 µM (Figure 13).

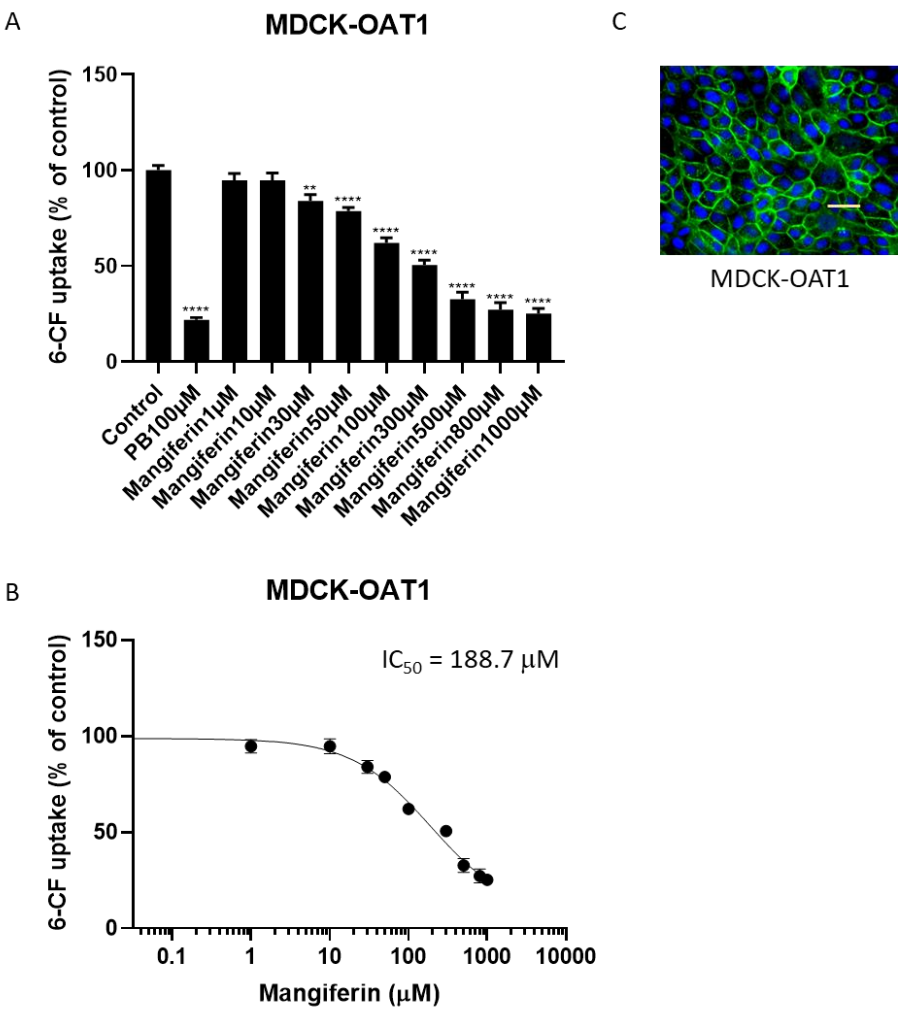


Figure 12. The uptake by OAT1-transfected MDCK cells (MDCK-OAT1). The cells were incubated with 3 μM 6-carboxyfluorescein (6-CF) in the absence (control) and presence of 0.1 mM probenecid (PB), an OATs inhibitor, and varying concentrations of mangiferin for 10 min at 37°C (A). The IC_{50} was calculated from the plot (B). The OAT1 protein on the membrane of the cells were stained in green and the nuclear staining using DAPI are in blue (C). Data are mean ± S.D., **p<0.01, ****p<0.0001, n=3-4.

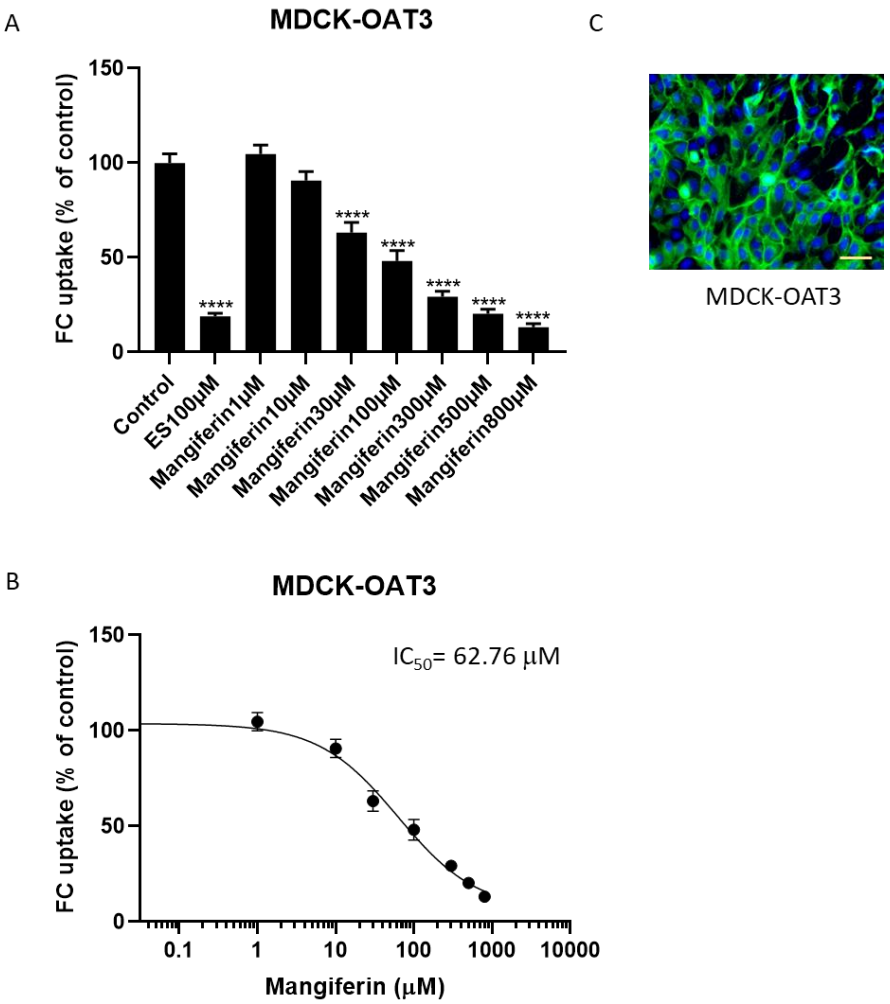


Figure 13. The fluorescein uptake by OAT3-transfected MDCK cells (MDCK-OAT3). MDCK-OAT3 cells were incubated with 5 μM fluorescein (FC) in the absence (control) and presence of 0.1 mM estrone-3-sulfate (ES), an OAT3 inhibitor, and varying concentrations of mangiferin for 10 min at 37°C (A). The IC_{50} was calculated from the plot (B). Immunofluorescence staining of MDCK-OAT3 are shown in (C). Data are mean ± S.D., one-way ANOVA with Bonferroni's multiple comparisons test, ****p<0.0001, n=3-4.

The radioactive glucose (^{3}H-glucose) uptake by RPTEC cells was significantly inhibited by phloretin, an inhibitor of GLUTs transporters, but not inhibited by phlorizin and dapagliflozin and empagliflozin, inhibitors of SGLTs and SGLT2, respectively (Figure 14). Mangiferin slightly inhibited glucose transport in RPTEC cells but not significant difference from control. The result indicates that there may be no or very low plasma membrane expression of SGLTs, but GLUTs function was detected in this cell line and was not inhibited by mangiferin. With this limitation of the expression, we could not conclude whether mangiferin inhibited glucose transport by SGLT2, or not. We tried to find other cells or over-expressing SGLT2 in cells to test this curiosity but the function of SGLT2 was not high enough to test the inhibition study.

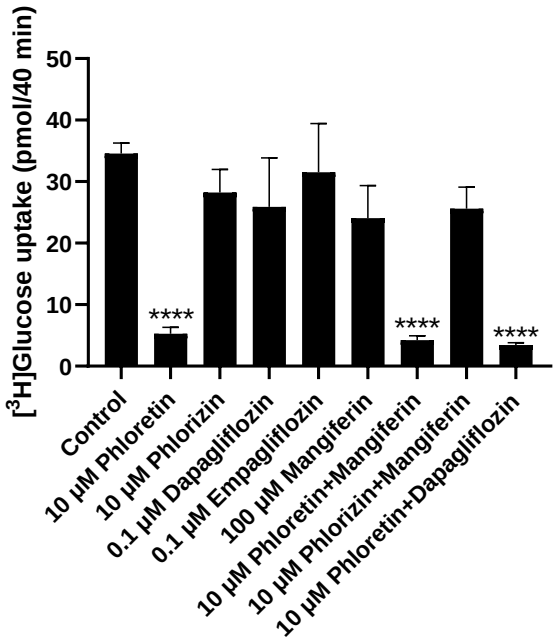


Figure 14. The [^{3}H] glucose uptake in RPTEC cells. The cells were incubated with 50 µM [^{3}H] glucose for 40 min at 37°C Various inhibitors as indicated in graph were added to the uptake solution together with [^{3}H] glucose. Data are mean ± S.D., one-way ANOVA with Bonferroni's multiple comparisons test, ****p<0.0001, n=3-.

4. The alteration of glucose transporters' expression in high glucose or diabetes mimicking conditions

The mRNA expression of glucose transporters, GLUT2, SGLT1 and SGLT2, was evaluated by real-time qPCR to detect the alteration after the RPTEC cells were exposed either with high glucose (HG) condition (30 mM D-glucose) or high glucose with other components imitated DM condition (in this study called condition media, CM), as mentioned in the part of methods, for 3, 7 and 14 days. There were no significant changes of mRNA expression of the glucose transporters (Figure 15). This may be due to the low plasma membrane expression of the glucose transporters in the cells and/or the mRNA inside the cell was also low and no increased synthesis, resulting in no movement of the protein into the membrane during the cells was exposed in high amount of glucose.

Together with this mRNA expression result in Figure 15 and the glucose transport function (Figure 14) were not changed with mangiferin treatment, thus, it is no advantage to test the action of mangiferin on the glucose transporter expression.

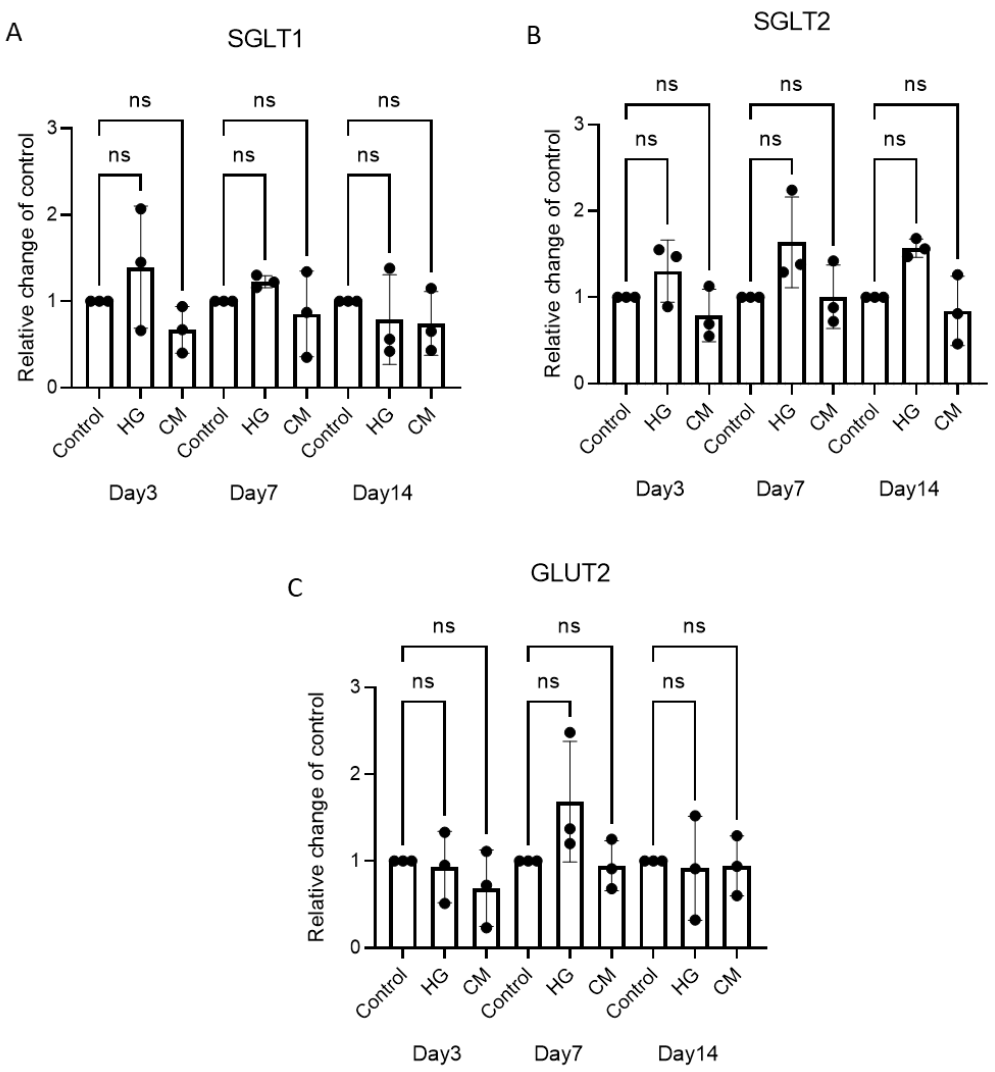


Figure 15. The mRNA expression of glucose transporters in RPTEC cells exposed to high glucose. RPTEC cells was exposed to high glucose (30 mM D-glucose, HG) or in the condition imitate diabetes condition using condition media (CM), as mentioned in the method, for 3, 7 and 14 days. The cell pellet was collected to extract total RNA and used for qRT-PCR. Data are mean $\pm$ S.D, n=3.

5. The alteration of kidney injury marker, proinflammatory and profibrotic markers in high glucose treated RPTEC cells

The effect of high glucose exposure in RPTEC cells on the proximal tubule cell injury and inflammation was evaluated by detecting the kidney injury marker proinflammatory and profibrotic markers in the cells treated with the high glucose (HG: 30 mM D glucose) or condition media (CM: 30mM D-glucose, 2ng/ml TNF-α, 1ng/ml IL6 and 100nM Insulin) in 2D-culture platform for varying durations (3, 7, and 14 days). Using real-time PCR, IL-1β was significantly increased in the cells treated with high concentration of glucose (30 mM) and condition media at day 3 (Figure 16A), whereas TGF-β1 was significantly increased only in the cells treated with condition media,

not with high glucose alone at day 3 (Figure 16B). The mRNA of KIM-1, fibronectin, collagen I and α-SMA were significantly increased in the cells treated with condition media after exposure to high concentration of glucose for 7 days (Figure 17). Collagen I was also increased in the cells treated with high glucose for 7 days as shown in Figure 17C.

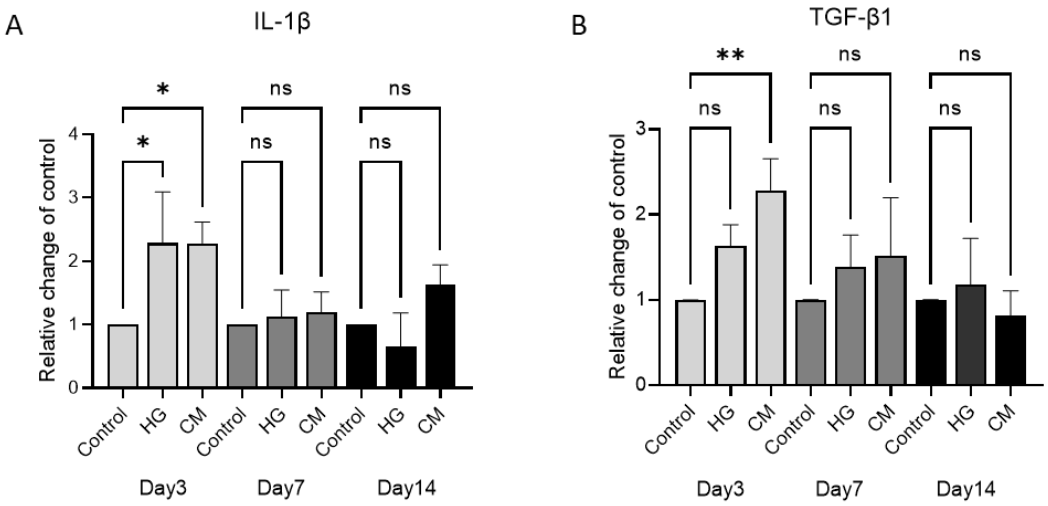


Figure 16. The mRNA levels of inflammatory cytokines and profibrotic markers. IL-1β (A), TGF- β 1 (B), in RPTEC cells treated with 30 mM D-glucose (HG), condition media (CM) compared to the control cells treated with normal medium containing 5 mM D-glucose for 3, 7 and 14 days were determined by quantitative RT-PCR. Data are mean ± SD, n=3. *p<0.05, **p<0.01 vs. control.

The elevation of TGF- β1, IL-1 β and IL-6 cytokines released from the cells was detected by ELISA assay using the supernatant of the cells treated with condition media. High glucose alone also significantly increased the release of IL-6 from the cells at 7 days of treatment (Figure 18). Osmotic control condition (25 mM Mannitol + 5 mM D-glucose) did not affect the released of cytokines in every time point of treatment. All treatment conditions had no effect on the cytokine release at day 3 and day 14 as shown in the Figure 18.

From these results indicate that the cells exposed to high glucose concentration for a certain period could induce the increased mRNA expression of cell inflammation, injury, and profibrotic markers, then the mRNAs of factor involved fibrogenesis were observed. The cytokine release was appeared after the mRNA production, in consequentially. However, at the longer duration (14 days) the cytokines might be degraded. The components that imitated the diabetes condition may be the factors that can activate the severity of the kidney disease progression together with high level of blood glucose.

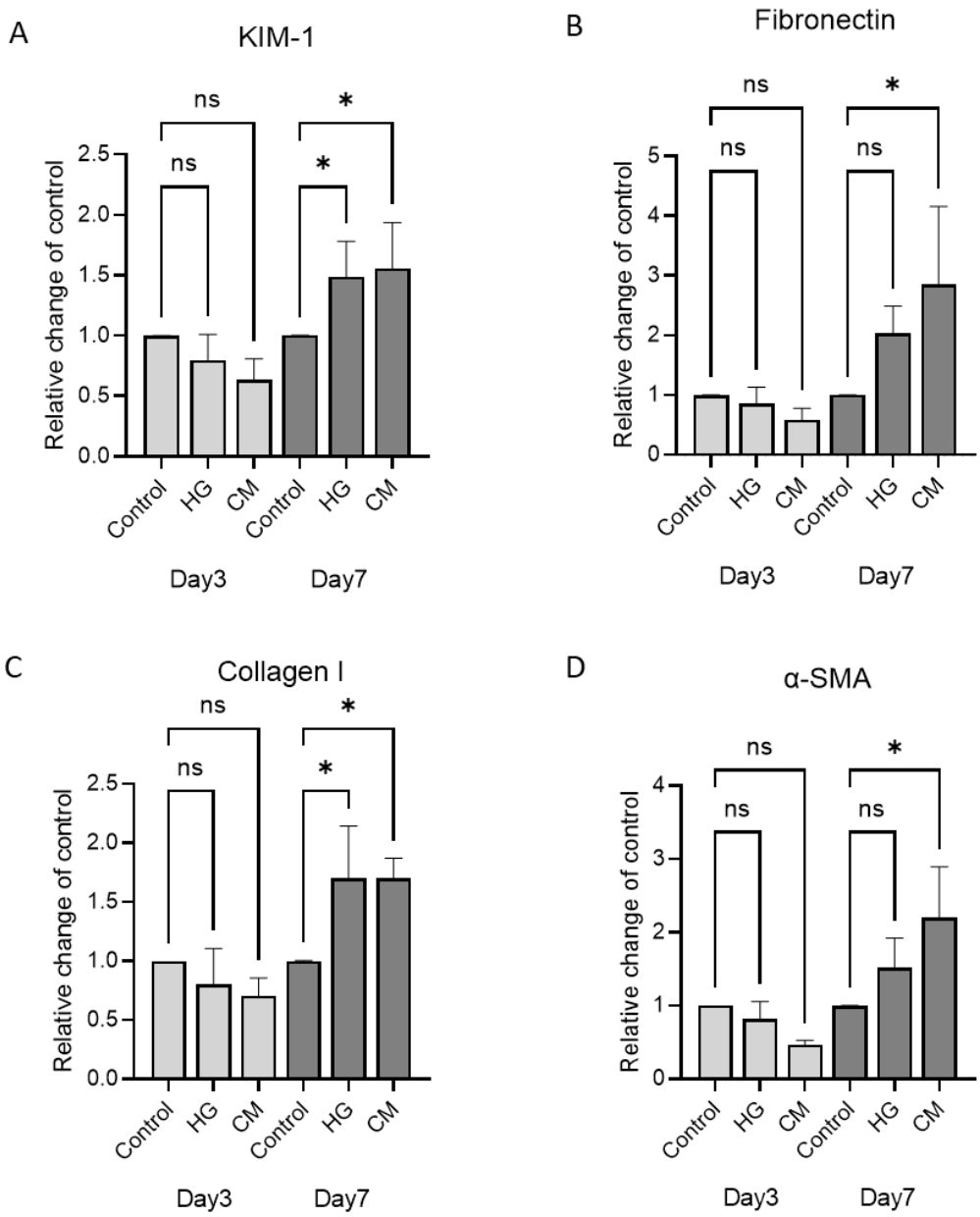


Figure 17. The mRNA levels of kidney injury and fibrogenesis markers. The kidney injury marker; KIM-1 (A), fibronectin; FN (B), collagen I; Col I (C) and α-smooth muscle actin; α-SMA (D) in the RPTEC cells treated with 30 mM D-glucose (HG), and condition media (CM) compared to the control cells treated with normal medium containing 5 mM D-glucose for 3 and 7 days were determined by quantitative RT-PCR. Data are mean ± SD, n=3-4. *p<0.05 vs. control.

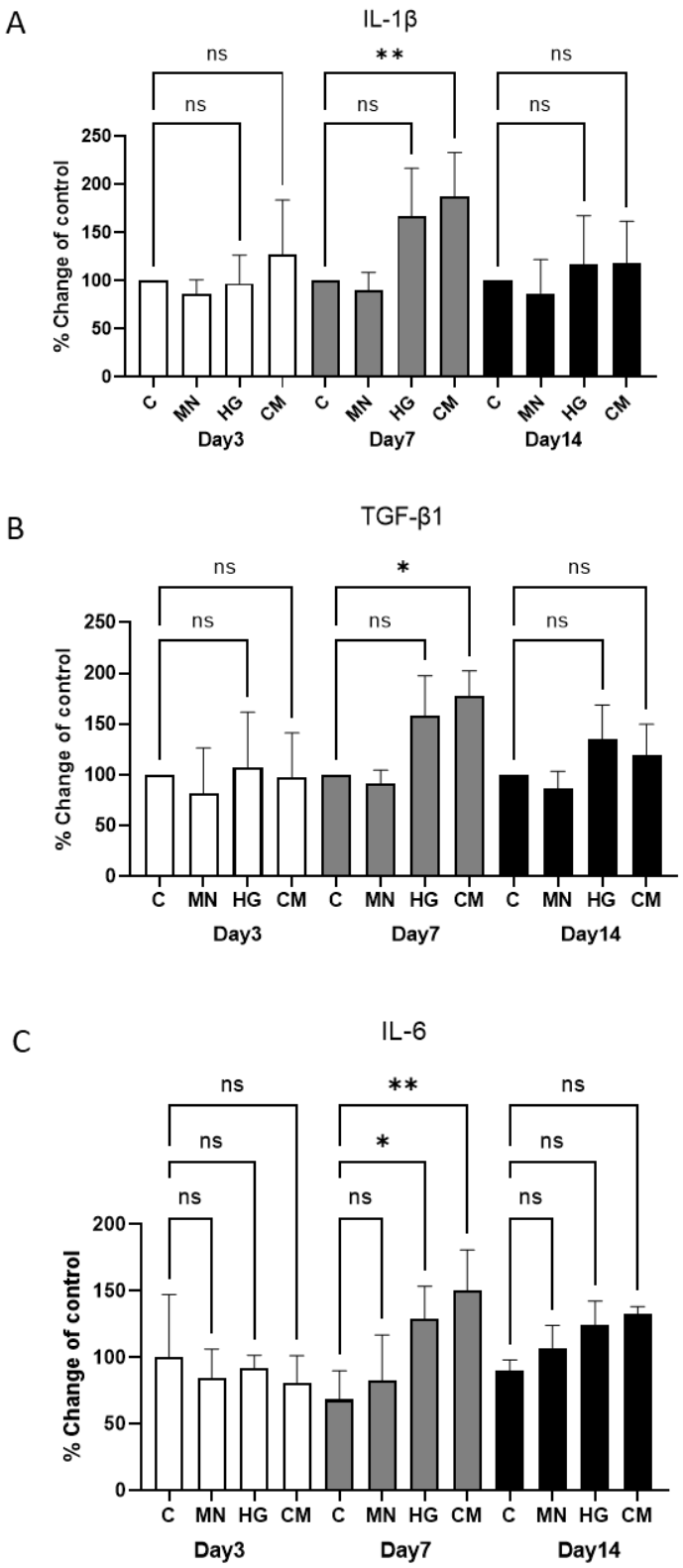


Figure 18. The levels of inflammatory cytokines detected in cell culture supernatant. IL-1□ (A), TGF-□1 (B), and IL-6 (C) in the RPTEC cells cultured in conventional 2D platform and treated with 30 mM D-glucose (HG), condition media (CM) and 5 mM D-glucose plus 25 mM mannitol (MN) compared to the control (C) cells treated with normal medium containing 5 mM D-glucose for 3, 7 and 14 days were measured by ELISA. Data are mean ± SD, n=3. *p<0.05, **p<0.01 vs. control.

When mangiferin (20 and 40 μM was applied into the culture media containing high glucose or condition media for 7 days, the induction of IL-6 release from RPTEC cells cultured in high glucose or condition media group were significantly inhibited as shown in Figure 19.

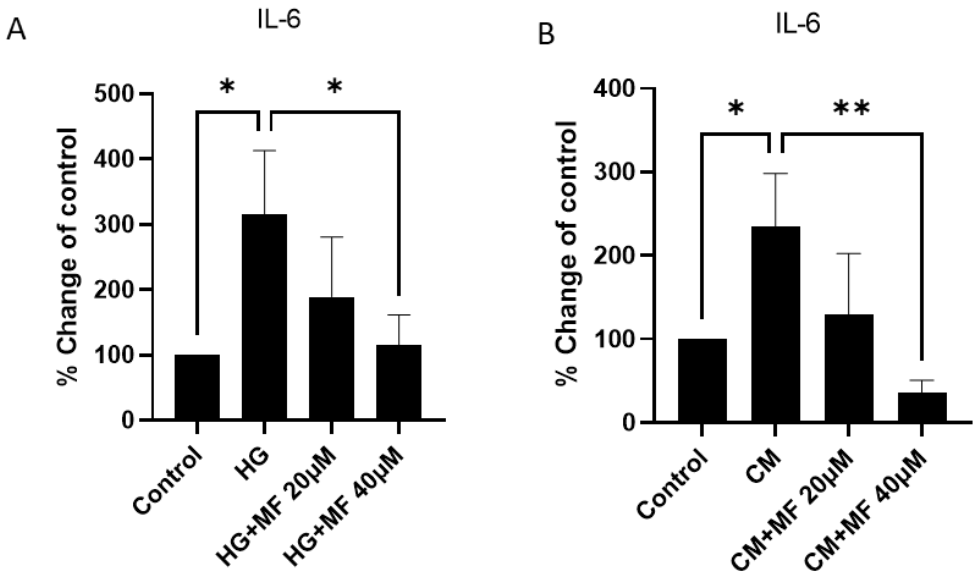


Figure 19. The effect of mangiferin on IL-6 release in RPTEC cells in conventional culture, treated with high glucose (HG) and condition media (CM). Mangiferin (MF) 20 and 40 μM were co-treated with high glucose and condition media for 7 days. The level of IL-6 in the supernatant of the cells treated with HG (A) and CM (B) were measured by ELISA. The data are the relative value compare with control, *p<0.05, **p<0.01, n=3-4.

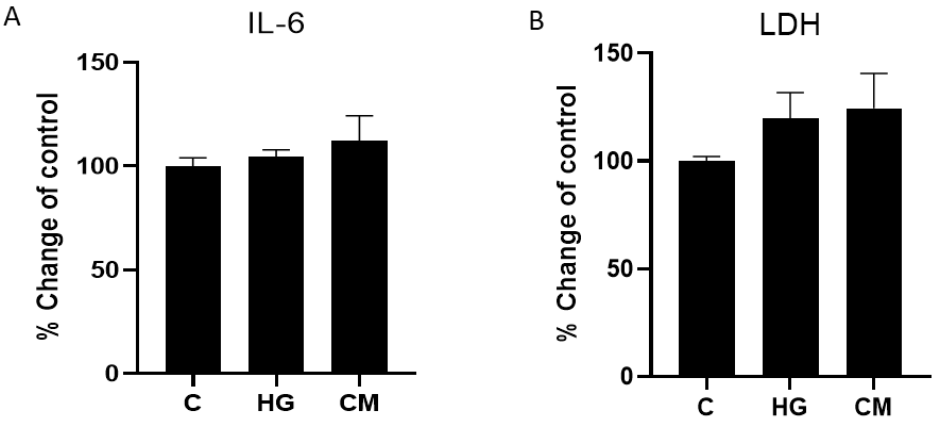


Figure 20. The IL-6 and LDH release in RPTEC cells in 3D culture platform of proximal tubule-on-a-chip, treated with high glucose (HG) and condition media (CM) for 7 days. The level of IL-6 and LDH was measured by ELISA and LDH assay kit, respectively. The data are the relative value compare with control. n=3

Surprisingly, the treatment with high glucose and condition media in the 3D microfluidic platform, a proximal tubule-on-a-chip showed the different result with the one in 2D

culture platform, as shown in Figure 20. There was no any significant effect of high glucose or diabetic mimicking condition on IL-6 and LDH release.

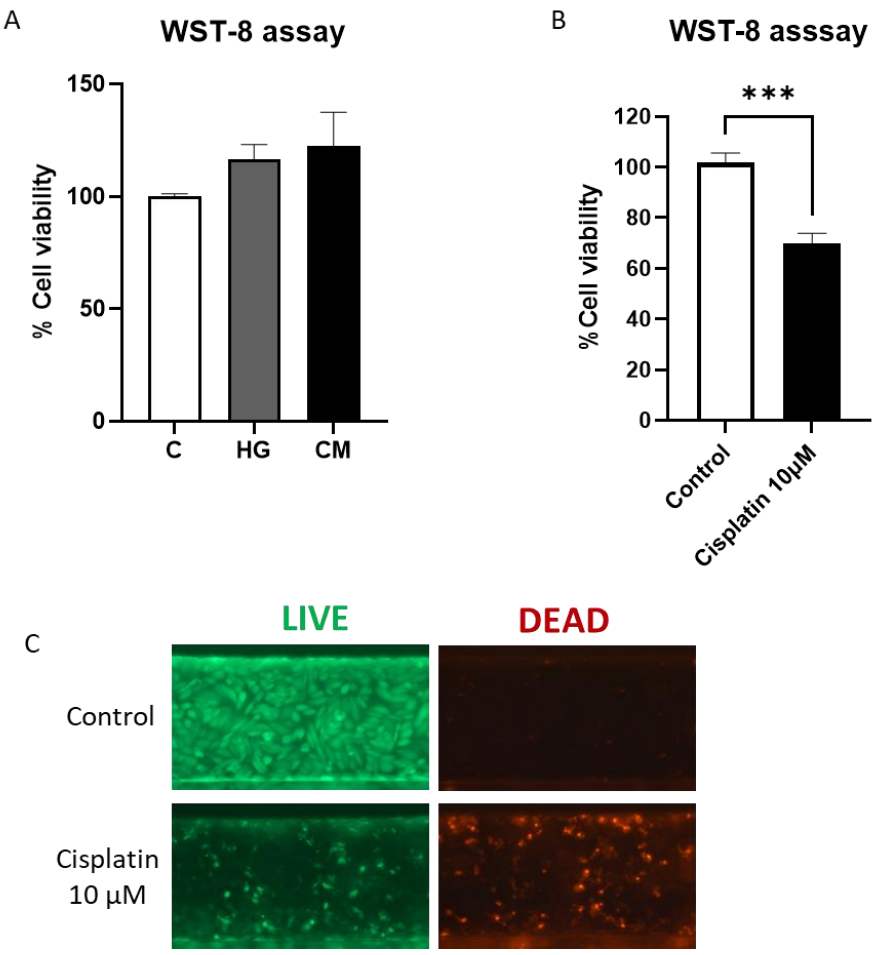


Figure 21. The RPTEC cell survival test in 3D culture platform of proximal tubule-on-a-chip. RPTEC cells were treated with high glucose (HG) and condition media (CM) for 7 days (A), or with 10 μM cisplatin (B) the cell viability was determined by WST-8 assay. The representative Live/Dead staining of the cells after 24-h cisplatin treatment was presented as green and red color for live and dead cells, respectively, under fluorescence microscope. ***p<0.001, n=3-4.

To check the possibility whether the materials of 3D platform or ECM may delay or had some unknown action on the sensitivity of the cells to treatment condition. We determined the survival of the cells in the high glucose (HG) and condition media (CM) using WST-8 assay since it is convenient to use in 3D platform. The cells exposed to HG or CM did not show the reduction of their survival (Figure 21A), whereas the survival of the cells treated with 10 μM cisplatin, an anti-cancer drug, was about 70% compared to control (figure 21B). The image of live/dead staining of the cells was shown in Figure 21C. Compare to cisplatin affected on RPTEC survival with an IC_{50} about 10 μM (our result obtained in other study in this cell) on 2D platform similar to the concentration used in this study, the survival rate in this 3D platform was higher

than the one in 2D culture platform. This may be explained by the effect of the accessibility of the treatment to the cells in 3D platform, it may need time that drug reach to the cells.

6. The induction of fibrogenesis using IL-1β and TGF-β1 and the anti-fibrotic activity test of mangiferin

Since the results in earlier part showed the increased IL-1β and TGF-β1 releases from the RPTEC cells treated with high concentration of glucose (Figure 18), The increase of these cytokines may be the ones induced fibrogenesis in patient with DM and result in the progression of the disease to ESRD. Therefore, we tried to develop fibrosis model using the induction by either IL-1β or TGF-β1.

As shown in Figure 22, after the varying concentrations and incubation times of IL-1β in RPTEC cells, IL-1β at the concentrations of 20 and 50 ng/mL after incubated with RPTEC cells for 24, 48 and 72h did not change the accumulation of collagen stained with Picro-Sirius Red (PSR) in the cells (Figure 22), whereas the cells treated with 2-20 ng/mL of TGF-β1 for 24 and 48h (Figure 23, 24) showed significant changes in collagen accumulation The collagen accumulation was abolished by the co-treatment with a TGF-β receptor 1 kinase inhibitor, ALK5 inhibitor.

TGF-β1 at the concentration of 5 ng/mL treated for 24h were used for testing the action of mangiferin, as shown in Figure 25. RPTEC cells were pre-treated with mangiferin at the concentrations of 50 and 100 μM for 3 days before giving 5 ng/mL TGF-β1 together with mangiferin. For control group, the cells were treated with vehicle during the time of mangiferin pre-treatment, instead, and 5 ng/mL TGF-β1 alone was used for 24h treatment in this control group. ALK5 inhibitor (10 μM) was used as positive control treatment, it blocks TGF-β receptor 1, thus the fibrogenesis was suppressed. The result in Figure 25 demonstrated the reduction of TGF-β1-induced collagen accumulation in the cells by the action of 10 μM ALK5 inhibitor and mangiferin at 50 and 100 μM. Furthermore, mangiferin also lowered the increased fibronectin mediated by TGF-β1, as demonstrated in Figure 26. E-cadherins, an adhesion molecule, expression was reduced with TGF-β1 treatment and its reduction was abolished with pre-treated mangiferin. As shown by Western blotting (Figure 26A and C). This indicates that mangiferin has an anti-fibrotic activity for the human renal proximal tubular cells.

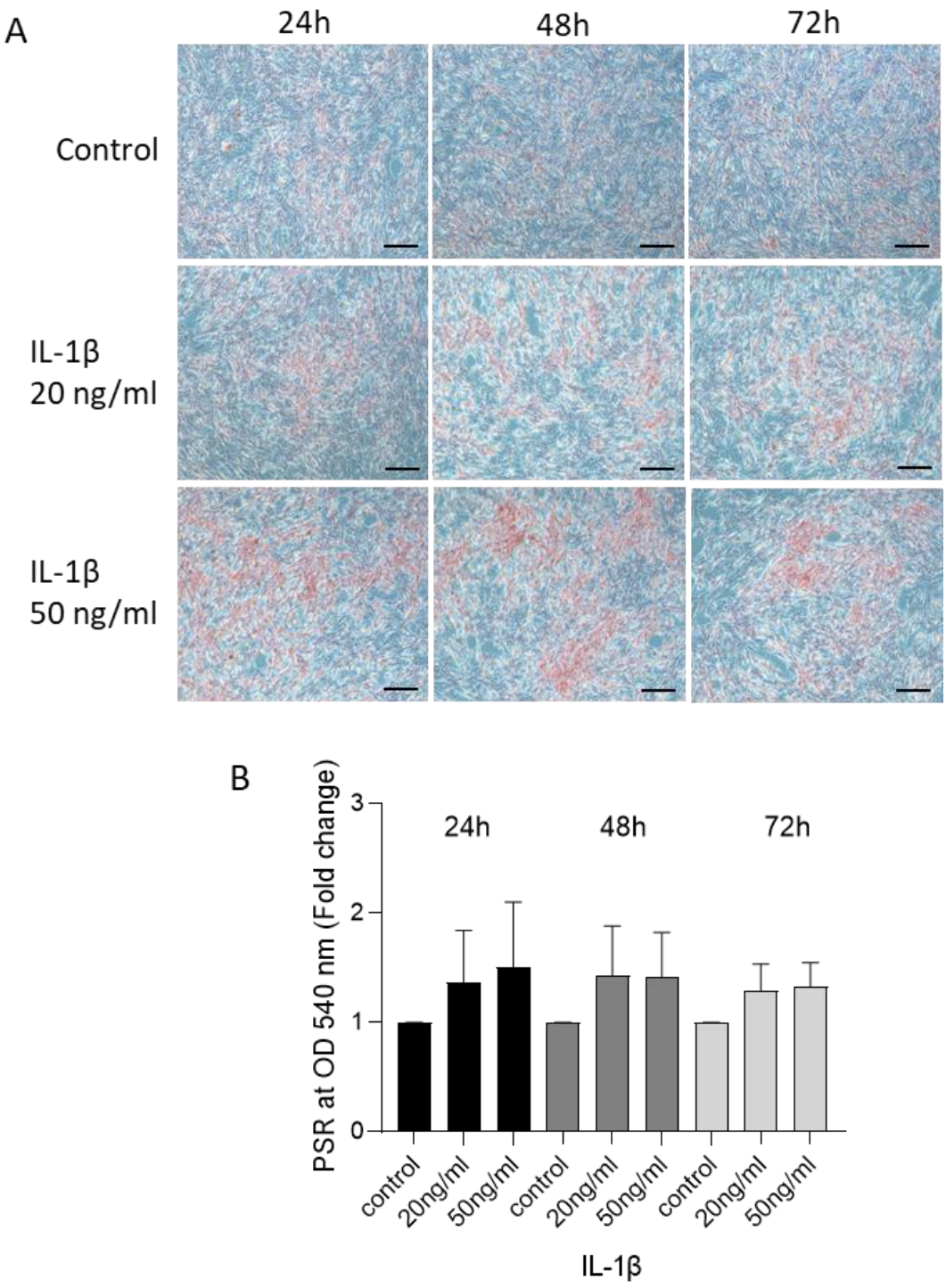


Figure 22. The induction of fibrogenesis with IL-1β in RPTEC cell. The cells were treated with IL-1β for 24, 48 and 72h and the collagen was detected with PSR staining and read the intensity using plate reader. n=3

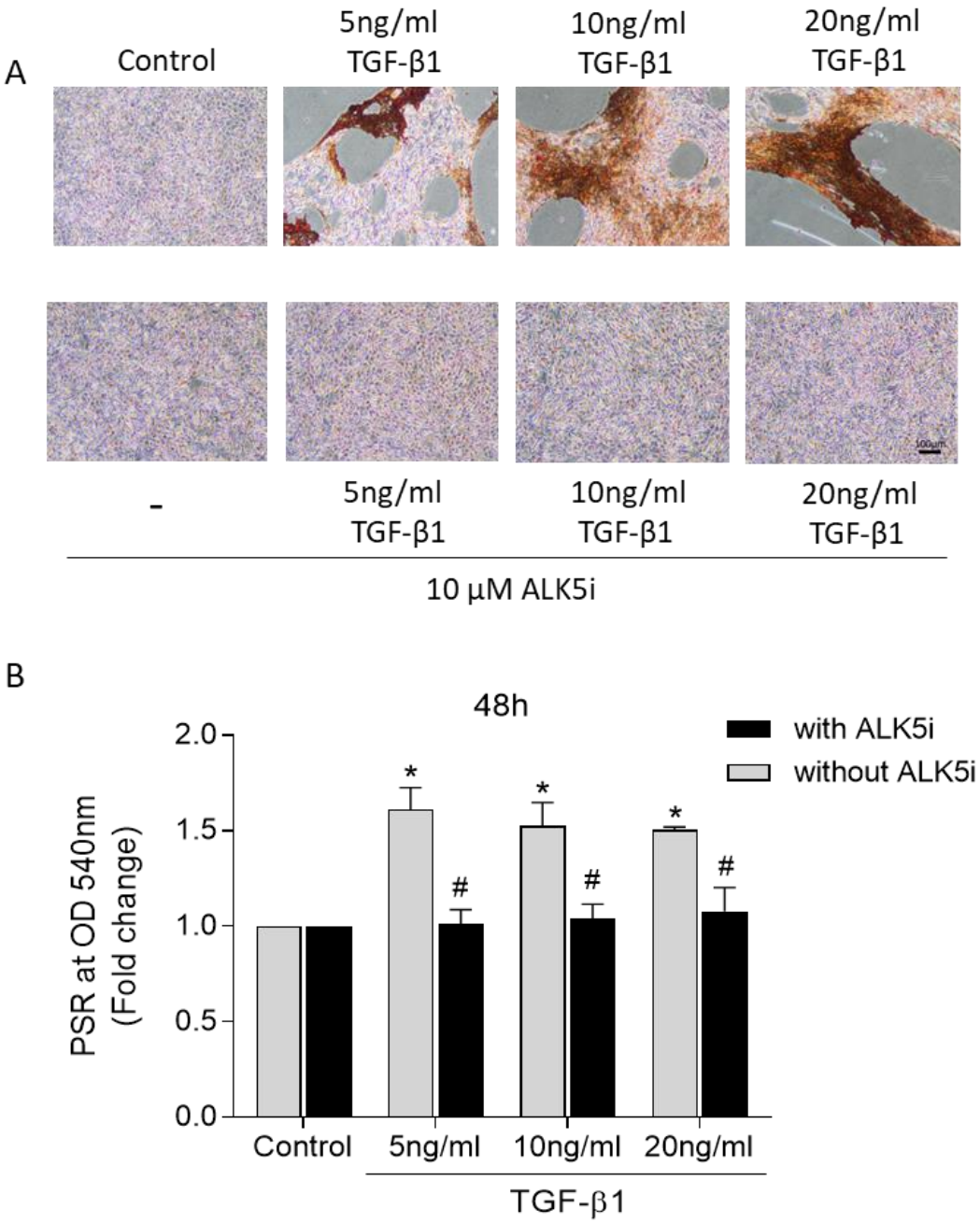
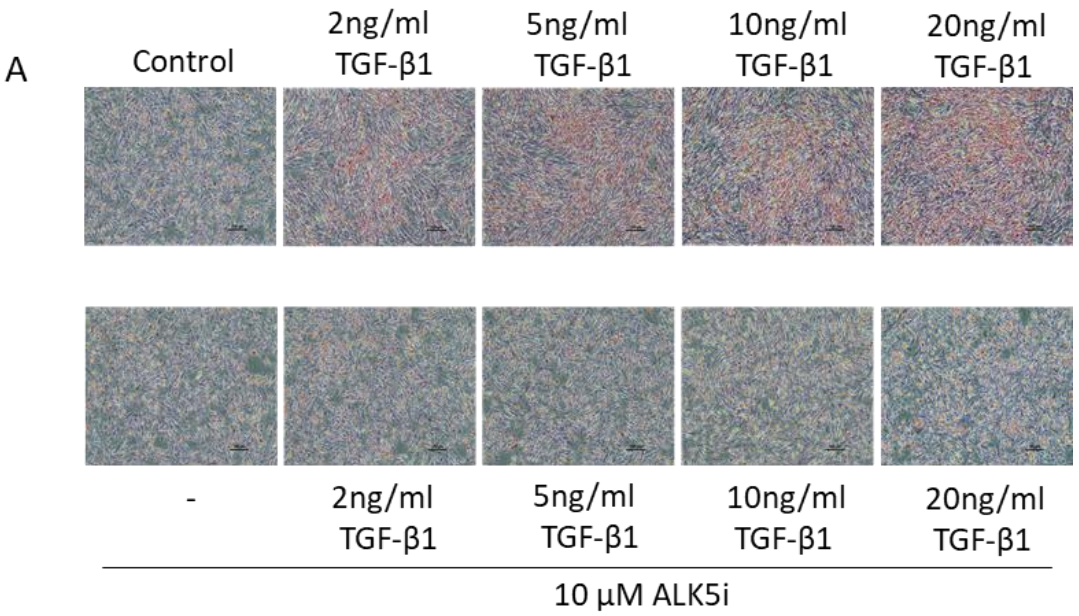


Figure 23. The induction of fibrogenesis with TGF-β1 for 48h in RPTEC cell. The cells were treated with 5, 10 and 20 ng/mL TGF-β1 for 48h and the TGF-β1 receptor 1 kinase inhibitor (ALK5i) at 10 μM were co-administered to the cells as a positive inhibitor. and the collagen was detected with PSR staining and the intensity of red color were determined using a plate reader. *p<0.05 vs. control, #p<0.05 vs. TGF-β1 alone. n=3



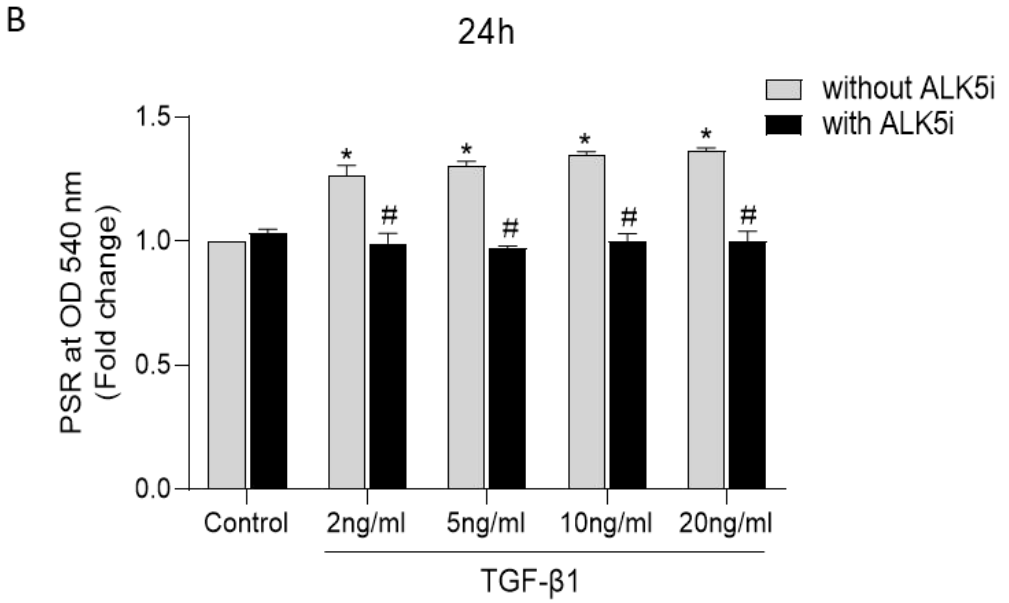


Figure 24. The induction of fibrogenesis with TGF-β1 for 24h in RPTEC cell. The cells were treated with 5, 10 and 20 ng/mL TGF-β1 for 24h and the TGF-β1 receptor 1 kinase inhibitor (ALK5i) at 10 μM were co-administered to the cells as a positive inhibitor. and the collagen was detected with PSR staining and the intensity of red color were determined using a plate reader. *p<0.05 vs. control, #p<0.05 vs. TGF-β1 alone. n=3

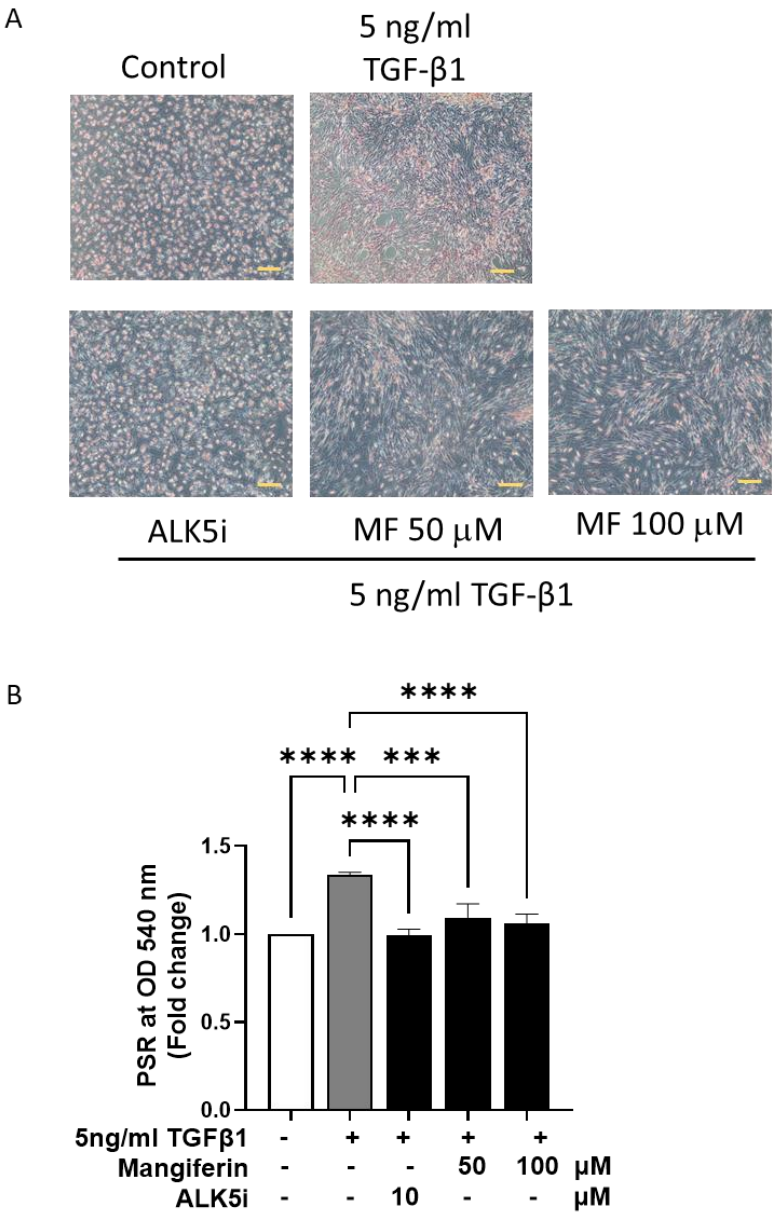


Figure 25. The effect of mangiferin on the reduction of TGF-β1-induced fibrosis. RPTEC cells were pre-treated with 50 and 100 µM for 3 days before 5 ng/mL TGF-β1 application for 24h. TGF-β receptor 1 kinase inhibitor (ALK5i) at 10 µM were co-administered with TGF-β1 to the cells as a positive inhibitor. The collagen accumulation was detected with PSR staining, the images of the cell staining was taken under light microscope (A) and the intensity of red color was determined using a plate reader (B). ****p<0.0001, Data are mean ± S.D., n=3.

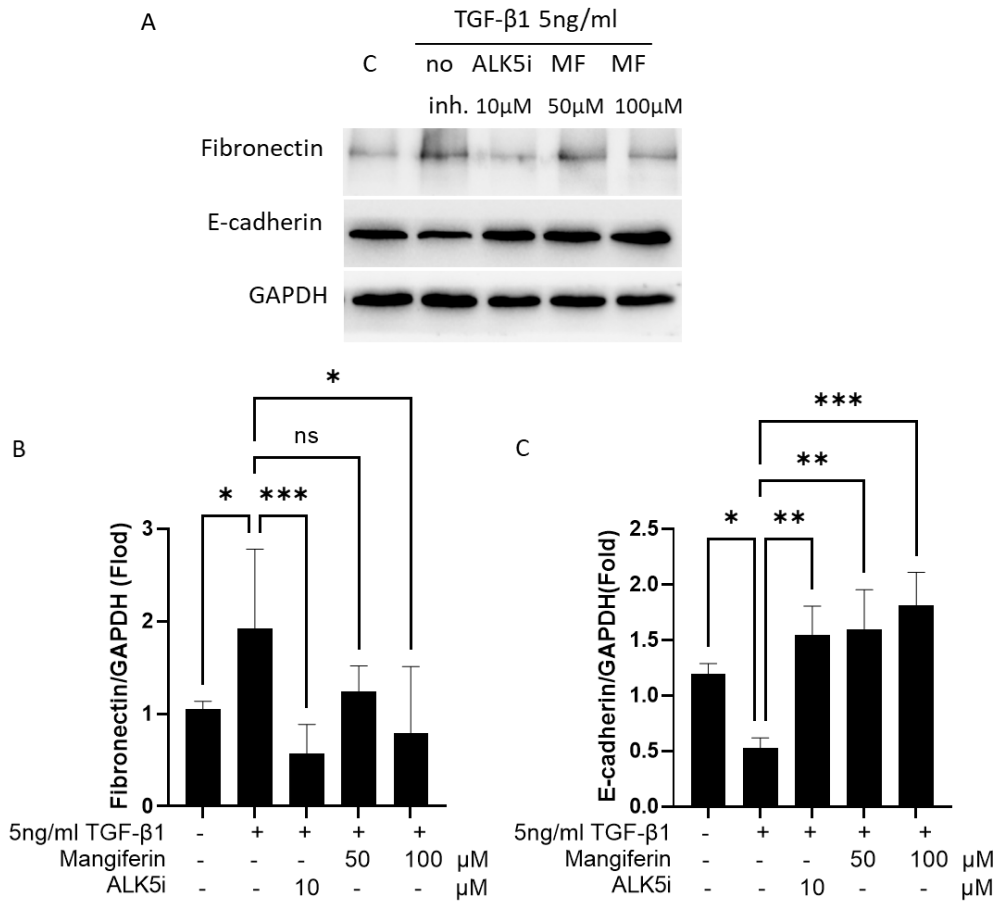


Figure 26. The effect of mangiferin on the reduction of TGF-β1-induced the alteration of fibronectin and e-cadherin. RPTEC cells were treated in the same protocol described in Figure 25. The fibronectin and e-cadherin expression were detected by Western blot analysis (A). The band intensity of fibronectin (B) and e-cadherin (C) from Western blotting were determined using ImageJ software. *p<0.05, **p<0.01, ***p<0.001. Data are mean ± S.D., n=3.

To figure out the site of mangiferin action, we examined the alteration of phospho-NF-ΚB and phospho-Smad3 in the cells that the fibrogenesis induced by TGF-β1. As shown in Figure 27, TGF-β1-induced fibrosis could activate the phosphorylation of Smad3 and mangiferin demonstrated the inhibition of this change. The increased phospho-NF-ΚB and total NF-ΚB was observed in the cells treated with TGF-β1 and their reduction was observed in the cells treated with mangiferin 50 and 100 μM in the presence of TGF-β1. Surprisingly, there was a reduction of the ratio of phospho/total NF-ΚB during 24h TGF-β1 treatment, this result was unexpectedly appeared, it was opposite effect from the our taught as consequence of increased chronic injury and inflammation before fibrogenesis. The occurrence may be from the direct activation of fibrosis by TGF-β1, not from the high glucose exposure that made the cell injury and inflammation before

induction of fibrosis from the elevation of TGF-β1 release. This finding can be explored in the next project.

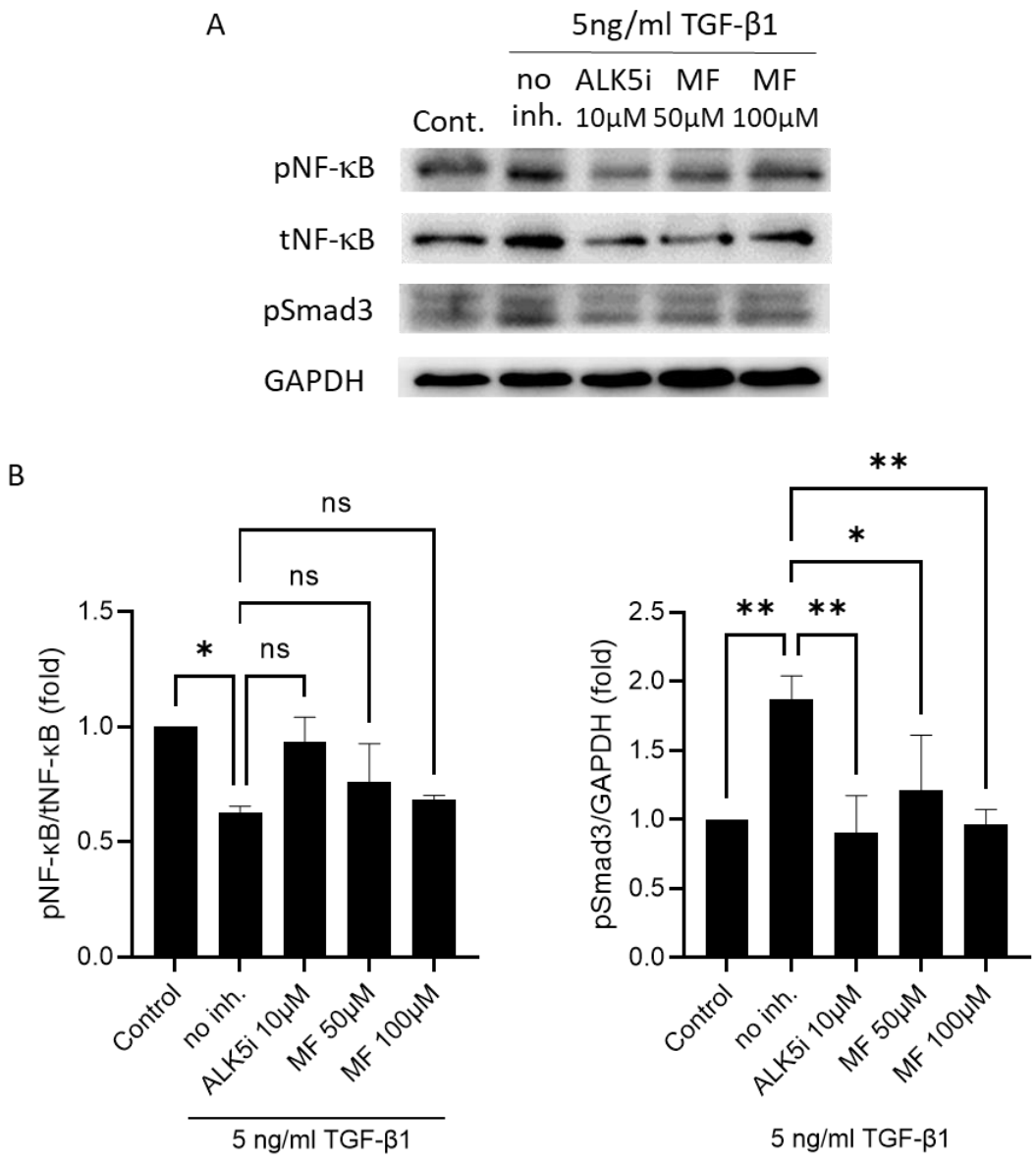


Figure 27. The effect of mangiferin on the mechanism of TGF-β1-induced fibrosis. RPTEC cells were treated in the same protocol described in Figure 25. The cell lysate was collected for determination of phospho-NF-κB and phospho-Smad3 proteins by Western blot analysis (A). The band intensity of NF-κB (B) and e-Smad3 (C) from Western blotting were determined using ImageJ software. *p<0.05, **p<0.01. Data are mean ± S.D., n=3.

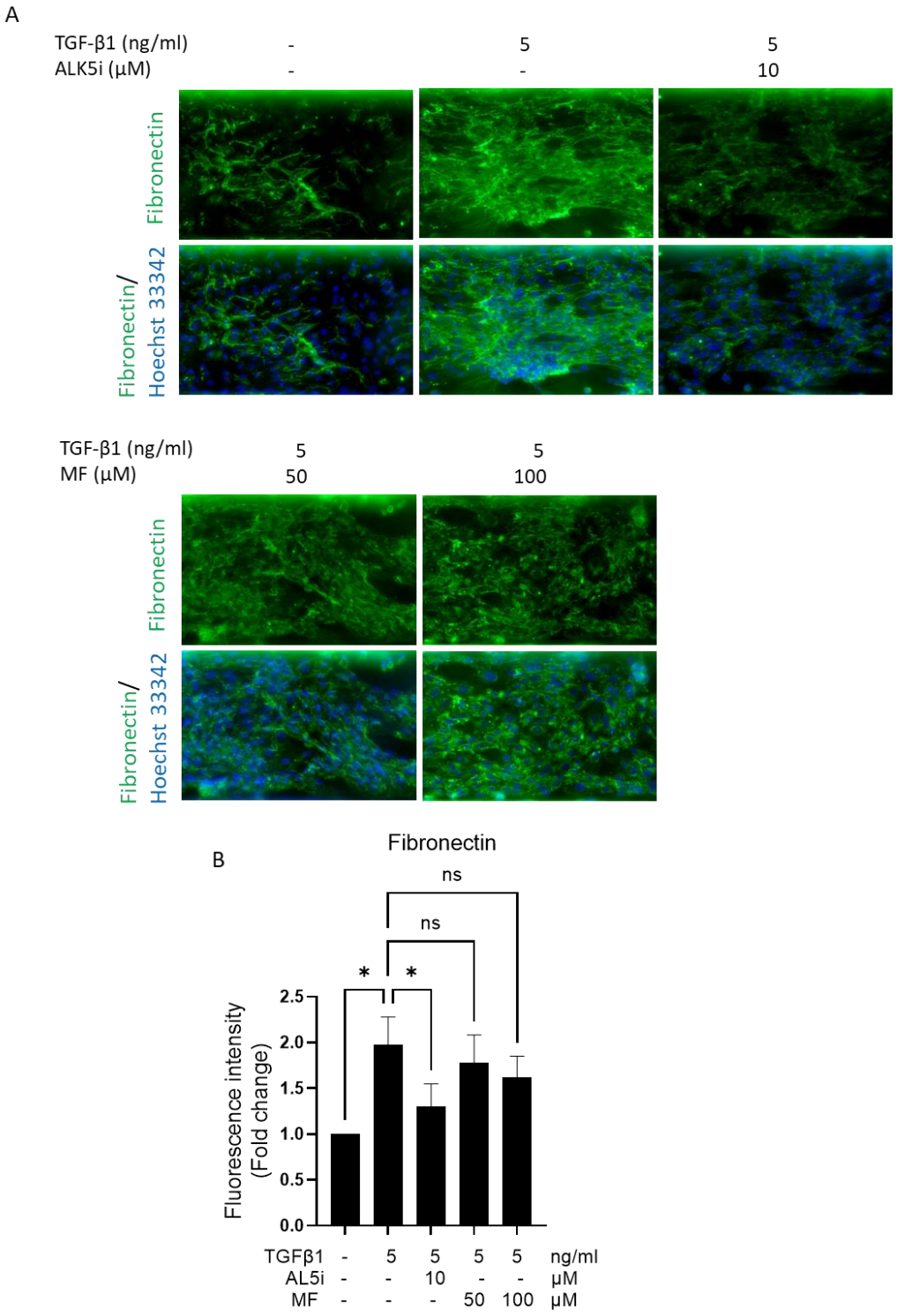


Figure 28. The effect of mangiferin on the expression of TGF-β1-induced the alteration of fibronectin in 3D platform of RPTEC cells. The cells were treated in the same protocol described in Figure 25 but the treatment was applied on both basolateral and apical sides of the cells. The fibronectin was detected by immunofluorescence (in green) under microscopy. The intensity of fibronectin (B) was calculated using ImageJ software. *p<0.05. Data are mean ± S.D.

The induction of fibrosis was also performed in 3D-micrifluidic platform of proximal tubule-on-a-chip. The fibronectin expression was detected by the anti-fibronectin antibody in this model. TGF-β1 induced the accumulation of fibronectin in this platform, the positive inhibitor ALK5 was inhibited the increased fibronectin by TGF-β1, however, pre-treated mangiferin for 3 days did not alter the TGF-β1-induced fibronectin expression, as seen in Figure 28. The failure of mangiferin treatment may be the accessibility of compound in this model.

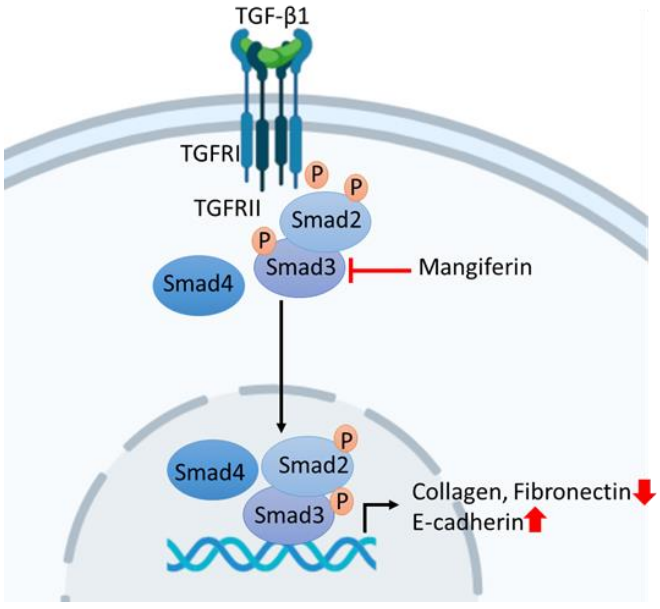
The action of mangiferin itself may not be strong enough if it was reached to the cells with lower amount in the 3D construct of the tubule, when compared to direct compound application to the cells in 2D platform. In addition, the RPTEC cell lines we used in this study did not express organic anion transporters OAT1 and OAT3 that may help to taken up mangiferin into the cells since our results of mangiferin interaction with OATs was demonstrated. RPTEC cells express OCT2, but mangiferin was less interact with OCT2. There may also have other factors that we could not investigate at this moment for the transport in 3D model and might be the pitfall of the study.

Conclusion and Suggestion

In this project, we aimed to study the pharmacological activities of mangiferin in 2 aspects, the first one is the action on glucose absorption in the cells and the second is the anti-fibrotic effect of mangiferin on the human renal proximal tubular epithelial cells.

The experiments were performed using renal proximal tubular epithelial cells cultured in conventional 2D platform and microfluidic 3D platform (proximal tubule-on-a-chip). The mangiferin action on glucose absorption in this RPTEC cells could not be concluded because of the lacking or very low SGLT2 protein expression in this cell type. The treatment of the cells with high glucose condition (30 mM D-glucose) and the condition that imitate diabetes (high glucose with insulin and TNF-α) in 2D culture platform caused the increase in inflammatory cytokines IL-1β, IL-6 and profibrotic markers TGF-β1. However, the treatment conditions did not affect the cells cultured in 3D platform. Mangiferin demonstrated the reduction of inflammatory cytokines induced by high glucose and diabetes conditions in 2D culture platform and also inhibited TGF-β1 induced fibrosis in 2D, not 3D platform.

The main finding from this project is the anti-fibrotic activity of mangiferin on the inhibition of cytokine release during the cells were exposed to high glucose (diabetes). The proposed mechanism of mangiferin is via the inhibition of phosphorylation of Smad3. In general, the binding of TGF-β1 on its receptor results in the receptor dimerization and activate phosphorylation of Smad2/3 complex and move to nucleus to increase the expression of ECM proteins involving for the induction of ECM accumulation resulting in cell fibrosis. The infographic summary is as follow:



The limitation of this project

RPTEC cells was used to established the disease model of the renal proximal tubule cell injury and fibrosis, even though the cells lack some drug transporters and also sodium-glucose co-transporters. The induction of cell injury by using high concentration of glucose and diabetic condition was possible in the 2D culture, but not in 3D culture platform that the polarization of the cells may exist. By the fact that exposure of the cells in high glucose condition at both apical and basolateral sides in 3D-platform should increase the ability of glucose entering the cells via the glucose transporters and should increase the possibility of cell inflammation/ injury but the finding in this study was in the opposite way. There may lack some factors to stimulate transporter expression, may be from contacting with other cell types. The 3D culture of renal proximal tubular cell with renal endothelial cells might be in the challenging to imitate the existing of peritubular capillaries for further studied to imitate the real environment of the tubule in our body.

With lacking glucose transporter expression, especially SGLT2, in the cell used in this study we cannot conclude the action of mangiferin on glucose absorption. The protective effect of mangiferin on the proximal tubule injury and fibrosis observed in this research project with conventional 2D platform culture of RPTEC might be the benefit to use it as lead compound for further drug development and may also be the scientific evidence supporting activities of mangiferin in the human renal cells that may be translate to human better than the study using other species.

The present study had many limitations to translate to real epithelial cells of human proximal tubule. The appropriate cell model is still needed and researchers are looking for it. If the establishment of the renal proximal tubular cells succeed in the near future, the information using proximal tubule will be expanded. That is the hope of researcher in this field.

References

1. Chen L, Magliano DJ, Zimmet PZ. The worldwide epidemiology of type 2 diabetes mellitus-present and future perspective. *Nat. Rev. Endocrinol*. 2012; 8: 228–236.
2. Lim A. Diabetic nephropathy - complications and treatment. *Int J Nephrol Renovasc Dis*. 2014; 7:361-81.
3. Satirapoj B. Tubulointerstitial biomarkers for diabetic nephropathy. *J Diabetes Research* 2018; 2852398.
4. Fioretto P, Mauer M. Histopathology of diabetic nephropathy. *Semin Nephrol*. 2007; 27(2): 195–207.
5. Gellman DD, Pirani CL, Soothill JF, Muehrcke RC, Kark RM. Diabetic nephropathy: a clinical and pathologic study based on renal biopsies. *Medicine* (*Baltimore*). 1959; 38: 321–367
6. Ziyadeh FN, Snipes ER, Watanabe M, et al. High glucose induces cell hypertrophy and stimulates collagen gene transcription in proximal tubule. *Am J Physiol*. 1990; 259(4 Pt2): F704–F714.
7. Xu Q, Norman JT, Shrivastav S, Lucio-Cazana J, and Kopp JB. In vitro models of TGF-β-induced fibrosis suitable for high-throughput screening of antifibrotic agents. *Am J Physiol Renal Physiol* 2007; 293: F631–F640.
8. Guang-Kai X, Chen-Yu S,Xiao-Ying Q, Yu H, Yi L, Guo-Yong X and Min-Jian Q. Effects of ethanol extract of Bombax ceiba leaves and its main constituent Mangiferin on diabetic nephropathy in mice. *Chin J Nat Med*. 2017; 15(8): 597-605.
9. Pal PB, Sinha K and Sil PC: Mangiferin attenuates diabetic nephropathy by inhibiting oxidative stress mediated signaling cascade, TNFα related and mitochondrial dependent apoptotic pathways in streptozotocin-induced diabetic rats. *PLoS One* 2014; 9(9): e107220.
10. Tang SC and Lai KN. The pathogenic role of the renal proximal tubular cell in diabetic nephropathy. *Nephrol Dial Transplant* 2012; 27(8): 3049-56.
11. Aschauer L, Carta G, Vogelsang N, Schlatter E, Jennings P. Expression of xenobiotic transporters in the human renal proximal tubule cell line RPTEC/TERT1. *Toxicol In Vitro*. 2015; 30(1 Pt A): 95-105.
12. Simon-Friedt BR, Wilson MJ, Blake DA, Yu H, Eriksson Y, Wickliffe JK. The RPTEC/TERT1 Cell Line as an Improved Tool for In Vitro Nephrotoxicity Assessments. *Biol Trace Elem Res*. 2015; 166(1): 66-71.
13. Shah H, Patel M, Shrivastava N. Gene expression study of phase I and II metabolizing enzymes in RPTEC/TERT1 cell line: application in *in vitro* nephrotoxicity prediction. *Xenobiotica.* 2017; 47(10): 837-843.

14. Secker PF, Luks L, Schlichenmaier N, Dietrich DR. RPTEC/TERT1 cells form highly differentiated tubules when cultured in a 3D matrix. *ALTEX.* 2018; 35(2): 223-234.
15. Wilmer MJ, Ng CP, Lanz HL, Vulto P, Suter-Dick L, Masereeuw R. Kidney-on-a-Chip Technology for Drug-Induced Nephrotoxicity Screening. *Trends Biotechnol*. 2016; 34(2): 156-170.
16. Weber EJ, Chapron A, Chapron BD, Voellinger JL, Lidberg KA, Yeung CK, Wang Z, Yamaura Y, Hailey DW, Neumann T, Shen DD, Thummel KE, Muczynski KA, Himmelfarb J, Kelly EJ. Development of a microphysiological model of human kidney proximal tubule function. *Kidney Int.* 2016; 90(3): 627-637.
17. Du S, Liu H, Lei T, Xie X, Wang H, He X, Tong R and Wang Y. Mangiferin: An effective therapeutic agent against several disorders (Review). *Molecular Medicine Report* 2018; 18: 4775-4786.
18. Rauf A, Imranb M and Patel S. Mangiferin: A phytochemical with panaceau potential. *Biomedicine & Pharmacotherapy* 2017; 96: 1562-1564.
19. Hou S, Wang F, Li YM, Li Y, Wang M, Sun D, Sun C. Pharmacokinetic study of mangiferin in human plasma after oral administration. *Food Chemistry*. 2012; 132: 289–294.
20. Kammalla AK, Ramasamy MK, Inampudi J, Dubey GP, Agrawal A, Kaliappan I. Comparative pharmacokinetic study of mangiferin after oral administration of pure mangiferin and US patented polyherbal formulation to rats. *AAPS PharmSciTech.* 2015; 16(2): 250-8.
21. Yoosook C, Bunyapraphatsara N, Boonyakiat Y and Kantasuk C. Anti-herpes simplex virus activities of crude water extracts of Thai medicinal plants. *Phytomedicine* 2000; 6(6): 411-419.
22. Chancharunee S. Structure modification of Mangiferin for using as biological active compounds. TRF grant #RDG5220049; August 1, 2009.
23. Miura T, Ichiki H, Iwamoto N, Kato M, Kubo M, Sasaki H, Okada M, Ishida T, Seino Y and Tanigawa K. Antidiabetic activity of the Rhizoma of *Anemarrhena asphodeloides* and active components, mangiferin and its glucoside. *Biol Pharm Bull*. 2001; 24(9): 1009-1011.
24. Liu YW, Zhu X, Zhang L, Lu Q, Wang JY, Zhang F, Guo H, Yin JL and Yin XX: Up-regulation of glyoxalase 1 by mangiferin prevents diabetic nephropathy progression in streptozotocin-induced diabetic rats. *Eur J Pharmacol.* 2013; 721: 355-364.
25. Wang X, Gao L, Lin H, Song J, Wang J, Yin Y, Zhao J, Xu X, Li Z and Li L. Mangiferin prevents diabetic nephropathy progression and protects podocyte function via autophagy in diabetic rat glomeruli. *Eur J Pharmacol.* 2018; 824: 170-178.

26. Zhu X, Cheng YQ, Du L, Li Y, Zhang F, Guo H, Liu YW and Yin XX: Mangiferin attenuates renal fibrosis through down-regulation of osteopontin in diabetic rats. *Phytother Res.* 2015; 29: 295-302.
27. Secker PF, Luks L, Schlichenmaier N, Dietrich DR. RPTEC/TERT1 cells form highly differentiated tubules when cultured in a 3D matrix. ALTEX. 2018; 35(2): 223-234.
28. Jang KJ, Mehr AP, Hamilton GA, McPartlin LA, Chung S, Suh KY, Ingber DE. Human kidney proximal tubule-on-a-chip for drug transport and nephrotoxicity assessment. *Integr Biol (Camb).* 2013; 5(9): 1119-1129.
29. Vormann MK, Gijzen L, Hutter S, Boot L, Nicolas A, et al. Nephrotoxicity and kidney transport assessment on 3D Perfused Proximal Tubules. *AAPS J.* 2018; 20(5): 90.
30. Vriend J, Peters JGP, Nieskens TTG, Skovronova R, Blaimschein N, et al. Flow stimulates drug transport in a human kidney proximal tubule-on-a-chip independent of primary cilia. *Biochim Biophys Acta - General Subjects*. 2020; 1864: 129433.
31. Vallon V and Thomson SC. The tubular hypothesis of nephron filtration and diabetic kidney disease. *Nat Rev Nephrol*. 2020.; 16**(**6): 317-336.
32. Gilbert RE. Proximal Tubulopathy*:* Prime mover and key therapeutic target in diabetic kidney disease. *Diabetes*. 2017; 66(4): 791-800.
33. Nihalani D and Susztak K. Sirt1-Claudin-1 crosstalk regulates renal function. *Nat Med*. 2013; 19(11): 1371-1372.
34. Simonson MS. Phenotypic transitions and fibrosis in diabetic nephropathy. *Kidney Int.* 2007; 71(9): 846-54.
35. Loeffler I and Wolf G. Mechanisms of interstitial fibrosis in diabetic nephropathy. In J.J. Roelofs JJ and Vogt L, editors. Diabetic nephropathy*:* Pathophysiology and clinical aspects. Cham: Springer International Publishing; 2019. p. 227-251.
36. Vallon V and Thomson SC. Renal function in diabetic disease models*:* the tubular system in the pathophysiology of the diabetic kidney. *Ann Rev Physiol*. 2012; 74: 351-375.
37. McVicker BL and Bennett RG. Novel anti-fibrotic therapies. *Front Pharmacol.* 2017; 8: 318.
38. Klinkhammer BM. Goldschmeding R, Floege J, Boor P. Treatment of renal fibrosis-Turning challenges into opportunities. *Adv Chronic Kidney Dis.* 2017; 24(2): 117-129.
39. Levey AS, Atkins R, Coresh J, Cohen EP, Collins AJ, et al. Chronic kidney disease as a global public health problem: Approaches and initiative – a position statement from Kidney disease improving global outcomes. *Kidney Int.* 2007; 72(3): 247-259.

40. Levey AS, Eckardt KU, Tsukamoto Y, Levin A, Coresh J, et al. Definition and classification of chronic kidney disease*:* A position statement from Kidney Disease*:* Improving Global Outcomes *(*KDIGO*). Kidney Int.* 2005; 67(6): 2089-2100.
41. Stevens LA, Coresh J, Greene T, Levey AS, et al. Assessing kidney function--measured and estimated glomerular filtration rate*. N Engl J Med*. 2006; 354(23): 2473-83.
42. Hallan SI, Coresh J, Astor BC, Asberg A, Powe NR, Romundstad S, Hallan HA, Lydersen S, Holmen J. International Comparison of the Relationship of Chronic Kidney Disease Prevalence and ESRD Risk*. J Am Soc Nephrol.* 2006; 17(8): 2275-2284.
43. Cho ME and Kopp JB. Pirfenidone*:* an anti-fibrotic therapy for progressive kidney disease*. Expert Opin Investig Drugs*. 2010; 19(2): 275-283.
44. Kok HM, Falke LL, Goldschmeding R, Nguyen TQ. Targeting CTGF, EGF and PDGF pathways to prevent progression of kidney disease*. Nat Rev Nephrol*. 2014; 10(12): 700-711.
45. Manson SR, Niederhoff RA, Hruska KA, Austin PA. Endogenous BMP-7 is a critical molecular determinant of the reversibility of obstruction-induced renal injuries*. Am J Physiol Renal Physiol.* 2011; 301(6): F1293-1302.
46. Herken R, Fussek M, Zarfl A. Localization of fucosyl moieties in the mouse renal cortex by lectin histochemistry using the fucose binding lectins LTA and UEA I and by autoradiography using 3H-labelled fucose. *Histochemistry*. 1988; 89(5): 505-508.
47. Silva FG, Nadasdy T, Laszik Z. Immunohistochemical and lectin dissection of the human nephron in health and disease *.Arch Pathol Lab Med.* 1993; 117)12 :(1233-1239.
48. Ishikawa I, Horiguchi T, Sikura N. Lectin peroxidase conjugate reactivity in acquired cystic disease of the kidney. *Nephron*. 1989; 51)2 :(211-214.

ภาคผนวก (Appendix)

การเผยแพร่ผลงาน

เนื้อหาบางส่วนของงานวิจัยนี้ เคยนำเสนอแบบโปสเตอร์ ในการประชุมวิชาการวิทยาศาสตร์การแพทย์ ครั้งที่ 30 ระหว่างวันที่ 22-24 มิถุนายน 2565 ณ โรงแรมริชมอนด์ นนทบุรี

บทคัดย่อ ที่ส่งไปนำเสนอแสดง ดังภาพด้านล่าง

P2-40 ฤทธิ์ของสารแมงจิเฟอรินต่อการลดการเกิดพังผืดในเซลล์ท่อไตส่วนต้นของคน

Protective effect of mangiferin on renal fibrosis in human renal proximal tubular epithelial cells

พร้อมสุข ชุตาภา[1,*], วันอังคาร พูลศรี[1], กรกมล อักษร[1], อุทัย วิชัย[2]

Promsuk Jutabha[1,*], Wanangkan Poolsri[1], Konkamol Aksorn[1], Uthai Wichai[2]

[1]สถาบันการแพทย์จักรีนฤบดินทร์ คณะแพทยศาสตร์โรงพยาบาลรามาธิบดี มหาวิทยาลัยมหิดล สมุทรปราการ

[2]ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยนเรศวร พิษณุโลก

[1]Chakri Naruebodindra Medical Institute, Faculty of Medicine Ramathibodi Hospital, Mahidol University, Samut Prakan

[2]Department of Chemistry, Faculty of Science, Naresuan University, Phitsanulok

บทคัดย่อ

การเกิดพังผืดที่ไตพบได้ในระยะสุดท้ายของการดำเนินของโรคไตเรื้อรัง ซึ่งอาจมีสาเหตุจากการบาดเจ็บหรือการอักเสบเรื้อรังที่ไต การป้องกันหรือชะลอการเกิดพังผืดที่ไตเป็นสิ่งสำคัญที่จะลดการสูญเสียชีวิตได้ การยับยั้งกระบวนการส่งสัญญาณของ transforming growth factor beta 1 (TGF-β1) ซึ่งเป็นปัจจัยเหนี่ยวนำให้เกิดการสร้างพังผืด อาจป้องกันหรือชะลอการเกิดพังผืดที่ไตได้ สารแมงจิเฟอรินซึ่งเป็นสารสำคัญที่พบได้ในมะม่วงและมีการศึกษาก่อนหน้านี้ว่ามีฤทธิ์ต้านการอักเสบและลดเบาหวานในหนูทดลอง แต่ยังไม่มีการศึกษาผลของสารนี้ในเซลล์ท่อไตของคน ในงานวิจัยนี้ศึกษาผลการลดการเกิดพังผืดในไตโดยใช้โมเดล TGF-β1 เหนี่ยวนำให้เกิดพังผืดในเซลล์ท่อไตส่วนต้นของคน พบว่าแมงจิเฟอรินที่ความเข้มข้น 50-100 µM สามารถลดการสะสมคอลลาเจน ไฟโบรเนคตินที่เป็นตัวบ่งชี้การเกิดพังผืดที่เซลล์ได้เมื่อเปรียบเทียบกับเซลล์ที่ได้รับ TGF-β1 แต่ไม่ได้รับแมงจิเฟอริน อีกทั้งยังพบว่าแมงจิเฟอรินสามารถเพิ่มปริมาณ e-cadherin ซึ่งเป็นโปรตีนเชื่อมเซลล์เยื่อบุผิวที่ลดลงไปเมื่อเกิดพังผืดให้กลับคืนมาได้เมื่อเทียบกับภาวะปกติ ดังนั้นการศึกษาครั้งนี้แสดงให้เห็นว่าสารแมงจิเฟอรินมีศักยภาพที่จะพัฒนาเป็นยาเพื่อลดการเกิดพังผืดที่ไต

คำสำคัญ: แมงจิเฟอริน, การเกิดพังผืดที่ไต, เซลล์ท่อไตส่วนต้น

Abstract

Renal fibrosis is known to be the end stage of chronic kidney diseases initiated by various causes including renal injury or chronic inflammation. The effective drug for delayed progression of renal fibrosis is needed. To attenuate the progression of renal disease to its end stage, the inhibition of transforming growth factor beta 1 (TGF-β1) pathway which induced fibrogenesis has believed to be a potential therapeutic for renal fibrosis. Mangiferin, an active ingredient found in mango tree, had previously reported for anti-inflammatory and anti-diabetic effects in rats. There is no study about anti-fibrotic effect of mangiferin in human renal epithelial cells. In this study, TGF-β1-induced fibrosis was performed in human renal proximal tubular epithelial cells (RPTEC) as a model for renal tubulointerstitial fibrosis. Mangiferin at 50-100 µM reduced collagen accumulation and profibrotic marker fibronectin in the model RPTEC compared with non-treated cells and also recovered e-cadherin, an adhesion molecule in epithelial cells, which lowered in epithelial fibrosis. Our study indicates that mangiferin has potential for further drug development for prevention of renal fibrogenesis.

Keywords: mangiferin, renal fibrosis, renal proximal tubule epithelial cells

Corresponding author: promsuk.jut@mahidol.ac.th

โปสเตอร์ที่นำเสนอในงานประชุมวิชาการวิทยาศาสตร์การแพทย์ ครั้งที่ 30 แสดงดังภาพด้านล่าง

Protective effect of mangiferin on renal fibrosis in human renal proximal tubular epithelial cells

Promsuk Jutabha[1,*], Wanangkan Poolsri[1], Konkamol Aksorn[1], Uthai Wichai[2]

[1]Chakri Naruebodindra Medical Institute, Faculty of Medicine Ramathibodi Hospital, Mahidol University, Samut Prakan,

[2]Department of Chemistry, Faculty of Science, Naresuan University, Phitsanulok *Corresponding author: promsuk.jut@mahidol.ac.th

Abstract

Renal fibrosis is known to be the end stage of chronic kidney diseases initiated by various causes including renal injury or chronic inflammation. The effective drug for delayed progression of renal fibrosis is needed. To attenuate the progression of renal disease to its end stage, the inhibition of transforming growth factor beta 1 (TGF-β1) pathway which induced fibrogenesis has believed to be a potential therapeutic for renal fibrosis. Mangiferin, an active ingredient found in mango tree, had previously reported for anti-inflammatory and anti-diabetic effects in rats. There is no study about anti-fibrotic effect of mangiferin in human renal epithelial cells. In this study, TGF-β1-induced fibrosis was performed in human renal proximal tubular epithelial cells (RPTEC) as a model for renal tubulointerstitial fibrosis. Mangiferin at 50-100 µM reduced collagen accumulation and profibrotic marker fibronectin in the model RPTEC compare with non-treated cells and also recovered e-cadherin, an adhesion molecule in epithelial cells, which lowered in epithelial fibrosis. Our study indicates that mangiferin has potential for further drug development for prevention of renal fibrogenesis.

Introduction

The transforming growth factor beta 1 (TGF-β1) is one of the major player of tubulointerstitial fibrosis induction. Moreover, the most potent anti-fibrotic agents targeting the TGF-β1 inhibition (1,2) but the effectiveness of anti-fibrotic therapies in humans has been limited. Thus, this study aim to figure out an alternative treatment for renal fibrosis. The natural compounds have been investigated for health benefits and disease prevention. Some have potential to be effective remedy for anti-fibrosis. Mangiferin is an active ingredient found in mango tree. According to the previous studies, the anti-inflammatory and anti-diabetic effect of mangiferin had been demonstrated in rats (3), but the efficacy of anti-fibrotic effect in human renal epithelial cells has not been reported. Therefore, the present study focuses on finding the therapeutic effects, anti-fibrotic effect of mangiferin for alternative prevention of renal fibrosis and may be useful information for the future treatment in kidney disease patients.

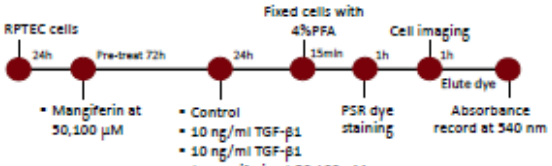
Objective

To investigate the anti-fibrotic effect of mangiferin in human renal proximal tubular epithelial cells.

Methods

- ❑ Investigation of toxicity of mangiferin by MTT assay
- ❑ Study of the mangiferin effect on collagen accumulation by Picro Sirius Red (PSR) staining
- ❑ Evaluation of mangiferin effect on profibrotic protein expression by WB

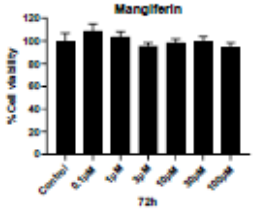
PSR staining is presented as a method for determination of collagen deposition.



ALK5i (ALK5 inhibitor): an inhibitor of TGF-β type I receptor kinase

Results

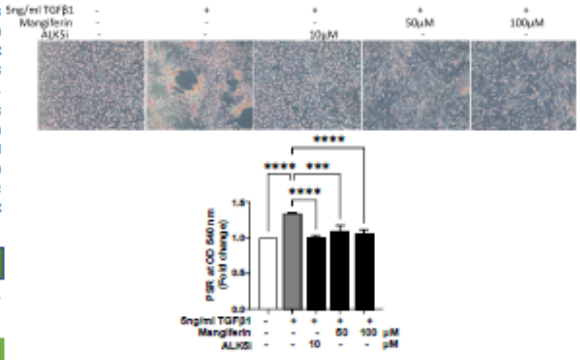
1. Effect of mangiferin on the cell viability



RPTEC cells exposed to various concentrations of Mangiferin (0.1, 1, 3, 10, 30 and 100 µM) for 3 days did not show the alteration of their cell viability (n=3)

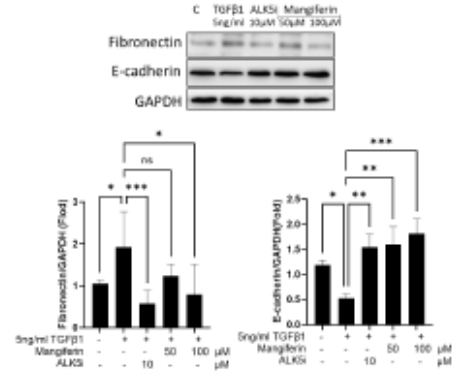
Results (cont.)

2. Effect of mangiferin on collagen deposition



Mangiferin at concentrations of 50 and 100 µM inhibited the collagen deposition in RPTEC cells. The collagen determination was performed using PSR staining assay (***p<0.001,****p<0.0001, n=3)

3. Effect of mangiferin on the protein expression of profibrotic markers



Mangiferin recovered proteins expression of fibronectin and E-cadherin in TGF-β1-induced renal fibrosis in RPTEC cells. (*p<0.05,**p<0.01, ***p<0.001 n=3)

Discussion and Conclusion

The present study exhibits the anti-fibrotic effect of mangiferin. It attenuated renal fibrogenesis through the inhibition of collagen deposition, the reduction of the profibrotic markers and the restoration of epithelial E-cadherin protein in human renal epithelial cells. The mechanisms in deep should be further clarified. The study indicates that mangiferin might prevent renal fibrogenesis and may be a potential lead compound for the development of future novel therapeutic agents for kidney disease patients.

Acknowledgements

This research work was supported by the Thailand Science Research and Innovation and National Research Council of Thailand (RDG6220034) and Faculty of Medicine Ramathibodi Hospital, Mahidol University.

References

1. Cho, M.E. and Kopp, J.B. (2010). Pirfenidone: an anti-fibrotic therapy for progressive kidney disease. Exp Opin Invest Drugs. 19(2): 275-283.
2. Kok, H.M., et al. (2014). Targeting CTGF, EGF and PDGF pathways to prevent progression of kidney disease. Nat Rev Nephrol. 10(12): 700-11.
3. Pal, P.B., Sinha, K. and Sil, P.C. (2014). Mangiferin attenuates diabetic nephropathy by inhibiting oxidative stress mediated signaling cascade, TNFα related and mitochondrial dependent apoptotic pathways in streptozotocin-induced diabetic rats. PLoS One. 9(9): e107220.

ตารางเปรียบเทียบวัตถุประสงค์ กิจกรรมที่วางแผนไว้ และ ที่ดำเนินการไปแล้ว ผลที่ได้รับตลอดโครงการ

1.2วัตถุประสงค์	กิจกรรมที่วางแผนไว้	กิจกรรมที่ดำเนินการแล้ว
1. To investigate the glucose lowering effect of mangiferin on human renal proximal tubular epithelial cells.	**1.1.Characterization of the RPTEC cells on 2D (transwell culture) and 3D microfluidic culture.** -Stain RPTEC cells with lotus lectin (proximal tubule marker), Na^+/K^+ ATPase, ZO-3, SGLT1 and 2, OAT1 and 3 and visualize them by confocal fluorescence microscopy	All are examined as the plan but ZO-1 was used instead of ZO-3
	1.2.Evaluating appropriate non-toxic concentrations of mangiferin to use in the glucose uptake experiment. -RPTEC cells will be cultured in 96-well plate and incubated with different concentrations of mangiferin at the varying incubation time to be test in further experiments. The cell viability will be determined using MTT assay.	Already performed the test
	1.3.Determination of glucose uptake by RPTEC cells and the inhibitory effect of mangiferin -The uptake of fluorescence-tagged glucose will be performed in 2D culture if there is SGLT2 transporter is detected in 1.1 and mangiferin inhibition of glucose uptake will be performed with varying concentration of mangiferin to determine its IC50, 0.5 µM empagliflozin (SGLT2 inhibitor) will be used as positive control. The inhibitory effect of mangiferin on glucose uptake will also be confirmed in 3D-microfluidic culture that mimic	Already performed the uptake of glucose in RPTEC cells but the IC50 with SGLT2 could not obtain because of very low expression of SGLT2 on the cell membrane The interaction of mangiferin with OATs and OCT2 were performed instead.

1.2วัตถุประสงค์	กิจกรรมที่วางแผนไว้	กิจกรรมที่ดำเนินการแล้ว
	physiological condition of renal proximal tubule **1.4. To test whether mangiferin reduce expression of glucose transporters in RPTEC cells that expose to normal and high glucose condition.** - RPTEC cells will be incubated in normo (5 mM) and high (30 mM) glucose conditions (osmolarity control with 5 mM glucose + 25 mM mannitol) for 0, 1, 3, 5, 7 days and determine expression of SGLT1, SGLT2 and GLUT2 by WB	Quantitative RT-PCR of glucose transporters was performed instead of western blot because of undetectable glucose transporters on the plasma membrane, the detection of mRNA was the best way for this situation.
2.To provide scientific evidence to support the protective effect of mangiferin on human renal proximal tubular epithelial cells	**2.1.Determination of the protective effect of mangiferin on high glucose-induced human renal proximal tubular epithelial cell injury (early stage before fibrosis)** -Pre-incubate RPTEC with 0, 10, 100 μM mangiferin for 1 wk then exposure to normo (5 mM) and high (30 mM) glucose for 7 days with continuing mangiferin treatment -Determination of proximal tubular injury marker KIM-1, LDH release and inflammatory cytokine IL-1, NF-kB, TGF-β1	The time points were varying to select an appropriate one and continued to check the alteration of each parameter during high glucose treatment and the condition imitate diabetic condition was added to the study in order to cover the component exist in blood of diabetes patient. IL-6 was also added to use instead of NF-kB The fibronectin, COL 1, a-SMA were also included in the parameters.
	2.2.To investigate the protective effect of mangiferin on IL-1 induced inflammation and fibrosis of renal proximal tubular epithelial cells -Establish condition for inducing fibrosis by exposure of RPTEC with recombinant IL-1 β -Pre-incubate RPTEC with 0, 10, 100 μM mangiferin for 1 wk, then	Already test condition with varying concentrations and exposure times but the consistency and the effect of IL-1 β to induce fibrosis during screening the accumulation of collagen was not significant

1.2วัตถุประสงค์	กิจกรรมที่วางแผนไว้	กิจกรรมที่ดำเนินการแล้ว
	expose RPTEC with recombinant IL-1β (concentration and time that will be used depending on result above) together with continuing mangiferin treatment -Measure COL1, COL4, FN, α-SMA expression by WB **2.3.To investigate the protective effect of mangiferin on TGF-β1 induced fibrosis of renal proximal tubular epithelial cells** -Establish condition for inducing fibrosis by exposure of RPTEC with TGF-β1 10 ng/ml for 24-48 hr -Pre-incubate RPTEC with 0, 10, 100 μM mangiferin for 1 wk, then expose RPTEC with TGF-β1 10 ng/ml for 24 or 48 hr depending on result above with continuing mangiferin treatment -Measure COL1, COL4, FN, α-SMA expression by WB	difference, so the effect of mangiferin was not performed with this condition The induction of fibrosis was already established and the effect of mangiferin on this condition was tested. And measured the alteration of COL1,4 by PSR staining, detected FN by WB and IF Also test the condition in 3D-platform.

ผลที่ได้รับตลอดโครงการ

กิจกรรม (activities)	ผลที่ได้รับ (outputs)
1.1-1.2	Check the expression of transporters and found that OATs and SGLTs are missing in RPTEC cells and the culturing on different platform did not improve transporter expression
1.3-1.4	- Glucose uptake was tested and only function of GLUT appeared but lack SGLT2 function related to the loss of expression checked by IF. Therefore, the action of mangiferin on SGLT2 was not obtained. We checked the interaction with OATs and OCT2, instead, and the IC50s with OATs and OCT2 were obtained.
1.4-2.1	- The early-stage model of fibrosis with high glucose was set up and protective effect of mangiferin can be tested and found the inhibition of cytokine release-induced by high glucose condition.
2.2-2.3	- The fibrosis condition was successfully set up with TGF-β1 and protective effect of mangiferin was observed by the inhibition of collagen accumulation and recovered the fibronectin and the alteration of e-cadherin.

กิจกรรม (activities)	ผลที่ได้รับ (outputs)
	- The addition of mechanism of mangiferin was examined and found the suppression of Smad pathway.