ROLE OF KCa3.1 CHANNEL ON RENAL FIBROSIS IN DOXORUBICIN-INDUCED FELINE
KIDNEY CELL LINE

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A Thesis Submitted in Partial Fulfillment of the Requirements
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บทบาทของ KCa3.1 CHANNEL ในการเกิดพังผืดที่ไตในเซลล์ไตแมวเพาะเลี้ยงที่ถูกเหนี่ยวนำด้วย
DOXORUBICIN

น.ส.เพ็ญพิชชา เกิดบ้านตะเคียน

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Thesis Title	ROLE OF KCa3.1 CHANNEL ON RENAL FIBROSIS IN DOXORUBICIN-INDUCED FELINE KIDNEY CELL LINE
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เพ็ญพิชชา เกิดบ้านตะเคียน : บทบาทของ KCa3.1 CHANNEL ในการเกิดพังผืดที่ไตในเซลล์
ไตแมวเพาะเลี้ยงที่ถูกเหนี่ยวนำด้วย DOXORUBICIN . (ROLE OF KCa3.1 CHANNEL ON
RENAL FIBROSIS IN DOXORUBICIN-INDUCED FELINE KIDNEY CELL LINE) อ.ที่ปรึกษา
หลัก : รศ. ดร.รสมา ภู่สุนทรธรรม, อ.ที่ปรึกษาร่วม : ศ.ฉัตรศรี เดชะปัญญา

การเกิดผังพืดในไตแมวเป็นรอยโรคที่พบได้มากที่สุดในแมวป่วยด้วยโรคไตวายเรื้อรัง ช่อง
ไอออนโพแทสเซียม ชนิดโพแทสเซียมแคลเซียม 3.1 มีบทบาทสำคัญในการรักษาในการเกิดภาวะผังพืด
ของไตในปัจจุบันในสัตว์หลายชนิด แต่ยังไม่มีการศึกษาเกี่ยวกับความสัมพันธ์ของช่องไอออนชนิดนี้กับ
การเกิดภาวะผังพืดในไตแมว การศึกษานี้ทำการศึกษาความสัมพันธ์ของช่องไอออนโพแทสเซียม ชนิด
โพแทสเซียมแคลเซียม 3.1 กับการเกิดผังพืดในเซลล์ไตแมวที่ถูกเหนี่ยวนำให้เกิดภาวะไตเรื้อรังด้วย
Doxorubicin และมีการใช้ไตรเอริลมีเทน-34 (Triarymethane-34) ซึ่งยับยั้งการทำงานของ ช่องไอออน
โพแทสเซียม ชนิดโพแทสเซียมแคลเซียม 3.1 และวัดการแสดงออกของโปรตีนที่เกี่ยวของกับการเกิดผัง
พืดในวิถี TGF-β/Smad ได้แก่ ตัวรับของ ทรานส์ฟอร์มมิง-โกรทแฟคเตอร์เบตา ชนิดที่ 2 (TGF-β
receptor type 2) โปรตีน Smad และ อัลฟาสมูทมัสเซิล แอคติน (α-Smooth muscle actin) ควบคู่
ไปกับการดูร้อยละของเซลล์มีชีวิตในระหว่างการทดลอง ผลการศึกษาพบว่ากลุ่มที่ได้รับ ไตรเอริลมีเทน-
34 ก่อนการเหนี่ยวนำให้เกิดผังพืดด้วยสาร Doxorubicin มีร้อยละของเซลล์ที่มีชีวิตไม่แตกต่างจากเซลล์
ในกลุ่มควบคุม ในขณะที่เซลล์ในกลุ่มที่ได้รับการเหนี่ยวนำด้วย Doxorubicin เพียงอย่างเดียวมีร้อยละ
ของเซลล์มีชีวิตลดลงอย่างมีนัยสำคัญทางสถิติ ส่วนทรานส์ฟอร์มมิง-โกรทแฟคเตอร์เบตา ชนิดที่ 2 และ
โปรตีน Smad ในแต่ละกลุ่มของการทดลองไม่มีความแตกต่างกันระหว่างกลุ่ม แต่โปรตีน อัลฟาสมูท
มัสเซิลแอคติน ลดลงอย่างมีนัยสำคัญในกลุ่มที่ถูกเหนี่ยวนำการเกิดผังพืดด้วยสาร Doxorubicin ทั้งใน
กลุ่มที่ได้รับและไม่ได้รับ ไตรเอริลมีเทน-34 ช่องไอออนโพแทสเซียม ชนิดโพแทสเซียมแคลเซียม 3.1 อาจ
ไม่เกี่ยวข้องกับการเกิดผังพืดในเซลล์ไตแมวที่ถูกเหนี่ยวนำด้วยDoxorubicin ผ่านทางวิถี TGF-β/Smad
และ Doxorubicin มีผลในการยับยั้งการสร้าง อัลฟาสมูทมัสเซิล แอคติน ดังนั้นหน้าที่ของ ช่องไอออน
โพแทสเซียม ชนิดโพแทสเซียมแคลเซียม 3.1 ในเซลล์ไตแมว และกลไกการเกิดพังผืดในโรคไตวายเรื้อรัง
ในแมวผ่านวิถีอื่นๆ จำเป็นต้องมีการศึกษาต่อไปในอนาคต

สาขาวิชา	อายุรศาสตร์สัตวแพทย์	ลายมือชื่อนิสิต ....
ปีการศึกษา	2565	ลายมือชื่อ อ.ที่ปรึกษาหลัก ....
		ลายมือชื่อ อ.ที่ปรึกษาร่วม ....

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KEYWORD: cats chronic kidney disease KCa3.1 channel renal fibrosis TGF-β

Penpicha Kuedbantakien : ROLE OF KCa3.1 CHANNEL ON RENAL FIBROSIS IN DOXORUBICIN-INDUCED FELINE KIDNEY CELL LINE . Advisor: Assoc. Prof. ROSAMA PUSOONTHORNTHUM, D.V.M., M.Sc., Ph.D., DTBVM Co-advisor: Prof. Chatsri Deachapunya, B.Sc., M.Sc., Ph.D.

Feline renal fibrosis is the most common outcome of chronic kidney disease in cats. Nowadays, there is no specific treatment and the mechanism of fibrosis in cats is still unknown. Intermediate conductance Ca_2^+ activated K^+ channel (KCa3.1) plays an important role in novel therapy for organ fibrosis in many species. Transforming growth factor- β (TGF-β)/Smad signaling pathway and KCa3.1 has been shown to relate to renal fibrosis. This study aimed to investigate the relationship between KCa3.1 and feline renal fibrosis via TGF-β/Smad signaling pathway. CRFK cultures were induced with doxorubicin (DOX) to generate feline renal fibrosis model. Triarymethane-34 (TRAM-34), selective inhibitor of KCa3.1 was used for blocking KCa3.1 function pretreatment before fibrosis induction. TGF-β receptor type 2 (TGF-βR2), Smad2/3, α-Smooth muscle actin (α-SMA) which are fibrotic markers in TGF-β/Smad signaling cascade were measured using western blot to compare the fibrosis production between the normal KCa3.1 expression and KCa 3.1 blockage CRFK cells. The cell viability assay was performed alongside with protein expression measurement. CRFK cells pretreatment with TRAM-34 before DOX induction has no significant difference of percentage of cell viability when compared to negative control group. CRFK with DOX incubation alone significantly declined the percentage of cell viability compared to negative control ($p<0.05$). Interestingly, the expression of TGF-βR2 and Smad2/3 were not significant difference when compared between all study groups but α-SMA level in DOX induction group with and without TRAM-34 pretreatment were significant decrease when compared to negative control group ($p<0.05$). These findings indicated that KCa3.1 may not be involved in fibrogenesis in feline kidney cells via TGF-β/Smad signaling pathway and DOX may affect to α-SMA production in CRFK cells. The further investigation of KCa3.1 in feline renal fibrosis on another fibrotic partway is needed to be evaluated in the future.

Field of Study:	Veterinary Medicine	Student's Signature	....
Academic Year:	2022	Advisor's Signature	....
		Co-advisor's Signature	....

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Penpicha Kuedbantakien

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CHAPTER I

INTRODUCTION

Importance and rationale

Chronic kidney disease (CKD) is the functional or structural impairment of one or both kidneys that has been presented more than 3 mouths (Reynolds and Lefebvre, 2013). This disease is commonly found in geriatric cats (Lawson et al., 2015; Sparkes et al., 2016). The clinical signs of feline CKD depended on disease's stage such as weight loss, vomit, polyuria, polydipsia, dehydration, anemia, seizure, and death. These signs might affect animal's quality of life. Nowadays, there is no specific treatment of feline CKD in veterinary medicine. The therapeutics of this disease are still focused on supportive therapy and management.

Nephron damage in CKD is usually progression and irreversible. The etiology of feline CKD is various (Lawson et al., 2015) and often cannot be identified (Reynolds and Lefebvre, 2013). Even though the etiology is still unknown, the most morphologic finding of CKD is tubulointerstitial fibrosis (Lawson et al., 2015; McLeland et al., 2015).

Renal fibrosis is characterized as the expansion of extracellular matrix, due to increased production and decreased degradation (Lawson et al., 2015) which gradually destroys the normal renal parenchyma (Lawson et al., 2018a). The major morphologic change of renal fibrosis is affected from the epithelial-to-mesenchymal transition (EMT). This process is the conversion of epithelium to mesenchymal fibroblast. Moreover, EMT is relevant to the inhibition of expression of protein that associate with tubular epithelial cells' function and impairment of tissue healing process by inducing cell cycle arrest (van Beusekom and Zimmering, 2018). Many fibrogenesis pathways have been reported in human medicine. Transforming growth factor beta or TGF-β, pro-fibrotic mediator is one of the most important key factors

in the progression of renal fibrosis (Lawson et al., 2015). Upregulation of TGF-β leading to the expansion of extracellular matrix that is outcome of the fibrotic desease (Meng et al., 2016). Fibrosis activation, that involved with TGF-β, is induced by Smad-based and non-Smad-based signaling pathways (Meng et al., 2016). Lawson et al. in 2018 revealed that the natural occurring renal interstitial fibrosis in cats is the result of pro-fibrotic effect of TGF-β and Smad-based signaling pathways (Lawson et al., 2018a). Furthermore, TGF-β was expressed in ischemia induced feline renal fibrosis (Lourenço et al., 2020).

Oxidative stress is a trigger of chronic kidney disease progression (Afsar et al., 2020). Doxorubicin (DOX), an antineoplastic drug, was reported as a causation of oxidative stress disorder in many organs. To study about fibrogenesis in academic field, some researchers often used DOX as an initiating agent to generate oxidative stress induced organ fibrosis (El-Agamy et al., 2018; Guo et al., 2019; Hazem et al., 2022). There is a report in rats that received DOX showed a higher level of TGF-β1, Smad2/3, and α-smooth muscle actin (α-SMA) expression which are fibrotic markers when compared to control group (Guo et al., 2019).

Previous reports in rats and mice have demonstrated the relationship between renal fibrosis and increased expression of intermediate conductance Ca_2^+ activated K^+ channel (Grgic et al., 2009b; Huang et al., 2018). Intermediate conductance Ca_2^+ activated K^+ channel (KCa3.1, KCNN4, SK4, IK1) can be stimulated by the increasing level of intracellular Ca_2^+ concentration, and this activation leads to hyperpolarization of the cell membrane (Daniel et al., 2016). This channel plays a major role in transepithelial ion transport (Daniel et al., 2016). Blockage of this channel by selective blockers or Triarymethane-34 (TRAM-34) in murine showed attenuation of disease progression (Grgic et al., 2009b; Menè and Pirozzi, 2013; Huang et al., 2018). In addition, inhibition, or knockdown of KCa3.1 channel shows the reduction of TGF-β, Smad phosphorylation, and downstream product of this pathway in mice (Huang et al., 2013a). Moreover, the study of blockage KCa3.1 on

feline kidney cells induced renal injury with doxorubicin has showed a protective effect when using TRAM-34 pretreatment for 24 hours with unknown mechanism (Nantarakchaikul, 2017).

However, the roles of KCa3.1 in renal fibrosis in cats with CKD is still unclear. The purpose of this study was to investigate the role of Kca3.1 in TGF-β/Smad signaling pathway and renal fibrosis in feline kidney cells. Better understanding about the association of KCa3.1 channel and TGF-β/Smad signaling pathway in feline renal fibrosis may help to develop the new therapeutic treatment for feline chronic kidney disease in the future.

Objective of Study

To investigate the role of Ca_2^+-activated K^+ channels 3.1 in TGF-β/smad2/3 signaling pathway and renal fibrosis in doxorubicin-treated feline kidney cells.

Hypothesis

The KCa3.1 protein, Smad2/3 phosphorylation protein, and α-smooth muscle actin levels in doxorubicin-treated feline kidney cells increase more than doxorubicin-treated feline kidney cells pre-treated with TRAM-34 and normal feline kidney cells.

CHAPTER II

LITERATURE REVIEW

Feline chronic kidney disease

Chronic kidney disease (CKD) is frequently reported in older cats (Lawson et al., 2015; Sparkes et al., 2016). This disease can be regcognized as the impairment of structure or funtion of one or both kidneys that have been presented for more than 3 months (Reynolds and Lefebvre, 2013). Clinical manifestations are usually associated with complications such as hyperphosphatemia, secondary renal hyperparathyroidism, anemia, proteinuria, systemic hypertension, metabolic acidosis, and uremia (Brown et al., 2016). Routine investigation usually bases on history and clinical findings such as weight loss, dehydration, polyuria, polydipsia, or low urine specific gravity (Sparkes et al., 2016). The conservative management of CKD in cats is to maintain adequate hydration status, restrict phosphorus and protein in diets, control systemic hypertension, proteinuria, and treat anemic condition (Sparkes et al., 2016). However, most CKD cats often progress to the end-stage kidney and death (Brown et al., 2016).

Even though CKD in cats has been recognized as an important disease in aged cats for many years, the prevalence of feline CKD has continuously increased especially in the current decade (Brown et al., 2016). The etiology of feline CKD is known to be heterogeneous (Lawson et al., 2015) and often cannot be identified (Reynolds and Lefebvre, 2013). Although the etiology is still unrevealed, the most morphologic finding of most CKD cats is tubulointerstitial fibrosis (Lawson et al., 2015; McLeland et al., 2015).

Nowadays, the diagnosis and treatment of CKD in veterinary practice is based on the recommendations of International Renal Interest Society (IRIS). CKD is staged by creatinine and SDMA concentration, blood pressure, and proteinuria status. The

treatment mainly aims to improve the quality of life and slow down the disease progression (Sparkes et al., 2016).

Renal fibrosis

Renal fibrosis is characterized as an expansion of extracellular matrix (Lawson et al., 2018a) due to an increased production and decreased degradation (Lawson et al., 2015) which gradually destroy the normal tissue parenchyma (Lawson et al., 2018a). It is the consequence from a failure of a common healing process (Lawson et al., 2018a). For acute and not severe kidney damage, the injured epithelium can be resolved by the proliferation of the same cell type and migration to the injury position (Lawson et al., 2015). Prolong severe injury can induce irreversible nephron loss and can stimulate profibrotic cytokine releasing which contributes to initiating fibrogenesis (Reynolds and Lefebvre.2013; Lawson et al., 2015). These events are the outcome of epithelial-mesenchymal transition (Zhou and Liu, 2016; Lawson et al., 2018a). Myofibroblasts play an important role in fibrogenesis. It is differentiated from various cell types through epithelial-mesenchymal transition. Myofibroblasts become activated and secrete extracellular matrix components leading to renal fibrosis (Reynolds and Lefebvre, 2013).

Sustained and
severe kidney injury

↓

Infiltration of
inflammatory cells

↓

Pro-fibrotic
cytokines releasing

↓

Myofibroblast
activation

↓

ECM production and
expansion

↓
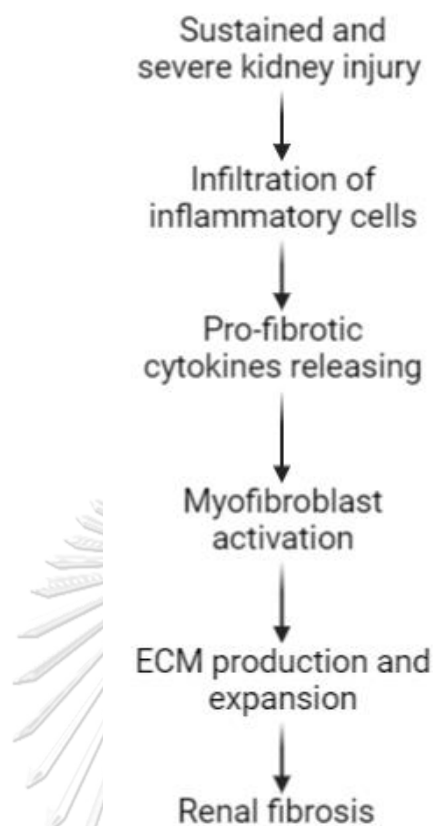
Renal fibrosis

Figure 1 Stage of renal fibrosis
(Modified from Reynolds and Lefebvre, 2013)

Epithelium-Mesenchymal transition

Epithelial–mesenchymal transition (EMT) is a cellular mechanism which cause a reversible changing in epithelial phenotypes of cells to mesenchymal features such as lose their apical-basal polarity, remodel their cytoskeleton, and reduce their adhesive properties (Yang et al., 2020). EMT involves in many process such as wound healing (Katsuno and Derynck, 2021), the development of embryo, cancer, and fibrosis (Gonzalez and Medici, 2014; Yang et al., 2020). The hallmark of EMT is a shift of the epithelial markers including E-cadherin, cytokeratins, zona occludens 1 (ZO-1), and occludin to the mesenchymal markers such as N-cadherin, vimentin, fibronectin, fibroblast specific protein 1 (FSP-1), and α-smooth muscle actin (α-SMA) (Gonzalez

and Medici, 2014). Myofibroblasts, spindle shape cells, are effector cells responsible for interstitial extracellular matrix deposition (Fintha et al., 2019). Myofibroblasts have been characterized as α-SMA expressing cells which have a higher rate of proliferation and migration. It can produce cytokines, inflammatory mediators, growth factors, as well as extracellular matrix proteins (Fintha et al., 2019). The dysregulation of myofibroblasts leads to impairment of tissue function and organ failure (Fintha et al., 2019).

In renal fibrosis, there is a suggestion that the renal tubular cell is converted to myofibroblast that promotes the progression of renal fibrosis. Moreover, a strong correlation between serum creatinine and the number of EMT presented renal tubular cells has been discovered (Fintha et al., 2019).

EMT is triggered by TGF-β, epidermal growth factor (EGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), bone morphogenetic protein (BMP), Notch, Sonic Hedgehog (Shh), Wnt, and integrin signaling (Gonzalez and Medici, 2014). Although there are several signaling pathways regulating in EMT, TGF-β signaling has been the most frequently recognized as the first and important pathway of EMT regulation (Steinestel et al., 2014). Moreover, there was a report that EMT is involved in renal fibrosis in both dogs and cats (Kutlu and Alcigir, 2019).

TGF-β/Smad signaling pathway

TGF-β1 is one of the most potent mediators in fibrogenesis (Lawson et al., 2015). It is a member of transforming growth factor (Ma and Meng, 2019). There are three mammalian isoforms of TGF-β: TGF-β1, TGF-β2, and TGF-β3. TGF-β1 contributes to the stimulation of intracellular signaling by binding to the TGF-β receptor complexes which consist of TGF-β receptor type 1 and TGF-β receptor type 2 (Chen et al., 2018; Ma and Meng, 2019). These receptors are transmembrane serine/threonine and tyrosine kinases (Chen et al., 2018; Vander Ark et al., 2018). In

most cell types, the active form of TGF-β directly binds to TGF-β receptor type 2 and then recruits TGF-β receptor type 1 into the complex and becomes phosphorylated (Chen et al., 2018). These complexes can regulate the cell signaling via Smad and non-Smad base signaling pathway (Chen et al., 2018).

Smads is a family of signal transducer proteins (Moustakas et al., 2001). There are three subfamilies of this protein (Moustakas et al., 2001). R-Smads are receptors-activated Smads, which are Smad 1, 2, 3, 5, 8 while I-Smads or inhibitory Smads consist of Smad 6 and Smad 7 (Moustakas et al., 2001; Macias et al., 2015). The last subfamily of Smads is Co-Smads which oligomerize with activated R-Smads (Moustakas et al., 2001). Both R-Smads and Co-Smads participate in the TGF-β/ Smad signaling pathway that induces fibrosis (Sureshbabu et al., 2016). Phosphorylation of Smads 2 and Smads 3 from R-Smads family by type I receptor kinases (Moustakas et al., 2001) are formed oligomeric complex with Co-Smads, namely, Smads 4 (Sureshbabu et al., 2016). Then this complex undergoes nuclear translocation to regulate the transcription of fibrosis-related proteins (Sureshbabu et al., 2016) such as α-SMA, collagen type 1α1, and fibronectin (Lawson et al., 2018b).

TGF-β/ Smad signaling are related with many fibrotic diseases. It is believed that this pathway is important for fibrogenesis which also correlated with impaired renal function. On the other hand, the inhibition of TGF-β signaling can attenuate EMT and tubulointerstitial fibrosis (Fintha et al., 2019).
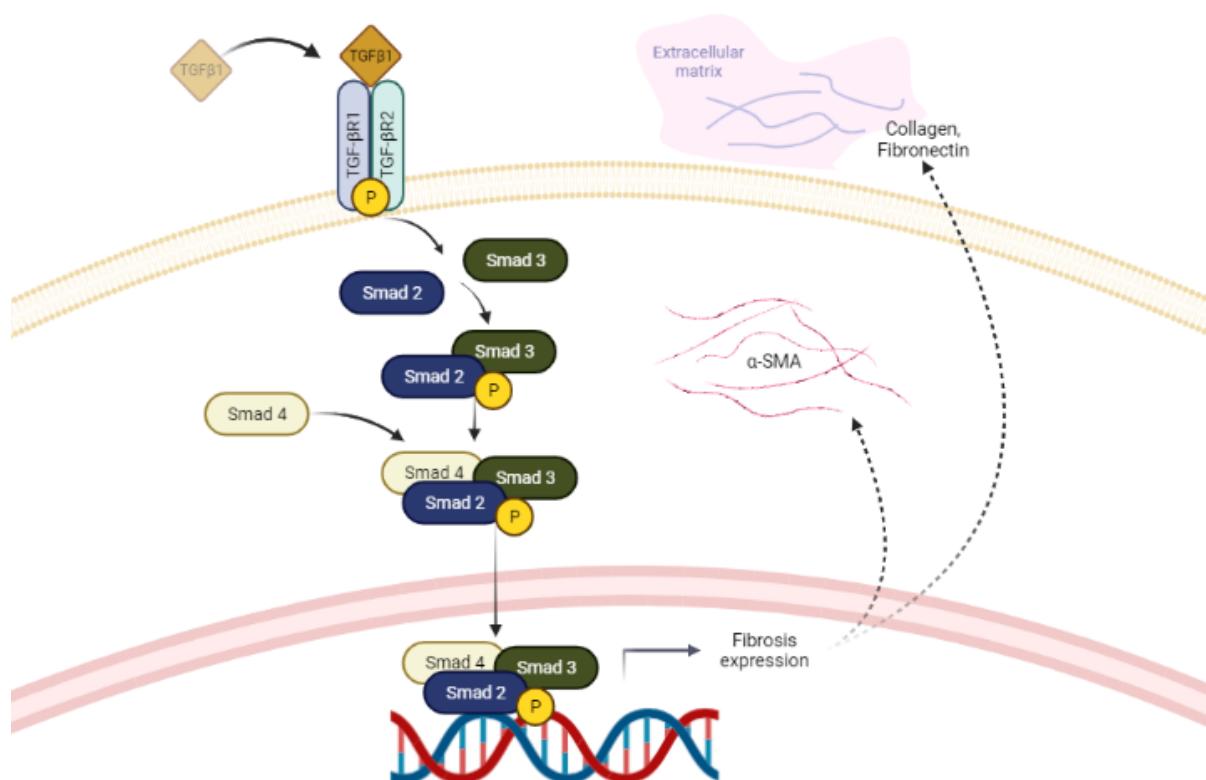
Figure 2 *TGF-β/Smad signaling pathway*
(Modified from Chen et al., 2018; Hu et al., 2018; Yu et al., 2022)

จุฬาลงกรณ์มหาวิทยาลัย

Oxidative stress CHULALONGKORN UNIVERSITY

Oxidative stress is defined as an imbalance between the pro-oxidants and the antioxidants (Pisoschi et al., 2021). Reactive oxygen and nitrogen species (RONS), the oxidative stress markers, are produced by endogenous and exogenous sources (Liguori et al., 2018). RONS have frequently founded as hydrogen peroxide (H_2O_2), superoxide (O_2^-), hydroxyl (OH), nitric oxide (NO), and peroxynitrite ($ONOO^-$) form (Pisoschi et al., 2021). The chemical reaction of RONS can impact the body through disrupts redox signaling and directly oxidizes macromolecules including proteins, carbohydrates, lipids, and DNA (Liguori et al., 2018; Forman and Zhang, 2021).

Oxidative stress is relevant to various pathologies by its toxicity and contribution of disease progression (Forman and Zhang, 2021).

In chronic kidney disease, oxidative was reported to play a role in disease progression leading to renal ischemia, glomerular damage, endothelial dysfunction, inflammation, and apoptosis in many species (Liguori et al., 2018; Pisoschi et al., 2021). Moreover, oxidative stress parameters were found to increase significantly in cats with natural occuring CKD when compared to the clinically normal cats (Piyarungsri and Pusoonthornthum, 2016).

Doxorubicin

Doxorubicin (DOX) is an antineoplastic antibiotics drug that is categorized as anthracycline class (Wallace et al., 2020). This drug is used in both human and veterinary medicine as the treatment of various malignancies such as lymphomas, sarcomas, leukemia, hemangiosarcoma, solid tumors and others (Batschinski et al., 2018; Tian et al., 2020). The mechanism is inhibition of DNA replication and suppression of topoisomerase II enzyme (Ibrahim Fouad and Ahmed, 2021; van der Zanden et al., 2021). Despite the highly effective chemotherapy, many adverse effects of doxorubicin have been reported. Doxorubicin can generate oxidative stress which damages the cells. Moreover, it is believed that it participates with apoptosis pathway (Afsar et al., 2020). Various experimental studies had previously use DOX to generate the oxidative stress models (Arafa et al., 2014; Szalay et al., 2015)

Kidney cells are very sensitive to oxidative stress because it is a high energy consuming (Guo et al., 2019). Oxidative stress in the kidney is the result from the decreased renal catalase (CAT), and increased renal malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) productions (Ibrahim Fouad and Ahmed, 2021). Doxorubicin can cause renal damage due to its accumulation in renal tissue leading to inflammation, glomerular capillary permeability amplification, and tubular

degeneration (Afsar et al., 2020; Ibrahim Fouad and Ahmed, 2021; Hazem et al., 2022).

Myofibroblast, an important key in fibrosis progression, is frequently activated by doxorubicin induced oxidative stress. It triggers profibrotic cytokines releasing especially TGF-β1 (Hazem et al., 2022). Doxorubicin has been used in many experiments to activate fibrosis. Doxorubicin as an oxidative induced renal fibrosis and cardiac fibrosis in both experimental animals and cell lines (Szalay et al., 2015; Narikawa et al., 2019; Ibrahim Fouad and Ahmed, 2021).

Doxorubicin induced feline kidney failure in CRFK cell was studied in 2014 by Piyarungsri et al. In that study, doxorubicin can reduce percentage of cell viability at the concentration of 2 µM and a significant dose that can reduce 50 percent of cell viability was 8 µM (Piyarungsri, 2014).

Potassium (K$^+$) channels

K$^+$ channels are one of the important ion channels that have been discovered in many organisms. It locates on cell membranes and controls the transportation of K$^+$ ions (Kuang et al., 2015). It is also associated with ions exchange in cellular mechanism. K$^+$ channels can be divided into four main classes which are inwardly rectifying K$^+$ channels, tandem pore domain K$^+$ channels, voltage-gated K$^+$ channels, and Ca^{2+}-activated K$^+$ channels. (Roach and Bradding, 2020).

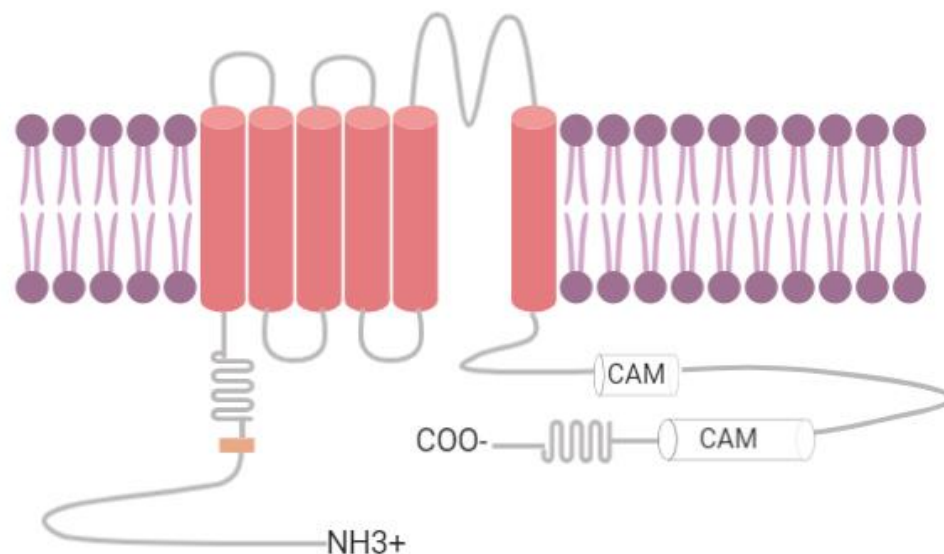**Intermediate-Conductance Ca^{2+}-Activated K$^+$ Channels**

Figure 3 *Structure of KCa3.1*

(Modified from Roach and Bradding, 2020)

Intermediate-Conductance Ca^{2+}-Activated K$^+$ Channels (KCa3.1, KCNN4, SK4, IK1) is one of the voltage independent potassium channels (Huang et al., 2018; Roach and Bradding, 2020). It has been found in many cell types such as hemopoietic cells, endothelial cells, epithelial cells, and fibroblasts (Huang et al., 2014; Roach and Bradding, 2020). KCa3.1 consists of six transmembrane proteins (S1-6) that are also known as homotetrameric protein (Roach and Bradding, 2020). The pore region of this channel locates between S5 and S6 (Roach and Bradding, 2020). KCa3.1 is Ca^{2+} dependent gate due to calmodulin (CaM) links with C-terminus which locates at intracellular site (Roach and Bradding, 2020). This channel has various functions including cell activation, proliferation, and migration through cell membrane potential regulation (Roach and Bradding, 2020). KCa3.1 controls Ca^{2+} entry and regulates Ca^{2+} signaling in cellular mechanism (Huang et al., 2015). This channel is also found to be associated with cell volume regulation in hepatic stellate cells in human patients (Sevelsted Møller et al., 2016). Furthermore, KCa3.1 was reported as

a molecular target of pharmacological intervention for urinary incontinence, prostate cancer, and autoimmune disease in human (Huang et al., 2013b).

KCa3.1 plays an important role in fibrogenesis in many organs. Blockade of KCa3.1 showed attenuated effect of renal fibrosis induced by unilateral ureteral obstruction in mice (Grgic et al., 2009a). Moreover, TRAM-34, a selective inhibitor of KCa3.1, can reduce the expression of fibrotic markers in TGF-β/ Smad signaling which are collagen type I, fibronectin, TGF-β1, and Smad2/3 in diabetic nephropathy mouse (Huang et al., 2018).

Many studies showed the successful treatment for fibrotic diseases by inhibition of KCa3.1 (table1). Currently, KCa3.1 has become a novel target therapy of organ fibrosis. However, there is no study on KCa3.1 that associated with renal fibrosis in cats. Our hypothesis is KCa3.1 participated in fibrogenesis in feline chronic kidney disease.
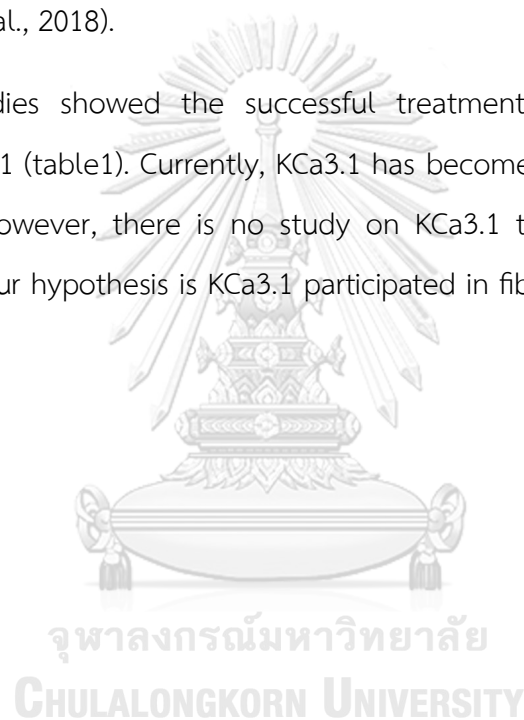
Table 1 *Examples of KCa3.1 inhibition reduced fibrotic diseases studies.*

| Organ of fibrosis	Fibrotic inducer	Experiment model	KCa3.1 blockage	Author
Corneal fibrosis	*in vivo*: a filter paper disc that presoaked in NaOH	*in vivo*: KCa3.1-/- mouse model	-	(Anumanthan et al., 2018)
	in vitro: rhTGFβ	*in vitro:* primary human corneal fibroblast cells (HCF)	TRAM-34	
Cardiac fibrosis	Angiotensin II	Rats	TRAM-34	(She et al., 2019)
Pulmonary fibrosis	Paraquat	Rats	TRAM-34	(Xie et al., 2018)
Liver fibrosis	*in vivo*: bile duct ligation (BDL)	*in vivo*: rats	TRAM-34	(Freise et al., 2015)
	in vitro: TGF-β1	*in vitro*: the rat hepatic stellate cell line CFSC	TRAM-34	
Renal fibrosis	Streptozotocin (induced diabetes)	eNOS-/- mice	TRAM-34	(Huang et al., 2018)
	TGF-β1	HK2 cells	TRAM-34	(Huang et al., 2014)
	Doxorubicin	CRFK epithelial cells	TRAM-34	(Nantarakchaikul, 2017)

CHAPTER III

MATERIALS AND METHODS

Conceptual framework

Evaluate role of KCa3.1 on renal fibrosis
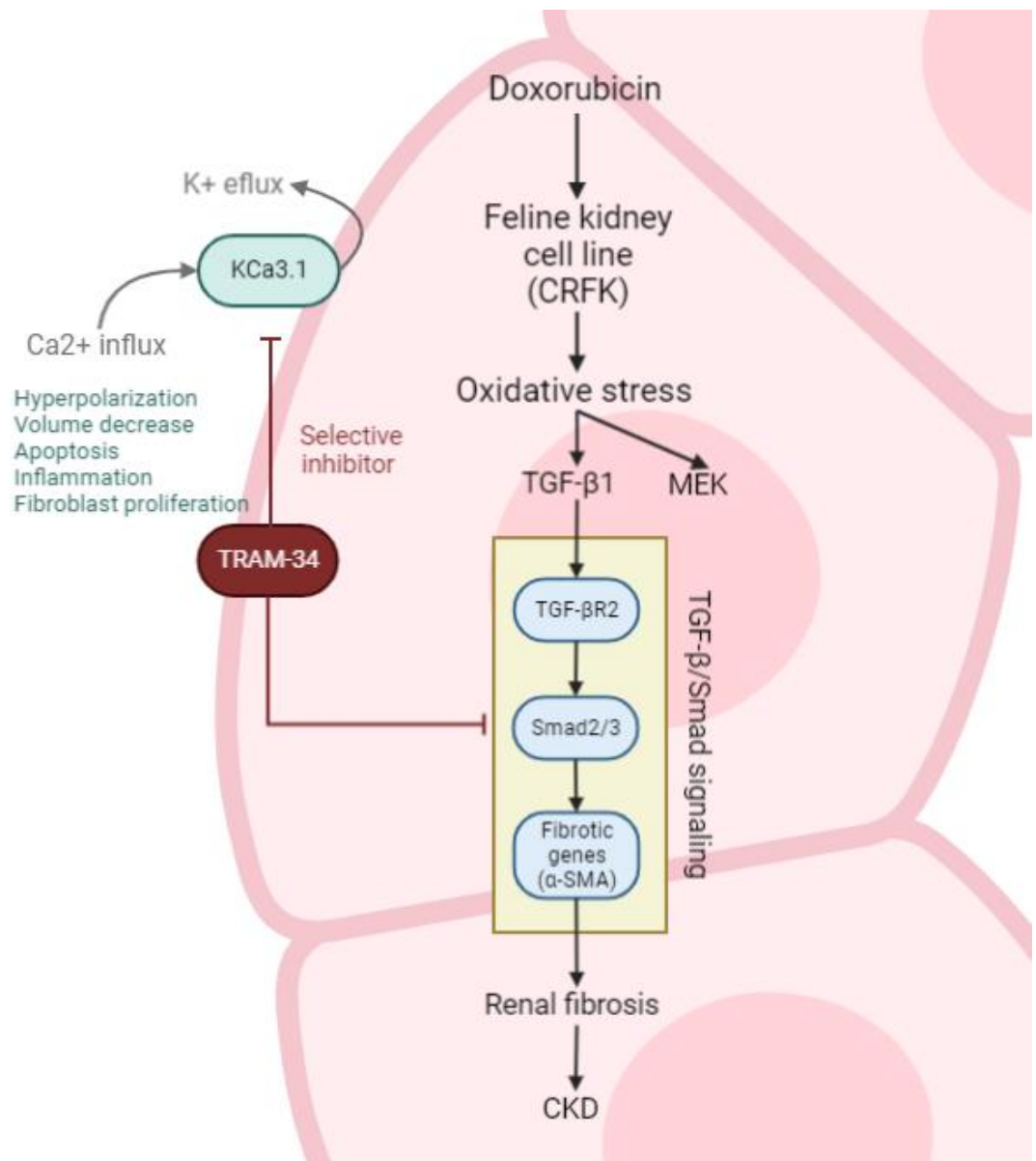
Study design
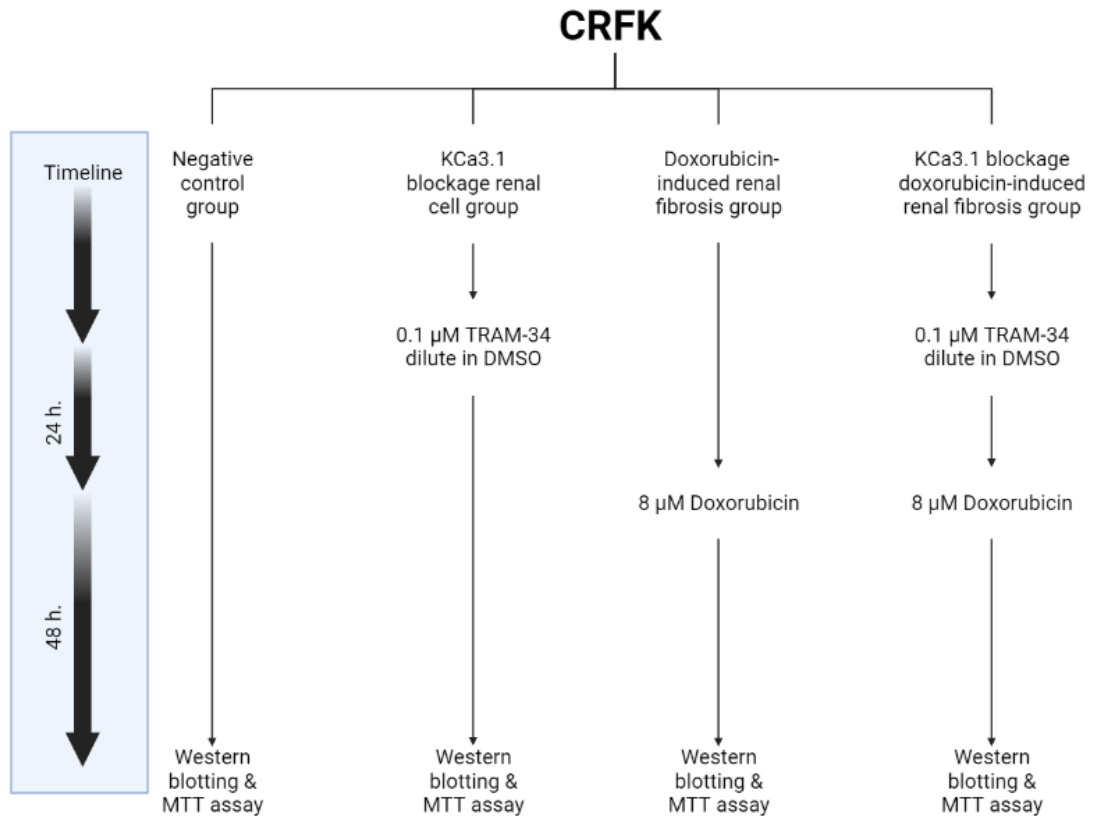
CRFK culture was obtained from cell bank (ATCC®). It was divided into 4 groups.

1. Negative control group: normal CRFK culture without any treatments served as negative control groups. The cell lysate was collected at 72 h after the beginning of the experiment.

2. KCa3.1 blockage renal cell group: normal CRFK culture was incubated with 0.1 μM TRAM-34 diluted in DMSO. The cell lysate was collected at 72 h after incubation.

3. Doxorubicin-induced renal fibrosis group: CRFK cells were cultured for 24 h before the cells expose to 8 μM Doxorubicin for 48 hours to induce cell injury (Piyarungsri, 2014). Then cell lysate was collected.

4. KCa3.1 blockage Doxorubicin-induced renal fibrosis group: CRFK culture exposed to 8 μM Doxorubicin for 48 hours to induce cell injury after pre-

incubation with 0.1 μM TRAM-34 diluted in DMSO for 24 hours (Piyarungsri, 2014; Nantarakchaikul, 2017). Then this cell lysate was collected.

TRAM-34

Triarymethane-34 or TRAM-34 (1-[(2-chlorophenyl) diphenylmethyl]-1H-pyrazole) is a clotrimazole analog characterized as the selective KCa3.1 blocker (Huang et al., 2018). TRAM-34 has been widely used to study the role of KCa3.1 in vivo and in vitro (Agarwal et al., 2013). TRAM-34 at concentration 0.1 μM has a protective effect against doxorubicin induced renal cell apoptosis and can decrease KCa 3.1 protein expression when using before induction of Doxorubicin 24 hours (Nantarakchaikul, 2017). In this study, TRAM-34 was prepared in dimethyl sulfide (DMSO) as a vehicle and added to feline kidney cells to suppress KCa 3.1 function.

Cell culture

Feline (*Felis catus*) renal cell line (CRFK ATCC® CCL-94™), derived from cortical portion of kidney tissue of 10-12 weeks old female cat (Crandell et al., 1973), was used as the experimental model. These cells are continuous cell line and grow as a smooth monolayer of epithelial-like cells (Crandell et al., 1973). CRFK cells were grown in DMEM supplement with 10% heat-inactivated fetal bovine serum and 100-unit penicillin-streptomycin at 37 ^{o}C under 5% CO_2 in a humidified atmosphere. The cells were subcultured every 2-3 days. Subculturing protocol started from discarding exhausted culture medium. Next, cells were washed by PBS and trypsinized by 0.25% (w/v) Trypsin-0.53 mM in EDTA solution. An inverted microscope was used to observe the dispersal of cell layer after trypsinization. Then cells were centrifuged at approximately 125 x g for 5 minutes. The supernatant in centrifuge tube was discarded. The remaining pallet was resuspended in complete growth medium (DMEM+10% FBS). Cell suspension was dispensed to new culture vessels.

To study fibrosis expression, CRFK cells were seeded at the density of 8×10^5 cells per 60 mm. TC dich in 3 mL medium. For cell viability assay, the cells were seeded into 96-well plate at 1×10^4 cells per a well in 200 μL medium.

Western blotting

Western blot assay was used to detect fibrosis pathway expression. TGF-β receptor type 2, S-mad2/3, and α-smooth muscle actin were used as the markers of fibrosis pathway.

To collect the cell lysates for western blot assay, CRFK cells in each group were discarded and washed with 4 degree celsius PBS. Then the cells were added 1 mL of 1X RIPA buffer (Millipore, USA) with protease inhibitor cocktail (SIGMAFAST™, Singapore) incubating on ice and scrapped with cell scrapper until almost cells were disaggregated. The cells were sonicated during incubation on ice for 40 minutes. After that, the cell solution was transferred into centrifuge tube and centrifuged at 27,000 g for 20 minutes. The supernatant was collected as cell lysates.

The cell lysates were collected to determine the concentration of total protein using Bicinchoninic acid (BCA) assay. SDS-PAGE of 10 mg/mg of total protein in cell lysates were performed. The samples of the study were prepared from the cell lysates by adding 6X loading buffer and 2-mercaptoethanol before boiled at 95 $^{\textbf{o}}$C for 5 minutes. The protein standard that was used in our study was Precision Plus Protein standards (Biorad, USA). The SDS-PAGE ran in running buffer at 70 V until the line of loading buffer reached the end of 8% acrylamide gel.

PVDF membrane was used for western blotting, the membranes were activated with absolute methanol for 5 seconds before blotting. Then the proteins that separated according to its molecular weight were transferred onto PVDF membrane using the semi-dry blotting machine following manufacturer's protocol. To visualize the embedded proteins on the membranes, PVDF membranes were

stained with Ponceau S dye. After that the membranes were destained with distilled water before washing with 5 ml TBS-T buffer 5 minutes for three times. To prevent non-specific binding, the non-specific proteins on the membranes were blocked using chemiluminescent Blocker (Immobilon®) for 30 minutes on shaker at room temperature. Blocked membranes were washed three times for 5 minutes with 5 ml TBS-T buffer.

To detect the expression of the proteins of interest, the primary antibodies; anti-TGF-β receptor type 2, anti-Smad2/3, and anti-α-smooth muscle actin were purchased from Cell Signaling Technology (USA). These primary antibodies at the concentration of 1:1,000 in TBS-T were incubated on the membranes overnight at 4 ^{o}C. The secondary antibody which was goat anti-rabbit antibody linked with HRP incubated on the membrane at room temperature on shaker after the membranes were washed by TBST-T for three times, the incubation period was 30 minutes.

The visualization system of this study was chemiluminescence. ECL(Bio-Rad) was used as a substrate for horseradish peroxidase. The signalment of protein on the membranes that were incubated with ECL for 5 minutes following manufacturer's protocol and were detected by Gel Documentation machine (Bio-Rad). Target bands were selected by the molecular weight and band intensity was determined by Image lab software.

Table 2 *Primary and secondary antibodies are used in western blotting.*

Protein name	Antibody	Dilution
TGF-β Receptor 2	Primary monoclonal rabbit anti-TGF-β receptor type 2	1:1000
	Secondary goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP)	1:5000
Smad2/3	Primary monoclonal rabbit anti-Smad2/3	1:1000
	Secondary goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP)	1:5000
α-smooth muscle actin	Primary monoclonal rabbit anti-α-smooth muscle actin	1:1000
	Secondary goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP)	1:5000
β-actin	Primary polyclonal rabbit anti-β-actin	1:1000
	Secondary goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP)	1:5000

Normalization method

Normalization method in this study was housekeeping protein which was β-actin. The same incubated membranes were stripped using commercial stripping buffer (StripPRO™) re-incubated with anti- β-actin antibody following the same protocol.

The protein intensity was calculated by image lab software. The protein in each lane was normalized with the normalization factor which was determined following this equation:

$$\text{Lane normalization factor} = \frac{\text{Observed signal of housekeeping protein for each lane}}{\text{Highest observed signal of housekeeping protein on the blot}}$$

Cell viability assay

To determine cell viability in each group, the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay was used in this study. MTT is colorimetric assay developed by the conversion of MTT to formazan crystal in living cells (van Meerloo et al., 2011).

CRFK cells were seeded into 3 wells of 96-well plate per study's group. Then the cells were treated as same as the fibrosis expression's study protocol. The culture medium was discarded and replaced with 5mg/mL of MTT in DMEM and incubated for 4 hours at 37 ^{o}C under 5% CO_2 in a humidified atmosphere. After incubation, 200 μL of DMSO was added into each well to dissolve formazan crystal. The absorbance was measured by a microplate reader at 570 nm. Percentage of cell viability was calculated following this equation:

$$\% \text{ Cell viability} = \frac{\text{Absorbance of the interested group}}{\text{Absorbance of control group}}$$

Statistical analysis

All groups' data were analyzed and compared with statistical methods. The number of protein expressions in each experiment is considered as a dependent variable. Data between groups were evaluated by one-way analysis of variance (ANOVA) with posthoc LSD's test and Kruskal-Wallis's test with pairwise comparison. Statistical significance was set at p-value <0.05 and statistical analyses performed using SPSS software version 22.

CHAPTER IV

RESULTS

Cell morphology and viability

The morphology of CRFK cells in each group after 72 hours of incubation under an inverted microscope was demonstrated (Figure 4). CRFK in all groups had elongated and bipolar shape. From visual observation, most cells in doxorubicin-treated CRFK cell group and KCa3.1 blockage with doxorubicin-treated CRFK cell group presented with more unattached and floating cells when compared with negative control and KCa3.1 blockage renal cell group.

MTT assay revealed that the living cells among study's groups, percentage of cell viability of KCa3.1 blockage renal cell group, doxorubicin-induced renal fibrosis group, and KCa3.1 blockage doxorubicin-induced renal fibrosis group were 98.56±1.97, 84.80±2.88, and 98.81±6.54, respectively. Percentage of cell viability in 8 µM of doxorubicin incubated CRFK cells were significantly lower than the negative controls ($p<0.05$).
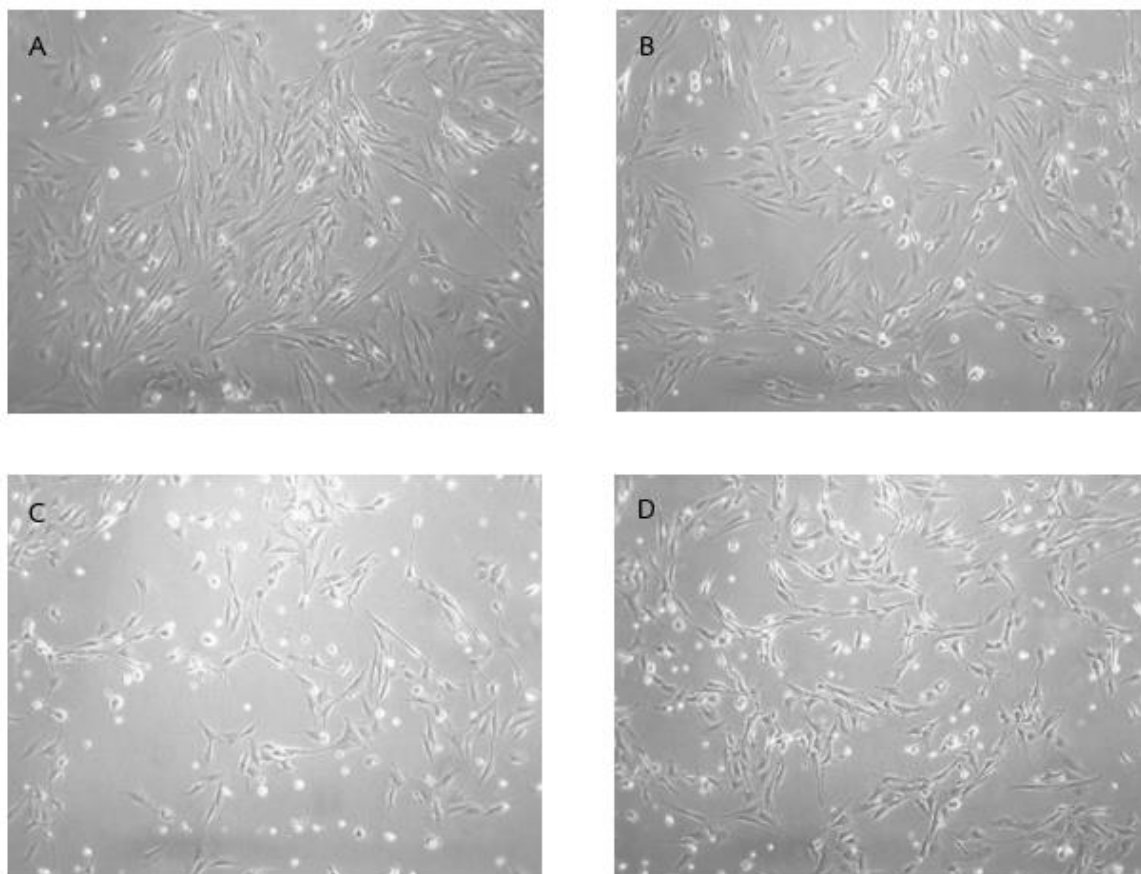
Figure 4 Cell morphology 20X

(A) Negative control group (B) KCa3.1 blockage renal cell group: 0.1 µM TRAM-34 incubation. (C) Doxorubicin treated group: 8 µM doxorubicin incubation for 48 hours. (D) KCa3.1 blockage with doxorubicin treated group: 8 µM doxorubicin incubation for 48 hours after 0.1 µM TRAM-34 pretreatment for 24 h.
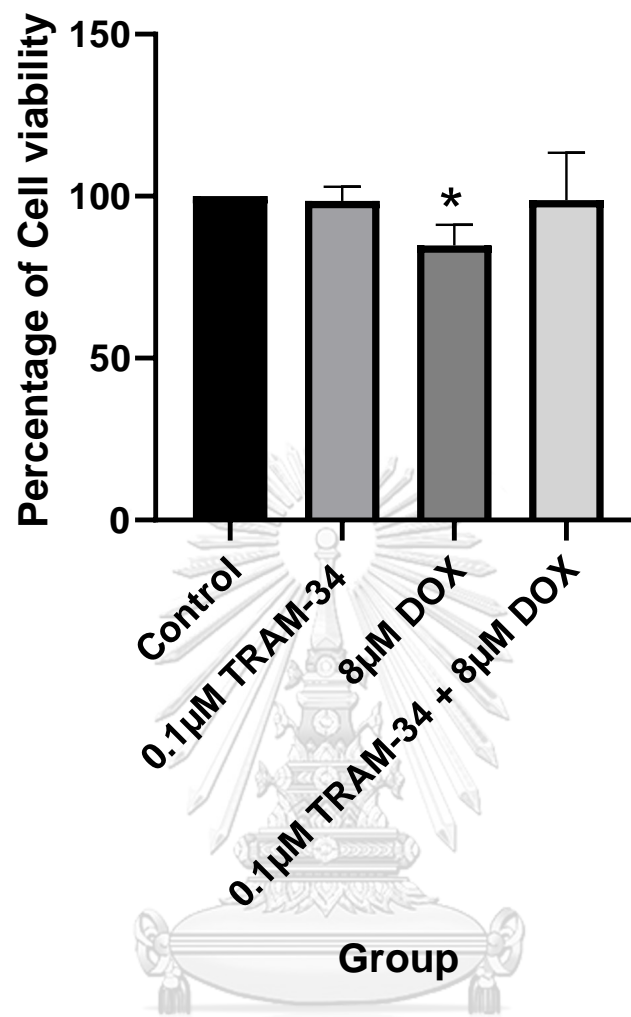
Figure 5 *The viability of CRFK cells after 72 hours of incubation with TRAM-34 and DOX.*

Data presented as mean±SE (n=5).

*Significant difference when compared between negative control and KCa3.1 blockage renal cell group (p<0.05) using Kruskal Wallis's test.

Study of protein expression of fibrosis markers

To investigate role of KCa3.1 in the oxidative stress induced feline renal fibrosis model by using doxorubicin, TRAM-34 was used to inhibit KCa3.1 function. The expression of proteins in TGF-β/smad signaling cascades and mesenchymal markers was observed by using western blotting. Protein expression was normalized with β-actin (housekeeping protein). The means of TGF-β receptor type 2, Smad2/3, and α-SMA were shown, respectively (Figure 6-8). There was no significant difference of TGF-β receptor type 2 and Smad2/3 expression among the study's group while α-SMA significantly decreased in 8 µM doxorubicin and 0.1 µM TRAM-34 pretreatment 8 µM doxorubicin incubated cells when compared with negative control group ($p<0.05$). This finding indicated that KCa3.1 may not involve with TGF-β/smad signaling in CRFK cells and doxorubicin at the concentration of 8 µM may reduce the expression of α-SMA.
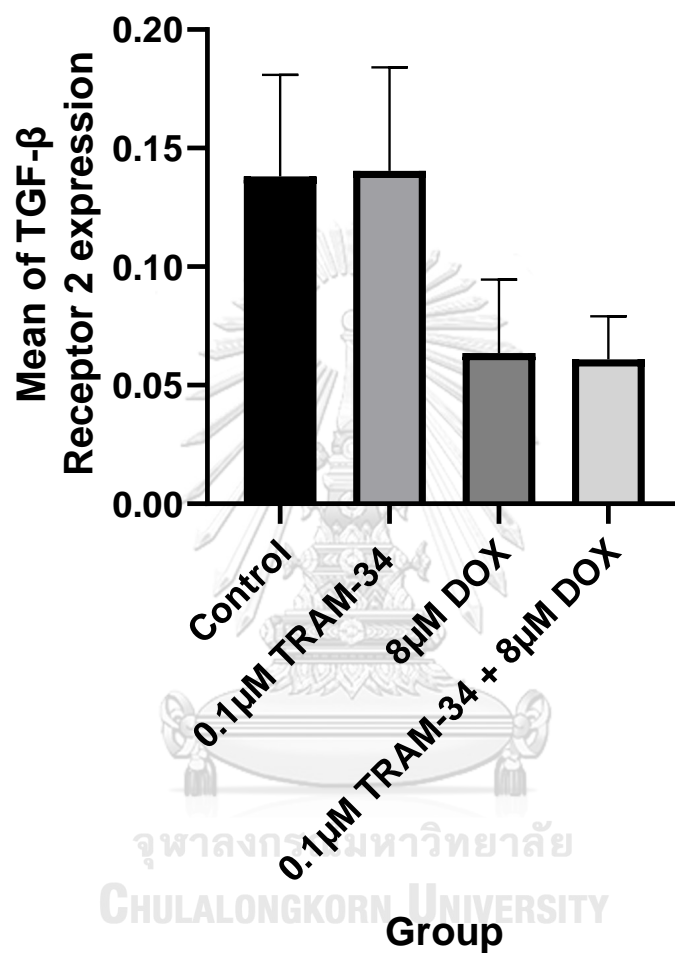
Figure 6 *Protein expressions of TGF-β Receptor 2 in different groups. β-actin was used as an internal control to normalize the volume of protein expression. Data presented as mean±SE (n=4).*

Figure 7 Protein expressions of Smad2/3 in different groups.
β-actin was used as an internal control to normalize the volume of protein expression. Data presented as mean±SE (n=4).
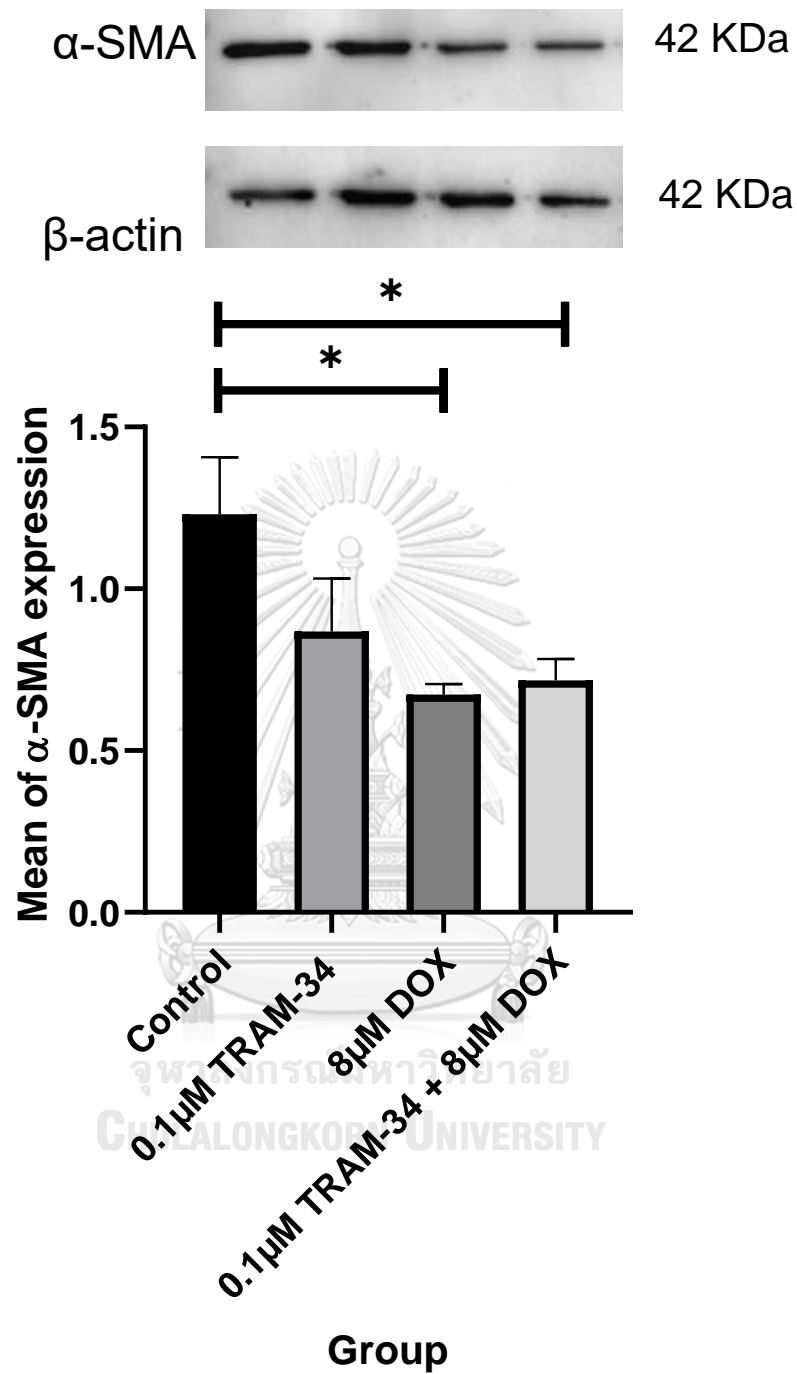
Figure 8 Protein expressions of **α**-SMA muscle actin in different groups.
β-actin was used as an internal control to normalize the volume of protein expression. Data presented as mean±SE (n=4).
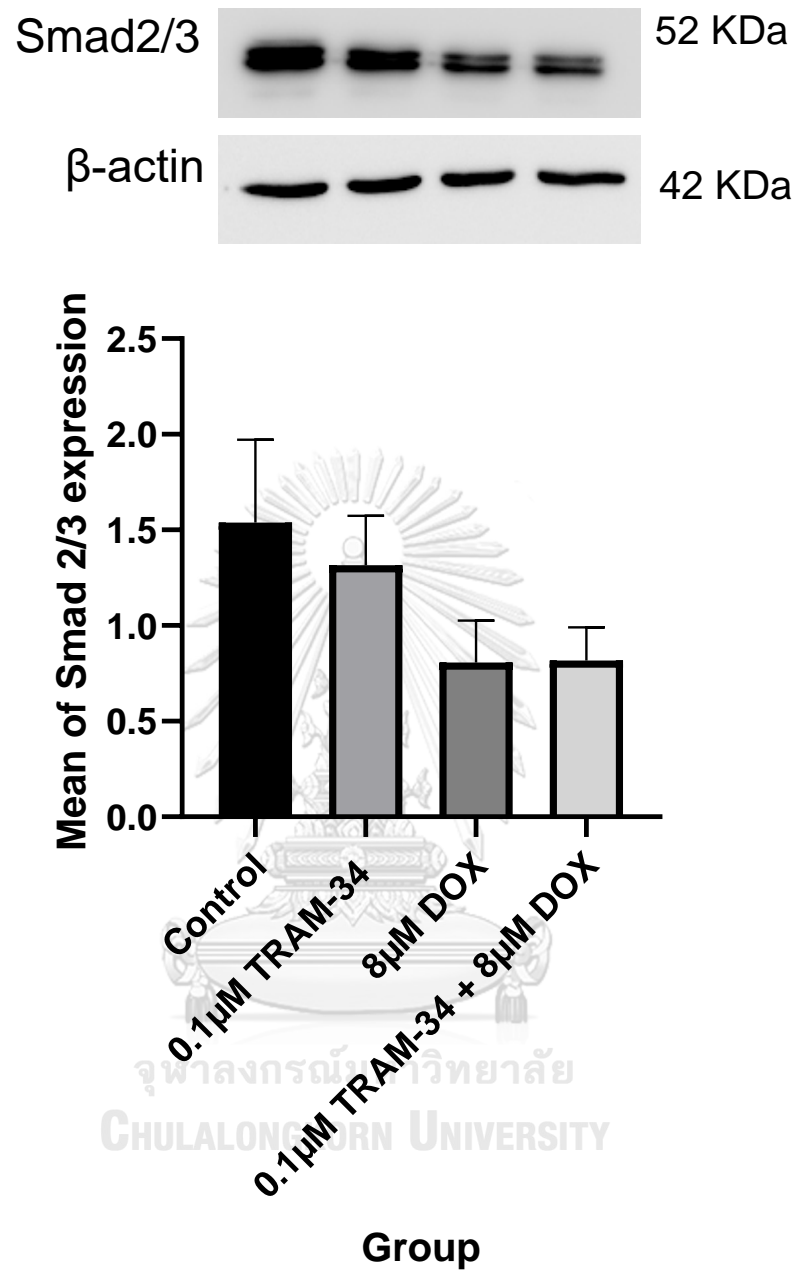
*Significant difference when compared to negative control group (p<0.05) using one way ANOVA.

Table 3 The protein expression of TGF-βR2, Smad2/3, and α-SMA.

Group	Ratio of protein expression / β-actin		
	TGF-βR2	Smad2/3	α-SMA
Control	0.1381 ± 0.0429	1.5381 ± 0.4346	1.2303 ± 0.1768
0.1µM TRAM-34	0.1405 ± 0.0437	1.3165 ± 0.2570	0.8676 ± 0.1649
8µM DOX	0.0635 ± 0.0310	0.8083 ± 0.2177	0.6731 ± 0.0331*
0.1µM TRAM-34 +8µM DOX	0.0610 ± 0.0181	0.8172 ± 0.1740	0.7174 ± 0.0653*

*Significant difference when compared to negative control group ($p<0.05$).

CHAPTER V

DISCUSSION

Our results showed that doxorubicin treated CRFK cells has significantly lower percentage of cell viability when compared to control group (p<0.05). The percentage of cell viability in DOX-treated CRFK cells that pretreatment with TRAM-34 for 24 hours tend to be higher than CRFK cell that received DOX alone (p=0.059). These results were similar to the results of cell viability part in study of Nantarakchaikul in 2017.

KCa3.1 has been regarded as a novel therapeutic target for fibrotic diseases (Roach and Bradding, 2020). The blockade of this channel with TRAM-34 showed the attenuated effects of fibrogenesis via TGF-β/Smad signaling pathway in the studies of other species (Huang et al., 2014; Freise et al., 2015; Huang et al., 2018)

Contrary to our expectation, the findings of our study demonstrated non-significant differences of TGF-β receptor 2 and Smad2/3 expression between study groups. The same observation was found in one previous study of pulmonary fibrosis in rats. The expression of TGF-β1 in rat's lung tissues in that study were not affected by TRAM-34 (Xie et al., 2018).. However, there is no obvious evidence of role of KCa3.1 about fibrotic disease in feline species via TGF-β/Smad signaling. The fibrotic process in feline kidney cells is still unclear.

Doxorubicin was used to induce renal failure through oxidative stress mechanism. Doxorubicin is an antineoplastic drug with the ability to breakdown double strand DNA by blocking DNA topoisomerase II activity (van der Zanden et al., 2021). Doxorubicin also has been reported that it is related to EMT expansion and TGF-β/Smad signaling activation in rats (Guo et al., 2019; Ibrahim Fouad and Ahmed, 2021). The present results showed that Doxorubicin at the concentration of 8 µM decreased the level of α-SMA expression with and without 0.1 µM TRAM-34

pretreatment for 24 hours. Doxorubicin showed an ability to inhibit the expression of α-SMA in feline kidney cell line independently and not associates with KCa3.1 inhibition. In other words, 8 μM of Doxorubicin incubation for 48 hours did not induce fibrosis in CRFK cells and it probably inhibits the outcome of EMT. These results were consistent with a study of doxorubicin's effect on TGF-β signaling in A549 cells which is isolated from human lung cancer tissue. In that study, it was found that doxorubicin can reduce the level of mRNA coding of TGF-β type II receptor and the expression of Smad proteins (Filyak et al., 2008). Moreover, one study of rat liver fibrosis induced by bile duct ligation found the antifibrotic effect of Doxorubicin. In that study, Doxorubicin can reduce the expression of α-SMA in liver (Greupink et al., 2006).

Our results demonstrated the reduction of α-SMA. α-SMA is widely recognized as myofibroblast marker (Rao et al., 2014). Eventhough many previous found that the KCa3.1 blocking agent such as TRAM-34 can reduce the expression of α-SMA (Anumanthan et al., 2018; Seo et al., 2021).

There are many limitations in this study. Firstly, there is a few evidence of the method of fibrosis induction in feline kidney cells. Doxorubicin is one of nephrotoxic drugs which can generate oxidative stress in the kidneys. Doxorubicin has been used for fibrosis induction in studies of organ fibrosis (Arafa et al., 2014; Szalay et al., 2015). Eventhough Doxorubicin has been used as initiating agent to induce fibrosis, there is no study on the effect of Doxorubicin in renal fibrosis in cats. The appropriate dose and duration of doxorubicin incubation which can induce fibrosis on CRFK cells should also be determined. The further investigation of another nephrotoxic drugs that can cause fibrogenesis is needed.

Secondly, there is no commercial antibody of fibrosis markers for western blot in cats. Antibodies used in our study were rabbit anti-human protein antibodies. Sequence blasting of target proteins must be required before performing the

experiment. Furthermore, the trial of antibody dilution should be performed to evaluate the suitable condition for protein detection method in western blot assay.

Third, the immortal characterization of CRFK cells may interrupt functions and appearance of normal renal tubular cells. Primary cell culture probably presents more typical features of feline kidney cells than cell line. The expression of KCa3.1 on CRFK cells that were changed their phenotype should be observed. Moreover, there is a report indicated that CRFK cells expressed more S100A4 gene which is metastasis and prognosis marker in renal cell carcinoma than feline proximal tubular cells primary feline and fibroblasts (Lawson et al., 2019). Therefore, using Doxorubicin, which is antineoplastic drug, probably caused inaccurate findings.

Finally, a single concentration of TRAM-34 that was used in this study may be inadequate to access its fibrosis attenuated effect on CRFK cells. Various concentrations of TRAM-34 levels should be used to determine the effect on KCa3.1 in future investigations about renal fibrosis in CRFK cells.

In conclusion, TRAM-34 at a concentration of 0.1 µM may improve percentage of cell viability in DOX induce CRFK cell but cannot significantly decrease the expression of fibrotic markers in TGF-β/Smad signaling cascade. Therefore, the role of KCa3.1 in feline renal fibrosis via TGF-β/Smad signaling pathway is still unclear. To understand the pathogenesis of feline CKD, another pathway of fibrogenesis in cats should be investigated. KCa3.1 is probably one of the keys in the progression of chronic kidney disease, but its role needs to be observed in many aspects for the development of feline CKD treatment in the future.

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