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Article

Unraveling TGF-β's Role in Feline Chronic Kidney Disease as a Key Driver to Fibrosis and Cell Death

Chanyanuch Intachat [1], Phongsakorn Chuammitri [1,2], Benjaporn Sornpet [3], Prapas Patchanee[1], Nawin Manachai [1] and Kakanang Piyarungsri [1,2,*]

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Simple Summary: Chronic kidney disease (CKD) is a significant disease associated with fibrosis and apoptosis. Transforming growth factor-beta (TGF-β) plays a role as a pro-fibrotic mediator. This study aims to evaluate the levels of *TGFβ, Bcl2,* and *MAPK* mRNA expression in doxorubicin-induced feline kidney cells and naturally occurring CKD-affected kidney tissues and to determine the protein expression of TGF-β and MAPK in doxorubicin-induced feline kidney cells. The result indicated a significant increase in *TGFβ* and a considerable decrease in *Bcl2* in the doxorubicin-treated feline kidney cells. The protein expression of TGF-β and MAPK was elevated in both doxorubicin-induced feline kidney cells and the kidney tissue of cats with CKD. The study concluded that TGF-β and Bcl2 may regulate renal fibrosis and apoptosis in feline kidney cells. In vivo, TGF-β and MAPK may be associated with renal fibrosis and apoptosis. Further research on these mediators could lead to the development of therapeutic medications that delay the progression of CKD.

Abstract: Chronic kidney disease (CKD) is increasingly common in older cats. The transforming growth factor-beta (TGF-β) pathway is associated with renal fibrosis. This study aimed to quantify the mRNA expression of *TGFβ, MAPK,* and *Bcl2* genes and the protein expression of TGF-β and MAPK in feline kidney cells and tissues. Gene expression analysis was conducted using relative gene expression, while protein expression was assessed through western blot analysis. The immunohistochemistry staining of TGF-β and MAPK was performed on feline kidney tissues. The result reveals the significant upregulation of *TGFβ* ($P = 0.001$) and considerable downregulation of *Bcl2* ($P = 0.010$) in doxorubicin-treated feline kidney cells. Protein expression level of TGF-β and MAPK also tended to increase in doxorubicin-induced feline kidney cells. The immunostaining levels of TGF-β and MAPK were higher in the kidney tissues of cats with CKD compared to the no lesions group. A deeper understanding of the TGF-β pathway could enable veterinarians to monitor disease progression and mitigate complications in feline CKD.

Keywords: Bcl-2; cat; chronic kidney disease; mitogen-activated protein kinase; transforming growth factor-β

1. Introduction

Chronic kidney disease (CKD) is an important disease in cats. The definition of CKD is an abnormality in the anatomy or function of one or both kidneys that persist for at least three months [1]. The prevalences of feline CKD were different in each country, with rate of 3.6% in the United Kingdom [2], 2.37 % in Chiang Mai, Thailand [3], and 50% in the United States [4]. Moreover, the prevalence increased with age; 6 months-5 years was 37.5%, 5-10 years was 40.9%, 10-15 years was 42.1%, and 15-20 years was 80.9% [2]. The glomerular filtration rate (GFR) measurement is the gold standard of renal function assessment. Nevertheless, this method is complicated to perform in routine health checks. The guideline for the feline CKD diagnosis was suggested by the International Renal

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Interest Society (IRIS). They suggest routine investigation of serum creatinine, blood urea nitrogen (BUN), urinalysis, symmetric dimethylarginine (SDMA), and ultrasonography. Criteria for CKD consist of creatinine greater than 1.6 mg/dl and/or inappropriate urine (USG less than 1.035) for several months [5]. The most common lesion of feline CKD is a tubulointerstitial lesion, including interstitial fibrosis, interstitial inflammation, tubular mineralization, and hyperplastic arteriolosclerosis. Interstitial fibrosis score was found to be increased with the IRIS stage [6]. In the renal fibrosis process, there are four pro-fibrotic mediators, including TGF-β, transglutaminase 2 (TG2), endothelin 1 (ET1), and the renin-angiotensin-aldosterone system (RAAS) components. The most crucial pro-fibrotic mediator is TGF-β, which changes various types of cells to myofibroblasts [7].

Transforming growth factor-beta (TGF-β) plays a role in regulating cell proliferation as either an inhibitor or stimulator, depending on signaling pathways. In addition, TGF-β is involved in wound healing, differentiation of cells, and the immune system. It is also associated with pathological processes, including connective tissue disorder, fibrosis, and cancer [8]. The TGF- β signaling pathway consists of 2 pathways: smad-dependent/canonical pathway and smad-independent/non-canonical pathway [9]. TGF-β is associated with apoptosis through these two pathways: the smad-dependent pathway affects BCL-2 family expression, and the smad-independent pathway induces apoptosis by activating p38 and JNK pathways [10]. In cats, previous study demonstrated that the urinary TGF-β1: creatinine ratio was significantly enhanced in CKD cats compared to healthy cats and positively correlated with creatinine in serum [11]. The study of feline kidney tissue found that TGF-β immunohistochemistry staining in CKD cats was stronger than in healthy cats [12]. While TGF-β concentration in CKD cats' blood was significantly less than in healthy cats. Moreover, they found that cats with lower circulating TGF-β had shorter life spans than those with higher circulating TGF-β [13]. The Mitogen-activated protein kinase (MAPK) is an enzyme that functions as a serine/threonine protein kinase. The MAPK was controlled by various extracellular stimuli, which then caused gene expression, cell division, and cell survival. The MAPK pathway contributes to acute kidney injury (AKI) and CKD [14]. Previous studies indicated the association between the MAPK pathway and renal fibrosis [15,30]. In mice, renal artery stenosis (RAS) surgery is a technique for inducing tubular atrophy and interstitial fibrosis. The result revealed increasing p38 MAPK signaling in mice undergoing RAS surgery compared to sham mice. Moreover, blocking p38 MAPK with the inhibitors in RAS mice led to a reduction in interstitial inflammation, interstitial fibrosis, and tubular atrophy compared to RAS without p38 MAPK inhibitors [15]. Furthermore, the MAPK pathway is involved in the apoptosis process, with the p38 MAPK and JNK pathways regulating the Bcl2 family [16]. The B-cell lymphoma 2 (Bcl-2) protein family plays a role in cell growth and apoptosis regulation. It is found to be associated with renal cell death during kidney injury [17]. There are three classes of the Bcl-2 protein family: anti-apoptosis (Bcl-XL, Bcl-2), pro-apoptosis (Bak, Bax), and BH3-only proteins (Bid, Bad) [18]. In cats, Bcl-2 expression in the blood of the CKD cats was significantly lower than in healthy cats. Bcl-2 immunoreactivity in feline renal tissues was found in glomerular and tubular epithelium cells. Previous studies suggested that Bcl-2 was an acceptable diagnostic marker for feline CKD. It was also indicated that higher CKD stages were associated with lower Bcl-2 concentration [19].

However, there were few studies of the TGF-β signaling pathway, which involves MAPK and Bcl-2, in cats with chronic kidney disease. This study aimed to evaluate TGFβ, MAPK, and Bcl2 gene expression in doxorubicin-induced feline kidney cells and kidney tissues of cats with naturally occurring CKD and to determine TGF-β and MAPK protein expression in doxorubicin-treated feline kidney cells.

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2. Materials and Methods

2.1. Sample

Feline kidney cell line

The Cradle Rees Feline Kidney (CRFK) cell line was procured from ATCC® (CCL-94), LOT 60980362. These cells originate from the cortex epithelium of the kidney of 12-week-old female domestic cats. Cells were grown in Dulbecco's Modified Eagle Medium (DMEM), 5% fetal bovine serum (FBS), 100 μg/ml streptomycin, 100 units/ml penicillin, and non-essential amino acids solution and incubated at 37 °C in 5% CO_2 in a humidified incubator. The cells used in this study were in passages 125-132 passages.

Kidney tissues

Kidney tissues were collected from cats with no kidney lesions (n = 6) and cats with CKD (n = 6) that died at the Small Animal Hospital, Faculty of Veterinary Medicine, Chiang Mai University. The samples were preserved in 10% formalin or frozen at -20 °C to keep them fresh. Paraffin block samples were prepared from 10% formalin-preserving tissues, cut into slides, and stained with Hematoxylin and Eosin. A pathologist examined the histological morphologies and categorized them as no kidney lesions or CKD. The Ethics Committee approved the Human and Animal Experimentation protocol, Faculty of Veterinary Medicine, Chiang Mai University, under the reference number S1/2566.

2.2. Sample Collection

Cytotoxicity test of doxorubicin-induced CRFK

CRFK cells were incubated with different concentrations and durations of doxorubicin (DOX) (Catalogue no. 324380). The concentrations of DOX used were 0, 1, 2, 4, and 8 μM, and the cells were incubated for 48, 72, and 96 hours [20]. The CRFK cells were grown in a 96-wells plate and kept overnight in a 37 °C, 5% CO_2 humidified incubator before adding DOX. Cell viability was determined using MTT colorimetric assay (Mosmann, 1983) [21]. The study applied the appropriate dose and duration that could decrease cell viability by 50%.

DOX-induced cytotoxicity test

CRFK cells were seeded into 6-well plates and then incubated at 37 °C in a humidified 5% CO_2 incubator overnight. The appropriate dose and duration of DOX were 8 μM for 48 hours. Next, DOX 8 μM was added to CRFK cells and incubated for 48 hours. After that, feline kidney cells with DOX-induced cytotoxicity were evaluated for the mRNA expression of *TGFβ*, *MAPK*, and *Bcl2*.

RNA extraction

The RNA was harvested from CRFK cells and kidney tissues. CRFK cells were grown in a 6-well plate before harvesting the RNA. RNA was extracted using TRIzol® reagent. First, the medium was removed, and then TRIzol® reagent was added to the plate for 15 minutes. The extraction was then transferred to the Eppendorf tube and centrifuged at 4 °C 12,000 rpm for 3 minutes. The supernatant was removed, and then isopropanol was added at room temperature for 10 minutes. The mixture was centrifuged at 4 °C 12,000 rpm for 3 minutes. The supernatant was removed, and 75% ethanol was added and centrifuged at 4 °C 8,000 rpm for 3 minutes. The supernatant was removed, and the RNA pellet was mixed with RNase-free water and transferred to a new Eppendorf tube. The RNA samples were then stored at -80 °C. For the kidney tissues, the kidney tissues were ground in liquid nitrogen and homogenized with TRIzol® reagent. Liquid sample was mixed with TRIzol® reagent at room temperature for 15 minutes. The process of RNA extraction was the same as cell line. The RNA quantification was measured using a NanoDrop spectrophotometer.

Protein extraction

The protein was extracted from CRFK cells in a 6-well plate. The growth medium was removed, and the cells were washed with PBS. Next, trypsin was applied to detach cells, and then growth medium was added. The samples were transferred to Eppendorf tubes and centrifuged at 4 °C at 1,200 rpm for 10 minutes. The supernatant was removed, and RIPA (lysis buffer) was added and left in an ice container for 30 minutes. The samples were centrifuged at 4 °C at 12,000 rpm for 10 minutes.

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The supernatant was collected and stored at -80 °C. Protein quantification was measured using the Bradford assay. The Bradford solution (Catalogue no. 500-0006) was diluted at 1:3 with PBS. The diluted-Bradford was added to the protein samples in 96-well plates. Then, protein lysates were added to each well and incubated at room temperature for 10 minutes. The protein quantification was assessed at 540 nm.

2.3. Sample Processing

Relative gene expression

The total RNA from CRFK cells and kidney tissues was extracted using TRIzol® reagent (Catalogue no. R4533). Real-time polymerase chain reaction was performed using iQ5 real-time PCR with IO SYBR green supermix (Catalogue no. 08-24-00001). The qPCR protocol involved an initial polymerase activation step to 95 °C for 12 min, followed by 40 cycles of denaturation at 95 °C for 15 sec and annealing/extension at 60°C for 30 sec. Data acquisition was performed at the end of the annealing/extension step. All reactions were conducted in triplicate using 96-well reaction plates (2 µl per reaction). In this study, the housekeeping gene was β-actin. The mRNA expression levels of *TGFβ*, *MAPK*, and *Bcl2* were calculated as relative gene expression ratio with β-actin using $2^{-\Delta CT}$. The primer sequences used in this study are detailed in Table 1.

Table 1. The primer sequences.

Gene name	Accession	Direction	Sequence	Annealing Temp. (°C)	Size (bp)
TGFβ	M38449.1	Forward	CCCTGGACACCAACTATTGC	60	163
		Reverse	TCCAGGCTCCAAATGTAGGG	60	
MAPK	XM_003994973.5	Forward	ACTGCTGAGCTAAGACCATGAG	60	119
		Reverse	AAGTCAATGCCACAGTGTGC	60	
Bcl2	NM_001009340.1	Forward	CCTATCTGGGCCACAAGTGA	60	123
		Reverse	TAAGAGACCACGGCTTCGTT	60	
β-actin	AB051104.1	Forward	CCATCGAACACGGCATTGT	60	147
		Reverse	TCTTCTCACGGTTGGCCTTG	60	

Bcl2 = B-cell lymphoma 2, *MAPK* = Mitogen-activated protein kinase, *TGFβ* = Transforming growth factor-beta.

Western blot analysis

The protein samples at a concentration of 30 µl were mixed with 2×Laemmli sample buffer (Bio-Rad) containing β-mercaptoethanol and heated at 95 °C for 5 min. Next, proteins were separated on 12% SDS-PAGE gels and transferred onto a polyvinylidene fluoride (PVDF) membrane 0.45 µm pores (Bio-Rad), utilizing a Trans-Blot® SD Semi-Dry Transfer Cell (Bio-Rad). Next, the membrane was washed with Tris-buffered saline with 0.05% Tween (TBST) and blocked with BSA at room temperature for 45 minutes. The membrane was incubated for 2 hours with primary antibodies for TGF-β, MAPK, or β-actin (direct-blot HRP mouse monoclonal anti-β-actin, BIOL-643808, BioLegend) and washed with TBST. After that, it was incubated with a secondary antibody labeled as HRP-conjugated antibody for 45 minutes at room temperature. The primary and secondary antibodies are shown in Table 2. The signal detection was performed using a DAB substrate (Bio Basic). Protein quantifying densitometry was determined using Image Studio™ Lite (LI-COR, Lincoln, Nebraska, USA) and then calculated as the ratio of TGF-β or MAPK to β-actin as loading control.

Immunohistochemistry

The kidney tissues from dead cats were collected through necropsy. The tissues were fixed in 10% formalin, and embedded in paraffin, then sliced into sections of 2-7 µm thickness using a rotary microtome. Deparaffinization was conducted using xylene, followed by 100% ethanol and 95% ethanol before staining. For heat-induced epitope retrieval (HIER), microwave 800 W was used for 20 minutes with sodium citrate buffer at pH 6. The tissue area was outlined using an

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immunohistochemistry pen, and 0.3% hydrogen peroxidase (H_2O_2) was applied for 5 minutes. The slides were washed with PBST for 5 minutes, three times. Next, 2.5% BSA in PBS was added to the samples at room temperature for 5 minutes and washed with PBST for 5 minutes, three times. Immunohistochemical staining was performed by incubating the tissue section with the primary antibodies at 37 °C for 2 hours, followed by washing with PBS for 5 minutes, three times. The tissue section was incubated with a dilution of PBS with normal goat serum (1:5) for 30 minutes and washed with PBS for 5 minutes, three times. The secondary antibodies were incubated at room temperature in a dark room for 45 minutes and washed with PBS for 5 minutes, three times. DAB solution was used to develop the staining at room temperature for 5 minutes, followed by washing the slide with tap water and staining with Hematoxylin and Lithium. The primary and secondary antibodies are shown in Table 2. CaseViewer program was used to evaluate the quality of immunoreactivity.

Table 2. Primary and secondary antibodies.

Protein name	Antibodies	Dilution	
		WB	IHC
TGF-β	Primary mouse monoclonal TGF-beta 1	1:1000	1:200
	Secondary goat anti-mouse	1:5000	
MAPK	Primary mouse polyclonal p38 MAPK	1:1000	1:200
	Secondary goat anti-mouse	1:5000	
β-actin	Direct-Blot HRP mouse monoclonal anti-β-actin	1:1000	

IHC = Immunohistochemistry, MAPK = Mitogen-activated protein kinase, TGF-β = Transforming growth factor-beta, WB = western blot analysis,.

2.1. Statistical Analysis

The data were presented as either mean ± standard deviation (SD) or median. We conducted a normality test using the Shapiro-Wilk test. The one-way analysis of variance (ANOVA) with the Bonferroni post hoc test was used to compare the mean between groups. An unpaired t-test or Welch two-sample t-test was performed to compare the mean between the two groups. Each experiment consisted of at least n = 5, where n was the number of monolayers or cat kidney tissues. A *P*-value less than 0.05 was considered significant.

3. Results

3.1. Cytotoxic Test of Doxorubicin-Induced Cytotoxicity in Feline Kidney Cells

The cell viability of CRFK was measured after being treated with DOX at concentration of 1, 2, 4, and 8 μM for 48, 72, and 96 hours. The results revealed that after 48 hours, the cell viability was 83.08%, 76.78%, 64.87%, and 53.83% for the respective DOX concentrations (Figure S1). After 72 hours, the cell viability percentages were 111.35%, 97.53%, 107.04%, and 59.37% for the corresponding DOX concentrations (Figure S2). The cell viability after 96 hours of treatment was 82.97%, 68.93%, 62.61%, and 61.04% for the respective DOX concentration (Figure S3). Our study focused on using DOX at a concentration of 8 μM for 48 hours.

3.2. TGFβ, MAPK, and Bcl2 Relative Gene Expression in Feline Kidney Cells

The results revealed that the mean fold-change of *TGFβ* gene expression in the DOX-treated group was 4.084. The difference in *TGFβ* gene expression fold change between the control and the DOX-treated group was 3.084 ± 0.668 (Figures 1 and 2). *TGFβ* gene expression in the DOX-treated group was significantly higher compared to the control group. The mean fold-change of *MAPK* gene

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expression in the DOX-treated group was 1.072. The difference in *MAPK* gene expression fold-change between the control and the DOX-treated group was 0.072 ± 0.509 (Figures 1 and 2). *MAPK* gene expression in the DOX-treated group did not differ from the control group. The mean fold-change of *Bcl2* gene expression in the DOX group was 0.596. The difference in *Bcl2* gene expression fold-change between the control and the DOX-treated group was -0.404 ± 0.125 (Figures 1 and 2). *Bcl2* gene expression in the DOX-treated group was significantly lower than in the control group. These results of *TGFβ*, *MAPK*, and *Bcl2* mRNA expression are available in Table 3.

Table 3. Fold-change and difference between mean ± SD of *TGFβ*, *MAPK*, and *Bcl2* gene expressions in controls and DOX-induced feline kidney cell toxicity.

mRNA expression	Fold-change		Difference between mean ± SD	ANOVA *P*-value	Unpaired t-test *P*-value
	Control (n = 6)	DOX-treated (n = 6)			
TGFβ	1	4.084	3.084 ± 0.668		0.001**
MAPK	1	1.072	0.072 ± 0.509	0.002**	0.890
Bcl2	1	0.596	-0.404 ± 0.125		0.010*

ANOVA = Analysis of variance, *Bcl2* = B-cell lymphoma 2, DOX = Doxorubicin, *MAPK* = Mitogen-activated protein kinase, SD = Standard deviation, *TGFβ* = Transforming growth factor-beta, * $P < 0.05$, ** $P < 0.01$.

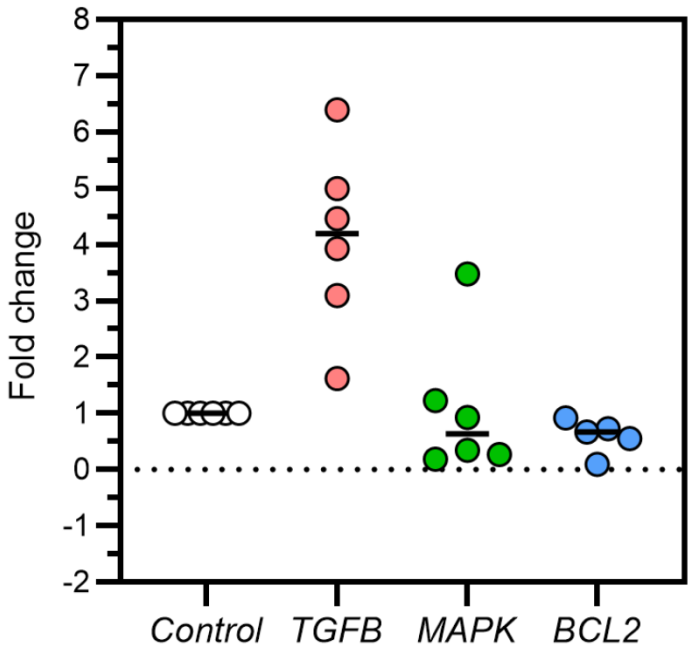

Figure 1. Fold-change of *TGFβ*, *MAPK*, and *Bcl2* gene expressions in controls and DOX-induced feline kidney cell toxicity.

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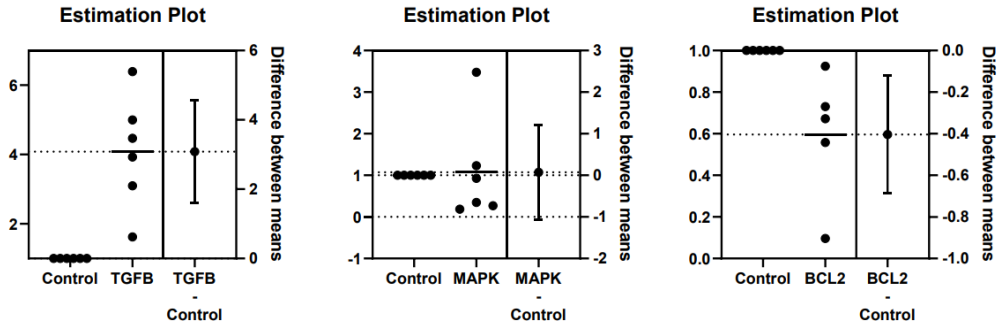

Figure 2. Difference between mean ± SD of *TGFβ*, *MAPK*, and *Bcl2* gene expressions in controls and DOX-induced feline kidney cell toxicity.

3.3. TGFβ, MAPK, and Bcl2 Relative Gene Expression in Kidney Tissues

The fold-change of *TGFβ*, *MAPK*, and *Bcl2* gene expression were 0.563, 0.925, and 0.683, respectively (Figure 3). The difference of *TGFβ*, *MAPK*, and *Bcl2* gene expression between cats with no kidney lesions and cats with CKD were -0.437 ± 0.222, -0.075 ± 0.373, and -0.317 ± 0.251, respectively (Figure 4). *TGFβ* and *Bcl2* gene expressions in CKD cats were slightly lower than in cats with no kidney lesions. However, the *MAPK* gene expression in the CKD cats was not significantly different from that in the cats with no kidney lesions. The results of gene expression in kidney tissues are shown in Table 4.

Table 4. Fold-change and difference between mean ± SD of *TGFβ*, *MAPK*, and *Bcl2* gene expressions in kidney tissues of cats with no kidney lesions and cats with chronic kidney disease (CKD).

mRNA expression	Fold-change		Difference between mean ± SD	ANOVA *P*-value	Unpaired t-test *P*-value
	No kidney lesions (n = 6)	CKD (n = 6)			
TGFβ	1	0.563	-0.437 ± 0.222	0.549	0.081
MAPK	1	0.925	-0.075 ± 0.373		0.846
Bcl2	1	0.683	-0.317 ± 0.251		0.238

ANOVA = Analysis of variance, *Bcl2* = B-cell lymphoma 2, CKD = Chronic kidney disease, DOX = Doxorubicin, *MAPK* = Mitogen-activated protein kinase, SD = Standard deviation, *TGFβ* = Transforming growth factor-beta.

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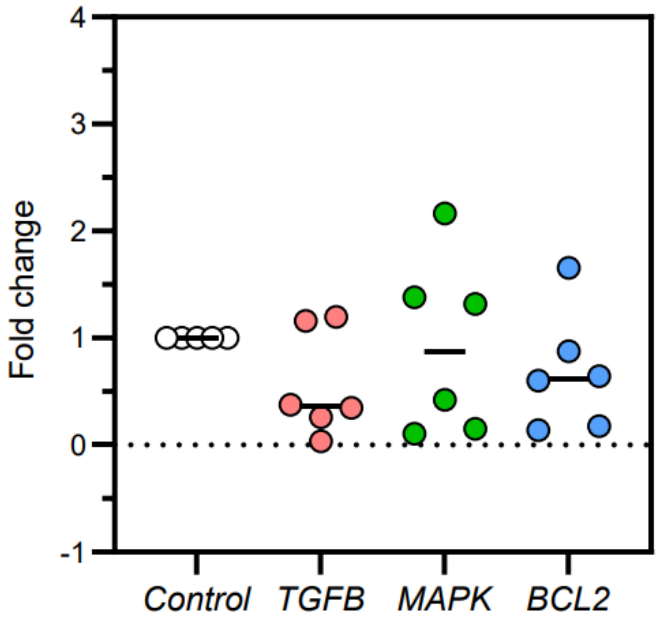

Figure 3. Fold-change of *TGFβ, MAPK,* and *Bcl2* gene expressions in kidney tissues of cats with no kidney lesions and cats with chronic kidney disease (CKD).

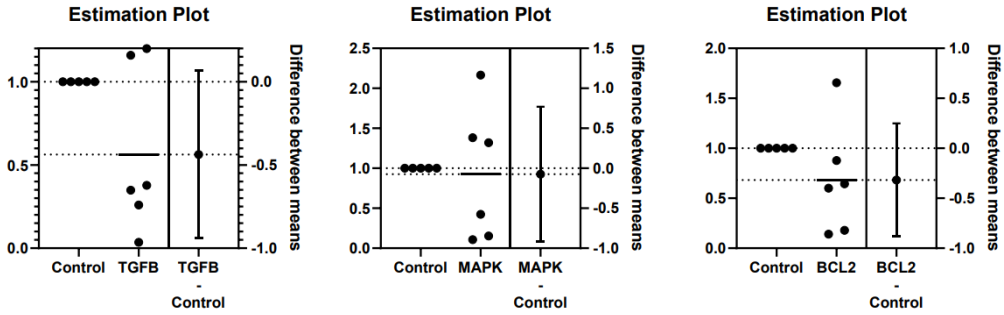

Figure 4. Difference between mean ± SD of *TGFβ, MAPK,* and *Bcl2* gene expressions in kidney tissues of cats with no kidney lesions and cats with chronic kidney disease (CKD).

3.4. Protein Expression of TGF-β and MAPK in Feline Kidney Cells

The protein expression of TGF-β was a trend to increase in the doxorubicin-treated group (0.06 ± 0.07) compared to the control group (0.04 ± 0.05) (Figure 5). MAPK protein expression of the doxorubicin group was 2.28 ± 2.64, and the control group was 0.44 ± 0.14. The protein expression of MAPK was a trend to increase in the doxorubicin-treated group (Figure 6). The results are shown in Table 5.

Table 5. Mean ± SD of TGF-β and MAPK protein expressions in control and DOX-induced feline kidney cell toxicity.

Protein expression	Control (n = 3)	DOX-treated (n = 3)	*P*-value
TGF-β	0.04 ± 0.05	0.06 ± 0.07	0.73
MAPK	0.44 ± 0.14	2.28 ± 2.64	0.68

DOX = Doxorubicin, MAPK = Mitogen-activated protein kinase, TGF-β = Transforming growth factor-beta.

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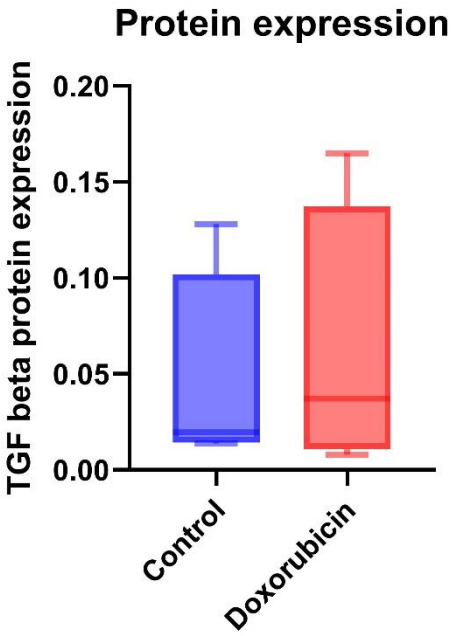

Figure 5. TGF-β protein expressions in control and doxorubicin-induced feline kidney cell toxicity.

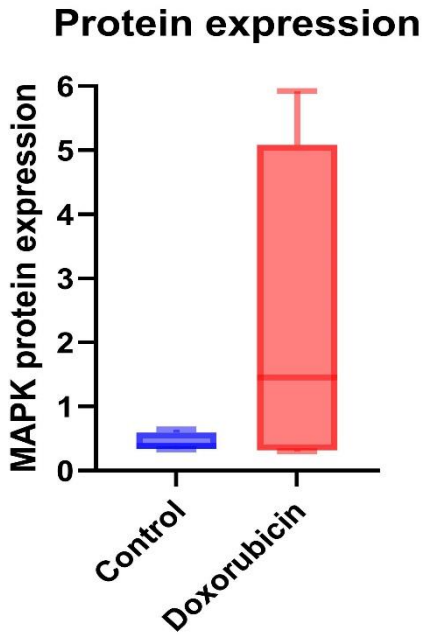

Figure 6. MAPK protein expressions in control and doxorubicin-induced feline kidney cell toxicity.

3.5. Immunohistochemistry of TGF-β and MAPK in Cat Kidney Tissues

TGF-β and MAPK immunostaining were observed in the tubulointerstitial area of the kidney tissue of CKD cat (Figure 7B,D), while no staining was present in kidney tissue with no lesions (Figure 7A,C).

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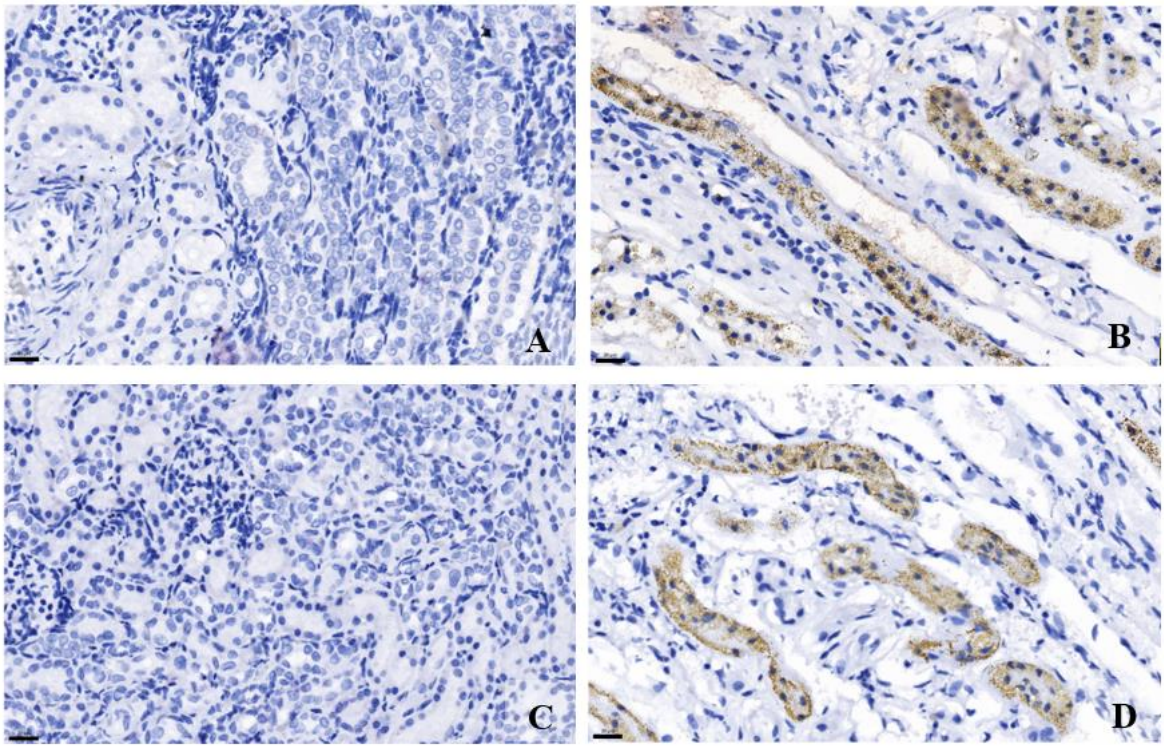

Figure 7. The immunohistochemistry staining from cats' kidney tissues. TGF–β immunohistochemistry staining in a cat with no lesion in kidney tissue **(A)** and a cat with CKD **(B)**. MAPK immunohistochemistry staining in a cat with no lesion in kidney tissue **(C)** and a cat with CKD **(D)**. Original magnification 400×. Scale bar = 20 μm.

4. Discussion

Doxorubicin can lead to oxidative stress in glomerular epithelial cells [22] and result in kidney damage [23]. An eight μM dose of DOX for 48 hours was determined to be the appropriate dosage and duration to induce cytotoxicity. This finding is consistent with a previous study in CRFK cells, which demonstrated a 50% decrease in cell viability of eight μM doxorubicin for 48 hours [24]. It is similar to the study of human kidney cells. The cell viability of HK-2 cells significantly decreased at a doxorubicin concentration of 8 μM for 24 hours, dropping to nearly 50% [20,25].

The significant lesion in CKD cats is tubulointerstitial fibrosis [6]. However, tubular atrophy is also markedly found in the kidneys of CKD cats [13]. Hence, fibrosis and apoptosis are associated with feline CKD development. The pro-fibrotic mediator associated with renal fibrosis is TGF-β [7]. The oxidative stress stimulates TGF-β production [26]. A TGF-β signaling pathway is composed of 2 cascades: smad-dependent (canonical) pathway and smad-independent (non-canonical) pathway [9]. Our study demonstrated the expression of the *TGFβ* gene significantly increased in the DOX-treated group compared to the control group. In feline kidney cells, a previous study found an increase in α-SMA, CTGF, TNC, TSP-1, and COL1 gene expression in the TGF-β-treated group [27]. In rat kidney cells, TGF-β treated group had higher α-SMA, COL1, and COL3 than the control group [28]. The previous studies in kidney cells demonstrated the pro-fibrotic mediator effect of TGF-β [27,28]. These results may explain that fibrosis was related to oxidative stress in feline kidney cytotoxicity.

The present study found that *TGFβ* gene expression in kidney tissues of CKD cats was lower than in cats with no kidney lesions. The gene expression of *TGFβ* revealed higher in the kidney tissues of mice with unilateral ureteral obstruction (UUO) when compared to sham mice [29]. In diabetic nephropathy mice, *TGFβ* mRNA expression in kidney tissue of diabetic mice with blood glucose fluctuation (BGF) was the highest compared to diabetic mice without BGF and control mice [30]. This difference in RNA stability in tissue can be attributed to temperature variations and collection procedures. It is important to note that the stability of RNA in the tissue is influenced by temperature

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[31]. A post-mortem study demonstrated that human kidney tissues were unsuitable for RNA evaluation [32]. Only one sample in our study was obtained via nephrectomy, while the other tissues were collected post-mortem. Urine TGF-β: creatinine in cats was correlated with fibrotic severity [33]. However, the kidney tissue in the immunohistochemistry part was derived from a cat with CKD stage II that may exhibit mild fibrosis. The CKD stage III tissue sample was from a cat with polycystic kidney disease (PKD) and severe subcapsular effusion. These kidney tissues in the CKD stage III group were compressed by fluid, possibly leading to reduced *TGFβ* gene expression.

Our study demonstrated that TGF-β protein expression in feline kidney cells tended to increase in the DOX-treated group compared to the control group. Immunohistochemistry of TGF-β in kidney tissue also indicated positive immunostaining in the tubular area of CKD cats. In agreement with a previous study, TGF-β immunostaining was predominantly located in the distal tubules and collecting ducts of kidney tissue of cats with CKD [13]. In contrast, normal feline kidney tissue showed weak immunostaining of TGF-β [12]. Additionally, kidneys tissues from patients experiencing renal fibrosis showed higher levels of TGF-β and α-SMA immunostaining compared to negative control [29]. The previous study reveals that the urine TGF-β to creatinine ratio was significantly higher in CKD cats than in healthy ones [11]. Moreover, a moderate correlation was identified between the urine TGF-β to creatinine ratio and interstitial fibrosis [33]. Analysis of TGF-β levels in the blood of cats indicated that the TGF-β concentration in those with CKD was significantly lower than in healthy cats. Furthermore, cats with low circulating TGF-β concentrations had shorter survival times [13]. Therefore, targeting the TGF-β pathway is important for developing therapies aimed at preventing the progression of fibrosis in the kidney [34]. In a previous study, IN-1130 showed potential in reducing renal fibrosis in rats subjected to UUO by inhibiting the TGF-β pathway [35].

Our study demonstrated that the *MAPK* gene expression response to DOX-induced cytotoxicity. MAPK protein expression in the DOX-treated cells was a trend to increase compared to the control cells. MAPK plays a role in signaling transmission of extracellular stimuli to intracellular response and apoptosis [16] and oxidative stress [36]. In cardiac cells, phosphorylated p38 MAPK protein expression was significantly increased in DOX-induced H9c2 cardiac cells. However, total p38 MAPK in DOX-induced cardiac cells was not different from control cells [37]. Moreover, the MAPK signaling pathway can be stimulated by TGF-β through TAK-1/MKK6/p38MAPK pathway [38], which was categorized as a TGF-β smad-independent pathway [39]. In a previous study, mice undergoing unilateral ischemia-reperfusion injury had higher *TGFβ* and *p38MAPK* mRNA expression than sham mice [40]. Our study also indicated that MAPK immunoreactivity in the kidney tissue of CKD cats was higher than in cats with no kidney lesions. Several previous studies revealed a high level of MAPK immunoreactivity in other species with kidney problems [41,42]. In patients with IgA nephropathy, p-p38MAPK immunostaining was predominantly in high-grade renal fibrosis [41]. In mice models, p-p38MAPK immunostaining was eminently in UUO mice [40]. In experimental nephrotic syndrome, p-p38MAPK staining was increased in podocyte, parietal epithelial cells, and glomeruli [42]. Therefore, MAPK may be associated with feline kidney disease through hypoxia and renal fibrosis, especially the TGF-β pathway.

The present study revealed that *Bcl2* gene expression in the DOX-treated group was significantly lower than in the control group. Moreover, the gene expression of *Bcl2* in the kidney tissue of CKD cats was a trend to decrease compared to cats with no kidney lesions. In the experiment-induced apoptosis, there was downregulation of Bcl-2 and upregulation of p38MAPK and Bax expression [43,44]. Additionally, the level of Bcl-2 in cats with CKD was lower than in healthy cats [19]. The study in leukemia cells found that treating cells with TGF-β promoted cell death, increased Bax, and deceased Bcl-2 expression [45]. In patients with glomerulonephritis, TGF-β had a negative correlation with Bcl-2 but a positive correlation with Bax [46]. These results may explain that apoptosis was related to feline kidney cytotoxicity and CKD development in cats. Bcl2 was regulated by the TGF-β smad-dependent pathway [39], and the downregulation of Bcl-2 may regulate apoptosis in feline CKD [19].

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Oxidative stress, renal fibrosis, and apoptosis play crucial roles in the progression of feline CKD [7,29,47]. The present study provided information on the TGF-β mediator that may be associated with renal fibrosis and apoptosis in feline CKD. Our study demonstrated the TGF-β mediator gene and protein expression in cats with naturally occurring CKD. This study may lead to further investigation into therapeutic medication that can delay renal fibrosis and apoptosis in CKD progression and benefit in prolonging the life span of cats with CKD. However, this study has limitations, including a small sample size of kidney tissue and incomplete blood profile data. Obtaining more blood samples and increasing the number of tissue samples could provide valuable information about these mediators.

5. Conclusions

The current study revealed that *TGFβ* was upregulated in feline kidney cells but downregulated in feline kidney tissues. Additionally, *Bcl2* was downregulated in both feline kidney cells and tissues. However, there was no significant change in *MAPK* gene expression in feline kidney cells and tissues. Protein expression of TGF-β and MAPK in the DOX-treated cells showed a trend to increase compared to control. The immunoreactivity of TGF-β and MAPK in kidney tissues of cats with CKD was highly stained compared to healthy tissue. This result indicated that TGF-β, MAPK, and Bcl-2 may regulate renal fibrosis and the progression of apoptosis in feline kidney cells (Figure 8). There have been studies on TGF-β inhibitors in organ fibrosis. However, knowledge regarding TGF-β inhibitors in renal fibrosis among animals is limited. TGF-β inhibitors have not been studied in cats with chronic kidney disease (CKD). Further research on these mediators could lead to the development of therapeutic medications that delay the progression of CKD.

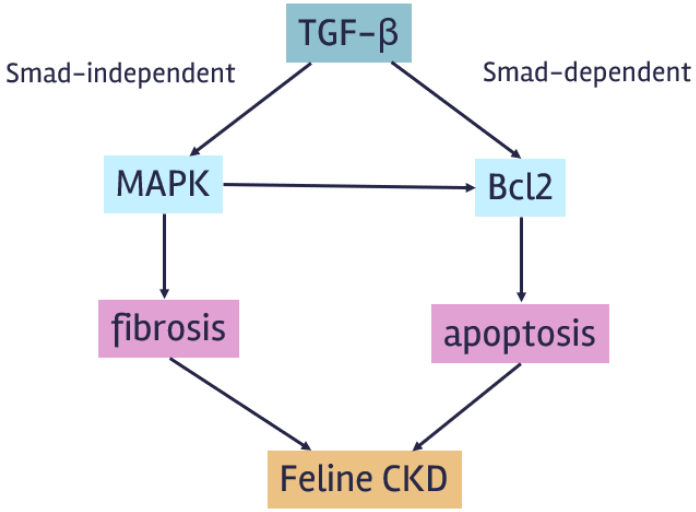

Figure 8. The summary of TGF-β pathway.

6. Patents

This section is not mandatory but may be added if there are patents resulting from the work reported in this manuscript.

Supplementary Materials: The following supporting information can be downloaded at: the website of this paper posted on Preprints.org.

Author Contributions: Conceptualization, K.P. and P.C.; methodology, C.I., K.P., N.M. and P.C.; software, C.I., K.P. and P.C.; validation, C.I., K.P., N.M. and P.C.; formal analysis, C.I., K.P. and P.C.; investigation, C.I., K.P., N.M. and P.C.; resources, C.I., B.S., K.P., N.M. and P.C.; data curation, K.P., P.C. and P.P.; writing—original draft preparation, C.I.; writing—review and editing, K.P and P.C..; visualization, C.I. and K.P.; supervision, K.P. and P.C.; project administration, K.P. All authors have read and agreed to the published version of the manuscript.

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