

Regulation of TGF- β 1-Mediated Wnt/ β -catenin Pathways Plays an Important Role in EMT-like Transformation Induced by Cadmium

Yanlin Zhang¹, Jiandong Wu¹, Lulu Ren¹, Guning Wang¹, Lihong Yuan¹, Zhihui Zou^{2,*}

¹Department of Environmental Monitoring, Guangdong Vocational College of Environmental Protection Engineering, Foshan, China

²School of Public Health, Guangdong Pharmaceutical University, Guangzhou, China

Email address:

zylin1980@126.com (Yanlin Zhang), zzhgyd@gdpu.edu.cn (Zhihui Zou)

*Corresponding author

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Abstract: Backgrounds Some studies have reported that different dosage of cadmium (Cd) had a biphasic effect on cells viability, Cd at a dose of more than 20 μ M can induce excessive apoptosis which past studies were mainly concentrated on while there has been few reports on a relatively lower dose of Cd causing abnormal proliferation. Kidney is the most sensitive target organ of Cd, however, the mechanism how Cd at a relatively lower dose affects the proliferation of renal tubular epithelial cells (RTEC) is not yet clear at present. Objectives To explore the toxic effect of Cd at a relatively lower dose on RTEC and study the mechanism how the TGF- β 1-mediated Wnt/ β -catenin pathways involved in EMT-like transformation induced by Cadmium. Methods The RTEC were isolated by mixed collagenase digestion methods and the effect of Cd at different dose on cell viability was detected by MTT assay. The effect of Cd at Hormesis zone dose on the expression of c-myc, cyclinD1, α -SMA, TGF- β 1, Wnt and β -catenin were determined by qRT-PCR. Results When exposed for 24h-72h, Cd at a dose of 2.5 μ M had maximum proliferation promoting effect. Cd at Hormesis zone dose could up-regulate the expression of cell cycle and proliferation key regulators c-myc and cyclinD1 in an obvious time-dependent manner. Cd at Hormesis zone dose could significantly promote α -SMA, TGF- β 1, Wnt and β -catenin mRNA expression. Conclusions Cd at Hormesis zone dose could induce RTEC significant abnormal proliferation and EMT-like transformation. The activation of TGF- β 1-mediated Wnt/ β -catenin pathways might play a key role in EMT-like transformation induced by Cd at Hormesis zone dose.

Keywords: Wnt/ β -catenin, TGF- β 1, EMT-like Transformation, Cadmium

1. Introduction

Cadmium (Cd) and its compounds are common industrial, environmental and occupational toxicants which can induce multiple organ damage [1]. Cd is one of the most harmful metal poisons to human beings and is classified as class I carcinogen by the International Agency for Research on Cancer [2].

The kidney is the most sensitive and the most easily accumulation target organ and the biological half-life of cadmium in the body's renal cortex usually lasts for 30 years, so renal tubular dysfunction is the critical effect of Cd exposure [3-4]. Tubular interstitial fibrosis (TIF) is the first

appearance and plays a key role during chronic renal injury caused by long-term cadmium exposure [5].

It is generally accepted that the core process of TIF induced by the exogenous toxins and drugs is epithelial mesenchymal transition (EMT), which is a process that pathogenic or toxic factors induce depolarization and phenotypic modulation of tubular epithelia cell [6-7]. The initiation of EMT involves the activation of muscle fibroblast (MF), the excessive synthesis and deposition of extra-cellular matrix (ECM), high expression and release of cell factors and growth cytokines [8-10].

Because Cd accumulated in the kidney by the reabsorption and resecretion of renal tubule, which is more affected than

other parts of the kidney [11]. In structure, renal tubular epithelial cells (RTEC) is closely linked to the MF, so RTEC is not only the target cells of cadmium toxicity but also the key participants in TIF [12]. Because about 30%~40% of MF originate from the EMT of RTEC [13-14], thus, it's very likely that the stimulating effect of Cd on RTEC proliferation is closely related to the activation of MF during chronic cadmium exposure. A lot of studies indicated that Cd had a biphasic effect on cell proliferation because of its exposure dose. Cd at a lower dose might cause non lethal injury, however, it could induce oxidative damage and excessive apoptosis when its exposure dosage increase more than 20 μ M [15]. Too much attention has been paid to the study on the latter in the past, but which is contradicted to the fact that human professional or environmental chronic Cd exposure dose is too low in most cases. Thus, it is of great significance to toxic effect on RTEC and pollution risk assessment of Cd at a lower dose [16-17]. However, the related research about effect of low-dose Cd on RTEC proliferation is rare and lack of report.

Because the initiation and occurrence of EMT induced by the exogenous poison is usually dynamic, RTEC was in an interim status characterizing as change of one or two specific phenotype-associated protein, high expression of few cytokines, which was described as EMT-like transformation (or partial EMT) [18-19]. Transforming growth factor β 1 (TGF- β 1) is known as yet to be the most important cytokine and its abnormal activation could be determined in almost all renal fibrosis models [20-21]. According to reports in some references, TGF- β 1 had a biphasic effect on the regulation of cellular biological effects [22]. Taken together, the activation of TGF- β 1 may be related to the EMT-like transformation induced by Cd at a certain low dose, which is of great significance for further understanding of renal toxic mechanism of Cd.

2. Experimental

2.1. Reagents

Cadmium chloride, trichloromethane, isopropanol, anhydrous ethanol and dimethyl sulfoxide were commercially analytical reagent. [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) was purchased from Sigma-Aldrich, China. RNAisoPlus RNA extraction kits, Prime Script reverse transcription kits and SYBR Premix Ex Taq II real-time fluorescent PCR Kits were supplied by TaKaRa Corporation from Japan.

2.2. Isolation and Culture of Rat Renal Tubular Epithelial Cells

Ten clean SD rats aged 5~6 weeks, half male and half female, weighing 190~210g, were supplied from the Experimental Animal Center of Sun Yat-sen University (the animal certificate number SCXK 2011-0029). The examination and acceptance number of the Laboratory Animal Ethics Committee is gdpulac2016050.

After extracting double kidney by aseptic manipulation, the mixture of 0.1% type I collagenases/0.1% type II collagenases (1:1, *V/V*) was applied to separate and purify the RTEC. The mixed enzyme digesting solution was added with 5ml per kidney ratio and shake out for 30 min at 37°C. After removal of flocculent cortical tissue through 100-mesh cell strainers, the harvest was centrifuged at 2000 r/min for 10 min using 22R high speed freezing centrifuge (Hettich Mikro Corporation, Germany). Finally the cell pellet was resuspended with Dulbecco's modified Eagle's medium (DMEM)/F12 medium (Gibco, USA) supplemented with 10% fetal bovine serum and routinely cultured at 37°C in a humidified incubator (RS Biotech Corporation, Britain) at 5% CO₂ [23-24].

2.3. Effects of Different Doses of Cd on Viability of RTEC

The RTEC in logarithm growth period were plated at a density of 5×10^4 cells/well in 96-well plates in 100 μ l and incubated for 24h, and then were exposed to DMEM/F12 medium with 10% fetal bovine serum containing 0 (control), 0.156, 0.312, 0.625, 1.25, 2.5, 5.0, 10.0, 20.0 and 40.0 μ M cadmium chloride for 24h, 48h and 72h, respectively. Before the end of exposure, 10 μ l of MTT (5 g/ml) was added to each well and continued to incubate for 4 h. The optical density at 560 nm (OD₅₆₀) was determined by xMark microplate reader (Bio-Rad, USA). Each experiment in different exposure time was performed in triplicate. The cell viability was expressed as a percentage of the control. The data were calculated by the formula: survival rate (%) = $(1 - OD_T / OD_C) \times 100\%$, where OD_T and OD_C represent the OD value in treatment groups and the control, respectively. Using logarithmic value of cadmium dose as abscissa and relative survival rate of RTEC as ordinate, the quadratic functional curve of the relation between cell viability and Cd exposure dosage were obtained, and finally determined the concentration of low-dose-cadmium which induced RTEC abnormal proliferation.

2.4. Detection of *c-myc*, *cyclind1*, α -SMA, TGF- β 1, Wnt, β -catenin mRNA Expression

The RTEC in logarithm growth period were plated at a density of 5×10^5 cells/well in 6-well plates in 1ml and incubated for 24h, and then were exposed to DMEM/F12 medium with 10% fetal bovine serum containing 0 (control), 0.625, 1.25, 2.5 μ M cadmium chloride for 24h, 48h and 72h, respectively. A Cd-free treatment was used as the control group and each treatment had 3 replicates. After exposure, discarded the original medium and washed the RTEC with pre-cooling PBS buffer for 2 times.

Total RNA was extracted using a commercial RNA isolation kit (TaKaRa code: 9108A, Dalian, China) according to the manufacturer's recommendations. Total RNA was dissolved in 20 μ l RNasefree dH₂O and stored at -80°C. Subsequently, the purity and concentration of extracted total RNA were tested with the NanoDrop2000 Nucleic acid spectrometer (Thermo Fisher Scientific Inc., USA). RNA of all samples had a ratio of optical density at 260 nm to that at

280 nm ranging from 1.8 to 2.0. Complementary DNA (cDNA) was synthesized using reverse transcription reagent kits (TaKaRa code: RR047A, Dalian, China) and each PCR reaction contained 4.0 μ l 5 \times RT buffer, 1.0 μ l RT enzyme mix, 1.0 μ l 100 μ mol/L random primers mix, 1 μ g total RNA and sufficient RNase-free dH₂O in a total volume of 20 μ l. The reverse transcription condition was 37°C for 15 min. The reaction was terminated by incubation at 85°C for 5s and the cDNA was stored at -80°C or used for real-time PCR immediately.

The quantity of c-myc, cyclin D1, TGF- β 1, Wnt and β -catenin mRNA expression in the Cd exposure groups relative to the control was determined with 7900 fast real-time

system (Applied Biosystems, USA). The primers of all tested gene and internal control, separated by at least one intron on the corresponding genomic DNA, were designed and synthesized (Invitrogen, Guangzhou, China) as in Table 1. The qPCR reaction components contained 2 μ l cDNA, 12.5 μ l SYBR Premix Ex Taq (TaKaRa, China), 0.5 μ l 10 μ mol/L forward/reverse primer, and sufficient RNase-free dH₂O in a total volume of 20 μ l. The qPCR conditions were 95°C for 60 s, 40 cycles of 95°C for 10 s and 57°C for 60 s, and 72°C for 30 s. GAPDH was chosen as an internal control and the relative quantification values for each gene were calculated with the $\Delta\Delta$ CT method [25-26].

Table 1. Real-time PCR primer sequence of target genes and internal control.

Gene	Primer sequence (5'-3')	Amplicon length (bp)
GAPDH	F 5'-CTGCGGGGATGGTTGGAAG-3' R 5'-CTCTCTCGGAGCCAATGCAA-3'	138
c-myc	F 5'-GCTGGACACGCTGACGAAA-3' R 5'-TCTAGCGCAAGCAGCTCTATTT-3'	135
cyclin D1	F 5'-GCGTACCCTGACACCAATCTC-3' R 5'-ACTGAAGTAAGATACGGAGGGC-3'	94
Wnt	F 5'-TCCAGACTCTTCGTGGACAGT-3' R 5'-CAGGTCCTTTTCGTGGAGGC-3'	113
β -catenin	F 5'-CTGCGGGGATGGTTGGAAG-3' R 5'-CTCTCTCGGAGCCAATGCAA-3'	62
TGF- β 1	F 5'-CTTCGACGTGACAGACGCT-3' R 5'-GCAGGGGCAGTGTAACCTTATT-3'	85

2.5. Statistical Analysis

Statistical analysis was performed with the SPSS 20.0 Software Package (SPSS Inc., USA). The data were analyzed using the paired-samples *t* test and were expressed by the mean values \pm standard deviation (SD). Two groups of independent samples were tested by *t* test to when satisfying normal distribution and homogeneity of variance. One Way ANOVA was used to compare the differences among groups, and Least-Significant Difference (LSD) was used to test the differences between groups. The effect of exposure time and dosage on the expression of related genes was analyzed by using analysis of variance of factorial design. The significant differences were compared between any two groups by Pearson correlation analysis ($\alpha=0.05$).

3. Results

3.1. Isolation and Culture of RTEC

All cells freshly isolated by mixed enzymes digesting began to attach wall and stretch after 36h, showed in Figure 1. After 72h, the adherence of RTEC basically completed and could exchange fresh medium. Some cells were spindle-shaped and the others were irregular polygon-shaped under TS100-F inverted microscope (Nikon Corporation, Japan), which had typical characteristics of epithelial cells.

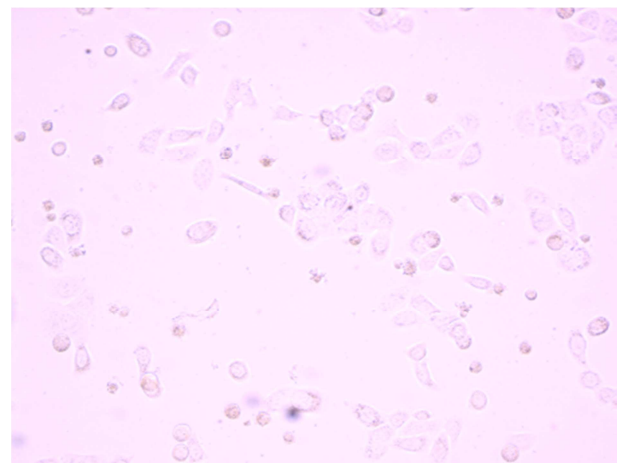


Figure 1. RTEC cultured for 72 h (400 \times).

3.2. Effects of Different Doses of Cd on the Viability of RTEC

The effects of different doses of Cd on the viability of RTEC showed in Figure 2. and the dose effect relationship resembled a special inverse U typed nonlinear quadric curve, with correlation coefficient (R) of 0.889, 0.913 and 0.959, respectively. In 24~72 h groups, dose-effect relationship described stimulatory effects at a low dose and inhibitory effects with Cd exposure dose, in a distinct Hormesis manner. The maximal low promotion effect occurred at a dose of 2.5 μ M and the maximal stimulation effect was 122.24% above the control group. It concluded that Hormesis zone dose of Cd

ranged from 0.63 to 2.5 μM.

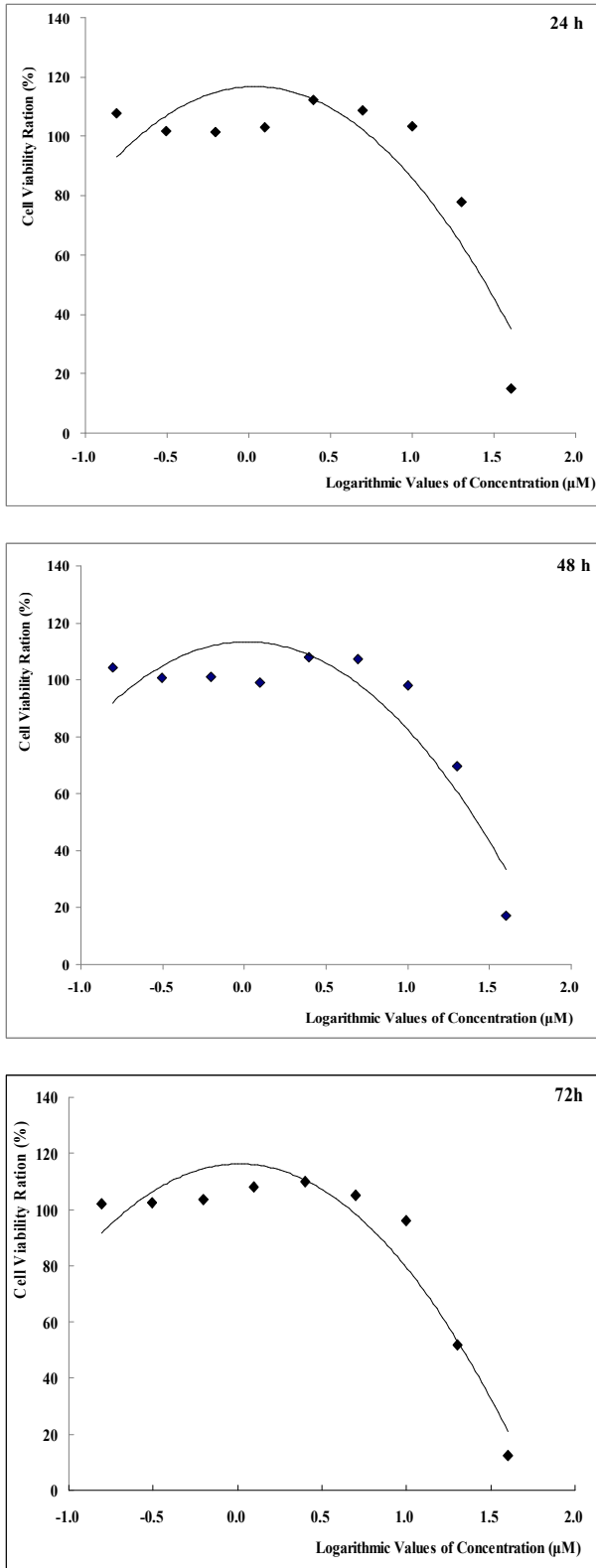


Figure 2. The Horemsis curve of proliferation induced by Cd.

3.3. Effect of Cd at Hormesis Zone Dose on the Expression of c-myc and cyclind1

Effect of Cd at Hormesis zone dose on the expression of

c-myc and cyclind1 was showed in Figure 3 and Figure 4. In 24 ~ 72 h groups, the level of proto-oncogene c-myc expression was significantly higher than that of the control group ($P<0.05$). As the Cd exposure dosages increased, the expression of c-myc in different exposure time increased in a significant dose-dependent manner ($r_{24}=0.966$, $r_{48}=0.982$, $r_{72}=0.985$, $P<0.01$), however, time effect in different exposure groups was not significant ($r_{0.625}=0.572$, $r_{1.25}=-0.567$, $r_{2.50}=0.556$, $P>0.05$).

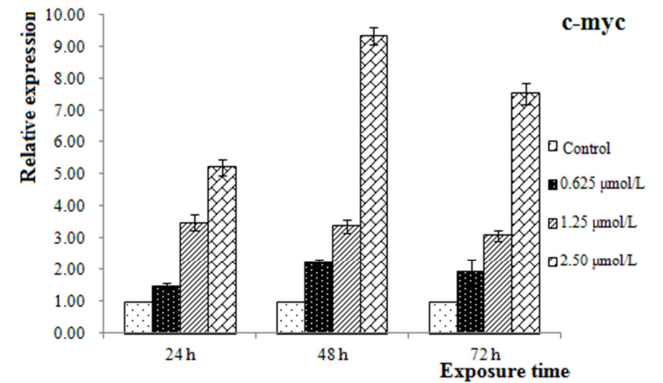


Figure 3. Effect of Cd at Hormesis zone dose on the expression of c-myc.

The data of qRT-PCR indicated that cyclinD1 mRNA expression in 2.50 μM groups at 24h and 1.25, 2.50μM groups at 48h up-regulated significantly compared with control ($P<0.05$). As the Cd exposure dosages increased, the up-regulation of cyclinD1 expression in a significant dose-dependent manner ($r_{24}=0.958$, $r_{48}=0.962$, $P<0.01$).

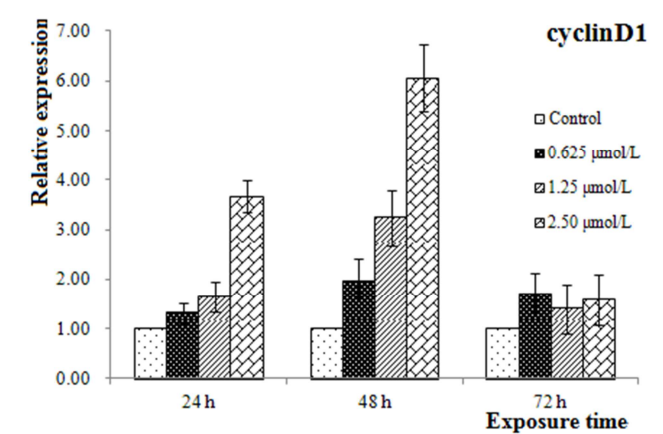


Figure 4. Effect of Cd at Hormesis zone dose on the expression of cyclinD1.

3.4. Effect of Cd at Hormesis Zone Dose on the Expression of α-SMA

As showed in Figure 5, 0.625 μM Cd could not induce significant changes of α-smooth muscle actin (α-SMA, characteristic protein of myofibroblast) expression. Compared with control, 1.25 and 2.50 μM Cd could induce significant increase of α-SMA expression ($P<0.05$), specifically, α-SMA expression in 2.50 μM group at 24h up-regulated by 6.7 fold more than control group.

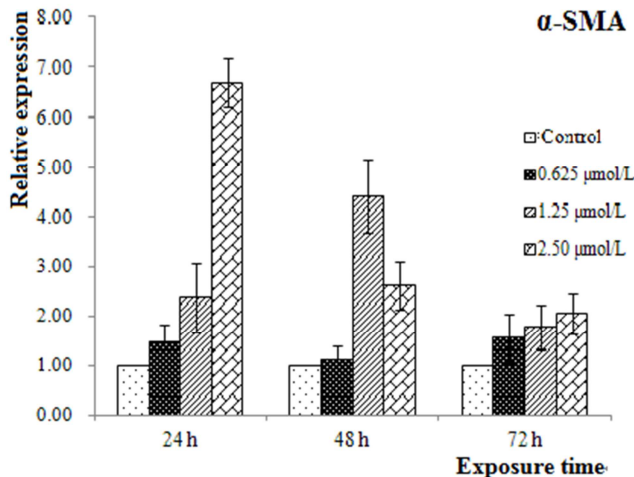


Figure 5. Effect of Cd at Hormesis zone dose on the expression of α -SMA.

3.5. Effect of Cd at Hormesis Zone Dose on the Expression of TGF- β 1

As showed in Figure 6, the expression of TGF- β 1 in 1.25 and 2.50 μ M exposure groups was significantly higher than that of the control group ($P < 0.05$). In 24~72 h, 0.625~2.50 μ M Cd could induce TGF- β 1 mRNA expression increase in a obvious dose-dependent manner ($r_{24}=0.890$, $r_{48}=0.958$, $r_{72}=0.893$, $P < 0.05$). With the prolonging of exposure time, the up-regulation of TGF- β 1 mRNA in all exposure groups in a obvious time-dependent manner ($r_{0.625}=0.812$, $r_{1.25}=0.860$, $r_{2.50}=0.952$, $P < 0.05$).

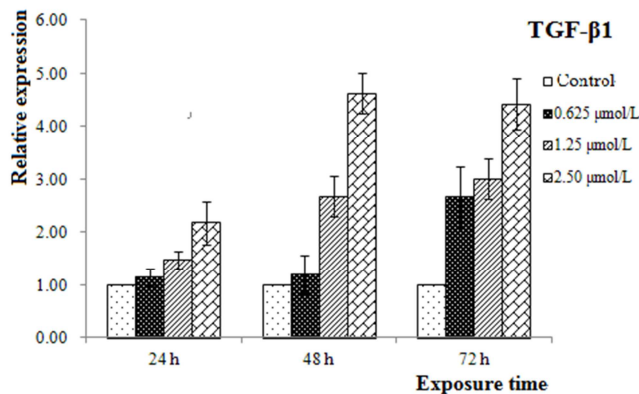


Figure 6. Effect of Cd at Hormesis zone dose on the expression of TGF- β 1.

3.6. Effect of Cd at Hormesis Zone Dose on the Expression of Wnt and β -catenin

As showed in Figure 7, in 24h and 48h, Cd at Hormesis zone dose could induce Wnt mRNA obvious up-regulation compared with control group, however, there was obvious effect on the Wnt expression after exposure for 72 h. The correlation analysis showed that the effect of Cd on the Wnt expression demonstrated obvious dose-effect relationship ($r_{24}=0.926$, $r_{48}=0.729$, $r_{72}=0.924$, $P < 0.05$) but not obvious time-effect relationship.

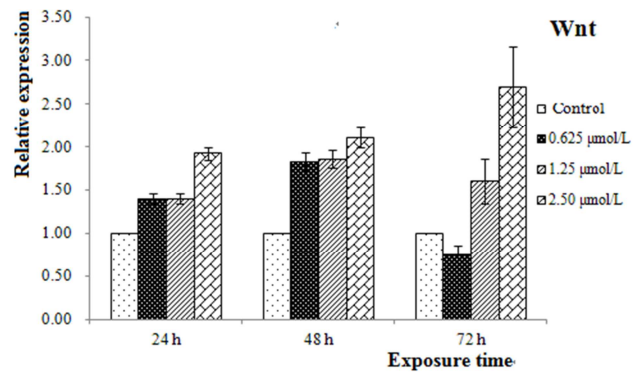


Figure 7. Effect of Cd at Hormesis zone dose on the expression of Wnt.

As showed in Figure 8, except 0.625 μ M exposure group in 72 h, β -atenin expression in all exposure groups up-regulated significantly. In 24 ~48 h, effect of Cd at Hormesis zone dose on the expression of β -catenin, like the expression of Wnt, it up-regulated in a obvious dose-effect manner ($r_{24}=0.910$, $r_{48}=0.830$, $P < 0.05$) while not in a time-effect manner.

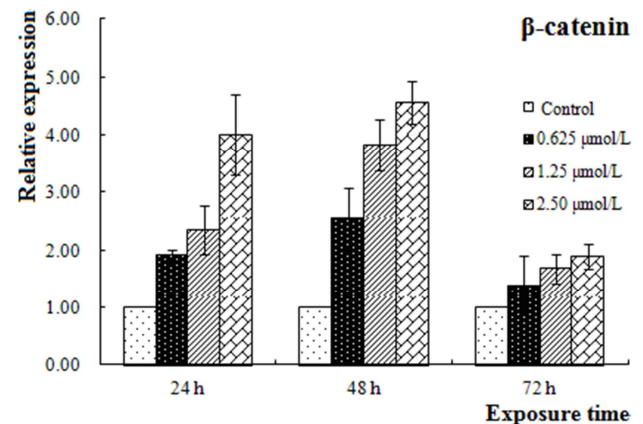


Figure 8. Effect of Cd at Hormesis zone dose on the expression of β -atenin.

4. Discussions

Some research has confirmed that the change of specific phenotype-associated protein mainly shows decrease of E-cadherin (characteristic protein of epithelial cell) expression and increase of α -SMA (characteristic protein of mesenchymal cell) expression when the start-up of EMT happens [27-28]. Generally, α -SMA in epithelial cells is absent or lowly expressed, so the expression induction or increase of α -SMA was one of the strongest predictor for initiation of cell trans-differentiation [29]. The results in this study showed that 0.625 μ M Cd could cause increase of α -SMA expression in dose-dependent manner, which indicated that Cd at Hormesis zone dose could induce RTEC to differentiate into myofibroblast with the function of secreting cytokines.

Transforming growth factor β 1 (TGF- β 1) is known as yet to be the most important cytokine to directly induce fibrosis and a variety of studies confirmed that the high expression and activation of TGF- β 1 could be determined in almost all renal fibrosis models [30]. The results in this study indicated that Cd

at Hormesis zone dose could cause significant abnormal proliferation, high expression of α -SMA and release of TGF- β 1. Taken together, it concluded that Cd at Hormesis zone dose could activate EMT-like transformation.

Cell cycle regulation is the most important step of cell proliferation. There exists two key detection points in cell cycle, that is, phase G1/S and G2/M, in which c-myc and cyclinD1 are two key members of cell cycle regulator. Scientific practice has been confirmed that the high expression of c-myc may induce the cell cycle to overpass G1/S restriction point and the over-expression of cyclinD1 can accelerate the translation from G1 to S phase and cell proliferation [31-32]. In this study, Cd at Hormesis zone dose could induce the sustained high expression of proto oncogenes c-myc and cyclinD1, which may be one of important regulatory mechanism for maintenance of cell homeostasis by proliferation activation. Activated RTEC cells would continue to release more TGF- β 1 through autocrine mode, which might further feed back on proliferation of RTEC and speed up process of EMT-like transformation.

At present, Wnt/ β -catenin, TGF- β 1 and integrin/ILK were regarded as 3 kinds of key signal pathway regulating organ fibrosis [33]. The interaction and crosstalk between Wnt/ β -catenin and TGF- β 1 plays an important role in biological effects including proliferation, differentiation, transformation, apoptosis, organ development, inflammatory infiltration and cell cycle. Although it is widely accepted that TGF- β 1 is the strongest fibrotic effect cytokine, some scholars holding views that the EMT process mediated by TGF- β 1 is indispensable to Wnt/ β -catenin pathway [34].

In general, Wnt/ β -catenin signaling pathway is silent in mature tissues and β -catenin is the key element in the pathway [35]. When Wnt signaling pathway is activated, the phosphorylation of β -catenin is inhibited and then results in cytoplasm accumulation, subsequently, a lot of β -catenin enters the nucleus by raising a series of synergistic cytokines which can bind nuclear transcription factor TCF/LEF family and start up the expression of target gene c-myc, cyclinD1 and α -SMA in Wnt pathway downstream [36]. In this study, there was a significant positive correlation between the expression levels of Wnt and that of c-myc ($r=0.998$, $P<0.05$), which indicated that a certain dose of Cd could promote RTEC survival by Wnt/ β -catenin pathway. From the animal models established by pathological factors (hyperuricemia, proteinuria, ischemia reperfusion) or the exogenous poisons (cyclosporine A, losartan) [37-41], it found that regulation of TGF- β 1-mediated Wnt/ β -catenin pathways played an important role in process of renal tubule fibrosis, which is in accordance with the results shown in this study.

Some cell biology research has been confirmed that, as the core element of the classical TGF- β 1 signaling pathway, smad3/4 can directly bind with β -catenin and TCF/LEF so that it plays an important role in mediating the interaction and crosstalk between TGF- β 1 and Wnt/ β -catenin pathway [42-44]. According to the results in this study, it could find that the expression of TGF- β 1 induced by Cd had obvious

consistency with the activation of Wnt/ β -catenin pathway [45]. From the role of chemical components of Chinese herbal in the intervention and mitigation of EMT, the expression of Wnt/ β -catenin downregulated and EMT induced by diabetes was reverse by target blocking the function of TGF- β 1, which had verified that regulation of TGF- β 1-mediated Wnt/ β -catenin pathways played an important role in process of EMT in another way.

By inference, the Wnt/ β -catenin signaling pathway mediated by TGF- β 1 played an important role in regulating α -SMA expression and activating abnormal proliferation, which might be an important mechanism of EMT-like transformation induced by Cd at Hormesis zone dose.

5. Conclusions

A certain dosage of Cd could induce RTEC normal proliferation in a manner of significant Hormesis effect and Cd at Hormesis zone dose could promote cell normal proliferation. In RTEC exposure model, high expression of TGF- β 1 and α -SMA means that Cd at Hormesis zone dose could induce EMT-like transformation *in vitro*. Thus, it could be seen that abnormal proliferation induced by low-dose Cd might be the primary step and early initiation period of EMT-like transformation. The activation of Wnt/ β -catenin signaling pathway mediated by TGF- β 1 played a key role in regulation of fibrosis-related gene α -SMA expression and abnormal activation of RTEC, which maybe an important mechanism of EMT-like transformation in RTEC induced by low-dose cadmium.

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