Bone Morphogenetic Protein-7 attenuates metabolic damage of kidney and pancreas under STZ-induced type 1 Diabetes model

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The Graduate School, Yonsei University

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Directed by Professor Jong In Yook

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Sang Hyun Song

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This certifies that the doctoral dissertation of
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구강악안면외과 수련기간 동안 대부분의 시간을 보내며 같이 공부하고 의지했던 권순모, 박슬마로, 윤승규, 최성환, 홍석환 선생과 의국 선후배님들께도 감사 인사를 전하며, 각자의 자리에서 저희가 받았던 가르침을 많이 베풀 수 있기를 바랍니다. 구강병리학 교실에 와서 실험과 그 외에 것들에서 도움을 받았던 차소영, 윤준섭, 강희은, 엄조은, 박경희, 황규호, 이재은 선생들께도 앞으로도 좋은 연구 결과 있길 바라며, 기초 의학에 종사하게 된 정철희 한다울 선생도 멋진 연구자로 성장하길 기대합니다.

친한 형이기도 하지만, 저의 수련 시작부터 지금까지 항상 제 마음속의 스승인 이승호, 김연호 선생님께도 특별한 감사의 마음을 전합니다. 가장 멋진 복서이자, 치과의사로서 존경하는

최병재 교수님께도 도전할 수 있는 용기를 주셔 감사하다는 말을 드리고 싶습니다.

마지막으로 항상 저의 편에 서서 지원을 아끼지 않았던 부모님과 동생에게도 감사의 마음과, 수련과 공부를 핑계로 소홀했던 미안한 마음을 전합니다.

2022 년 12 월

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ABSTRACT

Bone Morphogenetic Protein-7 attenuates metabolic damage of kidney and pancreas under STZ-induced type 1 Diabetes model

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(Directed by Professor Jong In Yook, D.D.S., Ph.D.)

Diabetes has various complications. Diabetic nephropathy is a representative micro-vascular complication, and about 30% of diabetic patients progress to diabetic nephropathy. Rental tubule damage by TGF-β1 is known to be a factor in diabetic nephropathy, and induces renal fibrosis, but the etiological mechanism has not yet been fully elucidated. Recently, it has been reported that a new type of cell death by iron metabolism called ferroptosis is involved in several kidney diseases. Ferroptosis is characterized by the accumulation of lipid peroxides and occurs when cystine uptake is inhibited or glutathione

peroxidase is inhibited. It has been reported that ferroptosis is partly involved in kidney damage in animal of diabetic nephropathy, and it has been reported that ferroptotic condition is induced by stimulation of TGF-β1 *in vitro* study. Therefore, this study aimed to find out that renal damage caused by ferroptosis is alleviated by BMP7 and endogenous antagonist of TGF-β1. In addition, based on the study that BMP7 is effective in regenerating pancreatic beta cells in diabetic animal models, we wanted to find out that pancreatic damage is alleviated.

We used the micellized prodrug type of BMP7(mPTD-BMP7) to facilitate delivery. In proximal tubular cell (NRK52E) of rodents (rat), it was proved that the lipid peroxidation was increased and glutathione was inhibited by TGF-β1 and Erastin, which was significantly improved by mPTD-BMP7. In addition, mPTD-BMP7 mitigated the inhibition of *Gpx4, Gpx3*, cystine transporter of proximal tubular cell (*Slc7a9*), and mitigated damage to iron metabolism markers *Ffh1* and *Hspb1* under ferropotsis. Further, it was demonstrated that markers of fibrosis in NRK52E were increased by stimulation of TGF-β1, but significantly decreased by mPTD-BMP7.

A diabetic animal model was induced using sterptozotocin, a drug that destroys pancreatic islets, particularly beta cells, and mPTD-BMP7 was delivered intraperitoneally to the treatment group for 16 weeks. In the kidney of diabetic animal model, lipid peroxidation was increased, and glutathione level and iron metabolism was inhibited, but these changes were improved by administration of mPTD-BMP7. Like the cell experiments, inhibition of *Gpx3, Gpx4* and *Slc7a9* was alleviated by mPTD-BMP7 in the diabetic kidney, and changes of *Fth1* and *Hspb1* was also mitigated. In addition, it was

confirmed through staining and qPCR that renal fibrosis was suppressed though administration of mPTD-BMP7, and it was confirmed through qPCR that the index of diabetes was significantly improved in the diabetic pancreas.

Ferroptosis seems to be involved in the process of renal injury and fibrosis in diabetes, which is expected to be alleviated by BMP7. In addition, it was confirmed that administration of BMP7 prevents continuous damage to the pancreas of diabetic patients and is also effective in regenerating the pancreas. Through this study, administration of BMP7 suggested that possibility of alleviating the metabolic damage of the pancreas as well as kidney in diabetic patients.

Key words: Diabetic nephropathy, Ferroptosis, Firbosis, BMP7, prodrug, beta cell regeneration.

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I. INTRODUCTION

Diabetic nephropathy(DN) is a major complication of diabetes which is causative factor of renal dysfunction, and the pathologic changes of DN include glomerulosclerosis, hypertrophy of basement membrane, extracellualr matrix(ECM) accumulation, tubulointerstitial fibrosis, tubular cell damage and death. About 30% patients with diabetes develop DN, however exact mechanism of pathogenesis remain unclear. Previous studies demonstrated that changes in renal hemodynamics, oxidative stress induced by hyperglycemia, inflammatory response, and enhanced activity of the renin-angiotensin-

aldosterone system are involved in pathogenesis of DN.(Sakuma et al., 2020; Zhang et al., 2021) Recently, TGF-β1 has been considered as main regulator and therapeutic target of kidney fibrosis and tubular cell damage in diabetic condition.(Hathaway et al., 2015; Sharma et al., 1996; Sun et al., 2016; Wang et al., 2021a; Zhao et al., 2020)

Ferroptosis is a newly proposed type of cell death that characterizes iron-dependent lipid oxidation. The key molecules are cystine transporter and glutathione peroxidase4(GPX4) that low level of intracellular cystine results in reduced glutathione synthesis and dysfunction of lipid oxidation degradation leading to ferroptosis.(Dixon, 2017; Dixon et al., 2012) Emerging evidence revealed ferroptopsis is involved in acute kidney injury(AKI) and in diabetic nephrology (Ni et al., 2022; Sha et al., 2021; Wang et al., 2021b) Further, recent study demonstrated ferroptosis is associated with pathogenesis of diabetic nephropathy. TGF-β1 stimulated tubular cells and Erastin stimulated tubular cells show similar ferroptosis condition, and streptozotocin(STZ) induced diabetic mice also shows ferroptotic condition in kidney.(Kim et al., 2021)

Multiple studies have reported that bone morphogenetic protein-7(BMP7) effectively inhibit fibrosis in diverse models such as retinopathy, liver fibrosis and peritoneal fibrosis.(Khan et al., 2011; Kinoshita et al., 2007; Yao et al., 2019) In diverse renal disease models, BMP7 counteracts fibrosis by downregulating multiple signaling pathways such as p38, p44/42 MAPK and TGF-β pathways, as well as reduction of ROS formation.(Isaka, 2018; Li et al., 2015; Wang et al., 2003) Also in STZ-induced diabetes model, BMP7 is demonstrated to be a therapeutic by protecting from diabetic kidney injury, revealing its

counteracts to TGF/SMADs signaling.(Peng et al., 2022; Wang et al., 2003) Further, several studies have identified the role of BMP in pancreas, and BMP7 has been found to be involved in supporting β-cell regeneration and differentiation in adult pancreas as well as prenatal pancreas development.(Ber et al., 2003; Ferber et al., 2000; Klein et al., 2015; Qadir et al., 2018; Zhou et al., 2008)

Since BMP(rhBMP)s has been limited by the rapid clearance and enzymatic degradation,(Lo et al., 2012) we designed protein transduction domain fused BMP7 in micelle(mPTD-BMP7) to enhance endosomal transduction *in vitro* and *in vivo*. We also investigated whether mPTD-BMP7 ameliorates metabolic damage of kidney and pancreas under diabetic condition induced by streptozotocin(STZ) *in vivo* and ferroptosis induced condition by TGF-β1 and Erastin *in vitro*.

II. MATERIALS AND METHODS

1. Cell culture and treatment of NRK52E cells

NRK52 cells (immortalized rat proximal tubular epithelial cells, cat# CRL-5171, ATCC, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum(FBS) and 1% Penicillin-Sterptomycin-Amphotericin B. Subconfluent NRK52E cells were starved in FBS-free condition for 24h, then the medium was replaced with DMEM with 0.5% FBS. For the TGF-β1 group, TGF-β1(10ng/ml; R&D Systems, Minneapolis, MN, USA) was applied, Erastin (Selleckchem) was applied for positive control of ferroptosis induction. Both groups were treated with mPTD-BMP7(250ng/ml)

2. Western blot analysis

For the Western blot analyses, NRK52 cells were treated with mPTD-BMP7 for 16h and intracellular transduction of recombinant protein was detected against BMP-7 (ab84684, Abcam, 1:2,000) and β-actin (a5441, Sigma-Aldrich, 1:5,000) were used.

3. Animal experiments

The animal model was 8-week-old male C57BL/6 mice were injected with either diluent (control; n=5) or streptozotocin (STZ; Sigma-Aldrich; 50mg/kg; n=20) intraperitoneally for five days consecutively. To confirm DM (fasting blood glucose > 300mg/dl), tail vein

blood glucose was measured and only thirteen mice were diagnosed DM. The five mice from control group and seven mice from DM confirmed group were treated with vehicle(10μg) every 72 hours intraperitoneaally. The other six mice from DM confirmed group were treated with mPTD-BMP7(10μg) every 72 hours intra-peritoneally. After 16 weeks, mice were anesthetized with Zoletil (10 mg/kg; Virbac, Carros, France), kidney and pancreas were extracted. Body weight, kidney weight, blood glucose, HbA1c, serum bilirubin, 24h urinary albumin and creatinine concentrations were determined at the time of sacrifice. Blood glucose was measured with a glucometer, and HbA1c was measured with glycolhemoglobin analyzer. 24h urinary albumin was assessed by ELISA assay (Nephrat II, Exocell Inc., Philadelphia, PA, USA), and 24h urinary creatinine was analyzed using a Cobas 8000 C502 (Roche, Mannheim, Baden Württemberg, Germany).

4. Quantitative real-time polymerase chain reaction

Total RNA was islolated from cells, whole pancreas and kidney samples using Trizol(Invitrogen) and cDNA was synthesized using a reverse transcriptase premix product (AccuPower CycleScript RT PreMix, Bioneer). Transcript levels were detected using the 7300 Real-Time PCR system(Applied Biosystems) with TB Green® Premix Ex Tqa TM II (Takara). mRNA expression level was calculated using △CT values. Results are shown as relative expression normalized by housekeeping gene (*r18s, Actb, Rpl13a*). The Primer sequences used in this study are presented in Table 1 and 2.

Table 1. Primer sequences used in qPCR analysis of animal experiment.

Gene	Species	Accession number	Primer sequences from 5' to 3'
Tgfb1	Mus musculus	NM_011577	Forward GCTTCAGCTCCACAGAGAAGA
			Reverse GACAGAAGTTGGCATGGTAGC
Col1a1	Mus musculus	NM_007742	Forward GTCAGACCTGTGTGTTCCCT
			Reverse TCCATCGGTCATGCTCTCTC
Ccn2	Mus musculus	NM_010217	Forward AAGAAGGGCAAAAAGTGCATC
			Reverse CGCAGAACTTAGCCCTGTATG
Acta2	Mus musculus	NM_007392	Forward CTGACAGAGGCACCACTGAA
			Reverse CATCTCCAGAGTCCAGCACA
Fn1	Mus musculus	NM_010233	Forward AGAAGTTTGTGCATGGTGTCC
			Reverse ACTTGGACAGGTCCAGTTGTG
Gpx3	Mus musculus	NM_008161	Forward ACCAATTTGGCAAACAGGAG
			Reverse TCTTTCTCCCCGTTCACATC
Gpx4	Mus musculus	NM_008162	Forward CCGGCTACAATGTCAGGTTT
			Reverse ACGCAGCCGTTCTTATCAAT
Slc7a9	Mus musculus	NM_021291	Forward GGATTCCTCTGGTGACCGTA
			Reverse GGACTACCCAAGATGCTGGA
Fth1	Mus musculus	NM_010239	Forward GAGAAGAGCCGAGACAATGG
			Reverse GAGCCTAAGCCTGAATGCAC
Hspb1	Mus musculus	NM_013560	Forward ACTGGCAAGCACGAAGAAAG
			Reverse AGGGAAGAGGACACTAGGGT
Ins	Mus musculus	NM_008387	Forward AGCGTGGCTTCTTCTACACAC
			Reverse CTGGTGCAGCACTGATCTACA
Pdx1	Mus musculus	NM_008814	Forward CGCGAATATCCTCCTGAAAG
			Reverse CCTGTTGGCAAAGAATGGTT
Gck	Mus musculus	NM_010292	Forward CTGGGCTACTTCTGCTTTGG
			Reverse TGCCAGGATCTGCTCTACCT
Prox1	Mus musculus	NM_008937	Forward TGAATCCCCAAGGTTCAGAG
			Reverse AAAGGCATCATGGCATCTTC
Krt19	Mus musculus	NM_008471	Forward GGTCAGTGTGGAGGTGGATT
			Reverse CCTCAATCCGAGCAAGGTAG
Slc2a1	Mus musculus	NM_011400	Forward GAGGCCACCTGAGAGTGTTC
			Reverse AGTGCCTAGCCTTGGACTGA
Syp	Mus musculus	NM_009305	Forward TGGAAATTCAACCCCAAGAG
			Reverse CCTTTTAGGCTCCACATCCA
Actb	Mus musculus	NM_007393	Forward CACCATGTACCCAGGCATTG
			Reverse CACACAGAGTACTTGCGCTC

Rpl13a	Mus musculus	NM_009438	Forward GAGGTCGGGTGGAAGTACCA Reverse TGCATCTTGGCCTTTTCCTT

Table 2. Primer sequences used in qPCR analysis of cell experiment.

Gene	Species	Accession number	Primer sequences from 5' to 3'
Tgfb1	Rattus norvegicus	NM_021578	Forward TGAGTGGCTGTCTTTTGACG Reverse TGGGACTGATCCCATTGATT
Tgfbr1	Rattus norvegicus	NM_012775	Forward TGAGTGGCTGTCTTTTGACG Reverse TGGGACTGATCCCATTGATT
Col1a1	Rattus norvegicus	NM_053304	Forward TTCTGAAACCCTCCCCTCTT Reverse CCACCCCAGGGATAAAAACT
Ccn2	Rattus norvegicus	NM_022266	Forward AGAGTGGAGATGCCAGGAGA Reverse CACACACCCAGCTCTTGCTA
Acta2	Rattus norvegicus	NM_031004	Forward TGTGCTGGACTCTGGAGATG Reverse GAAGGAATAGCCACGCTCAG
Fn1	Rattus norvegicus	NM_019143	Forward GTGGCTGCCTTCAACTTCTC Reverse TTGCAAACCTTCAATGGTCA
Gpx3	Rattus norvegicus	NM_022525	Forward GAGAAGAGCCGAGACAATGG Reverse GAGCCTAAGCCTGAATGCAC
Gpx4	Rattus norvegicus	NM_017165	Forward CCGGCTACAATGTCAGGTTT Reverse ACGCAGCCGTTCTTATCAAT
Slc7a9	Rattus norvegicus	NM_053929	Forward CAGGGGGTGAGTACCCCTAT Reverse TAAAAGGCCGCACACACATA
Fth1	Rattus norvegicus	NM_012848	Forward ATGATGTGGCCCTGAAGAAC Reverse TCATCACGGTCAGGTTTCTG
Hspb1	Rattus norvegicus	NM_031970	Forward CTGGTGTCCTCTTCCCTGTC Reverse GCTCCAGACTGTTCCGACTC
18s	Rattus norvegicus	NR_042637	Forward CAAGTAGGAGAGGAGCGAGC Reverse CATGTCTAAGTACGCACGGC

5. Measurement of lipid oxidation

Malondialdehyde(MDA) was measured using a lipid peroxidation assay kit (Abcam, Cambridge, United Kingdom). For live cell imaging, Image-iT® Lipid Peroxidation Kit

(Thermo Fisher Scientific) was used. For the MDA assay, cultured cells were homogenized in MDA lysis buffer with 5% butylated hydroxytoluene, and harvested kidney tissues were homogenized in distilled water with 5% butylated hydroxytoluene and the 2N perchloric acid was added. After centrifugation of samples at 13,000 × g for 10 minutes at 4 °C, supernatant of each sample was collected, and MDA levels were measured using the reaction of thiobarbituric acid at a wavelength of 532 nm. For the live cell analysis of lipid peroxidation, cells were seeded in four well chamber (3×10^4 per well) and mPTD-BMP7 was pre-treated 16h before TGF-β1 or Erastin stimulation. The Image-iT® lipid peroxidation sensor was added into each wells and incubated for 30 minutes at 37°C. Culture medium was removed, and samples were washed with PBS. Images were acquired using an LSM700 confocal microscope (Carl Zeiss Vision, Hallbergmoos, Germany) under ×40 magnification.

6. Glutathione assay

Glutathione concentration (reduced form of glutathione only) was measured using Glutathione assay kit(Sigma-Aldrich). Cultured cells and harvested kidney tissues were lysed in glutathione buffer and then 5% sulfosalicylic acid was added. Samples of cells were centrifuged at 700 × g for 5 minutes and samples of kidney were centrifuged at 8000 × g for 10 minutes and then supernatant was collected. NADPH generating mix were added to glutathione reaction buffer and incubated for 10 minutes at RT to generate NADPH. 20μl of supernatant and 160μl of NADPH solution were mixed and incubated for 10 minutes at

RT. Glutathione concentrations were determined using the reaction with DTNB at wavelength 415nm.

7. Immunohistochemistry

For histological and immunohistological examination, serial paraffin sections were stained with H&E for routine morphological observation. For immunohistochemical staining, tissue sections were deparaffinized with xylene, rehydrated in serial dilution ethyl alcohol, and immersed in 3% hydrogen peroxide. Antigen retrieval was carried out by microwave in the 10mM citric acid buffer (pH 6.0) for 20 minutes. Nonspecific binding of protein was blocked with 2.5% goat serum (MP-7451, Vector Laboratories) for 30 minutes at RT, and then incubated overnight at 4°C with primary antibody. After washing with PBS, the sections were incubated with a secondary antibody, horseradish peroxidase conjugated Goat Anti-Rabbit IgG (MP-7451, Vector Laboratories), for 30minutes at RT. After washing with PBS, DAB was developed with Envision Detection Systemic of Agilent (K5007, Agilent). Hematoxylin was used for counter staining and mounted.

4-hydroxynonenal (4-HNE; NBP2-59353, Novus Biologicals), MDA (NBP2-59367, Novus Biologicals), Fibronectin (ab2413, Abcam)

8. Statistics analysis

Statistical significance of qPCR, MDA assay and GSH assay was analyzed using paired t-test and Mann-Whitney test using Prism 5 (GraphPad, San Diego, CA). A P value < 0.05

was considered statistically significant as indicated in the text.

III. RESULTS

1. Endosomal transduction and secretion of mPTD-BMP7 in renal tubular cells.

To examine the cytotoxicity of mPTD-BMP-7, we treated various dose of mPTD-BMP-7 into NRK-52E cells, we found that mPTD-BMP-7 is not cytotoxic under excess dose exposure *in vitro* (Fig 1A). Further, we examined intracellular transduction of mPTD-BMP7, we treated mPTD-BMP-7 into NRK-52E cells for 16h and examined the transduction by Western blot analysis in Triton-insoluble fraction because denatured PTD- BMP7 forms insoluble aggregate in the micelle. As shown in Fig1B, transduction of mPTD-BMP7 was successfully detected.

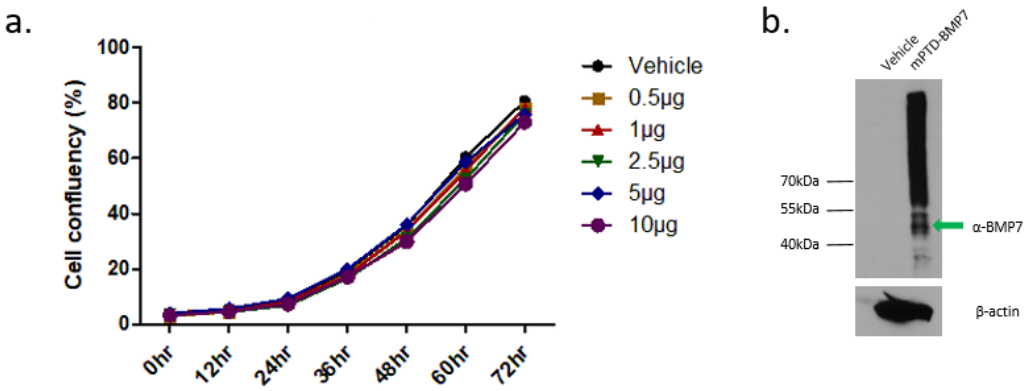


Figure 1. Endosomal transduction and secretion of mPTD-BMP7 in NRK cells.

(a) Cellular toxicity of mPTD-BMP7. The NRK-52E cells were treated by various concentration of mPTD-BMP7, and cell growth was observed by JuLi real-time live cell analyzer (NanoEntek). (b) NRK-52E cells were treated with 500ng mPTD-BMP7 for 16h, and the insoluble fraction of whole cell lysates were subjected to western blot to detect transduction of protein.

2. mPTD-BMP7 abrogates *TGF-β1 and Erastin – stimulated kidney tubular cell injury*.

Next, we investigated the impact of mPTD-BMP7 on kidney tubular cell damage induced by TGF-β1 and Erastin. Glutathione peroxidase (*Gpx3, Gpx4*) mRNA expression level was significantly attenuated by mPTD-BMP7 in TGF-β1 stimulated NRK52E cells. Also, cystine transporter(*Slc7a9*) mRNA expression level was also abrogated by mPTD-BMP7 administration. Furthermore, mRNA expression levels of ferritin heavy chain (*Fth1*) and heat shock protein beta-1(*Hspb1*) was significantly changed in TGF-β1 stimulated cells, however was abrogated with mPTD-BMP7 treatment. (Fig2A) Compared to TGF-β1 stimulated cells, similar findings were noted in NRK52E cells stimulated with Erastin, the ferroptosis inducer. (Fig2B) The increase of Lipid peroxidation caused by TGF-β1 was attenuated by administration of mPTD-BMP7 250ng/ml. Also in Erastin-stimulated NRK52E cells, mPTD-BMP7 abrogated lipid peroxidation (Fig2C,E,F). Reduced form of glutathione(GSH) level was significantly reduced in TGF-β1 and Erastin stimulated condition, but it was ameliorated by mPTD-BMP7 treatment. (Fig2D) Taken together, these findings suggest that TGF- β1-induced tubular cell injury is involved in ferroptosis, further mPTD-BMP7 is presumed to attenuate ferroptotic cell damage.

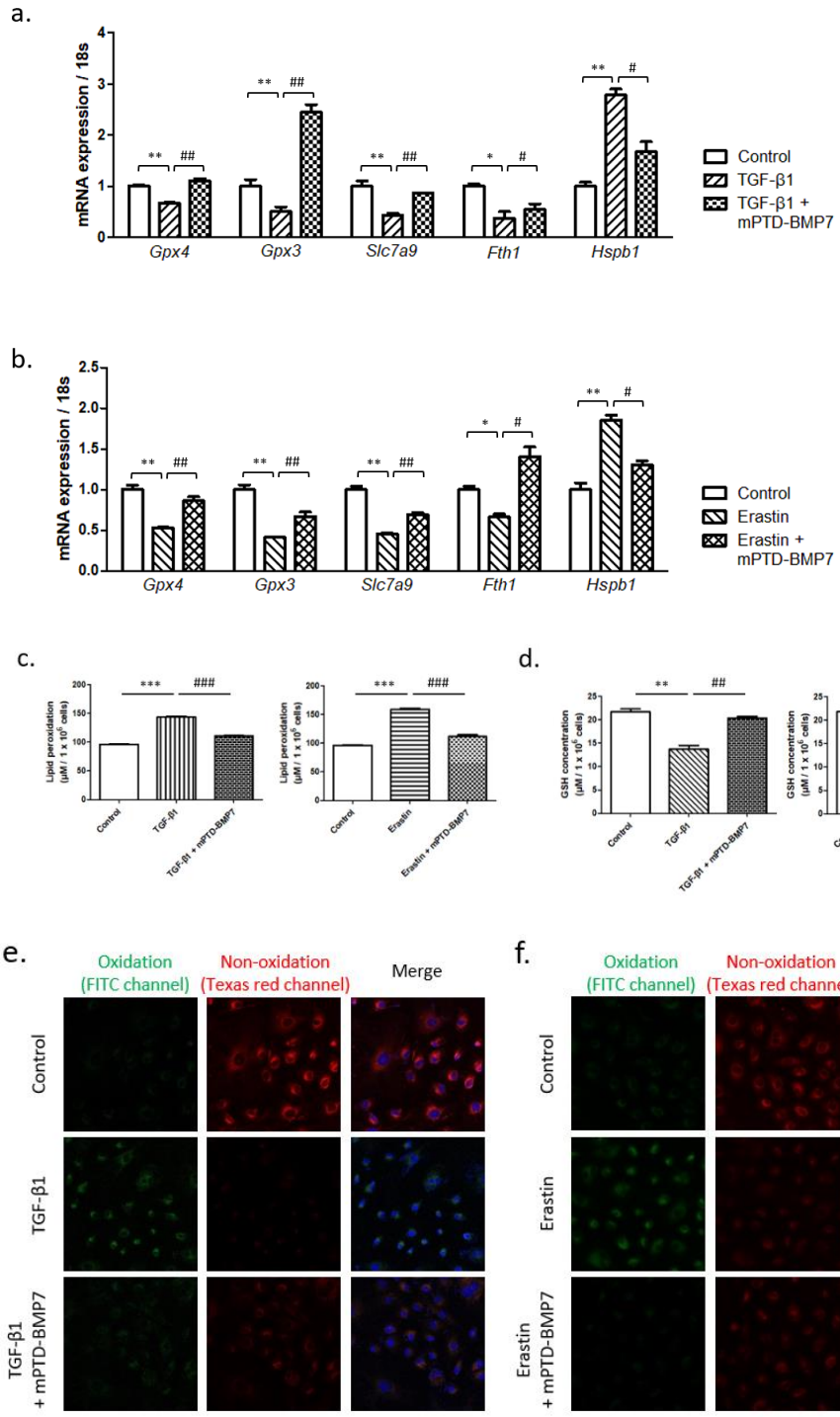


Figure 2. Effect of mPTD-BMP7 on changes in ferroptosis related markers in Erastin and TGF-β1 stimulated NRK cells after 12h.

(a) Significant changes in the mRNA expression level of ferroptosis-related genes observed in NRK52E cells stimulated by TGF-β1 were ameliorated after mPTD-BMP7 treatment. (b) Significant changes in the mRNA expression level of ferroptosis-related genes observed in NRK52E cells stimulated by Erastin were ameliorated after mPTD-BMP7 treatment. (c) Administration of mPTD-BMP7 significantly attenuated increase of lipid peroxidation in NRK-52E cells stimulated by TGF-β1 or Erastin. (d) Administration of mPTD-BMP7 significantly attenuated decrease of reduced glutathione(GSH) level in NRK-52E cells stimulated by TGF-β1 or Erastin. Lipid peroxidation assessed using Image-iT® showed that the increase observed in NRK-52E cells after exposure to TGF-β1(e) or Erastin(f) was significantly attenuated by mPTD-BMP7 treatment.

(*P <0.05; **P < 0.01; ***P < 0.001 versus Control group. #P<0.05; ##P < 0.01; ###P < 0.001 versus TGF-β1 or Erastin group.)

3. mPTD-BMP7 attenuates fibrosis of NRK cells.

In order to assess simultaneous change of kidney tubular cell, the expression level of fibrosis related genes were further evaluated. The expression levels of *Tgfb1* and *Tgfbr1* were significantly higher in TGF-β1 stimulated NRK52E cells for 96hr. According to preceded upregulation of *Tgfb1* and *Tgfbr1*, the down streaming genes (*Cola1, Fn1, Acta2*) were increased significantly. The upregulation of fibrosis related genes was alleviated by mPTD-BMP7.(Fig 3A)

a.

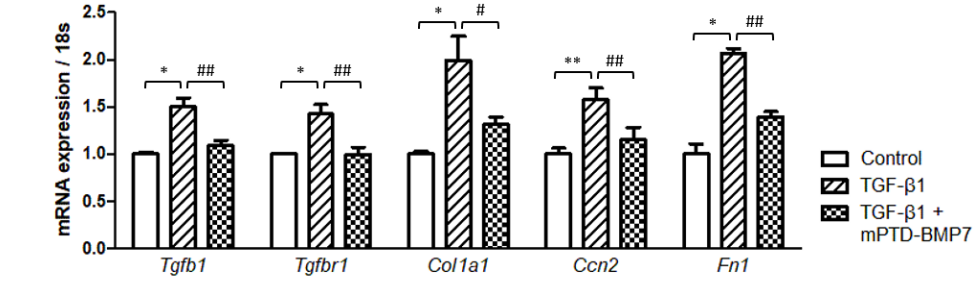


Figure 3. Effect of mPTD-BMP7 on changes in fibrosis related markers in TGF-β1 stimulated NRK cells after 96h.

(a) mPTD-BMP7 significantly ameliorates TGF-β1 down streaming genes in TGF-β1 stimulated NRK52E cells after 96hr.

(*P <0.05; **P < 0.01; ***P < 0.001 versus Control group. #P<0.05; ##P < 0.01; ###P < 0.001 versus TGF-β1 or Erastin group.)

4. Intraperitoneal administration of mPTD-BMP7 effectively is delivered to kidney and pancreas.

To test how mPTD-BMP7 is distributed, we covalently conjugated indocyanine green to mPTD- BMP7 and administered this compound to mice via intra-peritoneal injection. An ex vivo fluorescence analysis showed that much amount of mPTD-BMP7 accumulated in pancreas at 6h and persist mainly in pancreas at 72h.(Fig 4A)

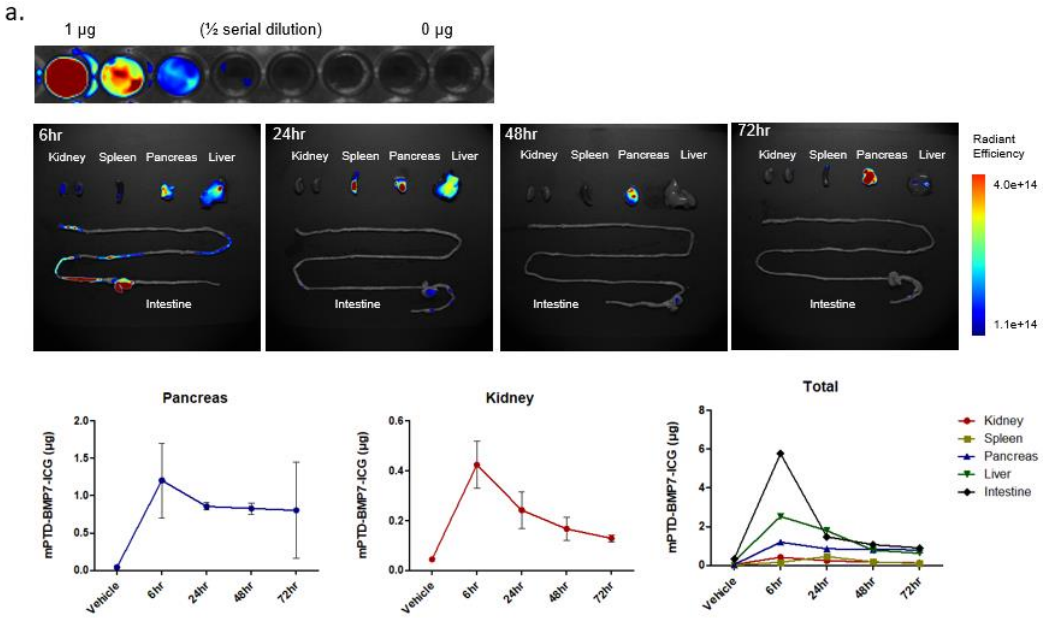


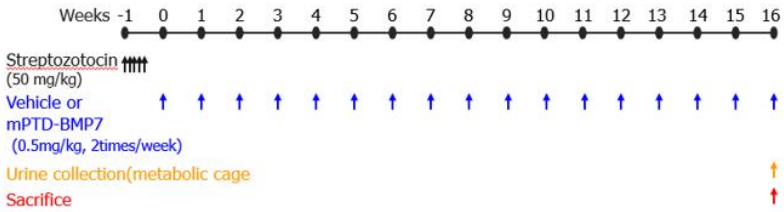
Figure 4. Bio-distribution assay of intra-peritoneal administration of mPTD-BMP7.

(a) Ten micrograms of indocyanine green(ICG)-labeled mPTD-BMP7 was injected intra-peritoneally, and the bio-distribution of mPTD-BMP7 was assessed by *in vivo* fluorescence imaging. The major organs in mice were dissected and ex vivo fluorescence images(upper) were taken for quantitative analysis(lower) with the passage of time.

5. *mPTD-BMP7 protects against kidney injury in an experimental animal model of diabetes.*

In animal model of diabetes, Kidney/body weight, blood glucose, serum bilirubin and urine albumin-creatinine ratio(UACR) was significantly increased, and these were improved overall, especially UACR and bilirubin, which improved significantly by mPTD-BMP7 treatment (P<0.05; Fig 5B). Further, H&E staining shows the vacuolization and destruction of renal tubules in diabetic condition, and it was prevented by mPTD-BMP7 administration (Fig 5C).

a.



b.

	Con (n=5)	STZ + Vehicle (n=7)	STZ + mPTD-BMP7 (n=6)
Body weight (g)	28.97 ± 0.63	21.09 ± 1.24	21.57 ± 2.26
Kidney weight (mg)	155.45 ± 17.56	185.69 ± 16.18	179.09 ± 29.60
Kidney/body weight (mg/g)	5.37 ± 0.66	8.80 ± 0.39	8.36 ± 1.39
Blood glucose (mg/dL)	181.20 ± 61.21	489.57 ± 144.00	437.17 ± 124.69
HbA1c (%)	5.92 ± 0.96	10.83 ± 1.43	9.60 ± 2.52
Serum Bilirubin (mg/dL)	0.03 ± 0.02	0.07 ± 0.03	0.04 ± 0.02
UACR (μg/mg)	16.05 ± 8.47	37.06 ± 10.79	31.60 ± 18.27

c.

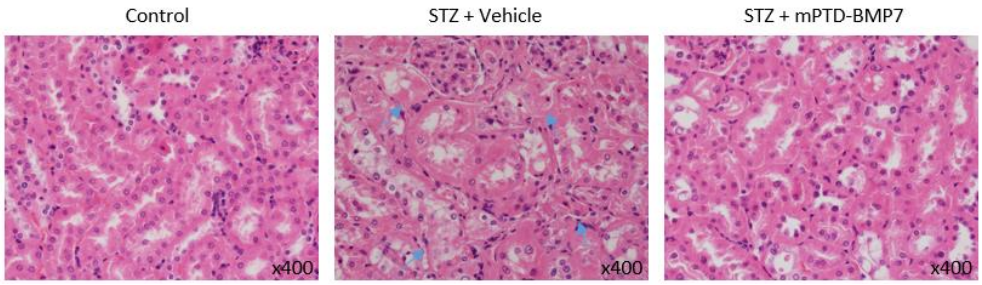


Figure 5. Effect of mPTD-BMP7 assessed by clinical parameters in animal models of diabetes.

(a) Experimental schematic of streptozotocin(STZ) induced diabetic mice. Diabetic condition was induced by intraperitoneal injection of STZ (50mg/kg) for five days consecutively. Intra-peritoneal injection of mPTD-BMP-7 (0.5mg/kg) twice per week, after induction of diabetic condition. (b) Establishment of STZ-induced diabetic condition is

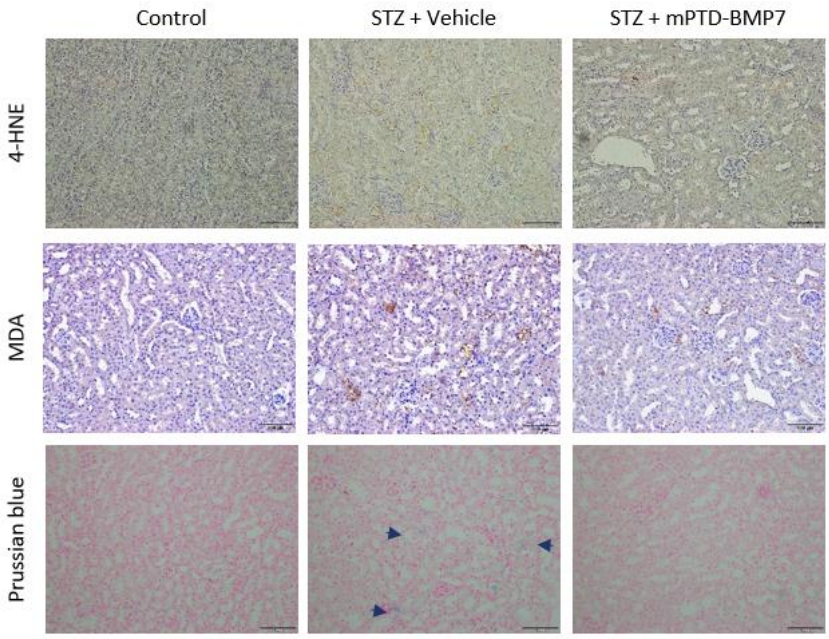
confirmed by clinical parameters, and some parameters was attenuated by mPTD-BMP7. (c) Kidney injury was confirmed by hematoxylin and eosin(H&E) staining and it was abrogated by mPTD-BMP7.

6. *mPTD-BMP7 ameliorates ferroptotic damage of kidney in diabetic condition.*

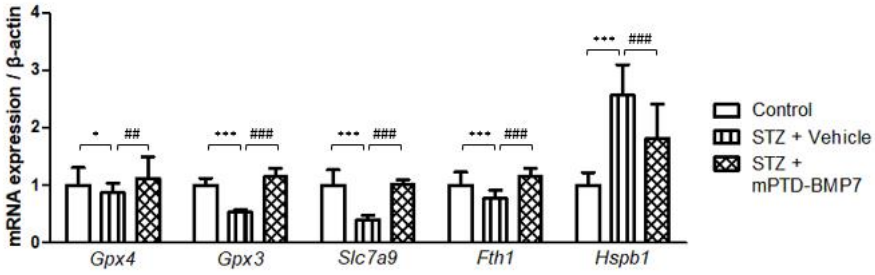
We sought to determine whether ferroptotic damage was taken in the kidneys of diabetic animals and whether mPTD-BMP7 could attenuate the kidney damage. First, MDA and 4-HNE staining showed increase of lipid peroxidation in the diabetic kidney, and attenuated by mPTD-BMP7 (Fig 6A). mRNA transcript levels of *Gpx3, Gpx4* and *Slc7a9* in diabetic kidney were significantly downregulated, and attenuated by treatment of mPTD-BMP7. Iron accumulation was confirmed in diabetic condition by Prussian blue staining, which is prevented by mPTD-BMP7(Fig 6A) showing significant rescue in mRNA expression levels of *Fth1* and *Hspb1*(Fig 6B).

GSH concentration was significantly decreased, whereas lipid peroxidation was significantly increased in the kidney of Vehicle group, and these changes were ameliorated by mPTD-BMP7 treatment significantly. (P<0.01 for GSH concentration and lipid peroxidation; Fig 6C, 6D)

a.



b.



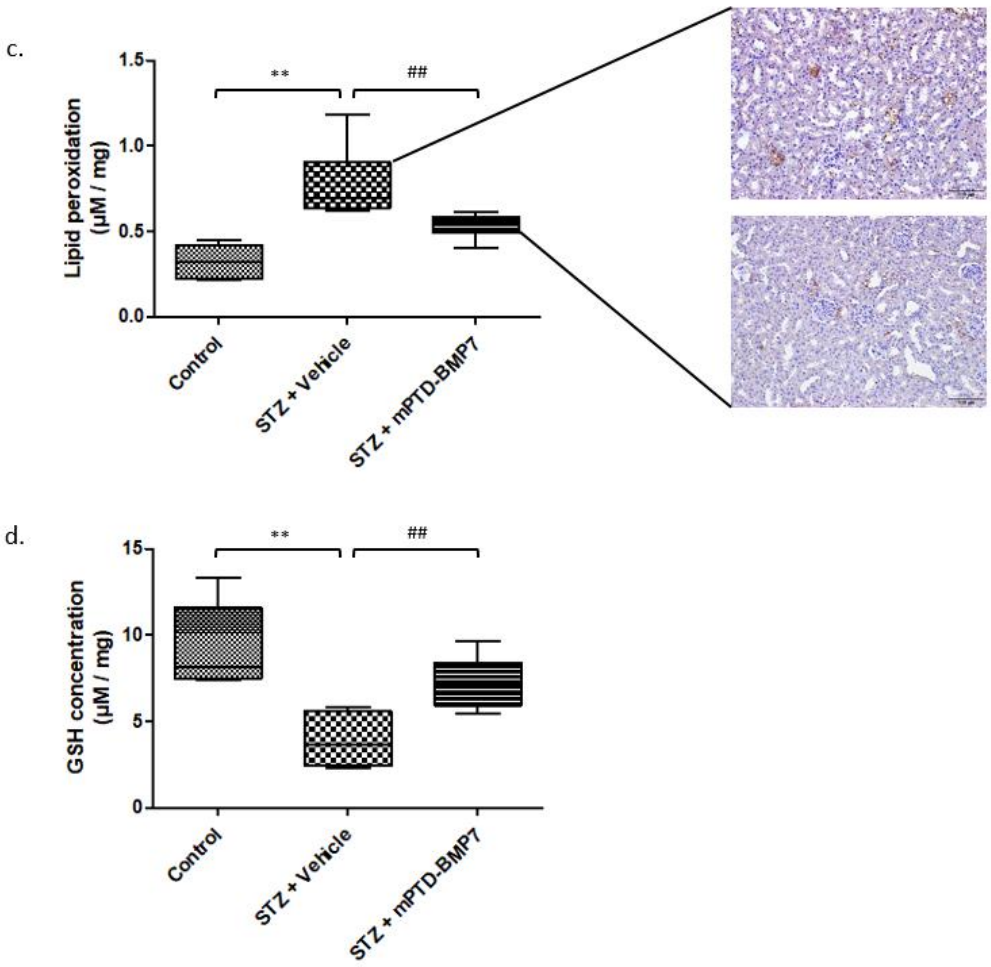


Figure 6. Effects of mPTD-BMP7 on ferroptosis related molecules in the kidney of diabetic mice.

(a) In immunohistochemistry, 4-HND and MDA show increase in the Vehicle group, and were abrogated by administration of mPTD-BMP7. Prussian blue staining revealed iron deposition in the Vehicle group, and was attenuated by mPTD-BMP7. (b) mRNA

expression of ferroptosis related genes were significantly changed in the diabetic kidney, and the changes were abrogated by mPTD-BMP7. (c) Lipid oxidation assessed by MDA levels in the kidney of STZ mice. (d) GSH concentrations in the kidney of STZ mice. (*P <0.05; **P < 0.01; ***P < 0.001 versus Control group. #P<0.05; ##P < 0.01; ###P < 0.001 versus Vehicle group.)

7. *Effect of mPTD-BMP7 on progression of diabetic renal fibrosis*

Masson's Trichrome, Sirius red and Immuhohistochemistry of Fibronectin revealed that STZ-induced diabetic condition advance kidney fibrosis, while intra-peritoneal injection of mPTD-BMP7 reduced DM-induced fibrosis. (Fig 7A). mRNA transcript level of *Tgfb1* was significantly increased in diabetic kidney, and downstream genes of *Tgfb1* which are related with fibrosis were elevated either according to *Tgfb1*. These fibrosis related genes were attenuated by mPTD-BMP7. (Fig 7B)

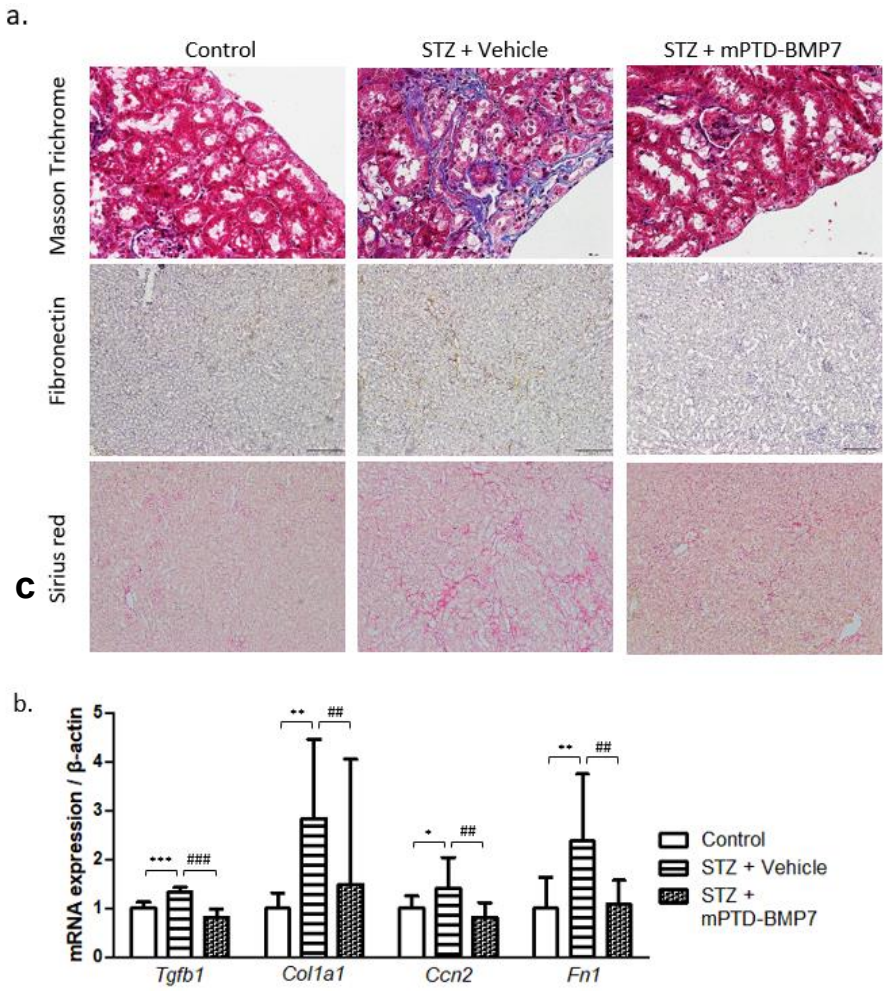


Figure 7. Effects of mPTD-BMP7 on fibrosis related molecules in the kidney of diabetic mice.

(a) Masson's trichrome and Sirius red staining were used to detect accumulated collagen in mouse kidney, and immunohistochemistry of fibronectin was used to measure the degree of fibrosis. (b) mRNA expression of fibrosis-related genes was attenuated by mPTD-BMP7 in the diabetic kidney.

(*P <0.05; **P < 0.01; ***P < 0.001 versus Control group. #P<0.05; ##P < 0.01; ###P < 0.001 versus Vehicle group.)

8. mPTD-BMP7 attenuate pancreas damage under diabetic condition.

To test mPTD-BMP7 affects pancreas, we examined qPCR of representative adult pancreas marker in diabetic pancreas tissue. Among them, *Pdx1* as β-cell putative progenitor was severely decreased with insulin, but improved significantly by mPTD-BMP7. mRNA transcript level shows decrease in Vehicle group, however many markers were attenuated by mPTD-BMP7 significantly. (*Ins, Pdx1, Gck, Prox1, Syp, Slc2a1, Krt19*)

a.

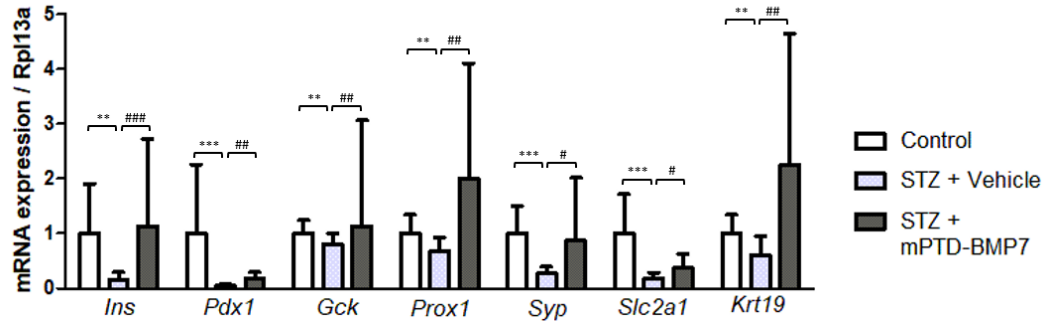


Figure 8. Effects of mPTD-BMP7 on adult pancreas markers and pancreatic developmental markers in diabetic pancreas.

(a) mRNA expression of adult pancreas and pancreas developmental markers was attenuated by mPTD-BMP7 in the STZ pancreas.

(*P <0.05; **P < 0.01; ***P < 0.001 versus Control group. #P<0.05; ##P < 0.01; ###P < 0.001 versus Vehicle group.)

IV. DISCUSSION

We demonstrated that *Gpx3, Gpx4* and *Slc7a9* expression was significantly downregulated in TGF-β1 or Erastin stimulated cultured tubular cells, also in the kidneys of diabetic mice. Downregulation of those genes results in low glutathione levels and enhanced lipid peroxidation, but were effectively mitigated by BMP7 which is designed as recombinant protein including prodomain and formulated in micelle (mPTD-BMP7). It also improved iron chelation attenuating expression level of *Fth1* and *Hspb1*. mPTD-BMP7 also significantly prevented kidney fibrosis, hypertrophy, albuminuria, and improved pancreatic damages ameliorating pancreatic development markers and adult pancreas markers.

In the pathogenesis of almost types of diabetes, β cell loss or dysfunctions is involved. There are many factors inducing β cell loss or dysfunctions like genetic mutation, extrinsic metabolic stress, injurious factors such as autoimmune antibodies or inflammation and diseases like pancreatitis, pancreatic cancer and Alzheimer.(Hart et al., 2016; Mittal et al., 2016; Wang et al., 2022) It is necessary to expand the population of β cell to compensate for the lack of insulin and improve glucose homeostasis. Insulin producing β cells can be generated from non-pancreatic somatic cells, pancreatic exocrine cells, or pancreatic islet cells by transdifferentiation.(Basile et al., 2019) Many studies demonstrated that β cells were obtained from hepatocytes by overexpression of pancreatic transcript factors that are important for β cell such as PDX1, NEUROD1, Ngn3, in murine hepatocytes by adenoviral delivery in diabetic animal models.(Ber et al., 2003; Ferber et al., 2000; Kojima et al., 2003;

Tang et al., 2013) Others focused on α-cells as a pool for generating β cells. As gene therapy has been proposed efficient protocol to convert α-cells into β cells, adenoviral delivery of transgenic constructs such as PDX1 and MAFA genes via pancreatic duct has proven to expand β-cells in STZ-treated and NOD mice.(Xiao et al., 2018) Nevertheless, acinar cells as a prospective source of β cell generation have mass abundance, close proximity and shared developmental origin with endocrine cells.(Basile et al., 2019) Not only overexpression pancreatic transcription factors like PDX1, Ngn3 and MafA or treatment with cytokines and growth factors were shown efficient transdifferentiation into β cells rodent *in vivo* and *in vitro*,(Baeyens et al., 2014; Rooman and Bouwens, 2004; Zhou et al., 2008) also evidence that β cell-like phenotype was induced in acinar cells after treatment of BMP-7 in humans was reported.(Klein et al., 2015) Further, BMP7 stimulated pancreatic progenitor cell proliferation by binding to ALK3 that is co-expressed with PDX1 in multipotent cells in exocrine pancreas.(Qadir et al., 2018) In our diabetic pancreas, we confirmed many adult pancreatic markers (*Ins, Gck, Syp, Slc2a1, Krt19*) and pancreatic development markers (*Pdx1, Prox1*) were attenuated by intraperitoneal administration of mPTD-BMP7 assuming pancreatic exocrine-endocrine conversion has partially occurred.

Until now, chronic kidney disease caused by diabetic condition is diagnosed as diabetic nephropathy with gradual kidney dysfunction by nephron loss, and is diagnosed by characteristic pathological findings, such as mesangial expansion, tubular cell death, nodular lesions, and tubulointerstitial fibrosis.(Tervaert et al., 2010; Yokoyama et al., 2000) Not only microvascular and glomerular injuries are mainly characterized in diabetic

nephropathy, also tubular atrophy and cell death have a role in this condition. Hyperglycemic condition promotes generation of free radicals and exerts oxidative stress on tubular cells, mediating hypertrophy, extracellular matrix deposition, and cell death.(Priante et al., 2019) Since the proximal tubule requires cell supplies of high-energy phosphate compounds and relies on aerobic metabolism, decreased oxygen supply to tubular epithelial cells leads to vulnerable condition to diabetic ischemic injury.(Martindale and Holbrook, 2002; Priante et al., 2019) Tubular cell injury and death has been recognized key findings of kidney damage in diseases such as unilateral ureteral obstruction (UUO), acute kidney injury(AKI), renal ischemia followed by reperfusion(I/R), chronic kidney disease(CKD) as well as in diabetic kidney.(Hoste et al., 2006; Jang and Padanilam, 2015; Kellum et al., 2011; Kennedy et al., 1997; Kennedy et al., 1994; Khan et al., 1999; Klahr and Morrissey, 2002; Linkermann et al., 2014; Nilsson et al., 2015; Price and Hodeify, 2012; Schumer et al., 1992; Thomas et al., 1998; Truong et al., 1996)

Up to date, studies have focused on modalities of apoptosis and necrosis in kidney disease, recent advances have enhanced the innovative concept of regulated necrosis described such as pyroptosis, ferroptosis, necroptosis, and mitochondrial permeability transition-related necrosis.(Priante et al., 2019) Unlike other types of cell death, ferroptosis is a form of regulated cell death first proposed by Dixon ex al, and characterized by the iron and O_2-dependent accumulation of lipid peroxides reaching lethal levels. It is triggered by restriction of influx of the amino acid cystine, or by dysfunction of repair of lipid peroxide.(Dixon et al., 2012) Disruption of this intracellular thiol antioxidant network

progresses to the non-apoptotic, iron-dependent, oxidative form of cell death termed ferroptosis.(Dixon, 2017) In many cells, GSH synthesis is dependent on the continuous import of cystine by the cell surface Cys_2/glutamate antiporter system x_c^-,(Bannai et al., 1977; Sato et al., 1999), the heterodimer of a membrane protein $b^{0,+}$AT/SLC7A9 and its auxiliary subunit rBAT/SLC3A1 has a key part of cystine reabsorption particularly in renal proximal tubules.(Chillaron et al., 2010; Correia et al., 2022; Nagamori et al., 2016; Okazaki, 2022) While, mutation of Slc7a9 has been reported to cause cysteinuria, losing function of protecting iron-induced oxidative damage in proximal tubular cells,(Chillaron et al., 2010; Correia et al., 2022; Nagamori et al., 2016) its function in ferroptosis remained unclear. Not only in Erastin-induced ferroptosis at proximal tubular cells, Slc7a9 showed significant decrease and rescued by mPTD-BMP7, also in STZ mice and TGF-β1 stimulated proximal tubular cells Slc7a9 showed significant attenuation by mPTD-BMP7. In accordance with our results, Slc7a9 is assumed to be involved in ferroptosis as a novel cystine importer in renal proximal tubular cell.

Among the factors that advance diabetic nephropathy, TGF-β1 is considered as key regulator in progression to diabetic nephropathy in type 1 and type 2 DM. (Sharma et al., 1996; Wang et al., 2021a; Zhao et al., 2020; Ziyadeh et al., 2000) It is a pleiotropic cytokine involved in angiogenesis, immunomodulation, and extracellular matrix (ECM) formation, stimulated by various factors including hyperglycemia and ROS in kidney cell,(Zhao et al., 2020) An abundance evidence that TGF-β plays a predominant role in kidney fibrosis was further confirmed by the fact that suppression of the TGF-β/Smads signaling pathway

attenuates kidney fibrosis and injury.(Sun et al., 2016) Recently, TGF-β has been reported to induce ferroptosis by inhibiting GPX4 and cystine transporter in hepatocellular carcinoma cell and renal tubular cell enhancing lipid-oxidation(Kim et al., 2020; Kim et al., 2021). Moreover, TGF-β and ROS interplay, not only by promoting their generation, but also by suppressing the expression of antioxidant enzymes such as catalase, glutaredoxin, superoxide dismutase, and glutathione peroxidase(Liu and Gaston Pravia, 2010) and function of GPX4 has been proposed to mediate alleviate the activation of the TGF-β/SMAD signaling pathway which accounts for the progression of myelofibrosis.(Li et al., 2022) In accordance with previous studies, we demonstrated GPX4 is involved both in ferroptosis and fibrosis showing rescue by mPTD-BMP7 significantly.

Iron overload is confirmed to induce ferroptosis that heat shock protein beta-1 (HSPB1) can reduce intracellular iron concentrations by inhibiting transferrin-receoptor-1(TFR1) expression, and is upregulated in environmental stress including erastin-induced ferroptosis.(Chen et al., 2020; Sun et al., 2015) Also, ferritin consists of ferritin heavy chain 1 (FTH1) and ferritin light chain (FTL). FTH1 has ferroxidase activity and reduce Fe^{2+} to Fe^{3+}, which prevents reaction to make lipid peroxides.(Chen et al., 2020) Up to date, BMP7 has been reported to regulate iron metabolism influencing signal to hepcidin via BMP/SMAD pathways associated with disorders of iron metabolism.(Babitt et al., 2007; Chen et al., 2020) Our results showed BMP7 could regulate iron metabolism sharing pathway of ferroptosis related with FTH1 and HSPB1.

Although underlying mechanisms that mediate tubulointerstitial lesions in diabetic nephropathy are not unveiled clearly, previous study demonstrated characterization of ferroptosis in renal tubular cell death under diabetic condition induced by TGF-β1, and was ameliorated by ferroptosis inhibitor Fer-1.(Kim et al., 2021) Our study demonstrated that BMP7 could protect renal tubular cells in diabetic condition protecting cell death involved in ferroptosis.

Taken together, we report that mPTD-BMP7 provides an alternative therapeutics against kidney and pancreas damage under diabetic condition. Moreover, mPTD-BMP7 help inhibit ferroptosis mechanism attenuating some ferroptosis markers. Further pharmacologic and preclinical studies are required for clinical therapeutics for diabetes mellitus.

V. CONCLUSION

1. mPTD-BMP7 attenuates kidney injury under diabetic condition.

   (1) mPTD-BMP7 inhibits kidney fibrosis progression under diabetic condition attenuating TGF-β and downstream genes of TGF-β.

   (2) mPTD-BMP7 is involved in amelioration of ferroptotic condition.

   : attenuating lipid peroxidation and glutathione level.

   : attenuating *GPX3, GPX4* and *Slc7a9* transcript level.

   : attenuating iron metabolism and related genes. (*Fth1, Hspb1*)

2. mPTD-BMP7 induces pancreatic exocrine-endocrine conversion in diabetic pancreas.

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ABSTRACT (IN KOREAN)

스트렙토조신으로 유도된 1형 당뇨 모델에서 골형성 단백질 7의 신장과 췌장에 대한 대사성 손상 완화 효과

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송 상 현

당뇨는 여러가지 합병증을 가지는데 당뇨병성 신증은 대표적인 모세혈관계의 합병증으로, 약 30%의 당뇨환자에서 당뇨병성 신증으로 진행된다. TGF-β1 에 의한 신장 세뇨관의 손상은 당뇨병성 신증의 요인으로 알려져 있으며, 동시에 신장 섬유화를 유도하지만 병인론적인 기전은 아직 완벽히 밝혀지지 않았다. 최근에 ferroptosis 라는 철 이온에 의한 새로운 형태의 세포 사멸이 여러 신장의 질병들에 연관되어 있다는 보고가 되었다. Ferroptosis 는 지질 과산화물의 축적이 특징이며, 시스테인의 유입이 저해되거나, glutathione peroxidase 가 저해되며 나타난다. 최근, 당뇨병성 신증 동물 모델에서 신장의 손상 과정에 ferroptosis 가 일부 관련되어 있다는 보고가 있었고, 세포 실험에서 TGF-β1 의 자극은

ferroptosis 가 일부 유도되는 것이 보고 되었다. 따라서, TGF-β1 의 내인성 길항제인 BMP7 에 의해 ferroptosis 에 의한 신장 손상이 완화되는 것을 밝히고자 하였으며, 더불어 BMP7 이 당뇨 동물 모델에서 췌장의 베타 세포 재생에 효과적이라는 연구를 바탕으로 췌장의 손상도 완화 되는 것을 밝히고자 하였다.

이 실험에서 전구체 약물 형태의 mPTD-BMP7 을 사용하여 전달을 용이하게 하였다. 설치류(Rat)의 근위세뇨관 세포(NRK52E)에서 TGF-β1 과 Erastin 에 의해 지질 과산화가 증가하고 글루타치온이 저해되는 것을 확인하였으며, 이는 mPTD-BMP7 에 의해 유의하게 개선되었다. 또한 mPTD-BMP7 은 ferroptosis 상태에서 *Gpx4, Gpx3* 와 신장 세뇨관의 시스테인 유입 경로인 *Slc7a9* 의 저해를 완화시켰으며, 철 대사 지표인 *Fth1* 과 *Hspb1* 의 손상도 완화시켰다. 그리고 TGF-β1 를 장시간 적용하여 세뇨관 세포에서 섬유화의 지표들이 증가하였다가, mPTD-BMP7 에 의해 유의한 감소를 보이는 것도 확인하였다.

당뇨 동물 모델은 Streptozotocin 이라는 췌장의 랑거한스섬과 베타 세포를 파괴하는 약물을 사용하여 유도하였으며, 치료 군은 16 주 동안 mPTD-BMP7 을 복강을 통해 전달하였다. 당뇨병성 동물 모델에서도 신장에서 지질 과산화가 증가되고 글루타치온 수준과 철의 대사가 저해되었으나, mPTD-BMP7 의 적용으로 이 현상이 개선되었다. 세포 실험에서와 마찬가지로 mPTD-BMP7 에 의해 당뇨 쥐의 신장에서 *Gpx3, Gpx4, Slc7a9* 의 저해를 완화시켰고 *Ffh1* 과 *Hspb1* 의 변화의 개선을 확인하였다. 또한, mPTD-BMP7 의 적용을 통해

신장에서 섬유화가 억제되는 것을 염색과 qPCR을 통해 확인하였으며, 췌장에서도 당뇨의 지표가 유의하게 개선 되는 것을 qPCR을 통해 확인하였다.

당뇨에서 신장의 손상과 섬유화의 과정에 ferroptosis가 관련되어 있으며, 이는 BMP7에 의해 완화가 가능한 것으로 보인다. 뿐만 아니라 BMP7의 적용은 당뇨 환자에서 췌장의 지속적인 손상을 방지하며, 췌장의 재생에도 효과가 있음을 확인하였다. 본 연구를 통해 BMP7의 적용은 당뇨 환자에서 신장뿐만 아니라 췌장의 대사 손상을 완화할 수 있는 가능성을 제시하였다.

핵심되는 말: 당뇨병성 신증, Ferroptosis, 섬유화, BMP7, 전구체, 베타 세포, 재생