THE ROLE OF BONE MORPHOGENETIC PROTEINS IN D-GALACTOSAMINE INDUCED HEPATIC FAILURE

BY

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A Thesis submitted to the Faculty of Graduate Studies of

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ABSTRACT

Bone morphogenetic proteins-2/4/7 (BMP-2/4/7) are important cytokines in systemic tissue morphogenesis. It has been demonstrated BMPs may have positive effects on liver repair and regeneration after hepatic injury. However, their function in the liver still remains unclear. D-galactosamine (D-gal) is a hepatotoxin used to induce hepatic failure. We employed D-gal and rat hepatoma cell line (1548) to investigate BMP-2/4/7 expression in hepatic injury induced by D-gal and probe their relations with liver repair and regeneration in hepatic injury. LDH release, mRNA and protein expression were detected. Results indicated that BMP-2/4/7 expression was activated by injury of rat hepatoma cells. It is indicative that repair and regeneration of the liver after hepatic injury and morphogenesis in early embryos seem to proceed through the same process. BMPs may be not only associated with hepatic injury after repair and regeneration, but also involved in chronic liver.

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LAST OF ABBREVITIONS

^{o}C	degree Celsius
AAP	acetaminophen
AIH	autoimmune hepatitis
ALF	acute liver failure
ALKs	activin-receptor-like kinases
BAMBI	BMP and activin membrane-bound inhibitor
BDL	common bile duct ligations
BMPR	bone morphogenetic protein receptor
BMPs	bone morphogenetic proteins
bp	base pair
CO_2	arbon dioxide
Co-Smads	common-mediator Smads
NAFLD	nonalcoholic fatty liver disease
D-gal	D-Galactosamine
DILI	drug induced liver injury
dpp	ecapentaplegic
EB	ethidium bromide
EDTA	ethylenediamine tetraacetic acid
FDA	food and drug administration
FBS	fetal bovin serum

FGFs	fibroblast growth factors
FLRG	follistatin-relatedgene
FSH	follicle-stimulating hormone
GAPDH	glyceraldehydes 3-phosphate dehydrogenase
GDF	growth and differentiation factor
GPI	glycosylphosphatidylinositol
GS domain	a domain rich in glycine and serine residues
HBV	hepatitis B viruses
HSCs	hepatic stellate cells
I-Smads	inhibitory Smads
JNK	jun N-terminal kinase
KDa	kilo Daltons
LDH	lactate dehydrogenase
LH	luteinizing hormone
Mad	mother against dpp
MAPK	mitogen activated protein kinase
MEM	minimal essential medium
MH1 and MH2	mad-homology domains 1 and 2
MIS	mu¨ llerian-inhibiting substance
M	molar
ml	milliliter
mM	millimolar

μl	microliter
MMLV	moloney murine leukemia virus reverse
NF-κB	nuclear factor kappa B
OD	optical density
OLT	orthotopic liver transplants
OP-1	osteogenic protein-1
PBS	phosphate buffered saline
PH	partial hepatectomy
PRDC	protein related to Dan and Cerberus
rhBMP7	recombinant human BMP7
RGM	repulsive guidance molecule
R-Smads	receptor-activated Smads
RT-PCR	reverse transcription polymerase chain reaction
SBEs	Smad1 binding elements
SDS	sodium dodecyl sulfate
SSXS motif	ser-ser-x-ser sequence SSXS
TAK1	tyrosine kinase 1
TBS-T	tris buffered saline-Tween 20
TGF-b	transforming growth factor-b
Tris	tris (hydroxymethyl) amino methane
Tsg	twisted gastrulation
XIAP	x-linked/inhibitor of apoptosis

Ⅰ. Introduction

1. Bone Morpogenetic Proteins

Bone morphogenetic proteins (BMPs) are a group of growth factors originally identified as proteins capable of inducing the formation of bone and cartilage when implanted at ectopic sites in rats. BMP-like molecules have been found in vertebrates as well as in invertebrates and they are now well known to exhibit a wide range of biological effects on various cell types. In addition to induction of bone and cartilage tissues, BMPs regulate embryogenesis, haematopoiesis, neurogenesis, skeletal formation, tooth, kidney, skin and hair development and maintain the iron metabolism and vascular homeostasis in vivo (Cheng, Jiang et al.2003；Katagir, Suda et al. 2008).

1.1 Discovery of BMPs

The potential of bone in repair and regeneration has been know since the time of Hippocrates. Senn, a surgeon at Rush Medical College in Chicago, described the utility of antiseptic decalcified bone implants during the treatment of osteomyelitis and certain bone deformities. Pierre Lacroix proposed there might be a hypothetical substance in bone to initiate skeletal development, which is osteogenin (Reddi. 1997).

Marshall R. Urist explained the biological basis of bone morphogenesis. Urist made the key discovery that demineralized, lyophilized segments of bone could induce new bone formation when implanted in muscle pouches in rabbits, published in 1965 in Science. In 1971, Urist proposed the name "Bone Morphogenetic Protein" in the scientific literature in the Journal of Dental Research (Reddi. 1997).

Bone induction is a sequential multistep cascade. The key steps in this cascade are

chemotaxis, mitosis, and differentiation. Early studies by Hari Reddi unraveled the sequence of events involved in bone matrix-induced bone morphogenesis. On the basis of the previous studies, it seemed that morphogens were present in the bone matrix. A systematic study was undertaken to isolate and purify putative bone morphogenetic proteins using a battery of bioassays for bone formation (Bessa, Casal et al.2008). A major stumbling barrier for purification was the insolubility of demineralized bone matrix. To overcome this hurdle, dissociative extractant such as 4M guanidine HCL, 8M Urea, or 1% SDS was used. The soluble extract alone or the insoluble residues alone were incapable of inducing new bone induction. This work suggested that the optimal osteogenic activity requires synergy between soluble extract and insoluble collagenous substratum. It not only represented a significant advance towards the final purification of bone morphogenetic proteins (BMPs) but also ultimately enabled the cloning of BMPs (Reddi, 1997； Bessa, Casal et al.2008).

1.2 Subgroups within the BMP Family

BMPs belong to the transforming growth factor-β (TGF-β) family, including 33 members in mammals such as TGF-βs, activins and inhibins, nodal and myostatin, etc (Derynck and Miyazono 2008). BMPs form the largest subgroup of the TGF-β family. More than a dozen molecules of the TGF-β family have been classified into the BMP family.

To date, over 20 members have been identified in humans. Members of the BMP family may be subdivided into different subgroups based on their gene homology and

similarity in protein structure (Table 1). These BMP subgroups are BMP-2/4 group, BMP-5/6/7/8 group (OP-1 [osteogenic protein-1] group), growth and differentiation factor (GDF)-5/6/7 group/BMP-12/13/14 group, BMP-9/10 group, BMP-3 group, BMP-11/GDF-8 group, and BMP-15/GDF-9 group. Proteins within the BMP-2/4 group, osteogenic protein-1 (OP-1) group, BMP-9/10 group and BMP-12/13/14 group share sequence similarities of more than 50%. They induce formation of bone and cartilage tissues in vivo while GDF-5/6/7 induce cartilage and tendon-like tissues. GDFs are morphogens named as growth and differentiation factors (GDFs). They are similar to some BMPs and could, therefore, be included in the BMP family. BMP-11 and BMP-15 are more distant members similar to growth differentiation factors -8 and -9, respectively.

Certain BMPs or GDFs were misnamed and they neither activate BMP type I receptors nor Smad1/5/8. BMP-3 and myostatin (also known as GDF-8) do not activate Smad1/5/8 and fail to induce bone and cartilage tissues in vivo. BMP-8 is a negative regulator of bone and muscle mass. Additionally, under the term BMP are BMP-16 to -18.. BMP-16 is a human homologue to murine Nodal and BMP-17/18 are related to Lefty. Both have important roles during embryonic patterning, mainly by antagonising the effect of BMP signalling in mesoderm formation (Nodal) or for the establishment of left-right embryonic asymmetry (Lefty) (Meno, Ito et al. 1997; Thisse, Wright et al. 2000). Since BMP-16 to -18 are more distantly related to BMPs than to other TGF-β superfamily members these should conceivably form a different group in the TGF-β superfamily distinct from BMPs. Among different BMPs and GDFs, BMP-2, BMP-6, and BMP-9 were shown to be the most potent in the induction of alkaline phosphatase activity and

Table 1. List of BMP subgroups

Members of TGF-β subgroups
BMP-2, -4
BMP-5, -6, -7, -8
BMP-9, -10
BMP-12, -13, -14
BMP-3
BMP-11
BMP-15

osteocalcin expression in C3H10T1/2 cells, and to induce differentiation of mesenchymal progenitor cells into osteoblasts (Cheng, Jiang et al. 2003).

1.3 Functions of BMPs

In general, biologically active BMPs are 30-38 kDa homodimers synthesized as prepropeptides with 400-525 amino acids in cells. The traditional functions of BMPs are related to embryonic development and bone formation. However, recent studies demonstrate that BMPs are also involved in other important cellular functions such as regulating cell proliferation and differentiation. Bone morphogenetic proteins (BMPs) exhibit a wide range of biological activities in various tissues, including bone, cartilage, blood vessels, heart, kidney, neurons, liver and lung (Table 2) (Reddi, 1997; Celeste and Murray 1999; Celeste and Murray 2000; Stephen, Duncan et al.2001; Cheng, Jiang et al.2003 ; Katagir, Suda et al. 2008; Bessa, Casal et al.2008; Bragdon, Moseychuk et al. 2011)

Apart from the functions above, BMPs play a critical role in hepatogenesis. BMPs are secreted from septum transversum mesenchyme. They can induce ventral endoderm to adopt a hepatic fate together with FGFs (fibroblast growth factors) secreted from cardiac mesoderm (Figure 1). Septum transversum mesenchyme locates right beside ventral endoderm prior to the regulation of cardiac mesoderm. After BMPs induce Gata-4 expression in foregut endoderm to make it competent to adopt a hepatic fate, BMPs (BMP-2/4) signalling from the septum transversum mesenchyme and FDFs and FDFs

Table 2. List of BMPs functions

BMPs	Human expression	Functions in Human	Mutations in mice
BMP-1	Thymus, bone marrow, spleen, brain, spinal cord, heart, skeletal muscle, kidney, lung, liver, pancreas, prostate	Metalloprotease that cleaves COOH–propeptides of procollagens I, II, and III/induces cartilage formation/cleaves BMP antagonist chordin	Heterozygous: reduced ossification of the skull, persistent herniation of the gut, abnormal collagen fibrils in the amnion, death at birth
BMP-2	Spleen, kidney, lung, pancreas	Skeletal repair and regeneration/heart formation	Heterozygous null: die at embryonic day 7.5–9 with failure of the proamniotic canal to close and abnormal development of the heart in the exocoelomin cavity
BMP-3 (osteogenin)	Thymus, bone marrow, spleen, brain, heart, skeletal muscle, pancreas, prostate	Negative regulator of bone morphogenesis	Homozygous: increased bone density
BMP-3b (GDF10)	Brain, spinal cord, skeletal muscle, pancreas, prostate	Cell differentiation regulation/skeletal morphogenesis	Homozygous: normal phenotype
BMP-4 (BMP-2b)	Thymus, bone marrow, spleen, brain, spinal cord, heart, skeletal muscle, kidney, lung, liver, pancreas, prostate	Skeletal repair and regeneration/kidney formation	Heterozygous null: abnormalities of the kidney and urinary tract. Targeted mutants: embryonic lethality, aberrant mesoderm differentiation, developmental retardation,
BMP-5	Thymus, bone marrow, spleen, brain, spinal cord, heart, skeletal muscle, kidney, lung, pancreas, prostate	Limb development/bone and cartilage morphogenesis/ connecting soft tissues	Homozygous recessive: shortened, slightly ruffled external ears due to a defective cartilage framework affecting the whole skeleton.
BMP-6 (Vrg1, Dvr6)	Thymus, bone marrow, spleen, brain, spinal cord, heart, skeletal muscle, kidney, lung, liver,	Cartilage hypertrophy/bone morphogenesis/ nervous system development	Homozygous: delayed ossification in the developing sternum, females smaller in size
BMP-7 (OP1)	Thymus, bone marrow, spleen, brain, spinal cord, heart, skeletal muscle, kidney, lung, liver, pancreas, prostate	Skeletal repair and regeneration/ kidney and eye formation/nervous system development	Homozygous: postnatal lethality, a wide range of skeletal and cartilage abnormalities, renal dysplasia and polycystic kidney, and eye defects
BMP-8a (OP2)	Thymus, bone marrow, spleen, brain, spinal cord, heart, kidney, lung, pancreas, prostate	Bone morphogenesis/ spermatogenesis	Homozygous targeted: spermatogenesis defects and germ cell degeneration

Table 2. List of BMPs functions

BMPs	Human expression	Functions in Human	Mutations in mice
BMP-8b	Bone marrow, spleen, brain, spinal cord, heart, skeletal muscle, kidney, liver, pancreas	Spermatogenesis	Homozygous: incidents of lethality. Heterozygous and surviving homozygous males: various degrees of germ cell deficiency and infertility
BMP-9 (GDF2)	Liver	Bone morphogenesis/development of cholinergic neurons	Data not found
BMP-10	Thymus, bone marrow, spleen, brain, spinal cord, heart, skeletal muscle, lung, liver, pancreas	Heart morphogenesis	Homozygous null: embryonic lethality with cardiac dysgenesis
BMP-11 (GDF11)	Thymus, bone marrow, spleen, brain, spinal cord, pancreas	Pattering mesodermal and neural tissues, dentin formation	Targeted KO: lethality, defects in urinary, renal, nervous system, vision and eye development
BMP-12 (GDF7/CDMP2)	Data not found	Ligament and tendon development/sensory neuron development	Targeted KO: defects in nervous system, embryogenesis
BMP-13 (GDF6/CDMP2)	Data not found	Normal formation of bones and joins/skeletal morphogenesi	Homozygous null: multiple joint and skeletal patterning defects affecting the extremities, inner ear, and skull
BMP-14 (GDF5/CDMP1)	Bone marrow, heart, kidney, liver	Skeletal repair and regeneration	Homozygous null: slightly shorter bones of the limbs, and drastically shorter bones of the feet, with some complete or partial fusions
BMP-15 (GDF9b)	None	Oocyte and follicular development	Homozygous: female infertility or reduced female fertility and smaller litter size, abnormalities in folliculogenesis, ovulation, and oocyte morthology
BMP-16	Data not found	Skeletal repair and regeneration	Data not found.
BMP-17	Data not found	Data not found	Data not found
BMP-18	Data not found	Data not found	Data not found

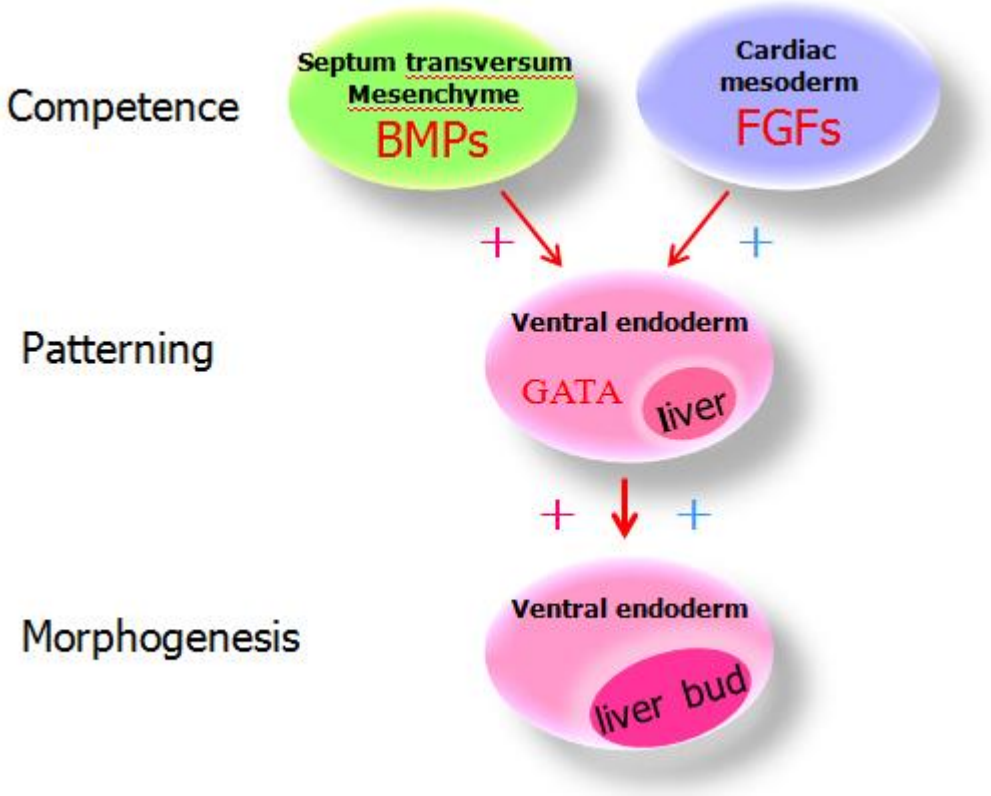

Figure 1. BMP can induce the ventral endoderm to adopt a hepatic fate. BMPs induce GATA-4 expression in ventral endoderm to make it ready to adopt a hepatic fate. Then BMPS and FGFs together induce a part of ventral endoderm to form liver bud. Their continuous actions are required for proliferation and outgrowth of the liver bud. It is clear BMPs will induce liver formation at embryonic stage of life.

signalling from the cardiac mesoderm together induce a portion of the foregut endoderm to initiate hepatogenesis, leaving the distal lip of endoderm to form the ventral pancreas. This results in the expression of transcription factors for subsequent developmental stages. The hepatic endoderm thickens to form the liver bud. The continued action of BMPs and FGFs is required for the proliferation and outgrowth of the liver bud (Stephen, Duncan et al. 2001).

1.4 BMPs signalling pathway

Multiple functions of BMPs depend on complex signal transduction mechanisms in cells. Signaling by BMP family members occurs through BMPR1A or BMPR1B and BMPRII serine/threonine kinase receptors (Ten and Hill 2004). The type I receptors are also referred to as Activin-receptor-like kinases (ALKs) (ALK3 for BMPR1A and ALK6 for BMPR1B). BMP binding induces the assembly of BMPR1A or BMPR1B with BMPRII into a complex within which BMPRII phosphorylates either BMPR1A or BMPR1B in the juxta-membrane region, or "GS domain" (a domain rich in glycine and serine residues). Different combinations of type II with any one of the type I receptors determines the system's specificity and results in different consequences.

There are two well-defined signaling pathways involved in BMP signal transduction (Derynck and Zhang 2003). The general BMP pathway is through receptor I mediated phosphorylation of Smad1, Smad5 or Smd8 (R-Smad). In this pathway two phosphorylated Smads form a heterotrimeric complex with a common Smad4 (co-Smad). The Smad heterotrimeric complex translocates into the nucleus, and binds to GC-rich

Smad1 binding elements (SBEs) within a target gene promoter or cooperates with other transcription factors to modulate target gene expression. A parallel pathway for the BMP signal is mediated by TGF-β1 activated tyrosine kinase 1 (TAK1, a MAPKKK) and mitogen activated protein kinase (MAPK) (Yamaguchi, Nagai et al. 1999), which are also involved in cross-talk between the BMP and Wnt pathway.

Group of natural antagonists of BMP has been found during the course of a search for the elusive neural inducer, which includes noggin, chordin and gremlin. These proteins were first identified in Xenopus followed by humans. They can bind BMPs to block the interaction with the BMP receptor. Therefore, the receptor bioavailability is modulated by these antagonists. Moreover, these proteins can be considered as BMP binding proteins (Reddi 2001)

1.4.1 BMP receptors

BMPs start signaling from the cell surface by interacting with two different serine/threonine kinase receptors: type I receptors and type II receptors. These two types of receptors share similar structural properties, including a relatively short extracellular domain, a single membrane-spanning domain and an intracellular domain containing a serine-threonine kinase domain. Both type I and type II receptors are important for signaling. BMPs bind to type I receptors first without type II receptors and then type II receptor kinases trans-phosphorylate the type I receptors. The type I receptors will transmit specific intracellular signals.

1.4.2 BMP type I receptors

Seven receptors (activin receptor-like kinases 1 through 7; ALK-1-7), have been identified as type I receptors for the TGF-β family in mammals. ALKs are classified into three groups based on the similarities in their structures and functions. They are the BMPR-I group (BMPR-IA and BMPR-IB; also denoted ALK-3 and ALK-6, respectively), the ALK-1 group (ALK-1 and ALK-2) and the TbR-I group (ALK-4/ ActR-IB, ALK-5/TbR-I, and ALK-7). The receptors of the ALK-1 group and those of the BMPR-I group activate Smad1/5/8 and transduce similar intracellular signals, while those of the TbR-I group activate Smad2/3. BMPR-IA and ALK-2 are widely expressed in various types of cells. In contrast, expression of BMPR-IB shows a more restricted expression profile, and that of ALK-1 is limited to endothelial cells and certain other cells.

Specificities of the binding of BMPs to type I receptors are affected by type II receptors. BMP-2 and BMP-4 bind to BMPR-IA and BMPR-IB, whereas BMP-6 and BMP-7 bind strongly to ALK-2 and weakly to BMPR-IB. BMP-9 and BMP-10 bind to ALK-1 and ALK-2. BMP type I receptors are shared by certain other members of the TGF-β family.

1.4.3 BMP type II receptors

Three receptors, BMPR-II, ActR-II and ActR-IIB, serve as type II receptors for BMPs in mammals, and are widely expressed in various tissues. BMPR-II is specific for BMPs, whereas ActR-II and ActR-IIB are shared by activins, myostatin and BMPs. These type II receptors appear to bind most BMP ligands and affect the binding preferences of

BMPs to type I receptors. BMPR-II has a unique, long C terminal tail with 530 amino acids after the kinase domain. The long form with the C-terminal tail is predominantly expressed in most types of cells, while the short form lacking the long C-terminal tail may be expressed only in certain types of cells.

1.5 Antagonists of BMP ligands

Members of BMP antagonists include Noggin, Chordin, Follistatin and Follistatin-related gene (FLRG), Ventroptin, twisted gastrulation (Tsg), and the Dan/cerberus family of genes. Dan/cerberus family of genes consist of the head inducer Cerberus, the tumor suppressor Dan, Gremlin and its rat homolog Drm, the protein related to Dan and Cerberus (PRDC), Caronte, Dante (Dte) and Sclerostin (Canalis, Economides et al. 2003).

Noggin and Chordin (Valenzuela, Economides et al. 1995) are proteins secreted by Spemann organizers (a small piece of tissue from the dosal mesodermic region of the early salamander embryo) and they are widely used to block the activities of BMPs because of their specific abilities to inhibit BMPs activities. Follistatin is expressed in the blastophore (Hemmati, Kellyet al. 1994), which was initially identified as an activin binding protein capable of precluding activin signaling. Follistatin binds BMPs with lesser affinity than activin. The antagonists prevent the interaction of BMPs with signaling receptors by binding to BMPs.

1.6 Smad family

Smads are the major signal transducers for the TGF-b family receptors (Heldin, Miyazon et al.1997; Derynck and Zhang 2003). After the binding of BMPs to receptor extracellular domains and activation of serine-threonine kinases, that is, Type I receptor kinases activated by the type II receptor kinases, phosphorylate R-Smads. R-Smads then form a complex with common partner Smad (co-Smad) and translocate to the nucleus (Figure 2B). The oligomeric Smad complexes regulate the transcription of target genes through interaction with various transcription factors and transcriptional coactivators or corepressors. Inhibitory Smads (I-Smads) negatively regulate the action of R-Smads and/or co-Smads.

1.6.1 Subtypes of Smads

Eight different Smads have been identified in mammals (Figure 2A). Smad proteins can be divided into three distinct classes: the receptor-activated Smads (R-Smads), the common-mediator Smads (Co-Smads) and the inhibitory Smads (I-Smads).

Smad 1, Smad 5 and Smad 8 are R-Smads in BMP signallijng pathways (BMP-specific R-Smads) and they are are phosphorylated by BMP type I receptors (Macias, Abdollah et al. 2002; Nakao, Imamura et al. 2002; Derynck and Zhang 2003). Smad1, Smad5 and Smad8 have highly similar structures and the difference of functions among them still remained to be discovered. Smad2 and Smad3 belong to the TGF-β/activin signalling pathways (TGF-β/activin-specific R-Smads). Smad4 is the only co-Smad in

A

Ligands	Type Ⅱ receptor	Type Ⅰ receptor	R-Smad	Co-Smad
BMP-2/4 group	**BMPR-Ⅱ**	**BMPR Ⅰ Group**	**Smad 1**	**Smad 4**
BMP-2/4		BMPR-ⅠA/ⅠB		
OP-1 group	**ActR-Ⅱ**	**ALK-1 Group**	**Smad 5**	
BMP-5/6/7/8		ALK-1/2		
BMP-9 group	**ActR-ⅡB**	**BMPR-Ⅰ Group**	**Smad 8**	
BMP-9/10		BMPR-ⅠB		
GDF-5 Group				
GDF-5/6/7				
GDF-5/6/7				

B

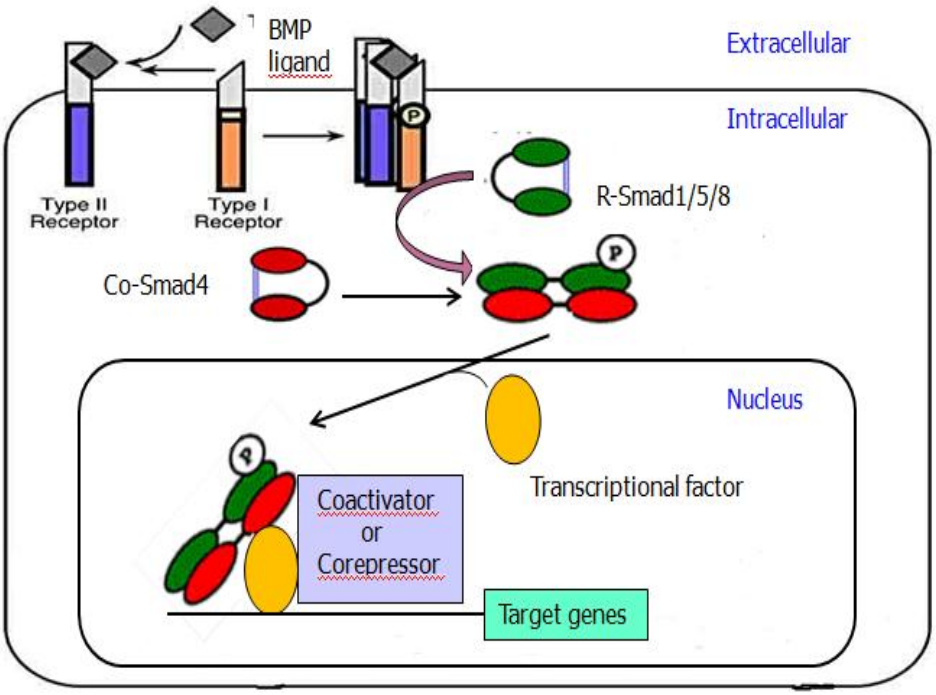

Figure. 2 Signal transductions by BMP receptors and Smads

Figure. 2 Signal transductions by BMP receptors and Smads.

(A) Relationships between BMP ligands, type II and type I receptors, and Smad proteins in signal transduction. Concerning the binding of BMPs to type I receptors, BMP-2/4 bind to BMPR-IA and BMPR-IB, whereas BMP-6/7 bind strongly to ALK-2, and weakly to BMPR-IB. BMP-9/10 bind to ALK-1 and ALK-2, and GDF-5 preferentially binds to BMPR-IB. Phylogenetic tree of the different BMP subgroups is adapted from.

(B) Signalling from BMP receptors at the plasma membrane to the nucleus by Smads. BMP ligands bind to heterotetrameric complex of type II and type I receptors, and RGM proteins serve as coreceptors for BMPs. R-Smads exist in the cytoplasm through interaction with membrane anchoring proteins, e.g.CD44 and endofin. Upon phosphorylation by type I receptors, R-Smads form complexes with co-Smad (Smad4), translocate into the nucleus and regulate transcription of target genes through interaction with transcription factors (DNA-binding proteins) and transcriptional coactivators.

mammals, shared by both BMP and TGF-β/activin signalling pathways. It functions as common partners, forming heteromeric complexes with the R-Smads. Smad6 and Smad7 are I-Smads, major negative regulators, downregulating signal transduction of TGF-β family. R-Smads and Co-Smads are expressed in most cell types, while the expression of I-Smads is regulated by extracellular signals

1.6.2 Smad signaling pathway

Smads are a group of intracellular proteins that transfer signals of TGF-β family members from the cell surface to the nucleus (Figure 2).

The binding of BMP ligands induce the formation of complex between type I receptor and type II receptor within which type II receptor will phosphorylate type I receptor in GS domain. BMP ligands bind to heterotetrameric complex of type II and type I receptors, and RGM proteins serve as coreceptors for BMPs. R-Smads exist in the cytoplasm through interaction with membrane anchoring proteins such as CD44 and endofin. Upon phosphorylation by type I receptors, R-Smads form complexes with co-Smad (Smad4), translocate into the nucleus and regulate transcription of target genes through interaction with transcription factors (DNA-binding proteins) and transcriptional coactivators. R-Smads are anchored at the cell membrane by interacting with various cytoplasmic proteins (Figure 2B). Endofin interacts with Smad1, enhances BMP signalling (Shi, Chang et al. 2007). In addition, CD44, a receptor for hyaluronan, was shown to interact with Smad1 in chondrocytes, and present Smad1 to the BMP receptors for activation (Peterson, Andhare et al.2004). The phosphorylation of R-Smads by type I

receptors is determined by the L45 loop of type I receptors and L3 loop of the MH2 domains of R-Smads (Figure 2B). Moreover, the α-helix H1 in the MH2 domain is also required for the interaction of BMP-specific R-Smads with type I receptors of the ALK-1 group.

2. Hepatic Failure

Liver failure is the inability of the liver to perform its normal synthetic and metabolic function as part of normal physiology, leading to acute or chronic liver failure.

2.1 Acute liver failure (ALF)

Acute liver failure (ALF) is a clinical syndrome due to fulminant necrosis of live mass, leading to the development of hepatic encephalopathy jaundice, coagulopathy, and multisystem organ failure and other severe impairment of hepatic function (Stravitz 2008; Khashab, Tector et al. 2007). ALF is the appearance of severe complications rapidly after the first signs of liver disease (such as jaundice), and indicates the liver has undergone severe damage (loss of function of 80-90% of liver cells). ALF is potentially reversible. It is characterized by the development of hepatic encephalopathy within eight week of the onset of acute hepatitis. Mortality rates as high as 95% have been reported in some North American centres. In 2005, ALF accounted for 6% of liver-related deaths and 7% of orthotopic liver transplants (OLT) in the United States (Kaita, Roberts et al.1998; Stravitz 2008).

2.1.1 Classifications

ALF was first defined by Trey and Davidson in 1970 as an onset of hepatic encephalopathy within 8 weeks of the first symptoms of illness in patients without preexisting liver diseases (Khashab, Tector et al. 2007). With the progress of medical research, people recognized that different patterns of ALF have similar etiologies and prognosises and some patients with liver failure may lead to chronic liver disease. The previous definition, as a result, was revised.

A number of classification systems have been developed for ALF. In one widely used classification system, the terms “hyperacute,” “acute,” and “subacute” are used to define the onset of encephalopathy after jaundice within 7 days, 8 to 28 days, and more than 28 days, respectively (Table 3). An alternative classification is fulminant and subfulminant liver failure (time from jaundice to encephalopathy less or more than 2 weeks) (Khashab, Tector et al. 2007).

2.1.2 Etiology

2.1.2.1 Infectious causes

Although acetaminophen overdose is the most common cause of ALF in the United States and the UK, viral hepatitis, including hepatitis A and B, remains the most common cause in other parts of the world, including France and Japan, whereas hepatitis E is the most common cause in India (Khashab, Tector et al. 2007; Hadem, Stiefel et al. 2008) (Lee and Seremba 2008).

Table 3. Classification of ALF

Interval from jaundice to encephalopathy	
Hyperacute	<7 days
Acute	8-28 days
Subacute	29 days to 12 weeks
ALF-acute liver failure	

Viral hepatitis leads to ALF in only a small number of cases (<1%). With the possible exception of HCV infection, each of the five primary hepatotropic viruses (A through E) has been implicated in ALF. The risk is lowest with hepatitis A (0.35% of cases), but it increases with age at time of exposure. Acute hepatitis B infection (HBV) may lead to ALF in 1% of patients, and greater than half of the cases ALF patients positive for HBV are due to delta virus rather than to hepatitis B alone. Reactivation of HBV is now a well-recognized complication in infected patients who undergo cytotoxic chemotherapy for cancer, with the clinical condition ranging from an asymptomatic rise in aminotransferases to ALF. Hepatitis E is common in parts of Asia and Africa, and the risk of developing ALF increases to over 20% in pregnant women in these areas, with the highest risk during the third trimester (Khashab, Tector et al. 2007).

2.1.2.2 Autoimmune hepatitis (AIH)

A recent study suggested that ALF is a common presentation of autoimmune hepatitis (AIH). Patients with acute presentation of AIH with ALF are significantly different from those with typical presentation of chronic hepatitis related with encephalopathy, albumin levels, and bilirubin levels. Liver biopsies in patients with acute presentation showed significantly less fibrosis and greater interface hepatitis, lobular disarray, lobular hepatitis, hepatocyte zone 3 necrosis, and submassive necrosis. Patients with ALF due to AIH had higher rates of death and more often required orthotopic liver transplantation (OLT), though four of 10 patients responded to standard therapy for AIH (Khashab, Tector et al. 2007).

2.1.2.3 Drug induced liver injury

Drug induced liver injury (DILI) is an important cause of death. It accounts for at least 13% of acute liver failure cases in the US. It is the leading cause of acute liver failure among patients received liver transplantation and the most common reason that drugs in development do not obtain Food and Drug Administration (FDA) approval. The incidence of DILI has been reported to be one in 10,000 to one in 100,000 patients; however, the actual incidence is probably higher partly due to the difficulty of diagnosis (Au, Navarro et al. 2011).

It is estimated that more than 1100 drugs, including herbal medicine and illicit drugs, are associated with DILI and drug-induced hepatotoxicity remains the leading cause of acute liver failure (ALF) from both acetaminophen (AAP) and non-AAP drugs (Table 4) (Stine and Lewis 2011).

It is widely admitted that drug-induced liver injury (DILI) is mediated by two main mechanisms: intrinsic and idiosyncratic hepatoxicity. The majority of drugs lead to idiosyncratic liver injury and can be classified into metabolic and immunological categories. In the former, the drug is metabolised into a toxic metabolite in predisposed individuals, while the latter is similar to ‘‘drug allergy’’ or hypersensitivity following sensitisation to the drug. Intrinsic hepatotoxins cause hepatocellular damage in a predictable dose-dependent manner directly by the drug or indirectly by its metabolite. Some drugs, such as acetaminophen, cause intrinsic hepatotoxicity. Because of the characteristic of intrinsic hepatotoxins, many clinical researchers use certain drugs to form animal models of specific diseases for experimental purpose, such as it did in the

Table 4. Drugs causing ALF

Common causes	Rarer causes
Paracetamol, halothane, NSAIDs, Isoniazid/rifampicin, sulphonamides, Flutamide, sodium valproate, Ecstasy, carbamazepine	Phenytoin, isoflurane, enflurane, tetracycline, allopurinol, methyldopa, gold amiodarone, propylthiouracil, ketoconazole, tricyclic antidepressants

case of D-Galactosamine (D-gal) induced hepatotoxicity.

2.2 Chronic liver failure

Chronic liver failure usually occurs in the context of cirrhosis. Cirrhosis is a consequence of chronic liver disease characterized by replacement of liver tissue by fibrosis, scar tissue and regenerative nodules (lumps that occur as a result of a process in which damaged tissue is regenerated, leading to loss of liver function) (Marthinez and Aamenta 1995; Brenner, Waterboer et al. 2000; Sato, Suzuki et al. 2003). Cirrhosis is generally irreversible, and treatment usually focuses on preventing progression and complications. In advanced stages of cirrhosis the only option is a liver transplant (Heidelbaugh and Bruderly 2006).

Single or multifactorial insults to the liver ultimately lead to cirrhosis. The most common insults are alcohol abuse, chronic hepatitis C, and obesity with concomitant nonalcoholic fatty liver disease (NAFLD) (Table 5). Risk factors include obesity, diabetes, hypertriglyceridemia, and profound weight after jejunoileal bypass (Heidelbaugh and Bruderly 2006; Starr and Raines 2011).

2.3 D-gal hepatotoxicity

D-gal is hepatotoxin widely used to produce hepatitis similar to human viral hepatitis in laboratory animals. Large doses of D-gal are used for fulminant hepatic failure models. During studies on hexosamine metabolism using rats, morphologic

Table 5. Etiologies of Hepatic Cirrhosis

Most common causes	Less common causes
Alcohol 60-70%	Autoimmune chronic hepatitis type 1, 2, and 3
Biliary obstruction	Drugs and toxins
Primary or secondary cirrhosis	Genetic metabolic diseases
Chronic hepatitis B or C 10%	Idiopathic/miscellaneous
Hemochromatosis 5-10%	Infection
NAFLD 10%	Vascular abnormalities
	Veno-occlusive disease

and functional features similar to acute human viral hepatitis were observed after intraperitoneal administration of D-gal. D-gal was widely used produce hepatitis similar to human viral hepatitis in laboratory animals since then (Kucera, Lotková et al. 2006).

D-gal is a hexosamine derived from galactose with the molecular formula $C_6H_{13}NO_5$. This amino sugar is a constituent of some glycoprotein hormones such as follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Other sugar constituents of FSH and LH include glucosamine, galactose and glucose. D-gal is metabolized by the galactose pathway in the liver. This leads to depletion of intracellular uridine moieties, which in turn disturbs hepatocyte RNA metabolism, ultimately leading to hepatocyte necrosis (Keppler, Lesch et al.1968; Maley, Tarentino et al. 1968; Keppler and Decker 1969; Farber, Gill et al. 1973; Shinozuka, Farber et al. 1973; Newsome, Plevris et al. 2000). Whether necrosis is caused solely by these biochemical changes or whether other factors are required, such as endotoxin or Kuppfer cell activation, iremains unclear.

2.4 Treatment

Most liver diseases lead to hepatic dysfunction with organ failure. Treatment is largely limited to supportive care until either transplantation can be arranged or sufficient hepatic regeneration occurs to restore essential hepatic functions. Liver transplantation is the best curative therapy but it has limitations such as donor shortage (Behbahan, Duan et al. 2011), possibility of rejection, and maintenance of immunosuppressant (Fausto 2001; Gill and Sterling 2001; Stravitz 2008).

Scientists have been in search for new therapies for the past decades while artificial liver support devices (Carpentier, Gautier et al. 2009; Rademacher, Oppert et al. 2011) and hepatocyte transplantation (Ito, Nagata et al. 2009; Behbahan, Duan et al. 2011) still remain experimental (Behbahan, Duan et al. 2011). Some progress is being made with stem cells in cell therapy. The capacity of stem cells to self-renewal and differentiation towards different lineage makes them applicable to human diseases. Rodent and human embryonic stem cells, bone marrow hematopoietic stem cells, mesenchymal stem cells, umbilical cord blood cells, fetal liver progenitor cells, adult liver progenitor cells, and mature hepatocytes have been reported to be capable of self-renewal and induce daughter hepatocytes both in vivo and in vitro (Kern, Eichler et al. 2006; Ito, Nagata et al. 2009; Behbahan, Duan et al. 2011). These cells can induce liver regeneration in animal models of liver injury and appear to be able to improve liver function. According to a recent report, the plasticity in adult bone marrow may offer exciting therapeutic opportunity for patients with chronic liver disease (Behbahan, Duan et al. 2011). Despite the great progress, challenges still exist before these cells can be used in humans, such as the lack of consensus about the immunophenotype of liver progenitor cells, uncertainty of the physiological role of reported candidate stem/progenitor cells, practicality of obtaining sufficient quantity of cells for clinical use, and concerns over ethics, long-term efficacy, and safety (Ito, Nagata et al. 2009; Behbahan, Duan et al. 2011).

In short, traditional therapy such as OLT is limited because of the donor liver shortage and long waiting list while cell therapy for liver failure cannot be applied in clinic within a long period of time. Therapeutic alternatives for patients with acute liver

failure are still in great demand.

2.5 Liver regeneration after hepatic injury

Although liver regeneration following the loss of liver mass has been realized for more than 3000 years, its underlying mechanisms still remain to be discovered (Taub 2004). Hepatocytes are quiescent, fully differentiated phenotype that seldom proliferates in adult and animal livers (Michalopoulos and DeFrances 1997). However, their innate capacity to replicate can be easily activated after liver resection or liver injury due to chemicals or viruses (Rabes, Wirsching et al. 1976). The liver can completely regenerate its function and amount of the hepatocytes when the loss of liver parenchyma is within a critical level (Zeisberg, Kramer et al. 2006). Several studies have demonstrated that the regenerative response of the liver is proportional to the loss of liver mass until the liver regains its normal weight, which correlates with the individual body size (Michalopoulos and DeFrances 1997). However, the regenerative capacity of the liver can not compensate for the loss of liver mass when the loss of viable hepatocytes is out of range (Gill and Sterling 2001). This will lead to hepatic failure.

To decrease the high mortality of hepatic failure, research on positive and negative regulators in liver regeneration have been carried out (Fausto 2001). Recently, the role of hepatic progenitor cells in the restitution of liver mass following fulminant liver injury has drawn attention (Kinoshita and Miyajima 2002). To date, activation and proliferation of hepatic progenitor cells has been described in rats receiving 2-acetaminofluorene (2-AAF) post partial hepatectomy followed by administration of the potent hepatotoxin

CCl_4 (carbon tetrachloride) (Alison, Goldinget al. 1996) and in the livers of rats following ethionine intoxication combined with a choline-deficient diet (Yang, Koteish et al. 2004; Knight, Akhurst et al. 2007; Tirnitz, Tonkin et al. 2007). Histological examinations of these laboratorial animal models revealed that increased amount of progenitor cells extended from the portal triad into adjacent hepatic parenchyma. The stimulus responsible for this progenitor cell activity remains to be identified.

2.6 BMPs in hepatic morphogenesis and regeneration

Bone morphogenetic proteins (BMPs) which belong to the transforming growth factor-β superfamily and were initially identified as a morphogenetic factor in bone, are known to have a wide potential in development of not only bone but also numerous organs (Hogan 1996; Nohe, Keating et al. 2004). Recent studies demonstrated that BMPs are also involved in other important cellular functions such as regulating cell proliferation and differentiation (Duncan and Watt 2001). BMPs can promote hepatic embryonic development and differentiation at an early stage of life. BMPs expressions were observed during the regeneration in hepatic injury.

2.6.1 BMP-2

During early embryonic hepatic genesis, BMP-2 appears critical for morphogenetic formation of hepatic endoderm into liver bud (Duncan and Watt 2001; Rossi, Dunn et al. 2001; Zaret 2001). BMP signalling derived from septum transversum mesenchyme is required for the morphogenetic activation, formation and expression of the liver gene in

ventral endoderm during the entire process of endodermal competence, patterning and morphogenesis (Rossi, Dunn et al. 2001; Zaret 2001). Autocrine BMP-2 signalling is also required for HEX and albumin expression in the endoderm during hepatic genesis (Zhang, Yatskievych et al. 2002; Zhang, Yatskievych et al. 2004)

Although it is considered that BMP-2 is involved in the generation of stem (progenitor) cell fate, the relation between BMP-2 and the hepatic regeneration after injury in mature rats remains unclear. The transient expression of BMP-2 in CCl_4 induced liver injury was observed in 2007 (Nakatsuka, Taniguchi et al. 2007). In CCl_4 induced hepatic injury rat model, the expression of albumin was significantly reduced at 24 hours post injection with CCl_4 and recovered at 48 hour time point. A transient expression of BMP-2 at 6-24 hours after the same treatment was also observed. This expression was also shown with depletion of Kupffer cell by $GdCl_3$, and immunostaining with anti-BMP-2 antibody showed BMP-2-producing cells interspersed in intralobular spaces of injured liver.

These observations indicated that hepatic repair and regeneration occurred soon after the hepatic injury. The transient expression of BMP-2 suggests a role of BMP-2 in hepatic repair and/or regeneration after hepatic injury.

2.6.2 BMP-7

BMP-7 is considered a multifunctional cytokine that mediates the growth and differentiation of many different cell types (Wozney 1998; Hruska, Guo et al. 2000; Zaret 2002; Davies, Lund et al. 2005). BMP-7 expression is absent in the liver while BMP-7

receptors are present in adult hepatocytes. It has been discovered that BMP-7 mediates the sprouting of the liver bud from the central foregut endoderm to develop into hepatocytes (Vukicevic, Latin et al 1994 ; Hogan 1996) and that BMP-7 promote the regeneration of injured kidney (Zeisberg, Shah et al. 2005).

Recent studies have revealed that the administration of rhBMP-7 (recombinant human BMP-7) after PH (partial hepatectomy) on mice significantly promoted liver regeneration that was associated with improvement of liver function. The administration of neutralization for circulating endogenous BMP-7 on mice after PH results in an inhibition of liver regeneration. These findings indicated that BMP-7 might function as an endogenous regulator for adult hepatocellular proliferation and hepatic homeostasis. These outcomes were mediated via circulating BMP-7 likely produced in the kidney and bone due to the absence of hepatic BMP-7 expression. Thus, BMP-7 might also function as a novel hormone to facilitate liver regeneration (Hikaru, Changqing et al. 2007).

2.6.3 BMP-4

Recently, our laboratory has carried out a series of experiments on BMP-4 and significant outcomes have been observed.

We discovered that the expression of BMP-4 was significantly elevated in the liver of common bile duct ligations (BDL) rats**.** BDL was performed on male Sprague-Dawley (SD) rats. The rats were then sacrificed at 1, 2, 3, 4, 5 and 6 weeks. Hepatic expression of BMP-4 at various time intervals was measured. We found that expression of BMP-4

significantly increased in the liver of BDL rats compared to the rats received sham surgery (Fan, Shen et al. 2006). Hepatic BMP-4 mRNA expression was observed to increase at week 2 and the increase in BMP-4 protein expression was observed at week 3 and week 6.

Expression of BMP-4 in HSCs (hepatic stellate cells) was also documented in our laboratory. We determined that the abundance of BMP-4 mRNA gradually increased during 24 days' culture of HSCs in vitro (Fan, Shen et al. 2006). The increase of BMP-4 mRNA expression was observed at day 9, and a significant increase was observed at day 24 during the culture in vitro.

In addition, we found that BMP-4 could induce rat hepatic progenitor cells (WB-F344 Cells) to differentiate towards a hepatic lineage. Adenovirus delivered BMP-4 and recombinant BMP-4 were employed to stimulate rat hepatic progenitor cells (WB-F344 cells). Hepatocyte markers (albumin, TAT-1, and G6Pase) and cholangiocyte markers (b4-integrin and CK19) were employed to examine the effects of BMP-4 on WB-F344 cell differentiation. After WB-F344 cells were infected with adenovirus-BMP-4, the expression levels of hepatocyte markers (albumin, TAT-1, and G6Pase) were all significantly elevated compared to control group without adenovirus-BMP4 infection. To examine the role of recombinant BMP-4, BMP-4, dexamethasone, and Noggin (BMP-4 antagonist) were employed in the other related experiment. BMP-4 and dexamethasone could stimulate the expression of hepatocyte markers (albumin, TAT-1, and G6Pase) in WB-F344 cells respectively, but no additive effect was observed when combined together. Neither of them induced the expression of

cholangiocyte markers. Noggin was able to prevent BMP-4 from inducing the expression of hepatocyte markers. This discovery revealed that BMP-4 could stimulate rat hepatic progenitor cells (WB-F344 cells) to differentiate towards hepatocytes (Fan, Shen, et al. 2009).

Based on the previous studies that BMP-2/4 induce hepatic genesis at an embryonic stage (Duncan and Watt 2001; Rossi, Dunn et al. 2001; Zaret 2001; Zaret and Grompe 2008; Huang, Ruan et al. 2008), and findings in our laboratory that BMP-4 expression increased after hepatic injury (BDL), BMP-4 expression increased during the HSCs culture in vitro and BMP-4 can induce the differentiation of hepatic progenitor cells towards hepatocytes, it is possible that BMPs may be important regulation factors in repair and regeneration after liver injury.

Ⅱ. Hypothesis and Objectives

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Hypothesis:

Based on the previous studies that BMP-2/4/7 induce hepatic genesis at an embryonic stage and the findings that BMP-4 expression increased after hepatic injury, BMP-4 expression increased during the HSCs culture in vitro and BMP-4 can induce the differentiation of hepatic progenitor cells towards hepatocytes, hypothesise that BMPs are important regulating factors in repair and regeneration after liver injury.

Objectives:

(1) To establish D-gal induced hepatic injury model in rat hepatoma 1548 cells.

(2) To examine the expression profile of BMP-2, BMP-4 and BMP-7 in D-gal induced hepatic injury.

Ⅲ. Materials and methods

1. Materials

Chemicals and reagents used in this study are listed in Table 6. Unless specified, all chemicals were purchased from Sigma-Aldrich.

Table 6. List of chemicals

Product	Company
Acrylamide	Fisher Scientific
Alcohol	Fisher Scientific
BCA protein assay reagent	PIERCE
BMP-4(3H2.3) antibody	Santa Cruz
CytoTox-ONE™ LDH Assay	Promega
D-galactosamine	Sigma-Aldrich
DNA ladder	Invitrogen
Ecl Mouse IgG, HRP-Linked Whole Ab	GE Healthcare
Ecl plus Western Detection Reagents	GE Healthcare
iQ™ SYBR® Green Supermix kit	Bio-Rad
iScript™ cDNA Synthesis Kit	Bio-Rad
L-Glutamine	Invitrogen
MEM/EBSS	HyClone
Methanol	Fisher Scientific
Non essential amino acids	Invitrogen
Penstrep	Invitrogen
Primers	Invitrogen
Protein ladder	Bio-Rad
Ptoteinase In hibitor	Roche
Tactin-HRP	Bio-Rad
TEMED	GIBCO/BRL
Tris-Base	Fisher Scientific
Trizol Reagent	Invitrogen

2. Methods

2.1 Cell line and cell culture conditions

Rat hepatoma 1548 cell line was provided by Dr. Frank J. Burczynski (University of Manitoba, Canada). Cells were grown in 10 cm diameter 10cm culture plates in MEM (minimal essential medium) medium containing 5% FBS (fetal bovin serum), 2mM/L L-glutamine, 1% non-essential amino acids (Invitrogen Canada Inc., Burlington, Canada), 100IU/L penicillin and 100 μg/ml streptomycin in a 37^{o}C humidified incubator in an atmosphere of 5% CO_2 and 95% air. Medium was changed every other day.

2.2 **LDH assay**

Lactate dehydrogenase (LDH) is an enzyme present in a wide variety of organisms, including plants and animals. Normal viable cells preserve LDH in endochylema with impermeable cell membranes. However, damage to the integrity of the cell membrane or cell death can lead to the leakage of LDH from the cytoplasm into the surrounding culture medium. Measurement of the leakage of LDH has been widely accepted as a valid method to test cytotoxicity and estimate the number of non-viable cells.

The LDH release assay is a rapid, fluorescent method to measure the release of LDH from cells with a damaged membrane, commonly used for testing cytotoxicity of various experimental compounds. Release of LDH from damaged cells is measured by supplying lactate, NAD+, and resazurin as substrates in the presence of diaphorase. The reaction is shown in Figure 3. After certain period of incubation, the fluorescent signal is measured. Fluorescence data are collected using a standard 96-well fluorometer.

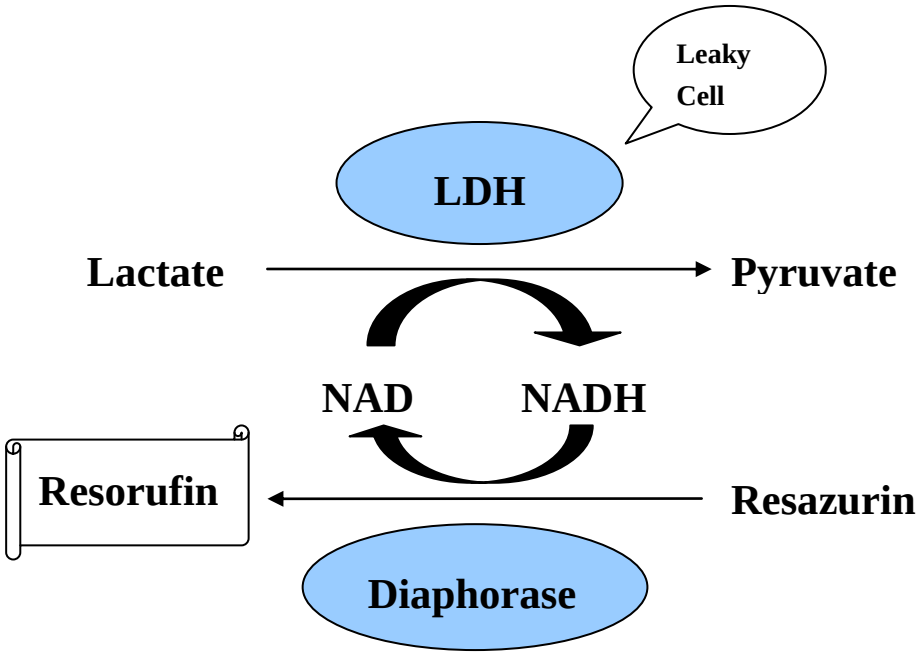

Figure 3. The principle of LDH leakage

Release of LDH from damaged cells is measured by supplying lactate, NAD+, and resazurizn as substrates in the presence of diaphorase.

Generation of fluorescent resorufin product is proportional to the amount of LDH as well as the number of lysed cells using a 96-well plate format.

2.2.1 Drug treatment

Rat hepatoma 1548 cells were seeded at a density of 300,000 cells per well in triplicate 96-well plates (SARSTEDT) in 100 μl fresh MEM. After the establishment of the monolayer, the MEM medium in each plate was removed and replaced with fresh complete MEM medium containing D-gal at concentrations of 0mM/L, 4mM/L, 20mM/L and 40mM/L. There were 6 wells reserved for each concentration. The medium was changed once every other day. LDH release tests were carried out on the three plates respectively after 24 hours, 48 hours and 72 hours of incubation. The LDH assays are described as below.

2.2.2 Procedure of LDH assay

CytoTox-One^{TM} Homogeneous Membrane Integrity Assay Kit was purchased from Promega (Madison, WI). The kit includes lyophilized Substrate Mix, Assay Buffer, Lysis Solution (a 9% (weight/volume) solution of Triton X-100 in water, and results in almost immediate lysis of most cell types and subsequent release of cytoplasmic LDH into the surrounding culture medium) and Stop solution (provided to rapidly stop the continued generation of fluorescent product and allow the plate to be read at a later time). The CytoTox-One^{TM} Reagent was formed on the day before the assay by mixing 11 ml of Assay Buffer with each vial of Buffer Substrate Mix after they had been equilibrated to

room tempreture (22°C) through a 37°C water bath. Control groups of this assay include No-Cell Control (negative control to determine background fluorescence that might be present), Untreated Cells Control (received PBS as the vehicle control) and Maximum LDH Release Control (positive control to perform a nearly 100% cell lysis control to determine the maximum amount of LDH present). Each of these controls was performed on each plate being assayed. Reagent preparation and the assay were carried out in dark room due to the light sensitivity of resazurin.

After the cells had been cultured for desired test exposure period (24 hours, 48 hours and 72 hours), the 96-plates were removed from 37^{o}C incubator and equilibrated to 22°C (approximately 20–30 minutes). 2 µl of Lysis Solution was added to the positive control wells before the addition of CytoTox-ONE™ Reagent. 100 µl of CytoTox-ONE™ Reagent was added to each well containing 100 µl MEM medium after D-gal treatment and mixed for 30 seconds. After 10 minutes of incubation of the cells at 22^{o}C, 50 µl of Stop Solution was added to each well in the same order as the CytoTox-ONE™ Reagent was added, The 96-well plate was then shaken for 10 seconds and the fluorescent signal was measured. Fluorescence data were collected using a standard 96-well fluorometer with an excitation wavelength of 560 nm and an emission wavelength of 590 nm (Figure 4).

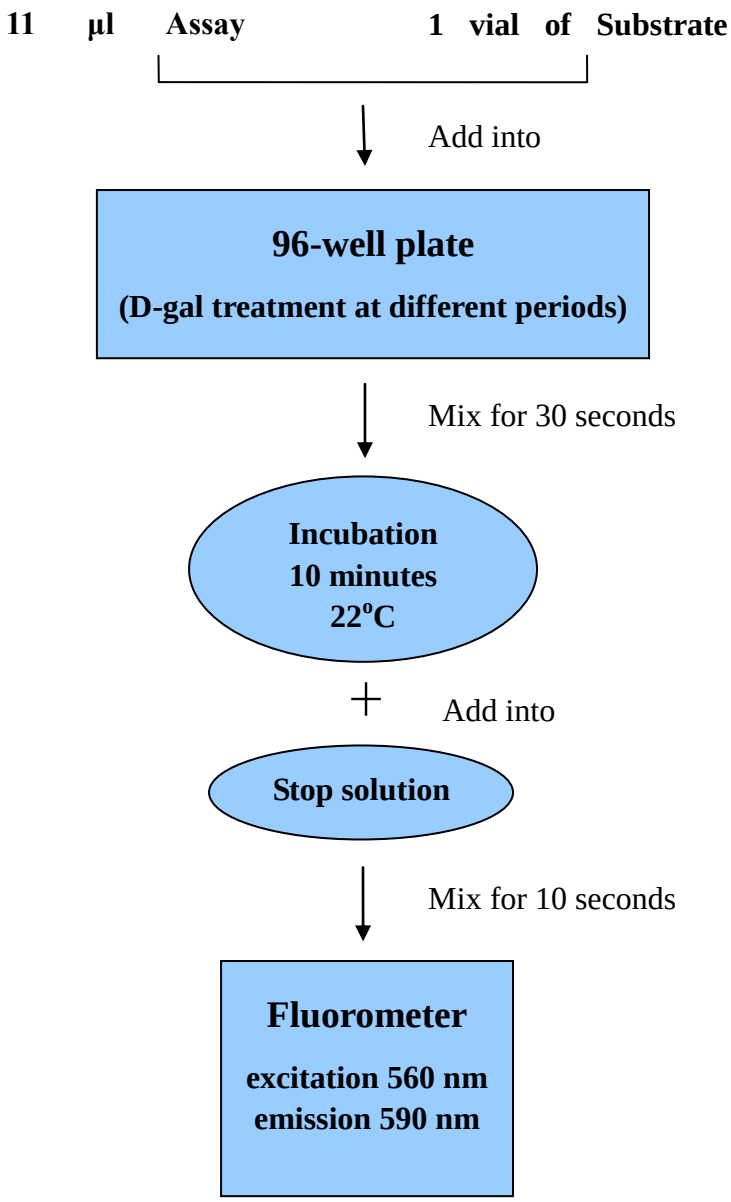

Figure 4. The CytoTox-ONE™ Homogeneous Membrane Integrity Assay procedure

2.3 PCR

2.3.1 Drug treatment

Rat hepatoma 1548 cells were seeded at a density of 1×10^6 per dish in 10 mm culture dishes and in 10 ml of complete MEM medium and incubated overnight. The MEM medium was replaced with fresh complete MEM medium every other day. The cells were incubated in a humidified incubator at 37 ^{o}C in an atmosphere of 5% $CO_{2.}$ After the establishment of the monolayer, the MEM medium was replaced with fresh complete MEM medium containing D-gal at different concentrations (0mM/L, 4mM/L, 20mM/L and 40mM/L) and the cells were incubated for 3, 6, 12, 24, and 48 hours. Total RNA was extracted at the end of the incubation and collected for RT-PCR (reverse transcription polymerase chain reaction).

2.3.2 RNA isolation

TRIZOL Reagent (Invitrogen Canada Inc, Burlington, Canada) was employed to isolate RNA from Rat hepatoma 1548 cells after the cells were washed two times with PBS buffer. Three ml of TRIZOL Reagent was added directly to the 10 cm diameter dish. The cell lysate passed several times through a pipette and was then transferred into a 50 ml tube afterwards. Samples were incubated at 15 to 30°C for 5 minutes to allow complete dissociation of nucleoprotein complexes and then 0.6 ml of chloroform was added. Sample tubes were shaken vigorously by hand for 15 seconds and were incubated at 15 to 30°C for 2 to 3 minutes. Samples were centrifuged at 12,000$\times$g for 15 minutes at 4°C. The upper aqueous phase containing RNA was transferred to a fresh tube after

centrifugation and 1.5 ml of isopropyl alcohol (0.5 ml/1 ml TRIZOL Reagent) was added to the sample tubes. After the addition of isopropyl alcohol, samples were mixed up and down 6 times, incubated at room temperature for 10 minutes and then centrifuged at 12,000×g for another 10 minutes at 4°C. The RNA precipitate was formed on the side and bottom of the tube. The supernatant was removed and the RNA pellets were washed once with at least 3 ml of 75% ethanol (at least 1 ml per 1 ml TRIZAL Reagent). The RNA pellet and 75% ethanol in each tube was vortexed and centrifuged at 7,500×g for 5 minutes at 4°C. The RNA pellets were briefly air dried for 5 minutes and RNA pellets were redissolved in 40 μl of RNase-free water in each tube. Samples were incubated in a 60°C water bath for 10 minutes. After the RNA pellets were completely dissolved in the water, the OD (optical density) value was read at 260 nm and 280 nm using a UV spectrophotometer (Bio-Rad SmartSpec 3000 Spectrophotometer, Life Science Research Division, Mississauga, ON). Good RNA samples had an $A_{260/280}$ ratio of 1.8 to 2.0. The RNA samples were stored at -80°C until required. The following equation was used for calculation of the concentration of the RNA solution：

$$Concentration\ (\mu g/\mu l) = \frac{OD \times k \times dilution\ \ factor}{1000}$$

OD = optical density reading　　k = 40 (constant)

dilution factor = 250 (2 μl of RNA solution in 498 μl of water)

2.3.3 Reverse transcription polymerase chain reaction (RT-PCR)

The reverse transcription reaction was performed following the iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Reaction components included Nuclease-free water, iScript Reverse Transcriptase (RNAse H+, a modified MMLV (Moloney murine leukemia virus reverse)-derived reverse transcriptase, provided pre-blended with RNAse inhibitor) and 5x iScript Reaction Mix (blend of oligo (dT) and random hexamer primers). Ten μg of total RNA was dissolved in Nuclease-free water to adjust the final volume to 15 μl. Four μl of 5x iScript Reaction Mix and 1 μl of iScript Reverse Transcriptase were added to achieve to a total volume of 20 μl. The reaction mixture was then incubated at 25°C for 5 minutes, 42°C for 30 minutes, 85°C for 5 minutes and held at 4°C. The synthesized complementary DNA (cDNA) served as DNA template in PCR amplification.

Polymerase chain reaction (PCR) was performed following the iQ™ SYBR® Green Supermix kit (Bio-Rad, Hercules, CA). The 2×SYBR Green Supermix contained thermostable iTaq DNA polymerase (50 units/ml), KCl, Tris-HCI, dNTP mix (dATP, dCTP, dGTP and dTTP), MgCl2). Gene-specific PCR primers were designed by Oligo 5 program based on BMP-2 and BMP-4 DNA sequences published at Genbank and synthesized by Invitrogen (Invitrogen, Burlington, Canada). Details of the PCR reaction and BMP-2 and BMP-4 primers are shown in Table 7 and Table 8 respectively. PCR amplification was carried out in a PCR Thermal Cycler (Bio-Rad S1000TM). After 2 minutes of initial denaturation, PCR amplifications were carried out in 35 cycles of denaturation at 95°C for 30seconds, annealing at 59.6°C (BMP4) and 61.3°C (GAPDH)

Table 7. RT-PCR primers and conditions

Genes	Primer sequences	Product (bp)	Tm (°C)
BMP-2	Sense: 5' CCCCTATATGCTCGACCTG 3'	248	57.3
	Antisense: 5' CCTGCATTTGTTCCCGAAA 3'		
BMP-4	Sense: 5' CCGGGAAAAGCAACCCAAC 3'	286	59.6
	Antisense: 5' GCCCACGTCACTGAAGTCCA 3'		
BMP-7	Sense: 5' AGGCCGTCTTCAGTACCCAG 3'	229	59.1
	Antisense: 5' CGCTCCCGGATGTAGTCCTT 3'		
GAPDH	Sense: 5' CAAAGTGGACATTGTGCCAT 3'	205	61.3
	Antisense: 5' ATACTCAGCACCAGCATCACC 3'		

Table 8. RT-PCR reaction components

Component	Volume per reaction	Final concentration
IQSYBR Green Supermix	12.5 µl	1X
Primer 1	1 µl	100 nM–500 nM
Primer 2	1 µl	100 nM–500 nM
Sterile water	8.5 µl	
DNA template	2 µl	
Total volume	25µl	

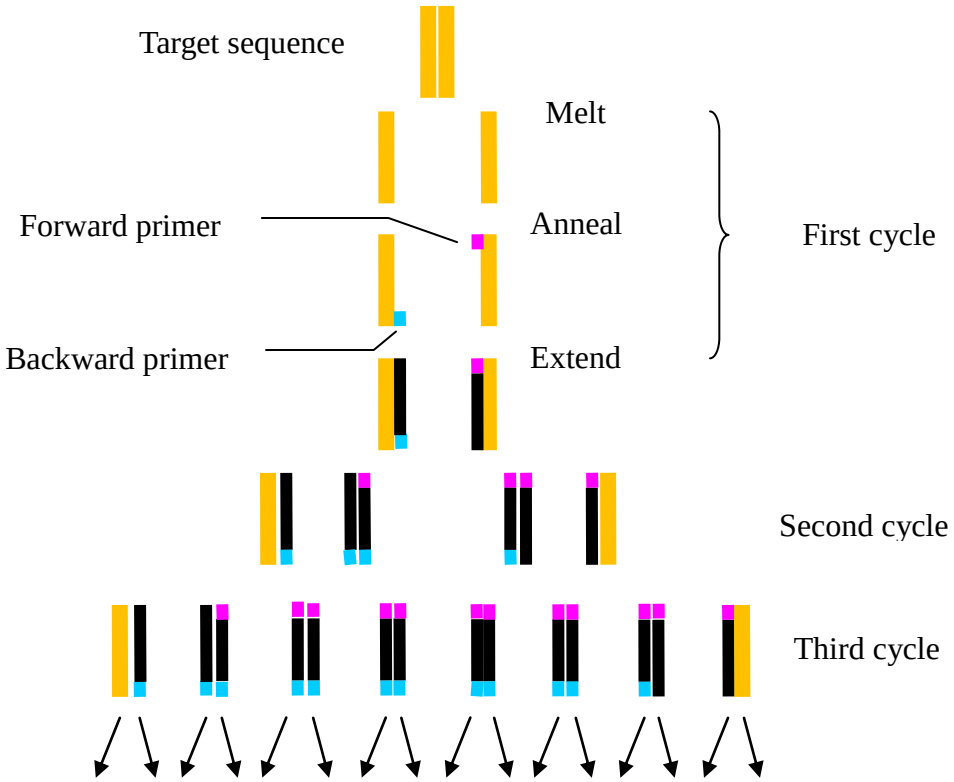

Figure 5. The principle of RT-PCR

for 30 seconds, elongation at 72°C for 60 seconds. After all the cycles were complete, a further elongation at 72°C for 5 minutes was carried out. PCR products were analyzed using a 2% agarose gel (Figure 5).

2.3.4 Agarose gel electrophoresis

Agarose powder was dissolved in 30 ml of 1×TAE buffer (1 L of 50×TAE buffer contained 242 g of Tris base, 100 ml of 0.5M EDTA (PH 8.0), 57.1 ml of glacial acetic acid) to make a 2% (1g/100ml) gel by heating in a microwave. The gel solution was stained with 1 μl of ethidium bromide (EB, 0.5 μg/ml) before it was poured into a casting tray with a comb and comb was removed from the gel after it solidified. A sufficient amount of 1×TAE buffer was added to cover the gel to a depth of 1 mm. 9 μl of DNA samples mixed with 1 μl of 10×DNA loading buffer (0.2 ml of 12.5% bromophenol blue, 0.2 ml of 12.5% xylene cyanol, 25 g of Ficoll and 100 ml of sterile water) were loaded into the wells. One μl of DNA ladder (Invitrogen, Burlington, Canada) was loaded beside the DNA samples. The voltage of electrophoresis was 100v. The PCR product was detected by a Gel Imager (Alpha Innotech, San Leandro, US) and analyzed by Image J program.

2.4 Western blot analyses

2.4.1 Drug treatment

Rat hepatoma 1548 cells were seeded at a density of 1×10^6 per dish in 10 mm culture dishes and in 10 ml of complete MEM medium and incubated overnight. The

MEM medium was replaced with fresh complete MEM medium every other day. Cells were incubated in a humidified incubator at 37 ^{o}C in an atmosphere of 5% $CO_{2.}$ After establishment of the monolayer, the MEM medium was replaced with fresh complete MEM medium containing D-gal at different concentrations (0mM/L, 4mM/L, 20mM/L) and the cells were incubated for 0, 6, 12, 24 and 48 hours. Protein was extracted at the end of the incubation and collected for western blot analyses.

2.4.2 Protein isolation

Protein was extracted using of RIPA lysis buffer (10 mM Tris-HCl pH 7.4, 10 mM NaCl, 1 mM EDTA, 5 μg/ml leupeptin, 10% sodium dodecyl sulfate). 1.5 ml of RIPA buffer was added into the 10 mm culture dish after the cells were washed twice with ice-cold PBS pH 7.4. Proteinase inhibitors (Roche, Laval, CA) were added into RIPA buffer prior to the addition of RIPA buffer to the cells to suppress the autologous enzymatic activity and preserve the biological features of the protein. The cell pellets were incubated in RIPA buffer on ice for 30 minutes (prevent protein from denaturing) and vortexed 3 times during this period for 1 minutes each time. The lysate was centrifuged at 13,000 g for 20 minutes at 4 ^{o}C then supernatant was transferred into a clean tube. Protein samples were stored at -80 ^{o}C and prepared for Western blot analysis. Protein concentrations were measured by BCA assay (PIERCE, Rockford, USA).

2.4.3 Western blot analysis

Protein samples used for Western blot analysis were extracted from rat hepatoma 1548 cells as describe above and 60 μg of proteins was loaded in each well mixed with 4×loading buffer (250 mM Tris-HCl, pH 6.8, 8% sodium dodecyl sulfate (SDS), 20% glycerol, 0.2% bromophenol blue, and 5% β-mercaptoethanol). After boiling for 5 minutes, proteins were separated on a 10% sulfate-polyacrylamide (SDS-polyacrylamide) gel electrophoresis at 120v for 2 hours. The separated proteins were transferred onto Nitroplus-2000 (Micron Separations Inc, Westborough, MA) membranes. In order to avoid nonspecific binding of the antibody, the membrane containing the proteins was incubated in 5% skim milk (to block the nonspecific protein binding sites on the membrane) in TBS-T (Tris buffered saline, 2.42 g Tris base, 29.25 g NaCl, and 0.5 ml Tween 20 per litter) for 1 hour at room temperature. After the blocking step, membranes were incubated overnight at 4 ^{o}C with primary antibody, mouse anti-BMP-4 (Santa Cruz Biotechnology, Inc., Santa Cruz, USA), at 1:200 and for mouse anti-β-actin at 1:1000 in 2% skim milk in TBS-T, respectively. After being washed with TBS-T, the membrane was incubated with secondary antibodies for sheep anti-mouse IgG (GE Healthcare, Pittsburgh, USA) for BMP-4 at 1:1000 and anti-mouse antibody for β-actin at 1:1000 for 1 hour at room temperature. The protein-antibody complexes were analyzed by Gel Imager (Alpha Innotech, San Leandro, US) and the optical density value of the targets were determined using Image J software.

2.5 Statistical analysis

Results are expressed as mean ± SD. Students' t-test and one-way analysis of variance (ANOVA) were carried out using GraphPad Prism 5 software. The control groups (0 mM of D-gal) was converted as 1 in the histograms and the other groups were relative to control groups. Statistical significance was taken at the $P<0.05$ level and the n value represents the number of replications in each assay.

Ⅳ. Results

1. The Cytotoxic Effect of D-gal in D-gal Treated Rat Hepatoma 1548 Cells

D-gal is a highly selective hepatotoxin frequently used in animal experiments to induce diffuse liver damage resembling hepatic failure. Toxic chemicals such as D-gal can affect basic functions of cells and the cytotoxicity can be measured by assessing cellular damage. One of the parameters often tested is the measurement of membrane integrity by measuring LDH in the extracellular medium. Normal cell membranes forms functional barriers around the cell and are impermeable to LDH, an enzyme normally present in the cytosol. When cells are damaged, the permeability of cell membranes increases and result in leakage of LDH into the extracellular medium. The amount of LDH release is related to the damage of cell membrane integrity and the cytotoxicity of cells after D-gal treatment.

LDH assay was employed to detect the cytotoxicity of D-gal during the process of cell damage. LDH reagent was added to the cells with different concentrations of D-gal treatment (0 mM/L, 4 mM/L, 20 mM/L, 40 mM/L) at various time points (24 hrs, 48 hrs, 72 hrs). Cells were then incubated for 10 minutes before measurement. Results are shown in Figures 6, 7, and 8. Figure 6 shows the result after 24 hours of D-gal incubation. The figure shows that the amount of LDH released from cells with a damaged membrane was elevated as the D-gal concentration was increased and reached a maximum at the highest dose of D-gal (40mM). A significant increase ($^{**}P<0.001$) in LDH release was observed when the concentration of D-gal was elevated to 20 mM/L and 40 mM/L compared to control group. This may indicate that the D-gal induced cytotoxicity of rat hepatoma 1548

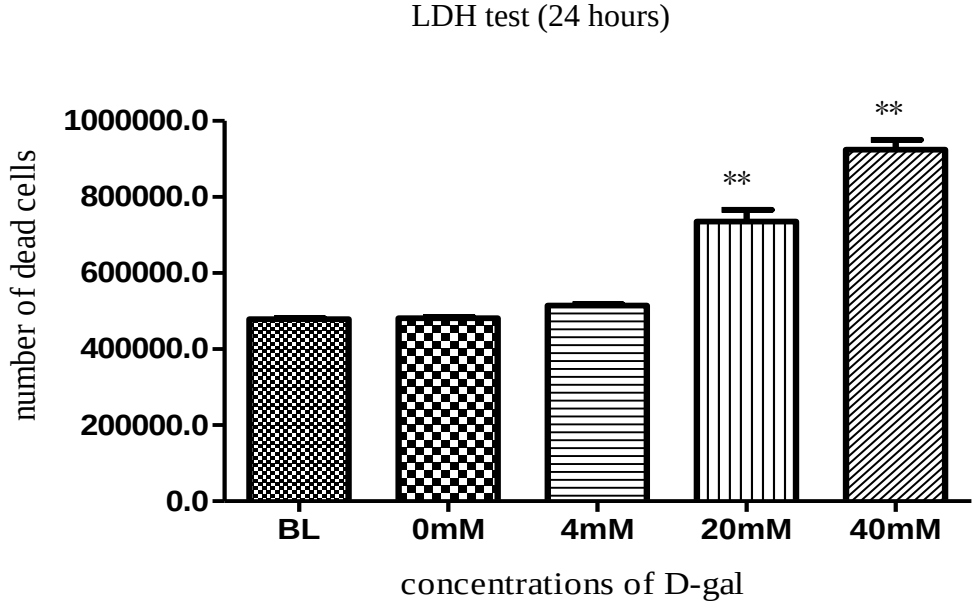

Figure 6. LDH assay following 24 hours treatment with different concentrations of D-gal. Cells were treated with D-gal at 0, 4, 20, 40 mM for 24 hours. The amount of LDH released from cells with a damaged membrane was elevated as the increased D-gal concentrations and reached the maximum at the highest dose of D-gal (40mM). Values are mean ± SD; n=6; ** $P<0.001$.

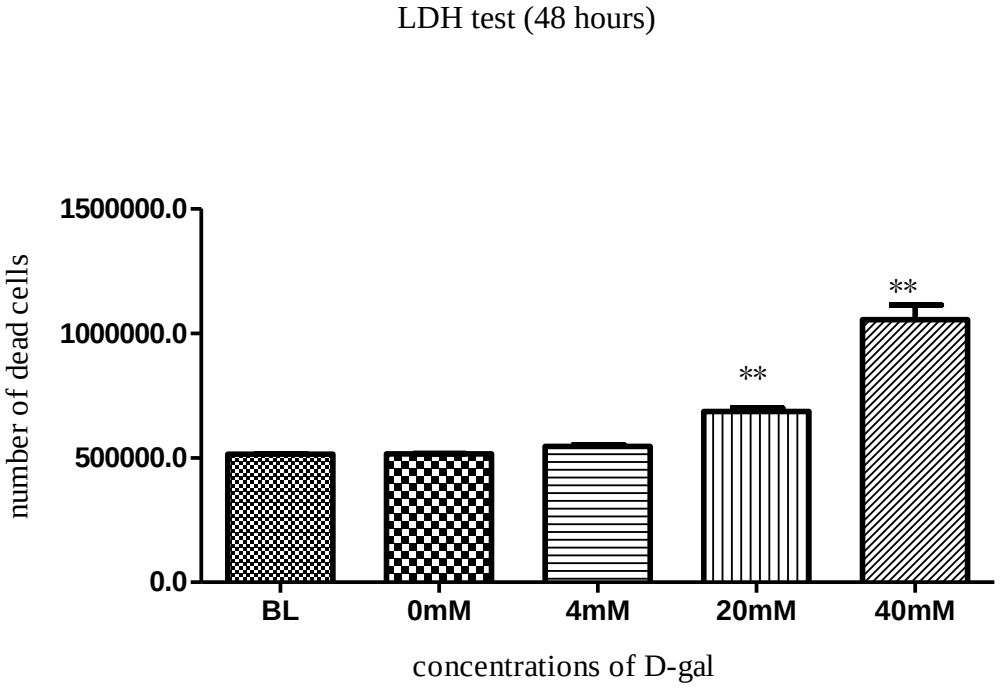

Figure 7. LDH assay following 48 hours treatment with different concentrations of D-gal. Cells were treated with D-gal at 0, 4, 20, 40 mM for 24 hours. The amount of LDH released from cells with a damaged membrane was elevated as the increased D-gal concentrations and reached the maximum at the highest dose of D-gal (40mM). Values are mean ± SD; n=6; $^{**}p<0.001$.

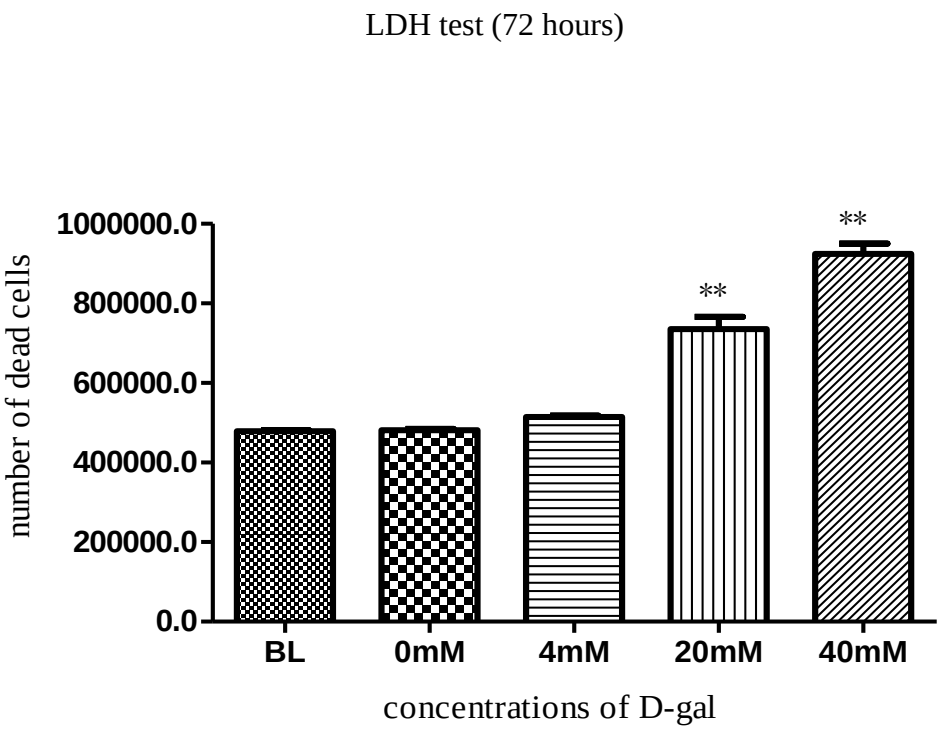

Figure 8. LDH assay following 72 hours treatment with different concentrations of D-gal. Cells were treated with D-gal at 0, 4, 20, 40 mM for 24 hours. The amount of LDH released from cells with a damaged membrane was elevated as the increased D-gal concentrations and reached the maximum at the highest dose of D-gal (40mM). Values are mean ± SD; n=6; ** $P<0.001$

cells is dose-dependent. Similar results of cytotoxicity changes after 48 hours and 72 hours of D-gal treatment was shown in Figure 7 and Figure 8.

2. The Expression of BMP-2 mRNA in D-gal Induced Hepatic Injury

RT-PCR was employed to detect the expression of BMP-2 in D-gal treated rat hepatoma 1548 cells. Cells were incubated with different concentrations of D-gal (0 mM, 4 mM, 20 mM and 40 mM), total RNA was extracted at different time points (3 hrs, 6 hrs, 12 hrs, 24 hrs and 48 hrs) and RT-PCR carried out. Results are shown in Figures 9, 10, 11, 12 and 13. GAPDH served as loading control. As shown in the Figures, the abundance of BMP-2 increased after treatment of D-gal for 6, 12, 24 and 48 hours. BMP-2 mRNA expression reached the maximum at 20mM of D-gal then decreased at 40mM of D-gal. In Figure 9, there was no significant increase in BMP-2 mRNA expression after 3 hours of incubation with 0 mM and 4 mM of D-gal but a decrease of BMP-2 with 40 mM of D-gal ($^{*}P<0.05$). Figures 10, 11, 12 and 13 shows expression of BMP-2 mRNA was elevated as the increased D-gal concentrations except 40 mM. The mRNA level reached the peak at 20mM of D-gal then drop down at 40 mM of D-gal following 6 hours, 12 hours, 24 hours and 48 hours treatment, respectively. The largest increase of BMP-2 was observed at 20 mM of D-gal after 6 hours treatment ($^{*}P<0.05$; Figure 10).

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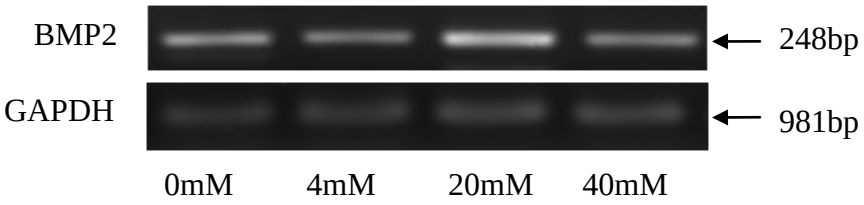

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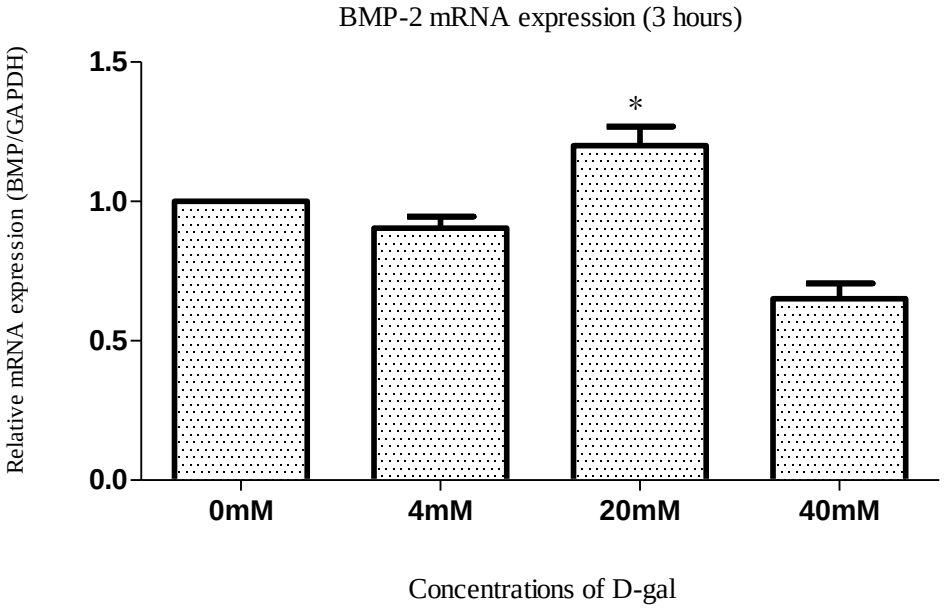

Figure 9. RT-PCR analysis of BMP-2 mRNA expression in rat hepatoma 1548 cells after 3 hours of D-gal treatment. Cells were incubated with 0, 4, 20, 40 mM of D-gal for 3 hours. No significant change of BMP-2 mRNA expression was observed at 4mM and 20 mM of D-gal compared with control group (0 mM) but the mRNA amount was reduced at 40 mM of D-gal. GAPDH served as loading control. Values are mean ± SD; n=4; $^{*}P<0.05$.

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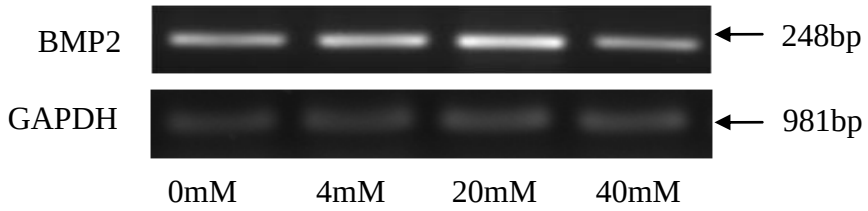

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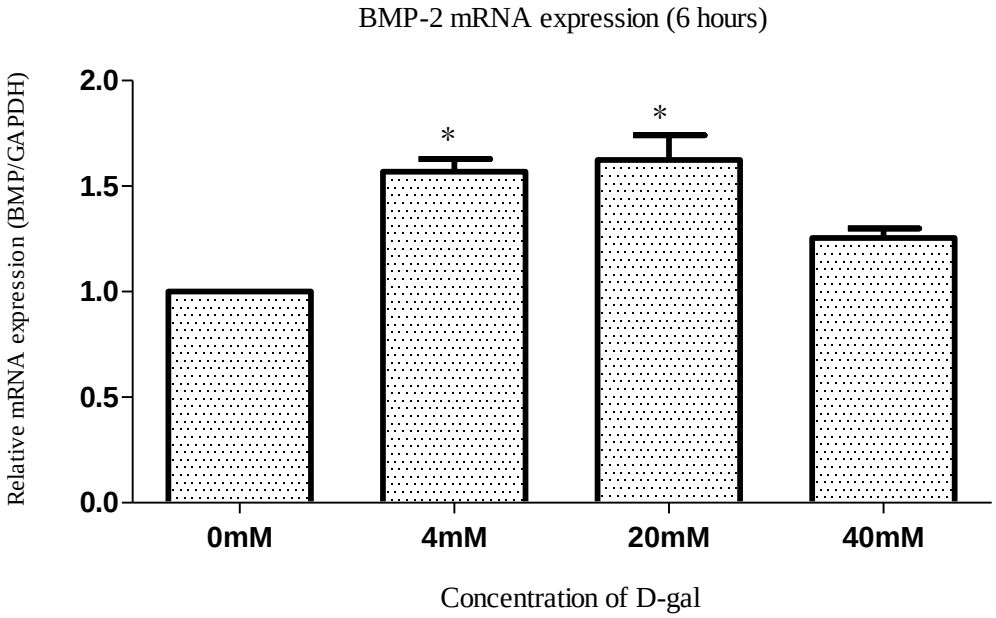

Figure 10. RT-PCR analysis of BMP-2 mRNA expression in rat hepatoma 1548 cells after 6 hours of D-gal treatment. Cells were incubated with 0, 4, 20, 40 mM of D-gal for 6 hours. Expression of BMP-2 mRNA was elevated as the increased D-gal concentrations. The mRNA level reached the peak at 20mM of D-gal then drop down at 40 mM of D-gal. GAPDH served as loading control. Values are mean ± SD, n=4, $^{*}P<0.05$.

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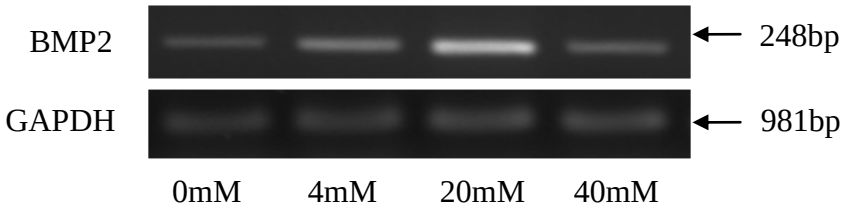

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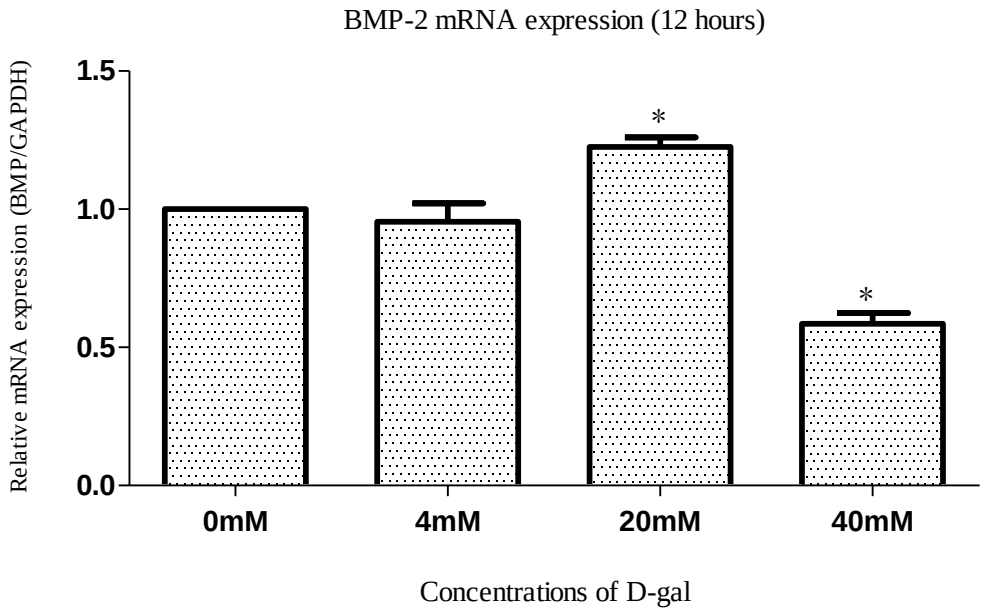

Figure 11. RT-PCR analysis of BMP-2 mRNA expression in rat hepatoma 1548 cells after 12 hours of D-gal treatment. Cells were incubated with 0, 4, 20, 40 mM of D-gal for 12 hours. Expression of BMP-2 mRNA was elevated as the increased D-gal concentrations. The mRNA level reached the peak at 20mM of D-gal then drop down at 40 mM of D-gal. GAPDH served as loading control. Values are mean ± SD, n=4, $^{*}P<0.05$.

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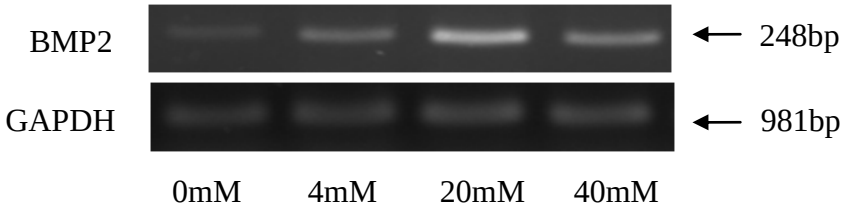

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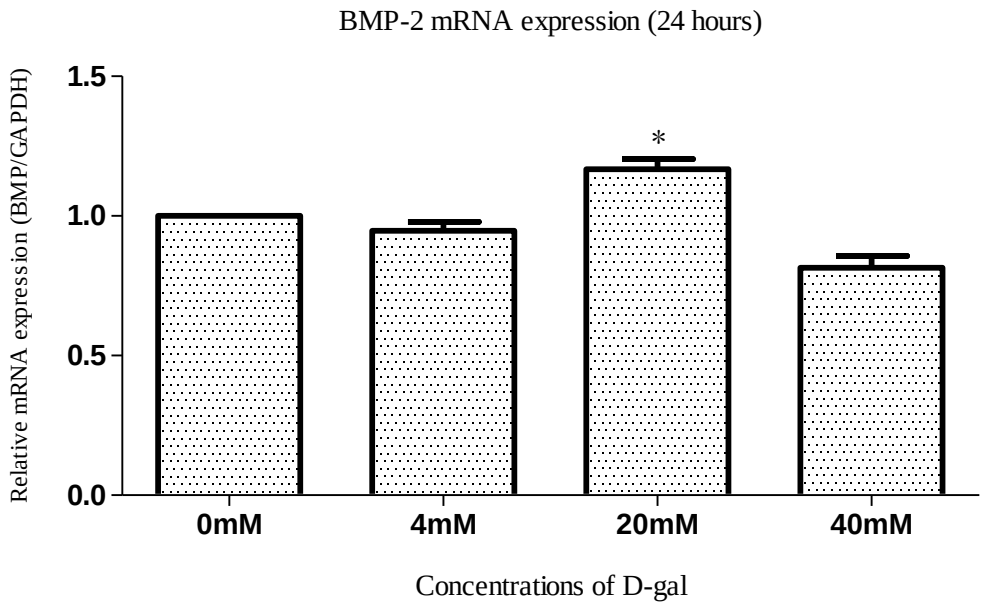

Figure 12. RT-PCR analysis of BMP-2 mRNA expression in rat hepatoma 1548 cells after 24 hours of D-gal treatment. Cells were incubated with 0, 4, 20, 40 mM of D-gal for 24 hours. Expression of BMP-2 mRNA was elevated as the increased D-gal concentrations. The mRNA level reached the peak at 20mM of D-gal then drop down at 40 mM of D-gal. GAPDH served as loading control. Values are mean ± SD, n=4, $^{*}P<0.05$.

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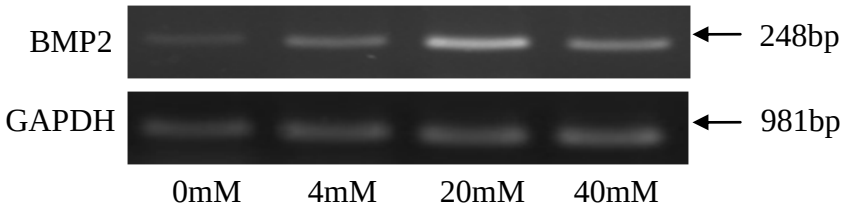

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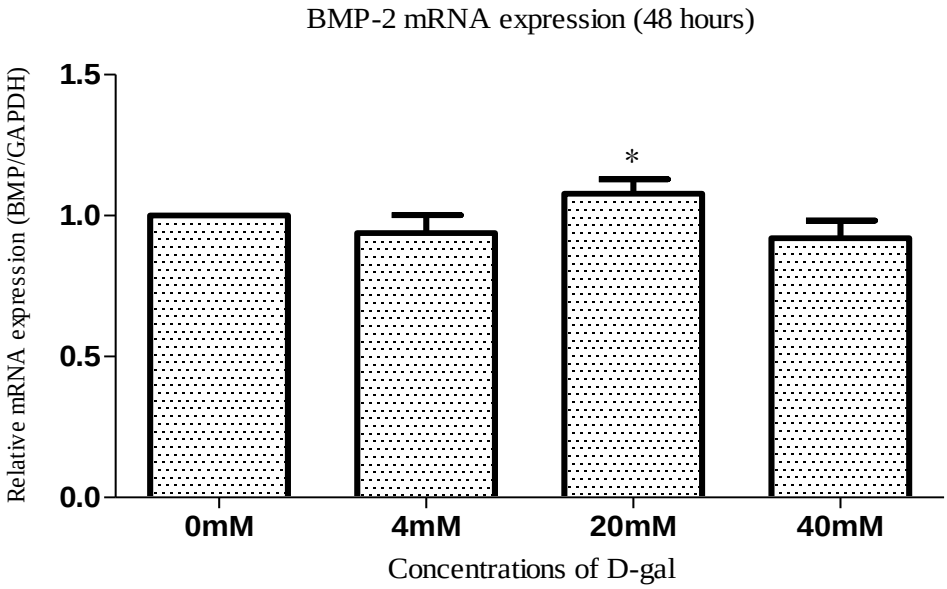

Figure 13. RT-PCR analysis of BMP-2 mRNA expression in rat hepatoma 1548 cells after 48 hours of D-gal treatment. Cells were incubated with 0, 4, 20, 40 mM of D-gal for 48 hours. Expression of BMP-2 mRNA was elevated as the increased D-gal concentrations. The mRNA level reached the peak at 20mM of D-gal then drop down at 40 mM of D-gal. GAPDH served as loading control. Values are mean ± SD, n=4, $^{*}P<0.05$.

3.The Expression of BMP-7 mRNA in D-gal Induced Hepatic Injury

RT-PCR was employed to detect the expression of BMP-7 in D-gal treated rat hepatoma 1548 cells. Cells were incubated with different concentrations of D-gal (0 mM, 4 mM, 20 mM and 40 mM), total RNA was extracted at different time points (3 hrs, 6 hrs, 12 hrs, 24 hrs and 48 hrs) and RT-PCR carried out. Results are shown in Figures 14, 15, 16, 17 and 18. GAPDH served as loading control. As shown in the Figures, BMP-7 mRNA was not statistically different from control values after treatment of D-gal but BMP-7 mRNA expression decreased with 40mM of D-gal treatment ($^{*}P<0.05$).

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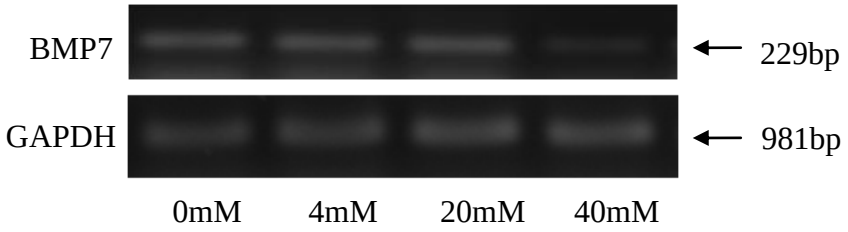

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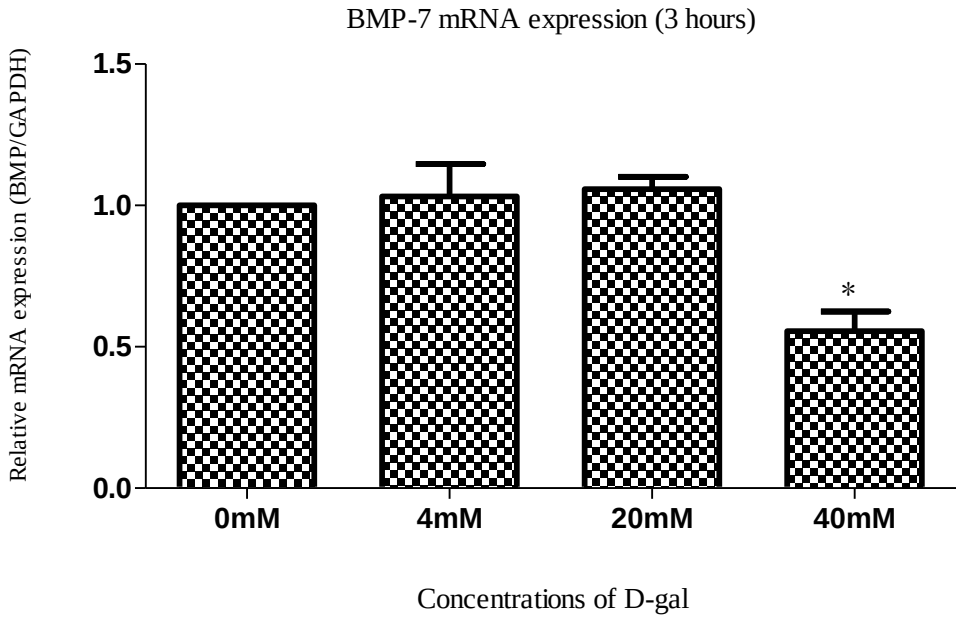

Figure 14. RT-PCR analysis of BMP-7 mRNA expression in rat hepatoma 1548 cells after 3 hours of D-gal treatment. Cells were incubated with 0, 4, 20, 40 mM of D-gal. Expression of BMP-7 mRNA was slightly elevated as the increased D-gal concentrations and dropped down at 40 mM of D-gal. GAPDH served as loading control. Values are mean ± SD, n=4, $^{*}P<0.05$.

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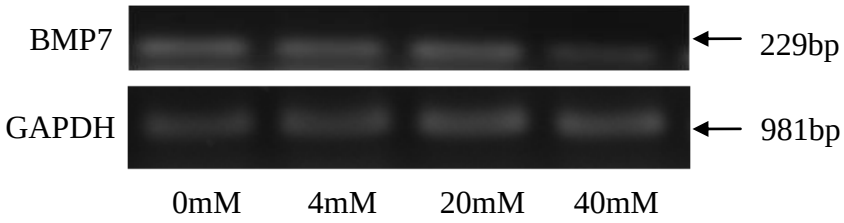

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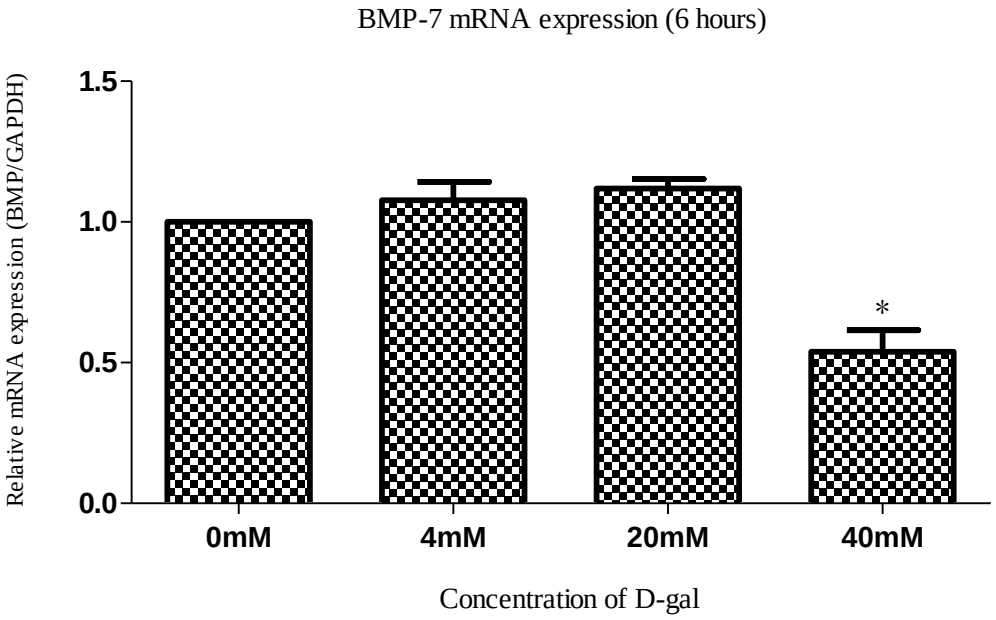

Figure 15. RT-PCR analysis of BMP-7 mRNA expression in rat hepatoma 1548 cells after 6 hours of D-gal treatment. Cells were incubated with 0, 4, 20, 40 mM of D-gal. Expression of BMP-7 mRNA was slightly elevated as the increased D-gal concentrations and dropped down at 40 mM of D-gal. GAPDH served as loading control. Values are mean ± SD, n=4, $^{*}P<0.05$.

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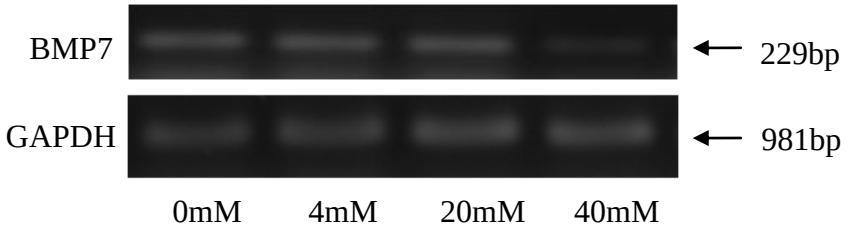

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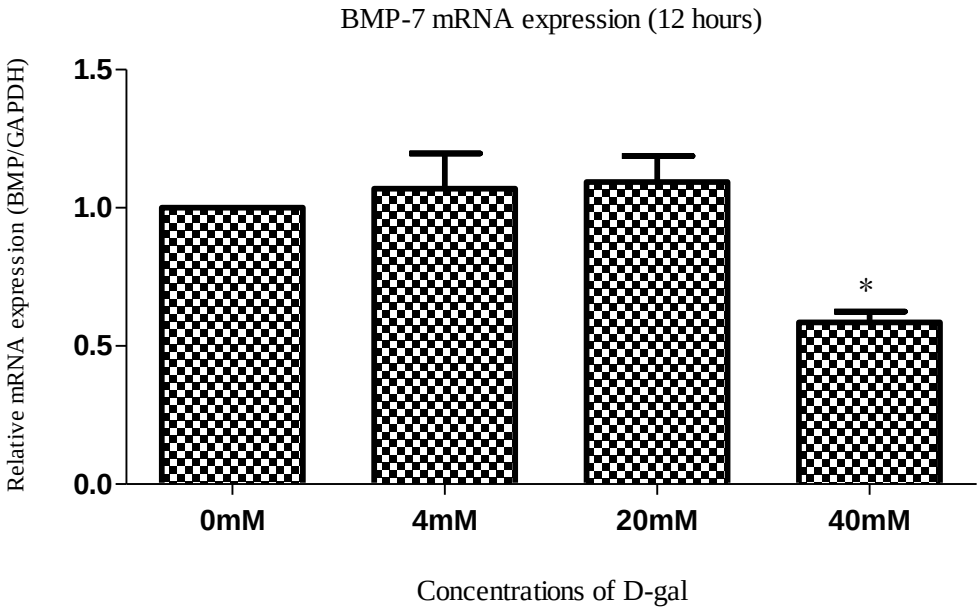

Figure 16. RT-PCR analysis of BMP-7 mRNA expression in rat hepatoma 1548 cells after 12 hours of D-gal treatment. Cells were incubated with 0, 4, 20, 40 mM of D-gal. Expression of BMP-7 mRNA was slightly elevated as the increased D-gal concentrations and dropped down at 40 mM of D-gal. GAPDH served as loading control. Values are mean ± SD, n=4, $^{*}P<0.05$.

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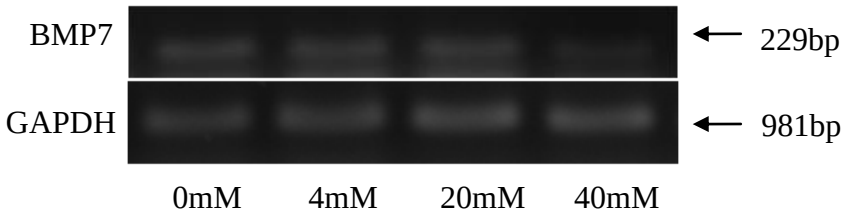

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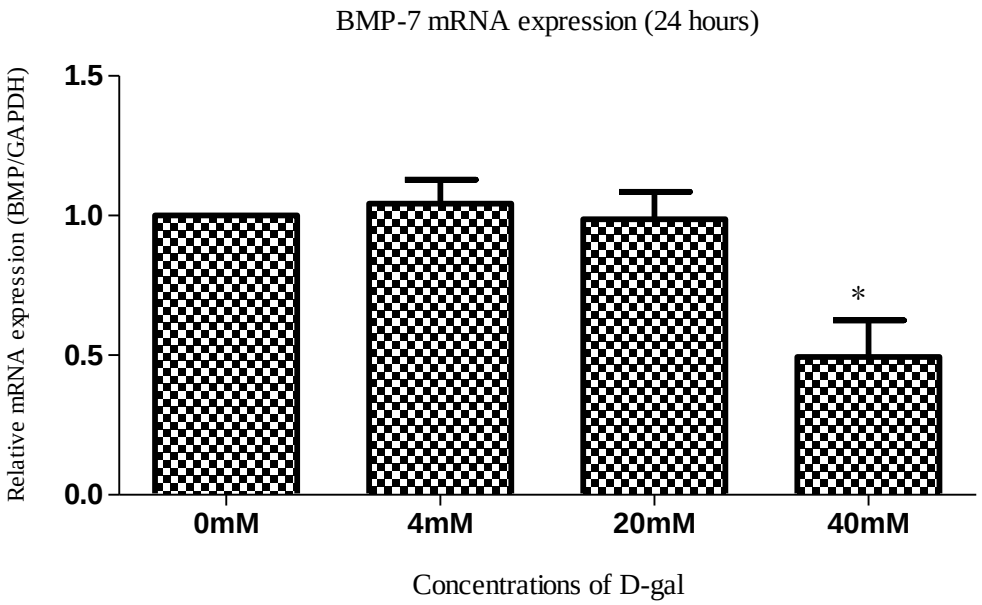

Figure 17. RT-PCR analysis of BMP-7 mRNA expression in rat hepatoma 1548 cells after 24 hours of D-gal treatment. Cells were incubated with 0, 4, 20, 40 mM of D-gal. Expression of BMP-7 mRNA was slightly elevated as the increased D-gal concentrations and dropped down at 40 mM of D-gal. GAPDH served as loading control. Values are mean ± SD, n=4, $^{*}P<0.05$.

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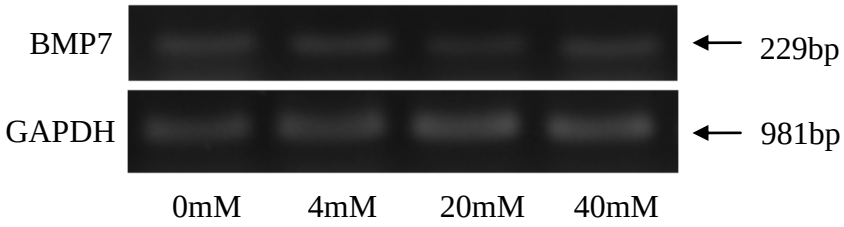

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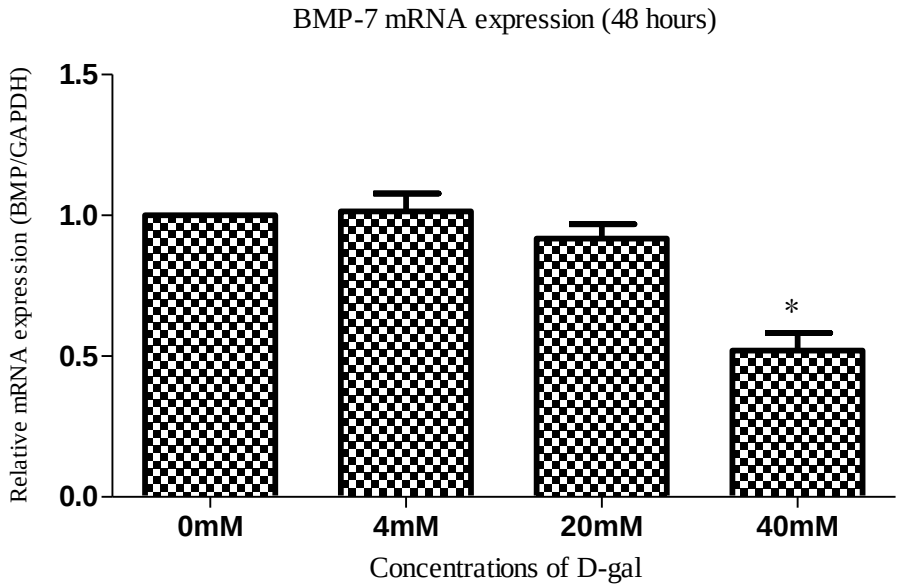

Figure 18. RT-PCR analysis of BMP-7 mRNA expression in rat hepatoma 1548 cells after 48 hours of D-gal treatment. Cells were incubated with 0, 4, 20, 40 mM of D-gal. Expression of BMP-7 mRNA was slightly elevated as the increased D-gal concentrations and dropped down at 40 mM of D-gal. GAPDH served as loading control. Values are mean ± SD, n=4, $^{*}P<0.05$.

4. The Expression of BMP-4 mRNA in D-gal Induced Hepatic Injury

RT-PCR was employed to detect the expression of BMP-4 in D-gal treated rat hepatoma 1548 cells. Cells were incubated with different concentrations of D-gal (0 mM, 4 mM, 20 mM and 40 mM), total RNA was extracted at different time points (3 hrs, 6 hrs, 12 hrs, 24 hrs and 48 hrs) and RT-PCR was carried out. Results are shown in Figures 19, 20, 21, 22 and 23. GAPDH served as loading control. As shown in the Figures, the abundance of BMP-4 increased after treatment of D-gal for 6, 12, 24 and 48 hours. BMP-4 mRNA expression increased as the concentration of D-gal increased. BMP-4 mRNA expression reached the maximum at 20mM of D-gal then decreased significantly at 40mM of D-gal. In Figure 19, there was no significant increase in BMP-4 mRNA expression after 3 hours of incubation with 0 mM and 4 mM of D-gal but a decrease of BMP-4 with 40 mM of D-gal ($^{*}P<0.05$). Figure 20, 21, 22 and 23 show expression of BMP-4 mRNA was elevated with an increase D-gal concentrations except at 40 mM. The mRNA level reached the peak at 20mM of D-gal then drop down at 40 mM of D-gal following 6 hours, 12 hours, 24 hours and 48 hours treatment, respectively. The greatest increase of BMP-4 was observed at 20 mM of D-gal after 6 hours treatment ($^{*}P<0.05$; Figure 20).

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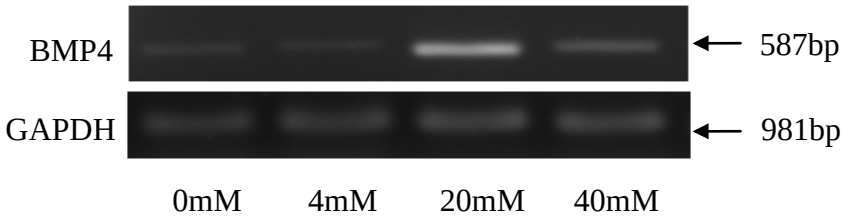

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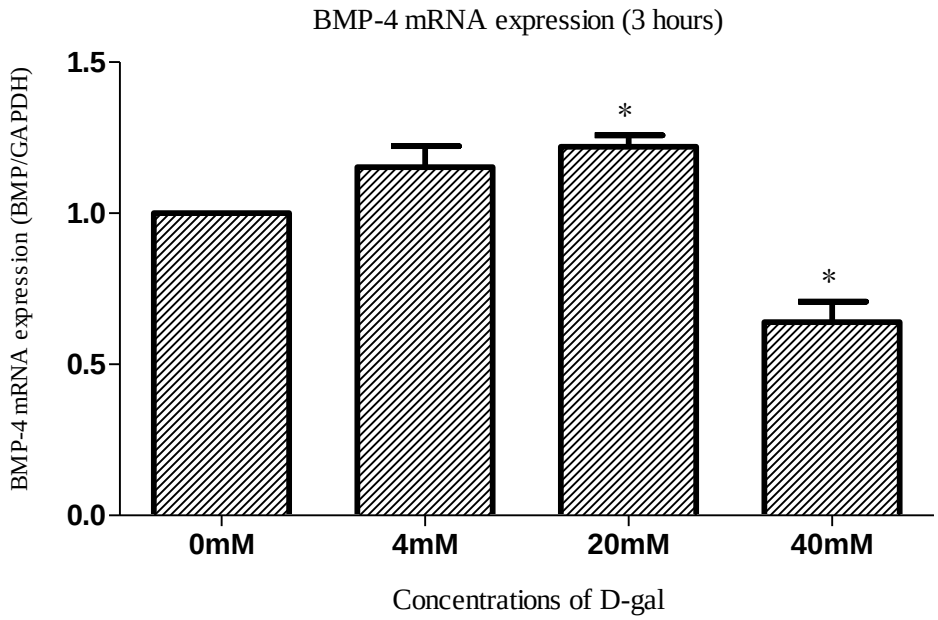

Figure 19. RT-PCR analysis of BMP-4 mRNA expression in rat hepatoma 1548 cells after 3 hours of D-gal treatment. Cells were incubated with 0, 4, 20, 40 mM of D-gal for 3 hours. No significant change of BMP-4 mRNA expression was observed at 4mM and 20 mM of D-gal compared with control group (0 mM) but the mRNA amount was reduced at 40 mM of D-gal. GAPDH served as loading control. Values are mean ± SD; n=4; $^{*}P<0.05$.

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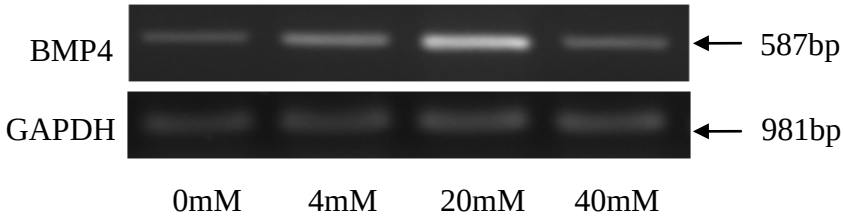

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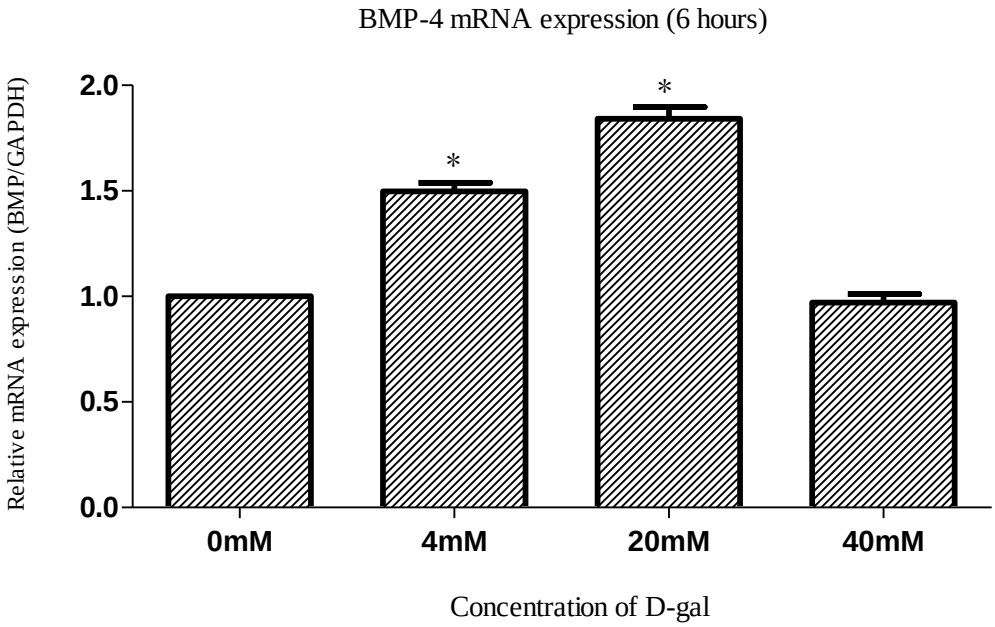

Figure 20. RT-PCR analysis of BMP-4 mRNA expression in rat hepatoma 1548 cells after 6 hours of D-gal treatment. Cells were incubated with 0, 4, 20, 40 mM of D-gal for 6 hours. Expression of BMP-4 mRNA was elevated as the increased D-gal concentrations. The mRNA level reached the peak at 20mM of D-gal then drop down at 40 mM of D-gal. GAPDH served as loading control. Values are mean ± SD, n=4, $^{*}P<0.05$.

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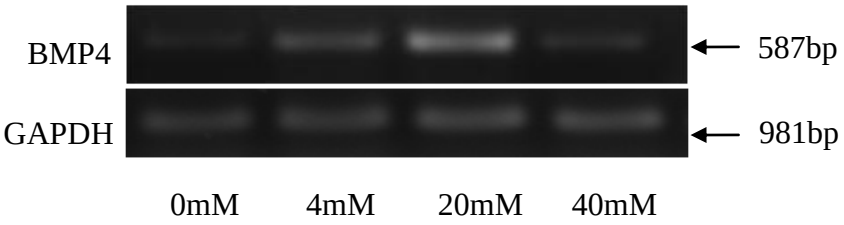

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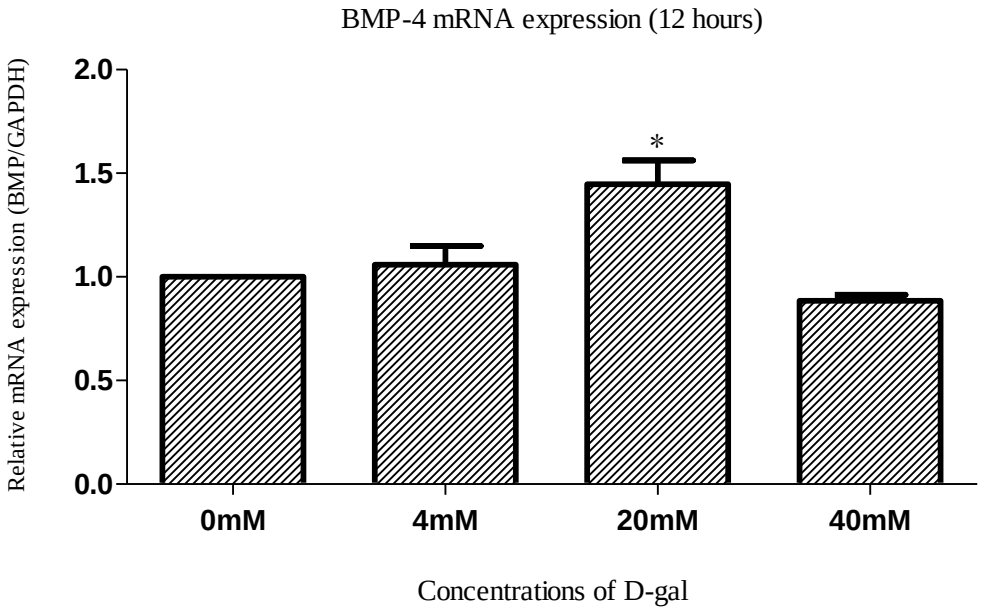

Figure 21. RT-PCR analysis of BMP-4 mRNA expression in rat hepatoma 1548 cells after 12 hours of D-gal treatment. Cells were incubated with 0, 4, 20, 40 mM of D-gal for 12 hours. Expression of BMP-4 mRNA was elevated as the increased D-gal concentrations. The mRNA level reached the peak at 20mM of D-gal then drop down at 40 mM of D-gal. GAPDH served as loading control. Values are mean ± SD, n=4, $^{*}P<0.05$.

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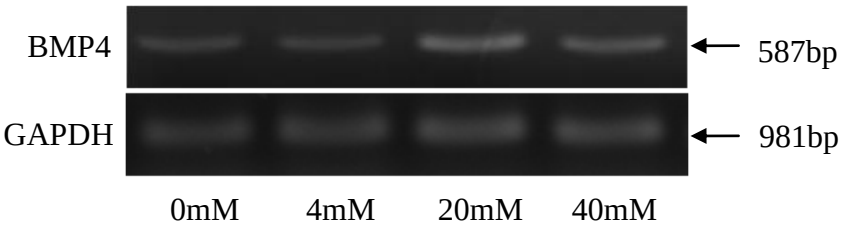

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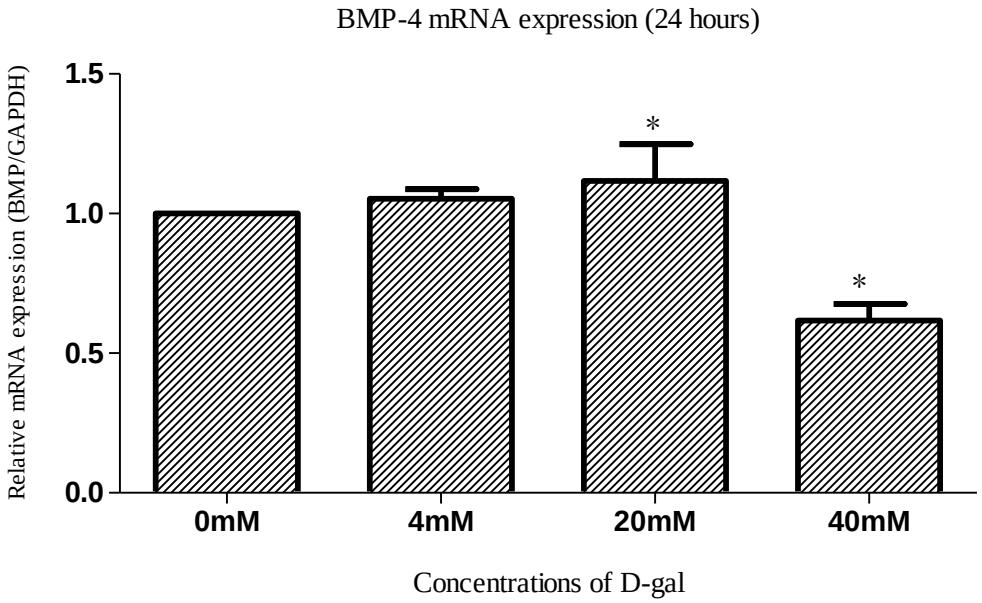

Figure 22. RT-PCR analysis of BMP-4 mRNA expression in rat hepatoma 1548 cells after 24 hours of D-gal treatment. Cells were incubated with 0, 4, 20, 40 mM of D-gal for 24 hours. Expression of BMP-4 mRNA was elevated as the increased D-gal concentrations. The mRNA level reached the peak at 20mM of D-gal then drop down at 40 mM of D-gal. GAPDH served as loading control. Values are mean ± SD, n=4, $^{*}P<0.05$.

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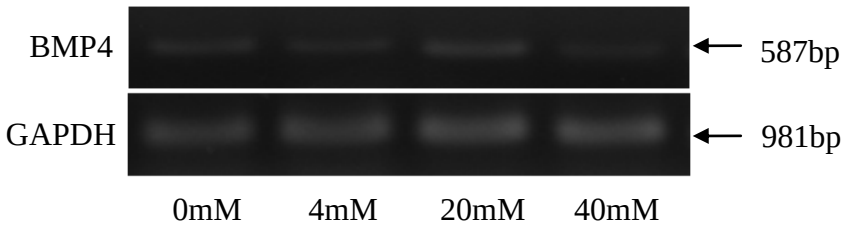

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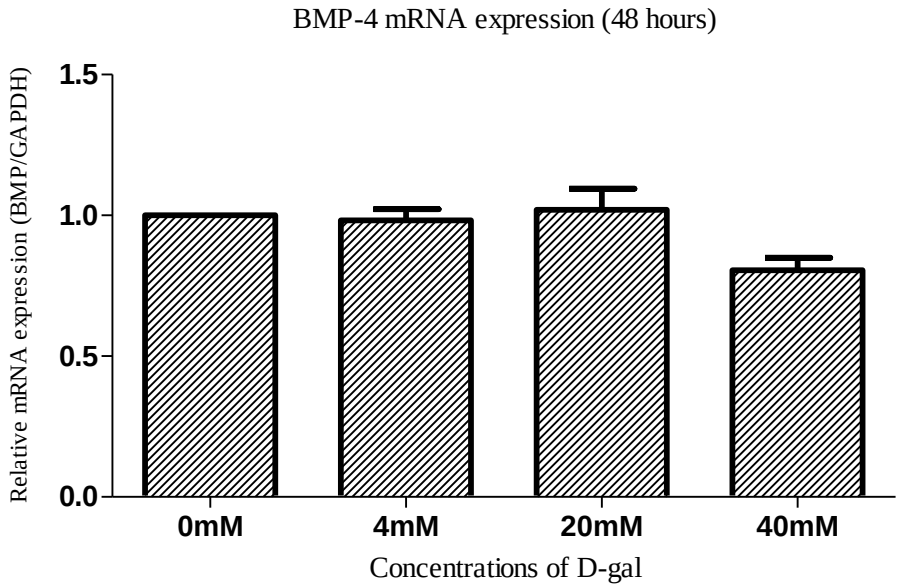

Figure 23. RT-PCR analysis of BMP-4 mRNA expression in rat hepatoma 1548 cells after 48 hours of D-gal treatment. Cells were incubated with 0, 4, 20, 40 mM of D-gal for 48 hours. Expression of BMP-4* mRNA was elevated as the increased D-gal concentrations. The mRNA level reached the peak at 20mM of D-gal then drop down at 40 mM of D-gal. GAPDH served as loading control. Values are mean ± SD, n=4, $^{*}P<0.05$.

5. The Expression of BMP-4 protein in D-gal Induced Hepatic Injury

Western blot was employed to detect the expression of BMP-4 in D-gal treated rat hepatoma 1548 cells. Cells were incubated with different concentrations of D-gal (0 mM, 4 mM, 20 mM) and protein was extracted at 24 hours and western blotting was carried out. The results are shown in Figure 24. Cells were also treated with 4 mM of D-gal and protein was extracted at different time point after incubation (0, 6, 12, 24 and 48 hours). β-actin served as loading control. Results were shown in Figure 25. As shown in Figure 24, the expression of BMP4 reached the peak at 4 mM D-gal after treatment of D-gal for 24 hours. In Figure 25, the amount of BMP-4 protein increased as the incubation period extended and reached the peak at 12 hours. Values are MEAN±SD, n=4, $^{**}P<0.05$.

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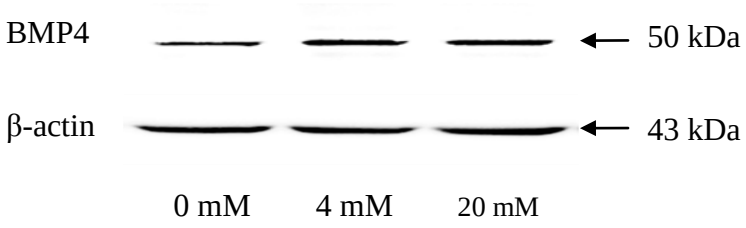

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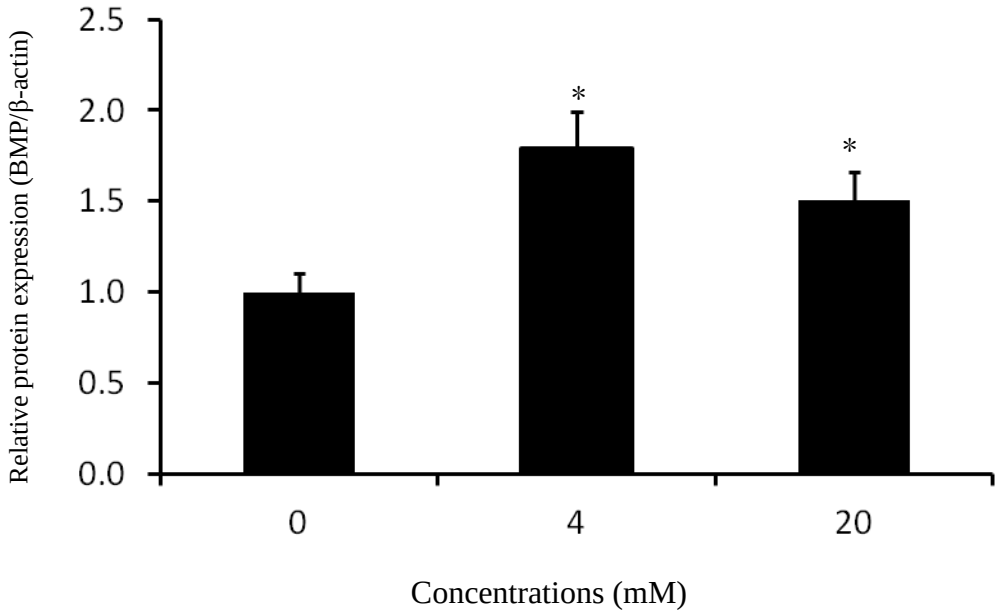
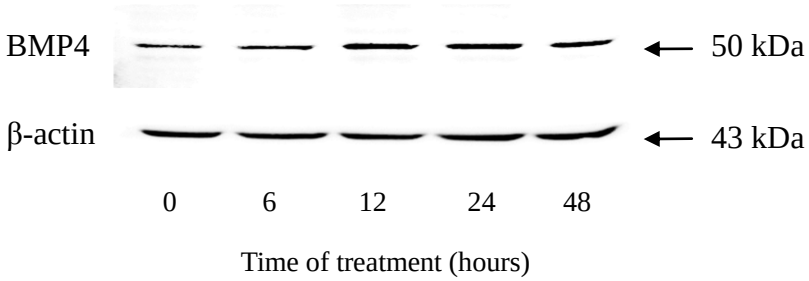


Figure 24. Western blot analysis of BMP-4 expression in rat hepatoma 1548 cells after 24 hours treatment of D-gal. Panel A displays the typical picture of western blot of BMP-4 and β-actin. Panel B represents histogram of the bands of BMP-4 versus β-actin. The zero concentration was converted as 1 and other concentrations were relative to zero concentration. β-actin served as loading control. Values are mean ± SD, n=4, $^{*}P<0.05$.

A



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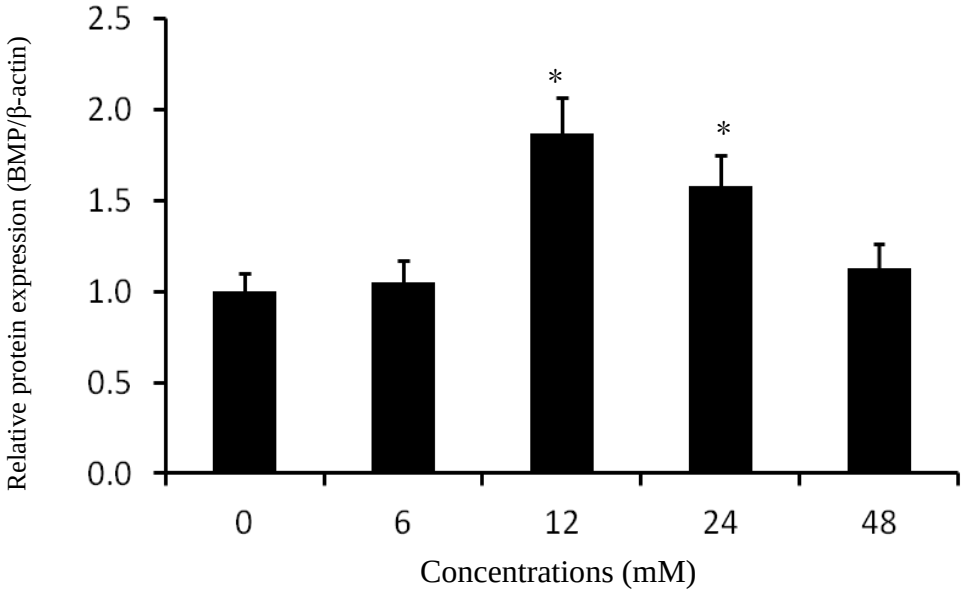

Figure 25. Western blot analysis of BMP-4 expression in rat hepatoma 1548 cells with 4 mM D-gal for 0, 6, 12, 24, and 48 hours. Panel A displays the typical picture of western blot of BMP-4 and β-actin. Panel B represents histogram of the bands of BMP4 versus β-actin. The zero time point was converted as 1 and other time points were relative to zero time point. β-actin served as loading control. Values are mean ± SD, n=4, $^{*}P<0.05$.

Ⅴ. Discussion

In embryonic development, the differentiation of hepatic progenitor cells towards mature hepatocytes depends on the cooperation of a series of complex mechanisms. It has been discovered that BMP2/4/7 signaling are essential to hepatic morphogenesis (Duncan and Watt 2001; Rossi, Dunn et al. 2001; Zaret 2001; Vukicevic, Latin et al. 1994; Hogan 1996; Zaret and Grompe 2008; Huang, Ruan et al. 2008). Studies in our laboratory have revealed that BMP4 can mediate hepatic progenitor cells to differentiate towards hepatocytes (Fan, Shen et al. 2009).

Hepatic injury can be caused by many factors. The changes of expressions of some growth factors and cytokines during the process of liver injury and regeneration have been detected. (Hikaru, Changqing et al. 2007; Nakatsuka, Taniguchi et al. 2007) Studies in our laboratory have revealed that increased BMP4 expression in acute liver injury may mediate positive regulation (Fan, Shen et al. 2006; Fan, Shen et al. 2009).

Based on the discovered role of BMPs on hepatic morphogenesis and hepatic injury, BMPs may also play an important role in hepatic repair and regeneration in the damaged liver. However, it is unclear now whether different factor-induced liver injury (liver resection, BDL, CCl_4, D-gal, etc) can stimulate the expression of different BMPs subtypes and whether multi-expression of different BMP subtypes exists during hepatic repair and regeneration after hepatic injury. This thesis has examined the expression of BMP-2/4 in D-gal induced acute hepatic injury in rat hepatoma 1548 cells.

1. The Evaluation of Cultured Cell Line Model of D-gal Induced Hepatic Injury

The establishment of an appropriate laboratory acute hepatic injury model can create a controllable environment for scientific research that can not be obtained from clinical patients. Animal experiments and cell cultures in vitro are most widely used as laboratory models. Compared to the disadvantage of animal models (Newsome, Plevris et al. 2000), cell cultures lack individual differences and are easier to use.

Among the various drugs and hepatotoxins (CCl_4, thiacetamide, nitrosamine, D-gal, acetaminophen, etc), D-gal and acetaminophen are most widely used to induce hepatic injury. Acetaminophen has been proved difficult to reproduce and to cause RA (refractory anemia) in animal models which can affect data interpretation. Employment of D-gal can prevent these disadvantages and the result is more predictable (Newsome, Plevris et al. 2000). D-gal has been employed in both animal and cell culture models. It has been used to induce hepatic injury in the primary cultures of rat (Abou, Siendones et al. 2002; Siendones, Fouad et al. 2003; Kucera, Lotková et al. 2006; Canová, Martínek et al. 2008) human hepatocytes (Rodríguez, López et al.2005; Ranchal, González et al. 2006; González, Ferrín et al. 2009).

In the present study, D-gal was applied to rat hepatoma 1548 cells at different concentratons (0mM, 4mM, 20mM and 40mM) and its cytotoxic effect was tested by the LDH assay. Results showed that the cytotoxicity of cells was proportional to the dose of D-gal. This indicated that the D-gal induced cytotoxicity was dose-dependent. Our results are consistent with the results published in reports using primary cultures of rat

hepatocytes (Kucera, Lotková et al. 2006) and primary cultures of human hepatocytes (González, Ferrín et al. 2009). This indicated that this cell culture model is reliable and could be used for further research on BMPs in our project as well as on other growth factors in hepatic injury in the future. To date, no published articles regarding D-gal induced hepatic injury in rat hepatoma 1548 cell line model' was available on PubMed.

2. The Potential Role of BMP-2 in D-gal Induced Hepatic Injury

BMP-2 influences not only skeletal development, but also systemic organic development (Hogan 1996). It is considered that BMP-2 may involve in the direction of progenitor (stem) cells. In early hepatic embryonic development, BMP-2 signalling is necessary for hepatic gene expression (Duncan and Watt 2001; Rossi, Dunn et al. 2001; Zaret 2001; Huang, Ruanet al. 2008). However, little information is known about the influences of BMPs in mature animal livers, or the relationship between BMP2 and hepatic repair and regeneration in damaged livers.

In 2007, Ryusuke Nakatsuka et al detected the expression of BMP-2 in CCl_4 induced acute hepatic injury in rats (Nakatsuka, Taniguchi et al. 2007). After the rats were injected with CCl_4, the expression of albumin significantly decreased 24 hours later but recovered at 48 hours. A transient but obvious expression of BMP-2 was detected 6-24 hours later and still existed after treatment with GdCl3 (gadolinium chloride, can destroy the functions of Kuffer cells, often used to study diseases related to Kuffer cells). BMP-2-producing cells were observed among intralobular spaces of injured hepatocytes using immunostainng with the anti-BMP-2 antibody.

It is still unclear whether BMP-2 also expresses with the treatment of other toxic factors. In the present study, we examined the expression of BMP-2 in D-gal treated rat hepatoma cells. Results showed that BMP-2 expressed with the treatment of D-gal and BMP-2 expression was in proportion to the concentration of D-gal within a certain amount. When the concentration of D-gal continued to increase, BMP-2 expression decreased significantly. This is probably because most cells were killed or badly injured due to large dose of D-gal. The strongest BMP-2 expression existed after 6 hours of D-gal incubation then dropped down gradually as the incubation time extended. Our result was consistent with the result published by Nakatsuka et al (Nakatsuka, Taniguchi et al. 2007). The difference between the previous study and our experiment was the toxic factors and laboratorial models. The BMP-2 expression in our experiment was induced by D-gal in vitro while in Nakatsuka study was induced by CCl_4 in vivo.

In general, our result indicated that changes of BMP-2 expression can be activated by hepatic injury induced by D-gal. The BMP-2 expression can be induced by different factors that can cause liver injury. Thus, BMP-2 may play an important role in hepatic repair and regeneration after hepatic injury.

3. The Potential Role of BMP-7 in D-gal Induced Hepatic Injury

BMP7 can mediate the sprouting of the liver bud from the central foregut endoderm to develop into hepatocytes (Vukicevic, Latin et al 1994; Hogan 1996). It is considered that BMP-7 might function as a novel hormone to facilitate liver regeneration through the circulation in vivo due to the absence of hepatic BMP7 expression in the liver (Hikaru,

Changqing et al. 2007).

In fact, minimal level of BMP-7 mRNA expression was detected in the livers of mice (Hikaru, Changqing et al. 2007; Nakatsuka, Taniguchi et al. 2007). However, some researchers considered that BMP-2 was mainly responsible for hepatic repair and regeneration in liver injury, therefore, the effect of BMP-7 can be ignored (Nakatsuka, Taniguchi et al. 2007). In the present study, the expression of BMP-7 mRNA was also detected in D-gal induced rat hepatoma 1548 cells, suggesting BMP-7 also may mediate liver regeneration after hepatic injury.

4. The Potential Role of BMP-4 in D-gal Induced Hepatic Injury

In early hepatic embryonic development, BMP-4 seemed to play an important role (Zaret and Grompe 2008). However, little was known about the physiological action of BMP-4 in normal liver,or its effect in hepatic injury.

In recent years, our laboratory has conducted a series of studies on BMP-4. In our work, BMP-4 expression in HSCs of BDL rats was observed (Fan, Shen et al. 2006). In the present study, the expression of BMP-4 was also observed in D-gal treated rat hepatoma 1548 cells. BMP-4 expression was in proportion to the concentration of D-gal within a certain amount. When the concentration of D-gal continued to increase, BMP-4 expression decreased significantly. This is probably because most cells were killed or badly injured due to the large doses of D-gal. The strongest BMP-4 expression existed after 6 hours of D-gal incubation then dropped down gradually as the incubation time

extended. This result showed that BMP-4 expression could be activated by hepatic injury induced by D-gal.

In general, our result indicated that changes in BMP-4 expression can be activated by hepatic injury induced by D-gal. The BMP-4 expression can be induced by different factors that can cause liver injury. Thus, BMP-4 may play an important role in hepatic repair and regeneration after hepatic injury.

In our present and previous work, both BMP-2 and BMP-4 expression was observed during the process of hepatic injury. In the BMP family, BMP-2 and BMP-4 share the most similarity in both structure and function. In the embryonic stage, BMP-2 and BMP-4 regulate liver formation (Zaret and Grompe 2008; Huang, Ruan et al. 2008). All these findings indicate that BMP-4 and BMP-2 signalling, which are essential in early embryonic hepatic morphogenesis, are activated or initiated by the injury of hepatocytes. BMP-2 and BMP-4 may be important regulators for the repair and regeneration of hepatic injury.

Furthermore, our results can further reveal the effects of BMPs in hepatic injury. In 2006 BMP4 expression was observed in BDL rats in our laboratory (Fan, Shen et al. 2006). In 2007, BMP2 and BMP7 expression was observed in CCl4 induced acute liver injury in rats by Nakatsuka et al (Nakatsuka, Taniguchi et al. 2007). In the present study, BMP2, BMP4 and BMP7 expression was observed in D-gal induced hepatic injury in rat hepatoma cells. All these findings indicated that (1) hepatic injury can induce BMP2, BMP4 and BMP7 expression both in vivo or in vitro experimental conditions; (2) different hepatic injury factors can induce expressions of different BMP subtypes. We can

predict that there may be expressions of different BMP subtypes during hepatic repair and regeneration after hepatic injury. Thus, there may be a variety of BMP subtypes that participate in the repair and regeneration of the liver following hepatic injury. BMPs may not be only associated with hepatic repair and regeneration after liver injury, but also involved in chronic liver diseases such as hepatic fibrosis or cirrhosis.

5. Future Direction

In the present study, we found changes in BMP2, BMP4 and BMP7 expression in D-gal induced hepatic injury. Different liver damage factors can induce the expressions of several different BMP subtypes. So far, all data about BMPs were from animals. Future studies, human hepatic cell lines could be employed to further examine the expression of BMP subtypes under different liver damage conditions. Exogenous BMP2, BMP4 and BMP7 could be employed in different factor-induced liver damage. Morphology and viability of the cells ought to be tested and compared with the cells without exogenous BMP2, BMP4 and BMP7. Relationships between BMPs and chronic liver diseases such as fibrosis or cirrhosis may then be truly revealed.

Ⅵ. Conclusion

BMP-2, BMP-4 and BMP-7 may be important regulators for the repair and regeneration after hepatic injury. Hepatic injury can induce BMP-2, BMP-4 and BMP-7 expression no matter in vivo or in vitro experimental conditions. Different hepatic injury factors can induce expressions of different BMP subtypes. The innate BMP-2, BMP-4 and BMP-7 signalling, which are essential in early embryos hepatic morphogenesis, are activated or initiated by the injury of hepatocytes. It is indicative that repair and regeneration of the liver after hepatic injury and hepatic morphogenesis in early embryos seem to proceed through a same process. There may be a variety of BMPs to participate in the repair and regeneration after hepatic injury. BMPs may be not only tightly associated with hepatic injury after repair and regeneration, but also involved in chronic liver diseases as liver fibrosis or cirrhosis of liver.

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