Bone Morphogenetic Protein-7 Attenuates Inflammation And Apoptosis And Improves Cardiac Function In Diabetes

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BONE MORPHOGENETIC PROTEIN-7 ATTENUATES INFLAMMATION AND APOPTOSIS AND IMPROVES CARDIAC FUNCTION IN DIABETES

by

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B.S. University of Central Florida, 2010

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in the Burnett School of Biomedical Sciences in the College of Medicine at the University of Central Florida Orlando, Florida

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Major Professor: Dinender K. Singla
ABSTRACT

Bone Morphogenetic Protein-7 (BMP-7) belongs to the transforming growth factor-β (TGFβ) family of cytokines has is known to have potent anti-inflammatory properties. It has been used in patients to treat osteoporosis clinically and has been reported to treat diabetic nephropathy in murine models. Moreover, studies show that inflammation is up-regulated in patients with pre-diabetes (PD). We, therefore, hypothesize that the administration of BMP-7 will attenuate inflammation in the heart of Streptozotocin (STZ)-induced PD mice. In this study, we divided C57Bl/6 mice into three groups: CONTROL, PD, and PD+BMP-7. CONTROL mice received intraperitoneal (i.p.) injections of Sodium Citrate Buffer while PD and PD+BMP-7 groups received i.p. injections of Streptozotocin (STZ) for two days. In addition, PD+BMP-7 mice received intravenous injections (i.v.) of BMP-7 (200µg/kg) on the last day of STZ injection and for the following two days. Animals were sacrificed 21 days post last injection and examined for levels of oxidative stress, inflammatory immune response, apoptosis, fibrosis and cardiac function. Our results indicate significant glucose intolerance in PD mice (p<0.05), which was attenuated in the PD+BMP-7 group (p<0.05). We also observed increased oxidative stress (p<0.001) and secretion of pro-inflammatory cytokines (p<0.05), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α), in PD mice as compared with the controls. PD+BMP-7 mice revealed significant up-regulation of M2 macrophages (p<0.05) and secretion of anti-inflammatory cytokines (p<0.05), interleukin-10 (IL-10) and interleukin-1RA (IL-1RA), as compared to PD mice. This was observed with a concomitant down-regulation of pro-inflammatory cytokines, IL-6 and TNF-α, as compared to the PD group. Moreover, we observed significantly increased
cardiac apoptosis and fibrosis in PD mice (p<0.001) as compared to the control group. These observations, however, were down-regulated upon treatment with BMP-7. Lastly, analysis of echocardiograms revealed significantly depressed cardiac function in PD mice as compared with controls, while the PD+BMP-7 group presented improved cardiac function compared to PD mice. In conclusion, our data suggest that treatment with BMP-7 is effective in alleviating cardiac inflammation, inhibiting apoptosis, blunting cardiac remodeling and improving cardiac function in the hearts of STZ-induced PD mice. This reveals the potential of BMP-7 as a therapy in PD patients who present an increased inflammatory immune response.
“Do not store up for yourselves treasures on earth, where moth and rust destroy,
and where thieves break in and steal.
But store up for yourselves treasures in heaven, where neither moth nor rust destroys,
and where thieves do not break in or steal;
for where your treasure is, there your heart will be also.”
— Matthew 6:19-21

I dedicate this and all my endeavors to my Lord and Savior, Jesus Christ.

To God be the glory.

“All that I am or ever hope to be,
I owe to my angel mother.”

-Abraham Lincoln

I am eternally grateful for the love and support of my wonderful mother, whose
sacrifices and determination have blessed me with a charmed life.

Maraming salamat po, mama. Mahal kita.

In loving memory of Lucio, Toribia and Tot-Tot Urbina.
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“Good timber does not grow with ease; the stronger the wind, the stronger the trees.”

-J. Willard Marriott

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“I’m glad we had the times together just to laugh and sing a song, seems like we just got started and then before you know it, the times we had together were gone.”

-Dr. Seuss

I am sincerely grateful for Reetu Singla for her kind words, encouragement, and expertise during my time in the lab. I would also like to thank my labmates, Hilda Merino-Chavez, Latifa S. Abdelli and Crystal M. Rocher, for believing in my dreams even when I could not. It has been my pleasure and honor to be in the company of such wonderful and talented people during this chapter of my life.
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LIST OF ABBREVIATIONS

Akt Protein Kinase B
BMP-7 Bone Morphogenetic Protein-7
C57BL/6 C57 Black/6 mice
DAPI 4′,6-diamidino-2-phenylindole
DHE Dihydroethidium
DM Diabetes Mellitus
EF Ejection fraction
ELISA Enzyme-linked immunosorbent assay
FS Fractional shortening
IFG Impaired fasting glucose
IGT Impaired glucose tolerance
IL-6 Interleukin-6
IL-10 Interleukin-10
IP Intraperitoneal
IV Intravenous
LV Left ventricle
LVIDd Left ventricular internal dimension-diastole
LVIDs Left ventricular internal dimension-systole
NIH National Institute of Health
MCP-1 Monocyte Chemoattractant Protein-1
MnSOD Manganese superoxide dismutase
p-Akt Phosphorylated Akt
p-PTEN  Phosphorylated phosphatase and tensin homolog
PBS   Phosphate buffer saline
PD    Pre-Diabetes
PTEN  Phosphatase and tensin homolog
PVDF  Polyvinylidene fluoride
RIPA  Radioimmunoprecipitation assay buffer
RNA   Ribonucleic acid
ROS   Reactive oxygen species
SEM   Standard error of the mean
STZ   Streptozotocin
T2DM  Type 2 Diabetes Mellitus
TBST  Tris buffer saline-tween-20
TGFβ-2 Transforming growth factor beta-2
TNF-α Tumor necrosis factor-alpha
TUNEL Terminal deoxynucleotidyl transferase dUTP nick end labeling
CHAPTER 1: INTRODUCTION

**Pre-Diabetes**

Diabetes Mellitus (DM) is a condition that affects about 25.8 million people in the United States (Blonde, 2012), with the majority of these patients suffering from Type 2 Diabetes Mellitus (T2DM). T2DM is characterized by insulin resistance despite relatively normal production and secretion from pancreatic β-cells. This results in hyperglycemia, which leads to increased risk for conditions such as heart failure (D’Souza et al, 2011) and nephropathy (Stolar, 2010). It has been shown, however, that even subclinical states of hyperglycemia can lead to adverse effects. Impaired fasting glucose (IFG) and impaired glucose tolerance (IGT) are markers for Pre-Diabetes (PD), which may affect upwards of 35 million people in the U.S (Nielson and Lange, 2005). PD is classified as fasting plasma glucose levels between 100-125 mg/dL and/or glucose levels between 140-199 mg/dL after a 2-hour glucose tolerance test (Bergman, 2010). It is estimated that up to 25% of those with abnormal fasting glucose levels develop full-blown diabetes, most within 2.5 years of initial hyperglycemia. Early detection of abnormal glucose levels is, thus, of utmost importance as it can prevent further development of the disease and complications such as diabetic cardiomyopathy.

**Inflammation and Cardiomyopathy**

The leading cause of death in diabetics is diabetic cardiomyopathy; however, it has been suggested that microvascular complications begin at lower glucose concentrations (Stolar, 2010). In fact, impaired glucose tolerance is associated with up to 54% greater risk of death due to cardiovascular disease (Saydah et al., 2001). A
strong association between oxidative stress and diabetic cardiomyopathy has long been established, however, accumulating evidence suggests that inflammation is also involved in cardiomyopathy.

It has been suggested that diabetes may indeed be related to a dysfunction of the innate immune response (Pickup, 1998). In fact, pro-inflammatory cytokines, such as IL-6 and TNF-α, have been found to be elevated in sera of patients with T2DM and IGT (Pickup et al., 2000; Muller et al., 2002). Furthermore, studies have reported that administration of IL-6 can stimulate gluconeogenesis and lead to hyperglycemia (Tsigos et al., 1997; Stith et al., 1994), which supports that IL-6 may be involved in the evolution of PD to full-blown T2DM. In fact, a prospective study suggested that IL-6 levels can act as markers of increased risk for the development of T2DM (Pradhan et al, 2001). Investigating therapies that target inflammation may, thus, result in treatments that attenuate hyperglycemia and associated complications.

**Bone Morphogenetic Protein-7**

BMP-7 belongs to the TGFβ-2 family of cytokines and is found in tissues including the thymus, bone marrow, and heart (Bragdon et al., 2010). It has been shown to regulate embryonic development as well as the growth, differentiation and apoptosis of endothelial, mesenchymal and cardiac cells (Boon et al., 2011). BMP-7 has also been shown to inhibit gene expression of cytokines such as TNF-α, IL-6 and MCP-1 (Chan et al., 2008; Gould et al., 2002). Moreover, our recent study suggests that BMP-7 may be able to inhibit inflammation specifically by polarizing monocytes into M2 macrophages in vitro (Rocher et al., 2012). M2 macrophages are a class of
macrophages that counteract inflammation by secreting high levels of IL-10 (Mantovani et al., 2004). IL-10 is a well-known anti-inflammatory cytokine that inhibits the expression of IL-6 and TNF-α from immune cells (Rajasingh et al., 2006). Thus, BMP-7-mediated up-regulation of IL-10 may be a potential therapy for conditions associated with inflammation, such as hyperglycemia. Since BMP-7 circulates in the blood (Zeisberg, 2006), it is reasonable to propose that BMP-7 may alleviate inflammation in organs typically affected by hyperglycemia. Thus, we investigated the effect of BMP-7 on inflammation in the PD heart.

**Hypothesis**

In the present study, we hypothesize that:

I. Administration of BMP-7 will inhibit cardiomyocyte apoptosis and improve cardiac function in a mouse model of streptozotocin-induced pre-diabetes.

II. The decrease in cardiomyocyte apoptosis is due to a BMP-7 mediated increase in M2 macrophage population and anti-inflammatory cytokine secretion.

**Aims**

Aim 1. Determine the effects of exogenous BMP-7 on apoptosis and cardiac function in streptozotocin-Induced pre-diabetic cardiomyopathy.

Aim 2. Determine the efficacy of which BMP-7 can increase M2 macrophage population and thereby decrease inflammation in the Pre-Diabetic heart.
CHAPTER 2: MATERIALS AND METHODS

Streptozotocin-Induced Pre-Diabetes

C57Bl/6 mice 8-10 weeks old were used in this study and maintained in a temperature controlled facility with access to food and water except when fasting for experiments. All animal studies were approved by the University of Central Florida Institutional Animal Care Use Committee. The mice were divided into three groups; Control, Pre-diabetic (PD) and PD+BMP-7 with n=7-10 animals in each group.

Multiple low doses of STZ (50mg/kg) have been previously used to create a T2DM model in rodents (Poucher et al, 2012; Zeng et al, 2012) while single high doses of STZ have been used to create T1DM models (Yin et al, 2006). As uncontrolled Pre-Diabetes has been shown to develop into T2DM, we used a modified low dose of STZ to create a PD model.

Mice in the Control group received intraperitoneal (IP) injections of Sodium Citrate Buffer for two consecutive days while the remaining two groups received i.v. injections of 50 mg/kg Streptozotocin (STZ) prepared in the vehicle. The PD+BMP-7 group received one i.v. injection of BMP-7 (200 µg/kg) on the second day of STZ induction and for the next two consecutive days.

Glucose Levels and Glucose Tolerance Test

Blood glucose levels were measured 7 and 21 days after the last injection using an OneTouch Ultra Mini glucose meter. On the 20th day, all groups underwent a glucose tolerance test. The mice were then fasted for 6 hours and their initial glucose levels were measured via tail-vein puncture. Intraperitoneal injections of 1g/kg glucose
were administered and blood glucose levels were checked every 30 minutes for a total of 120 minutes post-glucose injection.

**Tissue Processing**

Mouse hearts were harvested then washed with phosphate buffer saline (PBS) and cut transversely into two halves. The top half was kept in RNA Later solution for 24 hours in 4°C, decanted and placed in -80°C for future molecular studies. The apical half of the heart was kept in 4% paraformaldehyde at 4°C for 24 hours. The hearts were then rinsed with PBS three times for five minutes each, processed utilizing the Leica TP1020 tissue processing system and then embedded in paraffin wax with Tissue Tek TEC machines. Paraffin-embedded hearts were then sectioned into 5µm sections and placed onto Colorfrost Plus slides (Fischer Cat. #12-550-17).

**Immunohistochemistry**

Heart sections were deparaffinized and rehydrated as previously described. Heat-induced epitope retrieval was performed on the tissues for 20 minutes. Sections were then washed for five minutes three times with PBS and blocked with 10% goat serum (Vector Labs Cat. #S-1000) for one hour. Heart sections were incubated in primary antibody (CD206 [1:50, Abcam Cat. #ab64693], IL-10 [1:100, Abcam Cat. #ab33471], TNF-alpha [1:100, Abcam Cat. #ab6671], II-6 [1:100, Abcam Cat. #ab6672]) prepared in 10% goat serum overnight at 4°C. The sections were then washed for five minutes three times in PBS and incubated with secondary antibody (goat anti-rabbit 488 [1:50, Invitrogen Cat. #A11008], goat anti-rabbit 568 [1:50, Invitrogen Cat. #A11011 ] or goat anti-rat 594 [1:50, Invitrogen Cat. #A11007]) for one hour at room temperature.
Finally, the sections were washed for five minutes three times with PBS, the nuclei stained with DAPI (Vector Labs Cat. #H-1200) and images taken an Olympic IX-70 fluorescent and confocal microscope.

**Enzyme Linked Immunosorbent Assay (ELISA)**

On Day 21, mice were sacrifice and blood samples were collected in EDTA tubes (BD Microtainer, Cat. # REF 365973). Blood samples were centrifuged at 13,000 rpm for 10 minutes to isolate the sera, which were collected and stored at -20°C until needed. Commercial ELISA kits were used to determine levels of IL-10 (Raybiotech, Cat. #ELM-IL10-001), IL-1RA (Raybiotech, Cat. #ELM-IL1RA-001), TNF-α (Raybiotech, Cat. #ELM-TNFalpha-001), and IL-6 (Raybiotech, Cat. #ELM-IL6-001) and were followed according to manufacturers’ instructions.

**Terminal Deoxynucleotidyl Transferase dUTP-Mediated Nick-End Labeling Assay**

Heart sections were deparaffinized and rehydrated as previously described. The tissues were then incubated with proteinase-K (25 µg/ml) prepared in 100 mM Tris-HCL for 15 minutes at room temperature. Terminal deoxynucleotidyl transferase dUTP-mediated nick-end labeling (TUNEL) staining was then performed using a commercial cell death detection kit (Roche, Cat. #12156792910) according to manufacturer instructions. Sections were rinsed three times with PBS for five minutes each, the nuclei stained with DAPI and images taken with an Olympus IX-70 fluorescent and confocal microscope. Four images per section were analyzed by calculating the percent of apoptotic nuclei using NIH ImageJ software. In brief, cells with TUNEL positive (red)
nuclei that merged with DAPI (blue) were counted, divided by total nuclei and expressed as a percent.

**Western Blot**

Heart tissues were sonicated in RIPA buffer and centrifuged at 13,000g for 10 minutes. Supernatants were collected, prepared for protein quantification using Bio-Rad Protein Assay Dye (Bio-Rad Cat. #500-0006) and protein concentration was determined using a Biorad 680 microplate reader. Samples were run on either a 10% or 12% sodium-dodecyl sulfate polyacrylamide gel at 150V for one hour and then transferred onto a PVDF membrane (Bio-Rad Cat. #162-0177) at 15V for 35-60 minutes using a Trans-Blot Semi-dry transfer Cell. Blocking with 5% milk prepared in TBST was performed for one hour, membranes washed for five minutes three times with TBST and then incubated with primary antibody (p-AKT [1:1000, Cell Signaling Cat. #4058S], AKT [1:1000, Cell Signaling Cat. #4685S], PTEN [1:1000, Cell Signalling Cat. #9559S], p-PTEN [1:1000, Cell Signaling Cat. #9554S], MnSOD [1:5000, AbCam Cat. #ab13533] or Beta Actin [1:1000, Cell Signalling Cat. #4967L]) for one hour at room temperature or overnight at 4°C. The membranes were then washed for five minutes three times with TBST and incubated with secondary antibody (goat anti-rabbit [1:1000 or 1:5000, Cell Signaling Cat. #7074S]) for one hour at room temperature. Finally, the membranes were washed for five minutes three times with TBST, incubated with ECL for 2 minutes and then visualized via radiography.
**Masson’s Trichrome Staining**

Heart sections were deparaffinized, rehydrated and subsequently stained for interstitial and vascular fibrosis through Masson’s Trichrome staining as previously described (Singla et al., 2007). Fibrotic area (mm²) was assessed using the ImageJ software to measure collagen deposition (blue); interstitial fibrosis was calculated by measuring collagen within three areas of interstitial tissue per section while vascular fibrosis was measured as a ratio of vessel collagen deposition over total vessel area x 100% in six vessels per section.

**Dihydroethidium Staining**

Levels of radical oxygen species (ROS) were assessed by staining heart sections with dihydroethidium (DHE). In brief, the sections were deparaffinized and rehydrated as previously described (Singla et al., 2007) and then stained with 1 μM/ml DHE for 25 minutes at room temperature. Random images of each section were taken using an Olympic IX-70 fluorescent and confocal microscope. DHE intensity was then measured in each image using NIH ImageJ software and the average corrected integrated density was calculated for each animal.

**Echocardiography**

On Day 21, all mice underwent two-dimensional (2D) transthoracic echocardiography in order to assess their cardiac function. The animals were anesthetized with 2% isoflurane and laid on a heating pad. A 5500 Ultrasound System with a 15-6L Hockey stick transducer was used to take M-mode images of the left ventricle from which end diastolic volume (EDV), end systolic volume (ESV), ejection
fraction (EF%), left ventricular internal dimension-diastole (LVIDd), left ventricular internal dimension-systole (LVIDs), and fractional shortening (FS%) were measured. Following echocardiography, the mice were euthanized through cervical dislocation. The hearts were harvested, washed with PBS, separated with a transverse incision and either placed in formalin for sectioning or RNA Later for molecular analyses. Blood samples were collected in EDTA tubes (BD Microtainer Cat. # REF 365973) and kept in -20°C for future use.

**Statistical Analysis**

All samples were analyzed using one-way analysis of variance (ANOVA) followed by Tukey Test. Values are presented as a mean ± SEM with a p<0.05 considered to be statistically significant.
CHAPTER 3: RESULTS

Exogenous BMP-7 Improves Glucose Tolerance in Pre-Diabetic Mice

Glucose levels were measured via tail-vein puncture on days 7 and 21 after last injection. STZ treatment was effective in elevating the blood glucose levels of PD mice on both Day 7 and Day 21 as compared to the controls (Figure 1A-B). PD+BMP-7 mice, however, had reduced glucose levels as compared to the PD group. Impaired glucose tolerance is a marker for PD (Bergman, 2010), thus, we performed glucose tolerance tests on Day 20 to evaluate the extent to which the mice can metabolize glucose. Results show decreased glucose tolerance in the PD group at both 30 and 120 minutes (Figure 1C-D). PD+BMP-7 mice had reduced glucose levels at the 30 minute time point and statistically improved glucose tolerance at the 120 minute time point as compared to the PD group.
Figure 1. Effects of BMP-7 on glucose tolerance in PD mice. Glucose levels were measured via tail-vein puncture on days 7 and 21 after last injection (n=6-8 animals per group). (A,B) Pre-diabetic mice had significantly elevated glucose levels on both days 7 and 21 as compared to Control mice (*p<0.05) while treatment with BMP-7 significantly decreased glucose levels as compared to the PD group (#p<0.05). On day 20, mice underwent a glucose tolerance test (n=6-8 animals per group). Glucose levels at time points 30 and 120 minutes are shown. (C,D) The PD group resulted in impaired glucose tolerance as compared to the Control group (p<0.05). BMP-7 treatment resulted in increased glucose tolerance as compared to the PD group, which reached statistical significance 120 minutes post glucose injection (p<0.05).
BMP-7 Increases M2 Macrophages and Ameliorates Inflammation in the Heart

In order to evaluate the effects of BMP-7 on inflammation in PD mice, heart sections were incubated with antibodies against CD206, a marker for M2 macrophages. No significant difference was seen in M2 macrophage population between Control and PD mice; however, mice treated with BMP-7 showed a significant elevation in M2 macrophage population as compared to the PD group (Figure 2A-P).
Figure 2. Effects of BMP-7 on immune cell populations in the PD heart. Sections were stained for CD206, a marker for M2 macrophages depicted in red (A,F,K). Sarcomeric α-actin was stained and is shown in green (B,G,L), while nuclei were stained in blue with DAPI (C,H,M) (n=4 animals per group). A merge of the three pictures is shown (D,I,N) along with a magnified view (E,J,O). There was no difference in M2 macrophage populations between Control and PD mice (P), however, mice in the PD+BMP-7 group had significantly elevated levels of M2 macrophages as compared to the PD group (*p<0.05).
To determine the effects of increased M2 macrophages within the hearts of PD mice treated with BMP-7, we analyzed the levels of cytokine expression in heart tissues and in serum samples through immunohistochemistry and ELISA, respectively. Although not statistically significant, quantitative analyses of stained tissues showed decreased levels of IL-10 in PD mouse hearts as compared to the controls (Figure 3A-P). Furthermore, treatment with BMP-7 resulted in an observable increase in IL-10. Analyses of serum samples revealed a statistically significant decrease of IL-10 in PD mice, which was significantly elevated in mice treated with BMP-7 (Figure 3Q). In addition to IL-10, we also measured the levels of another anti-inflammatory cytokine, IL-1RA, in serum samples. Results indicated significantly decreased levels of IL-1RA in PD (*p<0.05) mice as compared to the controls (Figure 3R). Analyses also revealed that treatment with BMP-7 was able to significantly increase levels of the anti-inflammatory cytokine as compared to the PD group (#p<0.05).
Figure 3. BMP-7 regulates inflammation in the PD heart. Heart tissues were stained for IL-10 in red (A,F,K) (n=5 samples per group), sarcomeric α-actin in green (B,G,L) and nuclei blue (C,H,M). Merged (D,I,N) and magnified (E,J,O) pictures are also shown. PD mice had noticeably reduced levels of IL-10 as compared to the controls. Although not statistically significant, BMP-7 treatment observably increased IL-10 levels as compared to the PD group. (Q) Serum samples were analyzed for IL-10 expression using an ELISA kit (n=4-5 samples). PD mice had significantly (*p<0.05) decreased IL-10 in the serum as compared to the controls. Treatment with BMP-7 was effective in significantly (#p<0.05) restoring levels of IL-10. (R) Serum levels of IL-1RA were also measured using a commercial ELISA kit (n=5-7 samples). PD mice had significantly (*p<0.05) reduced levels of IL-1RA as compared to the controls, whereas PD+BMP-7 mice had significantly (*p<0.05) up-regulated levels of the cytokine as compared to the PD group.
In order to determine the effects of increased IL-10 in PD+BMP-7 mice, we measured levels of pro-inflammatory cytokines, TNF-α and IL-6. Analysis of heart sections showed significantly increased levels of TNF-α in PD mice as compared to the controls (Figure 4A-P). This increase was attenuated in the PD+BMP-7 group, which experienced significantly decreased levels of the pro-inflammatory cytokine as compared to the PD mice. Quantitative analyses revealed a similar trend, although not statistically significant, in our serum ELISA samples (Figure 4Q).
Figure 4. BMP-7 inhibits TNF-alpha in PD mice. Heart sections were stained for TNF-α in red (A,F,K), sarcomeric α-actin in green (B,G,L) and nuclei in blue (C,H,M) (n=4-5 animals per group). Merged (D,I,N) and magnified (E,J,O) pictures are also shown. PD mice had significantly increased levels of the pro-inflammatory cytokine (P) as compared to the Control group (*p<0.05). BMP-7 treatment resulted in significantly decreased levels of the cytokine as compared to the PD group (#p<0.05). (Q) Serum samples from mice analyzed to detect levels of TNF-α through an ELISA kit (n=5-7 samples per group). Although not statistically significant, there was an observable increase in the pro-inflammatory cytokine in PD mice. Levels of TNF-α in PD+BMP-7 mice were observably lower than those in the PD group and followed the trend seen in the tissue staining.
Levels of another pro-inflammatory cytokine, IL-6, were also measured in heart tissues and serum samples. Quantitative analyses of the stained tissues revealed increased levels of IL-6 in PD hearts, which was significantly alleviated with BMP-7 treatment (Figure 5 A-P). Our IL-6 ELISA results follow a similar trend. Serum samples from PD mice had increased levels of IL-6 as compared to the controls, while PD+BMP-7 had significantly decreased levels compared to PD mice (Figure 5Q).
Figure 5. BMP-7 inhibits IL-6 in PD mice. Mouse tissues were stained for IL-6 in red (A,F,K), sarcomeric α-actin in green (B,G,L) and nuclei in blue (C,H,M) (n=7-8 samples per group). Merged (D,I,N) and magnified (E,J,O) pictures are also shown. (P) PD mice had significant (*p<0.05) up-regulation of the pro-inflammatory cytokine as compared to the control mice. BMP-7 treatment effectively (#p<0.05) ameliorated inflammation in the heart as compared to the PD mice. (Q) Serum samples were analyzed for IL-6 expression using a commercial ELISA kit (n= 4-6 samples per group). PD mice had up-regulated (*p<0.05) levels of IL-6 as compared to the controls. Treatment with BMP-7 was effective (#p<0.05) in inhibiting IL-6 levels as compared to PD mice.
**BMP-7 Inhibits Apoptosis in Pre-Diabetic Mice by Regulation PTEN and AKT**

In order to assess the role of BMP-7 in cardiomyocyte apoptosis *in vivo*, we performed TUNEL staining on mouse heart sections (Figure 6A-J). Quantitative analyses reveal increased apoptotic nuclei in the hearts of PD mice as compared to the controls. Furthermore, our results show that treatment with BMP-7 significantly alleviated apoptosis as compared to untreated mice.

To further understand how BMP-7 inhibits apoptosis, we assessed the expression of a cell cycle regulator and tumor suppressor, phosphatase and tensin homolog (PTEN). In order to assess the effect of BMP-7 on PTEN, we measured the expression of phosphorylated PTEN in mouse heart homogenates (Figure 6K). Quantitative analyses of western blots show significantly up-regulated expression of phosphorylated PTEN in PD mice as compared to the controls. PD+BMP-7 mice showed significantly decreased levels of phosphorylated PTEN as compared to the PD mice. PTEN is a negative regulator for AKT, a survival protein. In order to determine the effects of BMP-7 on levels of p-AKT in PD mice, we performed western blots on mouse heart homogenates (Figure 6L). Quantitative analyses of results show that levels of p-AKT are down-regulated in PD mice, while treatment with BMP-7 significantly up-regulated levels of the survival protein.
Figure 6. BMP-7 inhibits apoptosis in the PD heart. Heart sections were assessed for apoptotic nuclei using TUNEL staining (A,D,G) (n=5-6 samples per group). Total nuclei were stained with DAPI in blue (B,E,H) and the images were merged (C,F,I). PD mice had a significantly (*p<0.001) higher percent of apoptotic nuclei (J) as compared to the controls. BMP-7 treatment was successful in reducing (#p<0.001) the percent of apoptotic nuclei as compared to the PD group. Heart samples were also analyzed for PTEN using WB (n=5-6 samples per group). Quantitative analyses show significantly (*p<0.05) increased phospho-PTEN (K) in PD hearts as compared to the controls. PTEN was significantly (#p<0.05) down-regulated in PD+BMP-7 hearts. Heart samples were also assessed for the expression of activated AKT (n=5-6 samples per group). PD mice had significantly (*p<0.05) reduced levels of phospho-AKT as compared to the controls (L). BMP-7 was effective (#p<0.05) in up-regulating the activation of AKT as compared to the PD group.
**BMP-7 Ameliorates Cardiac Remodeling in Pre-Diabetic Mice**

In order to determine the effect of BMP-7 on cardiac fibrosis in STZ-induced PD mice, we performed Masson’s Trichrome staining on heart sections (Figure 7A-G). Quantitative analyses show that both interstitial fibrosis and vascular fibrosis are significantly up-regulated in PD mice, while mice treated with BMP-7 had significantly down-regulated collagen deposition within the heart.
Figure 7. BMP-7 attenuates cardiac remodeling in the PD heart. Hearts were stained for interstitial fibrosis (A,B,D) and vascular fibrosis (B,C,E) using Masson’s Trichrome staining (n=7-9 samples per group). PD mice showed increase (*p<0.001) in both interstitial (F) and vascular (G) fibrosis as compared to the controls. BMP-7 was effective (#p<0.05) in blunting both interstitial and vascular collagen deposition as compared to the PD group.
**BMP-7 Reduces Cardiac Oxidative Stress through the Up-Regulation of MnSOD**

In order to assess the effect of BMP-7 cardiac oxidative stress in PD mice, tissue sections were stained with dihydroethidium as previously described (Singla et al., 2007). PD hearts revealed elevated levels of reactive oxygen species as compared to the controls (p<0.001), however, hearts from the PD+BMP-7 group had significantly reduced levels of ROS as compared to PD mice (Figure 8 A-J). Heart tissues were also analyzed for the expression of MnSOD. Quantitative analysis of our western blots revealed significantly down-regulated expression of MnSOD in PD mice as compared to controls (Figure 8K). Treatment with BMP-7, however, was successful in significantly up-regulating the expression of MnSOD as compared to the PD group.
Figure 8. Effects of BMP-7 on cardiac oxidative stress. Heart sections were stained for ROS using dihydroethidium (red) as depicted in red (A,D,G). Nuclei were stained with DAPI in blue (B,E,H) and the two images were merged (C,F,I). PD hearts revealed significant up-regulation of ROS levels (J) as compared to the controls (*p<0.001), however, treatment with BMP-7 effectively decreased levels of ROS as compared to PD group (#p<0.001) (n=6 animals per group). MnSOD levels were also assessed in heart tissues via western blot (n=5 animals per group). PD hearts resulted in decreased expression of MnSOD (K) as compared to the controls (*p<0.05). Levels of MnSOD were significantly up-regulated in PD+BMP-7 hearts as compared to those in prediabetic mice (#p<0.05).
**Exogenous BMP-7 Improves Cardiac Function in Pre-Diabetic Mice**

To assess the effect of BMP-7 on overall cardiac function in PD mice, the animals underwent echocardiography. Analyses shows significantly decreased ejection fraction (EF%) and fractional shortening (FS%) in mice treated with STZ (Figure 9A-B). Treatment with BMP-7 was able to restore cardiac function and significantly improve EF% and FS% as compared to PD mice.
Figure 9. BMP-7 improves cardiac function in PD mice. (A,B) Echocardiography was performed to assess cardiac function (n=6-9 samples per group). PD mice had significantly (*p<0.001) depressed ejection fraction and fractional shortening as compared to the control mice. Treatment with BMP-7 was able to significantly (#p<0.001) restore cardiac function and resulted in improved ejection fraction and fractional shortening as compared to the PD group.
CHAPTER 4: DISCUSSION

Diabetes Mellitus (DM) affects millions of people in the U.S. and is associated with increased risk for cardiovascular disease (D’Souza et al, 2011). It has been suggested that DM involves the innate immune system (Pickup, 1998) that leads to increased pro-inflammatory cytokines. Studies indeed support the role of inflammation in DM, however, even glucose concentrations below the diagnostic criteria for diabetes can up-regulate pro-inflammatory cytokines and initiate microvascular damage (Stolar, 2010; Pickup et al., 2000; Muller et al., 2002). BMP-7 has been reported to have potent anti-inflammatory properties (Chan et al., 2008; Gould et al., 2002). In this study, we investigate the effects of BMP-7 on inflammation in the Streptozotocin-induced PD heart.

PD is characterized by impaired fasting glucose and/or impaired glucose tolerance (Nielson and Lange, 2005). Mice in this study were made PD by injections with STZ and a 2-hour glucose tolerance test was performed to assess their reactions to glucose. Our results indicate that two hours post glucose injection, the PD group still presented elevated glucose levels as compared to the control mice. This suggests that the PD group were less able to metabolize glucose and exhibited glucose intolerance. Interestingly, the PD+BMP-7 group had improved glucose tolerance, and were better able to clear glucose as compared to the PD group.

It has been suggested that DM is actually a disease of the innate immune response (Pickup, 1998). In this study, analyses show that PD mice did not exhibit a significantly different population of M2 macrophages in the heart as compared to the control group. The PD+BMP-7 group, however, showed significantly increased M2
macrophages as compared to the PD group. Our recent study found that human monocytes treated with BMP-7 are able to polarize into M2 macrophages in vitro (Rocher et al., 2012). Considering this previous report, it is likely that BMP-7 up-regulates anti-inflammatory macrophage population in the PD heart by promoting the polarization of monocytes into M2 macrophages.

M2 macrophages are a class of immune cells that alleviate inflammation by secreting anti-inflammatory cytokines, such as IL-10 (Laskin, 2009). Studies suggest that IL-10 has a role in modulating inflammation by regulating levels of pro-inflammatory cytokines (Moore et al, 1993). We found decreased IL-10 levels in PD mice in both the heart tissue and serum, while BMP-7 treatment significantly increased serum levels of IL-10. We also determined that treatment with BMP-7 increases levels of IL-1RA, another anti-inflammatory cytokine, in the serum.

The anti-inflammatory properties of BMP-7 have been previously shown in various cell types. Gould et al. showed depressed levels of pro-inflammatory cytokines, TNF-α and IL-6, in proximal tubular epithelial cells treated with BMP-7. Furthermore, Chan et al. showed that human mesangial cells treated with pIgA expressed reduced levels of inflammation when pre-incubated with BMP-7. In this study, we found that treatment with BMP-7 attenuated pro-inflammatory cytokines, TNF-α and IL-6, both in the heart and serum. The inhibition of IL-6 may possibly help to alleviate PD hyperglycemia. Clinical and in vivo studies have shown that IL-6 stimulates gluconeogenesis and contributes to elevated glucose levels (Tsigos et al., 1997; Stith et al., 1994). These reports support the potential of BMP-7 to lower glucose levels and protect the PD heart from inflammation by regulating cytokine levels.
In addition to its role as a pro-inflammatory cytokine, TNF-α has also been shown to cause cardiomyocyte apoptosis in vitro (Dhingra et al., 2011). By binding to its receptors, TNF-α initiates the apoptotic cascade by triggering caspase-3 (Dhingra et al., 2011). Studies have shown that cardiomyocyte apoptosis is up-regulated in diabetic patients (Chowdhry et al., 2007) as well as in Streptozotocin models of diabetes (Li et al., 2007). Furthermore, a study has shown that bone morphogenetic proteins are able to inhibit apoptosis in rat cardiomyocytes in vitro (Izumi et al., 2001). Quantitative analyses of our TUNEL staining aligned with previous reports and showed increased percentage of apoptotic nuclei in PD hearts as compared to the controls. BMP-7 attenuated cardiac apoptosis as compared to the PD group, correlating to the in vitro data obtained by Izumi et al. It has been suggested that TNF-α-induced cardiomyocyte apoptosis can be mediated by anti-inflammatory cytokine, IL-10 (Dhingra et al., 2011). Since our results above indicated elevated levels of IL-10 in the PD+BMP-7 group, it is reasonable that BMP-7 may decrease apoptosis in the PD heart through IL-10 facilitated reduction of TNF-α.

We also investigated the effects of BMP-7 on proteins, Akt and PTEN. Akt is a known pro-survival protein that is regulated by tumor suppressor, PTEN. In this study, we found elevated levels of phosphorylated-pTEN (p-PTEN) in PD mouse hearts as compared to controls, which was observed with a concomitant decrease in levels of phosphorylated Akt (p-Akt). BMP-7 treatment was effective in inhibiting the activation of the tumor suppressor in the heart and up-regulating levels of p-Akt as compared to PD mice. A previous study has shown decreased levels of p-Akt in STZ models of diabetes (Jesmin, 2006). Furthermore, it is known that the over-expression of PTEN promotes
apoptosis in cardiomyocytes (Schwartzbauer and Robbins, 2001). Therefore, it is likely that BMP-7 blunts apoptosis in the PD heart by down-regulating the activation of PTEN and increasing the expression of p-AKT. Interestingly, a study suggested that PTEN knock-out mice are resistant to STZ-induced diabetes. Kurlawalla et al. proposed that PTEN decreases insulin sensitivity and may be a potential target for new diabetic therapies. In conjunction with our glucose data presented above, it is possible that BMP-7 may work to alleviate hyperglycemia by increasing insulin sensitivity through the inhibition of p-PTEN.

To further assess the anti-apoptotic effects of BMP-7 in the PD heart, we investigated its role in cardiac remodeling. It is known that following injury, cardiomyocyte apoptosis results in collagen deposition and fibrosis in the heart. We observed significantly increased levels of interstitial and vascular fibrosis in PD mice as compared to the controls. This aligns with a study which revealed that cardiac remodeling occurs during the PD state (D’Souza et al., 2011). The anti-fibrotic properties of BMP-7 have already been shown in the diabetic kidney (Boon et al., 2011). In this current study, we found that cardiac fibrosis is significantly attenuated upon treatment with BMP-7. This suggests that BMP-7 may be able to inhibit cardiac remodeling in the PD heart.

It is well known that hyperglycemia is associated with oxidative stress. In fact, we have previously shown that treatment with STZ results in significant up-regulation of reactive oxygen species (ROS) within the heart (Neel and Singla, 2011). In this study, we also show elevated levels of oxidative stress in the hearts of STZ-treated mice. Quantitative analysis showed that BMP-7 significantly reduced ROS as compared to the
PD group. This suggests that BMP-7 may promote antioxidant activity in the PD heart. To determine the mechanism by which BMP-7 ameliorated oxidative stress, we analyzed heart tissue for the expression of MnSOD, a well-known antioxidant. Quantitative analyses of our western blots showed decreased levels of MnSOD in PD mice, while mice treated with BMP-7 had significantly increased levels of the antioxidant. This aligns with a previous report by Xu et al. in which cardiomyocytes transfected with BMP-7 revealed increased levels of MnSOD activity. This up-regulation in antioxidant activity was observed with a concomitant decrease in malondialdehyde content, which suggests that BMP-7 may protect the heart from oxidative stress by attenuating lipid peroxidation.

Lastly, we investigated the effects of BMP-7 on cardiac function. Although DM is highly associated with heart failure, it has been shown that cardiac dysfunction can occur even during PD (Mizushige et al., 2000). In this study, we found depressed cardiac function in PD mice as compared to the controls. We found that treatment with BMP-7 was able to improve overall cardiac function as compared to the PD mice. Taken in conjunction with our fibrosis data above, it is likely that BMP-7 may improve cardiac function by preventing cardiac remodeling in the PD heart.

In conclusion, the data presented in this study suggests that BMP-7 treatment may inhibit apoptosis, decrease remodeling and improve cardiac function in the PD heart. Furthermore, this study suggests that the cardio-protective effects of BMP-7 lie in its ability to promote the polarization of monocytes into M2 macrophages and thereby reduce inflammation in the STZ-induced PD heart.
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LIST OF REFERENCES


