Amelioration of Diabetes Induced Inflammation Mediated Pyroptosis, Sarcopenia and Adverse Muscle Remodeling by BMP-7

Chandrakala Aluganti Narasimhulu, and Dinender K Singla*

Division of Metabolic and Cardiovascular Sciences, Burnett School of Biomedical Sciences, College of Medicine, University of Central Florida, Orlando, FL, 32816, USA

Running Title: BMP-7 inhibits diabetes induced muscle atrophy

*Name and complete address of corresponding author:
Dinender K. Singla, Ph.D.
Professor and Head-Division of Metabolic and Cardiovascular Sciences
Chair Endowed-Advent Health
Burnett School of Biomedical Sciences
College of Medicine
University of Central Florida
4110 Libra Dr
Orlando, FL, 32816, USA
E-mail: dinender.singla@ucf.edu
Phone: 407-823-0953
Fax: 407-823-0956
Abstract:

Background

Diabetic myopathy involves hyperglycemia and inflammation that causes skeletal muscle dysfunction; however, the potential cellular mechanisms that occur between hyperglycemia and inflammation, which induces sarcopenia, and muscle dysfunction remain unknown. In this study, we investigated hyperglycemia-induced inflammation mediating HMGB1 activation, which is involved in a novel form of cell death, pyroptosis, diabetic sarcopenia, atrophy and adverse muscle remodeling. Furthermore, we investigated the therapeutic potential of bone morphogenetic protein-7 (BMP-7), an osteoporosis drug, to treat pyroptosis, and diabetic muscle myopathy.

Methods

C57BL6 mice were treated with saline (control), Streptozotocin (STZ) or STZ+BMP-7 to generate diabetic myopathy. Diabetes was established by determining the increased levels of glucose. Then, muscle function was examined, and animals were sacrificed. Gastrocnemius (GM) muscle or blood samples were analyzed for inflammation, pyroptosis, weight loss, muscle atrophy and adverse structural remodeling of GM using histology, ELISA, immunohistochemistry, western blotting and RT-PCR.

Results

A significant (p<0.05) increase in hyperglycemia leads to an increase in inflammasome (HMGB1-TLR4-NLRP3) formation in diabetic muscle cells. Further analysis showed an upregulation of the downstream pyroptotic pathway with significant (p<0.05) number of positive muscle cells with pyroptotic specific markers (caspase-1, IL-1β, IL-18, and Gasdermin-D). Pyroptotic cell death, is involved in further increasing inflammation by releasing pro-inflammatory cytokine IL-6. Structural analysis showed the loss of muscle weight, decreased myofibrillar area, and increased fibrosis leading to muscle dysfunction. Consistent with this finding, BMP-7 attenuated hyperglycemia (~50%), pyroptosis, inflammation and diabetic adverse structural modifications as well as improved muscle function.

Conclusion

In conclusion, we report for the first time that increased hyperglycemia and inflammation involves cellular pyroptosis which induces significant muscle cell loss and adverse remodeling in diabetic myopathy. We also report that targeting pyroptosis with BMP-7 improves diabetic muscle pathophysiology and muscle function. These findings suggest that BMP-7 could be a potential therapeutic option to treat diabetic myopathy.

Key words: atrophy, inflammation, fibrosis, muscle dysfunction
Abbreviations

Introduction:

Diabetic patients are more susceptible than healthy people to the development and progression of sarcopenia muscle atrophy [1]. Diabetes is a major metabolic disorder usually accompanied with hyperglycemia, oxidative stress, and inflammation, leading to multi-organ diseases such as cardiomyopathy, nephropathy, neuropathy, periodontal disease, retinopathy, impaired wound healing and skeletal muscle dysfunction [1-5]. Skeletal muscle myopathy, especially when developed due to sarcopenia and muscle atrophy, has been considered as a major pathophysiological feature of diabetes [1, 2, 6]. Despite this, little attention has been paid to diabetes induced muscle sarcopenia, atrophy and progressed muscle dysfunction although these factors significantly contribute to impaired quality of life as well as increased morbidity and mortality in patients. The exact pathophysiological mechanisms involved in diabetic muscle dysfunction are complex and need further investigation.

Apoptosis, necrosis and autophagy have been reported in skeletal muscle dysfunction in aged humans [7-9]. Various studies reported these types of cell deaths in diabetic muscle dysfunction and suggest that they are generally mediated through oxidative stress [7-10]. Recent studies indicate that diabetes involves peripheral inflammation with increased macrophages in the blood of diabetic patients [11]; however, it remains unknown whether muscle tissue has increased inflammation that might trigger cellular mechanisms in the induction of diabetic sarcopenia, atrophy and muscle dysfunction. An inflammation-induced cell death, pyroptosis, has been reported in the gut and other organs involving infection [12]. Infection-initiated inflammation that causes pyroptosis has been considered to be a major mechanism that leads to organ dysfunction. Recent evidence suggests that non-cell dividing organs such as heart involve pyroptosis which is caused by sterile inflammation [13]. Pyroptosis, distinct from apoptosis and necrosis, is initiated by inflammation mediated through microbial infection or damage-associated molecular patterns (DAMPs). This leads to the formation of the inflammasome and a series of downstream activated pyroptosis markers such as caspase-1, IL-1β and IL-18 [14, 15]. It remains unknown whether diabetes-induced muscle dysfunction involves inflammation-induced pyroptosis in gastrocnemius muscle (GM) tissue.

Moreover, antioxidant and pharmacological agents such as myostatin and activin have been examined to treat skeletal muscle atrophy and dysfunction in disease conditions; however, these drugs have been reported to have off target effects, which raises concerns regarding their efficacy in patients [16, 17]. Therefore, alternative strategies are needed to attenuate diabetes-induced sarcopenia and atrophy. We recently reported that Bone morphogenetic protein-7 (BMP-7) an osteogenic protein-1, which belongs to the transforming growth factor β (TGF-β) superfamily, inhibits
apoptosis and inflammation in the diabetic heart [18]. Furthermore, we also demonstrated that BMP-7 inhibits plaque formation, monocyte infiltration and pro-inflammatory cytokine secretion in atherosclerosis [19, 20]. However, it remains unknown whether BMP-7 attenuates inflammation-induced pyroptosis, sarcopenia, skeletal muscle atrophy, and adverse remodeling.

This study will examine whether the presence of inflammation-induced cell death, pyroptosis, leads to sarcopenia, skeletal muscle atrophy and adverse muscle remodeling that causes skeletal muscle dysfunction in diabetic male and female mice. Furthermore, we also investigated whether BMP-7 would have a therapeutic effect on diabetes-induced pyroptosis, sarcopenia, muscle atrophy, and muscle dysfunction in diabetic animals.

Materials and Methods

Animal Model and Experimental Design

All animal procedures in the current study were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Central Florida. We used total 48 C57BL/6J mice age 10±2 weeks old, both male and female sexes as depicted in Figure 1A. Mice were divided into three groups control, streptozotocin (STZ), and STZ+BMP-7 (n=16; 8M+8F/group). Animals were administered 200 mg/kg STZ via intraperitoneal (i.p) injection whereas, recombinant mouse BMP-7 (Bioclone, cat#PA-0401) of 200 μg/kg body weight was administered via intravenous (i.v.) injection immediately after STZ injection. Additional injections of BMP-7 were given on two successive days. Total cumulative dose of BMP-7 given was 600 μg/kg body weight. Control animals were administered with saline injections. Body weight was measured prior to STZ injection and at the time of sacrifice. On Day-42 (D-42), muscle function was tested, and animals were euthanized under 4% isoflurane followed by cervical dislocation. Blood samples were collected by heart puncture via exsanguination for ELISA. GM muscles were collected, weighed and stored at -80°C in RNA later for western blotting and gene expression studies, or 4% paraformaldehyde (PFA) for histological staining.

Determination of Blood Glucose Levels

Blood prick method was used to measure the random blood glucose levels at D-42 using OneTouch Ultra Mini glucose meter [18]. Briefly, all mice were subjected for glucose test using 3-6µl of blood collected via tail vein puncture.

Immunohistochemistry (IHC) Staining

Double IHC staining was performed as published by us [18, 21]. GM tissue sections were deparaffinized followed by rehydration. Sections were blocked with 10% normal goat serum (NGS-Vector Labs). Following blocking, sections
were stained for skeletal muscle myosin using anti-myosin primary antibody and Alexa Fluor® 488 goat anti-rabbit (Invitrogen, Carlsbad, CA) secondary antibody. After myosin staining, a second blocking step was performed prior to co-staining with inflammasome primary antibodies [prepared in 10% GS] of High Mobility Group-Box1 (HMGB1), Toll-like Receptor-4 (TLR-4), Nucleotide-Binding Oligomerization Domain, Leucine Rich Repeat And Pyrin Domain Containing protein 3 (NLRP3)]. Sections were also stained for pyroptosis specific markers using Caspase-1, interleukin-1β (IL-1β), IL-18, and Gasdermin-D primary antibodies, and Alexa flour® 568-goat anti-rabbit antibody as secondary antibody. Finally, the sections were washed, and the nuclei were stained with mounting medium containing DAPI (4’,6-diamidino-2-phenylindole: Vector Labs Cat. #H-1200). Images (4-5 fields/section) were taken at 20X magnification using Keyence fluorescence microscope (Keyence, Itasca, IL) for quantification and representative images were recorded at 40X magnification. Quantitative data for pyroptotic cell death was calculated by dividing positive cells over total DAPI times 100 [(total cells+ve/total DAPI)*100] and Sigma Plot software was used for graphical representation. Details of antibodies were provided in Supplementary Table 1A.

cDNA Synthesis and RT-PCR
Total RNA was isolated from the GM tissue homogenate using Trizol™ (Invitrogen, Carlsbad, CA) and reverse transcribed into cDNA using the Superscript™ III First Strand Synthesis system (Invitrogen, Carlsbad, CA). Following cDNA synthesis, quantitative real time-PCR was performed using CFX96 C1000 Touch™ Thermal Cycler Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA) with SYBR Green (Invitrogen, Carlsbad, CA). PCR was carried out for pyroptotic initiator HMGB1, inflammasome marker NLRP3, pyroptotic markers (caspase-1, IL-1β, IL-18 and gasdermin-D), and for muscle atrophy marker muscle RING-finger protein-1 (MuRF1). The used list of mouse-specific primers are presented in Supplementary Table 1B. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) used as loading control. qPCR was performed with an initial step of denaturation at 50ºC for 2 min, 95ºC for 10 min followed by 40 cycles of 95ºC for 20 s and 60ºC for 20 s. Melt curves were established and normalized fold expression was calculated by using 2-∆∆Ct method.

Western blot analysis
Western blot was performed as reported previously [18, 21]. GM tissue (15-20 mg) was homogenized using Radioimmunoprecipitation (RIPA) lysis buffer, supernatant was collected after centrifugation, and protein concentration was estimated using Bio-Rad protein assay. Protein samples (50-80µg) were loaded and run on SDS–PAGE (10% or 15%) for 90–120 min at 150v. The gels were transblotted onto a polyvinylidene difluoride (PVDF)
membrane using Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA) for 60 min. Membranes were
blocked with 5% non-fat milk in 1x TBS-T at RT for 1 hr and then incubated with primary antibodies (1:1000 v/v
dilution) for HMGB1, NLRP3, GSDM-D, and β-actin (as a loading control) overnight at 4°C. Following primary
antibody incubation, membranes were washed with 1X TBS-T and incubated with HRP-conjugated goat anti-rabbit
IgG secondary antibody (1:1000 v/v dilution), for 1h at RT. Finally, membranes were exposed to enhanced
chemiluminescence reagent (Thermo Technologies, Rockford, IL) and the signal was detected using X-ray films.
Densitometric analysis was performed using Image J software on scanned X-ray films. All protein band intensities
were normalized to beta (β)-actin and expressed as arbitrary units (A.U.). Antibodies details were given in
Supplementary Table 1A.

**Enzyme-Linked Immunosorbert Assay (ELISA)**

Blood samples were centrifuged at 3000 rpm for 20 min. Serum was separated and stored at -80°C. Pro-inflammatory
cytokine levels of IL-6 were analyzed in mice serum samples using a sandwich ELISA kit (Ray Biotech, Inc.,
Norcross, GA, USA cat. No. ELM-IL6) following the supplier’s protocol. Data was plotted in a bar graph using Sigma
Plot software.

**Histological Analysis for Atrophy and Fibrosis**

**Hematoxylin and Eosin (H&E) Staining**

To evaluate myofibrillar loss GM sections were stained with H&E as we described previously [21, 22]. Sections were
deparaffinized, rehydrated and subsequently stained with hematoxylin (Thermo Fisher Scientific; cat# 7211), 1% acid
alcohol (Poly Scientific R&D Corp; cat# S104), bluing reagent (Thermo Fisher Scientific; cat# 73011) and eosin
(Thermo Fisher Scientific; cat# 7111) solutions. Nuclei were stained in blue/purple and muscle cells were in pink.
Images were recorded using Keyence microscope (Itasca, IL, USA). Image J software was used to quantify
myofibrillar size (mm²) at 20X magnification captured pictures and data was represented in a bar graph using Sigma
Plot software. Representative images were recorded at 40X magnification.

**Masson’s Trichrome Staining:**

To determine the interstitial and vascular fibrosis Trichrome staining was performed as we described previously [18,
21, 23, 24]. Using image J, Interstitial fibrosis (IF) was determined by measuring the collagen deposition (blue) in 6-
8 areas/section to quantify the fibrotic area in mm², while, vascular fibrosis (VF) was determined by measuring total
vessel area and fibrotic area by image J. Percent vascular fibrosis was calculated using [vascular fibrosis/total vessel
area] x 100 and graphs were plotted using Sigma Plot software. Representative images were recorded at 40X magnification.

Muscle function tests

To evaluate the effects of STZ induced diabetes with and without BMP-7 we determined the muscle function using Grip strength meter [25, 26], Rotarod [27, 28] and weights test [29] on D-42.

Grip-strength Test:
The grip strength of the four limbs of the mouse (combined) was assessed using Grip Strength Meter (Columbus Instruments, Columbus, OH) as described by Hakim et al. and Pasteuning-Vuhman et al.[25, 26]. Mice were placed on the mesh bar, allowed to hold with four limbs, and gently pulled away from the mesh bar in a horizontal fashion. The force applied by the mouse to hold the mesh was recorded as peak force in grams. The average grip force of 6-9 grip strength measurements was calculated and normalized with body weight (g). Data was represented in a bar graph using Sigma Plot software.

Rotarod Test:
To test the mouse endurance, mice were placed on the Rotarod apparatus (Columbus Instruments, Columbus, OH) following the protocol Beastrom et al.[27] and Mandillo et al.[28]. Briefly, mice were placed on the rotating rod with an initial speed of 4 RPM and gradually accelerated to 40 RPM over 5min. Three trials were performed, and each trial lasted approximately 5 to 8 minutes, with a resting period of 20mins between each trial. Once the mouse had fallen, time and speed were recorded. The results of three trials were averaged and calculated using Latency to fall/Duration in sec. Results were plotted as a bar graph using Sigma Plot software.

Weights Test:
Mice muscle strength was assessed by performing the weights test procedure as reported [29]. Mice were allowed to freely grasp the weights ranging from 5-65 g (15, 25, 35, 45, 50, 55, 60 and 65g) for 3s by holding the tail. Score [trial x time (TT)] was calculated by taking the number of links in the heaviest weight held for the full 3 sec, multiplied by the time (sec) it is held. Three independent trials were performed with 5 min rest, followed by average time (weight hold) in seconds was calculated and graph was plotted using Sigma Plot software.

Statistical analysis
Values are presented as mean ± standard error mean (SEM). To test the significance between the groups statistical analyses were performed using Student’s t-test and one-way analysis of variance (ANOVA) followed by Tukey test using Sigma Plot software, with p<0.05 considered as statistically significant.

Results

BMP-7 Treatment Attenuates Diabetes Induced Hyperglycemia

Figure 1B, shows a significantly (p<0.05) elevated blood glucose levels in diabetic male mice, as compared to control, whereas BMP-7 treatment significantly (p<0.05; ~34%) attenuated STZ induced hyperglycemia. However, female mice showed significantly (p<0.05; Figure 1C) elevated glucose levels in diabetic mice compared to controls, but less than diabetic males (~2.2 times), whereas, BMP-7 treatment showed a non-significant (~8%) reduction in glucose levels. The insignificant reduction in female mice with BMP-7 treatment might be due to an insufficient elevation of glucose levels in females. In addition, we also noticed an increase in glucose levels of control male mice compared to females, but the data was statistically insignificant. Further, both male and female mice data showed significantly increased (p<0.05; Figure 1D) hyperglycemia in diabetic animals, whereas BMP-7 treatment significantly (p<0.05; ~27%) attenuated diabetes induced hyperglycemia. Consistent with our previous studies [18] BMP-7 is able to attenuate diabetic hyperglycemia.

BMP-7 Treatment Improves Diabetes Induced Weight Loss:

To evaluate the effect of BMP-7 on weight loss in STZ induced diabetes, mice were weighed prior to the treatment as well as at the time of sacrifice (D-42). A significant (p<0.05) decrease in body weight was observed in STZ administered male mice as compared to the control (Figure 1E). While a non-significant increase in body weight was observed upon BMP-7 treatment in STZ administered male mice. Similarly, a significant reduction in body weight was observed in STZ administered female mice, whereas BMP-7 treated mice showed a significant gain in body weight (p<0.05; Figure 1F). Moreover, STZ administered female mice demonstrated an interesting phenomena of weight loss which was approximately 50% less than male mice in STZ group, suggesting the development and progression of diabetes is different in males and females. However, our male and female mice combined data showed a significant (p<0.05) reduction in body weight in STZ administered mice, whereas a significant (p<0.05) improvement in body weight was observed with BMP-7 treatment (Figure 1G), suggesting BMP-7 treatment attenuate weight loss observed following diabetes development.

BMP-7 Treatment Inhibits Pyroptosis Initiator HMGB1
IHC staining was performed to evaluate the effects of BMP-7 treatment on total number of positive cells for pyroptotic
initiator HMGB1. Figure 2i-A, images demonstrated the presence of a higher number of HMGB1 positive cells in the
STZ administered group (f-j) compared to control (a-e). Moreover, BMP-7 treatment significantly (p<0.05) reduced
the HMGB1+ve cells (k-o). Our HMGB1 data in male and female mice showed a significant (p<0.05; Figure 2i-B &
2i-C) increase in number of positive cells in diabetic group and this increase was significantly (p<0.05) attenuated
with BMP-7 treatment. Male animal diabetic changes on HMGB1 were similar in pattern as of female animals.
Combined data of male and female mice showed a significant (p<0.05; Figure 2i-D) increase in the number of positive
cells in diabetic animals, whereas a significant (p<0.05) reduction was observed with BMP-7 treatment.
To strengthen our findings, we performed RT-PCR (gene) and western blot (protein) analysis. A significant (p<0.05)
increase in both gene (Figure 2i-E) and protein expression (Figure 2i-H) was observed in diabetic male mice as
compared to controls, whereas, a significant (p<0.05) reduction was observed with BMP-7 treatment. Female mice
showed a significant (p<0.05; Figure 2i-F) increase in HMGB1 gene expression as compared to control, whereas
BMP-7 treatment reduced the HMGB1 gene expression, but the data was statistically non-significant. Western blot
analysis data revealed an increase in HMGB1 protein expression in diabetic female mice compared to control (Figure
2i-I) but the results are not statistically significant. Additionally, a non-significant reduction in HMGB1 protein
expression was observed with BMP-7 treatment. Noticeably, we observed HMGB1 protein expression in diabetic
female mice is less compared to male mice. Moreover, combined data of male and female mice showed a significant
(p<0.05; Figure 2i-G & 2i-J) increase in HMGB1 gene and protein expression in diabetic mice, which was significantly
reduced upon BMP-7 treatment suggesting the efficacy of BMP-7 in attenuating hyperglycemia induced pyroptosis
initiator HMGB1.

BMP-7 Treatment Inhibits TLR4 Expression in Diabetes

Published studies suggest the activation of intracellular signaling pathways via TLR4 in presence of damage associated
molecular pattern (DAMPs) protein HMGB1[30, 31]. IHC staining was performed to evaluate whether TLR4
expression is upregulated in diabetes. We further evaluated effects of BMP-7 on TLR4 expression. As shown in
Supporting information, Figure S1 A, representative photomicrographs specify the presence of higher number of +ve
cells for TLR4 in diabetic animal group (f-j) as compared to controls (a-e). A significant reduction in the number of
TLR4+ve cells (k-o) was observed upon BMP-7 treatment, suggesting the potential role of BMP-7 in the inhibition of
TLR4 expression. Moreover, quantitative analysis for TLR4 (Supporting information, Figure S1 B & C) in male and
female mice showed a significant increase in number of positive cells in diabetes, whereas BMP-7 treatment significantly diminished the TLR4 positive cells. Importantly, TLR4 positive cells in diabetic females were lesser (~22%) compared to males. Male and female mice combined data (Supporting information, Figure S1 D) showed a significant increase in TLR4 in diabetes, whereas BMP-7 treatment counteracted TLR4 expression suggesting the inhibitory potency of BMP-7.

**BMP-7 Treatment Decreases NLRP3 Inflammasome Formation in Diabetes**

NLRP3 is a downstream regulator as published by us and others [21, 32-34]. Therefore, we evaluated whether HMGB1 initiation would lead to inflammasome formation with TLR4 and NLRP3. To identify the role of BMP-7 in NLRP3 inflammasome formation, we performed IHC staining followed by gene and protein analysis. The fluorescent images (Figure 2ii-A) showed significantly (p<0.05) higher number of NLRP3 positive cells in STZ administered mice (f-j) as compared to control (a-e). However, a significant (p<0.05) reduction in NLRP3 positive cells (k-o) was observed upon BMP-7 treatment. Further quantitative analysis (Figure 2ii-B & 2ii-C) for males and females showed a significant increase in number of NLRP3 positive cells in STZ administered mice, which was significantly reduced upon BMP-7 treatment. IHC data for both male and female mice (p<0.05; Figure 2ii-D) showed a significant increase in NLRP3 positive cell in diabetes mice, and this increased number of positive cells was significantly (p<0.05) decreased with BMP-7 treatment.

Next, NLRP3 gene expression (RT-PCR analysis) was significantly (p<0.05; Figure 2ii-E) increased in diabetic male mice as compared to control, whereas BMP-7 treatment significantly (p<0.05) reduced diabetes induced NLRP3 gene expression. However, female mice showed similar pattern (Figure 2ii-F) as of males, but the data was not statistically significant.

Western blot analysis revealed a significant (p<0.05) increase in NLRP3 protein expression in STZ administered male and female mice as shown in Figures 2ii-H & 2ii-I. BMP-7 treatment significantly (p<0.05; Figure 2ii-H & 2ii-I) decreased the NLRP3 protein expression. Further, we noticed that the increase in NLRP3 gene and protein expression in diabetes female mice is less compared to male mice which is consistent with IHC data. Both male and female mice data showed a significant (p<0.05; Figure 2ii-G & 2ii-J) increase in gene and protein expression in diabetes mice, whereas, BMP-7 treatment significantly decreased the inflammasome formation and suggesting its therapeutic efficacy in attenuation of NLRP3 inflammasome formation.

**BMP-7 Treatment Reduces Pyroptosis Cascade Markers Caspase-1, IL-β, and IL-18**
TLR4 expression and inflammasome formation leads to the activation of pyroptosis cascade such as caspase-1, and downstream markers IL-1β, and IL-18 [35, 36]. We first established presence of these markers in diabetic mice tissue, then determined the potential role of BMP-7 on STZ induced pyroptotic cascade in GM tissue. Representative fluorescent images demonstrated significantly (p<0.05) higher number of pyroptosis markers caspase-1 (Figure 3i-A), IL-1β (Figure 3ii-A), and IL-18 (Figure 3iii-A) +ve cells in STZ administered mice (f-j) as compared to control (a-e). Diabetic mice treated with BMP-7, showed significantly (p<0.05) reduced number of +ve cells (k-o) specific for pyroptosis markers caspase-1, IL-β, and IL-18. Our pyroptotic cascade quantitative data for male and female mice showed a significant (p<0.05; Figure 3i, 3ii and 3iii-B&C) increase in number of positive cells in diabetic group and this increase was significantly (p<0.05) reduced with BMP-7 treatment. Combined data of male and female mice showed a significant (p<0.05; Figure 3i-D, 3ii-D, 3iii-D) increase in pyroptosis markers in diabetic mice, whereas BMP-7 treatment significantly (p<0.05) reduced all pyroptosis specific markers.

To strengthen our findings, we performed RT-PCR (gene) analysis. A significant (p<0.05) upregulation of caspase-1 gene expression was observed in diabetic male (Figure 3i-E) and female mice (Figure 3i-F) as compared to control, whereas BMP-7 treatment significantly downregulated caspase-1 expression. However, an insignificant increase in downstream markers IL-1β (Figure 3ii-E), and IL-18 (Figure 3iii-E) was observed in diabetic male mice vs control and a non-significant decrease was observed upon BMP-7 treatment. Whereas, a significant (p<0.05) increase in IL-1β (Figure 3ii-F), and IL-18 (Figure 3iii-F), gene expressions were observed in diabetic female mice while BMP-7 treatment significantly (p<0.05) reduced the gene upregulation.

Combined male and female mice data for gene analysis of caspase-1 (Figure 3i-G), IL-1β (Figure 3ii-G), and IL-18 (Figure 3iii-G) showed a significant (p<0.05) increase in all the pyroptosis markers in diabetes mice, whereas, BMP-7 treatment significantly attenuated all pyroptosis markers. Additionally, we also noticed that except IL-18, the increase in downstream markers gene expressions in diabetes females is less compared to males.

**BMP-7 Treatment Inhibits Pyroptosis Executioner GSDMD**

Recently, GSDMD has been considered as key pyroptosis executioner [15, 37]. Supporting information, Figure S2 A, IHC images demonstrated the presence of a higher number of GSDMD positive cells in the diabetic group (f-j) as compared to control (a-e). Moreover, BMP-7 treatment significantly (p<0.05) reduced the GSDMD +ve cells (k-o). Our quantitative GSDMD data in male (Supporting information, Figure S2 B) and female (Supporting information, Figure S2 C) mice showed of significant (p<0.05) increase in number of positive cells in diabetic group and this increase was
significantly (p<0.05) decreased with BMP-7 treatment. Diabetic changes of female mice on GSDMD were similar in pattern as of males. Combined data of male and female mice showed a significant (p<0.05; Supporting information, Figure S2 D) increase in the number of positive cells in diabetic animals, whereas a significant (p<0.05) reduction was observed with BMP-7 treatment.

To strengthen our findings, we performed RT-PCR (gene) and western blot (protein) analysis. A significant (p<0.05) increase in GSDMD gene expression was observed in diabetic male (Supporting information, Figure E) and female (Supporting information, Figure S2 F) mice as compared to controls, whereas, BMP-7 treatment significantly (p<0.05) attenuated the GSDMD expression. Western blot analysis data revealed a significant increase in GSDMD protein expression in diabetic male (Supporting information, Figure S2 H) and female (Supporting information, Figure S2 I) mice compared to control, and this increase was significantly (p<0.05) reduced upon BMP-7 treatment. Remarkably, we observed GSDMD gene and protein expressions in diabetic male mice are less compared to female mice. Both male and female mice data showed a significant (p<0.05) increase in gene (Supporting information, Figure S2 G) and protein (Supporting information, Figure S2 J) expression in diabetes mice, whereas, BMP-7 treatment significantly inhibited the pyroptosis executioner GSDMD, suggesting its potency in pyroptosis attenuation.

**BMP-7 Treatment Inhibits Diabetes Induced Pro-Inflammatory Cytokine IL-6**

To understand pyroptosis associated pro-inflammatory cytokine with or without BMP-7 treatment we performed ELISA in mice serum samples. As shown in Figure 4i, data analysis for male and female mice revealed a significant (p<0.05; Figure 4i-A & 4i-B) increase in IL-6 levels in diabetic mice suggesting inflammatory response. However, a significant (p<0.05; Figure 4i-A & B) decrease in IL-6 levels was observed upon BMP-7 treatment, suggesting the efficacy of BMP-7 in immune regulation. Moreover, we observed an interesting phenomenon that increased levels of IL-6 were lesser in diabetes female mice compared to male mice. Our results of male and female mice combined data showed a significant (p<0.05; Figure 4i-C) increase in IL-6 levels in diabetic mice, whereas BMP-7 treatment significantly (p<0.05) attenuated the IL-6 levels.

**BMP-7 Treatment Improves Diabetes Induced Sarcopenia**

To determine the impact of BMP-7 on GM muscle mass after STZ administration, the ratio of muscle weight to body weight was calculated. Our data show a significantly (p<0.05; Figure 4ii-A) developed sarcopenia (decrease in the GM mass) in male mice following STZ treatment as compared to control. A significant improvement in sarcopenia was observed upon BMP-7 treatment in male mice (p<0.05; Figure 4ii-A). Consistent with male mice, a significant
sarcopenia development was observed in STZ administered female mice, whereas treatment with BMP-7 showed a trend of improved sarcopenia but data was statistically non-significant. Moreover, both male and female mice combined data (Figure 4ii-C) showed a significant increase in developed sarcopenia in diabetic animals which was attenuated with BMP-7 treatment.

**BMP-7 Treatment Inhibits Diabetes Induced Muscle Atrophy**

Histological staining (H&E) was performed on GM tissue sections to determine the effect of BMP-7 on muscle atrophy in diabetes. In Figure 5A, representative photomicrographs demonstrated a significant decrease in GM myocyte area, suggesting muscle atrophy (Fig 5A-b) in STZ administered mice as compared to control (Figure 5A-a). Following, treatment with BMP-7, a significant (p<0.05; Figure 5A-c) increase in GM myocyte area was observed suggesting decrease in atrophy in diabetic mice. Further, quantitative analysis of male and female mice confirmed that, the cell size was significantly (p<0.05; Figure 5B & 5C) decreased in GM tissues of STZ received mice compared to control, whereas a significant (p<0.05) increase in muscle cell size was observed with BMP-7 treatment. Additionally, our male and female mice combined data showed significantly (p<0.05; Figure 5D) decreased muscle myocyte area in diabetes mice, which was significantly increased upon BMP-7 treatment (p<0.05; Figure 5D). Furthermore, to confirm attenuated atrophy by BMP-7 at the molecular level, we performed RT-PCR analysis for MuRF1 gene expression, which has been considered as a major atrophy gene in skeletal muscle [38]. In STZ administered male mice, MuRF1 gene expression was significantly (p<0.05; Figure 5E) increased as compared to control, whereas BMP-7 treatment significantly (p<0.05) reduced STZ induced MuRF1 gene expression. Noticeably, the MuRF1 gene expression was not statistically significant in diabetes female mice as compared to control. The exact reason for this discrepancy compared to histological finding on atrophy is not known. However, BMP-7 treatment significantly (p<0.05; Figure 5F) diminished the MuRF1 expression in female diabetic mice. Both male and female mice data showed significantly (p<0.05; Figure 5G) induced atrophy in diabetes animals, whereas BMP-7 treatment attenuated the diabetes induced muscle atrophy gene MuRF1. This set of data suggesting presence of atrophy in diabetes which is attenuated with BMP-7.

**BMP-7 Treatment Significantly Reduces Diabetes Induced Muscle Fibrosis**

Skeletal muscle fibrosis is an adverse remodeling mechanism that occurs in diabetes [18]. To determine whether BMP-7 attenuates interstitial and vascular fibrosis in STZ induced GM muscle, Masson’s trichrome staining was performed to quantify the presence of collagen. Blue area in representative photomicrographs (Figure 6) demonstrated...
significantly (p<0.05) increased interstitial (Figure 6A-b) and vascular fibrosis (Figure 6B-e) in STZ administered mice as compared to control (Figure 6A-a & 6B-d). Following BMP-7 treatment, a significant (p<0.05; Figure 6A-c & 6B-f) reduction in interstitial and vascular fibrosis was observed. Furthermore, our quantitative interstitial fibrosis (IF) data was significantly increased (p<0.05; Figure 6C,6D & 6E) in STZ induced diabetes mice compared to controls. Whereas BMP-7 treatment significantly (p<0.05) reduced IF in both male and female mice. Our vascular fibrosis (VF) quantitative data was significantly (p<0.05; Figure 6F, 6G & 6H) increased in STZ administered male and female mice as compared to control, whereas BMP-7 treatment statistically reduced VF in GM in diabetic males and females. These results suggest that reduction in collagen deposition and fibrosis further potentiate the therapeutic efficacy of BMP-7 in diabetic animals.

**BMP-7 Treatment Improves Diabetes Induced Muscle Dysfunction**

To assess, whether BMP-7 treatment can improve the muscle function, animals were subjected to three different types of tests. 1) grip strength for four limbs 2) rotarod test and 3) weights test for fore-limb muscle strength.

Grip strength analysis showed a significant (p<0.05) increase in grip strength of control female mice (~12%) as compared to control males. Further, our data showed a significant (p<0.05; Figure 7A & 7B) deficit in grip strength in diabetic male (~13%) and female (~11%) mice as compared to respective controls, whereas, BMP-7 treatment significantly (p<0.05) improved the grip strength. Noticeably, we observed a significant (p<0.05) improvement in grip strength in BMP-7 treated males (~29%) and females (~8%) as compared to diabetic mice. Combined data of male and female mice showed significantly (p<0.05; Figure 7C) reduced grip strength in diabetic mice, which was significantly improved with BMP-7 treatment.

Rotarod test: A significant (p<0.05) increase in latency to fall was observed in female mice (~42%) as compared to control males. Then, a significant (p<0.05; Figure 7D & 7E) decrease in muscle function performance i.e. the number of seconds stayed on a rotating rod (latency to fall) was observed in STZ administered male (~37%) and female (~30%) mice as compared to control. On the other hand, a significant (p<0.05) improvement in diabetic mice muscle function was observed with BMP-7 treatment, suggesting the potential impact of BMP-7 treatment on muscle dysfunction. Our result of combined data for male and female mice showed a significant (p<0.05, Figure 7F) decrease in muscle function in diabetic mice, whereas BMP-7 treatment significantly (p<0.05) improved the diabetes induced muscle dysfunction.

Weights test: A significant (p<0.05) decrease in forelimb muscle strength was observed in STZ administered male mice as compared to control (Figure 7G) using Trial x Time method. BMP-7 treatment showed an increase in the gain
of muscle strength, but data was statistically non-significant. A significant (p<0.05; Figure 7H) decrease in muscle strength was observed in STZ administered female mice as compared to control. Female mice lost 16% of muscle strength compared to male counterparts. Importantly, BMP-7 treatment significantly restored the diabetes induced muscle dysfunction. Male and female mice combined data showed a significant (p<0.05; Figure 7I) reduction in muscle strength in diabetic animals, whereas BMP-7 treatment significantly (p<0.05; Figure 7I) improved the forelimb muscle strength. These results confirm that BMP-7 significantly counteracts diabetes induced sarcopenia and atrophy further muscle dysfunction.

Discussion

Apoptosis and oxidative stress are key players in skeletal muscle sarcopenia, cachexia and in adverse muscle remodeling (fibrosis and atrophy) in aging, cancer, and diabetes [1, 2, 39]. Recent data shows that inflammation could be a triggering agent in the development and progression of sarcopenia and cachexia [40-42]. Moreover, in the diabetic condition, hyperglycemia dominates in the blood stream as well as various diabetic organs such as muscle, and causes diabetic muscle myopathy [3, 43]. Therefore, hyperglycemia and inflammation are detrimental in diabetic muscle myopathy. However, close association between hyperglycemia and induced inflammation in diabetic muscle myopathy is unknown. Additional questions that further arise are; 1) what the cellular mechanisms involved between the hyperglycemia and inflammation are, 2) and whether those cellular mechanisms are the key players that causes sarcopenia, fibrosis and atrophy that lead to diabetic muscle dysfunction and chronic muscle myopathy. The current study is undertaken to establish the close link between hyperglycemia and inflammation as well as the involved cellular level mechanisms that induce sarcopenia, fibrosis and muscle dysfunction. Furthermore, we will provide strong evidence on the use of BMP-7, an osteoporosis drug that attenuated induced hyperglycemia, inhibited cellular mechanisms involved in the inflammatory process, inhibited sarcopenia, fibrosis and improved muscle dysfunction.

In the present study, we developed an STZ-induced diabetic mouse model that shows a significant increase in hyperglycemia and weight loss. This data is consistent with previously published reports by us and others [2, 18]. Recent data shows that diabetic patients and animal models have increased hyperglycemia associated with increased levels of inflammation [44, 45]. Increase in hyperglycemia has also been observed in lipopolysaccharide (LPS)-induced septic rat model [46]. This increase in hyperglycemia and inflammation in the rat model is consistent with critically ill sepsis patients [47]. Increased sterile inflammation has been involved in the release of damage associated molecular patterns (DAMPs) such as high-mobility group box 1 (HMGB1). In addition, HMGB1 has been observed
in these patients and in the acute lung injury rat model, suggesting its association with inflammation. HMGB1 has been reported to be present in vascular smooth muscle cells, endothelial cells and monocytes in normal homeostasis [48]. However, an increased level of HMGB1 has also been observed in various tissue trauma, ischemia and inflammatory diseases [49, 50]. Consistent with these studies, we report in the present findings that HMGB1 was significantly increased in diabetic skeletal muscle cells. Moreover, we also report that increased HMGB1 in diabetic muscle myopathy induces sterile inflammation. To further understand the significance of increased HMGB1 in diabetic muscle cells we investigated the presence of its receptor, TLR4. Interestingly, we observed significantly increased levels of TLR4 (Supporting information, Figure S1), confirming that the increase in HMGB1 is associated with increased levels of TLR4 that may initiate further cell signaling and sterile inflammation. Therefore, we investigated activation of inflammation-induced cell death called pyroptosis in diabetic muscle myopathy. Pyroptosis is a newly investigated inflammation-induced cell death that was reported to occur with bacterial infections [51-53]. Recently, we and others have reported that Doxorubicin (anti-cancer drug) induced-cardiomyopathy, myocardial ischemia, and cancer involves sterile inflammation, which induces pyroptosis [12, 21, 32]. However, it was never investigated whether increased levels of hyperglycemia can induce the HMGB1 via TLR4 that triggers sterile inflammation as well as forms the NLRP3-associated inflammasome and the downstream pathway of pyroptotic cell death in diabetic skeletal muscle.

We report that HMGB1-TLR4 signaling gives rise to NLRP3-mediated inflammasome formation (Figure 2ii). This inflammasome formation has been reported in the in vitro Doxorubicin-treated H9C2 cells [32], and Sol8 cells [54], *Porphyromonas gingivalis* stimulated macrophages [55] as well as in vivo models of ischemia reperfusion injury [13], myocardial infarction [56], Dox-induced cardiomyopathy [21], and brain diseases [57]. Our data is in agreement with these findings on sterile inflammation and formed NLRP3 inflammasome as previously published in vitro and in vivo reports [13, 21, 32, 54, 56]. However, it would be interesting in the future studies to examine whether HMGB1-TLR4 complex contributes differently compared with the HMGB1-TLR4 signaling pathway in diabetic myopathy.

Moreover, these studies also suggest that formed inflammasome triggers upregulation of pyroptosis markers caspase-1, IL-1β, IL-18 that cause pore formation in the cells and induce cell death pyroptosis. To confirm the role of NLRP3 inflammasome in muscle pyroptosis, we examined the cascade of pyroptotic cell death markers caspase-1, IL-1β, IL-18 and GSDMD, using immunohistochemistry, western blotting, and RT-PCR techniques. We observed a
significant increase in pyroptosis markers caspase-1, IL-1β and IL-18 (Figure 3), suggesting the presence of the new form of cell death pyroptosis in diabetic muscle cells. Our data on pyroptosis death markers is consistent with previously published reports on bacteria-induced pyroptosis in macrophages and sterile inflammation-induced pyroptosis in the heart, kidney and brain [21, 58, 59]. Upregulation of caspase-1 and release of IL-1β mediated through caspase-1 a substrate of gasdermin-D are commonly reported in bacterial-induced pyroptosis in macrophages [60]; however, it remains elusive whether gasdermin-D is upregulated to execute pyroptosis in sterile inflammation-induced diabetic muscle cell pyroptosis. Our data reports an upregulation in diabetic muscle cells of the executor of pyroptosis, gasdermin-D (Supporting information, Figure S2) as reported by others in macrophages, and further confirms the presence of muscle cell pyroptosis [61].

Previous studies have shown that HMGB1 induces sterile inflammation in diseases such as septic shock, ischemia, and various neurological disorders including Parkinson’s, Multiple Sclerosis and Alzheimer’s [48, 62-67]. However, to confirm whether HMGB1-triggers inflammation in diabetic muscle myopathy, we studied the presence of inflammatory cytokine IL-6. Data reported in this study confirms the significant increase in pro-inflammatory cytokine IL-6 (Figure 4i) and presence of HMGB1 induced sterile inflammation. The increased level of IL-6 is detrimental due to its association in the development and progression of diabetic cardiomyopathy, inflammatory disease, and lung dysfunction [68-70]. Therefore, our data on inflammatory IL-6 is in agreement with these published reports on sterile inflammation.

Next, we determined the functional significance of pyroptosis and inflammation in the diabetic muscle. To establish that, this form of cell death has significance in diabetic muscle myopathy, we determined muscle weight loss, sarcopenia, muscle cell atrophy, fibrosis and muscle dysfunction. In the present study, we report that pyroptosis occurring in diabetic muscle cells shows a significant loss of muscle mass. Then, we examined whether loss of muscle mass leads to structural modification in the skeletal muscle such as atrophy and fibrosis as well as further contributes in sarcopenia development and progression. Noticeably, we observed a significant decrease in loss of myofibrillar area and increased levels of MuRF1 gene, suggesting the presence of muscle atrophy. Increased levels of HMGB1-induced pyroptosis and inflammation mediated muscle loss give rise to myofibroblast cell proliferation that provide structural adverse remodeling and stiffness in the diseased organ; therefore, we found presence of interstitial and vascular fibrosis following pyroptosis in the diabetic skeletal muscle. This data strongly suggests that a series of pathological events such as pyroptosis, sarcopenia and adverse muscle remodeling could lead to muscle dysfunction.
Further, our muscle dysfunction data showed a significant decrease in muscle function and confirmed the functional significance of these series of pathological events in STZ-induced diabetic muscle myopathy. Our muscle dysfunction data is consistent with earlier published studies, which implied that sarcopenia, muscle atrophy and fibrosis are key contributors in skeletal muscle dysfunction [39, 71-73].

Moreover, we mentioned above that HMBG1-induced pyroptosis and sterile inflammation leading to sarcopenia, atrophy and muscle dysfunction are the major contributors in the development and progression of diabetic muscle myopathy. Therefore, targeting HMGB1 directly or with a growth factor that can attenuate sterile inflammation and pyroptosis, which further attenuates a series of pathological events, could be a potential therapeutic agent to treat diabetic muscle myopathy, as there are limited options to treat this condition. We explored the potential of BMP-7, a commonly used drug to treat osteoporosis in patients and was examined in animal models of atherosclerosis, where it was found to polarize pro-inflammatory M1 macrophages into anti-inflammatory M2 macrophages and decrease atherosclerosis [19]. Moreover, BMP-7 has also been used to treat inflammation and fibrosis in kidneys [74]. However, the role of BMP-7 in the inhibition of HMGB1, pyroptosis, sarcopenia, fibrosis, atrophy and muscle dysfunction in diabetic muscle myopathy has never been established. We treated STZ-induced diabetic animals with BMP-7 and examined the levels of hyperglycemia, HMGB1 and sterile inflammation. Our data shows a significant decrease in hyperglycemia and sterile inflammation, which is consistent with our previously published report in diabetic cardiomyopathy [18]. Next, we examined levels of HMGB1 and its receptor, TLR4, which were significantly reduced along with the NLRP3 inflammasome and pyroptosis markers, suggesting that BMP-7 targets HMGB1 and its downstream pathway of pyroptosis (Figures 2 & 3). These novel findings reported raises further questions on the involvement of cell signaling pathways. In this report, involvement of cell signaling pathways was not examined. However, we anticipate that this opens a new avenue for investigation in the future studies in this subject matter by our lab or others. Our fibrosis data shows a significant reduction following BMP-7 treatment. This reduced fibrosis following BMP-7 treatment is in agreement with previously published report in kidney disease [74]. Interestingly, the present study shows that BMP-7 treatment significantly reduces muscle atrophy and sarcopenia while improving diabetic muscle dysfunction (Figure 8), which could be a future therapeutic drug to treat diabetic muscle myopathy patients.

Understanding the effects of sex differences on aging, disease development and progression is a current area of investigation. Specifically, it has been realized that sex hormones cause the difference in anabolic and metabolic
muscle rate in younger women who experience menopause [75]. Recent data suggest that in muscle regeneration, sex hormone testosterone acts via androgen receptor and enhances anabolic cell signaling in skeletal muscle cell [76]. Additionally, it has been observed that estrogen, another active hormone that plays a role in skeletal muscle cell catabolic and anabolic processes, decreases muscle atrophy, oxidative stress and inflammation in postmenopausal women [77, 78]. The current study does not investigate the role of testosterone or estrogen in menopausal animals. This study suggests that female animals at their young age, between 10-12 weeks, show lower levels of hyperglycemia, weight loss, inflammation, myofibrillar loss and atrophy specific MuRF1 gene compared with male animals of the same age group. This set of data suggests that females have a better recovery rate to improve hyperglycemia, atrophy and weight loss in diabetes. However, the exact reason for this improved recovery in diabetic females is not well understood or investigated in the present study. Nevertheless, based on published reports, we state that estrogen plays a pivotal role to decrease atrophy, oxidative stress and inflammation, which is in agreement with our findings. Therefore, this report starts a new area of investigation to understand whether estrogen directly initiates cell signaling and plays a role in the recovery of diabetic pathological alterations. Noticeably, we observed the same level of HMGB1-TLR4 signaling, inflammasome formation, and pyroptosis in male and female animals. Here, we could postulate that upregulated hyperglycemia and inflammation triggered the process of pyroptosis, but estrogen may not target the specific pathway of pyroptosis. This could be a potential reason that a difference in pyroptosis was not seen in these sex differences. Additionally, there could be more reasons that need further investigation to understand why the levels of pyroptosis are same in both sexes. Moreover, we observed that skeletal muscle function measurements with grip strength and rotarod tests were superior in diabetic female group compared with the male counterpart. As mentioned above, the decrease in hyperglycemia, inflammation and atrophy in female animals could be a potential reason to see improved muscle function. The effects of BMP-7 on the improvement of hyperglycemia and muscle function were the same in both sexes. Importantly, this study describes the association between hyperglycemia induced pyroptosis through the initiation of HMGB1/TLR-4 signaling, subsequent inflammasome activation, and downstream upregulation of pyroptosis in diabetic muscle cells. So, this study does not address the exact causative mechanistic role of BMP-7 in the attenuation of hyperglycemia and inflammatory cell death. Therefore, future studies are required to establish a causative mechanistic relationship on hyperglycemia induced pyroptosis and its attenuation by BMP-7 in diabetic myopathy. Moreover, this study design determines the effect of BMP-7 in STZ treatment and for 2 days after; therefore,
future studies are warranted to examine whether BMP-7 is effective in the developed acute and chronic stages of diabetic myopathy.

In conclusion, we report for the first time, to the best of our knowledge that diabetes induces hyperglycemia and inflammation, which triggers a cellular pathway mediated through upregulation of HMGB1 via TLR4 receptor. This in turn, further initiates NLRP3 inflammasome and downstream pathway of pyroptosis, which is executed by GSDM-D and pore-formation. These pyroptotic cells release inflammatory cytokines IL-18 and IL-6 that further causes atrophy, adverse muscle remodeling and muscle dysfunction. We also report that females recover much faster in hyperglycemia, inflammation, atrophy and muscle function. This study opens new areas of investigation; 1) what cell signaling mechanisms further pyroptosis, 2) how estrogen plays a role in diabetes, and 3) whether estrogen could be a future therapy for diabetes in females. Moreover, we provide the data and postulate that BMP-7, which was never tested to treat HMGB1-mediated pyroptosis could be a future therapeutic agent to treat diabetes.
Disclosures
The authors declare no competing interests

Author Contribution
D.K.S. designed and supervised the study. A.N.C performed the experiments, analyzed data, prepared figures and
drafted the manuscript. D.K.S revised the manuscript and approved final version of the manuscript.

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Figure 1. BMP-7 Treatment Attenuates Diabetes Induced Hyperglycemia and Improves Diabetes Induced Weight Loss

Schematic representation of injection schedule and study design (A). STZ induced diabetes was confirmed with elevated glucose levels. Bar graphs represent elevated glucose levels in both male and female mice of STZ administered mice whereas BMP-7 treatment potentially reduced the glucose levels on D42 (B: Male mice (n= 5-6); C: Female mice (n= 6); D: Both male and female mice (n=12-13). Similarly, histogram represents the gain in BW (body weight) upon BMP-7 treatment after 42 days of STZ administration (E: Male mice (n= 5-8); F: Female mice (n=5-8); G: Both male and female mice (n=11-13)]. Error bars = mean ± S.E.M. One-way ANOVA and Tukey tests were performed to assess statistical significance. * p<0.05; **p<0.01; ***p<0.001 vs. control, #p<0.05; ##p<0.01 vs. STZ
Figure 2i. BMP-7 Treatment Inhibits Pyroptosis Initiator HMGB1.

Hyperglycemia induced HMGB1 can initiate the inflammasome activation and subsequent pyroptotic cascade results in pyroptotic cell death. Representative photomicrographs of muscle sections stained with myosin and inflammasome markers. Individual boxes in panel A shows HMGB1+ve, cells shown in red (a, f, k), muscle cells in green (b, g, l), DAPI in blue (c, h, m), and merged images (d, i, n). Scale bar = 100μm. White dotted boxes and arrows indicates enlarged section of merged images (e, j, o). Quantitative analysis-derived in bar graphs of HMGB1+ve muscle cells quantified over total DAPI [B: Male mice (n=8); C: Female mice (n=7-8); D: Both male and female mice (n=16)]; Relative fold change in gene expression of HMGB1 [E: Male mice (n=5-7); F: Female mice (n=5); G: Both male and female mice (11-12)]; Representative blot and densitometric analysis of HMGB1 [H: Male mice (n=7); I: Female mice (n=4-5); J: Both male and female mice (13-14)]. One-way ANOVA and Tukey tests were performed to assess statistical significance. * p<0.05; **p<0.01; ***p<0.001 vs. control, #p<0.05; ##p<0.01; ###p<0.01 vs. STZ.

Figure 2ii. BMP-7 Treatment Decreases NLRP3 Inflammasome Formation in Diabetes

HMGB1-TLR4 signaling initiates the NLRP3 inflammasome activation. Representative photomicrographs of muscle sections stained with myosin and inflammasome markers NLRP3. Individual boxes in panel A shows NLRP3+ve cells
in red (a, f, k), muscle cells in green (b, g, l), DAPI in blue (c, h, m), and merged images (d, i, n). Scale bar = 100μm. White dotted boxes and arrows indicates enlarged section of merged images (e, j, o). Quantitative analysis-derived in bar graphs of NLRP3⁺ muscle cells quantified over total DAPI [B: Male mice (n=7-8); C: Female mice (n=8); D: Both male and female mice (n=15-16)]; Relative fold change in gene expression of NLRP3 [E: Male mice (n=5-7); F: Female mice (n=4-6); G: Both male and female mice (n=9-11)]; Representative blot and densitometric analysis of NLRP3 [H: Male mice (n=6-7); I: Female mice (n=5-6); J: Both male and female mice (n=14-15)]. One-way ANOVA and Tukey tests were performed to assess statistical significance. * p<0.05; ***p<0.001 vs. control, #p<0.05; ##p<0.01; ###p<0.001 vs. STZ.
Figure 3: BMP-7 Treatment Reduces Pyroptosis Cascade Markers Caspase1, IL-β, and IL-18

Activation of NLRP3 inflammasome furthers the pyroptosis cascade. Representative photomicrographs of muscle sections stained with myosin and pyroptosis markers (i: Caspase-1 ii: IL-1β and iii: IL-18 Panel A). Individual boxes in panel A shows caspase-1+ve, IL-1β+ve and IL-18+ve cells in red (a, f, k), muscle cells in green (b, g, l), DAPI in blue (c, h, m), and merged images (d, i, n). Scale bar = 100μm. White dotted boxes and arrows indicates enlarged section of merged images (e, j, o). Quantitative analysis-derived histogram of i) caspase-1+ve, ii) IL-1β+ve and iii) IL-18+ve muscle cells quantified over total DAPI [B: Male mice (n=7-8); C: Female mice (n=8); D: Both male and female mice (n=15-16)]; Relative fold change in gene expression of i) caspase-1 [E: Male mice (n=5-6); F: Female mice (n=4-5); G: Both male and female mice (n=12)], ii) IL-1β [E: Male mice (n=5); F: Female mice (n=4-5); G: Both male and female mice (n=10-11)] and iii) IL-18 [E: Male mice (n=4-5); F: Female mice (n=4-5); G: Both male and female mice (n=8-10)]; One-way ANOVA and Tukey tests were performed to assess statistical significance. * p<0.05; **p<0.01; ***p<0.001 vs. control, #p<0.05; ##p<0.01; ###p<0.001 vs. STZ. Fold change in gene expression was represented as A.U.
Figure 4i. BMP-7 inhibits Pro-inflammatory cytokine IL-6

ELISA was performed to determine the serum levels of pro-inflammatory cytokine IL-6. Bar graphs represent elevated IL-6 levels in A) Male mice B) Female mice C) Both male and female mice of STZ administered mice whereas BMP-7 treatment potentially reduced the IL-6 levels on D42. Error bars = mean ± S.E.M. One-way ANOVA and Tukey tests were performed to assess statistical significance. * p<0.05 vs. control, #p<0.05; ##p<0.01 vs. STZ, n=12-14 (both males and females) n=5-7 (male mice); n=6-7 (female mice).

Figure 4ii. BMP-7 Treatment Improves Diabetes Induced Sarcopenia

Bar graph demonstrating the ratio of muscle weight-to-body weight (in g) was significantly decreased in STZ administered on D42. (A: Male mice; B: Female mice; C: Both male and female mice). Whereas BMP-7 treatment significantly increased the muscle mass. Error bars = mean ± S.E.M. One-way ANOVA and Tukey tests were performed to assess statistical significance. * p<0.05; ***p<0.001 vs. control, #p<0.05 vs. STZ, n=11-16 (both males and females) n=5-7 (male mice); n=5-7 (female mice).
Figure 5: BMP-7 Treatment Inhibits Diabetes Induced Muscle Atrophy

A) Representative Photomicrographs (40X) are sections stained with H&E to detect atrophy in gastrocnemius muscle on day 42 after STZ administration in control and experimental groups. Stained sections were quantified at 20X magnification and magnified for visualization of atrophy. Bar graph represents quantitative analysis for muscle atrophy [B: Male mice (n=7-8); C: Female mice (n=8); D: Both male and female mice (n=16)]. Scale bar = 100μm.

Relative fold change in MuRF1 gene expression in bar graph [E: Male mice (n=6-7); F: Female mice (4-6); G: Both male and female mice (n=11-14)]. Error bars = mean ± S.E.M. One-way ANOVA and Tukey tests were performed to assess statistical significance. *p < 0.05; ***p<0.001 vs. control; #p <0.05; ##p<0.01; ###p<0.001 vs. STZ.
Figure 6. BMP-7 Treatment Significantly Reduces Diabetes Induced Muscle Fibrosis

Representative images of Masson’s Trichrome demonstrated interstitial (Panel A) and vascular fibrosis (Panel B) in gastrocnemius muscle on day 42 after STZ administration in control and experimental groups. Stained sections were quantified at 20X magnification and magnified for visualization of interstitial and vascular fibrosis. Quantitative analysis for intestinal fibrosis C: Male mice (n=7-8); D: Female mice (n=7-8); E: Both male and female mice (n=14-16). Percentage of vascular fibrosis quantified over the vessel area F: Male mice (n=5-6); G: Female mice (n=6-8); H: Both male and female mice (n=11-14). One-way ANOVA and Tukey tests were performed to assess statistical significance. *p<0.05; **p<0.01; ***p<0.001 vs. control; #p<0.05; ##p<0.01; ###p<0.001 vs. STZ. Scale bar = 100μm.
Figure 7: BMP-7 Treatment Improves Diabetes Induced Muscle Dysfunction

BMP-7 Treatment improves muscle function under hyperglycemic conditions. To evaluate this on day 42 after STZ administration, animals were subjected to different muscle function tests. Bar graphs represent the quantification and analysis for four-limb grip strength [A: Male mice (n=6-7); B: Female mice (n=6-7); C: Both male and female mice (n=14-15)], Rotarod performance test [D: Male mice (n=5-6); E: Female mice (n=5-7); F: Both male and female mice (n=12-13)] and Weights test-TT method [G: Male mice (n=5); H: Female mice (n=6); I: Both male and female mice (n=11-15)]; were analyzed and quantified. Error bars = mean ± S.E.M. One-way ANOVA and Tukey tests were performed to assess statistical significance. *p<0.05; **p<0.001 vs. control; #p<0.05; ###p<0.001 vs. STZ.
Figure 8: Schematic representation of overall study: BMP-7 attenuates diabetes induced pyroptosis, muscle atrophy and fibrosis.
References


