Cardiac Damage by Doxorubicin Is Mitigated in Smad3 Deficient Mice

**Methods**

Osteopathic Principles

Conclusion

**Significance**

• Cardiovascular disease is the leading cause of morbidity and mortality in long-term cancer survivors

• Anthracycline chemotherapy is the most common cause of cardiac toxicity in treated cancer patients

• Anthracyclines are used for various hematologic and solid-tumor malignancies

• Cardiovascular damage manifests months/years after initial exposure, causing cardiomyopathy and heart failure

**Hypothesis**

*Smad3 Deficiency will protect heart from doxorubicin toxicity*

**TGF-β Signaling Pathway**

- Increased production of TGF-beta cytokines in cardiomyopathy and Heart Failure
  - Endothelial cells are significantly effected by the TGF-beta signaling pathway
  - SB431542 is an inhibitor of TGF-beta signaling pathway
    1. Prevents phosphorylation of Smad2 and Smad3
    2. Prevents dimerization with Smad4
    3. Prevents translocation to nucleus → transcription of protein products

**Results 1**

**Results 2**

**Osteopathic Principles**

**Cellular Model of Doxorubicin Effects**

- Endothelial cells were co-cultured with fibroblasts
- Groups were divided into SB-treated and control endothelial cells
- Doxorubicin was added to co-culture to analyze its effects on angiogenesis
- Vascular network formation in live co-cultures was quantified using green fluorescent protein expressing endothelial cells
- Doxorubicin addition demonstrated a decrease in angiogenesis in control groups that was mitigated in SB-treated cells
- These results provided the rationale for in vivo study of the role of TGF-beta pathway in doxorubicin induced cardiac damage
Cardiac Damage by Doxorubicin Is Mitigated in Smad3 Deficient Mice


*, Kansas City University of Medicine and Biosciences; #, University of Nevada, Las Vegas School of Medicine; §, University of Kansas Medical Center

Introduction

Mouse cardiomyopathy model was used to test the hypothesis that cardiac damage by doxorubicin will be reduced in Smad3 deficient mice.

Smad3+/+, Smad3+/−, and Smad3−/− mice were treated with four weekly injections of doxorubicin:
- Single dose 5 mg/kg
- Total dosage 20 mg/kg

Methods

Strain Verification
- Mouse Strain – 129-Smad3tm1Par/J
- Western Blot

Experimental Timeline
- Mouse cardiomyopathy model was used to test the hypothesis that cardiac damage by doxorubicin will be reduced in Smad3-deficient mice.
- Smad3+/+, Smad3+/−, and Smad3−/− mice were treated with four weekly injections of doxorubicin.

Immunohistochemistry
- Hearts were fixed in paraformaldehyde, embedded in OCT
- Antibodies:
  - CD31 was used as an endothelial specific marker
  - Smooth muscle myosin heavy chain was used as a vascular smooth muscle cell marker
  - Microvessel perfusion
  - Vital Vascular Dye ILB4 DL 649
    - Injected intravenously 4 minutes prior to heart excision

Echocardiography
- Isoflurane anesthetized mice
- Mice were analyzed at baseline and at 9 weeks after the last doxorubicin injection
- Vevo 2100 Imaging System

Cardiac Fibrosis Analysis
- Cardiac tissue was fixed in Paraformaldehyde
- Cardiac tissue was embedded in Paraffin
- Trichrome Staining for collagen

Statistical Analysis
- Data presented as mean values +/- Standard Error
- Significance accepted with p < 0.05
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Introduction

Methods

Microvessels

- Smad3+/+
- Smad3+/-
- Smad3-/-

- Microvessel Diameter was analyzed at 9 weeks
- No statistically significant difference was found between Saline-treated and Doxorubicin-treated mice

Microvessel Density

- Microvessel Density was compared between Smad3+/+, Smad3+/-, and Smad3-/- mice using quantitative analysis
- Microvessel Density was analyzed at 9 weeks
- No statistically significant difference was found between Saline-treated and Doxorubicin-treated mice

Results

Results 1

- Microvessel Diameter was analyzed at 9 weeks
- Wild Type mice showed an increase in microvessel diameter in Doxorubicin-treated mice vs. Saline-treated mice
- Smad3 heterozygous and knockout mice showed no statistically significant change in microvessel diameter between treatment groups
- * p<0.05 as compared to saline-treated mice

Arteriole Density

- Mean arteriole density was quantified by normalizing the number of arterioles in each sample to the total cardiac tissue area for Smad3+/+, Smad3+/-, and Smad3-/- mice treated with Doxorubicin
- Arterioles staining: using antibodies for myosin heavy chain and troponin to visualize the arterioles and cardiac tissue, respectively
- No statistically significant difference was seen in arteriole density between the three groups

Results 2

- Microvessel Diameter was analyzed at 9 weeks
- Wild Type mice showed an increase in microvessel diameter in Doxorubicin-treated mice vs. Saline-treated mice
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Osteopathic Principles

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Introduction

Methods

Transthoracic Echocardiography Analysis

- Transthoracic echocardiography was performed at baseline and 9 weeks post-injections
- Increased Ejection Fraction and Radial Strain were detected at 9 weeks in doxorubicin treated Smad3 deficient mice, as compared to wild type animals
- Contractile decline was observed in doxorubicin treated wild type (Smad3+/+) but not Smad3 deficient (Smad3+/− and Smad3−/−) mice
- *, P<0.05 compared to saline treated mice of the same genotype

Cardiac Fibrosis Analysis

- Cardiac fibrosis was analyzed in Smad3+/+ and Smad3+/− mice
- Cardiac samples were stained with trichrome to identify regions of fibrosis
- Area of fibrosis was quantified in both groups as percent of total area

Cardiac Weight Analysis

- Heart weight was taken at 2-day and 9-week time points
- After normalization to body weight, there was no statistically significant change in heart weight

Conclusion

Results 1

Results 2

Osteopathic Principles
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Results 1

Results 2

Osteopathic Philosophy:

Human structure and function are united through four main principles:

1. The body is a unit. The person is a combination of body, mind, and spirit.
2. The body is capable of self-regulation, self-healing, and maintenance.
3. Structure and function are reciprocally interrelated.
4. Rational treatment is based on an understanding of the forementioned principles.

Osteopathic Approach to Chemotherapy Research:

• Pharmaceutical chemotherapy treats the immediate disease while harming the patient
• Many treatments cause delayed side effects that negatively impact the quality of the patient’s life
• Treating the entire patient means alleviating primary and secondary forms of disease
• Goal of this research is to improve long term quality of life for those treated with Doxorubicin

Conclusion
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Introduction

• Transthoracic echocardiography analysis demonstrated a decrease in contractility in Wild Type mice but not Smad3 deficient mice.
• Doxorubicin’s deleterious effect on cardiac function is partially mitigated in Smad3 deficient mice.

Methods

Cardiac Function

• Transthoracic echocardiography analysis demonstrated a decrease in contractility in Wild Type mice but not Smad3 deficient mice.
• Doxorubicin’s deleterious effect on cardiac function is partially mitigated in Smad3 deficient mice.

Microvessel Changes

• The microvessel diameter was increased in Doxorubicin-treated Wild Type mice.
• Microvessel density did not change upon administration of Doxorubicin in Wild Type, Heterozygous, or Homozygous Knockout mice.
• Microvessel diameter dilation appears to be a proximal event in Doxorubicin-induced vascular injury.

Cardiac Cachexia and Fibrosis

• There was no statistically significant change in cardiac cachexia and fibrosis upon Doxorubicin administration between Wild Type, Heterozygous, and Homozygous knockout mice.

Arteriole Density

• There was no statistically significant change in Arteriolar Density upon Doxorubicin administration between Wild Type, Heterozygous, and Homozygous knockout mice.
• Loss of smooth muscle may not be a proximal event in the pathogenesis of Doxorubicin-induced cardiomyopathy.

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Small molecular weight inhibitor SB431542

Smad3 knockout
Co-culture assay set-up

- Culture media
- Endothelial Cells
- Fibroblasts

Bar graph:
- control
- SB
- dox
- SB+dox

- Tube length (μm)/well

Images:
- -Dox
- +Dox
- -SB
- +SB

Scale bar: 100 μm
Smad3^{+/-}  Smad3^{-/-}  Smad3^{+/+}  Smad3^{+/-}  Smad3^{-/-}  Smad3^{-/-}  Smad3^{-/-}

Mutant allele

WT allele

Smad3

GAPDH

fold change in Smad3

Smad3^{+/-}  Smad3^{+/-}  Smad3^{-/-}  Smad3^{-/-}  Smad3^{-/-}