Investigating the Cardiotoxic Behavior of Doxorubicin on the ECM Through CCN2 Expression in Cardiac Fibroblasts

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BACKGROUND

The American Cancer Society® estimates over 1.9 million people in the US will be diagnosed with cancer, and roughly 600,000 people will lose their battle to cancer in 2022. Consequently, patients who undergo cancer treatment will present with an array of adverse drug reactions. One adverse effect, cardiotoxicity, negatively impacts cardiac function as a result of myocardial damage or ventricular function.[1] Doxorubicin (DOX) is an anthracycline chemotherapeutic that is commonly used and has shown to have a dose-dependent adverse effect on the heart through a variety of proposed mechanisms.[2] The extracellular matrix (ECM), an essential component of the heart, is a key regulator in cardiac remodeling, provides structural integrity, and helps maintain homeostasis of the heart.[3] Components that make up the ECM are produced in part from the expression of Cellular Communication Network Factor 2 (CCN2).[4] We investigated doxorubicin adverse effects on the cardiac extracellular matrix by means of disruption of ECM depositor, CCN2, in NIH3T3 mouse embryonic fibroblast and primary cardiac fibroblast.

METHODS

Cell Culture: NIH3T3 and mouse primary cardiac fibroblast cell lines were cultured. Brought to confluency prior to seeding experimental dishes using proper media and vitamin C spiked media during treatments. All treatments lasted 24 hours.

Cytotoxicity: NIH3T3 cells were seeded onto a 96-well plate and treated with an array of DOX concentrations. Using alamarBlue™ resazurin dye with microplate reader reading fluorescence at 560/590nm wavelength to achieve cell viability.

Protein Isolation: NIH3T3 and mouse primary cardiac fibroblast were lysed with RIPA buffer containing protease inhibitors and EDTA. Cell lysates were then sonicated and centrifuged at 14Gs at 4°C for 15 minutes. Supernatants collected and BCA assay performed to determine sample protein concentration.

Western Blot: Total protein cell lysate samples were loaded in 4-12% gradient SDS-PAGE, ran for 45 minutes, then transferred onto PVDF membranes. Membranes were immunoblotted with primary antibodies for CCN2 and related proteins along with house keeping protein, β-actin. Membranes were imaged on LiCor Odyssey CLx imager after secondary antibody incubation and wash steps. Densitometry analysis was used after imaging.

RNA Isolation: RNA was isolated from NIH3T3 cells using Qiagen RNeasy kit.

RT-qPCR: RNA was as transformed into cDNA using SYBR Green qPCR master mix. cDNA was loaded on precoated 96-well plate from Qiagen, RT[2] Profiler Array TGF-BMP signaling pathway plate.

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RESULTS

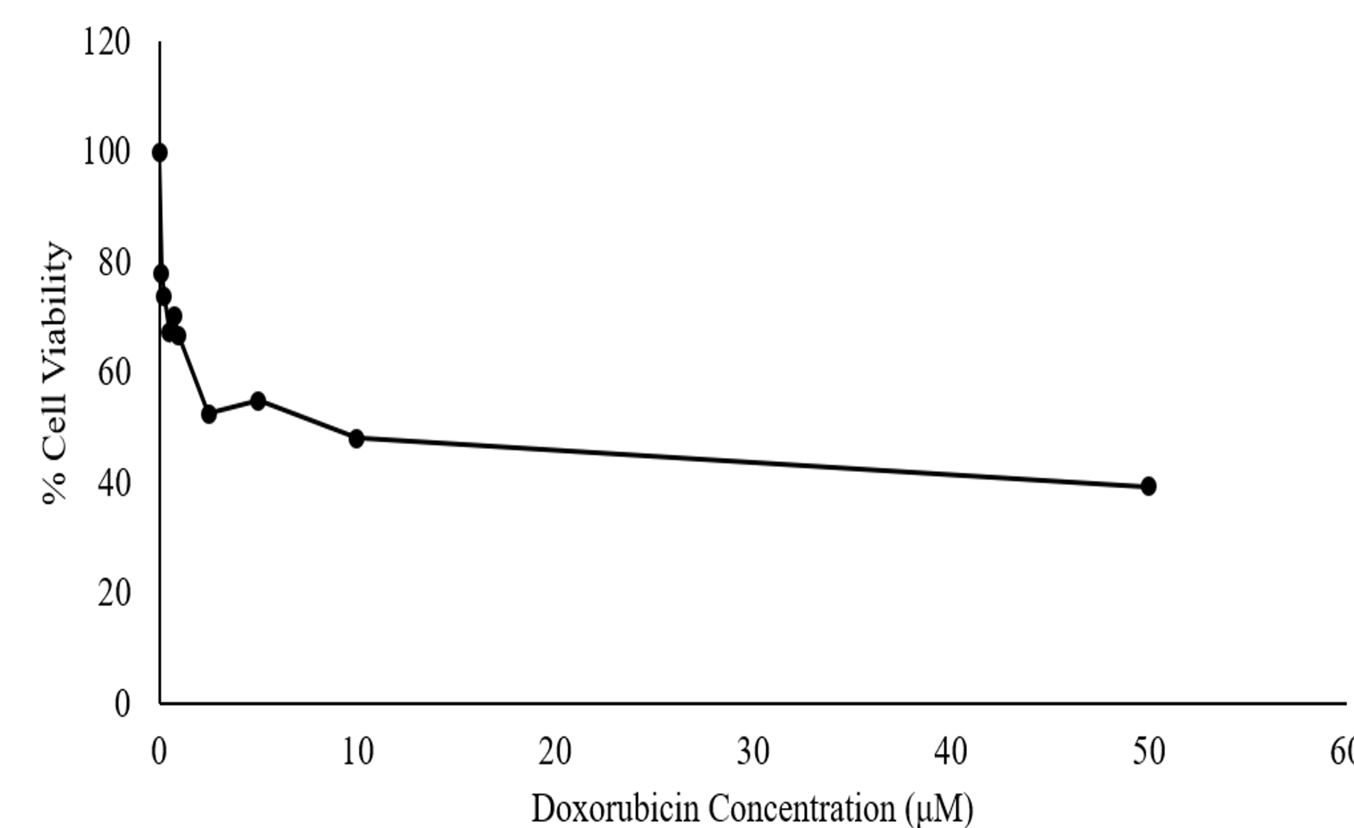

Figure 1. NIH3T3 alamarBlue™ cytotoxicity assay with increasing DOX concentrations. Cell viability is shown to decrease with increasing concentrations of DOX. (n=8)

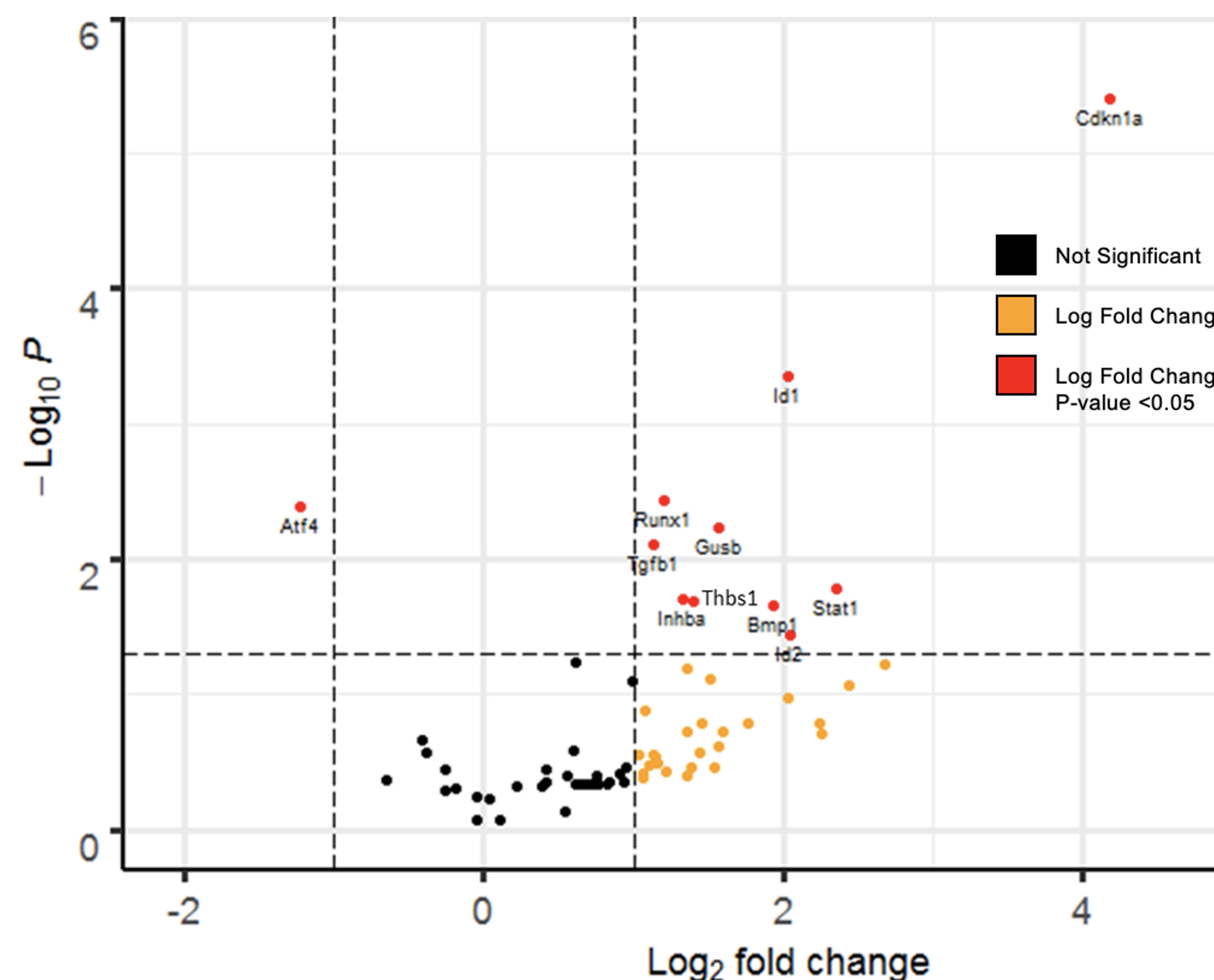

Figure 3. RT[2] Profiler Array TGF-BMP signaling pathway PCR plate use for NIH3T3 cells treated with and without DOX and TGF-β1. Ten genes showed upregulation in the presence od DOX while only one gene was downregulated.

Increased Gene Expression		
Gene	Fold Regulation	P-Value
Bmp1	3.80	0.022058
Cdkn1a	18.04	0.000004
Id1	4.09	0.000446
Id2	4.13	0.036558
Inhba	2.15	0.019780
Runx1	2.31	0.003689
Stat1	5.11	0.016429
Tgfb1	2.19	0.007815
Thbs1	2.63	0.020415
Gusb	2.95	0.005892
Decreased Gene Expression		
Gene	Fold Regulation	P-Value
Atf4	-2.32	0.004158

Table 1. Genes, p-values, and log fold changes for upregulated and downregulated genes from RT[2] Profiler Array. Of the 89 total genes, eleven genes were displayed for their significance (p<0.05).

RESULTS (CONT.)

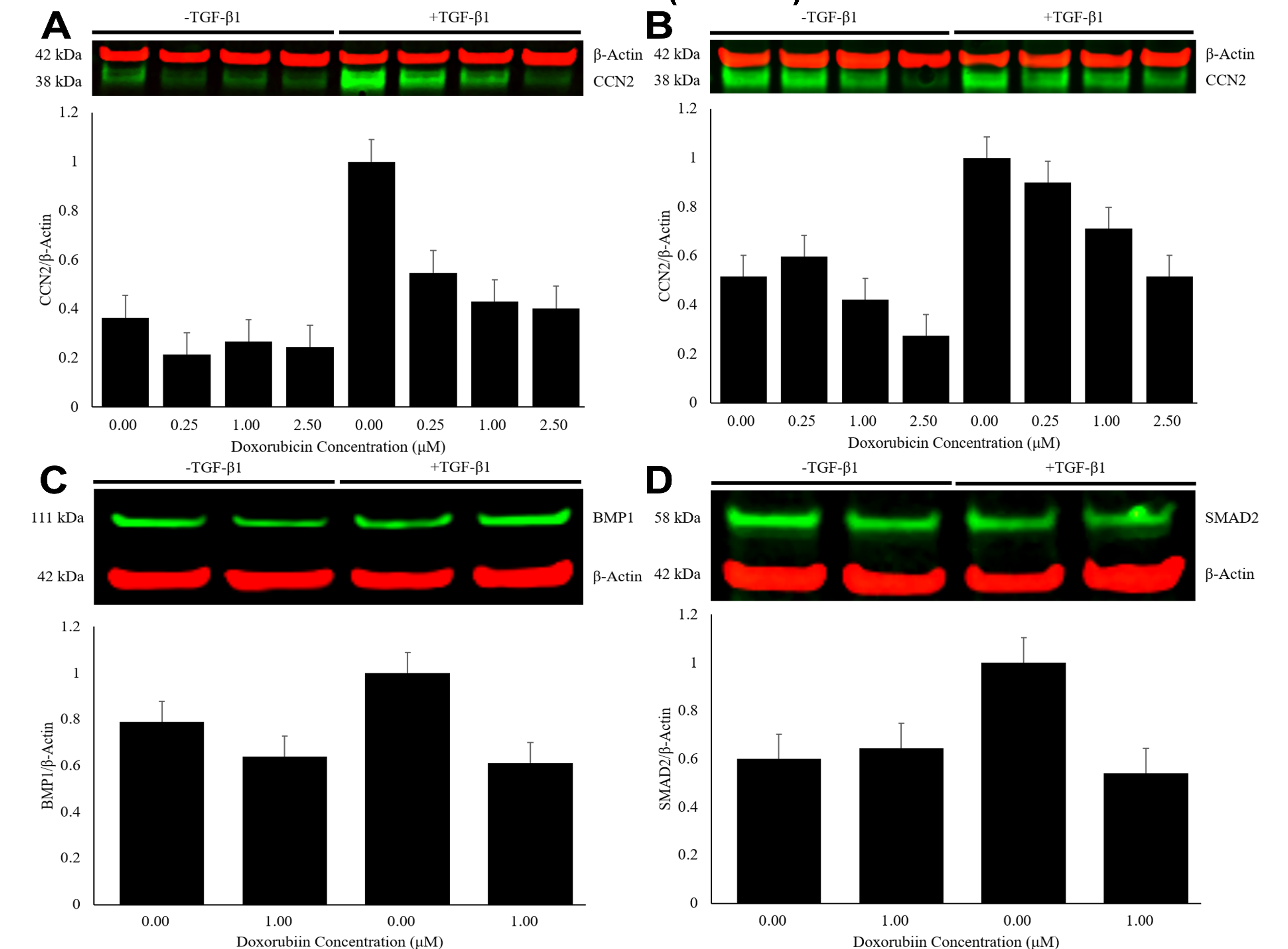

Figure 2. Effects of DOX treatments on CCN2 and related proteins. Western blot and densitometry analysis (A-D). β-actin utilized as housekeeping protein. TGF-β1 was used as a positive control. (A) NIH3T3 cells treated with varying doses of DOX for 24 hours. Densitometry reveals CCN2 decrease with increasing DOX concentrations. (B) Mouse primary cardiac fibroblast undergoing same treatments from (A). Similar trend in decrease in CCN2 expression as NIH3T3 cells. (C) NIH3T3 cells treated with and without DOX as well as TGF-β1 for 24 hours. Densitometry indicates decrease in BMP1 expression in the presence of DOX but requires statistical analysis to determine significance. (D) NIH3T3 cells undergoing same treatment as described in (C) probing for SMAD2. Densitometry reveals that DOX reduced SMAD2 expression.

CONCLUSION

DOX is a commonly used chemotherapeutic that has a potent effect on the heart. To better understand how potent DOX was to fibroblast, we conducted a cytotoxicity assay. In a cytotoxicity assay of NIH3T3 cells, we observed a maximum DOX concentration of 50 µM lead to >40% viability, allowed for maximum concentrations of 2.5 µM to be used in subsequent experiments. Western blots analyzing CCN2 for both NIH3T3 and primary cardiac cells indicated that CCN2 expression showed a similar trends when DOX concentrations increased. BMP1 and SMAD2 are inducers of CCN2 that appear to decrease in expression with DOX. Finally, RT-qPCR data from NIH3T3 cells on TGF-BMP signaling pathway shows a handful of genes that were upregulated and one gene that was downregulated. Future work would entail probing collagen and fibronectin proteins in western blots under same conditions to analyze ECM protein disruption from DOX.

References

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