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Article

Cytokine treatment for myocarditis may directly impact cardiomyocytes negatively

Jacob Kasner[1] and Leya Joykutty[1]
[1]American Heritage School, Plantation, Florida

SUMMARY

The purpose of our study was to determine if direct administration of CXCL1/KC to cardiomyocytes causes negative changes to cell density or proliferation. This molecule has been shown to reduce inflammation in certain instances. Homocysteine models the direct effect of an inflammatory agent on cardiomyocytes. Our question was whether these molecules directly impact cell density through an interaction with the cell proliferation process. We hypothesized that cells treated with CXCL1/KC would maintain the same cell density as untreated cells. In contrast, cells treated with Homocysteine or both Homocysteine and CXCL1/KC, were expected to have a higher cell density that than that of untreated cells. To test these hypotheses, HL-1 cardiomyocytes were cultured and treated in chambers on a glass slide. DAPI staining, which allows individual nuclei to be counted, was viewed under fluorescence and pictures were analyzed using Image J. Our hypotheses were rejected because there was a significant difference between the Homocysteine group and both the Homocysteine+CXCL1/KC and CXCL1/KC groups. This was extended to signify that the cell density of the CXCL1/KC and Homocysteine+CXCL1/KC groups was decreased cell proliferation had been reduced. The difference between the CXCL1/KC and Homocysteine groups likely indicates effects possessed by each treatment. Comparing results from the Homocysteine+CXCL1/KC treatment to the Homocysteine treatment, Homocysteine treatment increased cell density enough so that the decreased proliferation which may have been caused by the CXCL1/KC was significant compared to the Homocysteine sample. Future studies could examine additional applicable models such as mouse heart tissue or a living mouse.

INTRODUCTION

In general, inflammation is not a disease and can be beneficial in the healing process. For example, the inflammatory process is crucial when responding to an infection. In the most basic sense, it is the action of the immune system flooding the affected area with blood, fluid, and proteins that create swelling and heat to heal the damage. The impact of inflammation occurs at the cellular and organ system levels (1). The immune system begins the inflammatory process when sentinel cells signal to macrophages, B-cells, and T-cells that will directly address the infection. As this is occurring, the cardiovascular system leaks plasma from its capillaries to slow down the infection (1). This process indirectly involves cardiomyocytes. The issue arises when there is a constant low-level of inflammation caused by a hyperactive immune system that can result in myocarditis.

Myocarditis is an inflammatory disease of heart muscle. One of the most common causes for the disease is viral infection (2). Despite not being as widely known as atherosclerosis or tuberculosis, myocarditis is one of several diseases that threatens many members of today's society. Furthermore, myocarditis is known to cause dilated cardiomyopathy, which is more serious and better-known (3). According to one study, dilated cardiomyopathy can be defined as a loss of function in one or both ventricles and dilation that cannot be explained through the presence of other conditions such as coronary artery disease (4). In simpler terms, the disease encompasses the full or partial loss of contraction in at least half of the heart, as well as the enlargement of heart muscle. This loss of function can lead to blood clots, heart failure, or even sudden death. All these effects are consequences of the inflammatory process.

Myocarditis is difficult to diagnose because it presents with symptoms such as chest pain, shortness of breath, and abnormal rhythms which can be indicative of other cardiovascular diseases (5). As a result, many cases go undiagnosed. For some variants of myocarditis, there is less than a 20% chance of survival due to underdiagnosis and lack of treatment (5). Currently, the accepted treatment for myocarditis is immunosuppressive therapy, which has succeeded in improving the quality of life of afflicted patients (6). However, this treatment brings down the walls of protection that patients previously had against familiar pathogens like the common cold. Due to this, otherwise harmless infections can be lethal for immunosuppressed patients. To circumvent this issue, identifying another method of treatment would be ideal. Considering their expansive role in the immune system, we hypothesized that cytokines could be a potential treatment.

Many cytokines are known to have roles in the inflammation pathway. These cytokines are typically involved in the recruitment of neutrophils to increase inflammation. Chemokines, a variant of cytokines, interact with receptors to activate intracellular signaling pathways. Many of them recruit monocytes and are thus pro-inflammatory in nature. CXCL1/

KC is a chemokine that is not pro-inflammatory; rather it blocks antibodies and increases neointimal formation, reducing inflammation. CXCL1/KC was found to abrogate inflammation of cardiac muscle cells (7). Other researchers that have worked with CXCL1/KC in the context of the cardiovascular system have shown that 100 nmol of the cytokine is a physiologically relevant dose (personal communication). CXCL1/KC has been found to have the opposite effect on cardiac muscle cells. CXCL1/KC belongs to a group of cytokines known as inflammatory cytokines. These molecules have recently piqued the interest of the cardiovascular research field. They are now known to be involved in the development of diseases such as atherosclerosis, the cardiac dysfunction in systemic sepsis, and viral myocarditis (8)." Homocysteine was recognized as a risk factor for the presence of atherosclerotic vascular disease and hypercoagulability states in the early 1990s due to its molecular products, superoxide and hydrogen peroxide (9). Homocysteine causes oxidative damage to endothelial cells, the cells that line the walls of the blood vessels. The presence of increased levels of hydrogen peroxide recruits monocyte infiltration in vascular smooth muscle cells, contributing to vascular inflammation (10). Superoxide facilitates the hypertrophic remodeling of vascular smooth muscle cells (11). Hyperhomocysteinemia is known to be an independent risk factor for ischemic heart disease and stroke, as well as atherosclerosis (12).

At normal levels, Homocysteine has no negative effect on the body. It is only when Homocysteine levels rise above 15 umol/L that an issue arises. Blood Homocysteine levels can rise above 100 umol/L, a condition known as severe hyperhomocysteinemia (HHcy), through an enzyme mutation that occurs in one in every hundred thousand live births (13). The mutation is one of N5, N10-methylenetetrahydrofolate reductase (MTHFR). Because 5,10-MTHF is catalyzed into folate. the molecule utilized in the remethylation of Homocysteine, the mutation is a clear cause of hyperhomocysteinemia. A mutation of MTHFR is the most common inborn error of folate metabolism. It is also the most common genetic cause of hyperhomocysteinemia because it prevents the reduction of Homocysteine levels, leading to its accumulation in the heart (13).

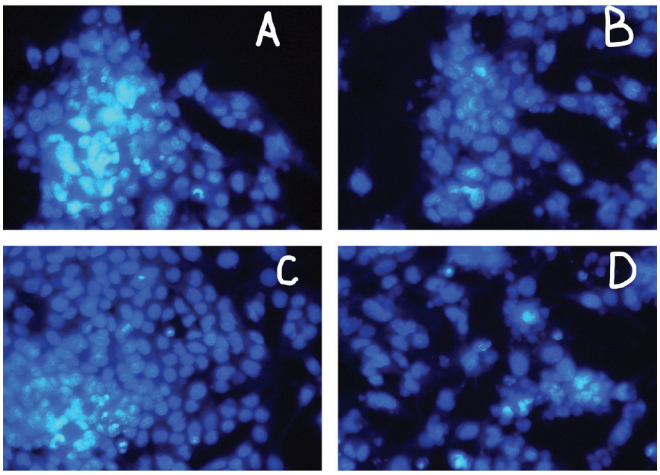

Figure 1: Fluorescent images of each experimental group. Images are labelled according to the experimental group they represent. A represents the control, B represents the CXCL1/KC treatment group, C represents the Homocysteine treatment group, and D represents the Homocysteine + CXCL1/KC treatment group.

Trial	Control	CXCL1/ KC	Homo- cysteine	Homocysteine + CXCL1/KC
1	61.026	25.050	70.743	22.417
2	51.239	28.075	83.737	34.603
3	51.405	25.834	57.788	21.080
4	60.407	78.769	59.814	64.667
5	83.286	37.371	72.112	54.715
6	46.118	65.960	71.297	71.042
7	31.782	71.657	75.772	58.015
8	54.112	31.083	44.638	48.793
9	60.833	12.364	78.481	66.866
10	68.061	72.980	56.727	45.231
11	35.415	58.318	66.875	44.417
12	33.843	59.745	79.869	49.003
13	63.731	72.205	61.655	63.750
14	65.789	38.264	55.946	82.382
15	75.546	49.854	75.979	77.667
16	83.205	56.899	67.429	22.417
17	53.577	74.396	79.639	34.603
18	61.026	51.544	48.515	21.080
19	74.146	63.758	82.348	64.667
20	84.175	25.834	52.859	54.715

Table 1: Average field fluorescence (AU) by trial. This table displays the average field fluorescence values for each trial obtained using ImageJ.

HL-1 cells can be serially passaged, yet they maintain the ability to contract and retain differentiated cardiac morphological, biochemical, and electrophysiological properties (14). For these reasons, HL-1 cardiomyocytes will be the test model for the following experiment. A classical immune response would involve the recruitment of T- and B-cells to the site of inflammation by sentinel cells (1). However, due to the lack of these immune cells in the culture, a classical immune response will not be produced. Therefore, this study will focus on the direct effect of CXCL1/KC on cardiomyocytes. Homocysteine will be used as a model for the direct effect of an inflammatory agent on cardiomyocytes. It is expected that cells treated with CXCL1/KC will maintain the cell density of untreated cells; if cells were treated with Homocysteine or both Homocysteine and CXCL1/KC, then they will have a cell density that was higher than that of untreated cells.

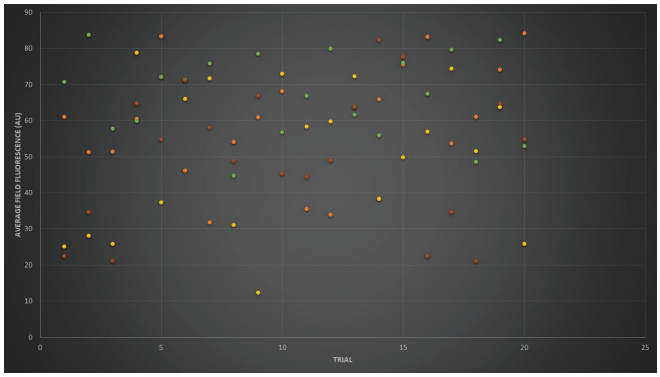

Figure 2: Average field fluorescence (AU) by trial. This scatterplot displays the data acquired through recording the mean fluorescence value as measured by Image J for each image taken by a fluorescent microscope following treatment. Orange represents the control, yellow represents the CXCL1/KC treatment, green represents the Homocysteine treatment, and brown represents the Homocysteine + CXCL1/KC treatment.

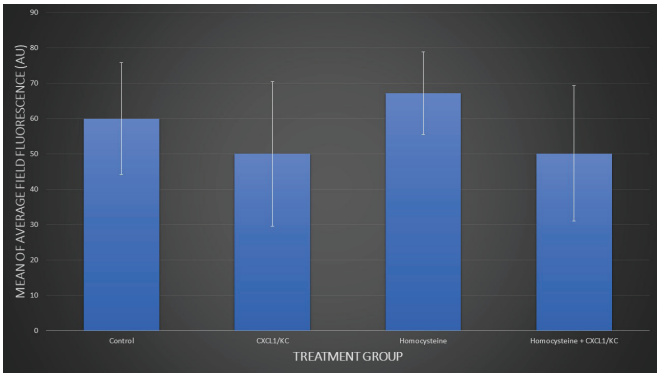

Figure 3: Mean average field fluorescence (AU) of each treatment group. This bar graph concisely displays what the effects of each treatment were on average field fluorescence by displaying the mean of each treatment group. The bars are representative of the standard deviation for each group.

RESULTS

The question of whether CXCL1/KC treatment negatively affects cardiomyocytes, signified by a decrease in cell density, was tested by measuring cell density of HL-1 cardiomyocytes treated with CXCL1/KC, Homocysteine, both in combination, or neither. The cells were visualized with DAPI, a blue nuclear stain, yielding a representation of cell density based upon presence of DAPI in the view window. Each field of view revealed cells with their nuclei as blue circular objects and the absence of cells as complete darkness (**Figure 1**). The average field fluorescence value, a relative measure of cell density, was recorded for twenty fields of view for the experimental groups with CXCL1/KC, Homocysteine, both, and neither which had ranges of average field fluorescence values of 12.364-78.769, 44.638-83.737, 21.080-82.382, and 31.782-84.175, respectively (**Table 1**).

The data collected varied greatly within each group due to the nonspecific method of measuring cell density. Viewed individually, the data points appear loosely correlated and the ranges for each group overlap (**Figure 2**). Furthermore, the standard deviations for the experimental groups with CXCL1/KC, Homocysteine, both, or neither are large at 20.409, 11.651, 19.186, 15.772 (**Table 2**). Still, when the values are averaged, distinct trends can be identified between the test groups. An order of mean average field fluorescence can be established from least to greatest as CXCL1/KC, CXCL1/KC + Homocysteine, Control, and then Homocysteine with means of 49.998, 50.107, 59.936, and 67.111, respectively (Table 2, Figure 3). The decrease in average field fluorescence (and thus cell density) in both the CXCL1/KC treatment group and the CXCL1/KC + Homocysteine treatment group compared to the control suggests that cardiomyocyte growth and survival is impeded by cytokine treatment. Additionally, the increase in average field fluorescence in the Homocysteine treatment group compared to the control suggests that cell density had increased. This likely occurred as a result of the non-classical immune response provoked by the administration of Homocysteine.

Group	Sum	Mean	Variance	Std Dev
Control	1198.72	59.936	248.76	15.772
CX	999.96	49.998	416.52	20.409
Hcy	1342.22	67.111	138.08	11.651
Hcy+CX	1002.13	50.107	368.10	19.186

Table 2: Summary of Statistics. This table shows the mean of the average field fluorescence values (AU) for each experimental group (N=20 for all groups), the sum of all values for each group, the mean average field fluorescence per group, variance, and standard deviation. This information summarizes the general statistics about the data collected. Ctrl=Control, CX=CXCL1/KC, Hcy=Homocysteine, and Hcy+CX=Homocysteine + CXCL1/KC.

Each group had a high standard deviation: 20.409, 11.651, 19.186, and 15.772 for CXCL1/KC, Homocysteine, both, and neither, respectively, and some overlap between standard deviation bars (**Table 2, Figure 3**). Additionally, one-way ANOVA analysis confirmed that there were significant differences between the groups ($F_{3,76}=4.72$, $P=0.0045$) (**Table 3**). Tukey HSD revealed that the significant differences existed between the CXCL1/KC group and the Homocysteine group, as well as the Homocysteine + CXCL1/KC and the Homocysteine group, for a Q critical value of 3.74 (**Table 4**). Although there were no significant differences between experimental treatments and the control, the differences amongst experimental groups can be extended to imply differences between an experimental group and the control group. Although the CXCL1/KC treated cells were expected not to differ from the control group, they had a significantly lower cell density compared to the Homocysteine group. From these results, we can discern that CXCL1/KC did influence cell density. Additionally, the CXCL1/KC + Homocysteine treatment group also had a significantly lower cell density compared to the Homocysteine group. This strengthens the finding that Homocysteine increased cell density and CXCL1/KC decreased it, even when CXCL1/KC was administered

with Homocysteine.

DISCUSSION

We initially hypothesized that if cells were treated with CXCL1/KC, then cell density would not differ from that of untreated cells. On the other hand, if cells were treated with Homocysteine or both Homocysteine and CXCL1/KC, we predicted that they would have a cell density that was higher than that of the untreated cells. We expected that CXCL1/KC would not alter the cell density because it was expected to only affect inflammation (not modelled here). We expected that Homocysteine would increase cell density because it has been shown to induce inflammation and thus cell proliferation. We also expected that combining Homocysteine with CXCL1/KC would have the same effect as Homocysteine alone because CXCL1/KC was predicted to have no effect.

The first part of our hypothesis, that cells treated with CXCL1/KC will maintain the cell density of untreated cells, was not supported because cell density in CXCL1/KC treated group was significantly decreased compared to the Homocysteine treated group. Given that the control group's average cell density was between the two, it implies that cell density decreased in the CXCL1/KC group and increased in the Homocysteine group compared to the control to create the significant difference between the CXCL1/KC and Homocysteine groups. The second part of our hypothesis, that if cells were treated with Homocysteine or both Homocysteine and CXCL1/KC, then they will have a cell density that was higher than that of untreated cells, was rejected because although treatment with Homocysteine alone resulted in a significant increase when compared to the CXCL1/KC group, the CXCL1/KC + Homocysteine group experienced a significant decrease in cell density when compared to the Homocysteine group.

The cytokine appears to have significantly decreased cell density both by itself and when administered to cells with Homocysteine treatment. Although the Homocysteine group only had a trending increase when compared to the control, this increase was compared to the other two experimental groups. The control itself was an intermediate between the two ends of the cell density spectrum. Both groups that had CXCL1/KC experienced a relative decrease in cell density, which may have been the result of slowed cell proliferation. The direct effect of CXCL1/KC seems to be beneficial, as it would counteract the cell proliferation associated with the inflammatory response to Homocysteine. Yet, it is concerning that even though Homocysteine treatment increased cell density alone, cell density was decreased overall with the combination treatment. If this result is not attributable to increased cell stress resulting from the application of two treatments, then it may signify that CXCL1/KC directly reduces cell density of cardiomyocytes.

Minor errors may have occurred due to the nature of the measuring instruments used. When using a micropipette to take up small volumes of 15 µl of treatment, we may have taken up slightly more or less volume due to the adhesion of the solution to the end of the pipette tip. Furthermore, when capturing images of the stained cell samples, the movement of the slide required repeated refocusing of the microscope. Due to an inability to refocus to the exact same level each time, although it was nearly the same each time, certain images may have been a little more focused than others, potentially having a small impact on the fluorescence readings.

CXCL1/KC may be a promising treatment for myocarditis. CXCL1/KC has previously been shown to successfully reduce cardiac inflammation, and this experiment revealed that the treatment of myocarditis with CXCL1/KC directly reduces cell proliferation. The treatment reduced cell density of the cardiomyocytes, which would be favorable in the context of reducing inflammation but may be detrimental at the level observed in this study. In the future, researchers could investigate the issue of inflammation induced by Homocysteine and the possibility of reducing this inflammation with CXCL1/

Source of Variation	SS	df	MS	Std Dev
Between	4144.49	3	1381.5	15.772
Within	22257.7	76	292.86	20.409
Total	26402.8	79		11.651
F	4.71719			
F crit	2.724944		p-value	0.004506

Table 3: ANOVA Test Results. This table shows the data from the ANOVA one-way statistical test including degrees of freedom and the F critical value.

Comparison	Abs. Difference	Std Error	Q stat	Null hypothesis	Q critical
Ctrl-CX	9.9381	3.827	2.597	Accept	3.74
Ctrl-Hcy	7.1751	3.827	1.875	Accept	
Ctrl-(Hcy+Cx)	9.8296	3.827	2.569	Accept	**df**
CX-Hcy	17.113	3.827	4.472	Reject	60
CX-(Hcy+CX)	0.1085	3.827	0.0283	Accept	
Hcy-(Hcy+CX)	17.005	3.827	4.444	Reject	

Table 4: Tukey HSD Results. This table shows the data from the Tukey HSD test and reveals that significant differences exist between the Homocysteine group and both the CXCL1/KC group and the Homocysteine + CXCL1/KC group. Ctrl=Control, CX=CXCL1/KC, Hcy=Homocysteine, and Hcy+CX=Homocysteine + CXCL1/KC.

KC in a more complex model than a simple cell culture. With either mouse tissue or a mouse heart in culture, a study could evaluate the classical immune response to Homocysteine and get a more comprehensive view of the effects of each treatment. Beyond this, a study could focus on the relationship between cardiomyocyte density and heart function since past research has shown that myocarditis reduces heart function. This would bridge the gap between the speculative results of the reduction of cell density seen in our study and more definitive conclusions regarding whether heart function is affected to determine if CXCL1/KC would be effective for reducing inflammation without negative consequences. Such a study would ensure that CXCL1/KC does not worsen heart function or confirm that it does have the detrimental effects suggested by this study.

MATERIALS AND METHODS

The HL-1 Cardiomyocytes were first cultured in T25 flasks (NEST) with the Claycomb's medium (Sigma Aldrich). The protocols found on the product data sheet were followed to supplement the medium with Fetal Bovine Serum, L-Glutamine, and Penicillin-Streptomycin. After several T25 flasks of HL-1 cardiomyocytes had reached confluency, the number of cells in each flask was determined using a hemocytometer (Thermo Fisher). Half a million cells were transferred into each chamber of a four-chambered cell culture slide (Pipette.com) with 500 microliters of Claycomb's medium.

The cells were left to grow in a 37° Celsius incubator with 5% CO2 and attach to the slide for three days in which most cells experienced attachment. Afterwards, each treatment was administered to its respective experimental group. The control group received no treatment. The CXCL1/KC group received CXCL1/KC (R&D Systems) solution, dissolved in 1X PBS, to make a 100 nanomolar concentration of CXCL1/KC in the medium. We determined this concentration was appropriate after consultation with other researchers that had experience working with CXCL1/KC. The Homocysteine group received DL-Homocysteine (Sigma Aldrich), dissolved in 1X PBS, to make a 100 micromolar concentration of DL-Homocysteine in the medium. This is a physiologically relevant concentration according to research done at the University of Nebraska (15). Finally, the CXCL1/KC + Homocysteine group received CXCL1/KC and Homocysteine dissolved in PBS to make a solution with the medium of 100 nanomolar CXCL1/KC and 100 micromolar DL-Homocysteine.

After 24 hours, all media was aspirated from the chambers and the cells were fixed using a 4% formaldehyde solution (Sigma Aldrich). Ten minutes later, formaldehyde was aspirated from each chamber and the cells were washed twice with ice cold 1X PBS. Then cells were permeabilized for 10 minutes using 0.01% Triton X-100 solution (Sigma Aldrich). Finally, the cells were incubated with a 1 microgram/milliliter concentration of DAPI (Biolegend) in 1X PBS for 15 minutes at 37 degrees Celsius and in darkness. After incubation, the DAPI solution was aspirated and any remnant DAPI was removed by 3 washes with 0.1% Tween 20 solution (Sigma Aldrich) in 1X PBS (PBST).

Before putting the slide under the fluorescent microscope, 100 microliters of 1X PBS were added to each chamber to prevent the samples from drying out. The slide was then placed on the stage of the fluorescent microscope in a dark room with the fluorescent violet light on. The cell imaging monitor (EVOS) was then used to visualize and capture images of each chamber. Ten images were taken of each chamber by moving to another area of the chamber and refocusing after each capture. The process was repeated twice to yield 20 images for each experimental group.

The images were analyzed using ImageJ software through Fiji (16). They were individually uploaded to the program and the mean feature of ImageJ was used to determine the average fluorescence of the field of view, referred to as average field fluorescence in this study. This value was used as a relative measure of cell density for the purposes of this research. Statistical analysis was performed by one-way ANOVA and Tukey HSD. A Q critical value of 3.74 was used for 60 degrees of freedom and a p-value of 0.05.

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