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Original Research Article

Effect of Aerobic Exercise on Left Ventricular Connexin43 Expression and Distribution in Juvenile and Young Adult Rats

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Abstract

Background: Gap Junction (GJ) plays a role in supporting the heart electricity. Connexin43 (Cx43) as the main protein constituent of GJ in left cardiac ventricle, will increase in number and slightly redistributed to the lateral sides of cardiomyocytes after aerobic exercise in adulthood. The effects of aerobic exercise that begin at childhood are not well known.

Objective: This study aims to observe the effect of aerobic exercise which started from childhood on left ventricle Cx43 distribution.

Methods: This study was conducted on 28 male Juvenile (4 weeks) and young adult (8 weeks) rats, divided into 7 groups: 1) Juvenile rats undergoing 4 weeks of exercise (E-J4); 2) Control E-J4 (C-J4); 3) Juvenile rats undergoing 8 weeks of exercise (E-J8); 4) Control EJ-8 (C-J8); 5) Juvenile rats undergoing 12 weeks of exercise (E-J12; 6); Young adult rats undergoing 8 weeks of exercise (E-Yo8); 7) Control E-J12 and E-Yo8 (C-JY128). Exercise group will undergo different length of duration, starting from week 4 until 12 weeks. Cx43 was identified by immunohistochemical staining (Anti-Connexin43, Abcam Ab11370) and analyzed with ImageJ software. Comparison was analyzed using independent t-test.

Results: Insignificant lower of total Cx43 expression in E-J4 (64200.45 $\pm$ 4243.676 total area, $p > 0.05$) compared to control. In contrast, a significant higher of total Cx43 expression was observed in EJ-8, EJ-12 and E-Yo8 (80152.95 $\pm$ 3760.481, $p = 0.001$; 75596.775 $\pm$ 3976.333, $p = 0.002$; 81216.85+ 2475.768, $p = 0.000$). Slightly higher of lateral Cx43 redistribution occurred in all aerobic exercise, with significant lateralization in E-J8 and E-Yo8.

Conclusion: Aerobic exercise increases Cx43 and slightly redistributed to lateral myocytes under normal condition both in juvenile and young adult rats.

Keywords: Aerobic exercise; left ventricle; connexin43; juvenile; young adult.
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INTRODUCTION

Aerobic physical activity can improve overall health of cardiovascular system.[1] WHO and the American Heart Association (AHA) recommends that aerobic physical activity should begin since childhood, in order to maintain cardiovascular health in adulthood.[2,3] Aerobic exercise is part of a programmed aerobic physical activity.[4] Aerobic exercise in children should be carried out with the intensity gradually increased and maintained at moderate intensity.[5]

Various physiological adaptations occur in the body of individuals who perform aerobic exercise.[6,7] Heart can adapt to aerobic exercise through increased size.[8] Cardiac hypertrophy can be broadly defined as an increase in cardiac mass.[8,9] Heart muscle adapts its size due to increased pressure and/or volume by hypertrophy.[10,11]

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Cardiac hypertrophy in response to exercise can be classified as physiological hypertrophy, partly reversible and characterized by normal morphology and cardiac function.[12] Communication between an increased cardiomyocytes size should be maintained.[13] An important communication between cardiomyocytes is conducted through gap junction (GJ).[13] GJ in the heart mainly serve to allow the rapid and coordinated heart electrical excitation travel from one cell to its adjacent cells, as a prerequisite for the rhythm of normal heart function.[13,14] GJ remodeling also occurs with increased formation of GJ in the response and adaptation to cardiac hypertrophy.[15]

GJ are normally found in many different structures and especially in the heart, are located at the end-site of the myocytes called intercalated disk.[13] This structure also contains many desmosomes and adherent junctions, which provides mechanical stability to the cardiac network.[14] Connexin (Cx) is a major protein constituent of gap junctions.[16] Cx43 is the major connexin isoform in GJ of left ventricular cardiomyocyte.[17] Impaired Cx43 expression and redistribution of location in heart's ventricles are observed in many cardiac diseases.[18] Disorders are often characterized by a down-regulation of total Cx43 expression and heterogeneously redistributed to the lateral sides of cardiomyocytes under histological observation,[19] these are referred as lateral Cx43.[20] Cx43 changes in expression and distribution can lead to changes in conduction velocity so as to contribute to cardiac arrhythmias.[20,21]

Aerobic exercise also causes changes in expression and distribution of Cx43.[22,23] Reviews on the benefits of physical exercise from childhood on cardiac function in adulthood has not been done, including the expression and distribution of Cx43 ventricles heart. The form of physical exercise done, including the frequency and duration of aerobic physical exercise is not very clear for children and younger age group, nor are the effects that will occur. The effects of physical exercise that are generally considered good in the adult group may present an opposite effect in younger age group. The aim of this study was to evaluate the effects of aerobic exercise that began in childhood (Juvenile in rats) and young adults, with different duration of exercise (but can still be considered as the chronic effects of aerobic exercise), on the expression and distribution of left ventricular Cx43.

MATERIALS AND METHODS

The protocol of this study has been approved by the Ethical Committee of the Faculty of Medicine, Universitas Indonesia/ Cipto Mangunkusumo Hospital (No.139/UN2.F1/ ETIK/2015).

Twenty-eight male Wistar rats (*Rattus norvegicus)* aged 4 weeks (juvenile) and aged 8 weeks (young adult) weighing 60-250 grams were divided randomly into seven groups: 1) Juvenile exercise group was given four weeks of aerobic exercise (E-J4); 2) Control E-J4 (C-J4); 3) Juvenile exercise group was given eight weeks of aerobic exercise (E-J8); 4) Control EJ-8 (C-J8); 5) Juvenile exercise group was given 12 weeks of aerobic exercise (E-J12); 6) Young adult exercise group was given eight weeks of aerobic exercise (E-Yo8); and 7) Control E-J12 and E-Yo8 (C-JY128).

Exercise group will undergo different lengths of training according to the age at which the physical exercise begins, starting from 4 weeks until 12 weeks, however the shortest duration of exercise may still be considered to provide chronic effects of exercise. This research was conducted in the Biomedical Science Laboratory of Health Ministry Republik Indonesia. Before and during intervention, the rats were maintained properly according to the ethics of experimental animal usage. Rats were given standardized food and water *ad libitum*. The cages were arranged 12 hours of light (18:00 pm to 6:00 am) and 12 hours of dark (6:00 am to 18:00 pm), thus aerobic exercise was conducted at night. Room temperature was set at 23°C ± 1°C. Before starting the exercise, rats were adapted to the environment for one week, then adapted to the treadmill (acclimation exercises) for 1 week. Rat exercise program started at aged 5 and 9 weeks old.

The acclimation stage is carried out before the physical exercise treatment begins, with a shorter duration than planned in aerobic exercise intervention. Running speed for juvenile and children (in humans) should be increased gradually and then maintained at a moderate intensity, with a frequency of five times a week.[23] All treatment groups followed the measured aerobic exercise protocol using an animal running treadmill at a speed of 9 meters/minute for 10 minutes each day and increasing until settling at a speed of 20 meters/minute for 10 minutes each day. All control groups are only placed on the non-operating animal treadmill for 10 minutes each day. This was done for the process of adapting the rats to physical exercise, and adapting the control by providing the same environment conditions. Treadmill speed was appropriately adjusted with rat's age.[24,25]

Furthermore, at the intervention stage of physical exercise, duration of exercise should be 20 minutes, with a 1.5 minute break every five minutes of running to avoid fatigue.[22] Training for juvenile group begins with a speed of 12 m/minute for the first two weeks, 15 m/minute for the following two weeks, and 20 m/minute for the next four weeks.[24] Exercises for the group of young adults were conducted at a speed of 20 m/minute from the first week until the last week.[24,25] Exercise implementation protocols are explained in table 1.

Aerobic exercise began at a speed of 12 meters/minute for 20 minutes, 5 times a week for the first and second week. The speed was then increased every 2 weeks and maintained after reaching a speed of 20 meters/minute (This speed is the limit speed for a moderate aerobic exercise). During the third and fourth week, aerobic exercise began at a speed of 15 meters/minute for 20 minutes, 5 times a week. On the fifth week, the exercise started at a speed of 20 meters/minute for 20 minutes, 5 times a week, until decapitated. Control groups: Rats were placed on the non-operating treadmill for 20 minutes. Protocols is modified from Hsu et al[24] and *Chondro et al.*[22]

Table 1. Aerobic exercise protocols

Group		Program (/week)											
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
E-J4	Speed (m/min)	12	12	15	15								
	Duration (minute)	20	20	20	20								
	Frequency (/week)	5	5	5	5								
E-J8	Speed (m/min)	12	12	15	15	20	20	20	20				
	Duration (minute)	20	20	20	20	20	20	20	20				
	Frequency (/week)	5	5	5	5	5	5	5	5				
E-J12	Speed (m/min)	12	12	15	15	20	20	20	20	20	20	20	20
	Duration (minute)	20	20	20	20	20	20	20	20	20	20	20	20
	Frequency (/week)	5	5	5	5	5	5	5	5	5	5	5	5
E-Yo8	Speed (m/min)	20	20	20	20	20	20	20	20				
	Duration (minute)	20	20	20	20	20	20	20	20				
	Frequency (/week)	5	5	5	5	5	5	5	5				

During the last aerobic exercise before decapitation, the rats were recuperated for 60 minutes to get the chronic effects of exercise and avoid the acute effects (22). The rats were then decapitated. The heart was taken, and the left ventricle was isolated. Left ventricles were placed in a container of 10% formalin buffer and allowed to stand at room temperature for 24 hours. After the tissues were fixed, tissue processing began with dehydration, clearing, and the making of paraffin blocks (embedding). The paraffin blocks were chilled on a cooling plate and the paraffin blocks were cut using a microtome with a thickness of 4-5 micrometers. Pieces of thin left ventricular tissues were attached to a glass slide. Slides were colored with immunohistochemical staining techniques (Abcam Ab11370 and Star Trek Universal HRP Detection System).

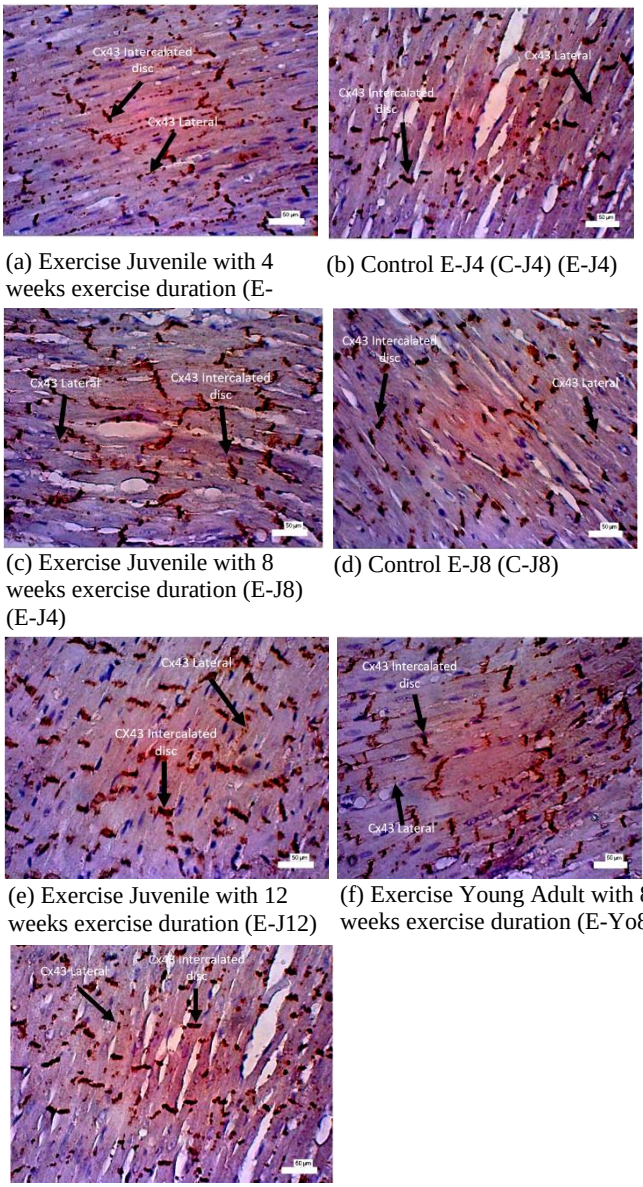
The positive cells expressing Cx43 were cells showing brown staining at the intercalated disk. The Cx43 expression found separately from the intercalary disc were called lateral Cx43. These slides were observed under a light microscope binocular (Olympus CH-20 binocular) with a 400x magnification and photographed by microscope build-in camera (Optilab), and five visual fields were taken randomly from each preparation. The photos were analyzed using imageJ software that will measure the total area of Cx43 expression and Cx43 distribution. This includes: Cx43 expression in the intercalary disc and Cx43 expression aside from the intercalary disc or defined as lateral Cx43. Cx43 distribution (in percentage) is obtained by dividing the total area of Cx43 with the area of Cx43 in the intercalary disc or non-intercalary disk area (lateral Cx43).

All data in the experiment were analyzed descriptive using averaged (mean), then the comparison between aerobic exercise group and the control were calculated using an independent t-test with $p < 0.05$ was taken as a limit of statistical significance.

RESULTS

Cx43 expression

Photographs of Cx43 expression with immunohistochemical staining in seven groups (exercise and control) are presented in Figure 1. The lower, although insignificant ($p = 0.153$) total Cx43 expression occurred in the EJ4 group compared to the control group (64200.45 $\pm$ 4243.676 total area).



(a) Exercise Juvenile with 4 weeks exercise duration (E-J4)

(b) Control E-J4 (C-J4)

(c) Exercise Juvenile with 8 weeks exercise duration (E-J8)

(d) Control E-J8 (C-J8)

(e) Exercise Juvenile with 12 weeks exercise duration (E-J12)

(f) Exercise Young Adult with 8 weeks exercise duration (E-Yo8)

(g) Control E-J12 dan E-Yo8 (C-JY128)

Figure 1. Immunohistochemical staining of left ventricle Cx43 after aerobic exercise in juvenile and young adult compared to the sedentary control. (Objective lens 40x).

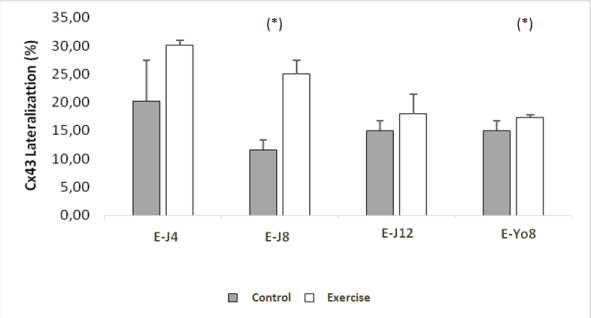
These photos represent each group, both from juvenile and young adult group and the control group. The result of this study showed a higher Cx43 expression in aerobic exercise group compared to the control, except in E-J4 group which show a non-significant lower Cx43 expression ($p > 0.05$). A higher

lateral Cx43 expression in lateral myocytes is also observed compared to the control group under normal conditions, due to an increased traffic of Cx43 production.

In contrast, a significant higher total Cx43 expression occurred in the E-J8, E-J12, and E-Yo8 (80152.95 ± 3760.481 total area, p = 0.001; 75596.775 ± 3976.333 total area, p = 0.002; 81216.85 ± 2475.768 total area, p = 0.000). The highest total Cx43 expression was found in the E-J8 group with a total value of 81216.85 total area (Figure 2).

Cx43 distribution

Figure 3 shows that the lateral Cx43 expression in all aerobic exercise juvenile groups (EJ4, EJ-8, EJ-12) and young adult EYo-8 (30.13%, 25.02%, 17.97% and 17.41%) is higher than the control groups (20.28%, 11.61%, and 15.08%). However, only the E-J8 and EYo-8 groups showed a significant difference with its respective control groups. Meanwhile, the difference was not significant in the E-J4 and E-J12 groups (p = 0.068, p = 0.187, p > 0.05) with the highest percentage of Lateral Cx43 observed in the E-J4 group (30.13%).



Mean value ± SEM, *Significant difference compared to the control group (p <0.05, unpaired t test)

Figure 3. Comparison of Lateral Cx43 expression between exercise group and sedentary control in juvenile (aged 4 weeks) and young adult (aged 8 weeks) rats.
Lateral Cx43 is presented as percentage from the Cx43 non-intercalary disc expression divided by total Cx43 expression of immunohistochemical staining measurement. This figure shows that a higher lateral Cx43 in all aerobic exercise compared to its control, with a significant difference in E-J8 and E-Yo8 (p < 0.05).

DISCUSSION

This study shows that remodeling of GJ, which is responsible for electrical conduction of the heart, caused by long-term aerobic exercise, is a dynamic and complex process. Moderate physical exercise is known to affect phosphorylated Cx-43 protein levels.[26] Experimental animals undergoing moderate aerobic exercise are known to have attenuation of Cx43 phosphorylation, then phospho-Cx43 can modulate gap junction performance.[26,27] Data analysis showed a higher total Cx43 expression (in a total area expression) compared to control group, along with a slightly higher lateral Cx43. Gap junctions can normally be found on the lateral side of cardiomyocytes in insignificant numbers and this is due to normal turnover of Cx43 production.[28] During Cx42 formation process, the Cx43 hemichannel will begin to enter the membrane from the lateral side, then trafficking towards the disc and joining the other Cx43 hemichannel from the adjacent cardiomyocyte cells (29). An increase Cx43 production can be followed by the increase a number of lateral Cx43. So, we still assumed that the slight increase in lateral Cx43 expression in this aerobic exercise program in juvenile and young adult, is a normal response.

Analysis results of this study showed a higher Cx43 expression after aerobic exercise both in juvenile (E-J8 and E-J12) and young adult (E-Yo8). These findings are consistent with the results in an experimental study that applied mechanical stretch stimulation to cultured cardiomyocytes. Several in vitro studies on cultured cardiomyocytes that studied the effects of mechanical stimulation on the GJ showed an increased Cx43 expression. Salameh, et al[30] reported that increased Cx43 expression is accompanied by an increase in conduction velocity in cardiomyocytes culture. Meanwhile, Salameh, et al[31] conducted a mechanical stretch stimulation research on cardiomyocytes resulted in a higher Cx43 expression compared to control. Animal studies on rats undergoing aerobic exercises also showed an increase in Cx43 expression. Bellafiore, et al[32] applied an aerobic exercise protocol of running at a speed of 4.8 meters/ minute for 300 minutes. Analysis of the data in the study also showed that the longer an exercise was carried out, the higher the Cx43 expression, measured using Western blot technique.[32] However, this study did not analyze the pattern of Cx43 expression distribution in cardiomyocytes of the rat's left ventricle tissue.

A higher Cx43 expression after aerobic exercise in the juvenile group (E-J8 and E-J12) and young adult in this study correspond to those reported by previous studies. In contrast, there are also rats that showed a lower Cx43 expression, although statistically insignificant, as seen in the exercising group E-J4. In contrast, to the study of Jepson, et al,[33] showed that exercise training can increased Cx43 expression in young and aged rats by using Western blot analysis. Differences in the findings in this study compared to other studies are difficult to explain. It may be caused by several factors such as the difference in the subject of studies (rats and mice), the sex of the subject, the difference in the study protocol (running and swimming; intensity of aerobic exercise), or the method of technical analysis used (western blot, polymerase chain reaction, or immunohistochemistry). This result is different with the downregulation Cx43 expression in the significance result which can lead to pathological hypertrophy within defined borders. Research on pathological cardiac hypertrophy by Jin et al[34] in mice after binding of the ascending aorta (aortic stenosis resembles condition in humans) also results in increased Cx43 expression during early hypertrophy (four weeks), accompanied by a moderate decrease in conduction velocity. Increased Cx43 expression accompanied by a decrease in conduction velocity is due to increased Cx43 phosphorylation. Whereas at a more advanced stage (eight weeks) decreased Cx43 expression and increased non-functional Cx43, led to a decreased conduction velocity.

There is no definite cause known as to why the expression of Cx43 in EJ4 is lower than controls. However, there are several possibilities that can be analyzed and can be investigated in future research. The strongest possibility cause for the lower total of Cx43 in

E-J4 is that the load of exercise implemented in this study did not comply with juvenile rats. Another possibility is stress experienced during early 4 weeks of aerobic exercise seen in group E-J4. A form of physical exercise recommended by AHA in the form of activities related to play and other fun activities with minimal stress. Weaknesses of this aerobic exercise protocol are not a voluntary exercise protocol; the possibility of stress can still arise and cannot be controlled even though all the rats have undergone an adaptation first.[35] Greenwood et al[36] have a concept of voluntary exercise. Rats allowed voluntary access to running wheels have constrained corticosterone responses to mild stressors and are protected against several behavioral consequences of uncontrollable stress which resemble symptoms of human anxiety and depression, including exaggerated fear and deficits in shuttle box escape learning.[36] Tiscornia, et al[37] conducted a research on trained and untrained mice by implementing voluntary aerobic exercise (swimming with a training load of up to two hours of swimming/day). This swimming exercise induced an increase in the protein level of Cx43 by 45–70% under resting conditions.

Cx43 as the main protein of GJ in the left ventricle is normally located in the intercalated disc. This study showed an increased Cx43 expression followed by an increase in the percentage of lateral Cx43. Cx43 in small amounts can normally be found aside from the intercalary disc or in other parts of myocytes which in this context is called lateral Cx43. This study showed an increased Cx43 expression in the exercise group both in juvenile and young adult, with an increased percentage of lateral Cx43. Research by Chondro et al[22] also showed that aerobic physical exercise in adult rats caused an increase in the lateral Cx43 distribution as measured by immunohistochemical stain. An increased lateral Cx43 expression together with an increased in the total Cx43 expression in normal conditions may be related to an increase in Cx43 production.[23,38] When Cx43 is produced, Cx43 will be released by the Golgi body in the form of a hemichannel which are then inserted in the lateral membrane and travel to the intercalary disc composing the complete gap junction.[28]

The increase in lateral Cx43 expression in this study is different from the increase in lateral Cx43 under pathological conditions. Whereas in pathological conditions, expression of Cx43 was dominant in the lateral area compared to that of intercalary discs.[29] Kostin, et al[39] conducted left ventricular biopsies of the heart in patients with pathological cardiac hypertrophy caused by aortic stenosis. Kostin, et al[39] reported that in patients with compensated pathological cardiac hypertrophy with ejection fraction >50%) an increased Cx43 expression occurred with a predominant redistribution of lateral Cx43 (a proportion of 40% lateral Cx43 compared to only 10% in control). Another study on pathological right ventricular cardiac hypertrophy in rats (caused by pulmonary resistance induced hypertension) revealed no increase in Cx43 expression.[40] However, redistributed lateral Cx43 was observed. The increased proportion of lateral Cx43 caused a significant decrease in longitudinal conduction velocity along the cardiomyocytes.[41] Seidel et al[42] examined the simulation study of cells experiencing hypertrophy with lateralization of Cx43 as happened in heart disease, showing an increased risk of arrhythmia.

A higher total Cx43 followed by a higher lateral Cx43 in this study is within normal limits. However, concerns about the possibility of adverse effects of an increase in the lateral Cx43 still remains. Some researchers claimed that the increased lateral Cx43 expression coincides with an increasing risk of cardiac electricity disorders. This study can be categorized as one of the many studies that illustrates the effects of aerobic exercise in several age groups of children and young adults with different durations. Thus, further research must be carried out. We recommend that in implementing an exercise program in children, a number of important things needs to be considered to avoid stress that will cause a negative effect. Children must do aerobic exercise program in the form of games or other voluntary exercise program. Voluntary exercise allows children free to choose when to start and when to stop when doing aerobics physical activity, for example, when playing chase, they are free to determine when to start playing, or stop because they feel tired. Exercise programs in children must also consider the condition of each child, so as not to cause fatigue or stress.

CONCLUSION

Aerobic exercise can improve both total Cx43 expression and lateral Cx43, whether the exercise began at juvenile or young adult age. This result in this study is considered normal and is related to increased Cx43 production. The slightest adverse effect that might occur still needs to be identified in association with the increased lateral Cx43 expression. Further research is needed to assess the effect increased Cx43 expression on other physiological parameters such as electrocardiography (ECG). Molecular research methods that can also be performed to aid analysis include: Immunofluorescence (cryosection, fluorescence microscope, 3D stacking with imaging software) can better accommodate the purpose of lateral shift of proteins on the cell membrane or in this case cardiomyocytes.

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