



# SUBSARCOLEMMA STRUCTURE OF CARDIOMYOCYTES AT PATIENTS WITH MITRAL VALVE DEFECT AND CORONARY ARTERIAL DISEASE

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Understanding of the mechanisms lying in the changes of the cardiomyocyte (CMC) subsarcolemma (SS) will give opportunities for searching the medicaments assuring maximal protection of cells. In this study, for the first time, we demonstrated coronary arterial disease (CAD) and mitral valve defect (MVD) influence on the myocardium leading to the alterations of CMC SS cytoskeleton thickness. The alterations of SS structures as well as the mitochondria of cells could be one of the main reasons leading to postsurgical damages of cardiomyocytes.

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## INTRODUCTION

It is well known that coronary arterial disease (CAD) and mitral valve defect (MVD) have a great clinical significance.<sup>1</sup> The drug therapy is not so effective and the prognosis of disease of the patients with CAD and MVD is unfavorable. The treatment of such diseases of heart is made by surgical way. However, it's still remaining unsolved one of the most important question: the mechanism of development of acute cardiovascular failure after elimination of hemodynamic.

The protection of myocardium viability during heart and vessels surgeries is very important and the intensive searching of the approach and pharmacological influence on myocardium function and metabolism are in progress.<sup>2</sup> Many biochemical and metabolic changes have been observed early after the onset of ischemia, but the precise cause of the transition to irreversibility has not elucidated. However, disruption of the plasmalemma (PM) of cardiomyocytes (CMC) is an early event indicates that ischemic myocytes are dead.<sup>3</sup> Consequently, many researchers aim to understand the underlying molecular mechanism of myocardial ischemia reperfusion injury to find therapeutic strategies ultimately reducing the final infarct size. The most striking basic science findings made during the past decades that are currently under clinical evaluation, with the ultimate goal to treat patients who are suffering from myocardial ischemia reperfusion-associated tissue injury.<sup>4</sup>

The cardiac cycle, cardiomyocytes depend on their highly evolved and specialized cytoskeletal apparatus. Defects in components of the cytoskeleton affect the ability of the cell

to compensate at both functional and structural levels in the long term.<sup>5</sup>

On the main causes leading to injury of CMC and other cells of the myocardium in cardio-pulmonary bypass application can be structural changes occurring in the cytoskeleton of PM.<sup>6</sup> It is well known that actin cytoskeleton function include support of plasma membrane, establishing and maintaining cell polarity, regulation of membrane protein distribution and activity and enhancing membrane vesicle trafficking. Consequently, the actin cytoskeleton contributed significantly to the cellular pathogenesis in a number of disease states.

Cytoskeleton is crucially involved in virtually all aspects of a cell's life, including cell shape changes, cell division, cell movement contacts and signaling between cells, and dynamic transport events.<sup>4,7</sup> And it's also provides support to the subcellular structures, organizes the cytoplasm, regulates the topography of the cell membrane and, finally, transmits mechanical and chemical signals both inside the cell and between cells.<sup>8</sup> Resisting sarcolemmal rupture: dystrophin repeats increase membrane-actin stiffness.<sup>9</sup>

Understanding of the mechanisms lying in the changes of the CMC subsarcolemmas (SS) will give opportunities for searching the medicaments assuring maximal protection of cells. In this study, for the first time, we demonstrated CAD and MVD influence on the myocardium leading to the alterations of CMC SS cytoskeleton thickness.

## MATERIALS AND METHODS

**Reagents:** Crystalloid cardioplegic solution (Na-147 meq L<sup>-1</sup>, K-19 meq L<sup>-1</sup>, Ca-4 meq L<sup>-1</sup>, Cl-155 meq L<sup>-1</sup>, HCO<sub>3</sub>- 25 meq L<sup>-1</sup>, Glucose-0,2%, pH-7,4, Mg -2 meq L<sup>-1</sup>); powdered paraformaldehyde; OsO<sub>4</sub>; sodium cacodylate trihydrate; 96° ethyl alcohol, acetone, Epon 812, Epon Hardener MNA, Epon Hardener DDSA, Epon accelerator DNP-30, uranyl acetate, sodium citrate, lead(II) nitrate, photoplates.

All reagent used were of analytical grade and purchased from Sigma Chemical Co. (USA).

**Human Subject:** All procedures involved human subject were approved by institutional review board/bioethical committee (Erevan State Medical University, RA) conformed to the Legal Aspects of Research Ethics and Science in European Community directive (2001/20/EC), (IRB Approval YSMU Bioethical committee N7 by 26.04.2011).

In this study the myocardium of right atrii of 14 patients: 4 with CAD and 10 with MVD was investigated.

Collecting biopsy material during cardiosurgical procedure of patients with CAD and MVD was performed during canulation. Right atria trabecula was obtained from adult patients aged (32-55 years).

**Treatment of material:** The bioplates taken during canulation (small pieces of the right atrii) have immediately put in cold 4 °C mix of paraformaldehyde in a sodium cacodylate buffer and glutaraldehyde for 12 hours with following post fixation in 1% OsO<sub>4</sub> solution during 2 hours; dehydration in ascending series of spirits; saturation in a mixture of acetone and Epon resins of different proportions and pouring in gelatinous capsules into epon.

**Obtaining of ultrathin slices and its treatment:** The ultrathin slices (up to 500 Å) were made using ultracut LKB (Swedish) and Reichert (Austria). Ultrathin slices were double contrasted with uranyl acetate and sodium citrate and lead(II) nitrate solutions.

**Observation under TEM:** Obtained ultrathin slices were observed under the transmission electron microscope (Phillips CM 10) with resolution X 10-40.000.

**Measurement of structures:** The electronograms of 6 patients (3 MVD and 3 CAD) were used for measurement of investigated structures by the Micro-ruler MR-1, Traceable and calculated by TED Pella ultrastructural size calculator.

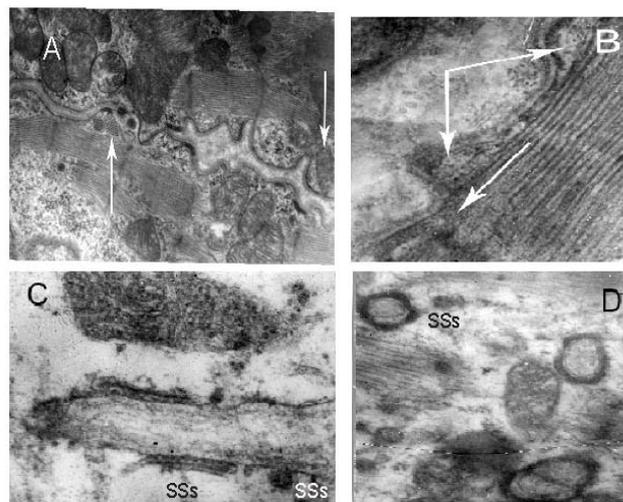
**Statistical Analysis:** Data were expresses as the mean +- S.E.M. All data were analyzed using a one-way analysis of variance (ANOVA) (SigmaStat 3.5 for Windows). Differences were considered as significant at P < 0.05.

## RESULTS

As have shown the results of our study, in the CMC of the trabecullas of right atrii of the patients with MVD and CAD, taken during canulation, were found some structures lay closely to sarcolemma (SL). These structure were changed depend on the progression of disease. They were not big by their size with a round and oval shape and were presented as a parallel, thin filaments (3-5), related to cross bridges and covered outside (Fig. 1) and are closely contacted not only with SL but also with nearby myofibrillas (MF).

As a rule, for one sarcomere of MF, there are 1-2 of such structures. The typical for SS structures is the ability to change their sizes, which varies, so it is possible to observe them at X 10.000 of microscope magnificence. The changes

of its sizes happen because of wideness as filamentous itself as well as its cross bridges, and alterations of its outer shell. These structures could change their sizes as in vertical and in horizontal direction.



**Figure 1.** The insignificant ultrastructural damages of CMC at MVD and CAD. SS round formations (see below: A x 10.000, B; C; D x 20.000). D – SS structures and microtubules observed on T system.

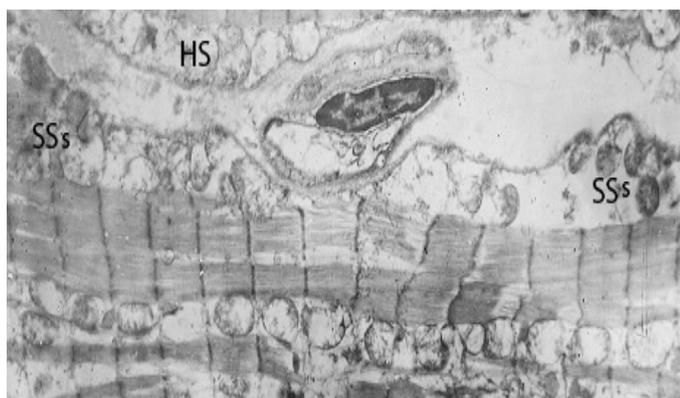
Mainly for myocardial of the right atrii trabeculae at MVD and CAD minor variations in the height and width of SS structures have been identified. At the maximum value of the length, with pronounced changes in the vertical direction SL forms arcade. For all patients in both types of heart disease the measurements of SS structures have shown that when the destructive damage of CMC are not very significant, the sizes of structures are varied mainly in the range from 150 nm to 200 nm in height and 120 - 200 nm in width. When the height of the structure reaches 230 nm, SL takes twisted character. In this case the width fluctuations can also reach its max up to 380 nm. When the damages of CMC are significant, SS structures have reached their max height of 350 nm and width of 250 nm, compared with the same structures in the less damaged cells (P<0,05).

Changes in the height and width have taken a more pronounced form when SL was in a process of vesiculation. At the same time it is specified the fact that the thin filaments in SS structures are very sensitive to the damaging effects leading to lysis. These structures often form vesicles, which makes difficult to identify them. Studies of past years have shown that due to the large number of vesicles the membrane takes a diffuse form.<sup>10</sup> Sometimes they are presented in a form of hollow structures with intact outer membrane, with the height of 350 nm and the width up to 480 nm (magnificence of electron microscope ranged from 10.000 - 20.000). At significant destructive changes of CMC the percentage of cavitated SS structures increases and the relatively intact structure in these CMC lose their contact with SL and separate from it. This process is irreversible.

It should be noted also that when such structures are observed and their sizes are expressed, the destructive damages of CMC affect the contractile apparatus of CMC -

myofibrils. In this case Mch form conglomerates slip to each other, while maintaining its internal structure safeness. The intracellular edema and swelling of the interstitium were expressed.

In one case study of CMC with lethal output at MVD SS structures were mainly with the lysis of thin filaments, expanded and had a form of hollow structures. At the same time, relatively intact, and small-sized structures lose contact with the SL or MF (Fig. 2).



**Figure 2.** Destruction of CMC, SS structures (SSs) lose contact with myofibrils and separated from sarcolemma, increase of the hollow structures (HS). X 20.000.

Furthermore, the sites where they were stored and close to SL were few. The destructive alterations of CMC were expressed. The cells were swollen, with damaged MF and energy producing apparatus of cells (Mch). Mch had no cristae, and had only the outer shell intact.

So in a relatively intact CMC, such structures could have different sizes around the perimeter of the cell. The similar structures have been also identified under the membranes of T-system, which in the case of destructive changes were also observed as outer contours. However, along with these structures the long tubular structures connected by thin filaments with the SL were observed (Fig. 1).

## DISCUSSION

It is well known that metabolic and functional changes take place within 8-10 seconds after coronary artery occlusion.<sup>11</sup> There are three potential initial causes of immediate reperfusion injury: cause 1, re-energization; cause 2, rapid normalization of tissue pH; and cause 3, rapid normalization of tissue osmolarity. Clinically this is accompanied with reduces systolic function, decreased myocardial diastolic compliance.<sup>12</sup> As we know by the studies of different scientists the final pathway of injury after reperfusion of ischemic tissue include three fundamental interrelating components: free radical formation, leukocyte infiltration and alteration of vascular permeability with seepage of macromolecules and toxic metabolites into the tissue with resultant tissue damage.<sup>13-15</sup>

In the last century the pathogenesis of SL disruption irreversibility was unknown.<sup>3,11</sup> Ultrastructural changes of CMC including cell swelling, evidence of generalized mitochondrial and marked SL damage.

By studying hypertrophied CMC we registered the phenomenon of membranes thickness alteration. In the next key part of our studies we made an ascent on the investigation of CMC SL thickness alterations during cardiac surgery.<sup>16</sup> It was shown, that the excitability of the membrane is accompanied by a change of its thickness. At depolarization, it decreases in size and at hyperpolarization takes place the process of its thickening.<sup>17</sup>

We offer a hypotheses that cytoskeleton of PM has the ability to change it's thickness when the changes of environments homeostasis take place. That can be the important key moment in the cell pathology.<sup>16</sup> For better understanding of this process we conducted investigation in deferent areas using as clinical and experimental models of diseases which gave us great opportunity to detect changes in the cytoskeleton of PM in general.<sup>6,18,19</sup>

The dates that we obtained during our studies indicate that cytoskeleton of CMC and cell's insides organelles have some unknown properties not revealed by electron microscopy before which gave us an idea to study relationship between the structural alterations of CMC PM cytoskeleton and the irreversible processes on it at injuring impact.<sup>6</sup>

Our dates have shown that at the different pathological influence become possible to observe some areas of the thickening, vesiculation and ruptures of SL of CMC.<sup>19</sup>

One of the most exciting principles to emerge from the last decade of research on actin is that the assembly of architecturally diverse actin structures is governed by highly conserved machinery and mechanisms. Cells can rapidly alter their cytoskeletons in response to internal and external cues. Actin has cable architecture. Cables are comprised of shorter filaments organized into bundle of uniform polarity. The F-actin cortex is a thin, membrane-bound F-actin network; the mechanical behaviors of the living F-actin cytoskeleton are largely unknown.<sup>20</sup> The rounded subsarcolemmal structures founded by us contain parallel arranged filaments which suggest that we visualize F-actin. These structures have a capacity to change their shape and size not only by changing the height of the filaments, but mainly by lengthening the cross-linking, and the fibrous structure bounding these structures.

It was suggested that  $\alpha$ -actinin has the ability to respond on the changes of the tension by lengthening and could do so within limits without dissociating from actin.<sup>21</sup> The role of  $\alpha$ -actinin of membranes, when actin filaments are oriented in the some direction is much less clear.  $\alpha$ -actinin is not simply a rigid spacer between actin filaments, but rather a flexible cross-linking, scaffolding, and anchoring protein.<sup>22</sup>

All this testifies to the fact that the changes in the size of new structures identified by us are provided by  $\alpha$ -actinin.

Moreover, this process is associated with the expenditure of energy. It should be noted that this process can be both pathological and physiological nature. In terms of physiological processes, these structures have a liability for rapid, reversible changes in size within certain limits in connection with the performance of electro-mechanical function of CMC.

From the standpoint of the pathological process of these structures size alteration, both vertically and horizontally can be accompanied by a violation of rapid reversibility of its changes that will disrupt the CMC function, respectively. All this indicates that such structures are involved in the mechanical processes on the membrane.

In addition to the rounded structures, the microtubules are also presented under SL, which are connected with its fiber structures as well. All this indicates that the cell surface contains all components of the cytoskeleton alternating with each other. All components of the cytoskeleton associated with the membrane are involved in the change of its thickness. It should be noted that the leading role in this process belongs to the new structures mentioned before. During damage of filamentous structures binding the components of the cytoskeleton to the SL occurs the process of detach of its fragments in those areas where the damage took place.

The ultrastructure of CMC of the right atrii with MVD and CAD corresponds to the severity of the disease. Destructive injuries mostly affect contractile apparatus. Variation in the size of SS structures mentioned before is slightly in all studied patients, and reaches its maximum value when the SL forms arcade.

In the practice of modern cardiac surgery the precondition during open heart surgery causing stress on the membrane. Reversibly injured myocardium develops adaptive changes that protect it to delay the development of cell death. On such change termed ischemic preconditioning persists for 1-2 hours and sever tissue is subjected to a new prolonged episode of ischemia.<sup>3</sup> IPC has been used in off-pump coronary artery bypass surgery reduce potential injury secondary to ligation of the target vessel.<sup>23-26</sup>

We suggest that the membrane responds to this mechanistically via these structures by changing of its thickness up to certain values. Thus, in one case with lethal outcome, the mentioned structure representing cavitated formation associated with the membrane, while the relatively normal structures separated from the membrane. At the same time there was widespread damage of the Mch.

Myocardial protection during cardiac procedures was performed by cardioplegic solution pumped in heart with regular intervals, which also affects the size of these SS structures. That's why, from the condition of these structures and Mch, depends patient myocardium state and the speed of recovery in the postsurgical period.

## CONCLUSION

The alterations of SS structures as well as in mitochondria of cells could be one of the main reasons leading to postsurgical damages of cardiomyocytes.

## ACKNOWLEDGEMENT

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