Research Article

Macrophages as the Homeostasis Regulators in the is chemically Damaged Myocardiumin Condition of the Use of Allogenic Biomaterial

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ABSTRACT

Macrophages as the effector cells play a key role in initiating the inflammatory process and they predetermine the manifestation of the post infarction cardiosclerosis. The population of these cells is heterogeneous and it is mainly represented by M1 and M2 phenotypes. Alloplant Biomaterial (ABM) is reabsorbed by the macrophages, which became the regulators of the cellular interaction in tissues.

Aim: The aim of the investigation is to reveal the peculiarities of the postinfarction healing of myocardium following the ABM insertion and to assess the population change dynamics of macrophages and c-kit+ cells.

Materials and methods: The experimental investigations were carried out on 100 male Wistar's rats weighing 0, 18-0, 25 kg. All the animals have had coronary occlusion by way of ligating the arteries. In the experimental group the ABM (12 mg) suspension was intra Myocardially inserted simultaneously with the vessel stricture formation. The histological, electron-microscopic, immunohistochemical (CD68, c-kit, Timp-2), Morphometric and statistical methods of the investigation were used in the work. The harvesting of hearts was carried out in 3, 7, 14, 30, 45 days.

Results of the investigation: In the experimental group the course of the inflammatory process was characterized by the onset of the early proliferative stage, whereas the colliquative necrosis was being developed in the control group. It was caused by different degrees of the macrophagic reaction expression. CD68+ cells in the rat reactive zone of the control group were more in number than the experimental one. In the experimental group the ABM induced macrophages of the mesenchyme origin were revealed and C-kit+ cells were considerably more in number than in the control one. After 45 days the scar area index in the experimental group made up9, 72±10, 8%,whereas in the control group it made up 26, 65±16,1%.

Conclusion: The Alloplant biological material had a histoprotective effect under the conditions of the acute myocardial ischemia thanks to the inhibition of macrophage migration and induction of cellular cardiomyogenesis.

INTRODUCTION

According to the WHO (2015) ischemic heart disease and stroke claimed the greatest number of human lives in 2015, a total of 15 million people. These diseases have consistently ranked top of all causes of death for the past 15 years. Myocardial infarction causes disability in more than 50% of patients and is fatal in 13% of cases. Myocardial infarction treatment is complicated by the fact that cardiomyocytes are

believed by many researchers to belong to the postmiotic group of cells which are impossible to duplicate. The stem cell pool involved is not designed to cope with the task of myocardium reparation due to its small size and inability to cover the whole zone of ischemia as well as its inability to reprogramme these cells to cardiomyogenic direction ***invivo***. The inevitable outcome of the damaged myocardium remodeling is scar formation. In the course of experiments using Alloplant Biomaterial (AAB) it was established that the key histion cells during the regeneration of fibrous connective and skeletal muscular tissue are macrophages with M1 phenotype. Their number markedly exceeds the amount of these cells in the control groups in which the defect infliction was not treated with the bio material in question [1,2]. It was shown that the use of AAB had a positive effect upon the cardiac muscle condition and improved its structure following ischemic damage [3]. The AAB biodegradates into the tissue and its resorption products are the Chemo attractant of the stem progenitor cells which induce the regeneration process [4,5]. There are conflicting views on the negative role of M1 macrophages in the healing process of the ischemic damaged Myocardium as key cells – the damaged promoters of Cardiomyocytes, manifestation of inflammation and fibrosis progression [6]. Consequently, the study of macrophage involvement in inflammatory and degenerative processes, developing in the cardiac muscle, following coronary occlusion experiments and when inserting the AAB, appears relevant.

Aim of the study

To understand the effect of AAB on the post-infarction Myocardium healing process and evaluate the dynamics behind the changing number of macrophage and c-kit+ cells.

MATERIALS AND METHODS

Experimental animal studies

Experiments involving AAB were carried out on 100 male Wistar rats weighing 0,18-0,25 kg. All the animals were divided into two groups. The Myocardium infarction modeling in the control group (n=50) was performed as follows: all the animals under general anesthesia (intra muscle injection of zoletil) underwent left-sided thoracotomy with further ligation in the upper third inter ventricular branch of the coronary artery (r. inter ventricularis paraconalis a. coronarii sin.) 1,2 mg of the AAB flurried in physiological solution was inserted into the cardiac muscle in its pool zone of the rats in the experimental group (n=50) immediately after the coronary artery ligation. The AAB dose was chosen arbitrarily. The rats in both groups were euthanised after the experiment by lethal insufflation of ether vapors after 3, 7, 14, 30, 45 days. 10 rats were taken from each point of the study.

The studies were conducted according to best practice laboratory guidelines of the Russian Federation in line with legislation adopted by the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (Strasburg, 1986). The studies were also carried out in accordance with the approved written protocol on standard researcher operating procedures as well as official laboratory guidelines on animal treatment and as per the alternative models in biomedical studies [7]. Alloplant® bio material was developed in the Federal State Budgetary Institution "The Russian Centre for Eye and Plastic Surgery" under the Russian Ministry of Health, in the city of Ufa. This bio material is produced according to technical specifications 42-2-537-87; it is certified and was approved for clinical use by the order of the USSR Ministry of Health №87 901-87 dated 22.07.1987.

Histological study

The allogeneic bio material in this study was made from rat tendons and enlarged to a size of 50-80 m cm. The ethical committee approved study protocol №31 dated 12.10.2015. For the histological investigation the hearts were fixed with 10% solution of neutral formalin, then dehydrated with increasing concentrations of alcohol and embedded in paraffin as per the generally accepted method. The sections were prepared with the use of LEICA RM 2145 microtome (Germany) and Mallory stained.

Immunohystochemical study

The 4m/cm thick paraffin sections were stained by Leica Microsystems Bond TM immune histostainer (Germany). CD 68, Timp-2 diluted in the proportion of 1:300 (Santa Cruz Biotechnology, USA) was used as the first antibodies. Single and double immune labelling of cells to the given antibodies was carried out. An indirect Leica Bond (NovocastraTM, Germany) streptavidin-biotin system of detection was used for unmasking. The reaction specificity estimate when staining the sections was conducted without the first antibodies. The

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calculation of the positively stained cells was carried out in 20 visual fields of each specimen (n=6) when magnified by X400. The study and visualization of the specimens were conducted with the use of Leica DMD 108 (Germany) light microscope equipped with specialized software to manage settings and capture images.

Electron-microscopic study

The Myocardium pieces 1-2 mm 3 in size fixed by 2,5% solution of Glutaraldehyde were used for the electron-microscopic study. The solution was prepared on Cacodylate buffer (pH 7,2 – 7,4) with further Postfixation by 1% OsO-4 solutions on the same buffer. The material was dehydrated in increasing concentrations of alcohol and embedded into Epon-812 according to the generally accepted method. EMUC7 (Leica, Germany) Ultratome was used to prepare semithin sections which were stained by toluidine blue solution based on 2,5% anhydrous soda solution. There were chosen areas on the specimens for the electron microscopic studies. The ultrathin sections were contrasted by 2% water solution of Uranyl acetate, lead citrate according to Reynolds and they were studied by JEM-1011 (Jeol& l t; Japan) transmission microscope.

Statistics

Each heart was cut into 5 sectors to determine the size of the post infarction scar. The scar area index (SAI) was measured against the specimens of the heart cross sections using the ITEM programme in the following way: the ratio between the scar area and the left ventricle wall area was multiplied by 100%.

The analysis of SAI values was performed using non-parametric methods, namely, the Univariate Kruskal-Wallis analysis of variance and comparison of un- correlated data by Mann-Whitney method [8].

RESULTS OF THE STUDIES

The study of the index area of the scar

The difference in healing between the ischemic damaged Myocardium in the control group and that in the experimental ones was significant. The SAI data in the experimental group insignificantly depended upon the follow-up periods (X 2 =5,7; P>0,12). However, the values of this parameter tended to reduce gradually. The distribution medians by the 7th day totalled 22, 7%, significantly, up to 13,4% (P&l t;0, 02). They decreased on the 14th day, whereas on the 30th and 45th days we recorded up to 16% and 5,2% (P>0,4 and P<0,02 correspondingly). The difference between the 14th, 30th and 45th days turned out to be statistically insignificant (P&g t; 0, 23 ÷ 0,75). It was proven that SAI zero values (absence of Myocardium scar) occurred after 7 days following the coronary occlusion. Comparisons between the control group and the experimental one in different follow-up periods showed that SAI was statistically less significant in the control one after AAB implantation than in the experimental group at any period. (P< 0,05÷<0,0001). By the end of the experiment, SAI in the control group exceeded the experimental group values by 2,74 times making up 26,65±16,1% against 9,72±10,8% respectively (Figure 1).

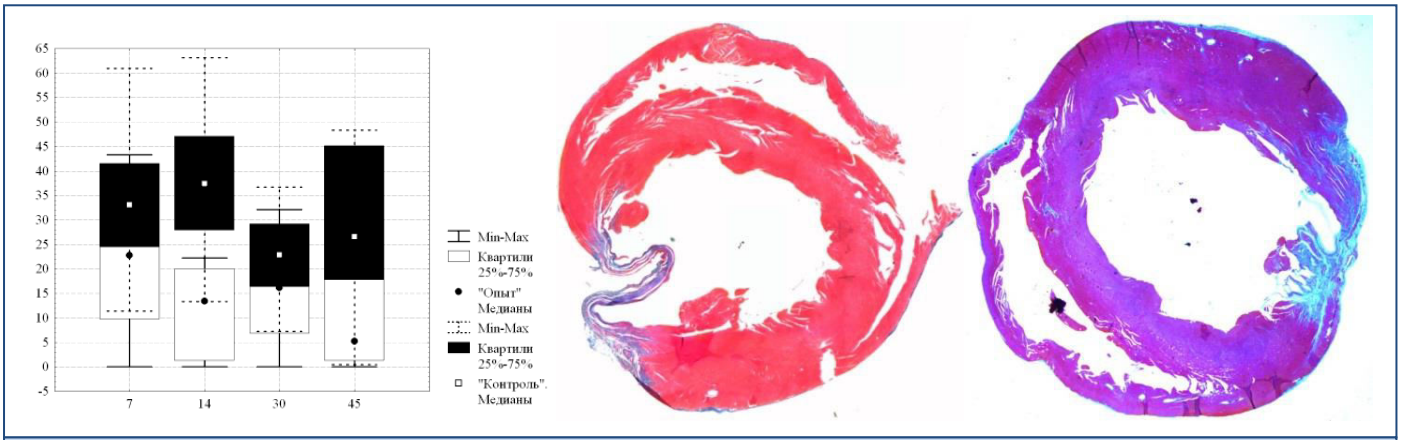


Figure 1: A-changes of SAI in the control and experimental groups. X-axis – Follow-up (days); Y-axis – SAI (%); B – Cross section of myocardium after 45 days in the control group. Mag. X40. C– Cross section of myocardium after 45 days in the experimental group. Mag X 40. Mallory's stain.

Identification of macrophages CB 68

Macrophage cells are of great importance in fibrous progression and scar manifestation [6]. The number of CD68 macrophages in the control group within the reactive zone of the ischemic damaged cardiac muscle exceeded the values of the experimental group almost throughout the experiment. In the control and experimental groups, the recurring rise and subsequent fall of the cells was, on the whole, highly significant (Chi-Square = 76,3 P<<0,0001 and Chi-Square = 45,2 P< < 0,0001 correspondingly). The number of CD68 + cells in the control group, statistically significant, was greater than the number in the experimental group during the follow-up period 3-14 days (P<0,003 and less). The remodeling attenuation process of Myocardium and scar formation took place over a period of 30-45 days. This caused a decrease in the number of macrophages in both groups (P> 0,12) and transformation from the exudative - proliferative phase of inflammation into the recovery stage (Figure 2).

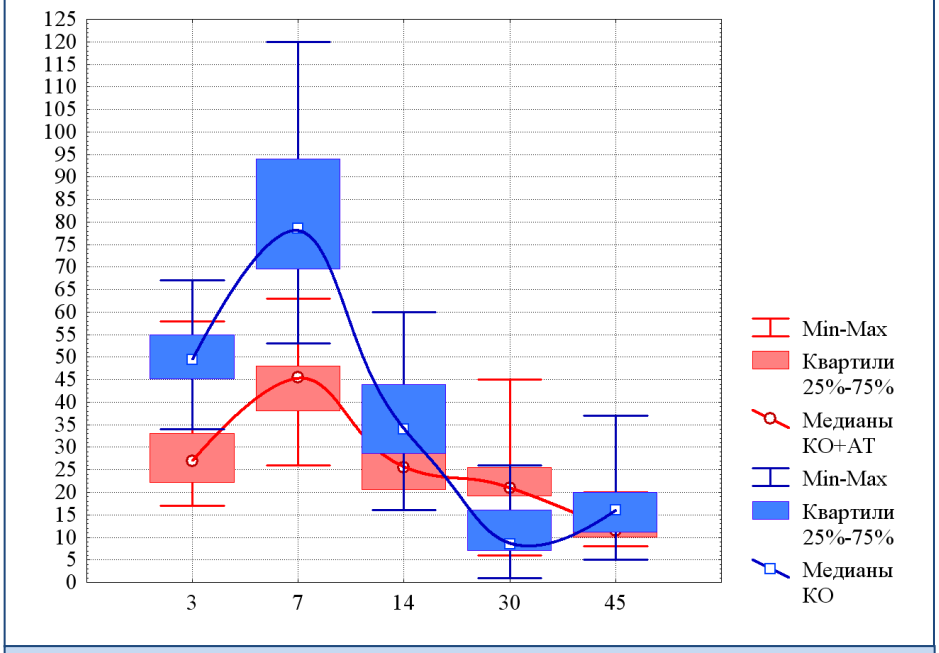
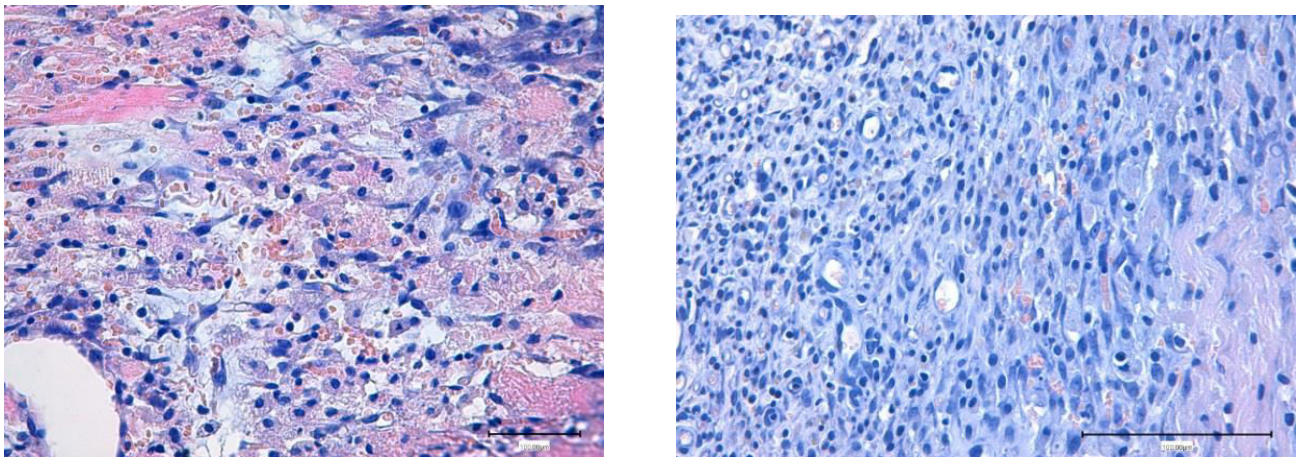


Figure 2: The number of macrophages CD68+ in the rat myocardium of the control group (KO) and after the AAB insertion (KO+AT). X-axis– Days; Y-axis– Number of cells. LCI– Limits of confidence intervals for mean area values, ± SE – Standard error of the mean value.

Histological study of the myocardial ischemic area

Through evaluation of the dynamics of pathomorphological changes, it was revealed that the initial stage of inflammation (3 days) was characterized by the early onset of the proliferative phase and the formation of the granulation tissue in the perifocal zone of the ischemic damaged myocardium. This is where thin collageneic fibres, mesenchymal and macrophagal-fibroblastic infiltration were observed (Figure 3A).



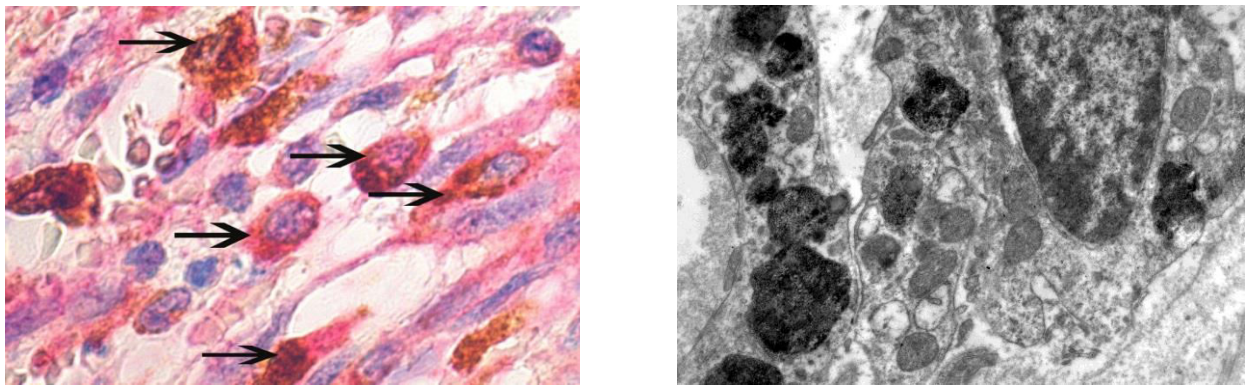
A B

Figure 3: A – formation of the granulation tissue in the perifocal zone, infiltration by macrophages, mesenchymal cells, fibroblasts in the rat myocardium after 3 days following coronary occlusion and AAB insertion. B – macrophagal-lymphocytic cell roll in the zone of necrotic changed cardiomyocytes after 3 days following coronary occlusion. Hematoxylin and eosin stained.

Phagocytosis of c-kit cells by macrophages

In the control group a wide cell rolls consisting of macrophages, lymphocytes, neutrophiles, was being formed in place of the decaying cardiomyocytes. In this study C-kit + cells in both groups were determined mainly in the perinfarction and perivascular zones. When doing it despite the autogenous origin of C-kit+ stem cells and absence of antigenicity factors they were subjected to phagocytosis by macrophages (Figure 4A). Numerous macrophages phagocyting undifferentiated cells on the electron microscopic level were also recorded.

Fragments of the cytoplasma and pycnotic nuclei were detected in phagocytic vacuoles and macrophagal cells showed signs of activation. The nuclei were oval-shaped and contained large amounts of euchromatin; numerous large mitochondria with a darkened matrix and parallel oriented lamellar crystal were observed in the wide cytoplasm rim. The cytolemma formed deep invaginations. Golgi apparatus was well developed with elongated flat piled up cisterns and uncoupled vesicles (Figure 4B).



A B

Figure 4: Phagocyting macrophages. A- CD68+ /C-kit+ cells after 7 days following coronary occlusion. CD68+ /C-kit+. Chromogen granules are revealed in macrophage cytoplasm (↑). Double indirect immunoperoxidase method of CD68+ /C-kit+ cell revealing with hematoxylin stain to finish with. Mag. X 600. B – phagocytic macrophage with vacuoles of cell detritus after 7 days following coronary occlusion. Mag. X 1000. Electron diffraction pattern.

Number of C-kit+ cells

When determining free C-kit+ cells, which were not subjected to macrophagal resorption, it was revealed that their number in the experimental group, statistically significant, had surpassed the control group during the follow-up period (P&l t; 0,001) (Figure 5). (KO+AT). X-axis –"days". Y-axis – number of cells. LCI – limits of confidence intervals for mean area values. ± SE – standard error of the mean value.

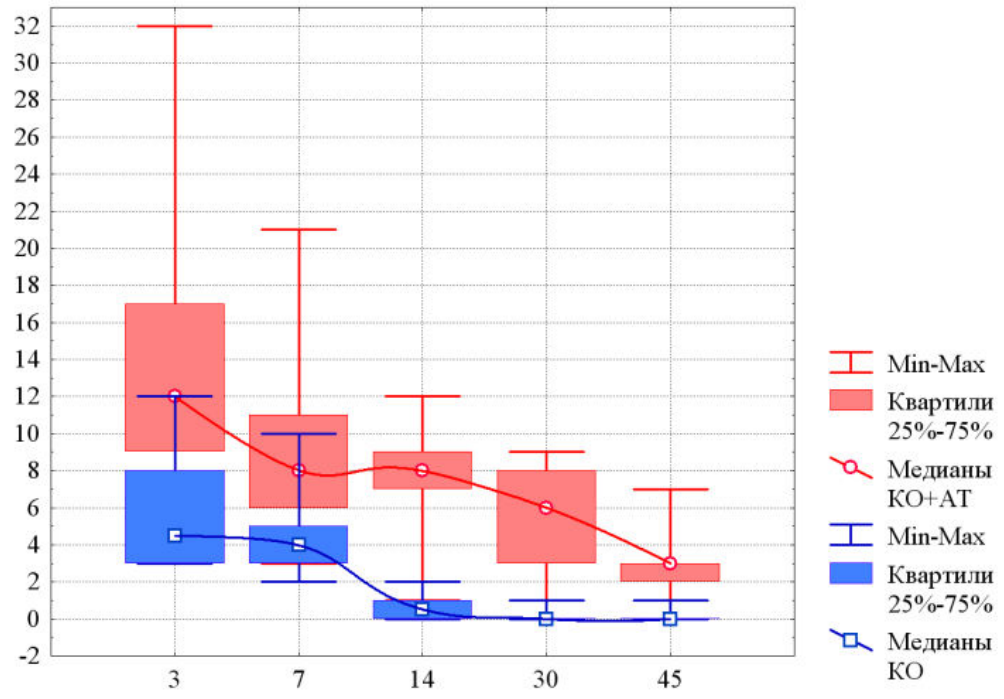
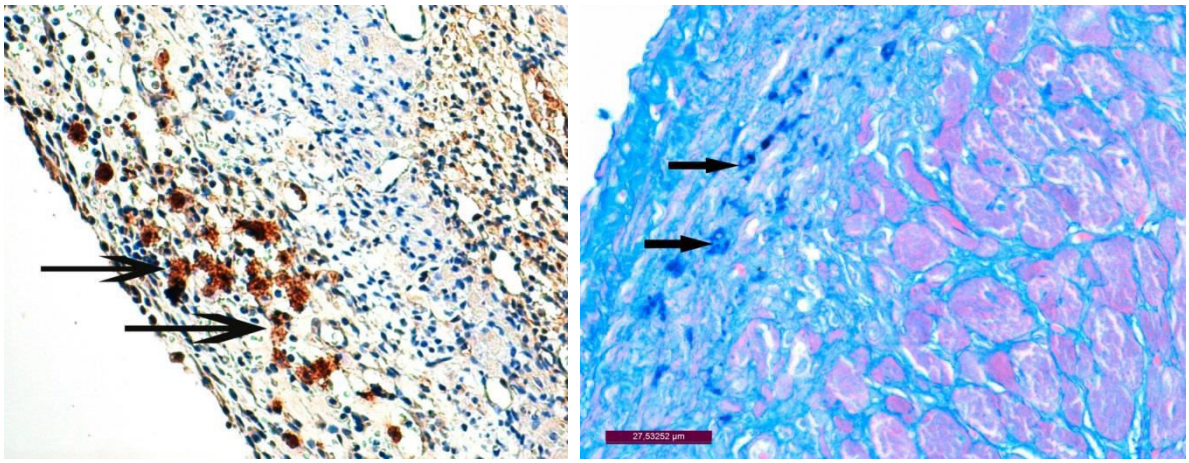


Figure 5: Number of free C-kit + cells in the rat myocardium in the control group (KO) and after the AAB insertion (KO+AT). X-axis– "Days". Y-axis – number of cells. LCI – limits of confidence intervals for mean area values. ± SE– Standard error of the mean value.

Phenotype of AAB - induced macrophages

Positive staining macrophages were revealed when stained according to Hale and expressing Timp-2 in the zone of implantation in the sub epicardial space (Figure 6).



A B

Figure 6: Macrophages in subepicardial space following the AAB implantation after 7 days. A – GAG-positive macrophages (↑). Hale stain. B – Timp-2+ macrophages (↑). Indirect immune peroxidase method of Timp-2 with hematoxylin stain to finish with.

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DISCUSSION OF THE OBTAINED RESULTS

Numerous factors, one of which being macrophage reaction, induced by AAB contributed more favorably to myocardial infarction healing in the experimental group. It has already been proven that the products of AAB biodegradation turn into chemoattractants of monocytes and macrophages during the connective tissue healing process followed by the inflammatory-destructive process and after inflicting damage [2]. Macrophagal cells displayed regeneration efficiency as a result of full-fledged phagocytosis and regulation of the inflammatory proliferative phase. They inhibited the fibroblastic activity by M1 stimulation of macrophages and prolongation of cytoxic phase [9,10]. We got the opposite result in this study in case of acute Myocardium infarction. Within 3 days the AAB particles were resorbed and they were not detected in the tissue. One can assume that after the biomaterial resorption, the AAB induced macrophages became the regulators of intercellular interactions and stimulated the onset of the early proliferative inflammation phase activating fibroblastic cells.

In the control group the ischemic damaged cardiomyocytes initiated a succession of inflammatory cell reactions which resulted in increased inflammation, expansion of the damaged zone and scar manifestation. This observation was confirmed by the data of other researchers who had illustrated that peak levels of the corresponding family of proinflammatory (CD14+) macrophages (monocytes negatively correlated with the restoration of the left ventricle function following the acute myocardium infarction [11]. Disregulated infiltration leads to the extension of myocardial infarction, expansion of the left ventricle and cardiac insufficiency. Monocytosis increases and extends the alteration and exudation stage due to the spectrum expression of inflammatory monokines (TNFa, IL1, IL6 etc) which, in their turn, induce the exudative stress spreading over the nearby cardiomyocytes, thus expanding the necrosis zone. As a result of inflammation the left ventricle remodeling is increased in the case of the ischemic damages of Myocardium [12].

Macrophages are the polymorphic cellular population, the phenotype of which is determined by the micro environment signals. In the experimental group after the use of AAB the products of its bio degradation create a certain micro environment; together with an anti fibrogenous effect [9], which induces TIMP-2 expression by random cells. This phenomenon helps to decrease inflammation when acute ischemia occurs [13]. The modulation approach of macrophages changes according to their environment. It was revealed that phenotypes and functions of macrophages had been formed by the corresponding organ micro environment [14]. The transplantation of differentiated peritoneal macrophages into the pulmonary medium, for example induced the transcriptional landscape reprogramming of those cells and acquisition by them of new specific tissue functions [11,12]. Thus, in case of acute ischemic myocardium damage, AAB has an anti-inflammatory effect and is a factor in the phenotype of macrophages switching from M1 to M2. Conversely the destructive Cardiomyocytes in the control group provoked a number of inflammatory reactions and also due to the pronounced expression of metalloproteinases MMP-9 [13]. It is known that the regenerative process participants in myocardium are not only effector fibroblastic cells but also cardiomyogenic progenitor cells. It is assumed that niche stem cells as well as epicardial cells, hematopoetic stromal cells etc., can be the source of stem cells [15,16]. The differentiation direction of progenitor stem cells is often unpredictable and this is due to the high probability of teratoma formation [17]. AAB stimulated migration of poorly differentiated cells C-kit+. Phagocytosis by macrophages of C-kit+ cells is probably connected with the genetically programmed mechanism of anti-tumorigenicity. In spite of this fact, the level of progenitor cells in the experimental group remained high enough which contributed to a more wholesome regeneration of myocardium and inhibition of scar tissue development. Macrophages populate heterogeneous cells.

Macrophages of mesenchymal origin, otherwise known as “matrix forming” have also been recorded during previous experiments with AAB [1,18,19]. They featured Vimentin+ /Hale+ /CD68+ /PCNA phenotype and secreted Glicosaminoglicanos (GAG); this phenomenon being typical of fibroblastic cells. Macrophages appeared to be of the mesenchymal origin. It was revealed in the study, that they had expressed tissue inhibitor of metalloproteinase Timp-2. Presumably these cells play a structural-informative role for

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cellular cooperations and create homeostasis in the inflammation focus. Their presence is connected with the hydrocarbon component synthesis. And Timp-2 has a histoprotective effect thanks to the anti-inflammatory mechanism in myocardium [20] which can also set in motion the early proliferative phase of inflammation and homeostasis regulation. The discovery of macrophages of the given phenotype is consistent with the observation that the adult human heart contains macrophages of embryonal origin capable of tissue restoration. It is noteworthy that though these families are present in the resting adult heart, the resident macrophages are lost after heart trauma in adults and are substituted by inflammatory monocytic macrophages of the medullary origin [14]. Thus, AAB had a histoprotective effect in the case of acute ischemic myocardium. Differences in number, composition and function of microphages allow for varying models of restoration and remodeling of the left ventricle observed in the given experiment.

CONCLUSION

1. Coronary artery stenosis alongside with AAB use allow to reduce the myocardium scar area by 2,74 times.
2. The AAB use decreases myocardial infiltration by macrophagal cells.
3. During the myocardium restoration, after the ischemic damage, macrophages are capable of actively phagocytising autogenetic stem cells.
4. There exists a GAG population in the AAB implantation zone – positive macrophages.
5. AAB usage ensures a substantial prevalence of C-kit^{+} cells compared with the control group.

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