Resveratrol pre- and post-treatment in doxorubicin-induced cardiac injury in relation to endogenous stem cell activation

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ABSTRACT

Background: Doxorubicin (DOX), is a highly efficient anti-neoplastic drug used for treatment of solid tumors including breast cancer (BC). Resveratrol (RES) is considered an interesting molecule in hormone dependent cancer therapy. Stem cell activation may induce less cardiomyocyte apoptosis, enhanced angiogenesis and increased proliferation of cardiomyocytes.

Materials and Methods: 27 adult male albino rats, were divided into: control group including 6 rats, group II (DOX group) including 7 rats that received intraperitoneal (IP) injection with DOX 3 mg /kg 3 times a week for 2 weeks. Group III (RES post-treatment group) included 7 rats that were injected with DOX followed by 50 mg/Kg RES daily orally for another 2 weeks. Group IV (RES pretreatment group) included 7 rats that were injected with DOX and received concomitantly 50 mg/Kg RES daily orally for 2 weeks and left untreated for another 2 weeks. By the end of the 4th week all animals were sacrificed. Cardiac muscle specimens were subjected to histological, immunohistochemical, morphometric and serological studies.

Results: Myocardial injury appeared as obvious congestion, apoptosis, degeneration, increased collagen fibers content, obvious caspase3 expression and minimal proliferating cell nuclear antigen (PCNA) +ve and CD44 +ve expression in DOX group. Regression of the previous changes was more remarkable in group IV than in group III except for PCNA and CD44 expression that was more obvious in both treatment groups.

Conclusion: RES proved amelioration of DOX induced myocardial injury that was more noticeable in RES early administration. RES post-treatment might have been more efficient if longer duration was applied.

Key Words: Doxorubicin, myocardium, resveratrol, stem cells,

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INTRODUCTION

Myocardial injury can be diagnosed by established histological, immunological, and immunohistochemical criteria and can cause sudden cardiac death in young people as shown in highly variable autopsy. Prognosis in cardiac patients also varies according to underlying etiology[1].

Anthracyclines, such as doxorubicin (DOX), are well-established, highly efficient anti-neoplastic drugs used for treatment of a variety of cancers, including solid tumors, leukemia, lymphomas, and breast cancer (BC). The successful use of doxorubicin has, however, been hindered by severe cardiotoxic side-effects[2].

Resveratrol (RES) is a well-known phytoestrogen that may be helpful as part of an overall strategy to defeat BC. The mixed agonist and antagonist role of resveratrol for the estrogen receptor makes it a controversial but interesting molecule in cancer therapy, especially in hormone dependent cancers[3].

Mesenchymal stem cells (MSCs) are multipotential cells and their differentiation potency into different cell lines such as cardiomyocytes and adipocytes has been intensely investigated[4]. Stem cell activation may induce less cardiomyocyte apoptosis, enhanced angiogenesis and increased proliferation of cardiomyocytes[5].

The present work was planned to investigate the possible protective and therapeutic role of resveratrol in DOX-induced myocardial injury. In addition, the possible role in the activation of endogenous mesenchymal stem cells, to ameliorate the resulting myocardial injury, was assessed in the adult male albino rat.
MATERIALS AND METHODS

Drugs Used:

Doxorubicin [(Adriblastina), Pharmacia Italia S.P.A, Italy] in the form of vials. Each vial contains 10 mg water soluble powder of doxorubicin. Each vial was dissolved in 5 ml distilled water and given by intraperitoneal injection (IP).

Resveratrol, Sigma Aldrich, St Louis, MO. Resveratrol is only commercially available as the trans-isomer, the most stable and pharmacologically active form of resveratrol. The required dose was weighed and dissolved in 0.5 ml saline solution.

Experimental Design:

This study included 27 adult male albino rats 180-200 grams body weight. They were housed in hygienic stainless steel cages and kept in clean well ventilated room. They were fed standard chow diet and allowed free access to water. The experiment was carried out at the Animal House of Histology Department, Faculty of Medicine, Cairo University. They were treated according to the ethical guidelines of Cairo University, Cairo, Egypt.

The animals were divided into four groups, kept in separate cages as follows:

Group I (Control Group):

Included 6 rats (2 sacrificed with the rats of each experimental group).

Two rats were given 0.3 ml sterile distilled water IP three times a week for two weeks then sacrificed at the end of the experiment.

Two rats were given 0.3 ml sterile distilled water IP three times a week then 0.5 ml saline was given orally daily for two more weeks, then sacrificed.

Two rats were given 0.3 ml sterile distilled water IP three times a week and 0.5 ml saline was concomitantly given orally daily during the same period. Rats were then sacrificed two weeks later.

Group II (DOX group)

Included 7 rats that were injected IP with DOX 3 mg/kg dissolved in 0.3 ml of sterile distilled water for 6 doses, three times a week for two weeks. Animals were then sacrificed after two more weeks.

Group III (RES post-treatment group):

Included 7 rats that were injected with DOX at the same dose, by the same route and for the same duration as in group II. In addition, each rat received 50 mg/Kg RES daily orally for the following two weeks then sacrificed.

Group IV (RES pretreatment group):

Included 7 rats that were injected with DOX at the same dose, by the same route and for the same duration as in group II. Concomitantly, each rat received 50 mg/Kg RES daily orally during the same period. Rats were then left for another two weeks then sacrificed.

The animals belonging to control and corresponding experimental groups were sacrificed under intraperitoneal anesthesia with sodium pentobarbital (100 mg/kg body weight).

Cardiac muscle specimens were removed by midline incision and exposure of the heart. The removed cardiac muscle specimens of control and experimental groups were fixed in 10% formol saline for 48 hours. Paraffin blocks were prepared and 5μm thick sections were subjected to the following studies:

A) Histological Study:

1. Hematoxylin and eosin
2. Masson’s trichrome stain

B) Immunohistochemical Study:

1. Caspase 3 immunostaining, the marker for apoptosis.
2. Proliferating cell nuclear antigen (PCNA) immunostaining.
3. CD44 immunostaining, marker of mesenchymal stem cells (MSCs).

Serial sections were cut and taken onto poly-lysine coated slides. Boiling of tissue sections in 10 mM citrate buffer pH 6.0. for 10-20 minutes was performed and then left to cool at room temperature for 20 minutes followed by washing twice in phosphate buffered saline (PBS). The following primary antibodies (1ry Ab) were applied:

a) 0.1 ml diluted rabbit polyclonal 1ry Ab (Caspase 3 3015 -100 Ab) (Biovision, Milpitas Boulevard, Milpitas, CA USA)

b) 0.1 ml at 200 microgram (μg)/ml concentrated 1ry Ab (PCNA) Ab-l (Labvision, USA).

c) 0.1 ml prediluted rabbit polyclonal 1ry Ab (CD44) Ab (IW-PA1021) (IHW, Ellicott City, USA).

The primary antibody was incubated at room temperature
in moist chamber for 60 minutes. The 1ry Ab used was 0.1 ml diluted. Dilution was used to reduce background and unspecific staining. Biotinylated goat antipolyvalent was applied and incubated for 10 minutes at RT in moist chamber. DAB chromogen mixture was incubated for 5- 15 minutes at RT. The slides were counterstained with Mayer Haematoxylin for 1- 3 minutes. Tonsils are considered positive control specimens which give a brown coloration. Cellular localization is cytoplasmic for caspase3, nuclear for PCNA, membranous for CD44. On the other hand, one of the cardiac muscle sections was used as a negative control bypassing the step of applying the primary antibody.

C) Morphometric Study:

Using Leica Qwin 500 LTD computer assisted image analysis (Cambridge, UK) assessment of the area of degenerated (deeply acidophilic) cardiac myocytes was measured in H&E stained sections using interactive measurements menu. The area% of collagen fibers, caspase3 +ve immunoexpression, that of PCNA +ve nuclei of cardiac myocytes and that of CD44 +ve MSCs were performed in immunostained sections using binary mode. The measurements were done in 10 high power fields (HPF) in control and experimental groups.

D) Serological Study:

CK-MB cardiac enzyme was measured 24 hours following the last injection and at the end of the experiment, just before sacrifice. Tail vein blood samples were collected for CK-MB cardiac enzyme estimation.

E) Statistical analysis(15):

Quantitative data were summarized as means and standard deviations and compared using one-way analysis-of-variance (ANOVA). Any significant ANOVA was followed by Bonferroni post-hoc test to detect which pairs of groups caused the significant difference. P-values <0.05 were considered statistically significant. Calculations were made on social package of statistical science (SPSS) software 16.

RESULTS

Histological results

1. H&E:

Control sections revealed normal architecture where cardiac muscle fibers were arranged in different directions with pale oval nuclei (Fig. 1a). Sections in the cardiac muscle of rats in DOX group (group II) showed widely spaced deeply acidophilic fibers including multiple disrupted, multiple thin attenuated and multiple fibers exhibiting dark peripheral nuclei (Fig. 1b). In addition, multiple congested blood vessels were evident among the muscle fibers (Fig. 1c). In RES post-treatment group (group III), some deeply acidophilic fibers, some thin attenuated and some fibers exhibiting dark peripheral nuclei were seen compared to (group II) (Fig. 1d). In RES pretreatment group (group IV), few congested blood vessels were detected among the muscle fibers, besides few deeply acidophilic and few thin attenuated fibers compared to groups II and III (Fig. 1e).

2. Masson’s trichrome results:

In group I fine collagen fibers were found among the muscle fibers (Fig. 2a), while in groupII dense collagen fibers were observed among thin fibers (Fig. 2b). In groups III and IV less dense collagen fibers were detected among the muscle fibers (Figs. 2c and 2d respectively).

Immunohistochemical results:

Negative immunostaining was noted in control sections of caspase3 immunostaining (Fig. 3a) and in control sections of PCNA and CD44 immunostaining as well.

1. Caspase3 immunostaining: In groupII, obvious caspase3 +ve immunoexpression was observed among the cardiac myocytes (Fig. 3b). In group III, less obvious caspase3 +ve immunoexpression was demonstrated among the cardiac myocytes (Fig. 3c). While in groupIV, sections revealed minimal caspase3 +ve immunoexpression among the cardiac myocytes (Fig 3d).

2. Proliferating cell nuclear antigen (PCNA) immunostaining: In group II few PCNA +ve nuclei (Fig. 4a), while in groupIII multiple PCNA +ve nuclei (Fig. 4b) and in groupIV some PCNA +ve nuclei (Fig. 4c) were found among the cardiac myocytes.

3. CD44 immunostaining: In groupII few CD44 +ve spindle cells (Fig. 5a), while in groupIII multiple +ve spindle cells (Fig. 5b) and in groupIV some +ve spindle cells (Fig. 5c) were found among the cardiac myocytes.

Morphometric results

Both the area of degenerated cardiac myocytes and area% of caspase3 immuno-expression measured reached their maximum values in groupII and showed a significant decrease in groupIV respectively. Furthermore, groupIV values continued to decline significantly in comparison to groupIII. On the other hand, groupII demonstrated a significant increase in the area% of collagen fibers in comparison to all the groups. For both, area% of PCNA +ve nuclei and that of CD44 +ve MSCs values of groupIII showed significant increase compared to groups II and IV. Moreover, a significant increase was found in groupIV
(Table 1) showing the mean values ± SD of the measured histological and immunohisto-chemical parameters in all groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>Area of degenerated cardiac myocytes (µm²)</th>
<th>Area% of collagen fibers</th>
<th>Area% of caspase3 immuno-expression</th>
<th>Area% of PCNA +ve nuclei</th>
<th>Area% of CD44 +ve MSCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (GpI)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>0.51±0.09</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Doxorubicin (GpII)</td>
<td>27.21±3.31</td>
<td>2.95±0.14*</td>
<td>5.95±0.81</td>
<td>0.29±0.01</td>
<td>0.67±0.08</td>
</tr>
<tr>
<td>Doxorubicin followed by resveratrol (GpIII)</td>
<td>12.92±2.82*</td>
<td>1.35±0.15</td>
<td>2.39±0.38*</td>
<td>2.42±0.26**</td>
<td>4.92±0.12**</td>
</tr>
<tr>
<td>Doxorubicin combined with resveratrol (GpIV)</td>
<td>7.24±0.61$</td>
<td>0.80±0.07</td>
<td>0.72±0.11$</td>
<td>0.51±0.04#</td>
<td>2.03±0.04#</td>
</tr>
</tbody>
</table>

* * significant decrease compared to Gp2
* $ significant decrease compared to Gp3
* * significant increase compared to Gp1, Gp3 and Gp4
* ** significant increase compared to Gp2 and Gp4
* # significant increase compared to Gp2

Serological Results

Table (2): showing the mean values of serum CK-MB ± SD in all groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Serum CK-MB measured just before sacrifice (ng/mL)</th>
<th>Serum CK-MB measured 24 hours following last injection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>129±6.4</td>
<td>119±6.2*</td>
</tr>
<tr>
<td>Group II</td>
<td>301±10.9#</td>
<td>258±7.9</td>
</tr>
<tr>
<td>Group III</td>
<td>164±9.1</td>
<td>264±12.4</td>
</tr>
<tr>
<td>Group IV</td>
<td>125±9.2</td>
<td>260±10.3</td>
</tr>
</tbody>
</table>

* significant decrease compared to other groups
# significant increase compared to other groups
RES Pre- & Post- treatment in DOX cardiac Injury.

Fig. 1: Photomicrographs of sections in the cardiac muscle of rats in (H&E, X200): a: control group (group I) showing muscle fibers arranged in different directions and exhibiting pale nuclei (arrows) b: DOX group (group II) showing widely spaced deeply acidophilic fibers including multiple disrupted (arrows), multiple thin attenuated (arrowheads) and multiple fibers exhibiting dark peripheral nuclei (n). c: group II showing multiple congested blood vessels (c) among the muscle fibers. d: Post-treatment group (group III) showing some deeply acidophilic fibers (arrows), some thin attenuated (arrow heads) and some fibers exhibiting dark peripheral nuclei (n). e: Pretreatment group (group IV) showing few congested blood vessels (c) among the muscle fibers, few deeply acidophilic (arrows) and few thin attenuated fibers (arrow heads).

Fig. 2: Photomicrograph of sections in the cardiac muscle of rats in (Masson's trichrome, X200): a: group I showing fine collagen fibers (arrows) among the muscle fibers. b: group II showing dense collagen fibers (arrows) among thin muscle fibers. c: group III showing less dense collagen fibers (arrows) among the muscle fibers. d: group IV showing less dense collagen fibers (arrows) among the muscle fibers.
Fig. 3: Photomicrograph of sections in the cardiac muscle of rats in (Caspase3 immunostaining, X200): a: group I showing –ve immunostaining among the muscle fibers. b: group II showing obvious caspase3 +ve immunoreexpression (arrows) among the cardiac myocytes. c: group III showing less obvious caspase3 +ve immunoreexpression (arrows) among the cardiac myocytes. d: group IV showing minimal caspase3 +ve immunoreexpression (arrows) among the cardiac myocytes.

Fig. 4: Photomicrograph of sections in the cardiac muscle of rats in (PCNA immunostaining, X200): a: group II showing few PCNA +ve nuclei (arrows) among the cardiac myocytes. b: group III showing multiple +ve PCNA +ve nuclei (arrows) among the cardiac myocytes. c: group IV showing some PCNA +ve nuclei +ve (arrows) among the cardiac myocytes.

Fig. 5: Photomicrograph of sections in the cardiac muscle of rats in (CD44 immunostaining, X200): a: group II showing few +ve immunostained spindle cells among the cardiac myocytes (arrows). b: group III showing multiple +ve spindle cells among the cardiac myocytes (arrows). c: group IV showing some +ve spindle cells among the cardiac myocytes (arrows).
DISCUSSION

Sections in the cardiac muscle of rats in doxorubicin (DOX) group (groupII) showed widely spaced deeply acidophilic fibers including multiple disrupted, multiple thin attenuated and multiple fibers exhibiting dark peripheral nuclei. These findings indicated degenerative and apoptotic changes developing in the cardiac myocytes secondary to DOX injection and were supported by a significant increase in the mean area of degenerated muscle fibers compared to groups III and IV. In support, a significant increase in the mean value of serum CK-MB was found. In accordance, Singla et al(16) stated that cytoplasmic vacuolation, cardiac myocyte apoptosis and myofibrillar loss were well established in response to DOX administration and were ascertained in DOX induced cardiomyopathy (DIC) in animal models and patients.

Zhang et al.(17) documented that oxidative stress and ROS production are key initiators of apoptosis. Fabbri et al.(18) proved DOX induced apoptosis by mediated deoxyuridine triphosphate in situ DNA nick end labeling (TUNEL) technique.

In groupII, dense collagen fibers were found among the cardiac muscle fibers that showed a significant increase in their mean area% compared to all groups. Going with the previous results, DOX was proved to induce interstitial and replacement fibrosis with cardiac myocyte decreased area(19).

In groupII, obvious caspase3+ve immunoexpression was observed among the cardiac myocytes. In agreement, caspase3 activation was correlated to enhanced apoptosis(20). Liu et al.(21) postulated that caspase3 activation promotes the elimination of genetically unstable or damaged cells.

In groupIII, less dense collagen fibers were found among the cardiac muscle fibers. This was confirmed by a significant decrease in the mean area% of collagen fibers compared to groupII. Res was found to exert anti-fibrotic effects against cardiovascular remodeling in doxycorticosterone acetate-treated rats(23).

In groupIII, less obvious caspase3+ve immunoexpression was demonstrated among the cardiac myocytes, this was confirmed by a significant decrease in the mean area% of +ve immunoexpression compared to groupII. Accumulated lines of evidence reported by Usta et al.(29) suggest that RES acts as an anti-apoptotic agent, providing cardioprotection through inhibition of caspase-3 expression and activity. Sin et al.(30) related the antiapoptotic effect to decreased level of acetylated p53 and of Bax.

Garcia et al.(23) recorded decreased PCNA proliferation marker in myocardial damage.

In groupII, several CD44 +ve spindle cells were detected, this finding can be related to caspase3 +ve immunoexpression in the same group as a direct relation. Li et al.(24) proved that postnatal stem/progenitor cells hold great promise to enhance repair of damaged tissues. Many of these cells are retrieved from bone marrow or adipose tissue. They were described as fibroblastic cells, plastic-adherent and exhibit a surface marker profile positive for CD73, CD44, CD90. They were defined by the International Society for Cellular Therapy as MSCs.

In RES post-treatment group (groupIII), some deeply acidophilic fibers, some thin attenuated and some fibers exhibiting dark peripheral nuclei were seen compared to groupII. The previous results indicated, regression of the degenerative and apoptotic changes developing in the cardiac myocytes secondary to DOX injection, in response to RES therapy. This was confirmed by a significant decrease in the mean area of degenerated cardiac myocytes. In support, a significant decrease in serum CK-MB was found before sacrifice. In line with, Tatlimde et al.(25) proved that RES treatment was shown to prevent the severity of DIC by alleviating the extent of oxidative stress, as demonstrated by increased levels of superoxide dismutase and decreased levels of malondialdehyde, suggesting its free radical scavenging capacity. Recently, it was confirmed that RES treatment of mice with established heart failure lessens cardiac fibrosis, improves molecular and structural remodeling of the heart, and enhances diastolic and vascular function(26).

Davatgaran-Taghipour et al.(27) commented that natural polyphenols have long been used for the prevention and treatment of several disorders due to their antioxidant, anti-inflammatory, cytotoxic, antineoplastic and immunomodulatory effects.

In group III, less dense collagen fibers were found among the cardiac muscle fibers. This was confirmed by a significant decrease in the mean area% of collagen fibers compared to group II. Res was found to exert anti-fibrotic effects against cardiovascular remodeling in deoxycorticosterone acetate-treated rats(23).

In groupIII, less obvious caspase3 +ve immunoexpression was demonstrated among the cardiac myocytes, this was confirmed by a significant decrease in the mean area% of +ve immunoexpression compared to groupII. Accumulated lines of evidence reported by Usta et al.(29) suggest that RES acts as an anti-apoptotic agent, providing cardioprotection through inhibition of caspase-3 expression and activity. Sin et al.(30) related the antiapoptotic effect decreased level of acetylated p53 and of Bax.

In group III, multiple PCNA +ve nuclei were evident confirmed by a significant increase in the mean area% of PCNA +ve nuclei compared to groupsII and IV. In accordance, Lee et al.(31) documented that sequential treatment with RES, upregulated PCNA in developing porcine embryos by regulating intracellular antioxidants.

In groupIII, multiple CD44 +ve cells were evident confirmed by a significant increase in the mean area% of +ve cells compared to groupsII and IV. In line with,
it was suggested that RES and adipose derived MSCs were successful in the prevention and treatment of DIC in rats. The hypothetical mechanisms of regeneration are multiple, including cell differentiation and autocrine/paracrine effects of MSCs. It was also evidenced that multipotent stem cells (SCs) develop into cardiomyocytes.

In RES pretreatment group (group IV), few congested blood vessels were detected among the muscle fibers, besides few deeply acidophilic and few thin attenuated fibers. This was confirmed by a significant decrease in the mean area of degenerated cardiac myocytes compared to groups II and III. In support, a significant decrease was found in the mean value of serum CK-MB before sacrifice. In agreement, a recent study performed by Chen et al. on diosgenin, a nutraceutical found in the edible tubers of one of the most used plants in the world, was proved to be a promising agent to prevent the DIC via its antioxidant, anti-apoptosis and anti-inflammation effects. Recently, cancer chemoprevention by phytochemicals as RES is thoroughly studied to assess their anti-inflammatory and antioxidant effects.

In group IV, fine collagen fibers were found among the cardiac muscle fibers, this was confirmed by a significant decrease in the mean area% of collagen fibers compared to group II. Concomitantly, resveratrol was proven to provide significant protection against DIC and fibrosis in rats. It was suggested to be used as a promising cardioprotective agent in patients treated with DOX due to malignant diseases.

In group IV, cardiac muscle sections revealed minimal caspase3 +ve immunoexpression among the cardiac myocytes, this was confirmed by a significant decrease in the mean area% of +ve immunoeexpression compared to groups II and III. It was stated that DOX in combination with RES was found to attenuate the DOX associated increased oxidative stress and apoptosis assessed by caspase3.

In group IV, some PCNA +ve nuclei were found among the cardiac myocytes. In agreement, Topinaga et al. demonstrated cellular proliferation in lung regeneration by increased expression of PCNA.

In group IV, some CD44+ve spindle cells were found among the cardiac myocytes, confirmed by a significant increase in the mean area% of +ve cells compared to group II. Going with, adult SCs were recorded to be present in most of the tissues and exhibit self-renewal and multipotency, contributing to tissue homeostasis. Among adult SCs, the mesenchymal ones have a greater availability and plasticity, likely candidacy them for applications in regenerative medicine. Liu et al. documented enhanced tissue regeneration by the use of chinese herbal extract through the antioxidant and anti-inflammatory. Elgebaly et al. confirmed activated liver regeneration following RES therapy.

CONCLUSION

Conclusion: RES proved amelioration of DOX induced myocardial injury that was more noticeable in RES early administration. RES post-treatment might have been more efficient if longer duration was applied.

CONFLICT OF INTEREST

There are no conflicts of interest

REFERENCES


الملخص العربي

العلاج المسبق و اللاحق بالريسفيراترول في حالة الإصابة القلبية المحدثة بواسطة عقار دوكسوروبيسين فيما يتعلق بتنشيط الخلايا الجذعية الذاتية

سحر جمال أبو الفضل1، مها شندي1، أحمد رضا2

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كلية الصيدلة - جامعة الشرق القريب - قبرص الشمالية - قبرص

المقدمة: دوكسوروبيسين (دوكس)، هو أحد الأدوية المضادة للأورام عالية الكفاءة والمستخدمة في علاج الأورام المصمتة بما في ذلك سرطان الثدي ويعتبر ريسفيراترول (ريس) عقار مثير للاهتمام في علاج الأورام المعتمدة على الهرمونات كما أوضح أن تنشيط الخلايا الجذعية قد يقلل من موت الخلايا المبرمج، ويعزز تكوين الأوعية الدموية الجديدة ويحسن من الإصلاح العددي لخلايا عضلة القلب.

مواد وطرق البحث:
تم تقسيم جرذان، المجموعة الثانية من ذكور الجرذان البيضاء البالغة إلى: مجموعة ضابطة تضم 6 جرذان، المجموعة التائية (مجموعة دوكس)، تحتوي على 7 جرذان تم حقنها بدوخ مقدار كل منها 3 مل/كجم من وزنها ثلاث مرات أسبوعيا لمدة أسبوعين. أما المجموعة الثالثة (مجموعة العلاج اللاحق بـ ريس) شملت 7 جرذان تم حقنها بدوخ ملعقة 50 مل/كجم من مادة ريس يوميا عن طريق الفم لمدة أسبوعين. وakhirًا المجموعة الرابعة (مجموعة العلاج المسبق بـ ريس) وشملت 7 جرذان تم حقنها بدوخ ريس وثقت أيضا 50 مل/كجم ريس يوميا عن طريق الفم لمدة أسبوعين، ثم تركت بدون علاج لمدة أسبوعين. نهاية التجربة، تم ذبح جميع الحيوانات في نهاية الأسبوع الرابع وتعريض عينات عضلة القلب للدراسات النسيجية والكيميائية المورفومترية، وكذلك التحليلات.

النتائج: في المجموعة الثانية (مجموعة دوكس) ظهرت إصابة عضلة القلب في صورة إحتقان الأوعية الدموية، زيادة موت الخلايا المبرمج، تحلل ارتفاع محتوى ألياف الكولاجين، ووضوح التعبير عن caspase 3 وانخفاض مستوى مستضد النواة PCNA والكراث CD44 (والتي كانت أجزاء محدودة من الخلايا الإيجابية لـ) و POSITIVE_VAUE (وتسمى في مجموعة المعالجة البيضاء المحفزة بـ) والكراث CD44 و (PCNA) الذي كان أكثر وضوحًا في مجموعتي العلاج.