RESEARCH ARTICLE

The Impact of Carvedilol on Organ Index, Inflammatory Mediators, Oxidative Stress Parameters and Skin Markers in D-Galactose-Induced Aging Mice

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Received: 22th March, 2023; Revised: 18th May, 2023; Accepted: 03rd June, 2023; Available Online: 25th September, 2023

ABSTRACT

Background: Aging processes are defined as those that increase the susceptibility of individuals, as they grow older, to the factors that eventually lead to death. It is a complex multi-factorial process where several factors may interact simultaneously and may operate at many levels of functional organization.. Accumulating evidence has suggested that active ingredients that possess antioxidative and anti-inflammatory proprties could decrease the incidence and development of aging-related diseases and promote longevity. Carvedilol is a non-selective third generation β-blocker/α1-blocker with antioxidant and inflammatory activity to evaluate its antiaging effect and to investigate if it is better to given carvedilol with aging induction or after aging induction is established.

Aim: To evaluate the effect of carvedilol on organ index, inflammatory mediators, oxidative stress parameters and skin markers in d-galactose-induced aging mice.

Method: Male (swiss albino mice) weighing 20 to 30 g and aged 3 to 6 months was randomly divided into six groups, each consisting of 10 animals (60 mice in total). Group I was given normal saline orally only for six weeks, group II was given d-galactose 500 mg/kg orally only for six weeks, group III was given d-galactose 500 mg/kg orally and vitamin C 100 mg/kg orally concomitantly for six weeks, group IV given d-galactose 500 mg/kg orally only for six weeks, then directly next day after ending of aging induction, vitamin c 100 mg/kg started for another six weeks, group V given d-galactose 500 mg/kg orally and carvedilol 10 mg/kg orally concomitantly for six weeks, group VI given d-galactose 500 mg/kg orally only for six weeks, then directly next day after ending of aging induction, carvedilol 10 mg/kg started for another six weeks. After that, animals were weighed, euthanized, and dissected, the heart was removed and the organ index was calculated. Also the heart tissue is divided in to two parts, one homogenated to measure interleukin-Iβ, tumor necrosis factor-alpha, glutathione peroxidase and malondialdehyde, while the other piece for histopathological analysis to determine the presence of hypertrophy. Skin tissue was taken and homogenated for ELISA assay (measurement of collagen I and III levels).

Results: Results showed a significant decrease in heart organ index in animals that received carvedilol orally administered compared to aged group, with a substantial decrease in inflammatory mediators, malondialdehyde level and a marked increase in glutathione peroxidase level, collagen I and collagen III levels. Furthermore, carvedilol reverses the myocardial hypertrophy induced by D-galactose.

Conclusion: The present study suggests that carvedilol has a positive antiaging benefit in reducing oxidative stress, decreasing inflammatory and skin aging markers and improving hypertrophic cardiac cells. Regarding the timing of drug administration, although carvedilol shows a benficial continuing impact when given with and after the initiation of aging. It's better to give at starting of the aging process to control the aging series as soon as possible.

Keywords: Carvedilol, D-Galactose, Aging.

International Journal of Drug Delivery Technology (2023); DOI: 10.25258/ijddt.13.3.41

How to cite this article: Shihab EM, Kadhim HM. The Impact of Carvedilol on Organ Index, Inflammatory Mediators, Oxidative Stress Parameters and Skin Markers in D-Galactose-Induced Aging Mice. International Journal of Drug Delivery Technology. 2023;13(3):1017-1023.

Source of support: Nil.

Conflict of interest: None

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INTRODUCTION

Aging is commonly defined as the accumulation of diverse deleterious changes occurring in cells and tissues with advancing age that are responsible for the increased risk of disease and death. The observation that most of the animals living in a natural environment rarely become senescent (because dying earlier for predation, disease, starvation, or drought) suggests that aging is a phenomenon unique to the human species.[1]

Different mechanisms contribute to the aging process. The main cause is due to the accumulation of random unrepaired molecular damage over time. This eventually leads to cellular defects, which gives rise to tissue dysfunction and aging.[2] These mechanisms include genomic instability,[3] Telomere attrition,[4] cellular senescence,[5] stem cell exhaustion.[5,6] altered intercellular communication.[7] These mechanisms work in a multi-layered model together to eventually result in the developing aging process.[8]

The foci of biological theories include explanations of deleterious effects leading to decreasing function of the organism, gradually occurring age-related changes that are progressive over time, and intrinsic changes that can affect all members of a species because of chronologic age.[9] Among the most important discussed theories of the inflammation-aging theory which is first described by Franceschi *et al*.,[10] which suggests there was a direct relation between age and macrophage activation (MACROPH-AGING).[11] Oxidative stress or free radical theory, suggested by Harman predicts that reactive oxygen species (ROS) are produced as an inevitable byproduct of metabolic activity. If these are not completely neutralized, oxidized lipids are potent ROS, autocatalyzing this process.[12] Unrepaired damage accumulates and results in the typical aging phenotype.[13,14] Alterations in the structure and function of arteries accompany aging and contribute to increased risks of developing CVD. Vascular modifications occurring with aging identify 2 main features: generalized endothelial dysfunction and central arterial stiffness.[15]

Carvedilol is s a third-generation, non-selective, competitive antagonist for the beta-adrenoreceptor (blocks both β1 and β2) with[16] peripheral vasodilatory properties due to α1-adrenoreceptor antagonism.[17] Carvedilol is used to treat hypertension heart failure, improve cardiac function after myocardial infarction, and reduce infarct size following myocardial ischemia and reperfusion injury.[18] Carvedilol has antioxidant, anti-inflammatory, anti-apoptotic, antiproliferative and cardiac remodeling attenuation effects. The antioxidant activity of carvedilol is due to its chemical structure that contains tricyclic moiety and its ability to directly scavenges oxygen free radicals.[19,20] It has also been reported that treatment with beta-blockers was negatively correlated with circulating levels of inflammatory cytokines such as tumor necrosis factor. Tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) in patients with CHF, indicating that inhibiting sympathetic nervous system activation might be associated with suppression of inflammatory reactions.[21] The aim of this study to evaluate the antiaging activity of carvedilol on several parameters related to the ageing process in experimentally induced aging mice

MATERIALS AND METHODS

Male (swiss albino mice) weighing 20 to 30 g aged 3 to 6 months was randomly divided into six groups each consisting of 10 animals (60 mice in total). The animals were identified by marking at different parts of the body. The mice were obtained from the National Center for Drug Control and Research, housed in polypropylene cages under a temperature-controlled environment (22 ± 2°C), with an inverted light-dark cycle (12/12 hours) and acclimated for two weeks before the study (Animal Facility of the Al-Nahrain University – Biotechnology research center, Baghdad, Iraq).

The animals were maintained on a standard pellet diet and free access to water *ad libitum* supplied by Al-Nahrain University – Biotechnology Research Center.

For animal allocation: Group I acted as (Control) given (normal saline) orally via gastric gavage once daily for six weeks (without D-galactose). Group II acted as an aging Induction model) by giving 500 mg/kg of D-galactose orally by gastric gavage once daily for six weeks.[22] Group III (Vitamin C started with induction of aging), given 500 mg/kg of D-galactose orally by gastric gavage once daily and concomitantly given Vitamin C 100 mg/kg orally by gastric gavage once daily for six weeks duration.[22,23] Group IV (Vitamin C started directly after the ending of aging induction), given 500 mg/kg of D-galactose orally by gastric gavage once daily for six weeks[22] and next day after aging induction was achieved, vitamin C 100 mg/kg was given orally by gastric gavage once daily and continued for another six weeks.[23] Group V (Carvedilol started with induction of aging) given 500 mg/kg of D-galactose orally by gastric gavage once daily and concomitantly given carvedilol 10 mg/kg orally by gastric gavage once daily for six weeks.[22,24] Group VI (Dapagliflozin started directly after ending of aging induction) given 500 mg/kg of D-galactose orally by gastric gavage once daily for six weeks[22] next day after aging induction achieved. Carvedilol 10 mg/kg was given orally by gastric gavage once daily for another six weeks.[24] D-galactose was supplied as powder preparation by Sigma Aldrich®, USA, (CAS no. 59-23-4). Carvedilol was supplied as powder preparation by Hangzhou Hyper Chemicals Limited®, China, (72956-09-3). Vitamin C was supplied as powder preparation by Hangzhou Hyper Chemicals Limited®, China, (CAS no.86404-04-8).

Induction and Assessment of Aging in Mice

Galactose in a dose of (500 mg/ kg) was given orally by gastric gavage once daily for 6 weeks to 70 male mice to induce aging. Assessment of aging of mice was carried out and its involved: ruffled fur and a more rounded general appearance, and they may seem less alert, less active, appearance of wrinkle skin and less responsive or more tentative in their movements than do young mice.[25]

Outcome Measures

Weight measurement for all mice was done at baseline and before euthanasia. Groups (I,II,III,V) mice were euthanized at the end of their substance administration period for 6 weeks. Groups (IV,VI) mice were euthanized at the end of their substance administration period for 12 weeks. The process of euthanasia for all mice was total anesthesia followed by exsanguination (cardiac puncture), a procedure suitable for tissue harvest and conservation.[26,27] After the end of each group experimental period, dissection has been done for the euthanized mice, and the heart was removed and weighted to determine the organ index based on the following equation described by Chen *et al.*[28]

$$Organ\ index\ (\%) = \frac{\text{organ weight (g)}}{\text{body weight (g)}}\ x\ 100\%$$

Also, a piece of 1-mm of the dorsal skin area has been harvested. Heart tissue was divided into 2 pieces one piece was used for histopathological analysis, first washed with "PBS, pH 7.4" then processed by the traditional processing procedure by the paraffin-embedded method.[29] The other piece of heart tissue together with the skin tissue were isolated and rinsed with cold phosphate buffer saline "PBS, pH 7.4", then the tissue was dried with filter paper and used for ELISA analysis by (ELISA reader, Diagnostic Automation/Cortez Diagnostics®, California, USA) and weighed by sensitive balance. For ELISA each 50 mg of tissue was put in an Eppendorf tube (Eppendorf®, Hamburg, Germany) containing 0.45 mL of chilled PBS and then minced into small pieces. The tube containing the tissue was then put in an ice-containing beaker to keep it cold and then homogenized by the homogenizer machine (Electrical tissue homogenizer, Staruar®, England). The homogenate was centrifuged for 20 minutes at 4°C and 2000 rpm in a cold centrifuge (Thermos scientific ®, USA). The supernatant was isolated using a micropipette (Bioevopeak®, China), and stored at -20°C until the day of analysis.[30]

Biochemical Analysis

The resultant stored supernatant of the sampled mice homogenated heart and skin tissues was then thawed and sent for biochemical analysis by double-sandwich ELISA technique for TNF-α by the kit (Mouse TNF-A ELISA KIT, product ID SL0547Mo, Sunlong biotech®, China), Interleukin-1Beta (IL-1β) by the kit (Mouse Interleukin1beta, IL-1beta ELISA Kit, product ID SL0316Mo, Sunlong biotech®, China), Glutathione peroxidase (GSH-Px) by the kit (Mouse GSH-Px ELISA Kit, product ID SL0241Mo, Sunlong biotech®, China), MDA by the kit (Mouse MDA ELISA Kit, product ID SL0370Mo, Sunlong biotech®, China), Collagen I (Col-I) by the kit (Mouse Col-I ELISA Kit, product ID SL0141Mo, Sunlong biotech®, China) and Col-III by the kit (Mouse COL-III ELISA Kit, product ID: SL0942Mo, Sunlong biotech®, China).

Light Microscopy

Applied by counting the number of hypertrophic cells in 1-mm area of tissue per 5 high power field (hpf) magnified by 400X. (modified from., Cree. *et al.* on WHO classification of tumor).[31] A light microscope **(**Olympus BX51 Microscope, Olympus Corporation®, Japan) was used to take five zones of a slide corner and the center, randomly at 40X magnification power[32] hypertrophic cells characterized by the presence of nuclei which are an increase in size, irregular, and hyperchromatic (Basso *et al*., 2021)

Ethical Approval

The study was approved by the ethical committee of Al-Nahrain University/College of Medicine (Approval no. :20210914, on 9th of December 2021).

Statistical Analysis

In the current study, GraphPad Prism version 10.0.1 used for the statistical analysis. The descriptive statistics were reported as mean ± standard deviation (SD). The analysis of variance (one-way ANOVA) test was applied to verify the significance of the difference between the studied groups, then followed by the post hoc Tukey test. The differences between the groups were considered significant statistically when the P-value was less than 0.05 ($p < 0.05$).

RESULTS

Regarding effect on Inflammatory mediators, tumor necrosis factor-alpha (TNF-α) and interleukin 1 beta(IL-1 β,). They showed similar patterns of changes, in summary, the means of inflammatory mediations were highly significantly elevated in the induction group (GII) compared to a control group (GI) ($p < 0.001$). There was no significant difference among groups GIII, GIV, and GV. Meanwhile, all these groups were significantly lower than GII, and significantly higher than G1. Furthermore, GVI was statistically significantly higher compared to the all other groups except GII (it was lower), as illustrated by Table 1.

Regarding the effect on GSH-px, the means of these markers was highly significantly reduced in the induction group (GII) compared to the control group (G1) ($p < 0.001$). There was no significant difference among groups GIII, GIV, and GV. Meanwhile, all these groups were significantly higher than GII and significantly lower than GI. Furthermore, GVI was statistically significantly lower than all other groups except GII (which was higher), as illustrated by Table 2.

Furthermore, MDA, it was highly significantly increased in the induction group (GII) compared to the control group (GI) ($p < 0.001$). There was no significant difference among groups GIII, GIV, and GV. Meanwhile, all these groups were significantly lower than GII and significantly higher than GI. Of notice, no significant differences among groups GV, and GVI, while GVI was significantly higher than GIII, and GIV, as illustrated by Table 2.

Regarding collagen I and collagen III levels, the means of skin markers were highly significantly reduced in the induction group (GII) compared to the control group (GI) ($p < 0.001$). There was no significant difference between GIII, and GIV. Of note, GV and GVI were significantly different from the other groups, as illustrated by Table 3.

Table 1: Evaluation of inflammatory mediators according to study groups

Groups	*TNF-α* *Mean ± SD*	*IL-1beta* *Mean ± SD*
GI: Normal control	27.70 ± 1.57 [a]	16.08 ± 3.17[a]
GII: Induction	299.60 ± 92.96 [b]	68.40 ± 6.20[b]
GIII: vitamin C 100 mg/kg with induction	87.92 ± 20.69 [c]	28.28 ± 5.58[c]
GIV: vitamin C 100 mg/kg after end of induction	87.66 ± 8.64 [c]	29.42 ± 4.42[c]
GV: carvedilol10 mg/kg with induction	129.32 ± 17.69 [c]	31.77 ± 5.75[c]
GVI: carvedilol 10 mg/kg after end of induction	217.26 ± 42.40[d]	50.54 ± 3.79[d]
p-value	<0.001[***#]	<0.001[***#]

Column with similar letter indicate no significant difference ($p \geq 0.05$), while different litters indicate significant difference ($p < 0.05$)
[#] One Way ANOVA (Post *hoc* Tukey test)
[***] indicate highly significant difference
SD: standard deviation

Table 2: Evaluation of study agents on oxidative stress markers

Groups	*GSH-Px* *Mean ± SD*	*MDA* *Mean ± SD*
GI: Normal control	5.10 ± 0.53[a]	22.92 ± 7.72[a]
GII: Induction	0.29 ± 0.15[b]	217.15 ± 55.25[b]
GIII: vitamin C 100mg/kg with induction	4.03 ± 0.43[c]	57.36 ± 11.71[c]
GIV: vitamin C 100mg/kg after end of induction	3.86 ± 0.54[c]	56.60 ± 7.94[c]
GV: carvedilol10 mg/kg with induction	3.52 ± 0.49[c]	65.87 ± 8.13[cd]
GVI: carvedilol 10 mg/kg after end of induction	2.28 ± 0.61[d]	91.14 ± 23.95[d]
p-value	<0.001[***#]	<0.001[***#]

Column with similar letter indicate no significant difference ($p \geq 0.05$), while different litters indicate significant difference ($p \geq 0.05$)
[#] One Way ANOVA (Post *hoc* Tukey test)
[***] indicate highly significant difference
SD: standard deviation

Table 3: Evaluation of study agents on skin markers (COL-I and COL-III)

Groups	*COL-1* *Mean ± SD*	*COL-III* *Mean ± SD*
GI: Normal control	3,095.34 ± 295.70[a]	2,895.34 ± 295.70[a]
GII: Induction	837.47 ± 244.55[b]	637.47 ± 244.55[b]
GIII: vitamin C 100mg/kg with induction	2,584.10 ± 216.80[c]	2,384.10 ± 216.80[c]
GIV: vitamin C 100mg/kg after end of induction	2,527.16 ± 323.56[c]	2,127.16 ± 323.56[c]
GV: carvedilol10 mg/kg with induction	1,752.80 ± 173.75[d]	1,552.80 ± 173.75[d]
GVI: carvedilol 10 mg/kg after end of induction	1,256.44 ± 151.89[e]	1,056.44 ± 151.89[e]
p-value	<0.001[***#]	<0.001[***#]

Columns with similar letter indicate no significant difference ($p \geq 0.05$), while different litters indicate a significant difference ($p \geq 0.05$)
[#] One Way ANOVA (Post *hoc* Tukey test)
[***] indicates highly significant difference
SD: standard deviation

Regarding Hypertrophic Changes in Myocardial Tissue

Histopathological Quantification of cardiomyocyte hypertrophy showed that the heart hypertrophic cell count was highly significantly increased in the induction group (GII) compared to the control group (GI) ($p < 0.001$). There was no significant difference GIII, GIV, GV and GVI. Additionally, all these groups were significantly different compared to GI, and GII, as illustrated by Table 4, while Figures 1 and 2 showed hypertrophic myocardial cells in comparison to normal myocardial cells in heart sections.

DISCUSSION

In the present study, D-galactose produces cardiac hypertrophy, but when the mice received carvedilol, this effect was attenuated, resulting in a lower heart index compared to group II. Carvedilol reduces the heart weight in mice treated for 16-week high fructose diet, indicating a reduction in cardiac hypertrophy.[33] Similar findings of reduction of hear weight for carvedilol treatment.[34,35] This could be attributed to its beta blocker and its antiproliferative effects since previous fundamental research showed that cardiac-specific overexpression of β1-receptors in mice caused cardiomyocyte hypertrophy,[36,37] selective β1-receptor blockers might show better regression on LVH like carvedilol.[38]

One of the most significant alterations that occurs with age is a deregulation of the immune response that results in a chronic systemic inflammatory state.[39] In the present study various inflammatory markers obtained from heart homogenate content was evaluated in different studied groups, as a result TNF-α, IL-1β was significantly improved in mice that received carvedilol (given both with and after induction phase) in comparison to the induction group. It was also noted that this improvement was more pronounced in mice given carvedilol with induction. In a comparable research, Raimundo Fernandes de Araujo *et al.* found that carvedilol dramatically reduced levels of the proinflammatory cytokines IL-1β and TNF-α.[40] Flori R. Sari *et al.* found that inflammation-mediated cardiotoxicity in daunorubicin-induced mice was greatly reduced by carvedilol therapy, as measured by cardiac production of TNF-α.[41] Both of these studies agree with the present findings.

Mechanistically, carvedilol's anti-inflammatory effect is linked to decreased levels of tumor necrosis factor alpha and interleukin 1 beta and increased levels of interleukin 10 (IL-10), a powerful anti-inflammatory molecule that has been demonstrated to prevent the activation of nuclear factor kappa B. The expression of adhesion molecules and chemokines is likewise suppressed by IL-10, and the generation of reactive oxygen intermediates is decreased in macrophages.[42]

In the present study various oxidative stress markers obtained from heart homogenate content was evaluated

Table 4: Evaluation of study agents on heart hypertrophy cells count

Groups	*Heart hypertrophic cell count. Mean ± SD*
GI: Normal control	21.70 ± 2.54^{a}
GII: Induction	90.30 ± 6.73^{b}
GIII: vitamin C 100 mg/kg with induction	51.60 ± 7.35^{c}
GIV: vitamin C 100 mg/kg after end of induction	56.10 ± 5.67^{c}
GV: carvedilol 10 mg/kg with induction	52.30 ± 7.33^{c}
GVI: carvedilol 10 mg/kg after end of induction	58.10 ± 7.22^{c}
p-value	$<0.001^{\#***}$

Column with similar letter indicate no significant difference ($p \geq 0.05$), while different litters indicate significant difference ($p \geq 0.05$)
$^{\#}$ One Way ANOVA (Post *hoc* Tukey test)
*** indicate highly significant difference
SD: standard deviation

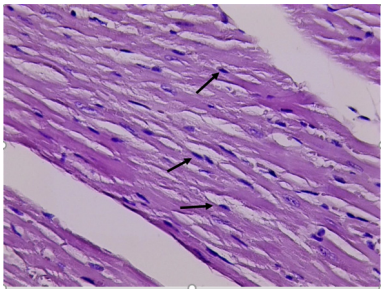

Figure 1: Longitudinal -sections of hematoxylin- and eosin-stained under light microscopy of the normal cardiac tissue in mice received normal saline only, showing normal cardiac cells and normal nuclei [Black arrow] (Olympus BX51 microscope, *X40*).

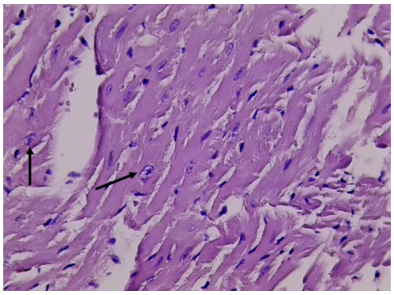

Figure 2: Longitudinal -sections of hematoxylin- and eosin-stained under light microscopy showed hypertrophied cardiomyocytes with bizarre, irregular, and hyperchromatic nuclei [Black arrow] (Olympus BX51microscpose, X40).

in different studied groups, as a result, GSH-Px, level was elevated while MDA level was decreased in mice receiving carvedilol both with or after the induction phase in comparison with the induction group. This change was more pronounced in the group receiving carvedilol with induction. Regarding carvedilol it had been shown that treatment with carvedilol reduced the levels of MDA and increased the activity of GSH-Px in mice model.[43] More recent study showed that carvedilol treatment decreased MDA level.[44] Another study also showed increased levels of GSH and reduced levels of MDA in mice treated with carvedilol.[45] These results came in accordance with our findings. Carvedilol keeps tissues under oxidative stress from losing their natural reserves of the antioxidants GSH and α-tocopherol (vitamin E).[46]

In the present study carvedilol with and after induction showed significantly higher skin levels of COL-1, and COL-3 compared to the induction group. Also, these changes were more pronounced in mice given carvedilol with galactose induction no single study examined the effect of carvedilol on skin aging in both animals and humans.

Carvedilol have mixed effect on beta-adrenergic system, it has more affinity towards β2 than β1 receptor.[47,48] Beta 2 adrenergic receptors are the dominant type of beta receptors in the skin.[49] In their study, Berg *et al.* showed that the addition of beta-adrenergic agonist caused an increase in cAMP and coupled with reduction in the levels of collagen later upon the administration of beta-adrenergic antagonist, this led to a reduction in cAMP coupled with increase collagen levels.[50] This reduction of collagen related to effect of cAMP on collagen degradation, in which increase cAMP led to increase its degradation and consequently reduction in collagen levels,[51] since in the current study the administration of carvedilol with/after induction increased collagen levels better than that for induction group, in the same time it was even better for carvedilol with induction compared to after induction. That means carvedilol increases collagen in skin tissue possibly via inhibiting cAMP pathway, a common mechanism for carvedilol and other beta-blockers effect.[52]

Most components of the cardiovascular system undergo some degree of change with aging.[53,54] The result of the current study showed that the heart hypertrophic cells count was highly significantly increased in the induction group compared to the control group and other treated groups, there was no significant difference among carvedilol (with and after induction). Additionally, all these groups were significantly different compared to induction and normal groups. The results of present study consist with previous studies that carvedilol repressed cardiac hypertrophy induced by aortic banding.[55, 56] Also in accordance with a study showed that carvedilol improves myocardial function and attenuates abnormal cardiac hypertrophy caused by daunorubicin.[57] Another study revealed that carvedilol reduced total cellular oxidative stress and myocyte apoptosis and cell hypertrophy in CHF was in agreement with current study.[58]

In addition to antioxidant anti-inflammatory effect of carvedilol, it has unique effect regarding to heart protection, since it has beta adrenoceptor and alpha adrenoceptor (AR-1) blocking properties.[59,60] AR-1 stimulated hypertrophy via activation of the Ras, mitogen/extracellular signal-regulated kinase (MEK) 1/2, and extracellular signal-regulated kinase (ERK) 1/2 signaling pathway.[61] Beta-adrenoceptors, play a key role in the rapid regulation of myocardial function. Meanwhile, chronic catecholamine stimulation of adrenoceptors involved in the adverse myocardial remodeling, including cardiac hypertrophy. A series of key signaling pathways involved in this process which are especially classical pathway Gs/AC/cAMP/PKA signaling cascades and MAPK signaling cascades.[62-64]

CONCLUSION

Carvedilol has a positive effect on several aging parameters in mice as shown by this study. It decreases the hypertrophy in cardiomyocytes, improves skin vitality, decreases the burden of inflammatory mediators and improve the impact of anti-oxidants both when administered in concomitance with or after the aging induction. But more effective when given with induction.

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