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Ameliorating role of whey syrup against ageing-related damage of myocardial muscle of Wistar Albino rats

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ABSTRACT

Age-ing is involved in gradual breakdown of biological structure and function of body organs. The heart represents the main organ responsible for pumping the main issues of life which involving oxygen, nutrients and bioactive molecules necessary for maintaining the body functions. The present study has been conducted to assess the anti-aging properties of whey syrup collected from fermented milk in 4, 18 and 30-months-old rats. The histopathological and histochemical changes of carbohydrates and proteins were investigated. Immunohistochemical expression of smooth muscle actin and P53 was performed to assess the function of cardiomyocytes. Furthermore, Annexin v and biochemical changes of different cardio-biomarkers were carried out to evaluate the effects of aging. The present result of 30 months-old rats revealed myocardial infarction assessed by widening of myocardial fibers, diffused with numerous blood capillaries and dense leukocytic infiltration. The assessed biochemical markers confirmed myocardial damage. Whey supplementation improved the myocardial structure, but less improvement was observed for the 30-months-old rats. The author recommended supplementation with whey is beneficial in giving a body the demand for amino acids and minerals essential for supporting the myocardium and also provides protection against age-ing.

1. Introduction

Aging is manifested by a programmed breakdown of fitness, which results from the increase of cellular damage related to the degradation of structure and loss of function due to chronic stress of small intensity. The cells undergo damage over life time as indicated by decreasing the growth rate and impairing the cell functions (Speakman et al., 2004). Examining the heart ventricle of 26–93 years old detected increased average of 74% mononucleated myocytes compared to 25.5% binucleated ones. Aging-related cardiac hypertrophy and ischemic cardiomyopathy were characterized by increased myocyte size and increased average of myocyte hypertrophy, compared to apparent myocyte cell loss in ischemic cardiomyopathy (Olivetti et al., 1996). Cardiac myocytes isolated from 6, 32–33 months old female F344xBN revealed an increase in the relative amount of b-myosin heavy chain during the ageing process and a significant rightward shift in the tension-pCa curve (Wahr et al., 2000). Collagens types I and III represent the important elements in facilitating muscle contraction. The myocardial fibroblasts are the important source of the collagenous materials (de Souza, 2002). Ageing caused excessive impairment of a mocardial structure including fibrosis and cardiomyocyte cell density, and cardiac function via cardiac output (Kwak, 2013). Moreover, it is involved in ischemic reperfusion, the main factor of myocardial and mitochondrial damage leading to the production of reactive oxygen species, which enhanced the altered myofibrillar Ca^{2+} and concomitant impaired the muscle contractile function (Zhou et al., 2018). Hypertensive C57BL/6J mice exhibited dilation and reduced contractility of myocardium. A marked reduction of myocytes coupled with an increase of interstitial fibrosis were observed. In addition, there was a detected increase of collagen deposition, and wall stiffness (Anversa et al., 1990; Lin et al., 2008). In normal myocardium, α-smooth muscle actin (α—SMA), is expressed and the amount of their transcripts has been shown to vary with species, developmental stage, aging and during pathological situations (Winegrad et al., 1990). During cardiogenesis, α-SMA makes their first differentiation during cardiomyocyte differentiation, and replaced by α-skeletal muscle actin and α-cardiac muscle actin isoforms (Woodcock-Mitchell et al., 1988). The α smooth muscle actin

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(α-SMA) is normally expressed in developing cardiomyocytes and overexpressed as a marker for myocardial hypertrophy in adult hearts. cTnI-ND transgenic mice at 2 and 3 months of age overexpressed α-SMA outside of the myofibrils (Kern et al., 2014). Cardiac α-smooth muscle actin (α-SMA) is expressed in mature myofibroblasts and become more abundant in murine myocardial infarction (Shinde et al., 2017). In addition, the tumor suppressor p53 regulated DNA repair and cell division, cellular aging and apoptosis (Riley et al., 2008). Patients with acute myocardial infarction showed increased level of P53 and 8-oxyhydroxydeoxy guanosine, which promoted apoptosis (Najar et al., 2010). P53 is more activated during aging and reduced tissue regeneration (Carrasco-Garcia et al., 2017). In a murine model of left ventricular pressure overload, increased expression of p53 in vascular endothelial cells and bone marrow cells promoted inflammatory cell infiltration into the heart, contributing to cardiac remodeling and systolic dysfunction (Katsuumi et al., 2016). Whey syrup of fermented milk is rich in lactic acid bacteria, which inhibit the growth of the pathogenic isolates *Salmonella enterica*, serovar Enteritidis and *E. coli* (Londero et al., 2011). Fermented Hazelnut milk exhibited increased DPPH radical scavenging activities and reducing power values (Maleki et al., 2015). Probiotic is rich in micronutrients, *Lactobacilli* and *Bifidobacterium pseudocatenulatum* which give a great help in synthesis of vitamin B complex and exhibited a great role in treating hypercholesterolemia involved in atherosclerosis and myocardial disease (Al-Sheraji et al., 2012; Paillard et al., 2015; El-Sayyad, 2017). *L. helveticus* from fermented milk exhibited improvement of blood pressure and myocardial function (Fuglsang et al., 2002). Protein in whey is rich in amino acids, which stimulate beta cells to secrete insulin, and consequently reduce higher glucose level and improve diabetes, the main contributing factor of diabetes (Mignone et al., 2015). Whey protein was found to decrease cholesterol level and soluble intercellular adhesion molecule 1 and soluble vascular cell adhesion molecule 1, the promoters of endothelial damage (Fekete et al., 2016). Taking into consideration the dramatic alteration of the biological process of breakdown of myocardial structure and function during ageing. The present study aimed to maintain the structure and function in the ageing myocardium via ongoing cellular repair and replacement, as well as survival of existing cardiomyocytes that generate contractile force. In whey syrup of fermented milk was used as dietary supplements to manage the histo-pathological, Immuno-histochemical and biochemical changes of aged myocardial muscle of rats at 18 and 30 months compared to 4 months old rats.

2. Materials and methods

Thirty-six male Wistar Albino rats (*Rattus norvegicus*) 6, 18 and 30-months-old **(n = 6)** were used during experimentation. The animals were obtained from Breeding Farm, Ministry of Health, Cairo, Egypt. The rats were acclimatized in well aerated room with controlled temperature (21 ± 2 °C), 50 ± 5% humidity and 12-h light and dark cycle. Free excess of food and water was allowed *ad libitum*. Rats were arranged into six groups; 18 male Wistar Albino rats remain feeding on standard diet. The other 18 rats, orally supplemented with freshly whey syrup (300 mg/kg body weight) collected from fermented buffalo milk, for 48 days. Rats were sacrificed and their hearts were incised. Half of the specimens were weighed and divided into two halves of which one was used for assessment anexin v for apoptosis. The other specimens were used for biochemical investigations via homogenization with phosphate buffer PH7.4, centrifugation and separation of their supernatants and keeping on a refrigerator. The other half was fixed in 10% phosphate buffered formalin. The heart samples were employed for the following investigations:

2.1. Histological & histochemical investigation

Heart samples of the mentioned ages were fixed in 10% phosphate buffered formalin (pH 7.4), dehydrated in ascending grades of ethyl alcohol, cleared in xylol, and mounted in molten pararplast 58–62 °C. Serial 5 μm thick sections were cut and stained with hematoxylin and eosin (H & E), mercuric bromophenol blue and periodic acid sciff reaction (PAS) (Drury and Wallington, 1967) and examined under bright field light microscopy, then photographed.

2.2. Immunohistochemistry

Five μm histological sections were cut from the previously prepared paraffin blocks of the heart at different age levels. They were mounted onto super frost plus glass slides (Fisher Thermo Scientific, Nepean, Ontario, Canada) and kept at normal room temperature. They were processed for antigen retrieval by digestion in 0.05 % trypsin (pH 7.8) for 15 min at 37 °C and incubated with antibodies against α-smooth muscle actin (Thermo Fisher Scientific, Fremont, CA, USA; Cat. No. A1-70007) and P53 (DAKO, clone MIB5, 1:50, mouse) and counterstained with Harris hematoxylin. The specimens were observed with a Leica BM5000 microscope (Leica Microsystems, Wetzlar, Germany) and photographed.

2.3. Biochemical investigation

The obtained serum samples were used to estimate colorimetrically lactic dehydrogenase, superoxide dismutase and malondialdhyde using Bio Vision kit cat. Nos; k726-500, k335-100 & k739-100. Troponin1 (cTn-1), Nuclear factor Kappa B (NF-kB), caspase 3 (casp3), tumor necrosis factor-α (TNF- α) and creatine kinase (CK-MB) were determined by ELISA Kit produced by HCHSA BIO

2.4. Assessments of annexin V

Flow cytometric analysis was carried out on FACScan (Becton Dickinson) using fluorescence 1 (FL1), 4 decades (logarithmic), detector 648 V, log amplifier, compensation 1.1%; fluorescence 2 (FL2), 4 decades (logarithmic), detector 496 V, log amplifier, compensation 22.8%. Data analysis was performed using lysis software (Becton Dickinson). Heart specimens were carried out, and cell suspension was prepared with Tris –EDTA buffer (pH 74) (Sigma–Aldrich Co.). They were fixed in ice-cold 96–100 % ethanol (Sigma) at 4 °C overnight, centrifuged at 1500 rpm for 10 min, and suspended in PBS containing 50 μg/mL propidium iodide (PI) (Sigma–Aldrich Co.). Single cell suspensions were prepared from at least of five samples of each of the aging rats, and $1.5–3 \times 10^6$ cells were stained with fluorescein isothiocyanate-conjugated annexin V (annexin V-FITC) and assayed after incubation for 15 min at room temperature.

3. Statistical analysis

Data are presented as mean ± standard error (SE). The statistical analysis was performed with one way post-hoc analysis of variance (ANOVA) using SPSS (version 13) software package for windows, post hoc analysis of comparing the variations between studied groups and $P < 0.05$ was considered statistically significant.

4. Results

4.1. Histological, histochemical & Immunohistochemical observations

Hematoxylin and eosin stained myocardium of the control ventricle of both control and whey supplemented 4-months-old

possessed regularly oriented muscle fibers with single or binucleated and centrally located. Intercellular spaces were enclosed by fine collagenous tissue containing fine blood capillaries (Fig. 1A).

In contrast, the ventricle of 18-months-old rats showed a slight histopathological alterations of damaged cardiomyocytes which almost improved post whey supplementation (Fig. 1B and C). In 30-months-old rats, the myocardium muscle fibers were irregularly arranged associated with numerous necrotic spots infiltrated by inflammatory cells. The necrotic fibers appeared eosinophilic and their nuclei dispersed in a zone of inflammatory cells. There were a detectable increase of newly formed blood capillaries of different sizes in between the muscle fibers. The distribution was associated with hyaline and degenerated the muscle fibers. The main blood vessels were atrophied and their lining layers tunica intima, media and adventitia attained a considerable atrophy (Fig. 1D). Following assay the qualitative protein content by staining with mercury bromophenol blue, intense protein staining was observed in 4 months old of 4 months old and or whey syrup supplementation (Fig. 1A1). Eighteen-months old rats exhibited intense blue staining affinity in cardiomyocytes. However, there was a detected of the presence of faintly stained cardiomyocytes (Fig. B). In eight moth-old supplemented whey, there was no observed change of the staining affinity (Fig. C). At 30 months old, the myocardial muscle irregularly oriented and possessed different patched of faintly protein stained affinity infiltrated by stretch of dense protein staining in a short thin sheath (Fig. D). Whey protein supplementation to 30 months old exhibited a decrease in fragility of muscle fibers, but gave no improvement of the faintly stained protein staining (Fig. E). Periodic acid staining exhibited increased PAS glycogen staining affinity horizontally oriented in parallel manner in both 4 months old and whey supplemented group (Fig. 1A2). Eighteen months old exhibited faintly stained PAS staining affinity (Fig. B2). However, whey supplementation increased the PAS glycogen staining affinity (Fig. C2). Thirteen months old possessed faintly staining affinity (Fig. D2) which moderately increased post-whey supplementation (Fig. E2). In 4 months old with or without whey supplementation, cardiomyocytes possessed negative immunoreaction of α-SMA (Fig. 2A & B). At 18 months old, intense patches of dark-brown immune reaction of α-SMA were sparse distributed in the muscle fibers (Fig. 2C). Whey supplementation to 18 months - old decreased the immunostaining reaction but was still exist with faint reaction (Fig. 2D). In 30 months old, intense immune reaction of the α-SMA were visualized distributed in the myocardial muscle fibers (Fig. 2E). Whey supplementation to 30 months old decreased the immune reaction but was still less dense (Fig. 2F). Image staining affinity attained markedly increased in the senile rate and improved to some extent after whey supplementation (Fig. 2G). Concerning P53, In 4 months old with or without whey supplementation, cardiomyocytes possessed missing of the immunoreaction of P53 (Fig. 3A & B). In 18 months old, moderate dark-brown immune reaction of P53 was distributed in the muscle fibers (Fig. 3C). Whey supplementation to 18 months-old decreased the immunostaining reaction but was still exist in faint reaction

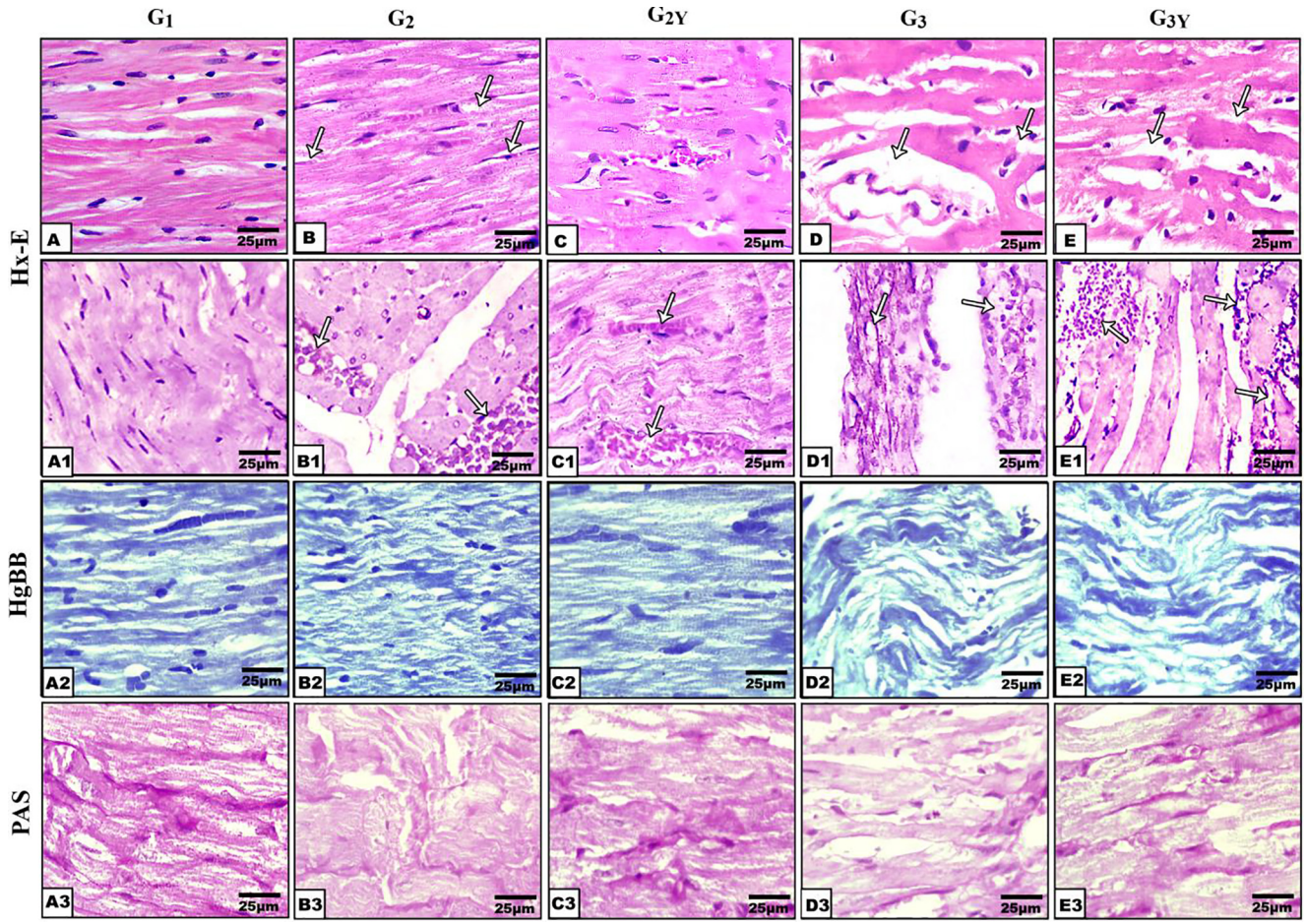

Fig. 1. photomicrographs of histological sections of myocardium of rat. A–A3. Four month-old. B–B3. 18 Month old. C–C3. 18 –Month old supplemented whey for 2 months. D–D3. 30 Month old. E–E. 30 Month old supplement whey. A–E & A1–E1. Hematoxylin and eosin stain myocardium. Note fragility of myocardial muscle and atrophied bood vessel (D & E) and widespread of blood capillaries inbetween muscle fibers in Fig. D1 and E1. A1–E1. Mercury bromophenol blue. A2–E2. Periodic Schiff stained. D. hyalinized amyloid materials fill the spaces in between the cardiomyofibrills.

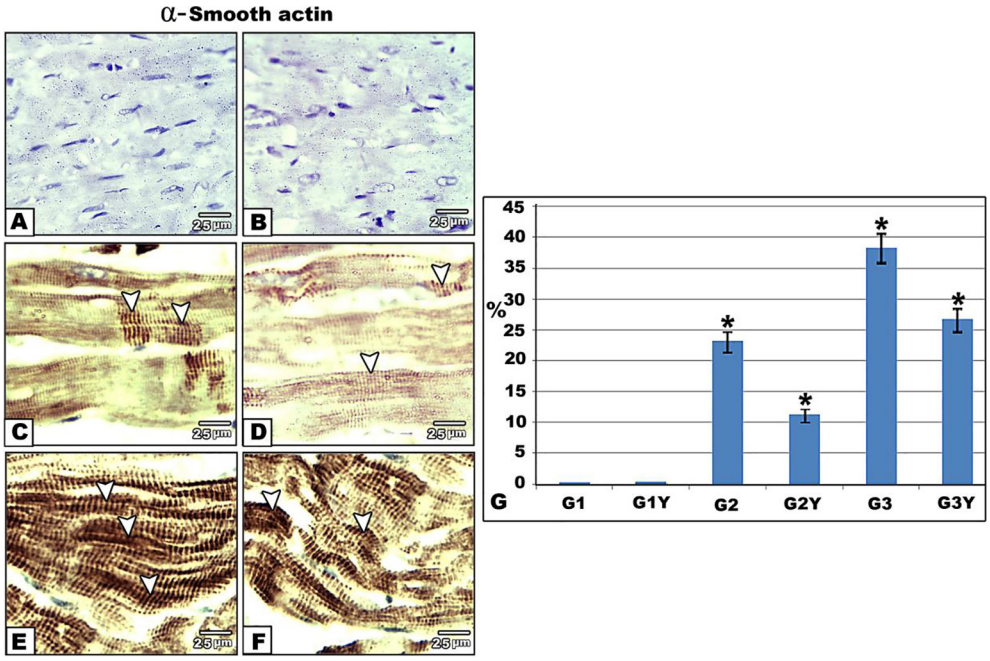

Fig. 2. Photomicrographs of formalin fixed histological sections of myocardium of rat immunostained with the antibody of α-smooth actin. A. Four month-old. B. 18 Month old. C. 18 –Month old supplemented whey for 2 months. D. 30 Month old. E. 30 Month old supplement whey.

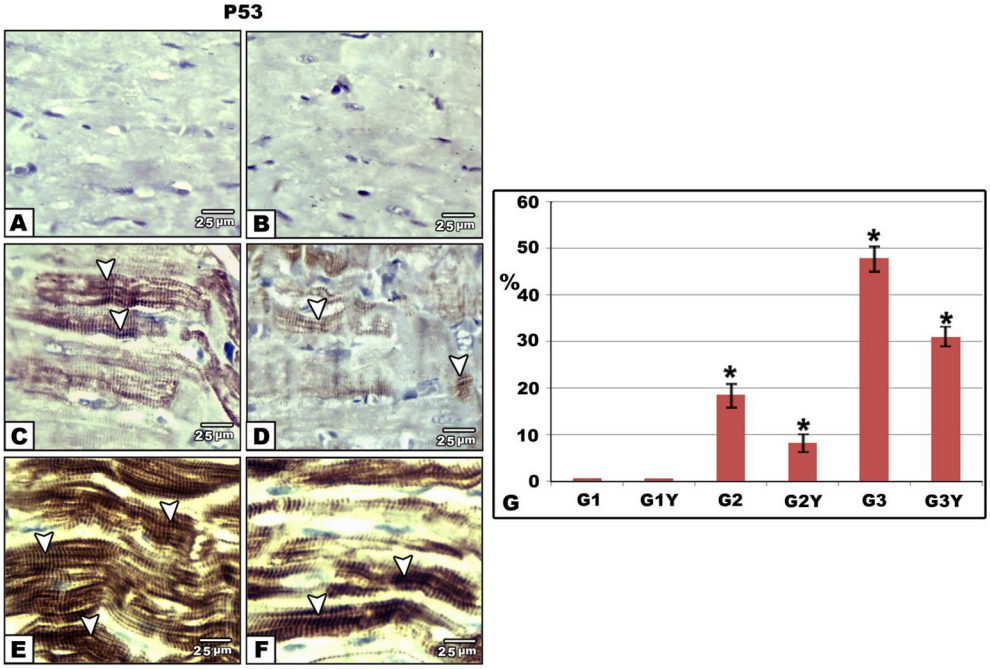

Fig. 3. Photomicrographs of formalin fixed histological sections of myocardium of rat immunostained with the antibody of P53 .A. Four month-old.B. 18 Month old. C. 18 – Months old supplemented whey for 2 months. D. 30 Months old. E. 30 Months old supplement whey.

(Fig. 3D). In 30 months old, intense immune reaction of the P53 which were visualized distributed in the myocardial muscle fibers (Fig. 3E). Whey supplementation to 30 months old decreased the immune reaction but was still less dense (Fig. 3F).

4.2. Biochemical observations

From Table 1, aging of rat at 18 and 30 months old exhibited a depletion of super oxide dismutase activities and vice versa increased the lipid peroxidation MDA. On the other hand, the inflammatory markers (TNF-α) and the enzyme activities of LDH, cTn1, CK-MB were markedly increased parallel with increased apoptosis assessed by increased casp3 and NF-kB.

4.3. Flow cytometric analysis of annexin v

From Fig. 4, Senile rat at 30 months -old exhibited a significant increase of the assayed annexin v of summation UR + LR compared of 4 months old. On the other hand, whey supplementation to aged group ameliorated the incidence of apoptosis of cardiomyocytes but was still more increase above the normal value.

Table 1
Anti-aging properties of whey syrup on myocardial biochemical markers on rats at 4 and 30- month **old**.

	LDH (mu/mgP)	TNF-α (Pg/mgP)	CAS-3 (Ng/mgP)	NF-kB (Pg/mg P)	cTnI (Pg/ml)	CK-MB (Ng/ml)	SOD (U/mg P)	MDA (Nmol/mg P)
4 M-old	3.75 ± 0.48	70.96 ± 3.43	3.54 ± 0.64	15.51 ± 0.67	72.01 ± 1.64	5.49 ± 0.42	16.85 ± 0.46	3.37 ± 0.08
4 M-old & whey syrup	3.67 ± 2.75	67.28 ± 0.55	2.75 ± 0.16	14.25 ± 0.65*	71.24 ± 0.65	4.43 ± 0.34	15.25 ± 0.65	3.29 ± 0.09
30 M old	9.98 ± 0.19*	114.65 ± 2.33*	7.81 ± 0.19*	46.34 ± 0.44*	131.02 ± 3.35*	11.44 ± 0.66*	9.49 ± 0.44	7.55 ± 0.66*
30 M old supplement whey syrup	8.12 ± 0.06*	102.21 ± 2.12*	6.45 ± 0.14	42.27 ± 0.63*	108.77 ± 1.51**	8.50 ± 0.47*	10.97 ± 0.64*	6.01 ± 0.31*
F-test	129.60	105.61	54.08	548.50	256.67	40.77	41.78	39.32

Each result represent the mean ± SE (n = 5). Abbreviations; Casp-3,Caspase 3; CK-MB, Creatine kinase; LDH, Lactic dehydrogenase; MDA, Malondialdhyde; NFkB, Nuclear factor Kappa B; SOD, Super oxide dismutase; cTn1, Troponin 1.
* Significant at P < 0.05

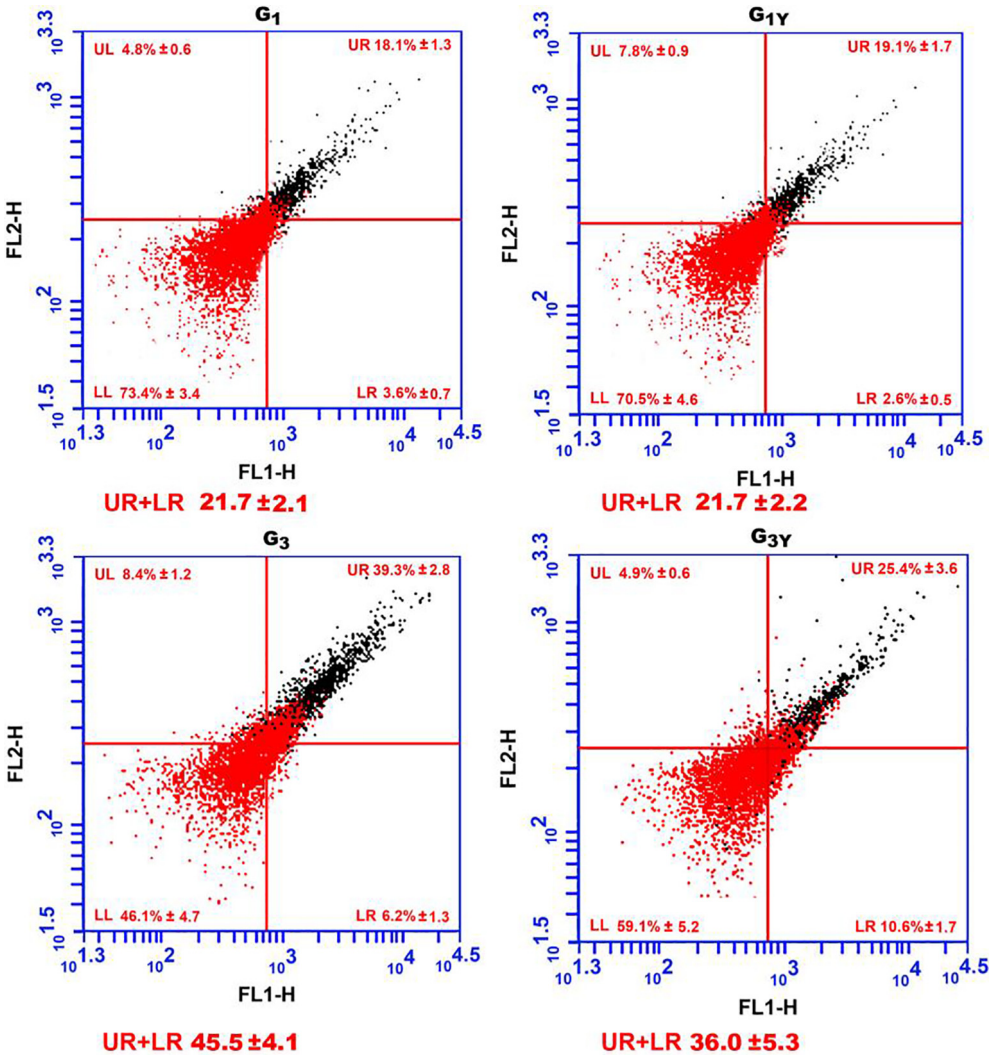

Fig. 4. Indicate the flow cytometric analysis of annexin v:

5. Discussion

Cardiomyocytes were negative for alpha-smooth muscle actin. α-SMA was slightly expressed and faintly expressed in myocardium of young age. Similar increased and diffused overexpression of α-SMA was reported in all layers of ventricular myocardium in myocardial hypertrophy and cardiomyopathies associated with increased myocyte stretch, increased wall stress, and pressure overload (Suurmeijer et al., 2003). The myocardial damage was associated with increased infiltration of angiogenesis in between the myocardial muscle bands. Also, leukocytes and spindle-shaped fibroblast cells were detected in between the muscle bands. The excessive distribution of fibroblasts may lead directly to biosynthesis of extra cellular matrix proteins or via the production of inflammatory cytokines, peptides, enzymes and matrix metallo proteinases (MMPs) which increased the extracellular matrix

(Fan et al., 2012). The observed myocardial damage was assessed by the increase of myocardium NF-kB. It is known that the NF-kB transcription factor complex is a cellular sensor, which responds to oxidative stress produced during aging of cardiomyocytes assessed by increased accumulation of lipofuscin (Helenius et al., 1996). It is associated with increased oxidative and inflammatory stresses and regulates expression of cytokines, growth factors, and genes that regulate apoptosis during aging (Tilstra et al., 2011). Lactate is regularly produced in myocardium and other tissues, even during completely aerobic conditions (Hashimoto et al., 2008). It has been shown that, under conditions of increased lactate production (i.e., exercise), the use of blood lactate as an energy source in the brain increases at the expense of blood glucose (van Hall et al., 2009). Lactate is a substrate for the mitochondrial TCA cycle, and its oxidation can produce a significant amount of ATP (Schurr, 2006). Lactate levels measured by ^{1}H NMR in 88- to 96-wk-old rats were significantly increased (Zhang et al., 2009). In contrast, long-lived Ames dwarf mice showed marked depletion of plasma lactate levels (Romanick et al., 2004). Ross et al. (2010) measured lactate and mitochondrial metabolism in hindbrain of aged mice, reported that lactate as a marker in aging, and markedly depleted although it is respond different from heart in decreasing oxidative phosphorylation. Similar increase of myocardial lactate dehydraogenase was observed in old rats (Spindler et al., 1983) and in patients with heart failure (Hu et al., 2015). The observed increase of myocardial lactate dehydrogenase reflected exhausted myocardia damage during aging parallel to that observed by El-Sayyad et al. (2012) in myocardium of hypercholesterolemic and diabetic rats. The observed increase of lactic dehydrogenase may lead to depletion of lactate as previously mentioned due to its characteristic function of catalyzes the interconversion of pyruvate and lactate via change of NADH and NAD^{+}. The observed findings revealed increased myocardial tumor necrosis-α assessed by increased leukocytic infiltration in between myocardial muscle bands which become infiltrated by blood capillaries of different sizes and hyalinization of myocardial fibers and presence of diffused necrotic patches. These was also reflected by increased myocardial caspase 3. Similar findings of increased TNF-α were reported by Bruunsgaard et al. (2000) in plasma of aged atherosclerotic patients. These factors activated the inflammatory cytokines leading to the myocardial disease (Bruuscaard et al., 2003). The observed breakdown of myocardial muscle and increased expression of α-mooth actin and p53 coincides with increased CK-MB and cTnI. Increased of both creatine kinase MB (CK-MB) and cardiac troponin I (cTnI) reflected the main indicator of myocardial infarction of older men (Welsh et al., 2002; Gupta et al., 2008; Joarder et al., 2011). It is known that mitochondria are the main aspects of cellular homeostasis such as source of energy, signaling of reactive oxygen species, and regulation of apoptotic pathways. Any alteration of mitochondrial structure may lead to damage of the myocardial muscle as assessed by annex v. The observed dramatic depletion of SOD and increase of MDA in aged myocardial tissue reflected the increase of reactive oxygen species induced oxidative stress and myocardial damage. Increased lipid peroxidation was reported during coronary by-pass surgery (Lazzarino et al., 1994) and patients with acute myocardial infarction (Madole et al., 2015). Increased release of free radicals during aging (Kuka et al., 2013) was attributed to aging related damage of cardiomyocytes causing a decreased number of mitochondria (Corsetti et al., 2008) and shape of mitochondrial (Cheng et al., 2013), and deterioration of their inner membrane (El'darov et al., 2015). Whey supplementation improved the assayed biochemical assayed parameters but were still markedly above the normal value especially SOD and MDA and caspase3. Prevention from oxidative damage when whey supplementation was applied was also detected by Garg et al., 2017. Probiotic supplementation showed a significant improvement in cardiomyocyte hypertrophy and an attenuation of heart failure (Ettinger, 2014). Whey syrup is rich in total 15 β-lactoglobulin, 1 α-lactoalbumin, and 6 β-casein, which have antioxidant activity against aging related changes in myocardial damage (Mann et al., 2015). The author concluded that dietary supplementation of nutrient of dietary sources like probiotic improved the aging related myocardial disease and decrease the oxidative stress.

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