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INHIBITION OF α-SMA, Bax AND INCREASE OF Bcl-2 EXPRESSION IN MYOCARDIOCYTES AS RESPONSE TO CHITOSAN ADMINISTRATION TO HYPERCHOLESTEROLEMIC RATS

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ABSTRACT

Hypercholesterolemia (**HPC**) cause biochemical alteration in lipid profile, disturbances in cardiac tissue, immunohistochemical expression of alpha-smooth muscle actin (α-SMA) and Bax were induced; while Bcl-2 expression was decreased. The present investigation aimed to evaluate the protective effect of chitosan (**COS**) in HPC rats by feeding on high fat diet for one month. Biochemical studies of total cholesterol, triglycerides, LDL and VLDL perceived high values and diminution in HDL levels in serum. Induced histopathological lesions in the cardiomyocytes was represented by detached muscle fibres, deposition of fats and presence of fibrotic areas; occupied by lymphocytes, macrophage and increase in the collagen fibres distribution throughout the cardiac tissue. **COS** supplementation to **HPC** rats, produced a counteractive effect on **HPC** rats, represented in the compensation of biochemical alteration and amended histological picture, and decrease in α-SMA and Bax expression and increase in Bcl-2 expression in the cardiac tissue. These findings were attributed to the significant inhibitory effect of **COS** remedy adjoining deleterious effects of **HPC**.

KEYWORDS: α-SMA, Bax, Bcl-2, Chitosan, Hypocholesterolemia.

INTRODUCTION

Hypercholesterolemia (**HPC**) is the major factor for cardiovascular disease, myocardial infarction which are the major cause of mortality in those adults with obesity and diabetes.[1] **C**hitosan (**COS**) is a polycationic polymer containing more than 5000 glucosamine units and is obtained commercially by alkaline deacetylation of chitin (N-acetyl-glucosamine polymer) from shellfish exoskeletons and from arthropods such as crabs and shrimps.[2] **COS** is cheap, non-toxic and have reactive amino functional groups. It has been used as an antifungal compound in agriculture, as a food additive in food industry, as a hydrating agent in cosmetics, beside, it is used as a pharmaceutical agent in biomedicine.[3] It is known to have a marked hypocholesterolemic effect in cholesterol-fed rats.[4] **COS** is not only a cholesterol-lowering effect; but also considering as a hypoglycemic agent.[5]

Alpha-smooth muscle actin (α-SMA) is the actin isoform that plays an important role in fibrogenesis.[6] Bcl-2 is critical for regulation of apoptosis across diverse cell types and acts along intrinsic mitochondrial apoptosis pathway that is activated in response to a number of stress stimuli including oxidative stress.[7] Babu et al.[8] & Song et al.[9] had been established that the expression of Bax and Bcl-2 has crucial roles in the progression of cell apoptosis.

The present study aimed to evaluate the cardioprotective effect of **COS** in hypercholesterolemic rats; and its immunomodulatory role on α-SMA, Bax and Bcl-2 expression in cardiac tissue.

MATERIAL AND METHODS

Animals and diets

Twenty four male rats (*Rattua rattus*) weighing 100±10 g were purchased from animal house of Vaccine and Serum Organization at Helwan, Egypt. The animals were housed in polyethylene cages in groups of six rats per cage in a controlled environment with 12 hour light-dark cycle. The animals were supplemented with water and fed *ad libitum* with a basal diet and water for one week, and were then randomly assigned to 4 groups (6 rats each): normal control group (**C**), receiving basal diet consisting of corn starch 65%, casein 10%, corn oil 10%, salt mixture 4%, vitamins mixture 1% and cellulose 10% (AOAC,2000), hypercholesterolemic group (**HPC**) receiving hypercholesterolemia-induced diet which prepared as basal diet preparation, except that the 10% corn oil portion was replaced with 10% sheep perineal fat and it was supplemented with 1% cholesterol and 0.25% cholic acid.[10] The fourth group is **HPC/COS** group. Rats belonging to this group received

hypercholesterolemia-induced diets supplemented with 3.6 g chitosan/kg diet.[11] Chitosan [C3646] was purchashed from Sigma-Aldrich Company. The experiment lasted for a total duration of one month; at the end of treatment period, rats were anaesthetized by ether and blood and cardiac tissue samples were collected. All animal procedures are in accordance with the recommendations of the Candian committee for care and use of animals.[12]

Lipid profile analysis

Blood samples were collected in heparinized tubes for the determination of total plasma cholesterol (TC) and triglyceride (TG) levels with an automated enzymatic colorimetric technique (Wiesbaden, Germany). High density lipoprotein cholesterol (HDLc), low density lipoprotein cholesterol (LDLc) and very low density lipoprotein cholesterol (VLDLc) levels were determined by a HDLc, LDLc and VLDLc Quantitation Kit (Sigma-Aldrich Company) via colorimetric tests (570 nm), according to the manufactured instructions.

Light microscopy studies

Left ventricle specimens were cut, washed in saline and fixed in 10% neutral buffered formalin. For general histopathology, paraffin blocks were cut at 4 m; slides stained with haematoxylin and eosin and examined under the light microscope. Cardiac muscle sections stained with Masson trichrome for collagen fibres content and thickness according to Bancroft and Stevens.[13]

Immunohistochemical investigations

Immunohistochemistry is the process of localizing proteins in tissues by exploiting the principle of antibodies binding specifically to antigens. The visualization of the antibody is commonly accomplished by conjugating an enzyme to the antibody. This can produce a color changing reaction. The advantage of this method is the ability to show exactly where an examined protein is located. The expression of α-SMA (ab5694, Abcam, Cambridge Science Park in Cambridge, England), Bax (ab7977, Abcam) and Bcl-2 (ab59348, Abcam) in cardiac tissue was determined immunohistochemically in formalin-fixed, paraffin-embedded tissue blocks were cut into 4 mm thick sections mounted on glass slides and then kept in an oven at 4°C overnight. Sections were deparaffinized in xylene and rehydrated. Endogenous peroxidase activity was blocked with 1% hydrogen peroxide for 20 min. To improve the quality of staining, microwave oven-based antigen retrieval was performed. Slides were probed with either anti- α-SMA (1:200), anti-Bax (1:100) or anti-Bcl-2 (1:50). Sections were washed with PBS for 10 min each and incubated with biotin-labeled anti-mouse IgG for 1 h

at room temperature. After washing, sections were stained with a streptavidin-peroxidase detection system.

Statistical analysis

Results are expressed as mean ± SD. One way ANOVA were performed by Tukey's honestly significant difference (HSD) test. A P value <0.05 was considered as statistically significant. All data were analyzed with the aid of statistical package program SPSS 16.0 for Windows.

RESULTS

Lipid profile analysis

The results obtained from the second experimental group (Table 1) showed that the increase in TC, TG, LDLc, VLDLc and the decrease in HDLc fractions as a result of feeding on high fat diet (**HPC** group) when compared to normal control (**C**) and chitosan group (**COS**). On the other hand, rats belonging to the **HPC/COS** group showed significant decrease in total cholesterol, triglycerides, LDLc and VLDLc levels while increase values in HDLc fraction were reported as compared to the **HPC** group.

Table (1): Biochemical and Cytokine Levels in Control and Different Experimental Groups.

Parameters Means±SD	Experimental Groups			
	C	COS	HPC	HPC/COS
TC	81.17±1.30	80.78±1.41	120.70±3.37^{a,b}	84.95±3.08^{c}
TG	67.08±1.52	60.51±1.85	105.16±1.74^{a,b}	84.64±1.47^{c}
LDLc	32.61±1.68	30.54±1.76	53.67±2.09^{a,b}	34.31±1.06^{c}
VLDLc	8.47±0.24	7.82±0.32	14.37±0.66^{a,b}	9.51±0.16^{c}
HDLc	50.58±1.22	58.07±2.80	20.77±1.15^{a,b}	48.58±1.39^{c}

Values are mean±SE. superscript letters denote the significant difference at (P<0.05).

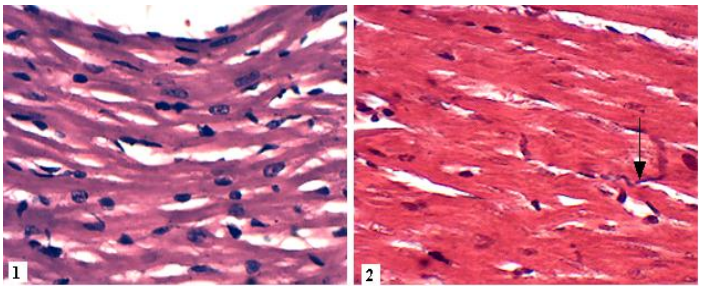
a: values are significantly different from control group.

b: values are significantly different from COS group.

c: values are significantly different from HPC group.

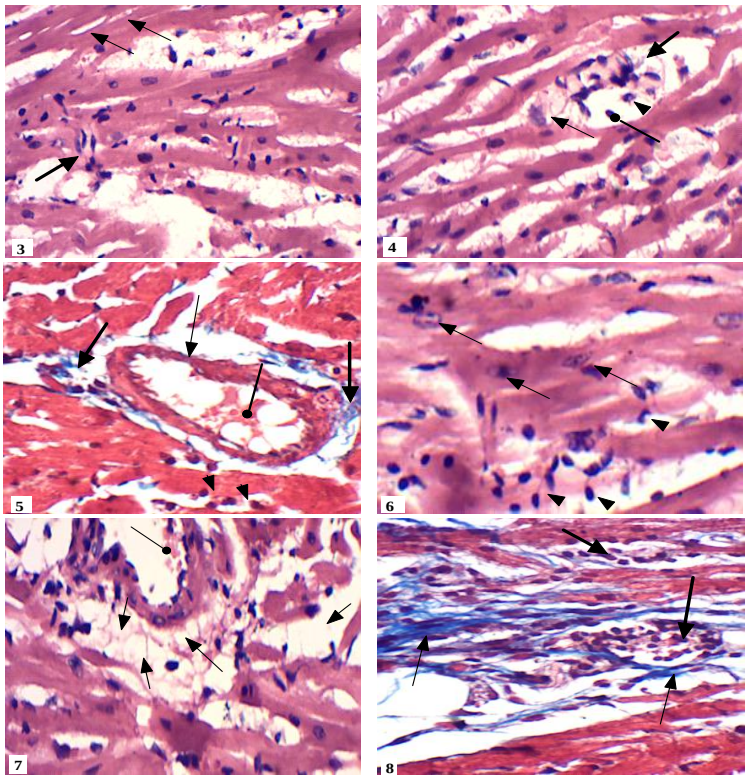
Histopathological studies

Cardiac muscles from **C** rats section revealed the normal anatomizing branching of the myocardiocytes (Fig. 1). No histological differences were found in **COS** treated group, where heart section showed normal histological profile of the cardiac tissue and the normal distribution of collagen fibres (Fig 2).



Figuers 1-2: Photomicrographs of heart section from C and COS groups showing: Normal histological packed branching myocardiocytes with central nuclei in C group (H-E, X400) and normal distribution of collagen fibres as appeared fron COS group (Masson, X400).

Regarding to the influence of the hypercholesterolemia (**HPC)** on cardiac muscles of rats, investigated sections showed myofibrils with cytoplasmic vacuolization and disarrangement of the nuclei. Also, fibroblasts are distributed throughout the cardiac tissue (Fig. 3). Loss of myofibrils, macrophages, fibroblasts infiltrated between the detached myofibrils, inflammatory cells such as monocytes and lymphocytes (Fig. 4). Dilated blood vessel with thickened walls and its lumen stuffed with RBCs. Increase in collagen fibres and inflammatory cells appearing around the blood vessel (Fig. 5). Myofibrils with degenerated nuclei and lymphocytic infiltration throughout the disarrangement myofibrils (Fig. 6). Invasion of various sizes of fat cells through the cardiac tissue and RBCs infiltration (Fig. 7). Increase of collagen fibers and fibroblasts invaded the cardiac tissue as appeared in figure (8).



Figures 3-8: Photomicrographs from heart section from HPC group showing myofibrils with cytoplasmic vacuolization (thin arrows) and disarrangement of the nuclei. Also, fibroblasts (thick arrow) are

distributed throughout the cardiac tissue (Fig. 3, H-E, X400). Loss of myofibrils, macrophages (thin arrow), fibroblasts (thick arrow) infiltrated between the detached myofibrils, inflammatory cells such as monocytes (head arrow) and lymphocytes (pointed arrow) (Fig. 4, H-E, X400). Dilated blood vessel with thickened walls (thin arrow) and its lumen stuffed with RBCs (pointed arrow). Increase in collagen fibres (thick arrows) and inflammatory cells (head arrows) appearing around the blood vessel (Fig. 5, Masson, X400). Myofibrils with degenerated nuclei (thin arrows) and lymphocytic infiltration (head arrows) throughout the disarrangement myofibrils (Fig. 6, H-E, X400). Invasion of various sizes of fat cells (thin arrows) through the cardiac tissue and RBCs infiltration (pointed arrow) (Fig. 7, H-E, X400). Increase of collagen fibers (thin arrow) and fibroblasts (thick arrow) invaded the cardiac tissue (Fig. 8, Masson, X400).

Sections of cardiac muscle fibres from **HPC/COS** group revealed decrease in fat vacuoles distribution and the arrangement of the fibres appeared near to normal structure (Fig. 9) and normal distributionof collagen fibres (Fig 10). Thus **COS** could protect the cardiac muscle fibres from **HPC** pathological changes, as appeared from the improvement picture of the cardiac tissue.

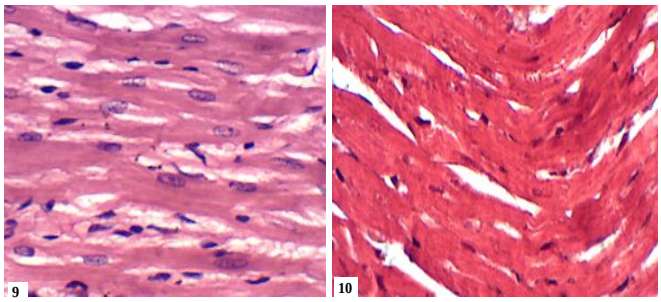
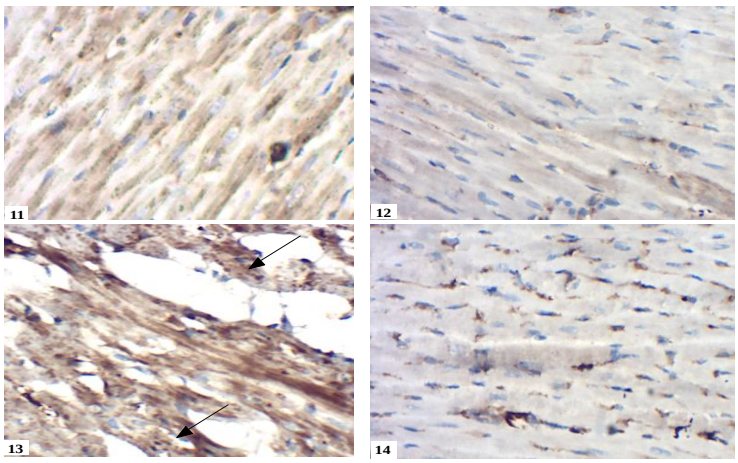


Fig. 9 - 10: Photomicrographs from heart section from HPC/COS group showing return to the normal arrangement of myofibrils with prominent nuclei as compared with HPC group. Well-arranged myofibrilis with slight number of collagen fibres (Masson, X400).

Immunohistochemical studies

α-SMA expression

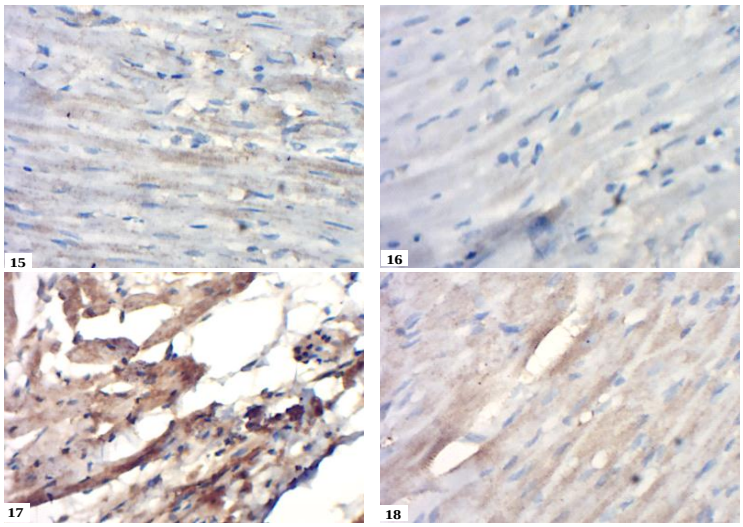
Heart sections from **C** and **COS** groups revealed normal distribution of α-SMA in the cardiac muscles (Fig. 11&12). An increase in the α-SMA expression in the cardiac tissue as appeared in the myofibrils from **HPC** group as shown in figure.[13] This increment revealed heart fibrosis which comes in harmony with the histopathological studies. On the other hand, cardiac tissue sections from **HPC/COS** group showed inhibition in the expression of α-SMA (Fig. 14).



Figures 11-14: Photomicrographs from heart sections immunostained for α-SMA expression showing normal distribution of α-SMA in myocardiocytes in C group (Fig. 11). Normal expression of α-SMA in the myofibrils in COS group (Fig. 12). Increase expression of α-SMA in cardiac especially in the fibrotic areas (arrows) in HPC group (Fig. 13). Slight expression of α-SMA in HPC/COS group (Fig. 14) as compared with HPC group. (Immunohistochemical stain, X400).

Bax expression

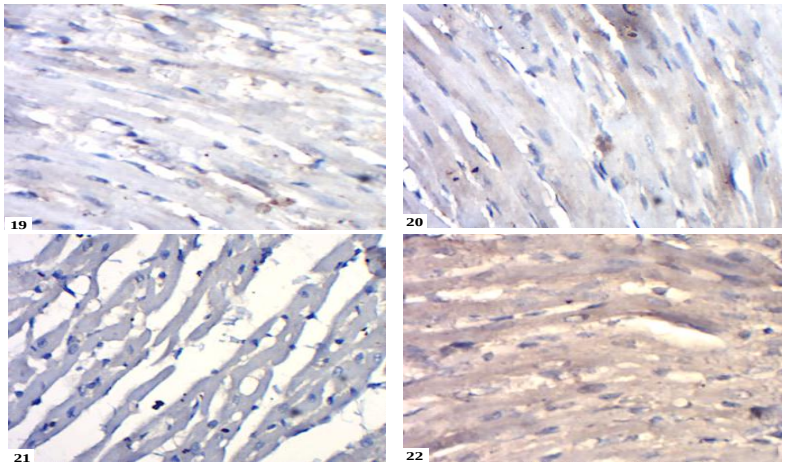
Cardiac tissue sections from **HPC** group revealed an increase in the Bax expression in the degenerated myofibrils; which is responsible for the apoptosis as shown in figure (17) as compared with normal **C** group in figure (15) and **COS** group (Fig. 16). On the other hand, cardiac tissue sections from **HPC/COS** showed inhibition in the expression of Bax (Fig. 18).



Figures 15-18: Photomicrographs from heart sections stained for the Bax expression showing: normal expression of Bax in the myofibrils cytoplasm from C group (Fig. 15). Normal Bax distribution in mycardiocytes cytoplasm from COS group (Fig. 16). Marked increment in Bax expression in the cytoplasm of degenerated mycocardiocytes from HPC group (Fig. 17). While, decrease Bax expression in myofibrils cytoplasm from HPC/COS group (Immunohistochemical stain, X400).

Bcl-2 expression

Heart sections from **C** and **COS** groups revealed normal distribution of Bcl-2 in the cytoplasm of the cardiac muscles (Fig. 19& 20). A decrease in the Bcl-2 expression was appeared in in the degenerated myofibrils belonged to the **HPC** group as shown in figure (21) as compared with **C** and **COS** groups. While, cardiac tissue sections from **HPC/COS** group showed increase in the expression of Bcl-2 (Fig. 22). From the above-mentioned results it can be concluded that hypercholesterolemia is responsible for the increased myocardial Bcl-2/Bax ratio, a well-known sign of cardiomyocyte apoptosis.



Figures 19-22: Photomicrographs from heart sections immunostained for Bcl-2 expression showing: from showing normal cytoplasmic expression of Bcl-2 in myocardiocytes from C and COS groups (Fig. 19 & 20). Inhibition of Bcl-2 expression in the degenerated myofibrils from HPC group (Fig. 21). Augmentation of Bcl-2 expression from HPC/COS group as compared to HPC group (Immunohistochemical stain, X400).

DISCUSSION

The present study revealed an increase in TC, TG, LDLc and VLDLc and a decrease in HDLc levels as a result of the feeding on high fat diet. These findings are in accordance with earlier reported studies[14,15], which showed that the free fatty acids liberated from adipose tissue and enters into the myocardium. Though heart can utilize free fatty acids for its energy requirements, the excess free fatty acid may be used for the synthesis of triglycerides, resulting in hypertriglyceridemia as observed in the present study.[16] The increase in TC, LDLc and VLDLc might be due to the increased mobilization of LDLc from the blood into the myocardial membranes, resulting in abnormal cholesterol deposition in the myocardium.[16] HDL cholesterol levels were significantly lower in HPC rats, which might be due to the increased mobilization of LDL-cholesterol from the blood into the myocardial membranes, resulting in abnormal cholesterol deposition in the myocardium.

The addition of **COS** significantly prevented the elevation in TC, TG, LDLc and VLDLc as compared to **HPC** rats. It also maintained the level of HDLc. These results proved its hypercholesterolamic effect. Sivakumar et al.[15] indicated that **COS** has a lowering effect on TC, LDLc and VLDLc levels in experimental animals, establishing the anticholesterolemic property of **COS**.[17] The cardioprotective effect of chitosan is probably related to its ability to inhibit the increased accumulation of lipids both in the systemic circulation and in the myocardium by its antilipidemic property. Qujeq & Ataei.[18] delineated that **COS** incline cholesterol absorption by binding cholesterol through inhibiting intestinal absorption of dietary fat. So, cholesterol cumulative in the cecum causing decreased TC levels.[14,19] Another factor for **COS** antilipidemic effect was reported by Park et al.[20] who have suggested that chitosan may eliminate various free radicals by the action of nitrogen on the C-2 position of the chitosan. While, Xie et al.[21] reported that the scavenging mechanism of **COS** is related to the fact that the free radicals can react with the hydrogen ion from the ammonium ions to form a stable molecule.[22]

The present study reported many disorder in **HPC** rats heart sections. This damage involved disturbance in myofiblis arrangement, fat deposition, presence of RBCs inside the cardiac tissue, increase in collagen fibres, fibroblasts and inflammatory cells infiltration. Ma et al.,[23] reported that inflammatory stress exacerbates the progression of cardiac fibrosis in high-fat-fed mice, suggesting that hyperlipidaemia and inflammation act synergistically to redistribute plasma lipids to cardiac tissues and accelerate the progression of cardiac fibrosis. Again, Tilellis et al.[24] delineated that high fat diet stimulates cardiac hypertrophy, inflammation, intramyocardial lipid accumulation and oxidative stress.

During fibrosis, bone marrow-derived fibroblasts and endothelial cells responsible for fibroblast accumulation via endothelial-mesenchymal transition (EndMT).[25] During End MT, resident endothelial cells divided from the organized cell layer and invade the underlying tissue. This mesenchymal phenotype is characterized by increased expression of mesenchymal markers, such as fibroblast-specific protein-1, collagen-I, or α-SMA. Thus, EndMT-derived cells function as fibroblasts in damaged tissue and may have an important role in fibrosis.[26] These data suggest that inflammatory stress and hyperlipidaemia in combination contribute to the progression of cardiac fibrosis via End MT. Ma et al.[23] demonstrated that hyperlipidaemia upregulated the protein expression of collagen I and α-SMA positive cells in high fat diet fed pigs.[27]

Apoptosis is a significant contributor to myocardial cell death as a result of reperfusion injury. The immunocytochemistry of the pro-apoptotic Bax showed increased expression, and a decreased anti-apoptotic Bcl-2 expression in the cardiac tissues from **HPC** group as compared with normal group. These results come in harmony with those of Guo et al.[28] and Kuo et al.[29] The significant increases in the levels of the pro-apoptotic proteins Bak and Bax suggests the presence of ongoing apoptosis.[30] The pro-apoptotic proteins, enhance cytochrome c release from mitochondria into cytosol, which is responsible for activating caspase 9, caspase 3 and facilitates the apoptosis. Bax can modulate the pro-apoptotic processes by inhibiting the expression of Bcl-2 members.[31] Hypercholesterolemia is associated with an increased myocardial Bcl-2/Bax ratio, not only by increasing the infarct size but also by increasing the extent of cardiomyocyte apoptosis.[32]

Supplementation of **COS** to high fat diet reduced the expression of α-SMA, Bax, and increased the expression of Bcl-2 in the cardiac tissue. To our knowledge this the first report concerning with the effect of **COS** on α-SMA, Bax and Bcl-2 expression in the cardiac tissue in **HPC** rats. Krishnan and his colleges[33], delineated that epigallocatechin-gallate (EGCG) a catechin member extracted from green tea reduced hepatic Bax expression in young hypercholesterolemic rats. On the other hand, Bcl-2 expression was up regulated significantly.

The inclusive cardioprotective effect of **COS** may be related to its ability to induce lipid accumulation by its hypolipidemic property and/or to its free radical scavenging ability. Thus **COS** responsible for the irreversible fibrosis, and degeneration of the cardiomyocytes. Also, the present study proved **COS** ability to modulate the expression of α-SMA, Bax and Bcl-2. This reducing fibrosis by inhibiting α-SMA expression, enhancement the pro-apoptotic and anti-apoptotic activities by decreasing Bax and increasing Bcl-2 expression respectively. **COS** was authentic in the protection of heart from hypercholesterolemia.

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