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Influence of mussels (*Mytilus galloprovincialis*) from polluted and non-polluted areas on some atherosclerosis indices in rats fed cholesterol

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ABSTRACT

The influence of diets supplemented with mussels, from polluted (MPoll) and non-polluted (MNPoll) areas, on some atherosclerosis indices in rats fed cholesterol (Chol) were studied. According to the results of our investigation *in vitro*, mussels from polluted areas had higher contents of proteins, metals and antioxidant compounds, mostly phenolics and higher antioxidant capacities. 28 male Wistar rats were divided into 4 groups of 7 and named Control, Chol, Chol/MPoll and Chol/MNPoll. The rats of the Control group received basal diet (BD) only, and the diets of the other 3 groups were supplemented with 1% of non-oxidized cholesterol (NOC), 1% of NOC and 5.6% of mussel dry matter (DM) from polluted and 1% of NOC and 5.6% of mussel DM from non-polluted areas for Chol, Chol/MPoll and Chol/MNPoll, respectively. The histology of the aorta and brain in rats fed cholesterol did not show any signs of atherosclerosis. Some differences were registered in the electrophoretic protein patterns of plasma in rats, with mussel-supplemented diets. In full plasma electrophoretic patterns of the Chol/MPoll diet group more proteins were detected than in both Chol and Control groups, and the differences were significant. In conclusion, in groups of rats fed cholesterol with mussels supplementation, a significant hindering in the rise of plasma lipid levels and also hindering in the decrease of plasma antioxidant activity were registered.

1. Introduction

Prevention of sea pollution is one of the important goals of mankind, especially due to the negative influence of industrial development (Gorinstein et al., 2005). In spite of the success in preservation of a healthy environment, the pollution problem is far from being solved (Moore, Icarus & McVeigh, 2006). Meanwhile, studies of polluted and non-polluted areas of the sea remain one of the important subjects (Jonsson, Schiedek, Grosvik, & Goksoyr, 2006; Khan, Parrish, & Shahidi, 2006; Zorita, Ortiz-Zarragoitia, Soto, & Cajaraville, 2006).

In recent years, the consumption of seafood has increased (Kalogeropoulos, Andrikopoulos, & Hassapidou, 2004; Perugini et al., 2007). Many patients suffering from coronary atherosclerosis include in their diets seafood products and, among them, mussels (Bethune et al., 2006; Imano et al., 2002; Schmidt et al., 2001; Valfre, Caprino, & Turchini, 2003). However, it is not known whether such patients benefit from seafood-supplemented diets.

The contents of bioactive compounds and the antioxidant capacity of mussels from polluted and non-polluted areas differ and in some cases significantly (Auffret et al., 2006; Devier et al., 2005; Oros & Ross, 2005; Pampanin et al., 2005). However, the influence of diets supplemented with mussels from polluted and non-polluted areas on laboratory animals is less understood (Gorinstein et al., 2005). Therefore, it was decided to compare contents of some bioactive compounds and antioxidant capacity of mussels from polluted and non-polluted areas *in vitro* and then to assess the influence on plasma atherosclerosis indices on rats fed with cholesterol.

In the *in vitro* experiment, the following procedures were performed: (a) the determination of the proteins, metals, polyphenols and antioxidant capacity in mussels from polluted and non-polluted areas using ferric-reducing/antioxidant power (FRAP) assay;

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and in the *in vivo* studies the following indices were determined: plasma lipid levels and total cholesterol in liver; plasma antioxidant activity; electrophoretic patterns; histological status of aortas and brains.

As far as we know there are no prior investigations.

2. Materials and methods

2.1. Reagents

Trolox (6-hydroxy-2,5,7,8,-tetramethyl-chroman-2-carboxylic acid), BHA (butylated hydroxyanisole), $FeCl_3 \times 6H_2O$, Folin-Ciocalteau reagent, cholesterol of analytical grade (USP), DPPH (1,1-diphenyl-2-picrylhydrazyl), metal standards, molecular marker and sodium dodecyl sulphate (SDS) were obtained from Sigma Chemical Co., St. Louis, MO, USA. 2,4,6-Tripyridyl-*s*-triazine (TPTZ) was purchased from Fluka Chemie, Buchs, Switzerland. All reagents were of analytical grade. Deionized and distilled water were used throughout.

2.2. Collection of mussel samples

Mussels were collected in March, 2005, in two regions of Mokpo coast, Mokpo bay, Republic of Korea: an ecologically non-contaminated (out of the port, 20 miles northwest of Mokpo bay) and a contaminated site (the Mokpo port, in the bay of Halla Ship large scale construction company, which belongs to Hyundai groups) at sea depth of 3–4 m. The collected mussels (*Mytilus galloprovincialis*) from both contaminated and non-contaminated sites were characterized by a similar maximum length and size of analyzed organisms (4.37 ± 0.5 cm): this was 75–85% of the maximum size reached within each population.

As previously shown, such an approach guaranteed that compared mussels had similar metabolic conditions and the influence of physiological differences between two populations was less pronounced (Regoli, 2000).

The samples were designated as follows: MNPoll, for non-polluted, and MPoll, for polluted sites. Whole soft tissue from 30 specimens of each population were rapidly frozen in liquid nitrogen and stored at −80 °C. Then the samples were dried in glass flasks on Finn – Aqua, Lyovac GT-2 equipment for 36 h.

2.3. Determination of proteins and metals in mussel samples

Protein content and the detection of metals and microelements were performed according to AOAC (1995). Microelements (Cd, Fe and Pb) and minerals (K, Cl, Ca, Mg, P) were determined by atomic absorption spectrophotometry with flameless atomization (Varian Spectra AA 300 Zeeman) from the entire soft tissue. Prior to the measurements, samples were digested with nitric acid (Gorinstein et al., 2005; Santamaria-Fernandez et al., 2004).

2.4. Extraction and determination of polyphenols

Defatted with acetone, lyophilized mussel samples were extracted from a 50 mg aliquot with 5 ml of 50% methanol/water with heating at 90 °C for 3 h for free polyphenols (FPOL) and under the same conditions, with 5 ml of 1.2 M HCl in 50% methanol/water, for total polyphenols (TPOL). The samples were cooled, diluted to 10 ml with methanol, and centrifuged for 5 min at 4000*g* with a benchtop centrifuge to remove solids (Vinson, Su, Zubic, & Bose, 2001). The Folin-Ciocalteu method was used and the measurements were performed at 765 nm with gallic acid as the standard (Singleton, Orthofer, & Lamuela-Raventos, 1999). The results were expressed as milligrams of gallic acid equivalents (GAE)/g DM.

2.5. Antioxidant capacity

For determination of the antioxidant capacity of the mussels' dry matter, the ferric-reducing/antioxidant power (FRAP) assay was used, which measures the ability of the antioxidants contained in the mussel samples to reduce ferric-tripiridyltriazine (Fe^{3+}-TPTZ) to a ferrous form (Fe^{2+}), which absorbs light at 593 nm. The ferro- and ferric-iron form complexes with TPTZ reagent are the main products of this reaction. The antioxidant capacity of 10 μl plasma was measured (Benzie & Strain, 1996).

2.6. Tests used in the in vivo experiment

2.6.1. Rats and diets

The Animal Care Committee of the Warsaw Agricultural University, Poland, had approved this study. The mean weight of the male Wistar rats ($n = 28$) at the beginning of the experiment was 111 g. The rats were housed in stainless steel metabolic cages and were divided into 4 groups of 7. These groups were named Control, Chol, Chol/MPoll and Chol/MNPoll. During first 5 days of the adaptation period to the new conditions, rats of all 4 groups were fed basal diet (BD) only, which included wheat starch, casein, soybean oil, vitamin and mineral mixtures. The rats of the Control diet group, for the following 30 days, continued receiving the BD. The diets of the other 3 groups were supplemented with 1% of non-oxidized cholesterol (NOC) of analytical grade (Chol group), 1% of NOC and 5.6% of mussels' dry matter (DM) from polluted (Chol/MPoll) and 1% of NOC and 5.6% of mussels' DM from non-polluted (Chol/MNPoll) areas, respectively. Our prior experiments on laboratory animals have shown that cellulose had no significant hypocholesterolemic effects (Leontowicz et al., 2007). Therefore, cellulose was used as a control fibre. The diets contained, as percentage of energy, 66% of carbohydrates, 25% of protein and 9% of fat. The calculated energy of the used diets was from 394.5 to 400.4 kcal/100 g, and the differences were statistically not significant.

At the end of the experiment, all rats were anaesthetized using diethyl ether, and the blood samples were taken from the left atrium of the heart. Plasma lipid levels and total cholesterol in liver and plasma antioxidant activity were determined as previously reported (Leontowicz et al., 2007).

2.7. Histology

Samples of the aorta and brains were analyzed. After a formalin fixation, segments of the organs were processed by a common paraffin technique. Each sample was cut into 72 serial sections (thickness of 5 μm) with a transversally oriented cutting plane, and stained with hematoxylin and eosin (HE) and green trichrome (Bobková & Tonar, 2005).

2.8. Electrophoresis

SDS-PAGE electrophoresis was performed according to Laemmli (1970). Full plasma (50 μl) and methanol-precipitable fraction (50 μl) were mixed with 100 μl of sample buffer, then extracted for 1 h, boiled for 5 min, and centrifuged for 5 min, 6000*g*.

Methanol precipitation was done by 50 μl of plasma and 100 μl of methanol. Extracts were stored in a freezer. Densitometry analysis of gels was performed with the use of BioGene software v. 11.9 Vilber Lourmat, France. The densitometry profiles of proteins of all examined lanes (20) were obtained for both extractions (full plasma/precipitated by methanol). The volumes under the detected peaks of bands were calculated (volume: sum of all protein intensities included in the defined area [window + separation]). The variance analysis of peaks volumes and test of homogeneity, Tukey-Kramer test (HSD) were performed with the use of SAS

software v. 9.1. The calculations were made at the significance level $\alpha = 0.05$ (Leontowicz et al., 2007).

2.9. Statistical analysis

The reported values of the experiment *in vitro* are means ± SD of five measurements. The analysis of variance and least significant difference tests were conducted to identify differences among means, while a Pearson correlation test was conducted to determine the correlations among means. Statistical significance was declared at $P < 0.05$.

3. Results

3.1. Bioactive compounds

The amount of proteins in MPoll was about 52.5% ± 3.14 DM and in MNPoll 50.3% ± 3.76 DM. The contents of Pb, Cd and Fe in MPoll were 0.021 ± 0.0005; 0.123 ± 0.001 and 0.076 ± 0.001 mg/kg, respectively. The contents of Pb, Cd and Fe in MPoll were 0.021 ± 0.0005; 0.123 ± 0.001; 0.076 ± 0.001 mg/kg, respectively. The contents of Pb, Cd and Fe in MNPoll were 0.018 ± 0.0008; 0.112 ± 0.001 and 0.056 ± 0.001 mg/kg, respectively. The amounts of total polyphenols (TPOL) were 28.5 ± 2.9 and 25.5 ± 2.7 mg GAE/g DM for MPoll and MNPoll areas, respectively. The free polyphenols (FPOL) ranged between 25.9 ± 2.5 and 23.6 ± 2.4 mg GAE/g DM, respectively. The related antioxidant activities in total polyphenol extracts (TFRAP) were about 14.3 ± 1.4 and 8.67 ± 0.9 μMTE/g DM for MPoll and MNPoll areas, respectively. Free polyphenol extracts (FFRAP) were about 12.7 ± 1.3 and 4.75 ± 0.5 μMTE/g DM for MPoll and MNPoll, respectively.

3.2. Plasma lipid levels

After the completion of the trial (Fig. 1), the increase in the plasma lipid levels, such as TC in the Chol diet group vs. Control group was significant ($P < 0.05$). In the group of rats, whose diets were supplemented with the mussels' DM (Chol/MPoll and Chol/MNPoll), the decrease in the AA was hindered. However, only in the rats of the Chol/MPoll group was the hindering significant ($P < 0.05$). The same patterns of changes in the LDL-C level were registered. The changes in the HDL-C and TG levels were not significant ($P > 0.05$). After completion of the trial a decrease in the plasma AA in all groups of rats fed cholesterol-containing diets (Chol, Chol/MPoll and Chol/MNPoll) was registered. In the group of rats, whose diets were supplemented with the mussels' DM (Chol/MPoll and Chol/MNPoll), the decrease in the antioxidant activity (AA) was hindered. However, in the groups Chol/MPoll and Chol/MNPoll, whose diets were supplemented with mussels' DW, the increase was significantly smaller than in the Chol diet group ($P < 0.05$).

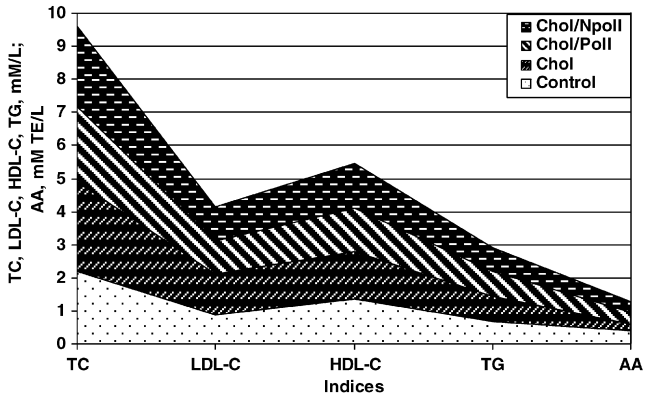

Fig. 1. Changes in plasma lipid levels and plasma antioxidant activity after completion of the experiment. Values are means ± SD, $n = 7$. Abbreviations used: Chol, cholesterol; Chol/MNPoll, diet group, supplemented with mussels' DM from non-polluted area, Chol/MPoll, diet group, supplemented with mussels' DM from polluted area, DM, dry matter, FRAP, Ferric-reducing/antioxidant power; Lipids (mM): HDL-C, high lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; TC, total cholesterol; TG, triglycerides, AA, antioxidant activity (mM TE).

3.3. Somatic index and total cholesterol concentration in liver

The increase in the liver somatic index (Table 1) in all cholesterol diet groups (Chol, Chol/MPoll and Chol/MNPoll) was significantly higher ($P < 0.05$) than that in the Control group. By contrast, there were no changes in the somatic index in other studied organs ($P > 0.05$).

The concentration of the total cholesterol in liver after completion of the experiment was 48.1 ± 3.1, 31.7 ± 2.3 and 33.3 ± 2.5 μmol/g for the Chol, Chol/MPoll and Chol/MNPoll diet groups, respectively. In all three cholesterol-fed diet groups, a significant increase in the total cholesterol concentration was registered. However, in the groups Chol/MPoll and Chol/MNPoll, whose diets were supplemented with mussels' DW, the increase was significantly smaller ($P < 0.05$) than that in the Chol diet group.

3.4. Minerals and Fe in the plasma of rats after trial

The contents of Cl and Ca (Table 2) were without significant changes ($P > 0.05$). The contents of K in all cholesterol fed groups and Fe in the Chol/MNPoll group were decreased significantly ($P < 0.05$). By contrast, the contents of Mg and P in the Chol/MPoll and Chol/MNPoll groups were increased significantly ($P < 0.05$).

3.5. Histological picture of aortas and brains

The histology of heart and brains in our study remained unaffected (Fig. 2A–C). The microscopic image of the tunica intima and tunica media did not differ between all investigated groups.

Table 1
The somatic index for liver, heart, kidney and spleen (%)

Groups	Liver	Heart	Kidneys	Spleen
Control	2.87 ± 0.16[a]	0.29 ± 0.04[a]	0.59 ± 0.04[a]	0.17 ± 0.02[a]
Chol	3.53 ± 0.24[b]	0.32 ± 0.08[a]	0.58 ± 0.04[a]	0.19 ± 0.03[a]
Chol/NPoll	3.22 ± 0.19[b]	0.30 ± 0.03[a]	0.64 ± 0.04[a]	0.16 ± 0.02[a]
Chol/MNPoll	3.25 ± 0.13[b]	0.28 ± 0.03[a]	0.66 ± 0.03[a]	0.17 ± 0.02[a]

Values are means ± SD, $n = 7$. Values with different superscripts in columns are significantly different ($P < 0.05$).
Abbreviations: Chol, cholesterol diet group; Chol/MPoll, cholesterol/polluted diet group; Chol/MNPoll, cholesterol/non-polluted diet group.

Table 2
Minerals (mmol/l) and Fe (μmol/l) in the plasma of rats after the trial

	Control	Chol	Chol/MPoll	Chol/MNPoll
K	5.31 ± 0.73[a]	4.85 ± 0.39[b]	4.61 ± 0.88[b]	4.67 ± 0.33[b]
Cl	101 ± 2.5[a]	102 ± 1.56[a]	103 ± 0.7[a]	102 ± 0.8[a]
Ca	2.55 ± 0.11[a]	2.59 ± 0.04[a]	2.63 ± 0.08[a]	2.49 ± 0.18[a]
Mg	0.87 ± 0.05[a]	0.91 ± 0.03[a]	1.08 ± 0.10[b]	1.02 ± 0.10[b]
P	2.95 ± 0.49[a]	2.91 ± 0.32[a]	3.31 ± 0.44[b]	3.31 ± 0.32[b]
Fe	17.0 ± 4.58[a]	17.3 ± 2.75[a]	18.1 ± 3.41[a]	15.1 ± 2.54[b]

Values are means ± SD ($n = 7$). Values with different superscripts in rows are significantly different ($P < 0.05$).
Abbreviations: Chol, cholesterol diet group; Chol/MPoll, cholesterol/polluted group; Chol/MNPoll, cholesterol/non-polluted group.

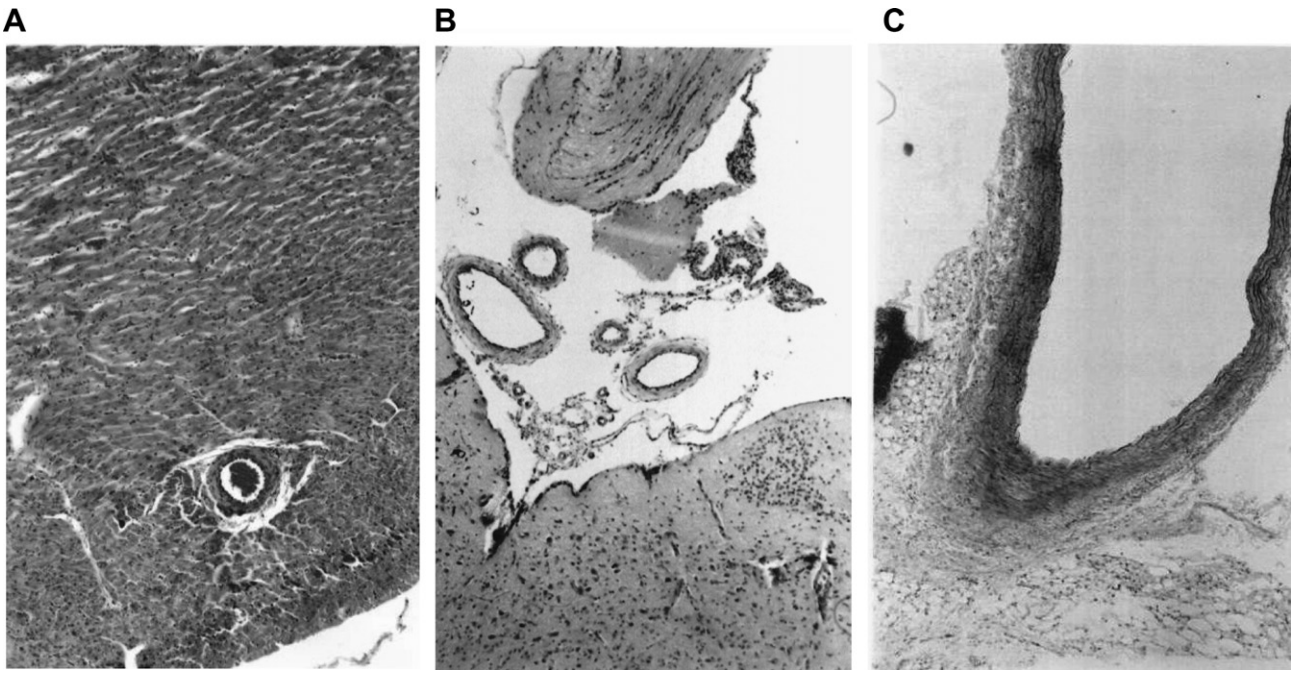

Fig. 2. Normal microscopic images of the coronary arteries of rats fed atherogenic diets with an addition of mussels. H.E. staining, ×100. (A) Heart muscle with the cross-section of the coronary artery seen at the bottom of the photo. The artery wall without atherosclerotic changes. Clotted blood inside the lumen. (B) Normal image of the brain tissue with the longitudinal sections of the basal arteries seen right side down. No atherosclerotic changes in the arterial walls. (C) Aorta of rats. There were no lesions found in the examined tissue.

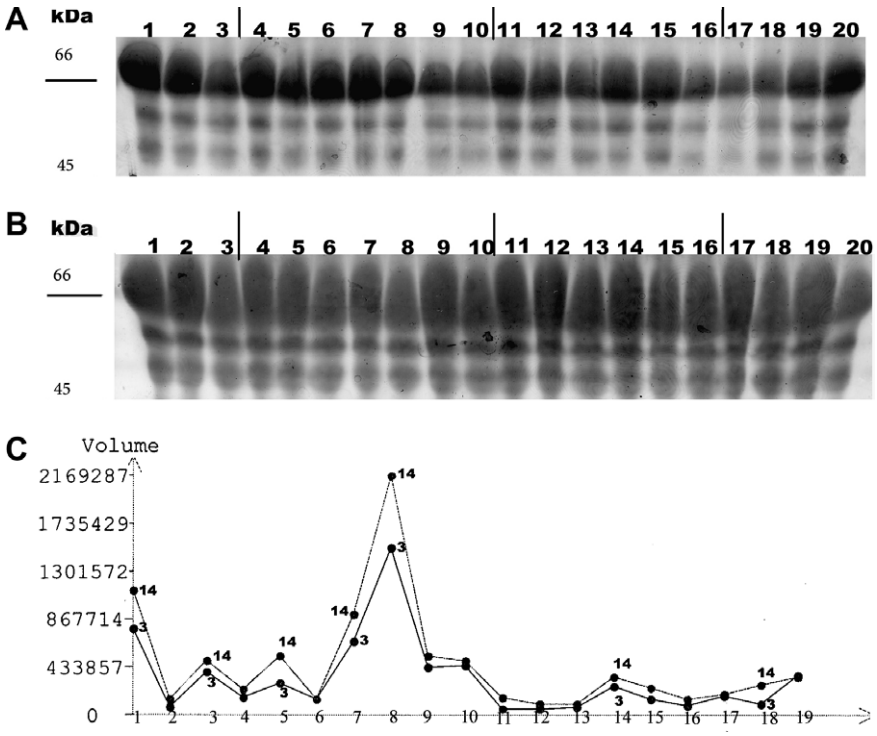

Fig. 3. Comparison of the band intensities of plasma proteins after different mussel diets were extracted with sample buffer containing SDS and 2-ME and separated by SDS-PAGE. Molecular markers (kDa): 66-albumin; 45-ovalbumin; (A) methanol precipitated, (B) full plasma: loading 2 μl: lanes 1–3 = Cont, control; lanes 4–10 = Chol, cholesterol; lanes 11–16 = Chol/MPoll, mussel polluted; 17–20 = Chol/MNPoll, non-polluted, (C) quantification results as volume curves of the volume peaks of lanes 3 (control) and 14 (Chol/MPoll) in full plasma: X, peaks (bands) corresponding to the lanes described above; Y, two curves of peak volumes (sum of the densitometric intensities in the defined peak area, arbitral units); each curve is formed by 20 peaks of bands of both analyzed lanes (3 and 14) by densitometry analysis.

In the microscopic appearance of brain tissue, as well as in heart, there were no endothelial lesions, cholesterol deposits, fibrosis or calcification seen in the intimal and medial layer of the brain arteries.

Table 3
Tukey-Kramer test (HSD) of the homogeneous groups of full plasma (FP) and methanol precipitated plasma (MPP) in arbitrary units of sum of intensities in the defined peak area of bands

Plasma samples	MPoll	MNPoll	Chol	Control
FP	8069594.0 ± 434062.9[b]	7639809.0 ± 588593.7[b]	7023603.1 ± 703198.6[a]	6594461.0 ± 502875.7[a]
MPP	6045283.8 ± 695551.8[a]	5341590.7 ± 854710.3[a]	5086074.9 ± 524249.2[a]	5951181.0 ± 812769.1[a]

Values are means ± SD of 7 measurements. Values with different superscripts in rows are significantly different ($P < 0.05$).
Abbreviations: Chol, cholesterol group; Chol/MPoll, cholesterol/pollutedgroup; Chol/MNPoll, cholesterol/non-polluted group.

3.6. Electrophoresis

Only major bands (approximately at 45–66 kDa) are shown (Fig. 3A–C). In the case of full plasma proteins we have a significant statistical difference between MPoll samples and both Chol and Control samples (Table 3). This means that MPoll samples have significantly more proteins than have either Chol or Control samples. From a statistical point of view MPoll and MNPoll samples were undistinguishable (Table 3). In the case of plasma proteins after methanol precipitation, we did not obtain significant statistical difference between any examined variants (Table 3). This means that all variants can be combined in the same homogeneous group. Full plasma spectrum proteins are more suitable for this purpose of differentiation than are precipitated samples (in full plasma there are more bands and they are more intense, Fig. 3C, Table 3).

4. Discussion

Some authors insist that seafood would resolve the food problem in the undeveloped countries (Imano et al., 2002). It has been shown that supplementation of seafood to common diets leads to some positive changes in atherogenicity and thrombogenicity indices (Imano et al., 2002; Valfre et al., 2003). These changes are attributed to mussel composition: proteins and antioxidant compounds, mostly phenolics. Therefore, it is of great interest to know whether supplementation of the mussel's dry matter to antiatherosclerotic diets could influence the levels of plasma lipids and plasma antioxidant activity in rats fed cholesterol.

The obtained results for proteins can be compared with other reports (Vernocchi, Maffei, Lanciotti, Suzzi, & Gardini, 2007). In this report the amount of proteins was lower than that in the cited data. This can be explained by the collection time and also by the geographical conditions. The same was shown in the concentration of metals. The results were similar to those of the same period of time, during March. The polyphenols and the antioxidants were higher in MPol than in MNPol. These results can be compared with our previous reports (Gorinstein et al., 2005).

The results of our investigation show that diets supplemented with mussel's dry matter hindered the rise in the level of plasma lipids and liver total cholesterol concentration and also hindered the decrease of the plasma antioxidant activity in rats fed cholesterol. These results were expected. Other authors have reported that cholesterol-rich diets have different effects on lipid peroxidation, cholesterol oxides, and antioxidant enzymes in rats and rabbits (Mahfouz & Kummerow, 2000).

Significant changes in the plasma contents of some studied minerals and Fe were registered. However, these changes could not lead to clear-cut conclusions.

In spite of 1% cholesterol feeding, the histology of aortas and brains in our experiment remained unaffected. In microscopic appearance there were no endothelial lesions, cholesterol deposits, fibrosis or calcification in the intimal or medial layers of the brain arteries. These findings can be explained by the relatively low content of cholesterol in the diets (1%) and the short duration of the experiment (30 days). Also, other investigators have shown that only prolonged cholesterol feeding for three and six months could induce changes in the histoarchitecture of aorta in the form of fatty streaks and atheromatous plaques followed by fibrous plaques (Bansal, Singh, & Bansal, 2002). Also Bobková and Tonar (2005) found that atherosclerotic lesions are significantly more developed in the experimental group fed a cholesterol diet for five months, than in the group fed the same diet for two months only. Our results can be compared with the data of Tokuno et al. (2002). These authors have investigated whether spontaneous ischemic events in mice with severe multi-organ atherosclerosis could adapt to ischemia. Mice were fed an atherogenic diet for 7–9 months. Signs of spontaneous ischemia occurred. One to two days later, hearts were excised, Langendorff-perfused with induced global ischemia medium, and compared with mice without signs of disease. In vivo heart or brain infarctions were verified by heart histology. Tokuno et al. (2002) suggest that spontaneous ischemic events in the brain and heart adapt the heart to ischemia. The administered high cholesterol diets, elicited in the adopted time, showed no atherosclerotic changes. Under the applied experimental conditions, the mice appeared to be resistant to diet-induced atherosclerosis. Electrophoretic patterns of proteins in plasma showed slight differences from full serum samples.

In conclusion, high levels of proteins, metals, polyphenols and antioxidants were found in mussels of MPol and MNPol groups. In groups of rats fed with cholesterol, supplemented with mussels' DM, a significant hindering of the rise of plasma lipid levels and also hindering of the decrease of the plasma antioxidant activity were registered.

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