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Inhibition of atherogenesis in rabbits by yoghurt enriched with olive mill waste extracts

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Αναστολή αθηρογένεσης σε κουνέλια από γιαούρτι ενισχυμένο με εκχύλισμα από υγρά απόβλητα ελαιουργίας

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AIM: The aim of the present paper is to investigate the possible antiatherogenic actions of a new functional food which is yoghurt 2% fat, enriched with the ethanolic extract of Olive Mill Wastes (OMW) rich in Biologically Active Lipids (BAL).

MATERIAL-METHODS: 30 New Zealand rabbits were divided into 3 groups, which were fed for 48 days an atherogenic diet, namely rabbit food enriched with 1% cholesterol (Group A), while one group was also given yoghurt 2% enriched with 500 mg of BAL/120g yoghurt (Group B) and the third group was also administered commercial yoghurt 2% (Group C) manufactured by MEVGAL SA. The OMW came from a mill using a two phage centrifugal system for olive oil extraction. After the intervention the animals were eu-

ΣΚΟΠΟΣ: Σκοπός της παρούσας εργασίας είναι η μελέτη των πιθανών αντιαθηρογόνων δράσεων ενός νέου λειτουργικού τροφίμου το οποίο είναι γιαούρτι 2% σε λιπαρά, εμπλουτισμένο με το αιθανολικό εκχύλισμα από Υγρά Απόβλητα Ελαιουργίας (ΥΑΕ) πλούσιο σε Βιολογικώς Δραστικά Λιποειδή (ΒΔΛ).

ΥΛΙΚΟ-ΜΕΘΟΔΟΣ: Στη μελέτη χρησιμοποιήθηκαν 30 κουνέλια Νέας Ζηλανδίας τα οποία χωρίστηκαν σε 3 ομάδες και οι οποίες τράφηκαν για 48 ημέρες με αθηρογόνο δίαιτα, δηλαδή τροφή εμπλουτισμένη με 1% σε χοληστερόλη (Ομάδα Α), σε μία συγχορηγήθηκε γιαούρτι 2% εμπλουτισμένο με 500 mg ΒΔΛ/120g γιαουρτιού (Ομάδα Β) ενώ στη τρίτη συγχορηγήθηκε γιαούρτι εμπορίου 2% (Ομάδα C) από τη ΜΕΒΓΑΛ Α.Ε. Τα ΥΑΕ προέρχονται από ελαιοτριβείο με διφασικό φυγοκεντρι-

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thanatized and their aortas were analyzed morphometrically after fixation in paraffin. Furthermore the blood lipids of the animals were measured before and after intervention.

RESULTS: The animals did not indicate any adverse effects from the consumption of the two diets containing yoghurt and grew normally. The animals of Group B showed a decrease in the maximum and average thickness of atheromatous plaques by 54% and 57%, respectively, compared with Group A. Group C also showed a reduction of atheromatous plaques by 32% and 30% respectively, that did not reach statistical significance. There was no difference in the serum lipid levels of any of the three groups.

CONCLUSIONS: The dramatic reduction of the atheromatous plaques is not related to changes of blood lipid levels among the three groups and can probably be attributed to anti-PAF, antiinflammatory and antioxidant activities of the BAL from the OMW.

κό σύστημα εξαγωγής του ελαιολάδου. Μετά το τέλος της παρέμβασης τα πειραματόζωα ευθανατώθηκαν και έγινε μορφομετρική αξιολόγηση των αθηρωματικών βλαβών στα αγγεία τους ύστερα από μονιμοποίηση σε παραφίνη. Επιπλέον μετρήθηκαν τα λιποειδή αίματος πριν και μετά τη παρέμβαση.

ΑΠΟΤΕΛΕΣΜΑΤΑ: Τα πειραματόζωα δεν εμφάνισαν πρόβλημα στην πρόσληψη της τροφής ενισχυμένη με γιαούρτι είτε εμπορίου ή ενισχυμένο με ΥΑΕ και αναπτύχθηκαν κανονικά. Τα πειραματόζωα της Ομάδας Β εμφάνισαν μείωση κατά 54% του μέγιστου και 57% του μέσου πάχους των αθηρωματικών πλακών σε σύγκριση με την Ομάδα Α. Η Ομάδα C επίσης εμφάνισε μείωση των αθηρωματικών πλακών 32% και 30% αντίστοιχα αλλά χωρίς στατιστική σημαντικότητα. Δεν παρατηρήθηκε καμία διαφορά στη συγκέντρωση των λιποειδών του αίματος σε καμία από τις τρεις ομάδες.

ΣΥΜΠΕΡΑΣΜΑΤΑ: Η δραματική μείωση των αθηρωματικών πλακών δεν σχετίζεται με τη μεταβολή των επιπέδων των λιπιδίων του αίματος ανάμεσα στις 3 Ομάδες και μπορεί να αποδοθεί πιθανότατα στις αντι-PAF, αντι-φλεγμονώδεις και αντιοξειδωτικές δράσεις των ΒΔΛ από τα ΥΑΕ.

Key words: Atherosclerosis, PAF, olive mill wastes, yoghurt.

Λέξεις ευρετηρίου: Αθηροσκλήρωση, PAF, υγρά απόβλητα ελαιουργίας, γιαούρτι.

1. Introduction

Cardiovascular diseases, which are different clinical outcomes of atherosclerotic disease is a leading cause of death in the "developed countries".

The Platelet Activating Factor (PAF) is the most potent today known inflammatory molecule showing its pathophysiological effects in concentrations of about 10–9 M which in some systems can reach even the 10–12M.[1] PAF is a large family of phospholipids with the general nomenclature of 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine.[2] PAF is produced by many cells types both in normal conditions and/or after stimulation, whereas PAF receptors are present in many cells.[1]

The strong effect of PAF as a cause but also as a mediator of inflammation makes it directly involved with atherosclerotic disease, one of the major inflammatory diseases.[3] The main stage for the theory of PAF involvement in atheromatous disease is the increased PAF levels in patients' blood, due to the presence of a cardiovascular risk factor like smoking, diabetes, etc., while its effects can be negated by PAF inhibitors/receptor agonists either endogenous or obtained through diet.[4]

Previous studies of our group have shown that lipids from olive oil, wine, fish, honey, milk and yogurt, which are traditional and basic foods of the Mediterranean diet, contain potent PAF inhibitors/agonists that have the ability to inhibit the PAF stimulated aggregation of washed rabbit platelets, while other researchers have demonstrated similar anti-PAF activities for garlic and onion.[5] Further *in vitro* studies of olive oil components against PAF induced platelets aggregation showed that the total olive oil lipids (TLO) and the fraction of polar lipids (PLO) had the major biological activity (PAF inhibition), while the neutral lipids fraction (NLO) had minimal or none.[6] The same olive oil fractions (TLO, PLO, NLO) were then tested *in vivo* to a rabbit atherosclerosis model. The rabbits fed only with atherogenic diet (1% cholesterol) acting as the control group and the rabbits fed an atherogenic diet enriched in NLO formed thicker atherosclerotic lesions against the groups fed atherogenic diet

enriched either in TLO or PLO.[7] The blood lipids (TC, HDL-C, LDL-C and TG) of the animals were elevated in all the groups except the group fed with PLO where there was a decrease of HDL-C, an observation that is consistent with other studies.[8]

The positive results of olive oil and its lipids fractions in inhibiting the atheromatous plaque formation led researchers to look for PAF inhibitors/agonists in the various stages of oil production.[9] Specifically the greatest biological activity was observed in the olive pomace and its subsequent polar lipid fraction (PLP). The co-administration of the PLP to rabbits fed an atherogenic diet greatly inhibited the atheromatous plaque formation compared to controls fed only an atherogenic diet.

These results correlate only (a) to the PAF levels bound in blood components (bound PAF) of the animals and (b) the increased concentration of PAF required to induce platelet aggregation in the platelet rich plasma (PRP) of the animals fed with the PLP, indicating that platelets in the control group were already activated and/or that the platelets of the animals fed the PLP had the PAF inhibitors present in the PLP agonizing PAF actions.[10] The PLP were further studied for their ability to regress already formed atheromatous lesions. The rabbits were fed an atherogenic diet for six weeks which was then replaced with standard rabbit food for 3 weeks acting as a control group that showed a significant increase in the thickness and the surface of atheromatous plaques compared to the groups fed a standard diet enriched with either PLP or Simvastatin that was the positive control group.[11]

Apart from the olive oil pomace, potent PAF inhibitors/agonists exist also in the Olive Mill Wastes (OMW), with similar structure to those isolated from olive oil and pomace.[9,12] OMW also contain a large number of phenolic molecules in very high concentrations, much higher than olive oil. The phenolic molecules exhibit numerous protective effects against atherosclerosis, including their inhibitory activity against PAF, strong antioxidant capacity and regulation of biochemical pathways.[5] The high concentrations of biologically active molecules in OMW have led many research groups to study methods for their recovery and utilization.[12,13]

The beneficial effects of dairy products in atheromatous disease are well known for many years and several studies have demonstrated reduction of atherosclerotic plaques in experimental models of atherosclerosis. One study showed that yogurt, milk and calcium were able to reduce cholesterol levels in the serum of rabbits fed an atherogenic diet (0.1 g cholesterol/Kg of body weight), but only the group fed with yogurt showed a reduction of atherosclerotic plaques in reality complete lack thereof.[14] In another study, the yogurt did not reduce the extent of atheromatous lesion but reduced cholesterol levels in the blood vessels of experimental animals.[15] A recently published epidemiological study showed that only the increased consumption of yogurt and not of other milk products associated with a reduced thickness of carotid arteries in elderly women.[16]

Utilizing these experimental data MEVGAL SA proceeded to enrich yogurt with the biologically active lipids contained in OMW in collaboration with the Laboratory of Biochemistry EKPA that tested and further studied this new functional food. The experiments have demonstrated that the biological activity detected in OMW was also detected in the enriched yogurt (unpublished results), while the molecules that are responsible for the emerging biological activity eluted in the same elution times with those from previous studies under the same chromatographic conditions.[6,9] These experiments have also proven that fermentation does not destroy the biological activity of molecules, while the extracts did not affect the production of yogurt and do not adversely affect the bacteria fermenting the yogurt.

The purpose of this study is to investigate *in vivo* the ability of this new fortified yogurt to inhibit the formation or development of atheromatous plaques in an animal model of atherosclerosis using New Zealand white rabbits.

2. Material and methods

The animals used were male New Zealand rabbits 2–3 kg. Hypercholesterolemia of animals was achieved by the administration of standard rabbit food (Viozokat SA, Katerini, Greece) with 1% cholesterol (Sigma, St Louis, MO, USA).

The biologically active lipids (BAL) used in the experiment were extracted from OMW obtained from the Nikolaou Family oil press (Megara of Attica, Greece) an olive mill that uses a two phases centrifugal system for the delivery of oil.

The fortified yogurts were manufactured by MEVGAL SA and contained 500 mg of BAL/120 g (one

cup of yogurt 2% fat), while the respective controls tested in the control group were plain (commercial) yoghurt 2% fat. The BAL were mixed with the low fat milk before the fermentation to yoghurt.

For the extraction of the BAL by OMW solvents of low toxicity to higher organisms were used and particularly absolute ethanol and n-hexane.

The xylazine was purchased from Rompun, Bayer, Leverkusen, Germany, cetamine from Fort Dodge Laboratories Inc., Fort Dodge, Iowa, USA and sodium thiopental (sodium pentothal) from Hospital Products Division, Abbott Laboratories Abbott Park, Illinois, USA.

Cholesterol was dissolved in diethyl ether (Carlo Erba, Rodano, MI, Italy). The morphometric analysis was performed using a microscope Zeiss Axiblab (Carl Zeiss, Jena GmbH, Jena, Germany) with attached CCD camera (Sony corp., Tokyo Japan) connected to a computer.

Blood tests were made in the biochemical analyzer of the Laboratory of Experimental Surgery and Surgical Research "N.S. Christeas".

For thickness and area (average thickness) of atheromatous plaques formed in the vessels was measured with the software program Image J v.1.46 (National Institutes of Health, USA).

The statistical analysis of the data was performed through the statistical software package IBM SPSS Statistics 19.0.0 (IBM Inc, Chicago, IL, USA).

2.1. Animal handling and treatment

The animals were maintained throughout the duration of the experiment in the animal house of the laboratory of Experimental Surgery and Surgical Research "N.S. Christeas", which is approved for this kind of experiments by the Veterinary Directorate of the Prefecture of Athens and Piraeus, which has also approved the realization of this experiment according to European Regulation 609/86. All blood tests and surgical operations were carried out in the facilities of the laboratory.

For the euthanasia animals were initially injected with xylazine 5 mg/kg body weight intramuscularly followed by cetamine 25 mg/kg body weight to enter anesthesia. After the anesthesia the animals were euthanatized by intravenous injection with sodium thiopental 20 mg/kg body weight.

2.2. Extraction of BAL from the OMW

The OMW were placed in a glass container and taking into account their content in water the necessary volumes of absolute ethanol and water were added, in order to achieve the final ratio of ethanol/water: 60/40. The whole mixture was left for 30 min with constant stirring in the dark and then the sample was filter through cloth in order to remove solid residues, receiving the polar lipids extract rich in BAL in the filtrate. The filtrate was then washed with equal volume of n-hexane twice. The extract was then condensed using rotary evaporator under reduced pressure. The concentrated solution was finally lyophilized and stored at –80 °C until used.

2.3. Normal rabbit food enrichment with cholesterol (atherogenic diet)

The cholesterol (30 g) was dissolved into the minimum possible volume of diethyl ether (600 mL), a solution which is then used to soak the necessary mass (3000 g) of normal rabbit food in order to achieve the 1% of cholesterol in food. The food is stirred continuously until the entire quantity the solution is absorbed by the food evenly. The food then is put on steel stainless sheets under a continuous air flow in hoods for 24 h for the evaporation of the solvent.

2.4. Yoghurt administration to animals

Yogurt is mixed with the atherogenic diet at a ratio of 120 g of fortified yogurt (1 cup) per 1 kg of food in order to achieve the final ratio of 50 mg BAL/100g food. The same ratio of plain yogurt/atherogenic diet was used for controls. The negative control group was fed only atherogenic diet. The food and water was provided ad libitum to the animals.

2.5. Histopathological examination and evaluation of atherosclerosis

The thoracic and peritoneal cavities were opened through a median longitudinal incision and the aorta was dissected from the aortic valve down to the aortic bifurcation.

Specimens of thoracic aorta were fixed in 10% buffered formalin solution, sectioned and embedded in paraffin wax using conventional techniques. For histopathological examination, 40–60 5 μm thick tissue sections per animal were cut, transferred on glass slides and stained with haematoxylin and eosin (H–E).

Extent (thickness, average thickness) of early atheromatous lesions (foam cell layers) in the wall of aorta was semiquantified using the afore-mentioned software package as previously described.[8,10]

2.6. Serum collection

Blood was collected from all the animals (10 mL) at the beginning (t=0 d) and the end of the dietary intervention (t=48 d) through the main ear artery and was placed in a glass tube without coagulant. The blood was allowed to clot at room temperature for 45 min followed by centrifugation at 1500×g for 10 min at 25 °C.

2.7. Statistical analysis

The Kruskal-Wallis test for nonparametric independent variables was used to assess differences among different groups. When statistically significant values were detected, the Mann-Whitney U-test was performed to evaluate differences between groups. The Wilcoxon signed rank to assess differences in the same group at different time intervals of time (0 and 48 d). Differences were considered to be statistically significant when P was less than 0.05.

3. Results

The rabbits were left for a week to acclimatize in the animal house before the initiation of the intervention. After the acclimatization period blood was collected from all the 30 animals, which were then divided in three equal groups of ten with matching serum lipid profile. The three groups were randomly chosen to be fed for 48 d with one of the following diets: Group A was fed with atherogenic diet, group B was fed atherogenic diet enriched with yoghurt containing BAL and Group C was under the atherogenic diet enriched with plain (commercial) yoghurt as mentioned in the methods section), presented schematically in figure 1. The animals did not indicate any adverse effects from the consumption of the two diets containing yoghurt (diarrhea, vomiting), but on the contrary they seem to favor them compared to the atherogenic diet as indicated from the daily food consumption data (table 1), suggesting a significant increase compared to Group A. The daily consumption of 173,5±11,9 g food/animal/day equals to receiving 77,5±5,3 mg of BAL/animal/day. All animals grew normally as indicated by the increment in their body weight. Moreover the animals in Group B had a significant body weight increase when compared to the control Group A. The morpho-

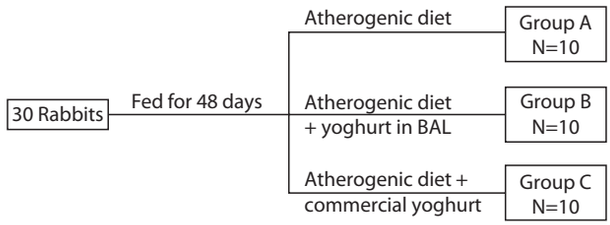

Figure 1. Schematic presentation of the dietary intervention.

metric analysis of the animal aorta is presented in figures 2 & 3 showing the maximum width and average width of the foam cell layer respectively. The dramatic reduction of both maximal (54%) and average thickness (57%) of the atheromatous lesions in Group B compared to control Group A can easily be depicted. Group C also showed a decrease in the lesion extend (32% reduction of the maximum thickness and 30% in the average thickness) but did not reach significance. No differences were observed in the blood sugars of animals either between the different groups before and after the intervention or in the same group at the time intervals. The consumption of atherogenic diet dramatically (and thus significantly) increased the total cholesterol in the blood of animals as expected in order to form the early atheromatous lesions. There was no difference in total cholesterol levels between the three different groups after the intervention. The triacylglycerols were also increased after the dietary intervention but no differences were observed between the groups. There was a significant increase in the serum HDL cholesterol of the animals in Groups A and B at the end of the intervention compared to the initial values, the significance was not reached in group C even though there was no difference between the HDL levels of the three groups at the end of the experiment. The LDL cholesterol levels were also dramatically elevated in all three groups compared to initial values but no difference was observed between the groups either before or after the intervention.

4. Discussion

Olive oil and its polar lipids fraction can both inhibit the progression of the atherosclerosis, the major cause of death in the "western" world.[7] During olive oil extraction several by-products are produced including olive pomace and olive mill waste water. Previous studies have reported the existence of several PAF inhibitors in the polar lipids fraction of both olive oil and its by-products. The *in vivo* testing of the afore-men-

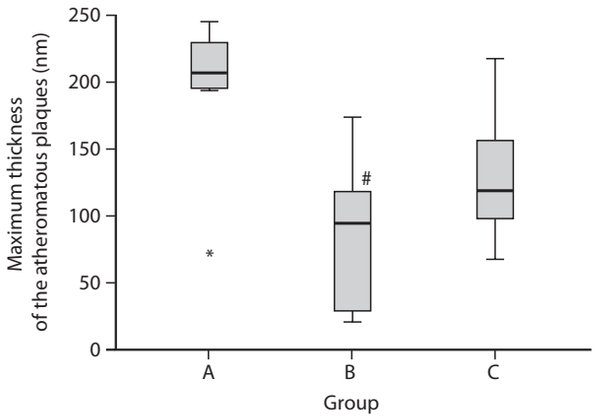

Figure 2. The maximum width of the atheromatous plaques in the three different animal groups. Group A: Rabbits fed only with atherogenic diet, Group B: Rabbits fed atherogenic diet with yoghurt enriched in BAL extracted from olive mill wastes, Group C: Rabbits fed atherogenic diet with commercial yoghurt, #: statistically significant difference with group A ($p<0.05$).

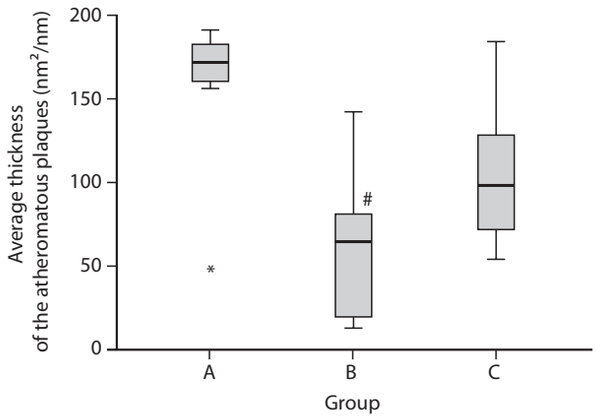

Figure 3. The average thickness of the atheromatous plaques in the three different animal groups. Group A: Rabbits fed only with atherogenic diet, Group B: Rabbits fed atherogenic diet with yoghurt enriched in BAL extracted from olive mill wastes, Group C: Rabbits fed atherogenic diet with commercial yoghurt, #: statistically significant difference with group A ($p<0.05$).

Table 1. Rabbit blood lipid profile before and after the dietary intervention.

	Time interval (d)	Daily Food Consumption (g food/day/animal)	Animal Weight (g)	Blood sugar (mg/dL)	Total Cholesterol (mg/dL)	Triacylglycerol (mg/dL)	HDL-Cholesterol (mg/dL)	LDL-Cholesterol (mg/dL)
Group A (Atherogenic diet)	t=0	155.6±16.5	2422±276	139±7	67±13	81±23	24±7	23±10
	t=48		3416±263a	115±7	2367±497a	136±36a	45±24a	2295±483a
Group B (Atherogenic diet+yoghurt enriched in BAL)	t=0	173.5±11.9b	2566±151	149±9	63±4	74±15	26±7	23±6
	t=48		3387±223a	118±8	2055±305a	146±52a	50±19a	1976±300a
Group C (Atherogenic diet+ commercial yoghurt)	t=0	185.6±2.4b	2542±139	145±12	63±8	74±18	26±4	22±9
	t=48		3625± 221[a,b]	112±12	1933±570a	148±33a	52±24	1852±545a

[a]: Significant difference for $P<0.05$ within the same group compared to 0 d, according to Wilcoxon signed rank test.
[b]: Significant difference for $P<0.05$ compared to control group A, according to Kruskal-Wallis test

tioned fractions in rabbit atherosclerosis models has exhibited increased anti-atherogenic activity, meaning reduced atheromatous lesion thickness compared to control groups fed only atherogenic diet and/or the neutral lipids fraction.[7,10,11] Dairy products and more specifically yoghurt has also proven anti-atherogenic activity both in rabbits but also in human as it is suggested by a recent epidemiological study.[14–16] The aim of the present study was to assess the ability of the yoghurt enriched in the ethanolic extract of the OMW in reducing the atheromatous lesions.

From the *in vivo* dietary intervention one can conclude that both the yoghurt enriched in BAL and the commercially available yoghurt have increased the appetite of animals leading them to consume larger quantities of food. No adverse effects like diarrhea or

vomiting were observed while animal growth measured as weight gain was normal. Animals of group C which were fed with commercially available yoghurt 2% in fat had significantly more weight than the control group A fed only an atherogenic diet.

The most important finding of this study is the beneficial effect of this new fortified yoghurt in the reduction of the atheromatous plaques development. Animals of group B fed an atherogenic diet with the yogurt enriched in BAL showed a dramatic decrease in maximal and average thickness of the plaques that reached up to 54% and 57% respectively, compared with control Group A. Animals of Group C also showed a decrease in the maximal and average thickness of the atheromatous plaques that reached up to 32% and 30% respectively compared to control group. The lack of significance can probably be attributed to the small number of animals used in this study.

The blood lipids of the animals did not exhibit any clinical significance. All animal groups showed a significant increase in the serum concentration of total cholesterol, triacylglycerol, HDL-C and LDL-C, after the dietary intervention but there was no difference between the three different groups either before or after the intervention.

The increase in HDL-C levels of groups A and B after the dietary intervention can be attributed to the large increase of total serum cholesterol, which proportionately increased also HDL-C, whereas in the case of Group C, the lack of significance is probably due to the large scattering of the values. Moreover, there was no difference in the HDL concentration among the three groups after the intervention.

The serum glucose concentration remained stable and equal between the groups throughout the experiment.

The results suggest that the enrichment of the yoghurt with the ethanolic extract from OMW can reduce atherosclerosis in animals. This effect can initially be attributed to the BAL of the extract with anti-PAF and anti-inflammatory effects in general, by the fact that the serum lipid profile of the animals was the same after the intervention in all three groups suggesting that the atherogenic potential of the animals was the same.

The effect of the BAL in the mechanism of atherosclerosis will be further tested by a series of experiments that will assess the effect of the molecules in the basic PAF metabolic enzymes of the three animal groups but also to their overall antioxidant capacity.

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