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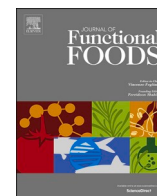
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Intake of coated EPA:DHA 6:1 nanoparticles improves age-related endothelial dysfunction by restoring the endothelial formation of NO and improving oxidative stress: Role of the local angiotensin system

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ABSTRACT

Omega-3 polyunsaturated fatty acids including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have been shown to reduce cardiovascular risk. This study investigated whether nanoencapsulation of EPA:DHA 6:1 is associated with an improved beneficial effect on age-related endothelial dysfunction. Thoracic aorta and main mesenteric artery from young and middle-aged rats that received daily by gavage 100 mg/kg of either native or coated form for 1-week were investigated. Vascular reactivity and level of oxidative stress and target proteins were assessed using organ chambers, dihydroethidium and immunofluorescence staining, respectively. Compared to the native form, the coated EPA:DHA 6:1 nanoparticles treatment improved to a greater extent NO-mediated relaxation and contractile response, and the level of vascular oxidative stress and expression of ACE/AT1R in the aged artery. The nanoencapsulation of EPA:DHA 6:1 promotes a greater beneficial effect on age-related endothelial dysfunction involving improved NO-mediated relaxations and normalization of the pro-oxidant local angiotensin system.

1. Introduction

The endothelium is a key regulator of vascular homeostasis through the release of several potent vasoactive factors that control vascular tone, smooth muscle cell proliferation, blood fluidity and inflammatory responses. The endothelium-derived relaxing factors, which promote vascular protection include nitric oxide (NO), prostacyclin, and endothelium-dependent hyperpolarization (EDH) (Vanhoutte, Shimokawa, Tang, & Feletou, 2009). Oxidative stress and blunted vascular relaxation, key mechanisms of endothelial dysfunction, often link risk factors to vascular disease, arterial stiffness and aging (Donato, Machin

& Mesniewski, 2018). The endothelial dysfunction associated with increasing age is thought to be the prime contributor to the development of cardiovascular diseases (CVDs), even in the absence of other recognized risk factors such as hypertension, hypercholesterolemia and diabetes (Lakatta, 2015). Hence, the age-related endothelial dysfunction is an important therapeutic goal for the prevention of CVDs in elderly people (Seals, Jablonski & Donato, 2011). Recent evidence suggests that endothelial senescence promotes blunted endothelium-dependent relaxation (Toda, 2012) involving NO and EDH, and an increased formation of endothelium-derived contracting factors (EDCFs) and vascular oxidative stress (El Assar et al., 2012).

Abbreviations: ACE, Angiotensin-Converting Enzyme; ACh, Acetylcholine; Ang II, Angiotensin II; AT1R, Angiotensin II Type 1 Receptor; BH4, Tetrahydrobiopterine; COX, Cyclooxygenase; CVDs, Cardiovascular Diseases; DAPI, 4',6-Diamidino-2'-Phenylindole Dihydrochloride; DHA, Docosahexaenoic Acid; DHE, Dihydroethidium; EDCFs, Endothelium-Derived Contracting Factors; EDH, Endothelium-Dependent Hyperpolarization; EPA, Eicosapentaenoic Acid; IK_{Ca}, Intermediate Conductance Ca²⁺ activated K⁺ channels; IMA, Internal Mammary Artery; Lev, Levocromakalim; L-NA, N^o-nitro-L-arginine; NO, Nitric Oxide; NOS, Nitric Oxide Synthase; PBS, Phosphate Buffer Saline; PE, Phenylephrine; PUFAs, Omega-3 polyunsaturated fatty acids; ROS, Reactive Oxygen Species; SK_{Ca}, Small Conductance Ca²⁺ Activated K⁺ Channels; SNP, Sodium Nitroprusside.

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Omega-3 polyunsaturated fatty acids (PUFAs) include two important components namely eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Both, EPA and DHA, have emerged as potential vaso-protective factors and their intake has been associated with lower cardiovascular mortality (Ajith & Jayakumar, 2019; Back, 2017), in part, by improving the endothelial function (Zehr & Walker, 2018).

In addition, purified EPA and DHA formulations induced endothelium-dependent relaxations by activating the NO and EDH pathways in several types of blood vessels including the rat mesenteric artery and the porcine coronary artery (Limbu, Cottrell & McNeish, 2018; Zghele et al., 2014). Our previous study has indicated that both the ratio and the purity of the EPA:DHA formulations are of major importance for the biological activity, as highly purified EPA:DHA 6:1 and 9:1 formulations demonstrated greater endothelium-dependent NO-mediated relaxation of porcine coronary artery rings than other ratios (Zghele et al., 2014). Moreover, intake of omega-3 EPA:DHA 6:1 has been shown to prevent endothelial dysfunction in angiotensin II-induced hypertensive rats (Niazi et al., 2017).

Since omega-3 PUFAs are highly sensitive to oxidation of the numerous double bonds, our previous study has evaluated the effect of nanoencapsulation of EPA:DHA 6:1 followed by coating at the vascular endothelium. The findings indicated that coated EPA:DHA 6:1 nanoparticles potentiated and prolonged the ability of the omega-3 PUFAs formulation to stimulate the formation of NO in cultured endothelial cells leading to a stronger anti-aggregatory response (Remila et al., 2021).

Therefore, the aim of the present study was to investigate the possibility that nanoencapsulation of EPA:DHA 6:1 followed by coating is associated *in vivo* with a greater beneficial effect on the endothelial function in middle-aged Wistar rats, and, if so, to determine the underlying mechanism.

2. Materials and methods

2.1. Ethics statement

This study conforms to the guide of animal care and use in laboratory, published by US institute of health (Bethesda, MD, USA; NIH publication number 85–23, revised 1996). The present protocol was authorized by the French Ministry of Higher Education, Research and Innovation (Authorization number #7626-2016111715542930) and by the local Ethics Committee (Comité Régional d’Ethique en Matière d’Expérimentation Animale de Strasbourg). All experiments were performed in a registered animal yard within Faculty of Pharmacy.

2.2. Preparation of omega-3 PUFAs

The omega-3 PUFAs EPA:DHA 6:1 (w/w) formulation was similar as that previously characterized and studied by the group (Zghele et al., 2014). It was kindly provided by Pivotal Therapeutics, Inc. (Woodbridge, ON, Canada). The coated EPA:DHA 6:1 nanoformulation was prepared by emulsification of omega-3 PUFAs into a surfactant solution containing Tween 80, Span 80 and lecithin with the MicroJet Reactor, a technology of impinging jets in a micro-sized chamber (particle size of 287.8 nm and a particle distribution index of 0.107; Gubday Tureli, Tureli & Schneider, 2016). The emulsion is further mixed with a gelatin and gummi arabicum solution. pH of the final emulsion was adjusted to 4.8 with 10% acetic acid.

2.3. In vivo treatment of rats

Male Wistar rats (Janvier-labs, Le Genest-Saint-Isle, France) were kept in the animal facility with controlled temperature (22 °C), 12 h light/dark cycle and were given free access to standard food (SAFE® A04) and water from the age of 12 weeks until they were 50 weeks-old (approximately equivalent to a 30-years old human, Quinn, 2005). They

were then randomly assigned to three groups and were administered daily by gavage with 100 mg/kg/day of either the native EPA:DHA 6:1 form or coated EPA:DHA 6:1 nanoparticles, or tap water for 7 days. A group of 12 week-old rats was used as young control. After treatment, rats were weighed and anesthetized by an intra-peritoneal injection of a mixture of ketamine/xylazine (120/10 mg/kg) before termination by thoracotomy and exsanguination by cardiac puncture for collection of blood and organs.

2.4. Vascular reactivity studies

Vascular reactivity studies were performed using the main mesenteric artery and the thoracic aorta. Both arteries were cleaned of connective tissue and cut into rings (2–3 mm in length). Rings were suspended in organ baths containing oxygenated (95% O₂, 5% CO₂) Krebs bicarbonate solution (composition in mM: NaCl 119, KCl 4.7, KH₂PO₄ 1.18, MgSO₄ 1.18, CaCl₂ 1.25, NaHCO₃ 25 and D-glucose 11, pH 7.4, 37 °C) for the assessment of changes in isometric tension. The rings were stretched to an optimal resting tension of 1 g for the main mesenteric artery and 2 g for the thoracic aorta. After the equilibration period, the rings were exposed to high K⁺-containing Krebs bicarbonate solution (80 mM) until reproducible contractile responses were obtained. To assess the endothelial function, the rings were precontracted with phenylephrine (PE, 1 µM) before the induction of a relaxation to acetylcholine (ACh, 1 µM). For the determination of contractile responses, rings were subjected to a concentration-contraction curve in response to PE. To assess the endothelium-dependent relaxations, rings were contracted with PE (1 µM) before the construction of concentration-relaxation curve in response to ACh.

To characterise the different pathways involved in the relaxation, rings were incubated for 30 min with a pharmacological agent before the construction of a concentration–response curve. The role of NO-mediated relaxation was studied in rings incubated with both indomethacin (10 µM, a nonselective COX inhibitor) and TRAM-34 plus UCL-1684 (1 µM each, inhibitors of IK_{Ca} and SK_{Ca}, respectively) to inhibit the production of vasoactive prostanoids and the EDH-mediated relaxation, respectively. To study EDH-mediated relaxation, rings were incubated with indomethacin and N^ω-nitro-L-arginine (L-NA, 300 µM, a NOS inhibitor) to prevent the release of vasoactive prostanoids and NO, respectively. To study the role of cyclooxygenase-derived prostanoids, rings were incubated with indomethacin (10 µM). To evaluate the vascular smooth muscle function, rings were incubated with indomethacin, L-NA and TRAM-34 plus UCL-1684, then contracted with PE (1 µM) before construction of a concentration-relaxation curve to either sodium nitroprusside (SNP, a NO donor) or levromakalim (Lev, an ATP-sensitive potassium channels opener).

2.5. Immunofluorescence studies

Rings of the thoracic aorta were embedded in histomolds containing frozen section media (FSC 22, Leica Biosystems, Nanterre, France) and were snap-frozen in liquid nitrogen. Rings were cryosectioned at 14 µm and stored at –40 °C until use. Sections were defrosted with phosphate buffer saline (PBS) and fixed during 30 min with 4% (w/v) para-formaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) and then incubated with blocking/permeabilizing buffer (PBS containing 1% bovine serum albumin (BSA) (w/v) and 0.5% Triton X-100 (w/v)) for 30 min at room temperature. After buffer removal, all sections, excluding negative controls, were incubated at 4 °C with a primary antibody against either eNOS (1/100, Cat. 610297, BD Transduction Laboratories, Le Pont de Claix, France), nitrotyrosine (1/500, Cat. 05–233, EMD Millipore, Billerica MA, USA), ACE (1/200, Cat. 250450, Abbiotec, San Diego, USA) or AT1R (1/200, Cat. ab124505, Abcam, Paris, France) for 1 h. All sections were washed 3 times with PBS prior to incubation with either an anti-rabbit or anti-mouse fluorescent secondary antibody (1/400, Alexa fluor 633 conjugate, Invitrogen) for 1 h at room

temperature in the dark. After washing 3 times with PBS, sections were incubated with 1 mg/ml 4',6-diamidino-2'-phenylindole dihydrochloride (DAPI, Thermo Fisher) during 5 min at room temperature, in order to counterstain nuclei. Slides were washed 3 times with PBS and mounted under coverslip using fluorescence mounting medium (Dako, Agilent Technologies France, Les Ulis, France) and dried for 20 min at room temperature. Slides were then analyzed with confocal laser-scanning microscope (Leica SP2 UV DM IRBE, Heidelberg, Germany) with a 20X magnification lens. The level of fluorescence was quantified by using Image J software (version 1.49 for Windows, NIH).

2.6. Determination of the vascular level of oxidative stress

The level of reactive oxygen species (ROS) in the thoracic aorta and in the mitochondria were determined using the redox-sensitive fluorescent probe dihydroethidium (DHE, Invitrogen, ThermoFischer) and MitoSox probe (MitoSox™, Invitrogen, ThermoFischer), respectively. Cryosections of thoracic aorta (25 µm) were defrosted with PBS and incubated with either DHE (2.5 µM) or MitoSox (2.5 µM) for 30 min at

37 °C in the dark. After washing 2 times with PBS, the sections were mounted under a cover-slip using fluorescence mounting medium (Dako, Agilent Technologies) and dried for 20 min at room temperature. Images were acquired using a confocal laser-scanning microscope (Leica SP2 UV DM IRBE) with a 20X magnification lens. Quantitative analysis was performed using Image J software (version 1.49 for Windows, NIH).

2.7. Statistical analysis

Data are expressed as means ± standard error mean (S.E.M) for n different experiments and analyzed by Graphpad Prism (Version 7 for Windows, Graphpad Software, San Diego, CA, USA). Statistical variance between different groups was determined by applying Two-way Anova test for vascular reactivity studies and One-way Anova test for quantitative confocal microscopy results followed by Bonferroni's Multiple Comparison *post hoc* test. Group differences were considered statistically significant at $P < 0.05$.

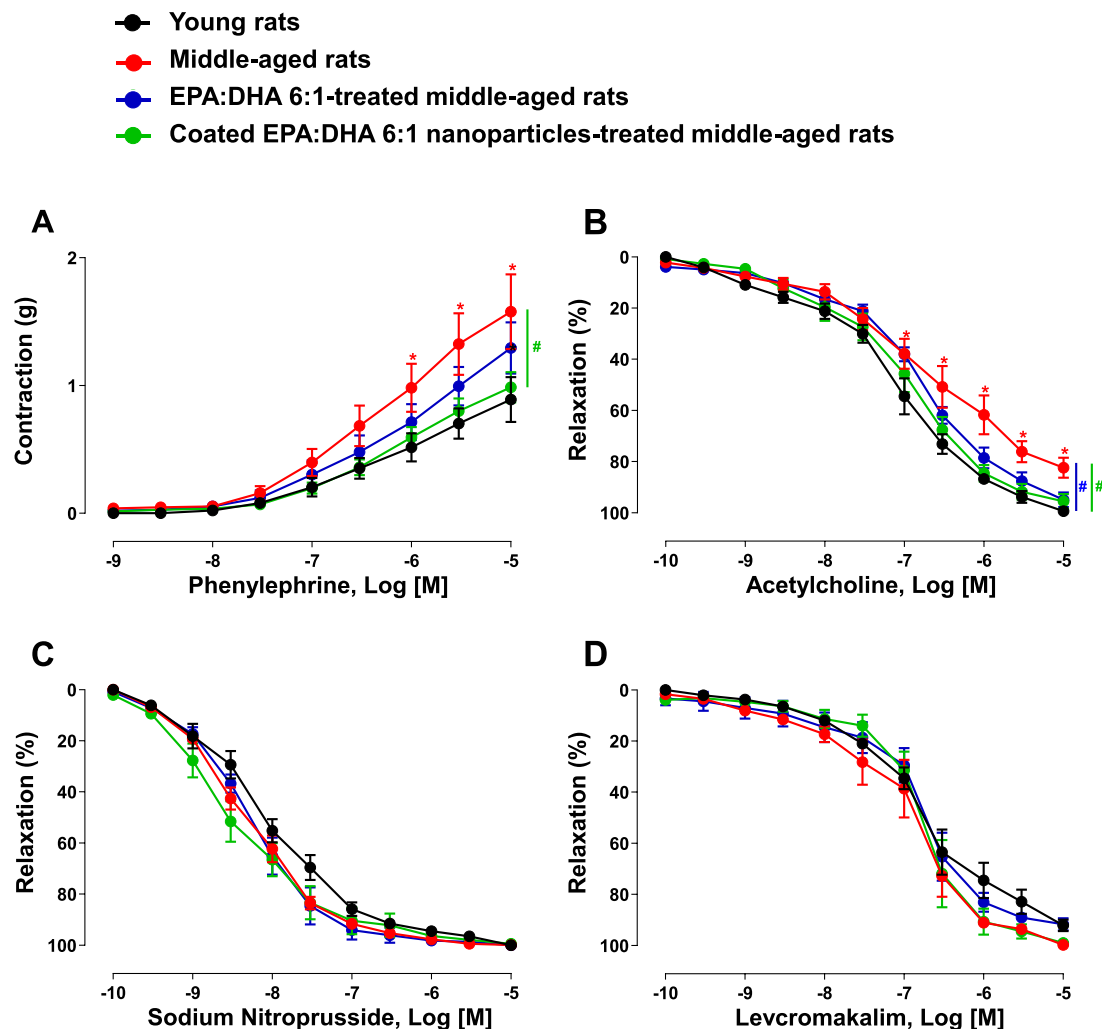


Fig. 1. Coated EPA:DHA 6:1 nanoparticles improve the age-related endothelial dysfunction in the main mesenteric artery. Rings were prepared from the main mesenteric artery and suspended in organ baths for the determination of changes in isometric tension. (A) To assess the contractile responses, rings were subjected to a concentration-contraction curve in response to phenylephrine. (B) To study the endothelium-dependent relaxation, rings were contracted with phenylephrine (PE, 1 µM) before the addition of increasing concentrations of acetylcholine (ACh). (C-D) The function of the vascular smooth muscle was assessed in rings with endothelium contracted with PE (1 µM) before the construction of a concentration-relaxation curve to either sodium nitroprusside (SNP, a NO donor) or levocromakalim (Lev, an ATP-sensitive potassium channels opener) in the presence of L-NA and TRAM-34 plus UCL-1684 and indomethacin to prevent the contribution of NO, EDH and vasoactive prostanoids, respectively. Results are expressed in grams of contraction (A) or in % relaxations (B, C-D) as means ± SEM of 5–7 rats per group. * $P < 0.05$ vs. Young rats, # $P < 0.05$ vs. Middle-aged rats.

3. Results

3.1. EPA:DHA 6:1 treatment improves the age-related endothelial dysfunction in the main mesenteric artery and the thoracic aorta

The endothelial and the vascular smooth muscle function were assessed by vascular reactivity studies of the main mesenteric artery and the thoracic aorta. Intact rings from middle-aged rats showed significantly decreased relaxations to acetylcholine and increased contractile responses to phenylephrine in comparison with those of young rats (Figs. 1, 2 A & B).

Treatment of rats with both types of EPA:DHA 6:1 formulations significantly improved acetylcholine-induced relaxations in the main mesenteric artery of middle-aged rats whereas only coated EPA:DHA 6:1 nanoparticles improved significantly phenylephrine-induced contractile responses (Fig. 1 A & B). In the thoracic aorta, the treatment with coated EPA:DHA 6:1 nanoparticles significantly improved the phenylephrine-induced contractile responses whereas relaxations to acetylcholine were minimally affected by both types of EPA:DHA treatments (Fig. 2 A

& B). In addition, neither ageing nor both types of EPA:DHA 6:1 treatments affected the function of the vascular smooth muscle as indicated by similar concentration-dependent relaxation curves to sodium nitroprusside and levocromakalim in the main mesenteric artery and the thoracic aorta (Figs. 1, 2 C & D).

3.2. Coated EPA:DHA 6:1 nanoparticles treatment improves the blunted NO component of the relaxation in middle-aged rats

In intact rings of the main mesenteric artery, the contractile response to phenylephrine was affected neither by indomethacin nor by the subsequent addition of L-NA but significantly reduced by TRAM-34 plus UCL-1684 in young and middle-aged rats (Fig. 3 A & B). Treatment with the coated EPA:DHA 6:1 nanoparticles, but not with the native EPA:DHA 6:1 form, significantly increased the basal formation of NO, as indicated by the pronounced enhancement of the phenylephrine-induced contractile response in the presence of L-NA (Fig. 3 C & D). In addition, although endothelium-dependent relaxations to acetylcholine were significantly improved in mesenteric artery rings of middle-aged rats by

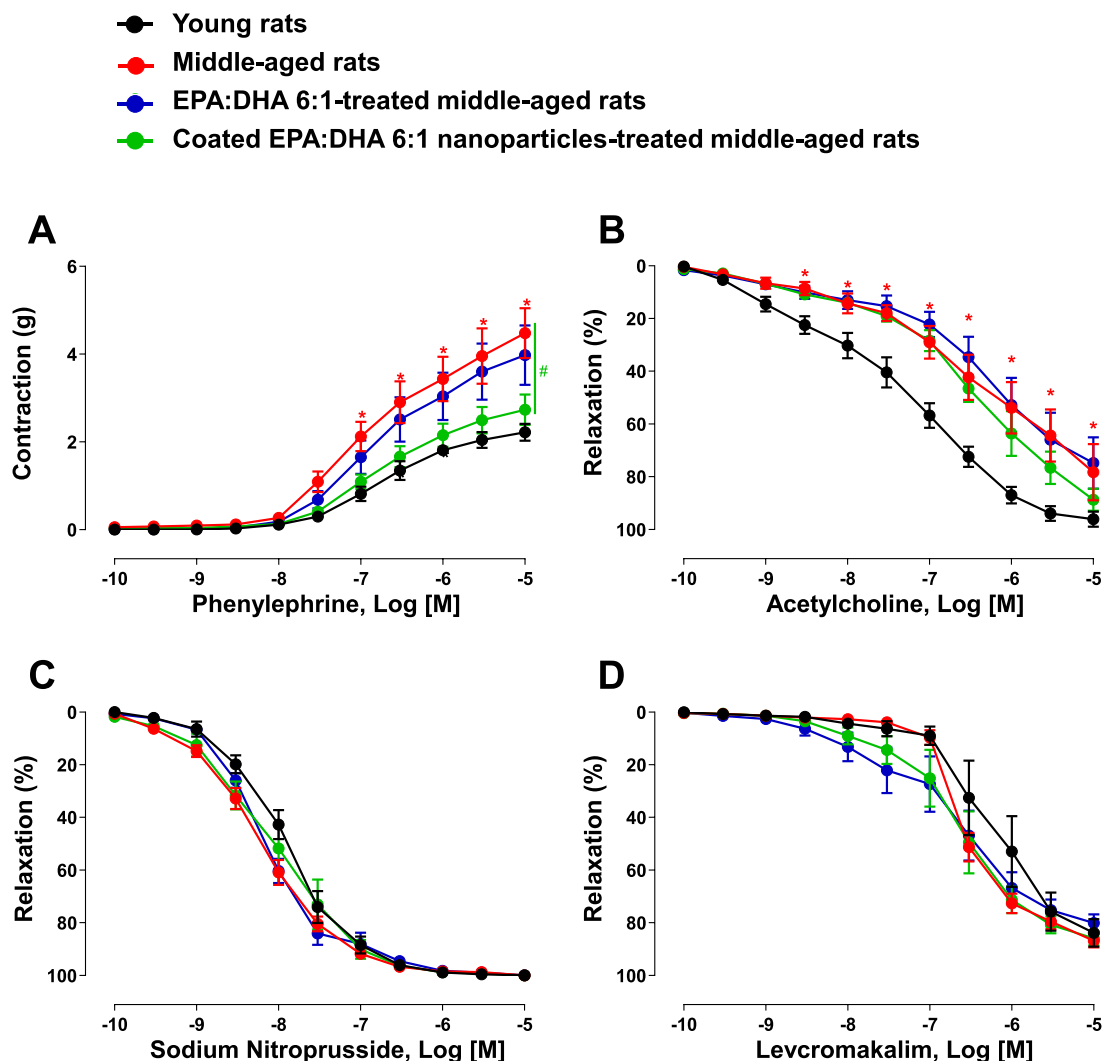


Fig. 2. Coated EPA:DHA 6:1 nanoparticles but not native EPA:DHA 6:1 improve the age-related endothelial dysfunction in the thoracic aorta. (A) To assess the contractile responses, rings were subjected to a concentration-contraction curve in response to phenylephrine. (B) To study the endothelium-dependent relaxation, rings were contracted with phenylephrine (PE, 1 μ M) before the addition of increasing concentrations of acetylcholine (ACh). (C-D) The function of the vascular smooth muscle was assessed in rings with endothelium contracted with PE (1 μ M) before the construction of a concentration-relaxation curve to either sodium nitroprusside (SNP, a NO donor) or levocromakalim (Lev, an ATP-sensitive potassium channels opener) in the presence of L-NA and TRAM-34 plus UCL-1684 and indomethacin to prevent the contribution of NO, EDH and vasoactive prostanoids, respectively. Results are expressed in grams of contraction (A) or in % relaxations (B, C-D) as means \pm SEM of 5–7 rats per group. * $P < 0.05$ vs. Young rats, # $P < 0.05$ vs. Middle-aged rats.

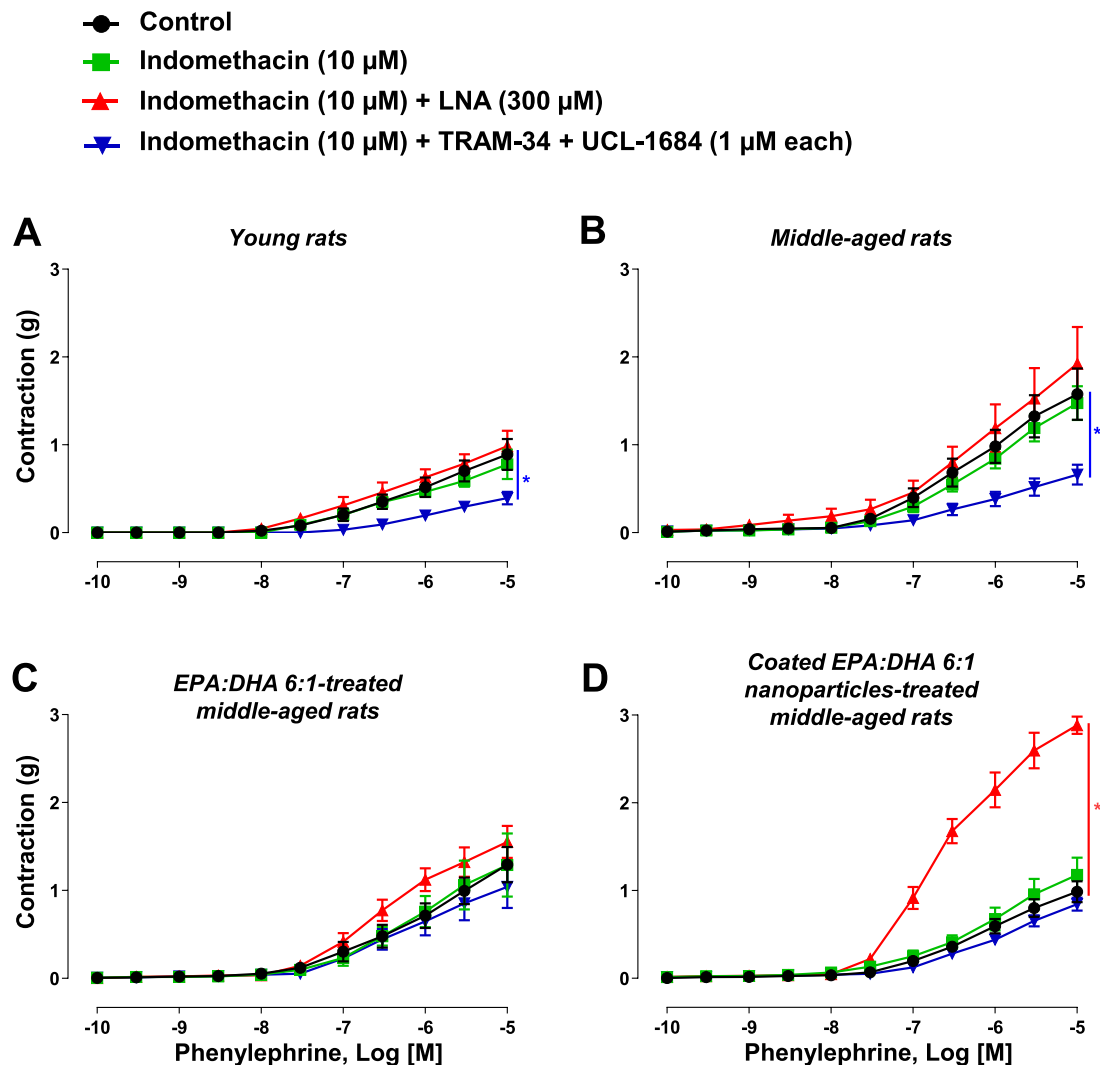


Fig. 3. Coated EPA:DHA 6:1 nanoparticles increase the basal formation of NO in the main mesenteric artery. (A–D) Rings were subjected to increasing concentrations of PE to construct a concentration-contraction response curve. In some baths, rings were incubated with either indomethacin (cyclooxygenases inhibitor, 10 μ M) in the absence or presence of either L-NA (eNOS inhibitor, 300 μ M) or TRAM-34 plus UCL-1684 (inhibitors of endothelium dependent hyperpolarization, 1 μ M each) for 30 min before contraction. Results are expressed in grams of contraction as means \pm SEM of 5–7 rats per group. * $P < 0.05$ vs. control without inhibitors.

both types of EPA:DHA 6:1 treatments, no such effects were observed in the presence of indomethacin, suggesting the involvement of vaso-relaxing prostanoids (Fig. 4 A & B). The small endothelium-dependent relaxation to acetylcholine observed in the presence of indomethacin plus L-NA was similar in all groups, indicating that the EDH-mediated component is a minor component in the mesenteric artery of both young and middle-aged rats, and that it is not affected by both EPA:DHA 6:1 treatments (Fig. 4 C). In contrast, relaxations to acetylcholine observed in the presence of indomethacin plus TRAM-34 and UCL-1684 were significantly reduced in mesenteric artery rings of middle-aged rats compared to young rats (Fig. 4 D). Treatment of middle-aged rats with the coated EPA:DHA 6:1 nanoparticles but not the native EPA:DHA 6:1 formulation significantly improved relaxations of acetylcholine suggesting an improved NO component (Fig. 4 D).

In intact aortic rings, the contractile response to phenylephrine in middle-aged rats was significantly reduced by indomethacin indicating an age-related contribution of vasoconstricting prostanoids (Fig. 5 B). Treatment of middle-aged rats with coated EPA:DHA 6:1 nanoparticles, but not with native EPA:DHA 6:1, normalized the contractile response that was insensitive to indomethacin, and increased the basal formation of NO as indicated by the increased phenylephrine-induced contraction

in the presence of L-NA (Fig. 5 C & D). In addition, in the presence of indomethacin the relaxation curves to ACh were similar in aortic rings of young and middle-aged rats indicating the involvement of vasoactive prostanoids in the age-related endothelial dysfunction. Moreover, the endothelium-dependent relaxation to acetylcholine was abolished by L-NA in all groups indicating a major role of NO (Fig. 6).

3.3. EPA:DHA 6:1 treatment reduces vascular and mitochondrial oxidative stress in the thoracic aorta of middle-aged rats

Since age-related endothelial dysfunction is associated with an increased vascular level of oxidative stress (Dal-Ros, Bronner, Auger & Schini-Kerth, 2012; El Assar, Angulo & Rodriguez-Manas, 2013; Khodja, Chataigneau, Auger & Schini-Kerth, 2012), the formation of ROS in the thoracic aorta was assessed using the redox-sensitive fluorescent probe dihydroethidium. The dihydroethidium fluorescence signal was significantly increased throughout the arterial wall in middle-aged compared to young rats, indicating an increased formation of ROS (Fig. 7 A). In addition, the level of mitochondrial oxidative stress was assessed using the redox-sensitive dye MitoSox. An increased signal was observed in the thoracic aorta of middle-aged rats compared to young rats (Fig. 7 B).

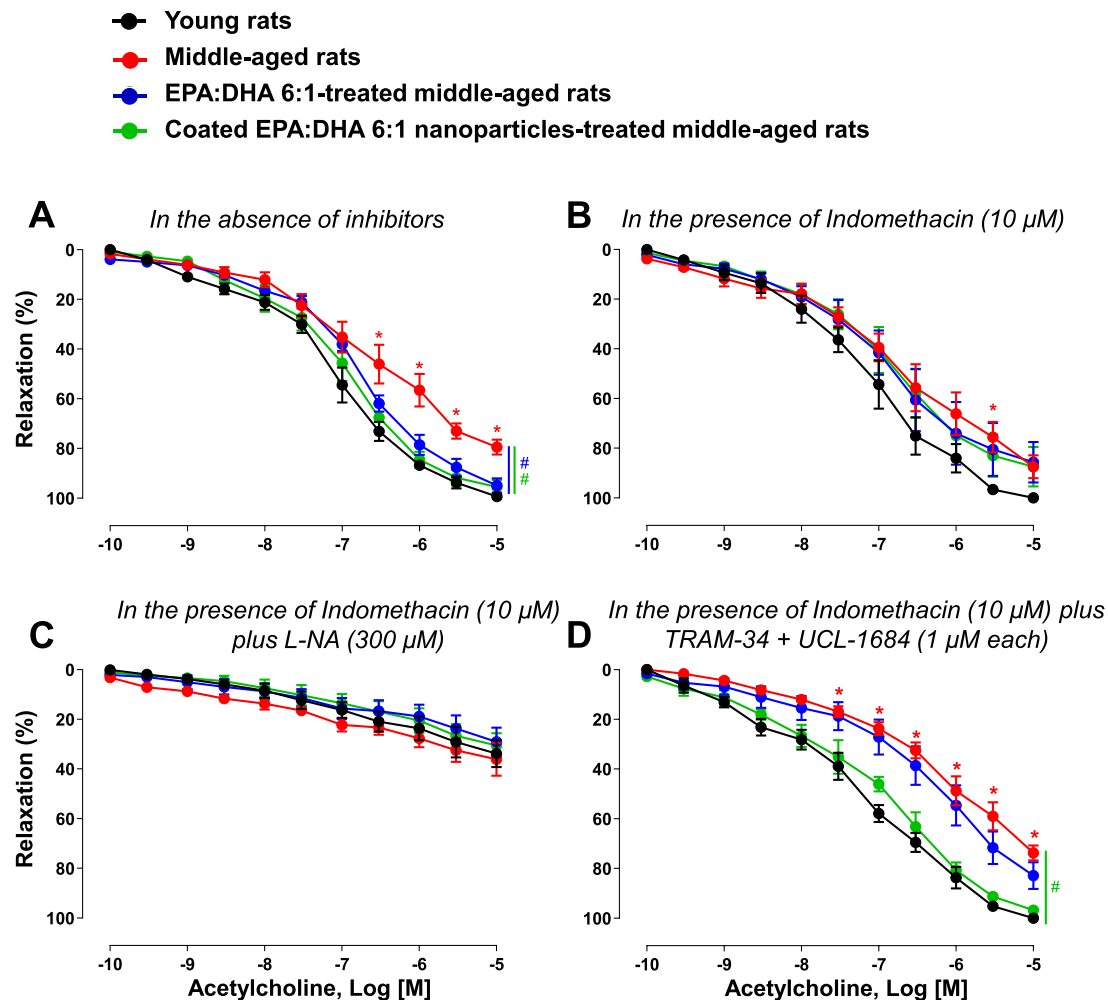


Fig. 4. Coated EPA:DHA 6:1 nanoparticles improve the blunted NO-component of the relaxation in the main mesenteric artery. Rings were contracted with phenylephrine (PE, 1 μ M) before the addition of increasing concentrations of acetylcholine (ACh). In some baths, rings were exposed to a pharmacological agent for 30 min before contraction. (B) To study the role of cyclooxygenases-derived prostanoids, rings were incubated with indomethacin (10 μ M). (C) EDH-mediated relaxations were studied in the presence of indomethacin and N^ω-nitro-L-arginine (L-NA, 300 μ M). (D) NO-mediated relaxations were studied in the presence of indomethacin and TRAM-34 plus UCL-1684 (1 μ M each). Results are expressed in % relaxations as means \pm SEM of 5–7 rats per group. * P < 0.05 vs. Young rats, # P < 0.05 vs. Middle-aged rats.

Intake of both EPA:DHA 6:1 formulations significantly reduced the level of vascular and mitochondrial oxidative stress (Fig. 7 A & B).

3.4. EPA:DHA 6:1 treatment normalizes the expression of eNOS and nitrotyrosine in the thoracic aorta of middle-aged rats

Since ageing is associated with a reduced endothelium-dependent NO-mediated relaxation, the expression level of proteins involved in the NO pathway was determined by immunofluorescence staining in the thoracic aorta. The eNOS signal is observed only in the endothelium whereas the nitrotyrosine signal, an indicator of the formation of peroxynitrites, is observed throughout the aortic wall in young and middle-aged rats (Fig. 7 C & D).

The eNOS signal in the aorta of middle-aged rats is significantly increased compared to that of young rats, demonstrating most likely a compensatory mechanism (Fig. 7 C). Similarly, the sections of middle-aged rats showed higher nitrotyrosine signals compared to young rats (Fig. 7D). Treatment of middle-aged rats with coated EPA:DHA 6:1 nanoparticles normalized the eNOS and nitrotyrosine signals whereas native EPA:DHA 6:1 improved significantly to some extent the nitrotyrosine level but not the eNOS level (Fig. 7 C & D).

3.5. EPA:DHA 6:1 treatment improves the age-related over-expression of two major components of the local angiotensin system in the thoracic aorta

Since an angiotensin-converting enzyme (ACE) inhibitor and an angiotensin II type 1 receptor (AT1R) antagonist improved age-related endothelial dysfunction and the excessive vascular formation of ROS (Khodja, Chataigneau, Auger & Schini-Kerth, 2012; Mukai et al., 2002), the expression level of ACE and AT1R was assessed by immunofluorescence staining in the thoracic aorta of young and middle-aged rats. Aortic sections of middle-aged rats showed higher fluorescence signals of both ACE and AT1R in the endothelium and vascular smooth muscle than those of young rats (Fig. 8 A & B). Intake of both types of EPA:DHA 6:1 significantly reduced the expression level of both AT1R and ACE (Fig. 8 A & B). In contrast to native EPA:DHA 6:1, the coated EPA:DHA 6:1 nanoparticles treatment normalized signals of both ACE and AT1R in the middle-aged aorta to a similar level as those observed in the young aorta (Fig. 8 A & B).

4. Discussion

With increasing age, the endothelium-dependent vasorelaxation is blunted due to an increased formation of endothelium-derived

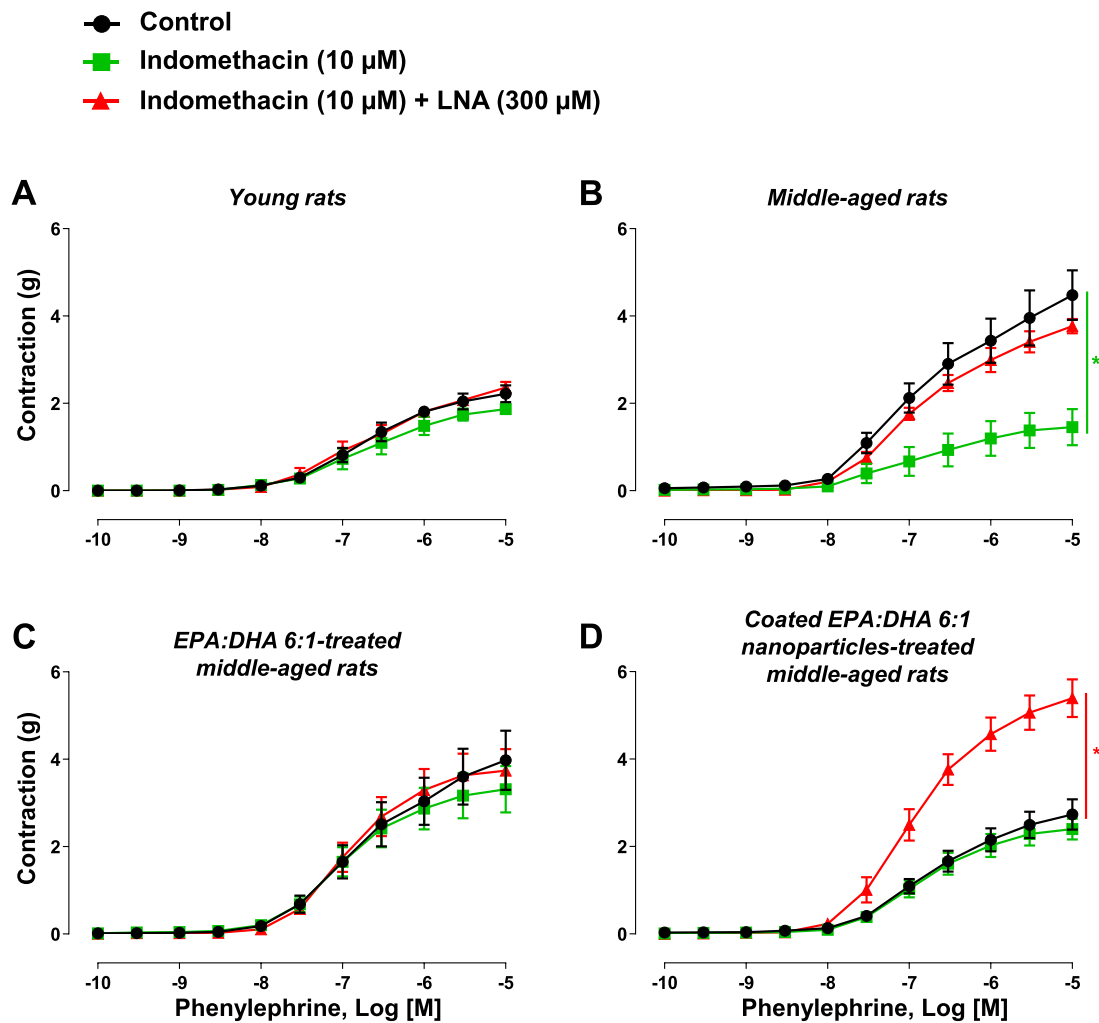


Fig. 5. Coated EPA:DHA 6:1 nanoparticles increase the basal formation NO and decrease the role of vasoactive prostanoids in the thoracic aorta. (A-D) Rings were subjected to increasing concentrations of PE to construct a concentration-contraction response curve. In some baths, rings were incubated with either L-NA (eNOS inhibitor, 300 μ M) or indomethacin (10 μ M) for 30 min before contraction to study the NO component and the vasoactive prostanoids component, respectively. Results are expressed in grams of contraction as means \pm SEM of 5–7 rats per group. * $P < 0.05$ vs. control without inhibitors.

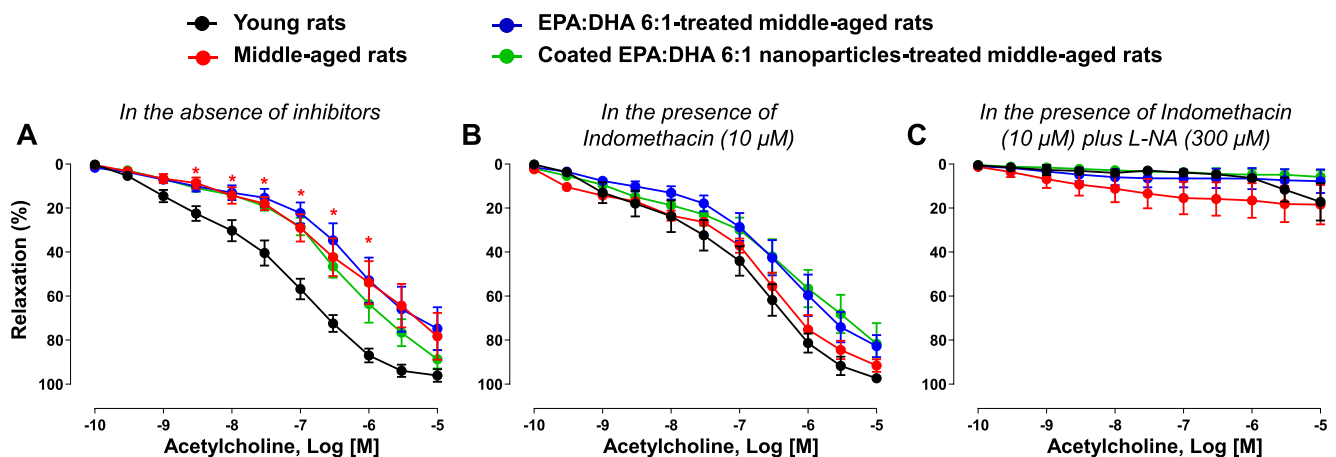


Fig. 6. Coated EPA:DHA 6:1 nanoparticles improve the blunted NO component of the relaxation in the thoracic aorta. Rings were contracted with phenylephrine (PE, 1 μ M) before the addition of increasing concentrations of acetylcholine (ACh). In some baths, rings were exposed to a pharmacological agent for 30 min before contraction. (B) To study the role of cyclooxygenase-derived prostanoids, rings were incubated with indomethacin (10 μ M). (C) EDH-mediated relaxations were studied in the presence of indomethacin and N^o-nitro-L-arginine (L-NA, 300 μ M). Results are expressed in % relaxations as means \pm SEM of 5–7 rats per group. * $P < 0.05$ vs. Young rats.

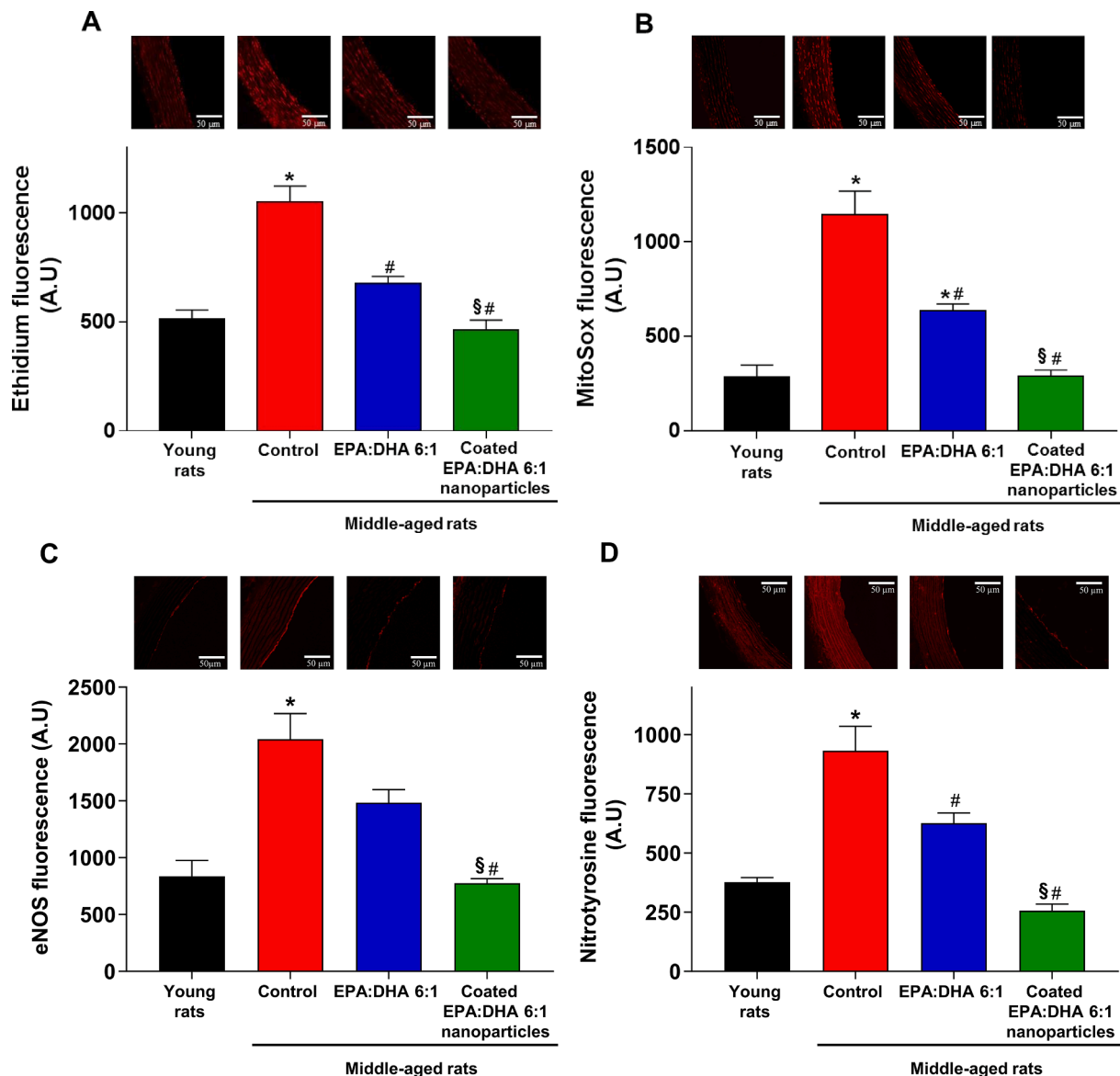


Fig. 7. Coated EPA:DHA 6:1 nanoparticles improve the age-related increased level of oxidative stress in the thoracic aorta. (A-B) The level of ROS in thoracic aorta and the formation of mitochondrial superoxide were determined by fluorescence histochemistry using the redox-sensitive fluorescent probe dihydroethidium (DHE, 2.5 μ M) and the MitoSOX probe (2.5 μ M), respectively. (C-D) The expression level of target proteins eNOS and nitrotyrosine were determined in cryosections of the thoracic aorta. Fluorescence signals were observed using a confocal laser-scanning microscope. Results are expressed as means \pm SEM of 4 rats per group. * $P < 0.05$ vs. Young rats, # $P < 0.05$ vs. Middle-aged rats, \$ $P < 0.05$ vs. Native EPA:DHA 6:1 form. Scale bar = 57 μ m (A & B) or 50 μ m (C & D).

contracting factors and oxidative stress, which can reduce the formation of both NO and EDH (El Assar et al., 2012). The major findings of the present study indicate that daily oral intake of the coated EPA:DHA 6:1 nanoparticles for one week is able to regenerate to a greater extent than the native EPA:DHA 6:1 formulation the protective endothelial function as indicated by improved endothelium-dependent relaxation, vascular and mitochondrial oxidative stress and expression of eNOS, nitrotyrosine and components of the local angiotensin system.

In the present study, the protective effect of a low dose of EPA:DHA 6:1 (100 mg/kg/day) compared to the 500 mg/kg previously studied on the endothelial function has been evaluated in a model of established age-related endothelial dysfunction (Farooq et al., 2020; Niazi et al., 2017). The dose of 100 mg/kg/day of native EPA:DHA 6:1 form or coated EPA:DHA 6:1 nanoparticles is equivalent to 1.14 g/day of omega-3 PUFAs intake by a 70 kg human (Reagan-Shaw, Nihal & Ahmad, 2008). This dose is within the range of doses reported in different clinical studies, ranging from 0.18 to 10 g/day (Appel, Miller, Seidler &

Whelton, 1993; Bhatt et al., 2019; Delgado-Lista, Perez-Martinez, Mopez-Miranda & Perez-Jimenez, 2012; Enns et al., 2014; Miller, Van Elswyk & Alexander, 2014).

The findings of the present study indicated that age-related endothelial dysfunction is associated with reduced endothelium-dependent relaxations and increased contractile responses in the main mesenteric artery and the thoracic aorta of 50-week old rats. They further indicate that age-related endothelial dysfunction is associated with an increased vascular level of ROS in the endothelium and the vascular smooth muscle of the aorta and in the mitochondria, and also an increased level of the local angiotensin system as indicated by higher signals of ACE and AT1R in the endothelium and the vascular smooth muscle.

Previous studies have indicated that ageing is associated with progressive blunted endothelium-dependent relaxations whereas endothelium-independent relaxations to sodium nitroprusside remained unaffected (Egashira et al., 1993; Matz & Andriantsitohaina, 2003; Taddei et al., 1995; Toda, 2012). The age-related decline in

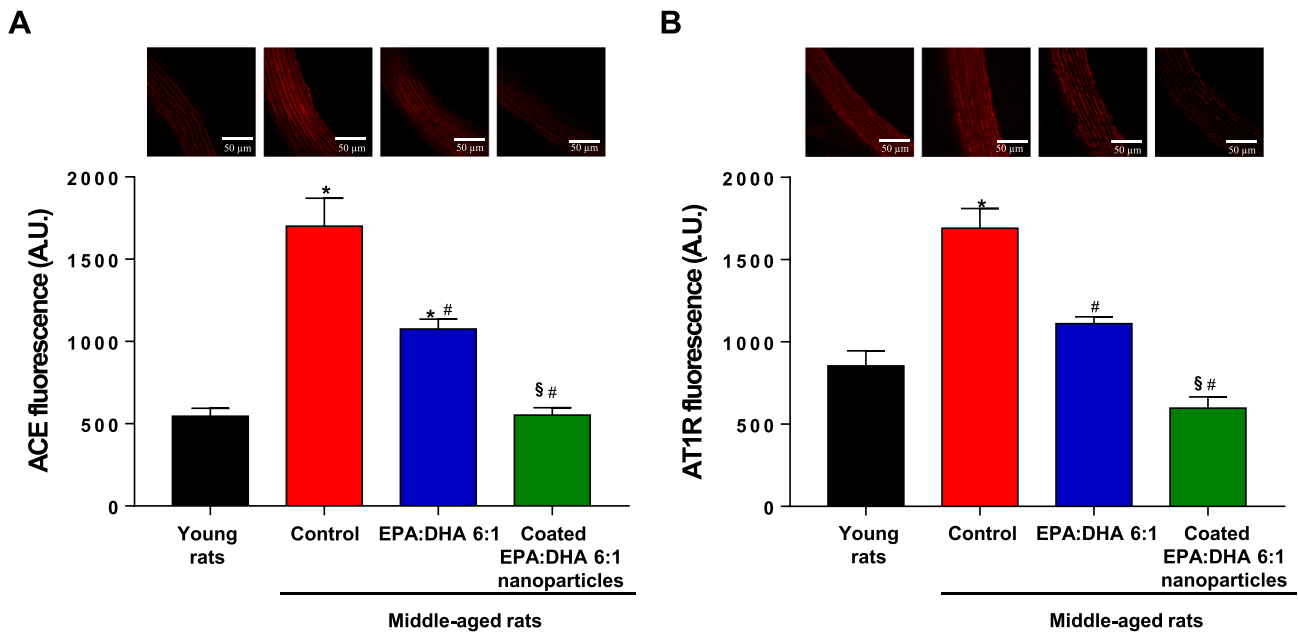


Fig. 8. Coated EPA:DHA 6:1 nanoparticles improve the age-related over-expression of the local angiotensin system in the thoracic aorta. The expression level of AT1 receptors (AT1R) and angiotensin-converting enzyme (ACE) was determined by immunofluorescence in cryosections of the thoracic aorta and analysed by a confocal laser-scanning microscope. Results are expressed as means \pm SEM of 4 rats per group. * $P < 0.05$ vs. Young rats, # $P < 0.05$ vs. Middle-aged rats, \$ $P < 0.05$ vs. native EPA:DHA 6:1 form. Scale bar = 50 μ m.

endothelium-dependent relaxations is attributable, at least in part, with a decreased production and/or an increased inactivation of vasorelaxing factors including NO and EDH, and an augmented level of oxidative stress (El Assar et al., 2012). Furthermore, aging and hypertension appear to have an additive effect on endothelial dysfunction as indicated by the progressive blunted vasodilatation observed with increasing age in both normotensive and hypertensive subjects, but with a more pronounced dysfunction observed in the hypertensive subjects (Taddei et al., 2001). Preclinical studies of different vascular beds of rats have shown that the age-related endothelial dysfunction is characterized by blunted NO-mediated relaxation in some arteries like the aorta (van der Loo et al., 2000), and also by a reduced EDH component in arteries like the mesenteric artery (Dal-Ros, Bronner, Auger & Schini-Kerth, 2012; Idris Khodja, Chataigneau, Auger & Schini-Kerth, 2012) and coronary arterioles (Csiszar et al., 2002). In addition, the age-related endothelial dysfunction appears to have features similar to those observed in several models of cardiovascular diseases such as in the femoral artery of spontaneous hypertensive rats (Puzserova et al., 2014), the mesenteric artery of angiotensin II-induced hypertensive rats (Niazi et al., 2017), and in the mesenteric artery of cirrhotic rats with portal hypertension (Rashid et al., 2018; Rashid et al., 2014).

A one-week intake of a low dose of either the native EPA:DHA 6:1 or coated EPA:DHA 6:1 nanoparticles by middle-aged rats significantly improved the acetylcholine-induced NO-mediated relaxation as well as the basal release of NO in the main mesenteric artery and thoracic aorta. Of interest, the coated EPA:DHA 6:1 nanoparticles induced a more pronounced beneficial effect on the endothelium than the native form. These findings are consistent with previous investigations showing that EPA:DHA 6:1 is a potent stimulator of the endothelial formation of NO in the isolated porcine coronary artery (Zgheel et al., 2014), the human internal mammary artery (Zgheel et al., 2019) and in the main mesenteric artery of 20-month old rats (Farooq et al., 2020), and that an additional beneficial effect can be observed following nano-encapsulation of EPA:DHA 6:1 followed by coating with gum (Remila et al., 2021).

An increased level of vascular oxidative stress promoting vascular dysfunction has been described in ageing and also in different cardiovascular diseases including hypertension and diabetes (Ungvari et al.,

2008). The age-related vascular pro-oxidant response is a major contributor to the development of endothelial dysfunction and can be due to either an increased formation of ROS and/or a reduced inactivation of ROS by the cellular defense mechanisms (El Assar, Angulo & Rodríguez-Manas, 2013).

Dal-Ros et al. and Khodja et al. have also observed an age-related endothelial dysfunction associated with oxidative stress in the mesenteric artery of middle-aged rats (Dal-Ros, Bronner, Auger & Schini-Kerth, 2012; Khodja, Chataigneau, Auger & Schini-Kerth, 2012). An increased level of ROS can promote (1) the inactivation of NO by converting it into peroxynitrite anion (ONOO⁻) and/or (2) decrease the formation of NO by oxidizing BH₄, which is an important cofactor of eNOS. Oxidized BH₄ can lead to uncoupling of eNOS, which, in turn, further increases the formation of ROS (Kirkwood & Kowald, 2012). Moreover, the increased expression level of eNOS in the old artery is mostly likely part of a compensatory mechanism subsequent to the reduced bioavailability of NO. When oxidative stress is increased, the bioavailability of NO is decreased resulting in the activation of a compensatory mechanism as indicated by an upregulation of the expression level of eNOS in the old artery. The findings of the present study indicated also that treatment of middle age rats with a low dose EPA:DHA 6:1 regenerated the endothelial function as indicated by improved endothelium-dependent relaxations mediated by NO and also contractile responses, normalized vascular and mitochondrial levels of oxidative stress and improved expression levels of eNOS and nitro-tyrosine in the thoracic aorta, and that these effects are more pronounced with the coated EPA:DHA 6:1 nanoparticles than the native EPA:DHA 6:1. In a previous study, we have shown that the intake of a higher dose of native EPA:DHA 6:1 (500 mg/kg/day compared to 100 mg/kg/day) by 20-month old rats caused an improvement of the endothelial function whereas no such effect was observed with isocaloric corn oil characterized by a low content of omega 3 PUFAs (Farooq et al., 2020).

The local vascular angiotensin system contributes to the induction of endothelial dysfunction and the associated vascular pro-oxidant response in *in vitro* and preclinical models of aging and hypertension (Dal-Ros et al., 2009; Harrison, Cai, Landmesser & Griendling, 2003; Rajagopalan et al., 1996). Ageing has also been associated with an

increased expression level of ACE in the aorta of 30-months-old rats (Challah et al., 1997), an increased expression level of Ang II and ACE in the aorta of non-human primates (Wang et al., 2003), and an increased expression level of AT1R, angiotensin II and ACE in the thoracic aorta of 24-month old mice (Yoon et al., 2016).

Moreover, a major role of the angiotensin system is underlined by the fact that treatment of old rats with either an ACE inhibitor or an AT1R antagonist resulted in an improvement of the endothelial dysfunction, at least in part, by a reduction of the level of oxidative stress (Goto et al., 2000; Kansui et al., 2002; Mukai et al., 2002; Kansui et al., 2002). In line with these studies, the present findings indicated a significantly increased expression level of AT1R and ACE in the endothelium and vascular smooth muscle of aortic sections of middle-aged rats in comparison with young rats, suggesting an activation of the local angiotensin system, which was improved partially by the native EPA:DHA 6:1 treatment and normalized by the coated EPA:DHA 6:1 nanoparticles treatment to a level similar to that observed in young rats. Similar observations with a higher dose of EPA:DHA 6:1 have been reported previously in old rats (Farooq et al., 2020) and in angiotensin II-induced hypertensive rats (Niazi et al., 2017).

Taken together, the findings of the present study indicate that ageing is characterized by the development of an endothelial dysfunction involving predominantly a reduced NO-mediated relaxation and an increased contractile response. Moreover, the endothelial dysfunction is related to an increased vascular level of oxidative stress and the local angiotensin system.

The chronic oral intake of coated EPA:DHA 6:1 nanoparticles for one week was able to restore to a greater extent than the native formulation the protective endothelial function. The beneficial effect involves an improved NO component, normalization of the local angiotensin system and the subsequent vascular pro-oxidant response. Thus, the nanoformulation of omega-3 PUFAs followed by coating appears as an interesting strategy to better protect the endothelial function and, hence, delay the development of cardiovascular disease.

To determine the clinical relevance of EPA:DHA 6:1, previous experiments have studied human internal mammary artery (IMA) rings obtained from patients undergoing bypass surgery (Zghele et al., 2019). These studies showed that EPA:DHA 6:1 inhibited the serotonin-induced contractile responses of IMA rings and that this effect is mediated by NO. Of interest, EPA:DHA 6:1 induced greater concentration-dependent relaxations of human IMA rings than acetylcholine or bradykinin that were abolished by L-NA, indicating a pivotal role of the NO (Zghele et al., 2019). Altogether, these previous findings in conjunction with the present ones suggest that nanoformulation of omega-3 PUFAs appears as an interesting approach to regenerate the endothelial function to better protect the vascular system and, hence, to promote healthy ageing.

Ethics statement

- Institutional Review Board Statement:

This study conforms to the guide of animal care and use in laboratory, published by US institute of health (Bethesda, MD, USA; NIH publication number 85–23, revised 1996). The present protocol was authorized by the French Ministry of Higher Education, Research and Innovation and by the local Ethics Committee (Comité Régional d’Ethique en Matière d’Expérimentation Animale de Strasbourg). All experiments were performed in a registered animal yard within Faculty of Pharmacy (Authorization number #7626-2016111715542930).

CRediT authorship contribution statement

Lamia Remila: Validation, Formal analysis, Investigation. **Nazende Guenday-Tuereli:** Software, Investigation, Data curation, Visualization. **Ursula Houngue:** Investigation. **Eugenia Belcastro:** Investigation. **Christophe Bruckert:** . **Thierry Vandamme:** Supervision. **Emre**

Tuereli: Investigation. **Paul Kerth:** Conceptualization. **Cyril Auger:** Methodology, Validation, Formal analysis. **Valérie Schini-Kerth:** Conceptualization, Methodology, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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