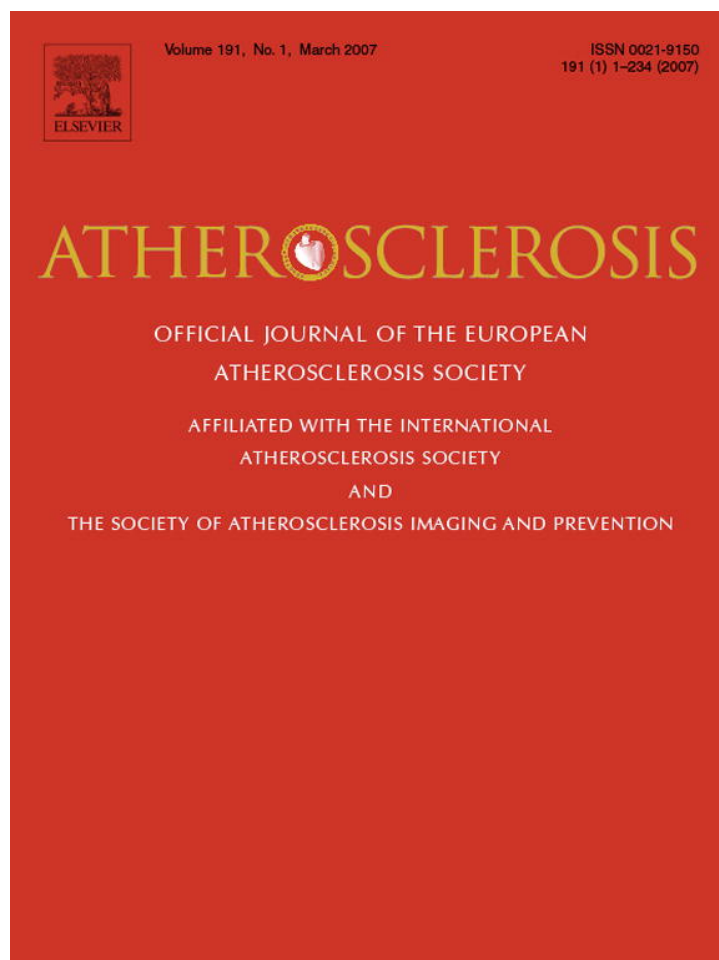


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Effect of a short-term diet and exercise intervention in youth on atherosclerotic risk factors

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Abstract

Early stages of atherosclerosis are commonly noted in youth. The present study was designed to examine the effects of lifestyle modification in 19 overweight children (age 8–17) who were placed on a high-fiber, low-fat diet in a 2-week residential program where food was provided ad libitum and daily exercise (2–2.5 h) was performed. In each subject, pre- and post-intervention fasting blood was drawn to measure serum lipids, oxidative stress marker 8-isoprostaglandin $F_{2\alpha}$ (8-iso-PGF $_{2\alpha}$) and generating enzyme myeloperoxidase (MPO), soluble intracellular adhesion molecule (sICAM)-1 and sE-selectin as indicators of endothelial activation, the inflammatory protein C-reactive protein (CRP) and total matrix metalloproteinase-9 (MMP-9). Using subject sera and human aortic endothelial cell (HAEC) culture systems, monocyte chemotactic protein-1 (MCP-1) production, as well as nitric oxide (NO), superoxide and hydrogen peroxide production were measured in vitro by fluorometric detection. After 2 weeks, significant reductions ($p < 0.05$) in all serum lipids (except HDL cholesterol), 8-iso-PGF $_{2\alpha}$, MPO, sICAM-1, sE-selectin, CRP, MMP-9, and cellular MCP-1 production were noted. Additionally, there was a significant decrease in cultured, serum-stimulated HAEC production of superoxide and hydrogen peroxide, and a concomitant increase in NO production (all $p < 0.01$). These results indicate amelioration of several traditional as well as novel factors associated with atherosclerosis after lifestyle modification, even in youth without documented disease.

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1. Introduction

It is well-established that cardiovascular risk factors may present during childhood and are predictive of future cardiovascular risk [1,2]. Although clinical cardiovascular disease occurs in later life, evidence of atherosclerosis is present in childhood [3]. Young adults with favorable levels of risk factors have lower mortality and greater longevity as adults [4]. Unhealthy lifestyles, such as physical inactivity, high-fat, high-refined-carbohydrate diet consumption, and tobacco use begin in childhood and contribute to the development of atherosclerosis.

Although serum lipid levels have been the focus to explain the incidence of atherosclerosis, other risk factors such as oxidative stress, endothelial cell activation, inflammation, and plaque stability are now recognized as important contributors. For example, levels of the oxidative stress markers 8-isoprostaglandin $F_{2\alpha}$ (8-iso-PGF $_{2\alpha}$) [5] and myeloperoxidase (MPO) [6], inflammation-associated proteins soluble intracellular adhesion molecule (sICAM)-1 and C-reactive protein (CRP) [7], and the matrix metalloproteinase MMP-9 [8] all independently predict early risk of cardiovascular disease or myocardial infarction. There are few studies examining the effects of combined physical activity and diet interventions on these novel mediators of inflammation, oxidative stress and plaque destabilization in children. The goal of this study was to investigate the effects of a short-term, daily physical activity and a low-fat, high-fiber diet

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intervention on traditional and novel cardiovascular risk factors in youth.

2. Methods

2.1. Subjects

Nineteen children, classified as overweight by the CDC sex-specific BMI-for-age percentiles, ages 8–17 (mean 13 ± 0.5 years) voluntarily participated in a 2-week residential lifestyle modification program at the Pritikin Longevity Center in Aventura, Florida. In the summer of 2002, the center offered a special program where parents were permitted to bring their children for a 1- or 2-week session. Eighteen children were enrolled in the 2-week session. Two families left early for unknown reasons, and thus pre- and post-data were obtained from 16 children, 10 males and 6 females. During 2003, the vast majority came for only 1 week and only 3 females provided 2-week data, for a total of 19 subjects. None of the subjects was under drug therapy, and none had prior histories of disease or injury that would prevent daily exercise. Consent to participate in a research program was obtained from the parents and the project was approved by the Human Subjects Protection Committee of the University of California, Los Angeles. Other measures from these subjects were previously reported [9].

2.2. Diet and exercise intervention

Participants in the program received a complete physical examination and underwent a 14-day diet and exercise intervention. From dietary analysis software (Food Processor SQL edition Version 9.8.1, ESHA Research, Salem OR), prepared meals contained 12–15% of calories from fat (polyunsaturated/saturated fatty acid ratio = 2.4:1), 15–20% of calories from protein, and 65–70% of calories from primarily unrefined-carbohydrate, high in dietary fiber (>40 g/day). The program is designed to allow the subjects ad libitum eating without control of calories, only restricting the type of foods. Carbohydrates were primarily in the form of high-fiber whole grains (≥ 5 servings/day), vegetables (≥ 4 servings/day) and fruits (≥ 3 servings/day). Protein was from plant sources, non-fat dairy (up to 2 servings/day) and fish/fowl (3.5 oz. portions 3 days/week and in soups or casseroles 2 days/week). The diet contained <100 mg of cholesterol/day. Tobacco and caffeinated beverages were not allowed during the program. Sodium intake was limited to less than 1600 mg/day. All foods except animal derived protein sources were served ad libitum. Subjects participated in one to two cooking classes daily, where small snacks were sampled. In addition, participants in the study attended twice daily lectures discussing nutrition, exercise, and general wellness.

The exercise intervention consisted of 2–2.5 h of supervised activity per day, including tennis, beach games, and

gym-based exercises intended to encourage physical activity in the subjects. This deviates from the standard Pritikin program recommendation of 45–60 min of exercise at the training heart rate (70–85% of maximal heart rate as determined by a graded exercise stress test), due to the age range of the subjects. The goal was to increase activity in these children, and as such heart rate was not measured during exercise.

Blood samples were drawn after a 12-h overnight fast on days 1 and 12 of the intervention. The blood was separated by centrifugation and serum was shipped on dry ice to UCLA where it was stored at -80°C until analysis. Height and body weight, for calculation of BMI were also assessed on these days. Body weight was measured using a scale from Pennsylvania Medical Scales (Model #7500). Height was measured using a stadiometer from Seca Inc., attached to the wall. BMI was calculated as weight (kg)/height (m^2). BMI-for-age percentiles were calculated as previously described [9].

2.3. Determination of serum lipids

Total cholesterol, TG, and HDL were measured at a national commercial laboratory (Quest Diagnostics, Miami, FL) using standardized techniques as previously described [10]. LDL was calculated as described by the Friedewald equation [11].

2.4. Determination of serum 8-iso-PGF_{2 α} , MPO, CRP, MMP-9, sICAM-1, sE-selectin, and MCP-1

Serum 8-iso-PGF_{2 α} was measured in duplicate using an enzyme immunoassay kit (Cayman Chemical). Serum MPO (Calbiochem), CRP (Diagnostic Systems Laboratories), MCP-1 (PharMingen, BDTM), total MMP-9, soluble ICAM-1 (sICAM-1), and soluble E-selectin (sE-selectin) (R&D Systems) concentrations were measured in duplicate with specific ELISA kits according to the manufacturer's instructions.

2.5. Cell cultures for *in vitro* studies

To ascertain the effects of the intervention on endothelial function, human aortic endothelial cell (HAEC) cultures were incubated with subject serum. HAEC were cultured as previously described [12,13]. In brief, the cells were subcultured and grown to 80% confluence in 75 cm² flasks in M199 medium (Invitrogen) supplemented with 20% fetal-bovine serum (FBS), 0.8 mL heparin and 2 mg endothelial cell growth factor/100 mL media (Becton-Dickinson), 1% penicillin–streptomycin–glutamine (Gibco BRL), and 1% sodium pyruvate (Gibco BRL). These cells were then counted and plated in a 96-well plate at approximately 1×10^5 cells/cm² and were allowed to grow forming a complete monolayer of confluent HAEC in 2 days.

2.5.1. MCP-1 production

HAEC cultures were washed with M199 medium containing 1% FBS three times. The second wash was incubated for 1–2 h at 37 °C and 95% O₂/5% CO₂; other washes were removed immediately. Washed cultures were then incubated with M199 medium containing 5% human serum pre- and post-intervention for 4 h. Subsequently, cultures were centrifuged and culture supernatants harvested and stored at –20 °C pending the MCP-1 measurement by ELISA (PharMingen, BD™ OptEIA ELISA Set #555179) following the manufacturer's instructions. Serum samples were incubated in wells with no cells as a control. Endothelial cell production of MCP-1 was determined by subtracting no cell control supernatants from supernatants incubated with cultures. All tests were run in triplicate.

2.5.2. NO production

All steps involving the probes were performed without direct light. The average of quadruplicate values for each condition was taken and the final values for each test condition reported as a percent of FBS control. The cell permeable fluorescent probe 4,5-diaminofluorescein diacetate (DAF-2DA, Calbiochem) was used to measure NO production by HAEC [14]. DAF-2DA is converted to DAF-2 by intracellular esterases, trapping the probe inside the cell. DAF-2 reacts with NO to form the highly fluorescent compound DAF-2T. After HAEC in 96-well plates reached confluency, growth medium was removed and the cells were washed with 200 µL serum-free medium (SFM) three times. The second wash was incubated for 1 h at 37 °C and 95% O₂/5% CO₂. SFM supplemented with 10% test serum (pre- or post-intervention) was added onto cells which were then incubated at 37 °C and 95% O₂/5% CO₂ for 18 h. Thereafter, 100 µL of 10 µM DAF-2DA dissolved in SFM was added to all cells except negative control wells. Negative control wells included cells with 100 µL of SFM containing either 10 µM 4 AF-DA (Calbiochem), a non-reactive DAF-2DA analog, or 300 µM *N*-nitro-*L*-arginine methyl ester (L-NAME, Sigma), an inhibitor of NO synthesis. The cells were then placed in a light protected incubator at 37 °C and 95% O₂/5% CO₂ for 1 h. After incubation, cells were washed three times with 200 µL of SFM to remove any residual extracellular DAF-2DA probe. Subsequently, 100 µL of SFM with 4 µM bradykinin (Calbiochem) was added to the cells. The DAF-2DA positive control included DAF-2T (Calbiochem) dissolved in SFM. After 10–15 min in a light protected incubator at 37 °C and 95% O₂/5% CO₂, fluorescence intensity was read and quantified in a fluorescence microplate reader at 495 nm excitation wavelength and 515 nm emission wavelength. The OD readings pre- and post-intervention were expressed as percentage of FBS control. Pictures of the DAF-2T fluorescence in the HAECs from the representative pre- and post-test conditions were taken with a camera connected to a fluorescent microscope (Zeiss Axiovert 135 microscope) and a computer, using Axiovision software. Picture (black and white) location within each

well was standardized through finding a cluster of cells indicated by a dark spot at the center of each well with low magnification (10×). A higher magnification (20×) and light wavelength filter was then used to identify the intracellular fluorescence of a group of cells within this cluster. The filter allowed the view of light only within the same wavelength range as the light emitted by the fluorescent probe. The color corresponding to the same wavelength was added to the black and white picture using Axiovision software. The picture files were converted to JPEG format using Adobe Photoshop.

2.5.3. Hydrogen peroxide production

The cell permeable fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA, Molecular Probes) was used to measure hydrogen peroxide production by HAEC [15]. For DCFH-DA to emit a fluorescent signal the diacetate group is first cleaved by cytoplasmic esterases to form DCFH. DCFH reacts with hydrogen peroxide to form a highly fluorescent compound, DCF, which can be used to detect enzymatic generation of reactive oxygen species [16]. In cultured endothelial cells, DCF is oxidized by H₂O₂, along with other intracellular processes involving reactive oxygen species, making it useful in determining changes in hydrogen peroxide and overall oxidant formation [17]. SFM supplemented with 10% test serum (pre- or post-intervention) was added onto cells which were then incubated at 37 °C and 95% O₂/5% CO₂ for 18 h. The hydrogen peroxide positive control included cells incubated for 18 h with 2 ng/mL TNF-α dissolved in SFM. Following incubation, the supernatant was removed from the cells and 100 µL of 10 µM DCFH-DA dissolved in SFM was applied to the cells which were then placed in a light protected incubator at 37 °C and 95% O₂/5% CO₂ for 1 h. Following DCFH-DA incubation, cells were washed three times with 200 µL SFM to remove residual extracellular probe. After washing, 100 µL SFM was applied to the cells which were then placed in a light protected incubator at 37 °C and 95% O₂/5% CO₂ for 30 min allowing time for the intracellular probe to be cleaved and oxidized as described above. The DCFH-DA probe positive control included the fluorescent oxidized form of DCFH in SFM. The negative control included SFM on cells without any probe application. The fluorescence intensity was then read and quantified in a fluorescence microplate reader with 485 nm excitation wavelength, 530 nm emission wavelength and 515 nm cutoff. The OD readings pre- and post-intervention were expressed as a percentage of FBS control.

2.5.4. Superoxide production

The cell permeable fluorescent probe dihydroethidium (DHE, Calbiochem) was used to measure superoxide production by HAEC [18]. DHE has blue fluorescence and after DHE is oxidized to ethidium by superoxide it intercalates with the cell's DNA staining the nucleus a bright fluorescent red. SFM supplemented with 10% test serum (pre- or

Table 1
Anthropometric and lipid measures in children undergoing a 14-day diet and exercise intervention

Parameter	Pre-intervention	Post-intervention	% Decrease
Body weight (kg)	92.0 ± 7.0	88.0 ± 6.8	4.3 [†]
BMI (kg/m ²)	33.2 ± 1.9	31.8 ± 1.9	4.3 [†]
BMI percentile	91.3 ± 2.1	87.1 ± 3.2	4.8 [†]
Systolic blood pressure (mm Hg)	130 ± 3.1	117 ± 1.8	10.4 [†]
Diastolic blood pressure (mm Hg)	74.3 ± 3.0	67.2 ± 2.3	10.0 [†]
Total cholesterol (mg/dL)	165 ± 7.8	127 ± 7.4	23.3 [†]
LDL cholesterol (mg/dL)	94.1 ± 8.2	68.5 ± 6.7	25.3 ^{*,†}
HDL cholesterol (mg/dL)	42.3 ± 2.4	40.8 ± 3.0	3.4
Total cholesterol/HDL cholesterol	4.16 ± 0.30	3.34 ± 0.30	19.8 [†]
LDL cholesterol/HDL cholesterol	2.27 ± 0.29	1.78 ± 0.23	22.9 [†]
Triglycerides (mg/dL)	146 ± 16.2	88.1 ± 8.1	39.5 [†]

All data are expressed as mean ± S.E.M.

[†] $p < 0.01$.

* $p < 0.05$.

post-intervention) was added onto cells which were then incubated at 37 °C and 95% O₂/5% CO₂ for 18 h. The superoxide positive control included cells incubated with 2 ng/mL TNF- α dissolved in SFM. Following incubation, the supernatant was removed and 100 μ L of 25 μ M DHE dissolved in SFM was applied to the cells which were then placed in a light protected incubator at 37 °C and 95% O₂/5% CO₂ for 45 min. Following DHE incubation, cells were washed once with 200 μ L SFM and then twice with 200 μ L HEPES to remove residual extracellular probe. After washing, 100 μ L HEPES was applied to the cells which were then placed in a light protected incubator at 37 °C and 95% O₂/5% CO₂ for 10 min. The negative control included HEPES buffer without any probe application. The fluorescence intensity was then read and quantified in a fluorescence microplate reader. A 518 nm excitation wavelength and 605 nm emission wavelength with 590 nm autocutoff filter was used to detect and quantify the fluorescence of the probe that reacted with superoxide. A 355 nm excitation wavelength and 425 nm emission wavelength with 420 nm autocutoff filter was used to detect and quantify the fluorescence of the remaining probe that did not react with superoxide. The average of the quadruplicate values was taken for each condition. The final data points were reported as a percent of the 10% FBS condition. Pictures of the ethidium fluorescence were taken in the same manner as for NO detection (above).

2.5.5. Statistical analysis

Statistical analyses were performed with Graph Pad Prism (GraphPad, San Diego, CA). Pre-intervention and post-intervention values were compared using matched paired t -tests. CRP pre- and post-intervention values were compared using matched paired Wilcoxon signed-ranks tests for non-parametric data, and was graphed using box plots with median and interquartile ranges. All data are expressed as mean ± S.E. unless otherwise noted. A $p < 0.05$ was considered statistically significant.

3. Results

3.1. Anthropometric data and serum lipids

Anthropometric and metabolic data are summarized in Table 1. All serum lipids improved significantly (>20% decreases, $p < 0.01$, Table 1), with the exception of HDL, which showed no significant change.

3.2. Serum 8-iso-PGF_{2 α} and MPO

After the diet and exercise intervention, 8-iso-PGF_{2 α} decreased (8.3 ± 3.3 pg/mL versus 44.6 ± 11.1 pg/mL,

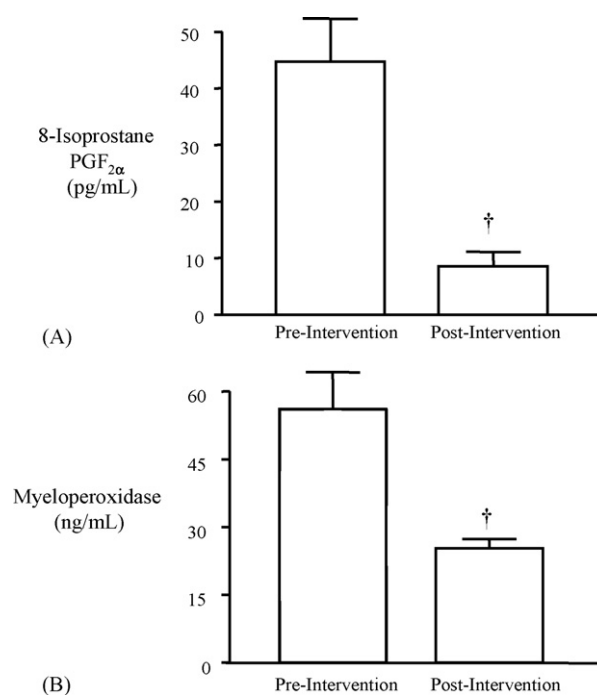


Fig. 1. (A) Effect of intervention on serum concentration of 8-iso-PGF_{2 α} . (B) Effect of intervention on serum MPO. All data are expressed as mean ± S.E. (†) $p < 0.01$ post-intervention vs. pre-intervention.

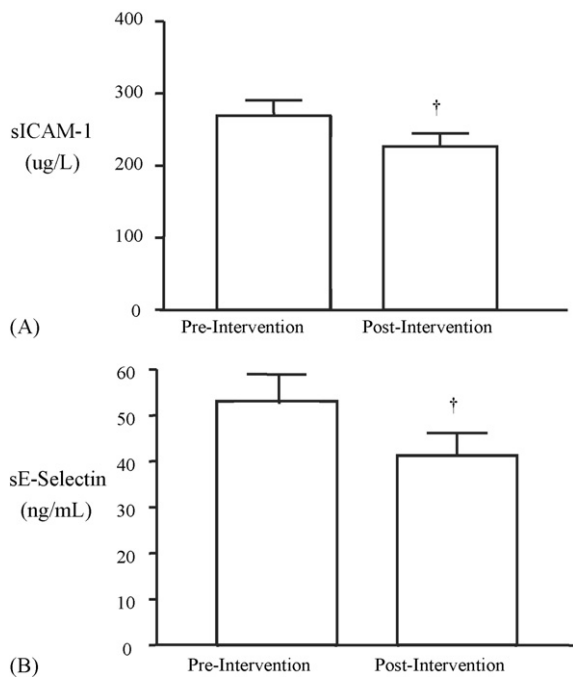


Fig. 2. (A) Effect of intervention on serum sICAM-1. (B) Effect of intervention on serum sE-selectin. All data are expressed as mean \pm S.E. (\dagger) $p < 0.01$ post-intervention vs. pre-intervention.

$p < 0.01$, Fig. 1A), indicative of a significant reduction in lipid peroxidation. Additionally, the enzymatic oxidant MPO was significantly reduced post-intervention (27.3 ± 3.6 ng/mL versus 57.6 ± 10.3 ng/mL, $p < 0.01$, Fig. 1B).

3.3. sICAM-1, sE-selectin, and MCP-1

We measured the serum concentration of ICAM-1 and sE-selectin as indicators of vascular endothelial cell activation. sICAM-1 (244.4 ± 11.9 ng/mL versus 270.7 ± 9.4 ng/mL, $p < 0.01$, Fig. 2A) and sE-selectin (41.8 ± 4.3 ng/mL versus 56.3 ± 6.1 ng/mL, $p < 0.001$, Fig. 2B) concentrations both decreased post-intervention. Serum levels of MCP-1 did not change (333.8 ± 24.7 pg/mL versus 324.3 ± 19.5 pg/mL).

3.4. CRP and MMP-9

As noted in Fig. 3, after the intervention, there was a reduction in serum concentration of the inflammatory protein CRP (2.14 ± 0.50 mg/L versus 3.61 ± 0.70 mg/L, $p < 0.05$, Fig. 3A). Serum total level of the gelatinase MMP-9, an index of plaque stability and progression was reduced post-intervention (760.8 ± 199.1 ng/mL versus 1484.1 ± 219.3 ng/mL, $p < 0.01$, Fig. 3B).

3.5. Cell culture studies

Since the serum MCP-1 did not change, we chose to measure the production of MCP-1 in a culture of HAEC as a marker of leukocyte chemoattraction. The addition of post-

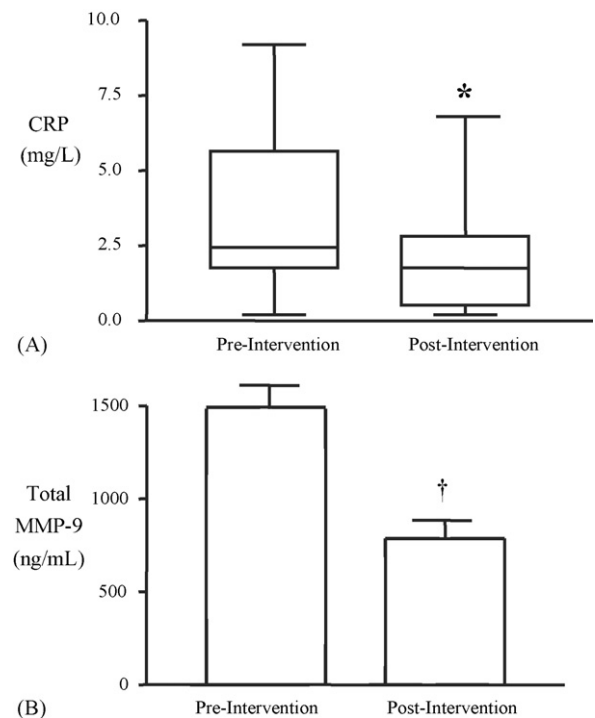


Fig. 3. (A) Effect of intervention on serum concentration of CRP. Data are expressed as median \pm S.D. Box plot demonstrates median, 25th, and 75th percentile values. (B) Effect of intervention on serum MMP-9. All data are expressed as mean \pm S.E. (*) $p < 0.05$, (\dagger) $p < 0.01$ post-intervention vs. pre-intervention.

intervention sera to cultures of HAEC for 4 h resulted in a lower production of MCP-1 compared to that noted with pre-intervention (1.09 ± 0.05 ng/mL versus 1.29 ± 0.08 ng/mL, $p < 0.05$, Fig. 4A).

Incubation of subject sera with cultured HAEC with fluorometric probe addition was used to detect production of NO and reactive oxygen species. DAF-2T (an indicator of NO production) quantitated fluorescence, increased significantly as a percentage of FBS control post-intervention versus pre-intervention ($108 \pm 2\%$ versus $101 \pm 2\%$ of FBS control, $p < 0.01$, Fig. 4B), indicating increased NO production from HAEC grown in post-intervention serum. Co-incubation of DAF2-DA with AF-DA or L-NAME abrogated NO production (data not shown). The probe DCF was used as an index of reactive oxygen species production, primarily as hydrogen peroxide. Post-intervention, there was a significant reduction in DCF fluorescence compared to pre-intervention, indicating a decrease in hydrogen peroxide formation ($94 \pm 3\%$ versus $110 \pm 5\%$ of FBS control, $p < 0.01$, Fig. 4C). Finally, DHE was used to detect superoxide production in HAEC incubated with subject sera. The ethidium fluorescence, an indicator of reacted DHE decreased significantly post-intervention ($83 \pm 3\%$ versus $96 \pm 3\%$ of FBS control, $p < 0.01$, Fig. 4D). Medium plus TNF- α (2 ng/mL) induced hydrogen peroxide and superoxide production (data not shown). These data demonstrate decreased subject sera-stimulated reactive oxygen species generation by HAEC.

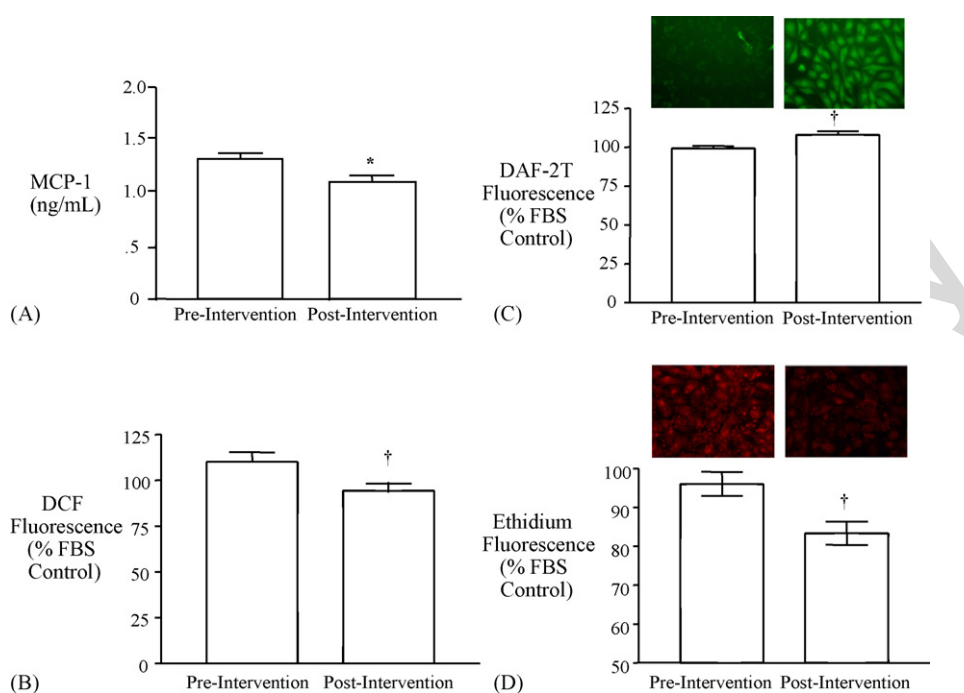


Fig. 4. (A) Effect of intervention on serum-stimulated production of MCP-1 in a HAEC culture system. (B) Serum-stimulated DCF fluorescence (% of FBS control), an index of peroxide production. (C) Serum-stimulated DAF-2T fluorescence (% of FBS control), an index of NO production. (D) Serum-stimulated ethidium fluorescence (% of FBS control), an index of superoxide production. Data are expressed as mean \pm S.E. (*) $p < 0.05$, (†) $p < 0.01$ post-intervention vs. pre-intervention.

4. Discussion

Atherosclerosis starts during childhood, may persist into adulthood [19] and leads to increased risk of cardiovascular mortality and decreased longevity [4]. For instance, the Pathobiological Determinants of Atherosclerosis in Youth (PDAY) study documented that intimal lesions were present in all aortas studied in the 15–19 age group [3] and that dyslipidemia and hypertension correlated with lesion progression [20,21]. Since lifestyle factors can mitigate the progression of atherosclerosis in adulthood [22], it is conceivable that risk is modifiable in childhood as well. Hence, the present study investigated a short-term, intensive diet and activity intervention in overweight children. The primary findings of this study provide evidence that even a short-term lifestyle modification program may (1) improve the lipid profile; (2) decrease production of the reactive oxygen species superoxide and hydrogen peroxide and increase NO production; (3) decrease endothelial cell activation and adhesion; (4) decrease inflammation; (5) decrease monocyte chemoattraction; and (6) decrease MMP-9, a marker of plaque destabilization, all of which may contribute to a reduction in atherosclerosis progression.

4.1. Lipids

A significant decrease in total-C, LDL-C, and TG levels were noted post-intervention. Evidence from a previous larger study using the same type of intervention in adults

[23] and studies in children [24,25] are in agreement with these findings. However, other studies [26,27] documented negligible changes in lipids with diet intervention, likely due to minor differences between control and intervention diets, as well as dietary adherence. The decreases in lipids are likely due to increased fiber/unrefined carbohydrate and decreased saturated fat/cholesterol consumption [28], as well as exercise [29]. Sudi et al. [30], using a restrictive diet (~ 1100 kcal/day) and substantially more physical activity (4.5 h/day), documented reduced total cholesterol, TG, body weight and insulin in overweight children over a 3-week span. We suggest that the ad libitum diet and lesser increase in the amount of physical activity may be more sustainable in this population, and offer that caloric restriction and large volumes of exercise are not required to achieve significant metabolic benefits. Moreover, Kelly et al. [31] noted modest improvements in blood pressure, HDL, insulin and flow-mediated dilation in overweight children with exercise alone, suggesting that superior results are achieved for many variables when a combined intervention as in the current study is utilized.

4.2. Oxidative stress and NO production

One enzymatic source for generation of NO-derived oxidants is MPO, a hemoprotein abundantly expressed in neutrophils and to a lesser extent by monocytes and macrophages, that displays potent proatherogenic properties. MPO can lead to oxidation of LDL cholesterol, thereby propagating

uptake by macrophages and perpetuating foam cell formation [32], and is markedly increased within human atherosclerotic lesions [33]. For example, MPO directly utilizes both NO and nitrite as substrates in vitro [34] and participates in both protein nitration and initiation of lipid peroxidation in vivo [35]. The observed decrease in MPO with lifestyle modification provides evidence for a decrease in oxidative stress. Interestingly, MPO has been shown to activate MMPs and promote remodeling, destabilization and rupture of the atherosclerotic plaque surface [36]. Thus, the reduction in MMP-9 in this study may be mediated, in part, by the reduction in MPO. Non-enzymatic oxidation products of arachidonic acid by reactive oxygen species, denote the presence of oxidative stress and are both inducers of inflammation and promoters of vascular endothelial cell and platelet activation. The diet and exercise intervention also resulted in a reduction in 8-iso-PGF_{2α}, providing evidence for decreased lipid peroxidation. Obesity in children is associated with increased 8-iso-PGF_{2α} [37] and thus, based on our findings, it is likely that elevated oxidative stress in obese children is mediated in part by diet and activity-related factors.

We utilized a HAEC culture system and specific fluorescent probes to study subject sera-stimulated NO and reactive oxygen species production (specifically superoxide and hydrogen peroxide). Noted was increased NO production by HAEC in vitro in agreement with previous data documenting improved urinary NO metabolite excretion with lifestyle intervention [38]. Additionally, both DCF and ethidium fluorescence decreased post-intervention indicating reduced serum-stimulated production of peroxides and superoxide, respectively. It is plausible that the improvement in endothelial function noted by Woo et al. [24] with diet and exercise in children was due, at least in part, to increasing endothelial cell NO production, decreased NO-scavenging reactive oxygen species production and/or MPO levels, which correlates inversely with endothelial dysfunction in humans [39].

4.3. Adhesion and inflammation

Adhesion and transendothelial migration of circulating leukocytes into the vessel wall involves various adhesion molecules and is thought to be a critical step in early atherogenesis. Proinflammatory cytokines and oxidized LDL activate the endothelium and induce the expression of adhesion molecules that are crucial to the recruitment of inflammatory cells to the vessel wall [40]. These adhesion molecules are released in soluble form into the bloodstream from proteolytic cleavage of membrane bound molecules, and thus are considered to be markers of endothelial cell activation and inflammation [41]. The present data indicate a marked reduction in both sICAM-1 and sE-selectin with diet and exercise. It should be noted that childhood obesity is associated with increased sICAM-1 and sE-selectin [37]. Our data in overweight children suggests that these elevations in overweight children are mediated, in part, by lifestyle, since our subjects were still overweight post-intervention.

In the past decade, atherosclerosis has been recognized not only as a disease of lipid accumulation, but also as a chronic inflammatory process [42,43]. CRP is currently well-established as an atherogenic catalyst, even in youth [44], where inflammatory cell presence in lesions has been noted [45]. The present study demonstrated a marked reduction in CRP (>40%) within 2 weeks, a finding unrelated to CRP instability [46] or circadian variation [47]. Receptor-mediated CRP uptake is associated with decreased nitric oxide bioavailability in human endothelial cells [48–50] and induces plasminogen activator inhibitor [51]. The observed increase in NO production and/or decrease in reactive oxygen species may be related to the reduction in CRP. Epidemiological studies [52] suggest that regular physical activity can also reduce inflammation and Liu et al. [53] have shown that increased glycemic load is associated with increased plasma CRP concentration.

4.4. MMP-9

Vulnerable plaques tend to have a lipid-rich core, with a thin fibrous cap and reduced extracellular matrix, as well as extensive macrophage-derived foam cells that produce MMP enzymes that degrade the collagen and elastin components of the matrix. Recent data suggest that MMPs play an integral role in human atherosclerosis and plaque disruption, as patients with CAD and history of myocardial infarction have elevated MMP-9 serum levels [8,54]. No studies to our knowledge have investigated the effects of lifestyle modification on MMPs in children. The decreased total MMP-9 post-intervention is in agreement with the reduction in oxidized lipids in our study, which have been shown to upregulate MMP-9 expression in monocyte derived macrophages [55]. Previously, Koh et al. [56] investigated the effects of diet and simvastatin on MMP-9 levels and noted that while an AHA Step I diet for 14 weeks had no effect, addition of simvastatin led to reduced MMP-9.

The current study has important strengths and limitations to consider. The major strength is the supervised nature of the study. Supervising food intake and physical activity removes the need to question compliance or to rely on food and activity questionnaires. Further, all exercise sessions were supervised and adherence to the diet and activities was essentially 100%. Conversely, the study was not randomized and the subjects were motivated to take part in the intervention; hence, we cannot extrapolate adherence to the general population. Nevertheless, the findings document that benefits are possible in motivated subjects. Further studies are needed to assess the ability of these changes to be durable in children in a home environment, as has been shown in adults previously [23]. Caloric intake was not determined with consumption of the ad libitum diet, except for animal protein. However, increasing fiber and reducing the fat content of the diet without specific efforts to maintain body weight has been reported to result in a spontaneous decrease in caloric intake and weight loss [57,58]. The present study was not

designed to investigate the independent effects of diet and physical activity, and thus cannot discern which aspect(s) of the intervention were responsible for the changes noted.

4.5. Significance

Overall, amelioration of cardiovascular risk factors occurred in with ad libitum dietary intake, as there were no restrictions on the quantity of food eaten other than animal protein; thus the diet quality likely contributed to the response. The idea of ad libitum diet consumption including improvement in cardiovascular disease factors is both novel and deserving of future investigation. Additionally, amelioration of risk factors occurred in children who increased their physical activity in the form of aerobic play. Children today experience reductions in physical education classes and recess, less play time due to unsafe places to play, and more time spent watching television and using computers, so an emphasis on increased physical activity is merited [59]. Additionally, the short time frame of the intervention suggests that protracted interventions are not required to induce changes. The present study found numerous factors associated with cardiovascular disease improved, suggesting that the changes are not likely spurious in nature. Furthermore, the changes noted in some measurements were very significant, as 8-iso-PGF_{2α} decreased 81%, MPO 53%, MMP-9 49%, and most other measurements $\geq 20\%$, at a significance of $p < 0.01$, which is impressive considering the small sample size and heterogeneity of the population.

In conclusion, reductions in numerous contributors to atherosclerosis progression can be achieved rapidly in childhood by an ad libitum diet and physical activity. Pathobiological Determinants of Atherosclerosis in Youth investigators have concluded that primary prevention must begin before age of 20 years [60]. The 2-week diet and activity intervention resulted in significant reductions in serum lipids, oxidative stress, inflammation, leukocyte–endothelial interactions, and leukocyte production of MMP-9. Intensive lifestyle modification in children with risk factors for CAD, if sustained, may mitigate the progression of atherosclerosis and its clinical consequences in adulthood. Given the challenge of implementing lifestyle changes in children, future studies should investigate how youth and their families can incorporate and sustain these changes.

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