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Does Intermittent Fasting Improve Microvascular Endothelial Function in Healthy Middle-aged Subjects?

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Abstract

Background: Reduced endothelial nitric oxide bioavailability, a hallmark of endothelial dysfunction, is commonly encountered in cardiovascular diseases. Intermittent fasting reduces serum markers of oxidative stress, while nitric oxide levels may rise. Whether this translates into persistent improvements in endothelial function is unknown. The aim of the study was to address the effects of intermittent «Ramadan-type» fasting on endothelial function, nitric oxide bioavailability, biological parameters and blood pressure.

Methods: We tested this hypothesis in fourteen healthy middle-aged male subjects, using a prospective case-controlled study design. Microvascular endothelial function of skin vessels was evaluated with a laser Doppler imager, Before-fasting, after thirty days of fasting, and one month thereafter (Post-fasting). Endothelial dependent and independent dilatations were assessed by acetylcholine and sodium nitroprusside iontophoresis, respectively. The hyperemic response to heating after a specific nitric oxide-synthase inhibitor L-N-arginine-methyl-ester administration, versus a saline solution, allowed further characterization of nitric oxide-mediated vasodilation. Blood pressure, body mass index, metabolic parameters were determined in all subjects.

Results: Blood pressure decreased, while blood glucose and LDL-cholesterol increased during fasting (all p<0.05 vs. Before-fasting). Body mass index did not change. Hyperemic skin reactions assessed by acetylcholine increased during Fasting and Post-fasting, while sodium nitroprusside-induced hyperemia and nitric oxide-related vasodilation in response to heating increased during Fasting only (all p<0.05 vs. Before-fasting). Rises in serum triglycerides and blood urea nitrogen during fasting blunted nitric oxide-related vasodilation improvement upon heating (r=-0.55 and -0.60 respectively, p<0.05). These parameters did not change over time in thirteen matched controls.

Conclusion: Intermittent fasting improved endothelial and non-endothelial dependent vasodilations and decreased blood pressure. Increased nitric oxide bioavailability during this period was negatively related to rises in serum triglycerides and blood urea nitrogen.

Keywords: Intermittent fasting; Microvascular endothelial function; Nitric oxide; Laser Doppler flowmetry; Ramadan fasting; Healthy middle-aged men

List of Abbreviations:

NF-kB: Nuclear factor kappa-light-chain-enhancer of activated B cells; BMI: Body Mass Index, NO: Nitric Oxide; Ach: Acetylcholine; SNP: Sodium Nitroprusside; LDI: Laser Doppler Imager; SkBF: Skin Blood Flow; BSL: Baseline; μA: Microamperes; L-NAME: L-N-arginine-methyl-ester; NaCl: Normal saline; TG: Triglycerides; Chol: Total Cholesterol; HDL: High-density Lipoprotein; LDL: Low-density Lipoprotein; BUN: Blood Urea Nitrogen; Cr: Creatinine; Tot. Bil: Total Bilirubin; PU: Perfusion Unit; AUC: Area under the curve; NS: Not Significant; BP: Blood Pressure; Sp: Saline; EDHF: Endothelium-Derived Hyperpolarizing Factor

Introduction

The vascular endothelium has a crucial role in the regulation of normal blood flow and platelet activity [1]. Increased oxidative stress is linked with endothelial dysfunction in atherosclerosis, and may have an important role in the pathogenesis of cardiovascular events [2]. There is growing evidence that fasting reduces oxidative damage and inflammation [3-7]. The exact mechanism responsible for this observation is however not known [6]. Reduced energy intake, which lessens oxidative stress formation in the mitochondria, and, as a result, reduces oxidative damage to the cells, may play a role [6-8]. The most striking evidence of the anti-inflammatory effect of fasting relies on at least four controlled studies in patients with rheumatoid arthritis, two of which were randomized [9-11]. This was also observed in asthma patients [12]. Experimental studies in mice maintained on intermittent fasting diet showed increased resistance to oxidative insults [13]. In rats, alternate-day fasting also protected their hearts against inflammation and fibrosis by inhibiting oxidative damage and NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells) activation [14]. These observations could explain why caloric restriction extends lifespan and delays the onset of age-related diseases in a wide spectrum of organisms [15]. These results are in contrast to the effects of recurrent religious fasting on human health, where studies provided either such heterogeneous results that no overall conclusion could be reached [16], while others reported improvements in body mass index (BMI), lipid profile, and blood pressure [17-20].

Reasons for these discrepancies are likely the lack of standardization of the meals taken from sunrise to sunset, the different moments of the year where fasting take place, and the variable durations of fasting, depending of the latitudinal distance from the equator. Uncontrolled studies have nevertheless been able to show that intermittent fasting decreased oxidative stress [4,5,21]. Lessened reactive oxidative species could plausibly cause a rise in nitric oxide (NO) levels and thereby improve endothelial function [22]. This was suggested by a previous uncontrolled study, where recurrent intermittent fasting for more than 25 days in cardiovascular disease patients increased serum NO, while serum asymmetric dimethylarginine, the naturally occurring endogenous inhibitor of NO synthase, decreased [23].

Whether fasting can result in functional improvements of microvasculature is not known. Using the previously cited studies as a background, we decided to test the hypothesis that recurrent intermittent «Ramadan-type» fasting improves endothelial microvascular function in healthy subjects. Endothelial function was evaluated Before-fasting, after 1 month of Fasting and 1 month after fasting cessation (Post-fasting). Endothelium dependent and independent vasodilation were assessed by iontophoresis of acetylcholine (Ach) and sodium nitroprusside (SNP), respectively [24,25], while vasodilatation in response to local heating (in the presence or absence of a NO synthase inhibitor) determined the contribution of NO to skin microvascular endothelial function [26]. These repeated measurements were also obtained in a matched non-fasting control group. We are not aware of a previous prospective controlled study on intermittent fasting and endothelial function.

Materials and Methods

Subjects

Assuming a level of significance at 5% and a study power at 80%, and according to previous validation and interventional studies in our laboratory [26,27], it was estimated that 15 subjects had to participate to demonstrate a threefold increase in the contribution of NO to the microvascular response to heating with fasting. Based on their health status, a total of 27 subjects were considered eligible to participate in the study, among of 60 volunteers. We compared 14 healthy male volunteers would intended to perform fasting during the Ramadan period, to 13 subjects matched for gender, ethnicity, age and BMI. The study protocol (reference: P2014/232; B406201421283, Registered June 16, 2014) was in agreement with the Ethics principles of Helsinki and was approved by the Ethics committee of the Erasme University Hospital. The study was conducted from June 20th to September 1st, 2014. The fasting regimen started on June 28th until July 27th, 2014. A written informed consent was obtained from all subjects.

Study design

We designed a prospective case-controlled study to test our working hypothesis. All subjects were older than 18 years, nonsmokers, had no concomitant disease and took no medications. This prevented that overnight smoking and medication intake affect our findings. Because the menstrual period may impede religious fasting and affect endothelial function [28-30], only male subjects were allowed to participate in the study. The fasting group underwent 19 hours of intermittent fasting for 26 ± 0.5 consecutive days, and was asked to consume no more than one large meal after sunset and one lighter meal before sunrise, in order to reduce caloric intake variability in our study [31]. All subjects were asked to keep their usual lifestyle and daily activities during the study. Experimental conditions were controlled by enrolling the subjects with similar lifestyle and activity, in order to minimize the impact of confounding variables.

Casual humeral blood pressure was determined by E.F. in the sitting position, using a device (WelchAllyn, USA) with a cuff size of 250-340 mm. After 5 minutes of rest, 2 measures on the non-dominant arm, separated by 1 minute, were averaged. Anthropometric measurements and fasting blood samples were obtained from all subjects on the day of the microvascular flow assessment. Three measures were obtained in the fasting group: before the beginning of the fasting regimen, after 26 ± 0.5 days of fasting, and 34.4 ± 2.5 days after fasting termination (i.e. these measurements are called respectively «Before-fasting», «Fasting» and «Post-fasting» throughout the manuscript). Two measures, separated by 28 ± 1.4 days, were performed in the control group. The moment of the day where these measurements were performed was kept identical in each subject and group throughout the study.

Microvascular blood flow was assessed by a laser Doppler imager (LDI). All subjects abstained from meals for 10 ± 2 hours and from alcohol and coffee beverages for at least 24 hours prior to each LDI session. They were asked not to wash their forearms on the morning of the experiment day and to avoid non-steroidal anti-inflammatory drugs for at least 3 days before each test. Two subjects could not participate to the Post-fasting measurements, because of a knee accident in one and vacation in the other one.

Microvascular endothelial function evaluation

All measurements were performed in a quiet room, in the supine position under carefully standardized conditions. The subjects were not allowed to sleep during the experiments. The ambient temperature in the room achieved by the air conditioner was 23 ± 1°C.

Cutaneous microcirculatory blood flow was assessed by a LDI (Moor Instruments, version 5.3d software, Axminster, United Kingdom) to measure the skin blood flow (SkBF) in a region of interest corresponding to a surface of skin of 3.8 cm². The reproducibility and accuracy of this method for endothelial function measurement has already been tested previously in our laboratory [26]. The servicing and calibration of the laser Doppler machine were made prior to beginning the measurements. Before beginning the measurements, and according to LDI guidelines, specific care was taken to create similar experimental conditions to ensure regional and temporal reproducibility [32]. Measures were performed at baseline (BSL) and during hyperemic tests. For each measure, 12 scans were acquired, where the 2 first scans corresponded to the BSL cutaneous flow.

Twenty minutes before the measurement, 5% EMLA cream® (2.5% lidocaine and 2.5% prilocaine; AstraZeneca, London, UK) was applied to the skin surface in order to limit any non-specific vasodilation induced by the electric current [33]. Firstly, we performed Ach and SNP- induced hyperemia by administering these molecules percutaneously using dedicated iontophoresis chambers (ION6; Moor Instruments Ltd, Axminster, United Kingdom). Ach and SNP solutions were prepared to obtain a final concentration of 2 g/dL in deionized water, and 2.5 ml of these solutions was introduced into the cathode (Ach electrode) and the anode (SNP electrode) chambers. Electric current was generated by an iontophoresis controller (MIC2, Moor Instruments Ltd, Axminster, United Kingdom), which was set to apply a current of 100 microamperes (μA) for 20 minutes. Ach and SNP iontophoresis were continued for 26 minutes in order to obtain a maximal skin vasodilation.

We also assessed skin hyperemia response to local heating according to our previously described methodology [26]. In summary, after a skin pre-treatment in 2 adjacent skin areas either by L-NAME (L-N-arginine-methyl-ester, 20 mmol/L) or NaCl (Normal saline 0.9 g/dL, Baxter®) iontophoresis, the skin was heated to 44°C using dedicated skin heater electrodes and a temperature monitor (SH02, Moor Instruments Ltd., Axminster, United Kingdom). Heating was continued for 26 minutes in order to obtain a maximal skin vasodilation.

Blood sample collection

About 6ml of venous blood were obtained each time. Fasting blood glucose, Triglycerides (TG), Total cholesterol (Chol), High-density lipoprotein (HDL), Low-density lipoprotein (LDL), Blood Urea Nitrogen (BUN), Creatinine (Cr), hepatic enzymes and Total bilirubin (Tot. Bil) were determined in all subjects.

Data analysis

All data analyses were performed in a blinded fashion as to the sequence of the measurement (i.e. Before-fasting, Fasting or Post-fasting). SkBF was automatically measured (LDI version 5.3D software, Moor Instruments Ltd, Axminster, United Kingdom) and was expressed in Perfusion Unit (P.U.), meaning arbitrary units of blood flow. The SkBF during BSL scans and hyperemia tests were calculated and expressed as the percentage of change from the BSL. The Area under the curve (AUC) was calculated by summing each of the 10 other measures of skin vasodilation in response to Ach and SNP induced hyperemia. We finally estimated the Ach-AUC to SNP-AUC ratio (Ach/SNP ratio), in order to determine the relative contributions of endothelium-dependent over the endothelium-independent

vasodilation. We also analyzed the effect of L-NAME iontophoresis Before-fasting, during Fasting and Post-fasting periods. The delta AUC, representing NO-mediated skin thermal hyperemia, was then calculated as the difference between the saline and L-NAME AUCs during the heating-induced hyperemia.

Statistical analysis

All statistical analyses were performed using SPSS software (PASW 18, Chicago, IL, USA). Data were expressed as mean ± SEM for quantitative variables and frequencies, and as percentages for qualitative variables. We used one way ANOVA repeated measure to determine the difference in descriptive characteristics and blood measurement among the 3 periods of test. Categorical variables were analyzed by Chi-square tests. Student t tests for independent samples were used to determine differences in normally distributed data. Correlation analyses using the Pearson coefficient, and a multivariate correlation were performed to determine the predictability of the dependent variable from the independent variables by linear combination. A P value <0.05 was considered statistically significant.

Results

Subjects' characteristics (Tables 1 and 2)

The participants were all males and non-smokers with a mean age of 42.4 ± 1.5 years and a BMI of 25.9 ± 1 kg/m² in the fasting group. The control group was 44.4 ± 0.8 years old and had a BMI of 26.2 ± 0.9 kg/m² (p=NS vs. fasting group). All experiments started at the same moment of the day (p=NS).

Parameters	Before-fasting (n=14)	Fasting (n=14)	P vs. Before-fasting	Post-fasting (n=12)	P vs. Before-fasting
Start of experiment (hours)	14:00 ± 0.83	14:32 ± 0.72	NS	14:21 ± 0.90	NS
BMI (Kg/m²)	25.9 ± 1	25.1 ± 1	NS	24.9 ± 0.7	NS
SBp (mmHg)	117 ± 3	104.3 ± 2.8	<0.001	109.2 ± 2.8	NS
DBp (mmHg)	72.5 ± 1.2	67 ± 1.5	<0.01	65.8 ± 2.6	NS
Cholesterol (<190 mg/dL)	180 ± 9.4	189.6 ± 10.4	NS	171.6 ± 9.2	NS
TG (40-150 mg/dL)	106.2 ± 20	119.6 ± 30.4	NS	110 ± 20	NS
HDL-Chol (>40 mg/dL)	51.8 ± 4.2	47.5 ± 3.2	NS	49.7 ± 3.7	NS
LDL-Chol (<115 mg/dL)	107 ± 7.5	118 ± 8	<0.01	99.8 ± 7.3	NS
Fasting Glucose (70-100 mg/dL)	85.6 ± 1.3	93.4 ± 2.5	<0.01	85.7 ± 2	NS
Uric. Acid (2-7.5 mg/dL)	5.7 ± 0.3	5.1 ± 0.3	<0.01	5.2 ± 0.2	<0.05
BUN (15-40 mg/dL)	31.1 ± 1.6	31.3 ± 1.9	NS	27 ± 1.6	<0.05
Cr (0.7-1.2 mg/dL)	0.93 ± 0.04	0.93 ± 0.05	NS	0.94 ± 0.04	NS
Tot. Bil (<1.2 mg/dL)	0.56 ± 0.06	0.46 ± 0.05	NS	0.51 ± 0.07	NS
Phos. Alk (53-128 U/L)	70.4 ± 3	65.7 ± 2.4	<0.05	68 ± 3	NS
ALT (<45 U/L)	26 ± 4.8	25 ± 4.7	NS	26.8 ± 5	NS
AST (<35 U/L)	21.3 ± 1.3	20.3 ± 1.8	NS	22 ± 1.6	NS

Days of fasting	0	26 ± 0.5	NA	NA	NA
Post-fasting days	NA	0	NA	34.4 ± 2.5	NA

ALT: Alanine Transaminase; AST: Aspartate Transaminase; BUN: Blood Urea Nitrogen; Chol: Cholesterol; Cr: Creatinine; DBP: Diastolic Blood Pressure; HDL: High Density Lipoprotein; LDL: Low Density Lipoprotein; NA: Not Applicable; NS: Not Significant; Phos Alk: Phosphatase Alkaline; SBP: Systolic Blood Pressure; TG: Triglycerides; Tot Bil: Total Bilirubin.

Table 1: Subjects' characteristics in the fasting group.

Changes in biological parameters during fasting (Table 1)

Body mass index did not change during the study (p=NS, Table 1). Both systolic and diastolic blood pressure (BP) decreased when compared to the Before-fasting session, albeit significantly only during Fasting period (p<0.05). Blood glucose and LDL-cholesterol increased, while phosphatase alkaline levels decreased, during Fasting (all p<0.05 vs. Before-fasting) but returned to the Before-fasting levels thereafter. Mean serum TG and BUN did not change during the Fasting period (both p=NS, vs. Before-fasting). Uric acid was lower during the Fasting and Post-fasting periods (all p<0.05 vs. Before-fasting). None of these parameters changed over time in the control group (Table 2).

| Parameters	1st test (n=13)	2nd test (n=13)	p
Start of experiment (hours)	11:43 ± 0.76	10:51 ± 0.71	NS
BMI (Kg/m²)	26.2 ± 0.9	26.4 ± 0.9	NS
SBp (mmHg)	121.5 ± 5	126.5 ± 5	NS
DBp (mmHg)	80.4 ± 3.7	80.8 ± 3	NS
Cholesterol (<190 mg/dL)	195 ± 7.6	197 ± 6.3	NS
TG (40-150 mg/dL)	90 ± 8.5	92 ± 11.4	NS
HDL-Chol (>40 mg/dL)	57.2 ± 3	56 ± 3	NS
LDL-Chol (<115 mg/dL)	119.6 ± 6.5	122.8 ± 5	NS
Fasting Glucose (70-100 mg/dL)	94.3 ± 4.6	98.2 ± 5.3	NS
Uric. Acid (2-7.5 mg/dL)	5.6 ± 0.2	5.5 ± 0.2	NS
BUN (15-40 mg/dL)	33 ± 1.6	31.8 ± 1.8	NS
Cr (0.7-1.2 mg/dL)	1 ± 0.03	1 ± 0.03	NS
Tot. Bil (<1.2 mg/dL)	0.7 ± 0.08	0.7 ± 0.09	NS
Phos. Alk (53-128 U/L)	57.2 ± 4.4	57.2 ± 4	NS
ALT (<45 U/L)	26 ± 2.8	24.6 ± 2.5	NS
AST (<35 U/L)	22.2 ± 1.7	21 ± 1	NS
Test interval (days)	0	28 ± 1.4	NA

ALT: Alanine Transaminase, AST: Aspartate Transaminase, BUN: Blood Urea Nitrogen, Chol: Cholesterol, Cr: Creatinine, DBP: Diastolic Blood Pressure, HDL: High-Density Lipoprotein, LDL: Low Density Lipoprotein, NA: Not Applicable, NS: Not Significant, Phos Alk: Phosphatase Alkaline, SBP: Systolic Blood Pressure, TG: Triglycerides, Tot Bil: Total Bilirubin.

Table 2: Subjects' characteristics in the control group.

Effect of fasting on Ach and SNP skin mediated hyperemia (endothelial dependent and independent vasodilations) (Figure 1)

The Ach and SNP baseline skin blood flows did not differ between the Before-fasting, the Fasting and the Post-fasting measurements (all p=NS). Intermittent fasting enhanced both Ach and SNP-induced vasodilatations (both p<0.05). The Ach hyperemic skin reaction was also enhanced at the Post-fasting session (p<0.05 vs. Before-fasting), however these changes were not significant anymore for the SNP hyperemic skin reaction (Post-fasting vs. Before-fasting, p=NS). As a result, the Ach/SNP ratio did not change during the time course of the study (Before fasting: 1.2 ± 0.2 vs. Fasting: 1.1 ± 0.1; and vs. Post-fasting: 1.4 ± 0.2, all p=NS). All of these parameters did not change over time in the control group (all p=NS).
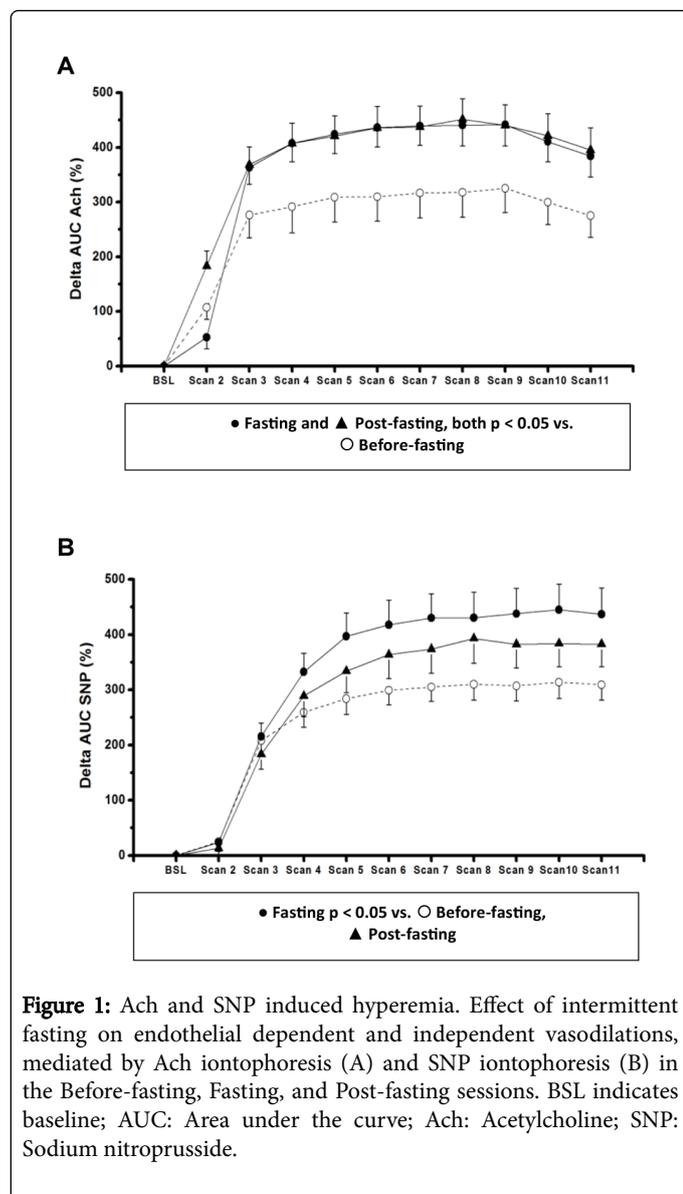
Figure 1: Ach and SNP induced hyperemia. Effect of intermittent fasting on endothelial dependent and independent vasodilations, mediated by Ach iontophoresis (A) and SNP iontophoresis (B) in the Before-fasting, Fasting, and Post-fasting sessions. BSL indicates baseline; AUC: Area under the curve; Ach: Acetylcholine; SNP: Sodium nitroprusside.
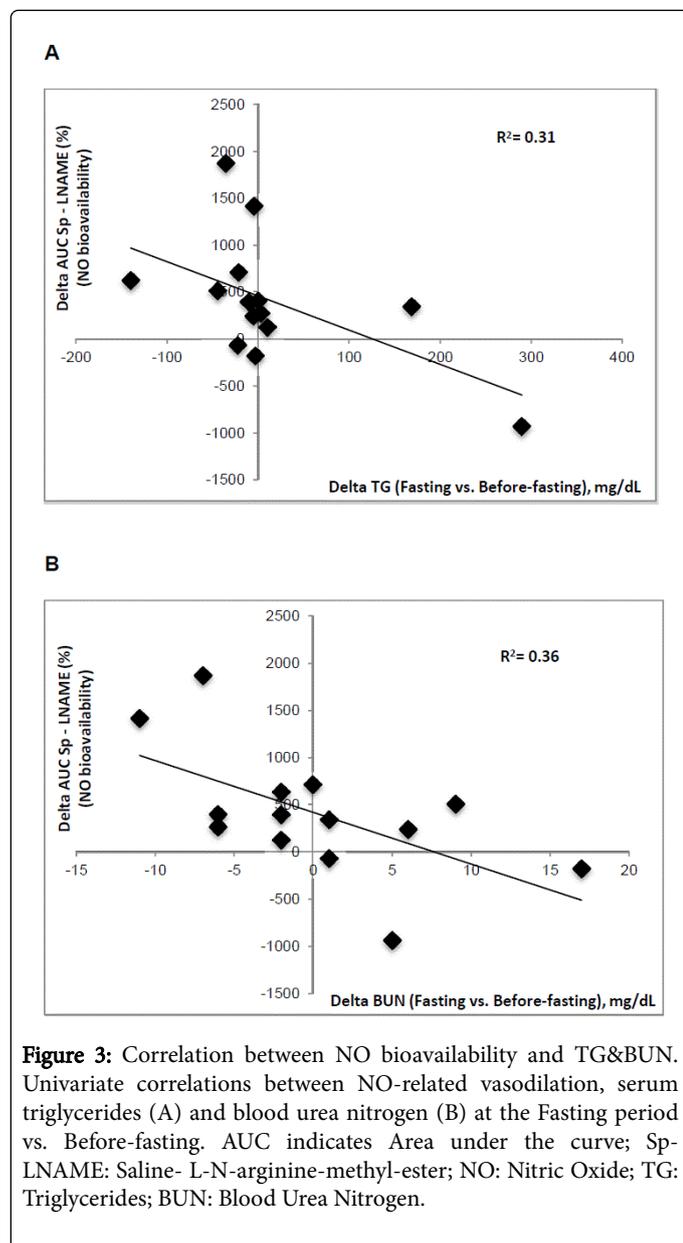
Effect of fasting on skin thermal hyperemia (endothelial NO bioavailability) (Figure 2)

Heating-mediated hyperemia responses in the absence of L-NAME were not affected in the Fasting and Post-fasting sessions, as compared to Before-fasting (p=NS). The hyperemic responses after L-NAME iontophoresis were reduced during the Fasting and Post-fasting sessions, when compared to Before-fasting (all p<0.05). As a consequence, the delta AUC between saline and L-NAME pretreated skin (Delta Sp-LNAME), which reflects the NO-related vasodilation, increased from 597.4 ± 279% to 1006.3 ± 322.3% (p<0.05, Figure 2B) during Fasting, and to 821.5 ± 281.3% during Post-fasting (P=NS vs. Before-fasting, Figure 2C).
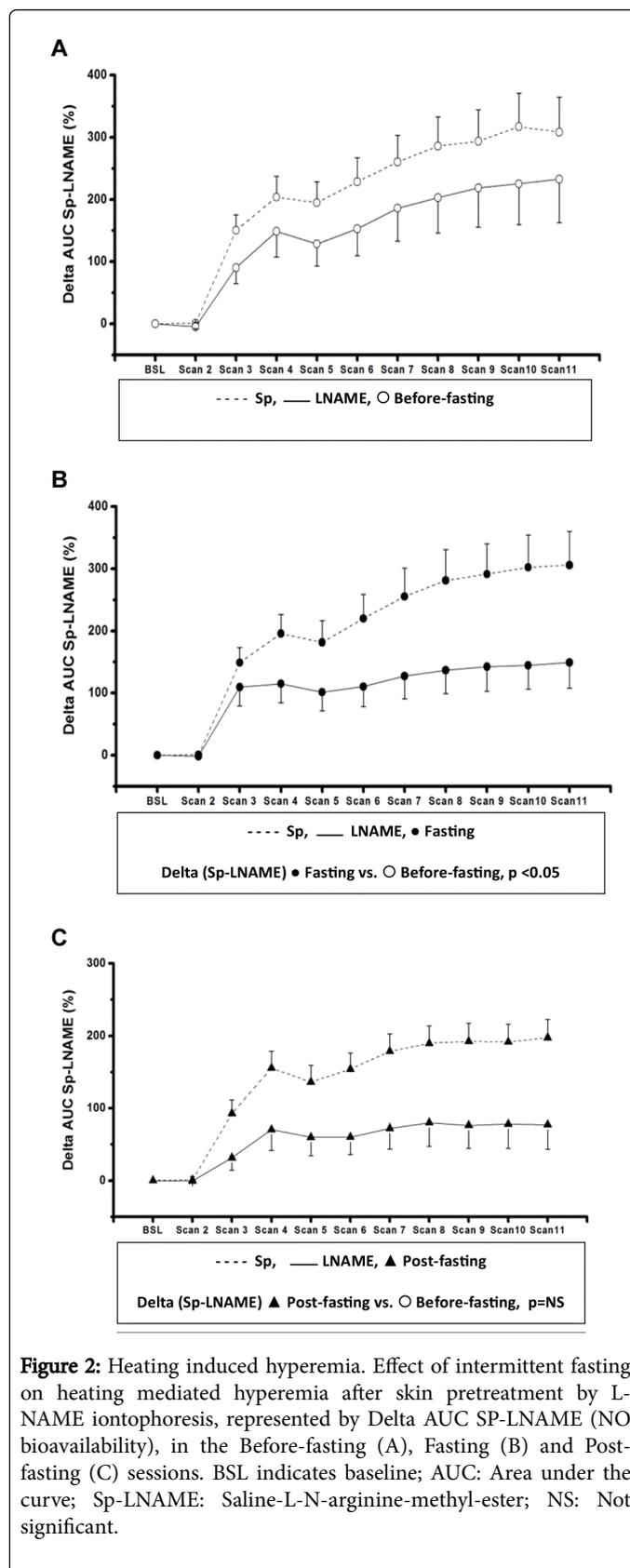
Figure 2: Heating induced hyperemia. Effect of intermittent fasting on heating mediated hyperemia after skin pretreatment by L-NAME iontophoresis, represented by Delta AUC SP-LNAME (NO bioavailability), in the Before-fasting (A), Fasting (B) and Post-fasting (C) sessions. BSL indicates baseline; AUC: Area under the curve; Sp-LNAME: Saline-L-N-arginine-methyl-ester; NS: Not significant.

These parameters did not change over time in the control group (all p=NS). Mean serum TG and BUN did not change during the Fasting period; however individual increases in their levels were adversely related to NO-related vasodilation enhancement (Figure 3A and B).

Figure 3: Correlation between NO bioavailability and TG&BUN. Univariate correlations between NO-related vasodilation, serum triglycerides (A) and blood urea nitrogen (B) at the Fasting period vs. Before-fasting. AUC indicates Area under the curve; Sp-LNAME: Saline- L-N-arginine-methyl-ester; NO: Nitric Oxide; TG: Triglycerides; BUN: Blood Urea Nitrogen.

Discussion

This study tested the hypothesis that a prolonged period of intermittent fasting improves endothelial function. The main new findings of our study are that: 1) After almost a month of 19 hours of daily fasting, both endothelial and non-endothelial microvascular functions were improved, in spite of momentary rises in blood glucose and LDL-cholesterol; 2) Increases in serum triglycerides and blood urea nitrogen hindered these favorable microvascular effects; 3) These changes were mostly apparent at the end of the fasting period, with the exception of the improved hyperemic response to Ach, which persisted 1 month after fasting cessation, while uric acid and blood urea

nitrogen were lower than Before-fasting; 4) Fasting also induced temporarily reductions in blood pressure.

This is, to the best of our knowledge, the first prospective and controlled study to assess the time dependent effects of intermittent fasting on microvascular function in humans. The study design of our study differs markedly from previous numerous uncontrolled trials on this topic, were some cardiovascular parameters and laboratory measures were often recorded before and after fasting without much standardization [19,20,34-36]. In our study, measures were carefully reiterated to determine if the effects of fasting persisted thereafter. A matched non-fasting group underwent also repeated measurements over a 1 month period, in order to rule out that non-specific mechanism, unrelated to fasting, contributed to our findings. All our subjects were male, healthy and non-smokers. Thus, changes in the menstrual cycle [28-30], as well as in the timing of medication intake and smoking, cannot explain our observations. We took also great care to ensure that the time of the day where the experiments were performed was kept constant throughout the study.

Microvascular function

We performed simultaneously several hyperemic assessments to improve our understanding of the effects of fasting on microvascular function. Since these tests elicit vasodilation through different pathways, they provide further insights on the mechanisms involved in the changes we observed [26]. After application of a local anesthetic to attenuate nonspecific neural mechanisms elicited by the iontophoresis and heating processes [33], thermal-induced skin vasodilation consists in a biphasic reaction characterized by an early peak followed by a plateau [37,38]. This late plateau is chiefly mediated by local NO generation [37,38]. The comparison of this response to the one elicited by thermal-induced skin vasodilation after L-NAME iontophoresis, revealed that fasting enhanced NO-related vasodilation. Because oxygen-free radicals or, more generally, reactive oxygen species, as well as reactive nitrogen species, are products of normal cellular metabolism, fasting-related decreased basal metabolic rate may explain our results [4,5,21]. The reduced production of superoxide anions during fasting may prevent rapid NO inactivation and thereby enhance NO bioavailability [39,40]. No changes occurred when thermal vasodilatation measures were repeated over the same time in the matched control group. This is in accordance with a previous study on the reproducibility and selectivity of thermal-induced skin vasodilation after L-NAME iontophoresis [26].

The vascular response to Ach iontophoresis involves endothelium-derived hyperpolarizing factor (EDHF), NO, and prostaglandins [38]. NO synthase inhibition with L-NAME decreases the cutaneous heating-induced vasodilation by 20% to 50%, but it reduces the Ach-induced hyperemia only by 0% to 15% [27]. This may explain the different time courses of changes in the responses to skin vasodilation in our study, which were significant for the Fasting and Post-fasting session for the less NO-dependent Ach response, but achieved significance during the Fasting only for the more NO-dependent thermal response [37,38]. There are also reasons to believe that intermittent fasting had a global favorable effect on vascular function [13,18,23,41], because fasting increased the endothelial-independent vasodilation in response to SNP. Thus not only did fasting increase NO generation, but it also enhanced smooth muscle sensitivity to a NO donor. Reduction in basal metabolic rate after fasting could also account for this latter observation [4,5,21]. Indeed, less superoxide production may result in fewer reactions with NO to generate

cytotoxic peroxynitrite [42]. This may reduce protein nitration, prevent potassium channel inhibition, lessen vascular cells hyperpolarization, and thereby improve endothelium-independent relaxation [43,44]. The fact that the relative contributions of the endothelium-dependent over the endothelium-independent vasodilation, assessed by the Ach/SNP ratio, did not change argues also in favor of a global improvement in endothelium function as a result of intermittent fasting. The Ach hyperemic skin reaction was enhanced during the Post-fasting session, when compared to the Before-fasting period. The reason for this observation is unknown. It could be speculated that the lower uric acid and BUN during the Post-fasting period indicate that some of the dietary changes during the fasting period persisted during this latter period, and played a role in this finding. Changes in uric acid levels may also have played a role in our findings throughout the study. Before fasting uric acid level of 5.7 ± 0.3 mg/dl decreased by approximately 0.5 mg/dl during the study. Excess uric acid has adverse endothelial effects, but acts also as a reducing substance [45-49]. In our study, the acid uric levels remained within the 4.5 mg/dl to 6.2 mg/dl (or 6.0 mg/dl in the NHANES III cohort [46]) range where the J curve which relates uric acid concentrations to cardiovascular disease is flat [47,48]. The human vascular smooth and endothelial cells contain urate transporters (URAT1 [SLC22A12], URATv1/GLUT9 [SLC2A9]) and are sensitive to oxidative stress changes [49]. Depletion of uric acid due to SLC22A12 (URAT1) loss-of-function mutation alters flow-mediated dilatation [49]. However, conceivably, less stringent reductions in uric acid could improve microvascular function.

Metabolic parameters and blood pressure

Mean serum TG and BUN did not change during the Fasting period; however individual variation in their levels allowed us to show that their increases were adversely related to an enhanced NO-related vasodilation. Previous studies have demonstrated that endothelial function is inversely related to glucose and TG concentrations [50-52]. Although not novel, the relationship between TG level and endothelial function we observed is worthwhile to mention as it may be regarded as internal control of scientific data quality. Less known are the endothelial effects of BUN, also inversely related to endothelial function improvements in our study. Uremic levels of BUN did not induce nitric oxide deficiency in rats with normal renal function, but did so in cultured human and bovine endothelial cells [53]. Elevated BUN may also reflect a negative effect of dehydration on endothelial function and oxidative stress in our study, since Ramadan fasting dictates that no liquids are ingested from sunrise to sunset [54,55]. This could participate to the reduction in BP we observed. Moreover, one month of intermittent fasting decreased markers of sympathetic activity in animal studies [56]. A similar change in our study would also translate into a lower BP and an improved endothelial function [57].

Changes in meal composition occur frequently during Ramadan fasting [16,58,59]. While one meal is taken after dawn and the other before sunset, they contain usually more calories and larger amounts of sweet and fatty foods. This likely explains the rises in blood glucose and LDL-cholesterol observed in our study. Albeit these changes, endothelial function was improved during Fasting. Low carbohydrate diets result in short-term weight loss and some metabolic benefits [60], but have a deleterious effect on endothelial function [61]. Regarding to current dietary recommendations, 45-60% of daily energy intake should be provided from carbohydrates [62]. The carbohydrates consumed during intermittent fasting, such as dates, honey and home-made pastry, instead of refined and processed foods and commercial high glycemic index foods, could play a role in our findings, since for vascular protection, carbohydrate quality could be more important than quantity [63].

Limitations

The sample size of our study is small and our results cannot be extrapolated to other subjects than the middle-aged non-smoker male healthy subjects investigated in our study. In mitigation, however, it should be remembered that the Ramadan fasting started and ended in all volunteers on the same day. Much larger studies, also using time-consuming measurements similar to ours, are impractical because all experimental sessions must occur during a very limited period of time. Another limitation of our study is that the Ramadan fasting performed in our study likely differs from the one performed in other regions in the world, under different latitudes, and during different seasons. Shorter or longer intermittent fasting periods may yield different result. Finally, further studies are clearly needed to better understand the mechanisms involved in the changes in endothelial function we observed.

Conclusions

Intermittent fasting improved endothelial and non-endothelial dependent vasodilations and decreased blood pressure. Increased nitric oxide bioavailability during this period was negatively related to rises in serum triglycerides and BUN.

Acknowledgments

Conflict of interest: The authors declare to have no financial or personal interests.

Authors' contributions: EF designed and conducted research; EF and VBP analyzed data; EF and VBP wrote the paper; EF had primary responsibility for final content. All authors read and approved the final manuscript.

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