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Impact of a Multi-Component Dietary Supplement on Blood Testosterone, Nitrate/Nitrite and Physical Performance in Resistance-Trained Men

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Authors' contributions

This work was carried out in collaboration between all authors. Authors LJR, JHMS and TAG were responsible for subject recruitment, data collection, data entry and assistance with manuscript preparation. Author RJB was responsible for the study design, biochemical work, data analysis, and manuscript preparation. All authors read and approved the final manuscript.

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ABSTRACT

Both D-aspartic acid (DAA) and nitrate have received considerable attention in recent years. Vitamin D_3 is also considered important for overall physical health and has been associated with elevated blood testosterone. The present study evaluated the impact of a DAA-nitrate-vitamin D_3 containing dietary supplement on anaerobic exercise performance, blood testosterone and nitrate/nitrite in men.

Methods: 24 resistance-trained men (mean age: 23 years) were assigned to ingest a DAA-nitrate-vitamin D_3 supplement or a placebo for 28 days. Exercise performance (upper body muscle power, force, and endurance; Wingate cycle sprints), in addition to blood total and free testosterone and nitrate/nitrite was measured before and after 14 and 28 days of supplementation.

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Results: No increase in total or free testosterone was noted at either measurement time (p>0.05), with values remaining stable or decreasing slightly following intake of the supplement. Nitrate/nitrite was increased significantly following intake of the supplement (p<0.05), from 19.1±2.1 μmol·L^{-1} (pre) to 70.0±12.4 μmol·L^{-1} at 14 days and 68.6±7.7 μmol·L^{-1} at 28 days. Despite this increase in nitrate/nitrite, no performance variable was impacted in a statistically significant manner by supplementation (p>0.05). However, the cumulative number of repetitions performed during a five-set bench press challenge was 11.3% higher after 28 days of supplementation, as compared to 3.6% higher for placebo.
Conclusion: Twenty-eight days of treatment with a DAA-nitrate-vitamin D_3 supplement increases blood nitrate/nitrite and can moderately improve repetitive bench press performance. However, this supplement does not result in an increase in total or free testosterone or any other performance measure.

Keywords: D-aspartic acid; nitrate; vitamin D_3; dietary supplements; exercise; ergogenic aid.

1. INTRODUCTION

Dietary supplements aimed at improving physical performance are of great interest to athletes and coaches alike [1-3]. This is partly fueled by the recent scrutiny surrounding performance enhancing drugs within athletics [4], such as anabolic steroids, growth hormone, and amphetamine-like compounds. Certain isolated dietary ingredients have been reported to provide a benefit in terms of improving physical performance, with the focus centered on those ingredients thought to promote gains in muscle strength, as well as cardio respiratory endurance [5].

In terms of muscle mass and strength, ingredients targeting heightened testosterone are of main interest. While many ingredients are touted as testosterone precursors, few actually result in a measureable increase in this hormone in a reliable and repeatable manner. One such ingredient that has received a great deal of attention recently is D-aspartic acid (DAA)—an endogenous amino acid that has been reported to increase blood testosterone concentrations by 42% in young (27-37 year old), healthy men following daily supplementation at 3120 mg over the course of a 12 day period [6]. More recently, Willoughby and Leutholtz [7] reported conflicting findings to those of Topo and colleagues. In this study, young men (mean age of 22.8 years) ingested either a placebo or 3 grams of DAA for 28 days, in conjunction with a resistance training program. No increase in total or free testosterone was noted, with values remaining near identical to pre-supplementation. As expected due to the resistance training program, muscle strength was increased from pre to post intervention in both groups; however, it was not differently impacted by DAA supplementation. Aside from the above work, we are unaware of any human studies using DAA, despite multiple animal studies demonstrating increases in testosterone [6] and improvements in reproductive health [8-9]. The increase in testosterone may have implications for improving muscle strength and overall physical performance, as the link between testosterone and muscle health and performance is very strong [10-12].

Beyond DAA supplementation, recent work has indicated a link between vitamin D_3 and testosterone levels [13-14]. For example, the Pilz and coworkers [13] reported that 31 healthy, overweight men who supplemented with 3332 IU of vitamin D daily for one year were noted as having a significant increase in total, bioactive, and free testosterone levels. Men assigned to a placebo were noted as having no measureable increase in these variables following the same one year intervention. While this finding requires confirmation through additional research, coupled with the recent interest in vitamin D3 as an overall health-enhancing nutrient [15], supplementation with this vitamin is commonplace and continues to gain momentum.

In addition to the focus on serum testosterone and associated physical performance outcomes, there has been increasing attention given to dietary nitrate and its role in aiding cardiorespiratory and muscular endurance [16]. Specifically, investigators have used either sodium nitrate (~500 mg) or beetroot juice (containing nitrate at a dosage of approximately 500 mg) as an ergogenic aid, reporting improvements in exercise performance [17] Jones 2014), in addition to a lowering in blood pressure [18]. These effects may be mediated by increased nitric oxide (NO), which acts as a vasodilator to open vessels and allow for greater

blood flow. Theoretically, this enhanced blood flow may allow for greater oxygen and nutrient delivery to active tissues (i.e., skeletal muscle), leading to enhanced physical performance.

Considering the available evidence, it is possible that the combination of DAA, vitamin D_3, and nitrate may allow for improved muscular performance. This may be mediated by the increase in circulating testosterone and NO. The purpose of the present study was to determine the impact of a DAA-nitrate-vitamin D_3 containing dietary supplement on physical performance, blood testosterone and blood nitrate/nitrite (the metabolites of nitric oxide) in healthy, resistance-trained men.

2. MATERIALS AND METHODS

2.1 Subjects

A total of 24 healthy and recreationally active men completed this study (age range: 19-38 years). The sample size is similar to many other investigations involving dietary supplements. All subjects had a history of performing resistance exercise (i.e. weight training) for a minimum of the past 6 months. This was to provide adequate adaptations to the skeletal, muscular, and neuromuscular systems, all of which were taxed when performing the exercise test battery as a component of this study. Men were recruited to participate via informal word of mouth conversations, email communications, and recruitment flyers posted on campus. All subject recruitment was performed by the investigators. Subjects were not current smokers and were considered to be in good overall health, without a history of cardiovascular, neurological, or metabolic disorders. Subjects were not using hormonal replacement therapy or dietary supplements aimed at enhancing hormone production (such as growth hormone or testosterone precursors); nor were subjects using dietary supplements containing nitrate or isolated high dose vitamin D_3. Subject characteristics are presented in Table 1. Health history, medication and dietary supplement usage, and physical activity questionnaires were completed by all subjects and reviewed by an investigator to determine eligibility. Subjects were informed of all procedures, potential risks, and benefits associated with the study through both verbal and written form. The study procedures were approved by the University Institutional Review Board (IRB) for Human Subjects Research. Subjects who completed the study were compensated $100 for their time.

2.2 Testing

During the initial lab visit subjects completed all paperwork, including the informed consent. Subjects' heart rate and blood pressure, height, weight, waist, and hip circumference were measured. Subjects were provided with food logs and instructions and examples regarding how to complete these logs during the two days before each test day. Subjects also completed a 1-repetition maximum (1RM) test in the bench press exercise and completed a familiarization trial for all performance tests.

Within a few days of this familiarization trial, subjects reported back to the lab to practice all performance tests one additional time—using the exact procedures as described below for the performance tests. This was necessary to eliminate any learning effect.

Table 1. Characteristics of men assigned to placebo or supplement

Variable	Placebo (n=12)	Supplement (n=12)
Age (years)	24.8±1.5	22.5±0.6
Height (cm)	180.6±1.3	179.0±1.8
Body weight (kg)	82.4±2.1	81.5±2.4
Body mass index ($kg \cdot m^{-2}$)	25.3±0.8	25.4±0.8
Waist circumference (cm)	84.5±1.6	85.2±1.6
Hip circumference (cm)	98.5±1.2	98.6±1.9
Waist:Hip	0.86±0.01	0.90±0.01
Weekly aerobic training (hrs)	2.8±0.7	1.8±0.4
Aerobic training history (yrs)	5.0±1.1	5.6±1.2
Weekly anaerobic training (hrs)	4.7±0.4	3.8±0.5
Anaerobic training history (yrs)	6.3±0.7	6.0±0.8
1-Repetition maximum (kg)	153.0±5.4	136.1±6.4

Values are mean±SEM. No differences of statistical significance noted (p>0.05)

Subjects then returned to the lab on three occasions (before supplementation, after 14 days of supplementation, and after 28 days of supplementation) to complete the assessments indicated below. On each occasion subjects reported to the lab following a minimum 10 hour overnight fast. Upon arrival:

1. Subjects rested quietly for 20 minutes while seated in a chair.
2. Subjects completed a questionnaire related to their perceived level of energy and associated variables.
3. Resting heart rate (via palpation of the radial artery) and blood pressure (via auscultation) was measured.
4. A blood sample was taken and processed as described below.
5. Subjects were provided with a standard liquid meal consisting of orange juice (8 ounces) and protein powder (approximately 20 grams), proving approximately 225 kcal. Other than the standard liquid meal, no food or calorie containing beverages were allowed during this time. However, subjects were allowed to consume as much water as they desired.
6. Subjects begin warming up for the exercise performance tests and begin testing approximately 20 minutes following the consumption of the liquid meal.
7. Performance testing began in the order listed below, with three minutes of rest allowed between each different performance test. The same procedures were followed for each of the three test days (pre, post 14 days, post 28 days) and the time of day for each subject was matched for all visits (6:00-9:00 am).

    a. Bench press throws to assess upper body power: Using a weight equivalent to 30% of predetermined 1RM using a ProSpot® device. This apparatus utilizes a self spotting mechanism that contains an electronic sensor which allows the computerized system to "catch" the barbell when thrown. We have used this same device in many past research studies. Three attempts were provided, with one minute of rest between attempts. The best attempt was used for data analysis.
    b. IIsometric bench press to assess muscular force: Using a standard bench fixed atop a force plate, subjects were asked to exert as much force as possible for a period of 3-4 seconds against the barbell, while their upper and lower arm angle was fixed at 90º. Three attempts were provided, with one minute of rest between attempts. The best attempt was used for data analysis.
    c. MMuscular endurance in the bench press exercise: Subjects performed 5 sets using a load equal to 50% of 1RM. Each set was performed using a full range of motion on a Hammer Strength® flat bench press apparatus. Each set was carried to a point of momentary muscular failure, using a controlled speed. Subjects were given 120 seconds of rest between each set. Rating of perceived exertion (RPE: using the standard 6-20 scale) was assessed at the end of each set.
    d. MMuscular power and endurance in cycle sprinting: Subjects perform a total of three, 30 second Wingate cycle sprints using a computer-integrated Monark anaerobic testing cycle ergometer. Subjects were given 180 seconds of rest between each sprint. RPE was assessed at the end of each sprint.

2.3 Supplementation

Following the above assessments on day 1, subjects were evenly assigned to one of two conditions, in double-blind manner: Placebo (cellulose) or Supplement (D-Pol™, Purus Labs, Dallas, TX; containing DAA at a dosage of 3120 mg; sodium nitrate at a dosage of 480 mg; vitamin D3 at a dosage of 4000IU). Both the placebo and supplement tablets were very similar in appearance and were provided to subjects in unlabeled bottles. Subjects were instructed to consume a total of three tablets of their assigned condition each day with a meal, 60 minutes prior to exercise. If no exercise was to be performed that day, subjects were instructed to take their three tablets with their breakfast meal. All tablets were produced under standard Good Manufacturing Practices by a dietary supplement contract manufacturer. The blinding code was retained by the study sponsor until all data collection was completed. Subjects returned capsule bottles to investigators so that capsule counts could be used to determine compliance to intake. Compliance to intake was not different between placebo (95.5±2.0%) and supplement (98.2±0.8%) (p=0.22).

2.4 Blood Collection and Analysis

Venous blood samples (~15 mL) were taken from subjects via needle and Vacutainer®. Blood samples were collected before starting supplementation (Pre) and after 14 and 28 days of supplementation. Following collection, samples were processed accordingly and the plasma and serum was stored at -70ºC until analyzed within three months of collection. Samples for analysis of total testosterone (catalogue: TE187S), free testosterone (catalogue: FT178S), and estradiol (catalogue: ES180S) were analyzed in serum using enzyme linked immunosorbent assay (ELISA) procedures according to the instructions provided by the manufacturer (Calbiotech, Inc. Spring Valley, CA). Nitrate/nitrite was analyzed in plasma using a commercially available colorimetric assay kit (catalogue: 780001) according to the procedures provided by the manufacturer (Caymen Chemical, Ann Arbor, MI). Prior to analysis, all sampled were mixed thoroughly following thawing. All samples were analyzed in duplicate and on first thaw. The coefficient of variation for the assays was as follows: Total testosterone 5.4%, free testosterone 4.8%; estradiol 7.9%; nitrate/nitrite 10.3%.

2.5 Questionnaire

Due to the potential influence of circulating testosterone and NO on health-specific feelings, a questionnaire was completed by subjects on the morning of each test day. Using a scale of 1-5, with 1 representing the lowest rating and 5 representing the highest rating, subjects rated a number of variables pertaining to overall health with regards to how they felt over the past two weeks.

2.6 Dietary Intake and Physical Activity

All subjects were instructed to consume their usual diet and to record dietary intake during the two days prior to each day of testing. Subjects were asked to mimic this intake during the days preceding each test day. Dietary records were analyzed using nutritional software (Food Processor Pro; ESHA Research, Salem, OR). Subjects were instructed not to consume alcohol or caffeinated beverages (such as “energy drinks”, coffee, tea, or soda) or dietary supplements containing caffeine or other stimulants, during the 48 hours prior to each test day. Subjects were asked to refrain from strenuous physical activity for the 48 hours prior to each test day.

2.7 Statistical Analysis

Data were analyzed using a 2 (condition) by 3 (day) analysis of variance (ANOVA). Tukey post-hoc testing was used as needed. The data are presented as mean ± SEM. All analyses were performed using JMP statistical software. Statistical significance was set at $p \leq 0.05$.

3. RESULTS

All 24 subjects successfully completed the four-week study. The following protocol deviations were noted. Placebo condition: The day 14 visit occurred on day 15 for one subject and on day 17 for another, due to scheduling conflicts; two subjects completed the study after 27 days of treatment rather than after 28 days of treatment, due to scheduling conflicts. Supplement condition: The day 14 visit occurred on day 18 for one subject, due to scheduling conflicts.

The following data are missing from the analysis, primarily due to a lack of obtaining the data from the subject. Placebo condition: Sprint 1 at the pre visit for one subject; sprint 2 and 3 at the 28 day visit for one subject; sprint 2 at the 14 day visit for one subject; questionnaire data at the pre visit for one subject. Supplement condition: no performance, heart rate/blood pressure, or questionnaire data for day 28 for one subject; power at the day 28 visit for one subject; sprint 2 and 3 at the pre visit for one subject; blood at day 4 visit for one subject; nitrate/nitrite at day 28 visit for two subjects.

Subject characteristics were not different between conditions ($p>0.05$), as can be viewed in Table 1. Compliance to capsules was excellent for subjects in both conditions (placebo: 95.5±2.0; supplement: 98.2±0.8). Aside from a higher vitamin C intake in the placebo group ($p=0.02$), dietary intake was not different between subjects or across time ($p>0.05$; Table 2), nor was resting heart rate or blood pressure ($p>0.05$; Table 3). Although vitamin C was close to twice as high with treatment as compared to before treatment began, values were still below the RDA (90 mg/day) and the difference across time was not of statistical significance ($p>0.05$). No interaction effect was noted ($p>0.05$) for subjects’ perceived feelings associated with use of the placebo and supplement (Table 4). However, a condition effect was noted for Muscle Strength ($p=0.05$) and Physical Appearance ($p=0.01$), with values higher for supplement compared to placebo. In addition, a day effect

was noted for Muscle Endurance (p=0.04) and Sleep Quality (p=0.005), with day 28 higher than Pre.

Blood testosterone, estradiol, and nitrate/nitrite data are presented in Table 5. No effects were noted for total or free testosterone, or for estradiol (p>0.05). An interaction effect was noted for blood nitrate/nitrite (p=0.008), with values higher for supplement at day 14 and day 28 as compared to Pre. A day effect was also noted (p=0.02), with values higher at day 14 compared to Pre. No other effects of statistical significance were noted (p>0.05).

Table 2. Dietary data of men assigned to placebo or supplement

Variable	Placebo pre	Placebo day 14	Placebo day 28	Supplement pre	Supplement day 14	Supplement day 28
Kilocalories	2554±239	2386±202	2519±330	2624±214	246 4±231	2519±227
Protein(grams)	153±18	142±18	129±12	123±14	120±15	10 8±13
Carbohydrate (grams)	261±30	270±26	270±34	294±37	259±32	266±36
Fat (grams)	108±12	83±10	105±21	110±14	87±7	106±19
Vitamin C* (mg)	96±19	98±26	86±16	32±8	64±20	71±21
Vitamin E (mg)	4±1	6±2	5±2	3±1	4±1	5±2
Vitamin A (RE)	6944±3363	5440±2165	4228±845	4478±1624	5485±2198	7786±3068

*Data are mean±SEM. * Condition effect for vitamin C (p=0.02). No other differences of Statistical significance noted (p>0.05).*
Note: Values are for the 48-hour period immediately preceding each test day

Table 3. Resting heart rate and blood pressure of men assigned to placebo or supplement

Variable	Placebo pre	Placebo day 14	Placebo day 28	Supplement pre	Supplement day 14	Supplement day 28
HR (bpm)	60.9±2.9	62.2±2.6	61.1±2.8	65.6±2.0	65.3±2 .3	65.1±3.0
SBP (mm Hg)	113.0±2.2	107.8±3.1	110.2±2.6	108.8±2.8	109.1±3.2	1 12.6±2.3
DBP (mm Hg)	70.3±1.2	70.5±3.6	67.9±2.7	66.5±2.5	67.6±2.2	71.2±2 .4

Data are mean±SEM. No differences of statistical significance noted (p>0.05).
Note: HR=Heart Rate; SBP = Systolic Blood Pressure; DBP = Diastolic Blood Pressure

Table 4. Perceived feelings of men assigned to placebo or supplement

Variable	Placebo pre	Placebo day 14	Placebo day 28	Supplement pre	Supplement day 14	Supplement day 28
Alertness	3.4±0.2	3.5±0.3	3.8±0.1	3.8±0.2	3.8±0.2	3 .8±0.2
Energy levels	3.4±0.2	3.6±0.2	3.7±0.1	3.6±0.3	3.8±0 .1	3.8±0.2
Vitality	3.5±0.2	3.6±0.3	3.9±0.2	3.8±0.2	3.8±0.1	4. 0±0.2
Libido	3.6±0.2	3.8±0.3	4.2±0.2	3.8±0.3	3.9±0.2	4.2± 0.2
Workout effectiveness	3.9±0.3	3.8±0.1	3.9±0.1	3.7±0. 3	4.3±0.3	4.1±0.2
Muscle strength*	3.5±0.3	3.7±0.1	3.7±0.1	3.5±0.2	4.2± 0.2	4.1±0.2
Muscle endurance**	3.5±0.2	3.8±0.1	3.8±0.2	3.4±0.2	4. 0±0.2	4.0±0.2
Sleep quality**	3.1±0.3	3.2±0.2	3.4±0.3	2.8±0.2	3.7 ±0.2	4.0±0.2
Mental outlook & mood	4.2±0.3	3.8±0.2	3.8±0.2	3.9±0 .2	3.9±0.2	4.3±0.1
Physical appearance *	3.6±0.2	3.7±0.2	3.7±0.1	3.8±0. 2	4.1±0.2	4.1±0.2

*Data are mean±SEM. * Condition effect for Muscle Strength (p=0.05) and Physical Appearance (p=0.01).*
*** Day effect for Muscle Endurance (p=0.04) and Sleep Quality (p=0.005); Day 28 > Pre. No other differences of statistical significance noted (p>0.05)*
Note: Using a rating of 1-5, with 1 representing the lowest rating and 5 representing the highest rating, subjects were asked to rate each variable in the above table, with consideration of how they felt during the prior two weeks

Performance data are presented in Table 6 (bench press assessments) and Table 7 (Wingate cycle tests). No differences of statistical significance were noted for any variable (p>005). The cumulative number of repetitions performed during the five-set bench press test was 11.3% higher at day 28 of supplementation as compared to Pre, while values for the placebo condition increased 3.6% during the same time period.

4. DISCUSSION

Findings from the present study indicate that daily ingestion of a DAA-nitrate-vitamin D_3 supplement for 28 days results in an approximate 3.5-fold increase in blood nitrate/nitrite and can moderately improve repeated bench press performance. This supplement does not, however, result in an increase in total or free testosterone, nor does it result in an improvement in other physical performance measures. These findings are in reference to a relatively small sample of young, healthy, exercise-trained men with moderate to high basal testosterone values. Further study of this supplement in middle-aged to older men with low-normal testosterone values is needed, possibly inclusive of a larger sample size.

Prior work involving DAA supplementation in human subjects has yielded mixed findings with regards to increasing circulating testosterone. For example, Topo and colleagues noted an approximate 42% increase in total testosterone following just 12 days of DAA supplementation in young (aged 27-37 years) and healthy men [6]. Interestingly, the finding of increased testosterone was observed in 20 out of 23 subjects. To the contrary, Willoughby and Leutholtz [7] recently reported that the same dosage of DAA (~3 grams) for a 28 day period failed to yield an increase in total or free testosterone in young (aged 18-23 years) and healthy men. It should be noted that DAA treatment was compared to a placebo and provided in conjunction with a resistance training program. These results of Willoughby and Leutholtz parallel those of the present investigation and suggest that DAA does not increase serum total or free testosterone in young and healthy men. With only three published studies to date to evaluate the impact of DAA supplementation to stimulate testosterone production, more work is needed in this area in order to more fully elucidate the role of DAA to stimulate testosterone production in otherwise healthy men with normal testosterone values.

In prior literature, DAA has been reported to increase the synthesis and release of testosterone in animals [6,19-20]. In a study using adult male rats, three groups of 10 animals each were treated for 12 days with a solution containing 20 mM of DAA. After 12 days of treatment, testosterone increased significantly, from a baseline mean value of 5.1±0.8 (ng/ml) to 10.4±1.2 (ng/ml), reported as mean ± SEM [6]. In a study by D'Aniello et al. [19], injections were administered to adult male rats and blood samples were collected at five hours post injection.

Testosterone and luteinizing hormone increased from baseline 3-fold and 1.6-fold, respectively. It has also been demonstrated that DAA acts to stimulate N-Methyl-D-aspartic acid (NMDA) synthesis, leading to a small hormonal release from the hypothalamus [21].

Table 5. Biochemical data of men assigned to placebo or supplement

Variable	Placebo pre	Placebo day 14	Placebo day 28	Supplement pre	Supplement day 14	Supplement day 28
Testosterone Total ($ng \cdot mL^{-1}$)	5.3±1.2	5.3±1.1	5.3±1.2	6.2±2.2	5.5±1.3	5.1±1.3
Testosterone Free ($pg \cdot mL^{-1}$)	10.6±1.7	10.6±1.7	11.2±2.0	11.4±1.8	10.2±0.9	9.7±0.9
Estradiol ($pg \cdot mL^{-1}$)	14.7±5.1	15.4±4.8	14.4±5.0	29.0±14.6	26.0±10.6	22.5±8.2
Nitrate/Nitrite† ($\mu mol \cdot L^{-1}$)	40.4±12.0	39.9±11.5	34.2±7.6	19.1±2.1	70.0±12.4	68.6±7.7

Data are mean±SEM. † Interaction effect (p=0.008); Day 14 and Day 28 > Pre for Supplement. No other differences of statistical significance noted (p>0.05)

Although the above findings in animals, coupled with the findings obtained from human subjects by Topo and colleagues [6] indicate a beneficial effect of DAA treatment on testosterone, our findings and those of Willoughby and Leutholtz [7] are in disagreement. We believe that the age and basal testosterone values of subjects may be responsible for these discrepancies. For example, our sample consisted of a group of young men between the ages of 18-39 years whose total testosterone levels were on average, in the mid-range of "normal" (6.2 $ng \cdot mL^{-1}$); considering the normal range of ~ 3-9 $ng \cdot mL^{-1}$ [22]. Subjects in the study of Willoughby and Leutholtz [7] were also young (18-23 years) and presented with a mean basal total testosterone value of 8.1 $ng \cdot mL^{-1}$. In comparison, subjects in the work of Topo and coworkers ranged in age from 27 to 37 years and had a mean basal testosterone value of 4.5 $ng \cdot mL^{-1}$. The significantly lower mean basal testosterone value of subjects in the Topo et al. study could have been responsible for the differing findings for testosterone elevation with DAA treatment. In support of this, we have recently noted that when middle-aged men (mean age: 41 years) with low basal testosterone values (e.g., 3 $ng \cdot mL^{-1}$) use DAA at the same dosage as provided in the present study, serum testosterone concentrations increase following treatment (unpublished findings). The collective findings suggest that DAA may be more beneficial for middle-aged men, in particular those with depressed basal levels of testosterone. More work is needed to confirm this hypotheses.

Aside from the focus on testosterone, it has been reported in animal studies that DAA may lead to increased estradiol—a result of increased aromatase activity and the conversion of testosterone to estradiol [23-25]. To our knowledge, at the time of designing the present investigation, this finding had only been observed using animal models. Since that time, a lack of estradiol increase following DAA supplementation has been reported by Willoughby and Leutholtz [7]. Our findings agree with those of the aforementioned authors, noting no increase in estradiol with DAA supplementation for a period of 28 days. In fact, in both our study and the study of Willoughby and Leutholtz, values decreased slightly over the course of treatment. It should be noted that two of the 12 subjects in the present study who were assigned to the DAA condition had very high levels of estradiol at baseline, influencing the group mean. The fact that estradiol was lower following treatment with DAA eliminates the possibility that DAA initially promoted an increase in testosterone, only to have this increase abolished by a rapid conversion toward estradiol. Our findings coupled with those of Willoughby and Leutholtz indicated that DAA has no impact on either testosterone or estradiol in a sample of young and healthy men, with mid- to high-normal basal testosterone levels.

Aside from DAA and its potential influence on testosterone, the supplement contained sodium nitrate, which resulted in an approximate 3.5-fold increase in plasma nitrate/nitrite—a surrogate marker of NO levels. In relation to sport nutrition, NO plays a role in regulation of vasodilation, blood flow, and mitochondrial oxidative phosphorylation [26]. Prior studies involving nitrate ingestion, typically delivered either as sodium nitrate or as beetroot juice, have reported an increase in circulating nitrite levels, with most studies reporting an improvement in certain aspects of physical performance [16]. That said, the majority of work involving nitrate supplementation has focused on aerobic exercise and not resistance exercise. In the present study, we noted an improvement in repetitive bench press exercise with the supplement (11.3%) as compared to placebo (3.6%); however no other improvements were noted in physical performance (Tables 6 and 7). It is possible that nitrate ingestion and the resulting increase in circulating NO metabolites (nitrate/nitrite) may have more application to aerobic exercise conditions as opposed to those involving anaerobic work—as used in the present study. This is supported by the recent work of Hoon and colleagues [27], who failed to note an improvement in high intensity cycling performance following nitrate ingestion. Additional studies involving nitrate supplementation and resistance exercise are needed to more fully understand the potential impact of nitrate as an ergogenic aid.

Although not a primary outcome measure in the present study, we noted a small improvement in the subjective feeling of muscle strength in subjects assigned to the supplement (Table 4). However, it is unknown which component of the supplement (DAA, vitamin D_3, or sodium nitrate) was responsible for this enhanced feeling of muscle strength. Additional work involving supplementation of each ingredient alone would be needed to confirm the independent role of each on subjective feelings of muscle strength and related variables.

Table 6. Upper body bench press performance data of men assigned to placebo or supplement

Variable	Placebo pre	Placebo day 14	Placebo day 28	Supplement pre	Supplement day 14	Supplement day 28
Power (W)	1213.5±104.8	1221.7±103.8	1095.1±60.6	1237.9±111.7	1197.2±83.3	1114.1±99.6
Force (N)*	1275.4±71.7	1337.1±91.6	1182.3±51.7	1134.8±40.7	1179.7±63.9	1120.7±83.0
Reps: Set 1	23.0±1.0	23.6±1.0	24.0±1.0	21.5±1.0	23.1±1.1	22.5±1.2
Total Reps (sets 1-5)	63.3±5.1	63.8±4.2	65.6±3.9	59.4±3.1	62.6±2.6	66.2±2.3
Total Volume Load (kg)*	8331.8±820.8	8373.2±698.7	8614.8±676.8	6613.9±468.1	7049.5±542.3	7362.1±553.1
RPE	14.4±1.1	14.9±1.1	14.5±1.2	16.1±0.7	16.3±0.8	16.2±0.8

*Data are mean±SEM. * Condition effect for Force (p=0.004) and Total Volume Load (p=0.004). No other differences of statistical significance noted (p>0.05).*
Note: RPE is the mean of all 5 sets of bench press exercise

Table 7. Wingate performance data of men assigned to placebo or supplement

Variable	Placebo pre	Placebo day 14	Placebo day 28	Supplement pre	Supplement day 14	Supplement day 28
Sprint 1						
Peak Power (W)	822.7±34.1	776.1±34.7	763.4±24.2	787.1±48.3	772.1±59.9	764.5±58.1
Mean Power (W)	633.7±17.9	612.3±25.4	595.0±19.7	595.5±20.5	578.5±24.8	564.4±26.5
Total Work (J)	19011.8±536.5	18368.1±761.0	17849.0±591.9	17865.3±614.0	17353.7±743.2	16932.7±796.4
RPE	16.5±0.9	16.7±0.8	16.4±0.9	16.3±0.8	16.3±0.7	16.5±0.7
Sprint 2						
Peak Power (W)	726.6±33.5	716.4±36.9	649.4±40.3	662.0±27.4	729.2±57.2	635.9±35.8
Mean Power (W)	528.0±18.7	535.9±21.0	496.6±28.4	514.2±22.2	517.1±22.5	483.0±20.6
Total Work (J)	15839.7±560.6	16077.1±629.1	14899.1±852.3	15426.2±665.2	15512.2±674.9	14489.2±619.0
RPE	18.0±0.6	18.2±0.6	17.6±0.8	17.6±0.6	17.8±0.6	17.8±0.5
Sprint 3						
Peak Power (W)	642.6±37.0	608.1±30.4	638.4±76.7	602.5±39.6	616.2±26.4	599.8±35.4
Mean Power (W)	470.8±23.9	455.2±21.9	466.5±43.2	449.3±28.7	452.7±16.5	445.1±23.8
Total Work (J)	14125.0±717.1	13655.9±657.2	13993.9±1296.0	13479.9±860.5	13579.8±494.0	13352.0±713.3
RPE	18.9±0.6	18.9±0.4	18.8±0.6	18.7±0.4	19.0±0.4	18.4±0.5
Mean of Sprints 1-3						
Peak Power (W)	730.8±27.7	705.3±31.6	681.8±37.7	681.3±31.1	705.8±42.2	666.8±32.9
Mean Power (W)	544.3±16.6	540.2±19.4	519.4±24.1	521.1±21.5	516.1±19.3	497.5±19.9
Total Work (J)	16328.9±496.9	16206.1±582.1	15581.5±724.0	15631.9±643.8	15481.9±579.1	14924.7±597.7
RPE	17.8±0.7	18.1±0.5	17.5±0.8	17.5±0.6	17.7±0.5	17.6±0.5

Data are mean±SEM. No differences of statistical significance noted (p>0.05)

5. CONCLUSION

We report that twenty-eight days of treatment with a DAA-nitrate-vitamin D_3 supplement increases blood nitrate/nitrite and results in a modest improvement in repetitive bench press performance. The supplement does not increase circulating free or total testosterone values, likely due to the fact that subjects were young and had basal testosterone within the mid to high range of normal. Differences in basal concentrations of testosterone, estradiol, and nitrate/nitrite between the supplement and placebo condition may have confounded the outcomes and this should be considered. Future work involving middle-aged and older men (and perhaps women), in particular those with low-normal testosterone values, is needed to further our knowledge of DAA treatment for purposes of elevating circulating testosterone.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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