Coconut oil and enriched environment: effects on memory in nourished and overnourished rats

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Abstract

Background: Postnatal overnutrition has been associated with lasting impairment of cognitive function. On the other hand, virgin coconut oil (CO) and environmental enrichment (EE) have been associated with the improvement of memory function. Objectives: To evaluate whether CO and EE could counteract any possible memory impairment caused by overnutrition during rats’ postnatal period. Methods: Rats were suckled in litters of either 9 (N, nourished) or 3 (ON, overnourished) pups. At 7 to 30 days of life, N and ON animals were treated with CO (10 ml/kg/d) or vehicle (V). At 36 days old, rats were exposed to EE during a 4-week period. Recognition memory was investigated in young and adult groups to evaluate rats’ memory after CO supplementation period and after exposure to an EE, respectively. Lastly, murinometric evaluation and blood collection were performed. Results: ON young rats displayed a treatment-dependent impairment of memory (p < 0.001). Additionally, CO coupled with overnutrition had age-dependent effects on memory. At adulthood, CO&ON rats were not able to recognize novel object placement (novel versus familiar, p > 0.05); however, CO&ON rats that were exposed to EE performed this task successfully (p < 0.0001). Conclusions: The etiology of diseases characterized by memory impairment can date back to lactation. Nutrition during this critical period can influence cognitive function. Early-life overnutrition, coupled with CO, has age-dependent effects on recognition memory. Our data suggests EE can rescue the memory impairment found in CO&ON adult rats.

1. Introduction

Memory function is crucial to daily life and encompasses several abilities that enable information to be stored, accessed, and retrieved. Its impairment can be associated with age-cognitive decline and functional impairment among young adults (1). Due to this, there is growing interest in better understanding the potential use of natural enhancers and its application in the episodic memory, in particular, enables the use of contextual information regarding distinct episodes from the personal past to guide behavior (2). The rats’ capability to recognize object characteristics depends on the preservation of aspects related to the episodic memory function. For example, different memory processes, such as acquisition, consolidation, and retrieval, can be investigated through tools to study memory, like the task of object recognition (3). Nowadays, some strategies have been proposed to improve memory, such as coconut oil intake and environmental enrichment. There is growing interest in understanding how these approaches may benefit brain function, as well as the possibility of counteracting either the incidence or the complications of memory disorders.

Virgin coconut oil (CO) was declared as a potential cognitive strengthener (4). Assessment of cognitive and memory-enhancing effects of CO is very important, since this oil is accessible in many regions worldwide and demonstrates less side effects when compared to other vegetable oils (5). Besides CO putative effects on memory, it has also been recognized as a functional oil, that can optimize antioxidant levels, enhance cholinergic activity, and reduce oxidative stress in rodents (6). The objective of this study was to evaluate whether CO supplementation early in life could improve memory immediately after chronic treatment and...
if this possible effect would last until adult life. If so, early life CO supplementation could represent an interesting strategy to improve memory during the whole life, as well as could counteract the cognitive age-imposed decline.

In human development, a critical window exists from conception to the second anniversary. This phase is referred to as ‘the 1,000 days period’. In rats, this similar phase comprehends mainly the weaning period, the three first weeks after birth. During this critical window, epigenetic DNA imprinting activity is considered the most active. In this way, nutrition can play a key role in developmental programming during this period of life (7). Early dietary intake can persistently influence brain functions and affect the way aging-related cognitive decline occurs (8). Another strategy related to memory improvement is the environmental enrichment (EE). Data demonstrate that EE can lead to permanent or at least long-lasting changes in behavior (9). Rats that were housed with EE presented improved memory and spatial learning (10). Beneficial effects of EE are well documented in brain areas involved with memory and cognition, including the hippocampus and cortex (11; 12). On the other hand, overnutrition during lactation can promote memory impairments later in life (13). Therefore, the present study investigated whether supplementation with CO during lactation could influence metabolic changes caused by early-life overnutrition, as well as if CO and EE interfered with the aftereffects of neonatal overnutrition on memory performance.

2. Materials and methods

2.1 Animals and experimental design

All experiments were carried out with the offspring of an outbred colony strain of Wistar rats obtained from Departamento de Nutrição at Universidade Federal de Pernambuco (UFPE, Brazil). The experimental design was performed in accordance with the guidelines of the Ethics Committee for Animal Research of UFPE (approval protocol no. nº 23076.048535/2015-78), which comply with the “Principles of Laboratory Animal Care” (National Institutes of Health, Bethesda, USA).

Male rats were reared in a room with a temperature of 23±1 °C and a 12-h light/dark cycle (lights on from 7:00 am to 7:00 pm), with free access to water and food – a commercial laboratory chow diet (Purina do Brazil Ltd., Paulinia, São Paulo, Brazil) with 23% protein. The total trial period was 10 weeks.

2.2 Supplementation with coconut oil

An early postnatal overnutrition model was induced by reducing the litter size, according to Davidowa and Plagemann (24). During the first three days of lactation, the amount breast milk produce is relative the number of rats birthed in standard litters (9 pups). After these days, milk production is the same. Thus, increasing the availability of milk for the remaining pups in the litter when the number of pups was reduced. The animals were randomly distributed to be suckled in litters of either 9 pups (group N, nourished) or 3 pups (group ON, overnourished) to represent the two distinct lactation conditions that differentially affect the pups’ nutritional state (25).

Half of the animals from each group (N or ON) received virgin coconut oil by gavage (CO, 10 ml/kg/d and 10 ml/kg/d of the vehicle solution - Copra®) and the other half of the animals received an equivalent volume of the vehicle solution (V, 20 ml/kg/d - 0.009% cremophor – Sigma®) on a daily basis from the 7th through the 30th day of life, as adapted from Costa et al. (18). The CO dosage was chosen according to previously published data (26; 27), showing antioxidant and anti-stress effects of CO \textit{in vivo} (6).

Either nourished (N) or overnourished (ON) young rats (7 days old) were randomly assigned into 4 different experimental groups: supplemented with coconut oil and nourished (CO&N, n=23); supplemented with coconut oil and overnourished (CO&ON, n=19); supplemented with vehicle solution and nourished (V&N, n=22), and supplemented with vehicle solution and overnourished (V&ON, n=23).

2.3 Behavioral analysis

Behavioral tests were performed in young and adult rats (28). Young animals were 33 days old [CO&N (n=13), CO&ON (n=11), V&N (n=12), V&ON (n=13)]; adult animals were between 67 and 70 days old.
Initially, the rats were placed in the open field apparatus for five minutes, to explore and become familiar with the experiment’s environment. The open field apparatus consisted of a circular arena whose floor was divided into 17 fields which were separated by black lines. In this arena, there were three concentric circles, and the apparatus was in a sound-attenuated room, with reduced lighting. The sessions were recorded, and after each test session, the open-field test’s arena was cleaned with 70% alcohol.

Two experiments were performed to test the novelty recognition paradigm, regarding the object’s novel shape or location. Over three days, these behavioral evaluations were assessed on an individual basis. 24 and 48 hours later the habituation phase, each rat was returned to the circular arena, which now contained two equal objects made of clear glass. These objects were explored by each rat for five-minutes, which constituted the trial session. After a fifty-minute interval, the animal returned to the arena to perform the first five-minute test session. The test session assessed the rat’s capability to recognize novel object shapes, or novel object locations (dislocated object) during the second and third day, respectively. Each experiment was recorded by a camera installed in the ceiling.

The recorded data was analyzed with the ANY-Maze Software (version 4.99) by two previously trained observers who were “blinded” regarding the previous treatment of the animal. The videos were analyzed to assess the time spent by the rat in exploring each object. The criteria to define the time spent by the rats were based on the “active exploration”, defined as touching objects with the vibrissae, snout, or forepaws, as previous published by Akkerman et al. (29). In each trial session, preference ratio was calculated for each animal as time spent in exploring each object/total exploration time. The trial sessions described above ensured that rats were given equal time in exploring each of the two objects and did not exhibit a preference for one single item. All rats presented a ratio near 0.5 during the trial session, indicating equal exploration of the two objects, therefore, they were eligible to realize the test session.

In each test session, the rats were expected to recognize the familiar object, or the familiar (stationary) position previously presented (in the trial session). The preference ratios for the familiar, the novel shape, or the object placement (dislocated) were calculated for these animals. The animals were expected to spend more time exploring the objects that represented a novel shape or a dislocated position in the arena.

The rat performance was represented by a discrimination index (%) which consisted of the exploration time for each analyzed criterion (familiar versus novel shape and stationery versus dislocated position), expressed as a percentage of the total time of exploration. After each session, the objects and the apparatus were made ready for the next rat by being thoroughly cleaned with 70% ethanol solution.

2.4 Environmental enrichment: housing conditions

Animals at 36 days old were exposed to the enriched environment (EE) for three hours per day for four weeks. Thus, four groups were created: CO&N (n=11), CO&ON (n=9), V&N (n=10), V&ON (n=11). Meanwhile, a separate group of rats [CO&N (n=12), CO&ON (n=10), V&N (n=12), V&ON (n=12)] were housed for the duration of the experimental period in the standard environment but not exposed to the EE (30).

The EE was designed as previously published by Andin et al. (31). The EE was comprised of 8 rats housed together in large acrylic cages, (100×60×35 cm) containing a variety of objects, including: running wheels, toy balls, plexiglass tunnels as well as various objects of differing sizes and shapes. The objects were changed once a week. The control group remained in the standard environment (SE), where groups of 3–4 rats were housed per cage (51×35.5×18.5 cm) with only sawdust bedding and no additional objects in the cages.

2.5 Murimetric parameters

The measurements were made immediately before blood and tissue collection. The abdominal circumference (AC, immediately anterior to the forefoot), thoracic circumference (TC, immediately behind the foreleg), body weight, and body length (BL, muzzle-to-anns length) were determined in all rats, as described by Novelli et al. (22). Body weight, (BL), (AC) and (TC) were used to determine the following anthropometric
index: Body mass index (BMI) = body weight (g) / length² (cm²) and Lee index = cube root of body weight (g) / muzzle-to-anus length (cm) AC/TC ratio.

2.6 Blood analyses

Fasted (8–12 h) adult rats (n=6, per experimental group) were anesthetized with 63.5 mg/kg of Ketamine (Vetnil®, Louveira / SP, Brasil) and 9 mg/kg of Xylazine (Dopaser®, Hertape S. A. Juatuba/MG, Brasil). Blood samples were obtained by cardiac puncture and collected immediately in separate tubes. Approximately 4 ml of blood was placed in a 10 ml tube and gently inverted for 30 seconds to mix. After 20 minutes, the samples were centrifuged at 8000 rpm for 10 min. The serum was frozen at -20 degC until assayed for content of lipids and glycemia. The analyses of cholesterol (total cholesterol and high-density lipoprotein, HDL) and triglycerides (TG) were performed as previously described (32). Very low-density lipoprotein (VLDL = triglycerides /5) and atherogenic index were also calculated [log (triglycerides /HDL-cholesterol)]. After the blood samples were obtained by cardiac puncture, the anesthetized animals were then sacrificed.

2.7 Statistical analyses

All data were plotted, and the statistical analysis performed using GraphPad Prism 6.0 software (GraphPad Software Inc., La Jolla, CA, USA). Two-way ANOVA test followed by Tukey test was performed to assess significant differences between the groups. Data were considered as statistically significant for \( p < 0.05 \).

3. Results

3.1 Behavioral assays

Young animals were able to recognize the novel object identity [two-way ANOVA, \( F_{[1, 82]} = 645, p < 0.0001 \)] and novel object placement (\( F_{[1, 90]} = 166.4; p < 0.0001 \)).

Regarding the object identity (Figure 1A), the intergroup analysis demonstrated that overnutrition impaired the discrimination index of the novel object (V&ON versus V&N groups, \( p < 0.001 \)). CO seems to prevent this impairment, since there is no difference between the groups CO&ON and CO&N.

Concerning the intragroup comparisons of object placement recognition, note the discrimination index in the group V&ON was below 60% and CO ameliorate this index (group CO&ON). However, no intergroup difference in the recognition of the object placement was displayed amongst the young rats (Figure 1B).

Please place Figure 1 HERE

Adult rats were also able to recognize the novel object identity (\( F_{[1,72]} = 325.9 p < 0.0001 \)) according to higher discrimination indexes (novel versus familiar). Regarding the recognition of the novel placement, most of the adult rats kept under standard environment conditions were able to identify the novel object placement, as shown by the higher discrimination indexes (novel versus familiar placement, \( F_{[1,70]} = 129.1 p < 0.0001 \)). However, CO&ON rats were unable to perform the task properly. The intergroup analysis displayed a significant impairment in the discrimination indexes of CO&ON rats, when compared to the indexes for the identification of familiar and novel placement of the other groups (\( F_{[3,70]} = 14.74, p < 0.0001 \)). These results are presented in Figures 2A and 2C.

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3.2 Murinometric parameters

Adult rats that had been previously exposed to EE presented increased discrimination indexes for the novel, when compared to the familiar objects for each respective experimental group [\( F_{[1,64]} = 828.4, p < 0.0001 \)]. Moreover, in this environmental condition (EE), all the experimental groups could perform the object placement task successfully (novel placement versus familiar, \( F_{[1,74]} = 322.4, p < 0.0001 \)). Figures 2B and 2D.

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3.2 Murinometric parameters
Neither CO [BMI, \( F\{1,39\} = 0.02890, p = 0.8659\); LI, \( F\{1,34\} = 0.2900, p = 0.5937\); AC/TC ratio \( F\{1,28\} = 0.3864, p = 0.5392\)], nor the EE (Figure 3A-C, SE vs EE groups) significantly influenced murinometric parameters (two-way ANOVA test, \( p > 0.05\)). However, overnutrition during lactation increased: (i) body mass index (BMI \( F\{1,39\} = 16.41, p = 0.0002\), Figure 4A); (ii) Lee index (LI \( F\{1,34\} = 20.14, p < 0.0001\), Figure 4B), and (iii) the AC/TC ratio \( F\{1,28\} = 27.05, p < 0.0001\), Figure 4C) among rats from the different experimental groups (MEAN+-SD, two-way ANOVA test followed by Tukey, \( p < 0.05\)).

PLEASE PLACE FIGURE 3 AND 4 HERE

### 3.3 Glycemia, lipid profile and atherogenic index

CO supplementation reduced \( F\{1,18\} = 5.672, p = 0.0285\) the amounts of total cholesterol in both nourished and overnourished groups (Figure 5D). When the overnourished groups were compared to the nourished controls, data show significant difference in the values (MEAN+-SD) of the fasting glucose [FG \( F\{1, 19\} = 4.915, p = 0.039\)], very low density lipid [VLDL \( F\{1,18\} = 10.63, p = 0.0043\)] and triglycerides [TG \( F\{1,18\} = 10.63, p = 0.0043\)] (Figure 5A-C). The high-density lipid (HDL) and atherogenic index (AI) were not significantly different (two-way ANOVA, \( p > 0.05\), Figures 5E and 5F) among rats from different experimental conditions.

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### 4. Discussion

Our data suggest CO ameliorates the damage overnutrition-imposed on episodic-like memory of young rats. Moreover, EE demonstrated protective memory effects in adult rats, regardless of previous overnourishment. Data clearly present that neonatal overnutrition increases the murinometric and biochemical parameters, as well as promotes mnemonic impairment in the rats. CO was able to reduce the total cholesterol in the animals.

About the memory tests, all young rat groups performed the object recognition tasks effectively. The animals recognized the novelty aspect of the tasks, showing preservation of episodic-like memory. However, young overnourished rats presented mnemonic impairment. Overnourished young rats also displayed a treatment-dependent impairment in their performance to recognize the novel object identity when compared to the control group. Interestingly, young rats that received CO supplementation presented a similar episodic-like memory to the control groups.

Rahim et al. (4) demonstrated that treatment with CO enhances and consolidates memory of rodents. Prior findings have suggested that neonatal overnutrition may lead to impaired memory (14), as early postnatal overfeeding may stimulate the establishment of a chronic pro-inflammatory profile. For instance, a high-fat diet can induce microgliosis after only 14 postnatal days of overfeeding (15). This microgliosis can cause synaptic remodeling, neuronal apoptosis, and impairment of neurogenesis in the hypothalamus.

In rodents, the episodic-like memory is supported by a combination of familiarity with previously experienced stimuli and recollection of specific episodes involving those stimuli. The recollective component of recognition memory depends on the hippocampus (12). Therefore, it is possible that the postnatal overfeeding used in the present study induced microgliosis. However, further studies would be necessary to confirm the proposed causal relationship.

Our adult animals – previously overnourished during lactation – did not present significant impairment on their performance in identifying the novel object, compared to the respective control groups. These results suggest that there might be age-dependent effects of postnatal overfeeding on episodic-like memory. With respect to this potential age-dependent effect, the literature shows that postnatal overfeeding can be associated with enhanced maternal care due to the reduced number of pups per litter, as well as sensory stimuli from the mother during the perinatal period which can program the adult brain (16).

Pups that receive enhanced maternal care may present a better synaptic development of the neural systems that mediate the cognitive processes, as well as an increase in the survival of hippocampal neurons. This is
reflected in later behavioral and physiological benefits (17). This may be, in part, the reason why the adult rats in the present work did not have a significant impairment in their ability to recognize the identity of objects. Besides the age-dependent effects of neonatal overfeeding on episodic-like memory, adult rats that had been previously overnourished and supplemented with CO also presented impaired episodic-like memory. CO&ON rats had a significant impairment in the discrimination indexes of object placement. However, EE reversed this treatment-task impairment caused by CO&ON.

As previously mentioned, Rahim et al. (4) suggest CO as a natural memory enhancer in normal adult rats. They treated Wistar rats (7–8 weeks old) with 1, 5 and 10 g/kg CO for 31 days, and then used the Morris Water Maze Test to evaluate memory function. Even given the fact that the rats in our study received CO supplementation during a critical period of brain development, as opposed to adulthood, in contrast to the study by Rahim et al. (4), we found CO has a potential role as memory enhancer, as judged by the performance of our young animals as well as our normally nourished adult rats.

Supported by reports in the literature that enriched environment favors neuroprotective responses, we believe the positive effects of EE in our CO&ON group were due to antioxidant benefits promoted by the environmental enrichment. Living in an EE is related to the reduction of markers for oxidative stress in the hippocampus and cerebral cortex (11). Environmental enrichment is also associated with an increase of hippocampal neurogenesis and improvement of spatial memory (10). Studies have shown EE to be a useful experimental model for stimulating brain plasticity, which causes neurochemical and neuroanatomic alterations associated to long-lasting improvements in memory and learning tasks (18).

The overnourished adult rats presented increased Lee index, body mass index and AC/TC ratio when compared to the nourished controls. It is reported that both, the litter size reduction model, and high-fat diet, are associated with increased body fat and body weight (19). Reduction of litter size is an effective experimental approach to induce overweight in rats, corroborating previous findings (20). Neither supplementation with coconut oil (CO), nor environmental enrichment influenced the effects of overnutrition on the muri-nometric parameters. This increased caloric intake during lactation is associated with rapid increase in fat deposition, persistence of overweight and hyperphagia throughout lifespan (20). The induction of postnatal overnutrition has been related to complex functional and metabolic alterations like those that occur when this metabolic syndrome affects humans (21).

Overnourished adult rats also presented elevated fasting glycemia and dyslipidemia, as judged by the increased amounts of triglycerides and VLDL-C. These differences between overnourished and nourished rats were similar in previous data (22). Thus, taking all above together with the persistent abnormal muri-nometric parameters found in this present study, our results confirm the reliability of the experimental model for inducing overnutrition in a long-lasting manner.

The supplementation with CO reduced total cholesterol. However, this decrease was not associated with any effect on the levels of HDL-C and atherogenic index. The effect of CO on the levels of total cholesterol could be related to the increased amount of phytosterols in this oil, since phytosterols competitively block the absorption of cholesterol and increase fecal excretion of bile acids and neutral sterols, therefore improving circulating lipid profile (23).

In conclusion, our results demonstrate that a combination of supplementation with CO and exposition to EE can rescue the memory impairment found in CO&ON adult rats. Reports in the literature suggests that the etiology of diseases characterized by memory impairment can date back to lactation, and type of nutrition during this critical period can influence cognitive aftereffects. These are the first findings demonstrating that neonatal overfeeding is related to age-dependent effects on memory, as well as to studies showing early-life overnutrition coupled with CO exerted lasting negative effects on episodic-like memory, and EE was able to reverse these effects. Because of the worldwide epidemic prevalence of overnutrition, these results reinforce the need to improve the understanding of the role of overnutrition on neurodevelopment, as well as the use of adjunctive therapies that could ameliorate aftereffects of overnutrition on cognitive function and memory.
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Conflicts of interest

The authors report no competing interests.

Author contributions

MGBT designed and conducted research as well as wrote paper. DCS and HMB conducted research and provided essential materials. CVSS conducted research. RDLB help in the writing of the manuscript. MBOH designed research, analyzed data, performed statistical analysis, and wrote paper. AAS participated in project conception and study oversight as well as conducted research. All the authors have read and approved the manuscript.

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Data availability

Data generated or analyzed during this study are available from the corresponding author upon reasonable request.

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Environmental enrichment on steady-state mRNA levels for EAAC1, AMPA1 and NMDA2A receptor subunits in hippocampal neurons from the long-lasting effects of protein malnutrition early in life. Behav Brain Res. 2017; 29, 335:55-62.


Captions

Graphical abstract. Nourished and overnourished rats received virgin coconut oil during lactation and enriched environment at adulthood. Both approaches improved episodic memory.

**Figure 1.** Discrimination indexes with the performance of young rats on the tests of object identity and placement recognition presented in the left (A) and right (B) panels, respectively. There is a significant effect on the discrimination indexes of the novel object (A) identity (F [1, 82] = 645, p < 0.0001)) and novel (B) placement (F [1,90] = 166.4 p<0.0001), when compared to the respective familiar object characteristics. In regards of the recognition of object identity (A), young rats presented a significant difference for the treatment-task interaction (F [3, 82] = 19.65, p < 0.0001). These analyses were performed with two-way ANOVA followed by Tukey test. The (*) indicate intragroup differences (novel versus familiar), and (#) marks intergroup differences (V&ON versus V&N, novel and familiar).

**Figure 2.** Effect of the standard (SE, A & C) or enriched (EE, B & D) environment on the ability of rats to identify object characteristics. Adult rats performed two recognition tasks: object identity or object placement (C-D). Two-way ANOVA showed significant effects on the discrimination indexes of the novel object (A) identity in both living conditions: SE (F [1,72] = 325.9 p < 0.0001) and EE rats (B) (F [1, 64] = 828.4 p < 0.0001). Additionally, ANOVA demonstrated that most SE rats (C) (F [1,70] = 129.1 p < 0.0001) and EE rats (D) (F [1,74] = 322.4 p < 0.0001) were able to identify the novel placement, as judged by the higher discrimination indexes, when compared to the respective familiar object characteristics. CO&ON rats kept in SE had impaired episodic memory ((A), (F [3,70] = 14.74, p < 0.0001)). The (*) indicate intragroup differences (novel versus familiar), and (#) marks intergroup differences (CO&ON versus the other experimental groups).

**Figure 3.** Murinometric parameters of adult rats (n=46, MEAN±SD). These animals were subdivided into overnourished or nourished groups and received one single daily dose of either coconut oil or vehicle solution from the 7th to the 30th day of life. At adulthood, the body mass index (A), Lee index (B), and abdominal and thoracic circumferences ratio (C) were determined, as described by Novelli et al., (2007). Two-way ANOVA test followed by Tukey found significant differences of body mass index, Lee index, and the AC/TC ratio among adult rats from distinct experimental groups. Asterisks indicate values that were significantly different from the corresponding control group (*p<0.05, ***p<0.001). CO&ON (n=10) represent rats that were supplemented with coconut oil and overnourished; CO&N (n=12), supplemented with coconut oil and nourished; V&ON (n=12), supplemented with vehicle solution and overnourished; V&N (n=12) supplemented with vehicle solution and nourished during lactation.

**Figure 4.** Murinometric parameters of adult rats (MEAN±SD). Data of BMI (A), LI (B) and AC/TC ratio (C) of rats that lived with a standard (SE) or environmental enrichment (EE). Asterisks indicate values that were different from the corresponding control group (*p<0.05, ***p<0.001). Two-way ANOVA test did not find differences of body mass index, Lee index, and the AC/TC ratio. CO&ON (n=10, SE and n=9, EE); CO&N (n=12, SE and n=11, EE); V&ON (n=12, SE and n=11, EE); V&N (n=12, SE and n=10, EE).

**Figure 5.** Fasting glucose, lipid profile and atherogenic index of adult rats (MEAN±SD). A) Fasting glucose. B) Very low-density lipid (VLDL). C) Triglycerides (TG). D) Total cholesterol (TC). E) High density lipoprotein-(HDL). E) Atherogenic index (AI). Asterisks indicate difference (two-way ANOVA, followed by Tukey test p<0.05).