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To cite this article: Juliana Azevedo de Meneses, Liana de Araújo Trugilho, Samantha de Aguiar Lima, Amanda Carneiro Ferreira Freitas, Henrique Saldanha Melo, Maitê Rocha Ferreira, Luis Guillermo Coca Velarde, Felipe Zandonadi Brandão, Gabrielle de Souza Rocha & Gilson Teles Boaventura (2017): The influence of a diet based on flaxseed, an omega-3 source, during different developmental periods, on the blood pressure of rats submitted to stress, The Journal of Maternal-Fetal & Neonatal Medicine, DOI: 10.1080/14767058.2017.1407309

To link to this article: https://doi.org/10.1080/14767058.2017.1407309

Accepted author version posted online: 29 Nov 2017.
Published online: 12 Dec 2017.

Article views: 3

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ABSTRACT

Objective: This study aimed to investigate a flaxseed diet during different developmental periods, and its effect on the blood pressure of rats submitted to stress.

Methods: Fifty-six male rats (F₁), born from 14 rats (F₀), were divided into seven groups (n = 8): flaxseed group (FG); flaxseed group gestation and lactation (FG-GL); flaxseed group weaning (FG-W); flaxseed group weaning and stress (FG-WS); flaxseed group gestation lactation and weaning (FG-GLW), and control Group (CG). Stress protocol was undertaken for 1 month. Blood pressure was analysed before and after the stress protocol. The left adrenal glands and serum corticosterone levels were analysed.

Results: Systolic blood pressure before stress was lower in all groups with flaxseed diet compared with the CG (p = .00001). After stress, CG showed higher blood pressure compared with FG, FG-GL, and FG-GLW (p = .004). The levels of corticosterone were lower in the FG between all groups (p < .000001) and the CG showed higher compared with FG-W, FG-WS, FG-GL, and FG-GLW (p < .0001). The adrenal gland did not show differences.

Conclusions: Results suggest a possible factor from a flaxseed diet against the effects of stress on a blood pressure in all periods of life but especially in the gestation and lactation periods.

Introduction

Stress is defined as a state of homeostasis that is threatened or perceived to be threatened [1]. It is a daily experience in which the human subject and all organisms undergo changes in its internal environment to face the stressing agents. These agents activate a wide range of neural and hormonal systems, inducing physiological and behavioural changes that promote adaptation to the new situation [2]. Compared with a physical threat, this is an efficient answer to ensure survival as well as to increase the body’s ability to escape. The stressing agents that are typically involved in human and animal lives may change the organisms function and contribute to aging and degenerative effects, such as, cardiovascular, metabolic and infectious diseases, arthritis, cancer, and depression [3,4].

Neurophysiological responses to a stressor initially involve the activation of the sympathetic nervous systems, resulting in rapid elevation of circulating catecholamine, which prepares the body for survival. Thereafter an endocrine response occurs, which is slower. The hypothalano–pituitary–adrenal (HPA) axis is activated with the sequential release of corticotrophin hormone (CRH) from the hypothalamus, adrenocorticotropic hormone (ACTH) from the anterior pituitary, and glucocorticoids (cortisol in humans and corticosterone in rats) from the adrenal cortex. The cortisol has effects on metabolism within most body cells [5–7].

Evidence from animal and human models has demonstrated the existence of dietary effects, with polyunsaturated fatty acids (PUFA) omega-3 (n-3) having influence over reduction of the effects stress...
induced [5,8–11]. The n-3 PUFA is precursor for some substances with physiological and pharmacological activity called eicosanoids including thromboxane, prostaglandins (which have hypotensive effects, inhibit platelet aggregation and increase high-density lipoprotein), and leukotrienes. The appropriate balance between the production of prostaglandins and thromboxane protects against the development of cardiovascular disease [12]. The n-3 PUFA are generally considered essential because mammals are unable to synthesize them and, in this way, supplementation in the diet is necessary [13].

From a nutritional perspective, the flaxseed (Linum usitatissimum) is a well-known seed due to its excellent source of oil, fibre, lignan, and others [14] and is also considered one of the best vegetable sources of n-3 PUFA. For these reasons, interest in flaxseed has been increasing due to its favourable physiological effects on organisms [15]. There are many studies that analyse the effect of its consumption on the reduction and prevention of various diseases like diabetes, hypercholesterolemia, cardiovascular diseases, bone density loss [12,14,16,17] and the beneficial effects of flaxseed consumption and its components on blood pressure [18,19].

Intrauterine and postnatal nutrition may influence the risk of chronic disease in adults, suggesting that nutrition in early life may have a marked effect manifesting at later ages. Thus, a good supply of these PUFAs is essential for normal fetal and neonatal development [20], development and neurological function [21–23], and learning and behaviour [24].

According to these findings and considering the potential consumption effect of flaxseed on life quality, we hypothesized that a diet based on flaxseed could promote a positive response in the behaviour of systolic blood pressure in the state of stress.

### Materials and methods

#### Flaxseed

Flaxseed was purchased by the Laboratory of Experimental Nutrition (LabNE) from the company Arma Zen Produtos Naturais, Ltda, Rio de Janeiro-Brasil, chosen in order to ensure the safety and quality of the seeds. The seeds were weighed and crushed in a blender to obtain the seed flour which was used as a supplement for lipids and fibre during the diet preparation.

#### Diet

All diets were prepared at LabNE of the Fluminense Federal University – Brazil. They were supplemented with minerals and vitamins according to Committee Laboratory Animal Diet, 1979, modified according to the recommendations of AIN-93G and AIN-93M [25] (Table 1).

#### Animals

The protocol used to deal with experimental animals was approved by the Ethics Committee on Animal Research of Fluminense Federal University, Niterói-RJ, Brazil (protocol 373/2013). All procedures were in accordance with the provisions of the Brazilian Society of Science and Laboratory Animals and the Guide for the Care and Use of Laboratory Animals published by the USA National Institute of Health (NIH Publication no. 85-23, revised in 1996).

The biological assay used 14 pregnant females Wistar rats (F₀) which generated 56 male pups (F₁) which were submitted to the study protocol. All rats stemmed from LabNE and were housed in a temperature-controlled room (23 ± 1°C), with controlled light/dark cycle (12 h/12 h) and food and water ad libitum.

#### Experimental design

Pups were divided into seven groups (n = 8) according to the diet and scenarios studied:

### Table 1. Nutritional composition of experimental diets.

<table>
<thead>
<tr>
<th>Ingredients g/100 g</th>
<th>17% protein diet</th>
<th>10% protein diet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control Flaxseed</td>
<td>Control Flaxseed</td>
</tr>
<tr>
<td>Casein a</td>
<td>20 14.12</td>
<td>11.8 5.9</td>
</tr>
<tr>
<td>Flaxseed b</td>
<td>0 25</td>
<td>0 25</td>
</tr>
<tr>
<td>Corn starch c</td>
<td>52.95 45.83</td>
<td>61.2 54.1</td>
</tr>
<tr>
<td>Sucrose d</td>
<td>10 10</td>
<td>10 10</td>
</tr>
<tr>
<td>Mineral mix e</td>
<td>3.5 3.5</td>
<td>3.5 3.5</td>
</tr>
<tr>
<td>Vitamin mix e</td>
<td>1 1</td>
<td>1 1</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>7 0</td>
<td>7 0</td>
</tr>
<tr>
<td>Cellulose</td>
<td>5 0</td>
<td>5 0</td>
</tr>
<tr>
<td>Choline bitartrate h</td>
<td>0.3 0.3</td>
<td>0.3 0.3</td>
</tr>
<tr>
<td>Cystine h</td>
<td>0.3 0.3</td>
<td>0.3 0.3</td>
</tr>
<tr>
<td>Tert-Butylhydroquinone (BHT)</td>
<td>0.0014 0.0014 0.0014 0.0014</td>
<td></td>
</tr>
<tr>
<td>Total g</td>
<td>100 100</td>
<td>100 100</td>
</tr>
<tr>
<td>Energy (kcal/100 g)</td>
<td>355.6 386.9</td>
<td>355.1 387.2</td>
</tr>
</tbody>
</table>

*Comércio e Indústria Farmos Ltda. (Rio de Janeiro, RJ, Brazil).
*bArma Zen Produtos Naturais Ltda. (Rio de Janeiro, RJ, Brazil).
*cMaízena da Unilever Bestfoods Brasil Ltda. (Mogi Guacu, SP, Brazil).
*dUnião (Rio de Janeiro, RJ, Brazil).
*ePrag Soluções Comércio e Serviços Ltda-ME (Jau, SP, Brazil).
*fLiza da Cargill Agricultura Ltda. (Mairinque, SP, Brazil).
*gMicrocel da Blanver Ltda. (Cotia, SP, Brazil).
*hM. Cassab Comércio e Indústria Ltda. (São Paulo, SP, Brazil).
1. Flaxseed group (FG), received flaxseed diet (F₀ and F₁) for the whole duration of the experiment (until 88th day).
2. Flaxseed group gestation and lactation (FG-GL), received flaxseed diet just during gestation and lactation (F₀).
3. Flaxseed group weaning (FG-W), received flaxseed diet after weaning (21st day) (F₁) until the stress protocol began (60th day).
4. Flaxseed group weaning stress (FG-WS), received flaxseed diet after weaning (21st day) until the end of experiment (88th day) (F₁).
5. Flaxseed group stress (FG-S), received flaxseed diet as the stress protocol began (60th day) until the experiment end (88th day) (F₁).
6. Flaxseed group gestation lactation and weaning (FG-GLW), received flaxseed diet during gestation and lactation (F₀) and after weaning until the stress protocol began (60th day) (F₁).
7. Control group (CG) received control diet (F₀ and F₁) for the whole duration of the experiment (88th day).

At all times when animals did not receive the flaxseed diet, they received the control diet (Table 2).

During gestation and lactation the diets contained 17% protein according to the AIN-93G recommendation, after weaning and until the end of experiment, the diets contained 10% protein in accordance with AIN-93M recommendation.

All animals underwent the stress protocol and blood pressure measurement. After the 88th day and undergoing these procedures, they were euthanized, having their organs and blood collected and stored for further analysis.

**Stress protocol**

To promote restraint stress, the rats were removed from their home cages and placed in a transparent Plexiglas tube (inner diameter, 5 and 20 cm length) for a period of 20 min each day from the 60th–88th day, 5 times per week. The tube had perforated ends to allow ventilation and avoid overheating. The tubes maintained the animals in a standing position with slight compression of the body [5].

**Systolic blood pressure (SBP)**

Blood pressure (BP) was measured in the animals of all experimental groups by tail plethysmography, a non-invasive method (Tail Plethysmography V1.10 – Insight®,) on the 59th day before starting the stress protocol (60th day) and on the last day of the stress protocol (88th day).

In the BP recording by tail plethysmography, loss and return of the pulse signals occur during the process of insufflation and deflation of the cuff. For this reason, the BP was considered as the pressure at the time of the return of the first pulse signal. Three consecutive measurements were recorded and the mean of the three measurements was used for the analysis. The animals were always handled by the same person.

**Serum corticosterone levels**

After the last day of tests (88th day), the animals received, intraperitoneally, a lethal dose of Thiopentax® (sodium thiopental) 1 g (DOSe ml = 0.15 × animal weight g/100). After sedation, the animals had blood taken by cardiac puncture. The blood was collected and placed in vacuum tubes without anticoagulant and centrifuged at 4 °C at 1500 × g for 15 min. The serum was frozen at −80 °C until analysis. The analysis was completed in the Hormonal Dosing Laboratory located in the Animal Reproduction Sector at Veterinary College of the Fluminense Federal University, Niterói – RJ, Brazil, using a solid phase radioimmunoassay technique with a commercial kit (Immunotech/Beckman Coulter®, Prague, Czech Republic).
Dissection

The adrenal glands were excised and weighed in an analytic scale with an accuracy of 0.0001 g to obtain the relative weight of the organ. Relative weight was calculated by dividing the adrenal gland weight by body weight and multiplying this result by 100.

Statistical analysis

The analysis of variance (ANOVA) was used, considering values of \( p \) less than 0.05 being statistically significant. The statistical significance was confirmed by the Tukey test. Software S-Plus 8.0 was used.

Results

Systolic blood pressure

Systolic blood pressure before the beginning of the stress protocol (60th day) for the studied groups are presented as follows: the groups FG (103.8 ± 20.4 mmHg) and FG-GLW (93.5 ± 17.4 mmHg) presented lowest SBP value. The other group’s results were FG-WL (112.9 ± 18.3 mmHg); FG-WS (118.2 ± 20.7 mmHg); FG-W (127.0 ± 10.9 mmHg); FG-S (128.5 ± 15.6 mmHg); and CG (137.2 ± 21.8 mmHg). Statistically significant differences were found between FG-GLW and CG; FG and CG; FG-GLD and FG-S; and FG-W and FG-GLW (\( p = 0.0001 \)) (Figure 1(a)).

After the end of the stress protocol (88th day), the studied group’s SBP were the CG (166 ± 24.3 mmHg) showed the highest value after the stress, followed by groups FG-S (139.6 ± 9.7 mmHg), FG-W (133.7 ± 14.0 mmHg); and FG-WS (134.1 ± 14.9 mmHg). There were significant difference (\( p = 0.004 \)) between FG (124.6 ± 34.7 mmHg) and the groups FG-GL (131.4 ± 23.3 mmHg) and FG-GLW (123.4 ± 22.8 mmHg) (Figure 1(b)).

Serum corticosterone levels

The FG (54.3 ± 16.4 nM) were the group that presented the lowest values of serum corticosterone levels with big statistical difference (\( p < 0.00001 \)) compared with the other studied groups. The CG (198.1 ± 77.1 nM) were the group that presented highest serum corticosterone levels that exhibited difference not just with the FG group, but with the FG-W (130.0 ± 36.6 nM), FG-WS (99.9 ± 32.1 nM), FG-GL (101.6 ± 19.9 nM), and FG-GLW (91.1 ± 28.2 nM) (\( p < 0.0001 \)). There was no statistical difference with the FG-S group (156.7 ± 41.7 nM) (Figure 2).

Relative weight of left adrenal (RWA)

The relative weight of the left adrenal glands does not show statistically significant difference between the studied groups (\( p = .49 \)), as shown in Table 3. The weights analysed for the groups were: CG (0.0084 ± 0.0023 g); FG (0.0073 ± 0.0008 g); FG-GL (0.0084 ± 0.0018 g); FG-W (0.0095 ± 0.0034 g); FG-GLW (0.0082 ± 0.0020 g); FG-S (0.0090 ± 0.0012 g); and FG-WS (0.0085 ± 0.0013 g).

Discussion

Flaxseed has proved to be a nutritionally appealing food given its high content of alpha-linolenic acid.
(ALA), dietary fibre, the high quality of its proteins, and the abundance of lignans, of which the secoisolariciresinol (SDG) is present in greater quantity. Flaxseed contains about 55% ALA, 28–30% protein and 35% fibre [15]. Due to its potential health benefits, flaxseed has been a focus of growing interest among nutritionists and medical researchers [26] including its use in heart disease and hypertension preventions [18].

In the present study, the SBP of the animals studied presented the lowest value in the FG-GLW and FG groups before starting the stress protocol. These groups received the flaxseed diet from gestation during lactation and maintained it until the beginning of the stress protocol, confirming the importance of an n-3 rich diet in the pre- and postnatal development phase [19]. It has been described that n-3 PUFA deficiency, especially during the prenatal period, can cause hypertension in adult life [27,28]. The other groups, FG-GL, FG-WS, FG-W, and FG-S, who received the flaxseed diet at differing periods of their life before the stress protocol, maintained their SBP values within normal limits, which for rats is between 100 and 130 mmHg [29], although numerically higher than the FG and FG-GLW groups.

This suggests that although n-3 PUFA deficiency tends to raise the pressure, its supplementation can avoid this, normalizing BP [27,30]. Only the CG, who received the casein-based diet, presented a higher SBP before the beginning of the stress protocol. This observation leads us to understand that, in fact, a diet rich in n-3 fatty acids can positively influence the prevention of hypertension or the normalization of blood pressure.

Figure 2. Serum corticosterone levels. CG: control group; FG: flaxseed group; FG-W: flaxseed group weaning; FG-WS: flaxseed group weaning stress; FG-S: flaxseed group stress; FG-GL: flaxseed group gestation and lactation; FG-GLW: flaxseed group gestation, lactation, and weaning.

Table 3. Relative weight of left adrenal gland (RWA) (g/100 g body weight).

<table>
<thead>
<tr>
<th>Groups</th>
<th>RWA (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FG</td>
<td>0.0073 ± 0.0008</td>
</tr>
<tr>
<td>FG-GL</td>
<td>0.0084 ± 0.0018</td>
</tr>
<tr>
<td>FG-W</td>
<td>0.0095 ± 0.0034</td>
</tr>
<tr>
<td>FG-GLW</td>
<td>0.0082 ± 0.0020</td>
</tr>
<tr>
<td>FG-S</td>
<td>0.0090 ± 0.0012</td>
</tr>
<tr>
<td>FL-WS</td>
<td>0.0085 ± 0.0013</td>
</tr>
<tr>
<td>CG</td>
<td>0.0084 ± 0.0023</td>
</tr>
</tbody>
</table>


After the stress protocol, all groups presented higher SBP values relative to those obtained before the protocol. In fact, the stress state tends to raise BP. However, this possibly damaging increase did not occur in the FG, FG-GL, and FG-GLW groups. The FG-S, FG-W, and FG-WS groups presented higher SBP values after stress. However, these values probably would not cause damage to these animals, since they were close to normal. On the other hand, the CG presented an increase in the BP value, characterizing arterial hypertension, as it was above 150 mmHg [29], confirming the negative influence that the stress state can exert on the individual. Some studies using a flaxseed diet have observed important results in reducing blood pressure [18,19,28].

A disturbing feature of western diets is the growing imbalance between n-3 and n-6 fatty acids, which may limit the supply of PUFA to tissues and lead to n-3 deficiency [11]. A combination of stress and lack of n-3 may be an important factor for cognition and mood disorders, and n-3 food supplements could improve brain resistance to stress-induced responses. The physiological response anticipated to stress is the activation of hypothalamic-pituitary-adrenal (HPA) axis, raising circulating glucocorticoid concentrations. Chronic or repeated exposure to stress situations may have influence, through deregulation of the HPA axis, on the development and severity of various diseases [31].

Serum corticosterone levels in animals in the FG group were much lower than those observed in the CG, showing that, in fact, n-3 supplementation is of great importance and may provide some protection against the effects of stress in the body. Ferraz [5] showed that n-3 supplementation promoted the reduction of corticosterone plasmatic levels in rats submitted to the restraint stress protocol when compared with a control group, which did not receive such supplementation. Another study [11], corroborating our results, showed that an n-3 enriched diet...
attenuates some of the harmful effects of chronic stress, such as sustained cortisol peak. In another study published by our group, rats fed the same flaxseed diet in gestation, lactation and up to 60 d after birth presented less stressed behaviour than the control group [24].

The FG-W, FG-WS, FG-GL, and FG-GLW groups also showed significant differences when compared with the CG, which means that the period in which n-3 is supplemented is important for its incorporation as a possible protective factor against stress. These findings suggest that n-3 supplementation is essential in the early stages of development, as also observed in the studies by Velasco [32]. The FG-S did not present a statistical difference when compared with the CG, only a lower absolute value, showing that although the difference was not significant, somehow the flaxseed supplementation as a source of n-3 during the stress protocol promoted a reduction in serum corticosterone secretion.

The left adrenal gland weight did not present significant differences between the studied groups, even though the CG had a higher serum corticosterone value. Borsonelo [33] obtained a similar result, that is, a higher serum secretion of corticosterone in the CG. However, as in our study, adrenal gland weights were similar to those in the experimental group, which received n-3 supplementation. There are few data in the literature associating adrenal weight to stress, but a higher level of corticosterone would lead us to believe in hypertrophy of this gland, contrary to what was observed. Further studies are required to state that, in fact, increased secretion of cortisol would lead to hypertrophy of the adrenal glands.

Conclusion

A flaxseed diet contributes to the maintenance of systolic blood pressure, and its supplementation, independent of the developmental phase, helps maintain SBP in the normal range even under stress conditions. Serum levels of corticosterone in stressed animals appear to remain under control when a diet with n-3 PUFA is consumed, especially in the gestation and lactation stages, suggesting that this diet is a possible protective factor against the effects of stress on the body. The weight of the adrenal gland was not related to the amount of corticosterone secreted in response to the stress.

Disclosure statement

The authors declare no conflict of interest.

Funding

This research was supported by the National Council for Scientific and Technological Development (CNPq) and the Rio de Janeiro State Foundation for Scientific Research (FAPERJ).

References


