Maternal flaxseed diet during lactation changes adrenal function in adult male rat offspring

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Abstract
Flaxseed (Linum usitatissimum L.) has been a focus of interest in the field of functional foods because of its potential health benefits. However, we hypothesised that maternal flaxseed intake during lactation could induce several metabolic dysfunctions in adult offspring. In the present study, we aimed to characterise the adrenal function of adult offspring whose dams were supplemented with whole flaxseed during lactation. At birth, lactating Wistar rats were divided into two groups: rats from dams fed the flaxseed diet (FLAX) with 25% of flaxseed and controls dams. Pups received standard diet after weaning and male offspring were killed at age 180 days old to collect blood and tissues. We evaluated body weight and food intake during development, corticosteronaemia, adrenal catecholamine content, hepatic cholesterol, TAG and glycogen contents, and the protein expression of corticotropin-releasing hormone (CRH), adrenocorticotropic hormone (ACTH), 11-ß-hydroxysteroid dehydrogenase type 1 (11ß-HSD1) and adrenaline B2 receptor at postnatal day 180 (PN180). After weaning, pups from the FLAX group had a higher body weight (+10%) and food intake (+10%). At PN180, the FLAX offspring exhibited higher serum corticosterone (+48%) and lower adrenal catecholamine (+23%) contents, lower glycogen (−30%), higher cholesterol (+4-fold increase) and TAG (3-fold-increase) contents in the liver, and higher 11ß-HSD1 (+62%) protein expression. Although the protein expression of hypothalamic CRH was unaffected, the FLAX offspring had lower protein expression of pituitary ACTH (−34%). Therefore, induction of hypercorticosteronaemia by dietary flaxseed during lactation may be due to an increased hepatic activation of 11ß-HSD1 and suppression of ACTH. The changes in the liver fat content of the FLAX group are suggestive of steatosis, in which hypercorticosteronaemia may play an important role. Thus, it is recommended that lactating women restrict the intake of flaxseed during lactation.

Key words: Flaxseed; Lactation; Programming; Adrenal function

Flaxseed (Linum usitatissimum L.) is the richest source of α-linolenic acid and lignan secoisolariciresinol diglycoside (SDG)1,2. Flaxseed has been a focus of interest in the field of functional foods because of its potential health benefits, such as cardiovascular system protection, antioxidant activity and hypoglycaemic effects2,3. However, some researchers have suggested caution when flaxseed is consumed during pregnancy and lactation4,5. The risk of developing chronic diseases during adult life may be influenced by exposure to diet and/or bioactive food compounds in early life that may interfere with or act as hormones, which is called metabolic programming2,6.

We have previously demonstrated that maternal intake of flaxseed (25%) during lactation leads to lower body fat mass, hyperleptinaemia, hypoinsulininaemia, increased insulin sensitivity, increased pituitary expression of leptin receptor, phosphorylated signal transducer and activator of transcription 3, and lower serum T3 concentrations in weaned pups. In adulthood, these animals were programmed for an increased adipocyte area in both visceral and subcutaneous adipose tissues, increased leptin sensitivity, hypercalcaemia, hyperinsulinaemia, hypoadipocinaemia and increased insulin resistance, lower serum T4 (thyroxine), type 1 deiodinase (D1) and type 2 deiodinase (D2) activities in the thyroid, higher D2 activity in brown adipose tissue, and increased expression of leptin receptor in the hypothalamus5,6,8–10. The majority of these effects were mediated by bioactive compounds present in flaxseed, such as α-linolenic acid and SDG.

As changes in adiposity and glucose homeostasis are dependent on normal adrenal function, we hypothesised

Abbreviations: 11ß-HSD1, 11-ß-hydroxysteroid dehydrogenase type 1; ACTH, adrenocorticotropic hormone; CON, control diet; CRH, corticotropin-releasing hormone; FLAX, flaxseed diet; GR, glucocorticoid receptor; HPA, hypothalamic–pituitary–adrenal; SDG, secoisolariciresinol diglycoside.

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that maternal flaxseed supplementation during lactation could affect adrenal function in the offspring later in life. Therefore, the present study aimed to evaluate the effects of maternal whole flaxseed intake during lactation on adrenal function in adult male offspring. We studied the hypothalamic–pituitary–adrenal (HPA) axis by evaluating the protein expression levels of corticotropin-releasing hormone (CRH), adrenocorticotropic hormone (ACTH), corticosterone, glucocorticoid receptor (GR)-α in the hypothalamus, pituitary gland and liver and 11-β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) in the liver, which converts 11-dehydrocorticosterone into the active hormone corticosterone in rats to supply local demand(11). Furthermore, we studied catecholamine content in adrenal glands and adrenaline B2 receptor in the liver. As both hormones influence liver metabolism, the contents of cholesterol, TAG and glycogen in the liver were also measured.

Materials and methods

Animal experiment

Wistar rats, aged 3 months old, were maintained in a temperature-controlled room (25 ± 1°C) with a 12 h dark–12 h light cycle. Virgin rats (200–220 g) were mated, and each female was placed in an individual cage with free access to water and food until parturition. Animal use and experimental design were approved by the Animal Care and Use Committee of the Biology Institute of the State University of Rio de Janeiro (no. 230/2008).

At birth, sixteen lactating rats were randomly assigned to one of the following experimental groups: (1) control dams (CON), with free access to a diet containing 17 % protein, 52 % carbohydrate, 7 % lipid and 5 % fibre; (2) flaxseed dams (FLAX), with free access to a diet containing 17 % protein (12 % casein and 5 % flaxseed), 54 % carbohydrate, 10 % lipid exclusively from flaxseed and 5 % fibre exclusively from flaxseed (Table 1). We ground the whole flaxseed before adding to the diet. The diets were started at birth, which was defined as day 0 of lactation, and ended at weaning (day 21). After birth, litters were adjusted to six males per dam, as this has been shown to maximise lactation performance(12).

After weaning, two pups from each dam received a standard laboratory diet (Nuvilab; Nuvital Nutrientes) containing 22 % protein, 66 % carbohydrate and 11 % lipid until they were 180 d old (Table 1). All offspring were killed at 180 d of age with a lethal dose of pentobarbital (0.06 g/kg body weight). Blood was collected by cardiac puncture, and tissues (hypothalamus, pituitary gland, liver and adrenal gland) were stored at −80°C until analysis.

Evaluation of nutritional status

Body weight and food intake of the offspring were evaluated once every 4 d after weaning until they were 180 d old.

Measurement of serum hormone concentrations

Blood samples were centrifuged to obtain serum, which was individually kept at −20°C until assay. All measurements were performed in one assay. Serum corticosterone concentration was measured using a specific commercial RIA kit (ICN Biomedicals, Inc.) with an assay sensitivity of 50 ng/ml and an intra-assay CV of 7 %.

Quantification of tissue catecholamine content

Right adrenal glands were homogenised in 10 % acetic acid and centrifuged (1120 × g, 5 min), and supernatants were kept frozen for later analysis. Total catecholamines (adrenaline and noradrenaline) were quantified by the trihydroxyindole method(13). Adrenaline was used as the standard. Briefly, 50 μl

<table>
<thead>
<tr>
<th>Table 1. Composition of the control, flaxseed and standard diets</th>
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<tr>
<td>Ingredients (%)</td>
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<tr>
<td>Control diet</td>
</tr>
<tr>
<td>Casein†</td>
</tr>
<tr>
<td>Flaxseed‡</td>
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<tr>
<td>Soyabean + wheat</td>
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<tr>
<td>Maize starch</td>
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<tr>
<td>Sucrose</td>
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<td>Mineral mix§</td>
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<td>Soyabean oil</td>
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<td>Fibre</td>
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<tr>
<td>Choline bitartrate</td>
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<td>L-Cystine</td>
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Macronutrient composition (100 g/diet)

| Protein         | 17.35         | 17.34          | 23.00       |
| Carbohydrate    | 52.12         | 54.33          | 66.00       |
| Fat             | 7.64          | 10.57          | 11.00       |

Total energy (kJ/100 g)

| Control diet    | Flaxseed diet | Standard diet* |
| 1451.62         | 1601.77       | 1704.70       |

* Standard diet for rats (Nuvilab; Nuvital Nutrientes).
† Commercial casein was acquired from Kauffman Company.
‡ Armazen Produtos Naturais LTDA.
§ Formulated to meet the AIN-93G recommendations.
of the standard supernatant were mixed with 250 μl of 0.5 M-buffer phosphate (pH 7.0) and 25 μl of 0.5% potassium ferricyanate, followed by incubation in an ice bath for 20 min. The reaction was stopped with 500 μl of 60 mg/ml of ascorbic acid per 5 ml-NaOH (1:19). Fluorescence was measured at 420 nm (excitation) and 510 nm (emission) using a fluorometer (Victor 3; PerkinElmer).

Measurement of liver TAG, cholesterol and glycogen contents

Liver samples (50 mg) were homogenised in 1 ml isopropanol (Vetec) and centrifuged (5000 rpm, 10 min, 4°C). Total TAG and cholesterol levels were measured by a colorimetric method using a commercial kit (Bioclin) (14).

The glucose produced by glycogen hydrolysis was measured using a commercial kit (Glucox; Doles). Liver was homogenised with 4 ml TCA (10%). After centrifugation (1000 g, 4°C, 10 min), 2 ml of supernatant were added to 5 ml of absolute ethanol and frozen. After 24 h, the mixture was centrifuged (1000 g, 4°C, 10 min), and the supernatant was discarded. Glycogen was hydrolysed by boiling the pellet for 30 min with 1 M-HCl. After addition of 1 ml of 1M-NaOH to neutralise the mixture, glucose was measured in 200 μl of supernatant as described previously (15).

Western blot analysis

For Western blot assays, samples of the liver, hypothalamus and pituitary gland were taken from rats and immediately frozen in liquid N₂ (10). Briefly, to obtain cell extracts, tissues were homogenised in ice-cold lysis buffer (50 mM-HEPES, 1 mM-MgCl₂, 10 mM-EDTA, Triton X-100 1 %, pH 6.4) containing the following protease inhibitors: 10 μg/ml of aprotinin; 10 μg/ml of leupeptin; 2 μg/ml of pepstatin; 1 μg/ml-phenylmethylsulfonyl fluoride (Sigma-Aldrich). β-Actin was used as an internal control. The total protein content in homogenates was determined by the BCA protein kit assay (Thermo Scientific), and cell lysates were denatured in sample buffer (50 mM-Tris–HCl, pH 6.8, 1 % SDS, 5 % 2-mercaptoethanol, 10 % glycerol, 0.001 % bromophenol blue) and heated at 95°C for 5 min. Samples (30 μg total protein) were separated by 10 % SDS-PAGE and transferred to polyvinylidene fluoride membranes (Hybond-P; Amersham Pharmacia Biotech). Molecular-weight markers (Amersham Biosciences) were run in parallel. Membranes were blocked with 5 % non-fat milk in Tween-Tris-buffered saline solution (20 mM-Tris–HCl pH 7.5, 500 mM-NaCl, 0.1 %, Tween-20). The following primary antibodies (Santa Cruz Biotechnology, Inc.) were used: anti-ACTH (1:500) in the pituitary gland; anti-CRH (1:500) in the hypothalamus; anti-11β-HSD1 in the liver; anti GR α (1:500) in the liver, pituitary gland and hypothalamus; anti-adrenaline β2 receptor adrenergic receptors (1:500) in the liver. Membranes were then washed three times with Tween-Tris-buffered saline solution (0.1%), followed by incubation for 1 h with an appropriate secondary antibody conjugated with biotin (Santa Cruz Biotechnology, Inc.). Then, the membranes were incubated with streptavidin-conjugated horseradish peroxidase (Caltag Laboratories). All Western blots were allowed to react with horseradish peroxidase substrate (ECLplus; Amersham Pharmacia Biotech) and then exposed to X-ray film for 10 s to 1 min. Images were obtained, and bands were quantified by densitometry using Imagej software and normalised to the bands obtained for β-actin.

Statistical analysis

Data were analysed by the statistical program GraphPad Prism 5 (GraphPad Software, Inc.), and reported as means with their standard errors. Body weight and food intake were analysed using ANOVA. Other parameters were analysed by Student’s unpaired t test. Differences were considered significant at P < 0.05.

Results

After weaning, pups from FLAX mothers had significantly higher body weight at days 34, 38 and 42 (approximately...
14%, $P < 0.05$), on days 46, 50 and 53 (approximately 10%, $P < 0.05$), on day 57 (approximately 15.5%, $P < 0.05$) and on days 84, 116, 128 and 144 (approximately 9%, $P < 0.05$) (Fig. 1(a)). Similarly, pups from FLAX mothers had higher food intake at days 27–53 and days 72–132 (approximately 10%, $P < 0.05$; Fig. 1(b)). No changes were observed in weight gain between pups from FLAX and CON mothers (Fig. 1(c)).

The adult offspring from FLAX mothers exhibited higher serum corticosterone (+48%, $P < 0.05$; Fig. 2(a)) and lower adrenal catecholamine (−23%, $P < 0.05$; Fig. 2(b)) contents compared with the offspring from CON mothers.

The offspring from FLAX mothers exhibited lower glycogen (−30%, $P < 0.05$; Fig. 3(a)), higher cholesterol (4-fold increase, $P < 0.05$; Fig. 3(b)) and higher TAG (3-fold increase, $P < 0.05$; Fig. 3(c)) contents in the liver compared with the offspring from CON mothers. A higher expression level of hepatic 11β-HSD1 (62%, $P < 0.05$) was observed in these offspring; however, there were no changes in hepatic GR-α expression and ADR-B3 (Fig. 4(a)–(d)).

There was no change in CRH expression in the hypothalamus of the offspring from FLAX mothers, but there was a higher expression level of GR-α (2-fold increase, $P < 0.05$; Fig. 5). However, lower ACTH expression (−34%, $P < 0.05$) was found in the pituitary gland of the offspring from FLAX mothers, but no changes in GR-α expression were observed (Fig. 6).

**Discussion**

Flaxseed was added to the diet because it is usually ingested by humans in their food as part of a healthy lifestyle to provide fibre and α-linolenic acid, especially during pregnancy, when fibre intake might be increased from 25 to 28 g/d, according to the United States Food and Nutrition Board (Food and Nutrition Board, 2005). The maternal flaxseed diet that we used here during lactation had flaxseed as the exclusive source of oil and fibre and represented a dose of 25% flaxseed in the diet. This dose was based on a previous experimental study conducted only during gestation or from gestation until 90 d old, which used 20–40% flaxseed (6). Our experimental animals received roughly one-quarter of their diet as flaxseed, i.e. about 8.8% oil, 5% protein, 5% carbohydrate, and 5% fibre exclusively from flaxseed. For women who eat about 800 g of food per d, this would represent...
200 g of flaxseed and would be equivalent to about nine spoonfuls of flaxseed per d. This amount is easily reached if women ate three spoonfuls of flaxseed with each main meal. Daily consumption of 200 g of flaxseed would represent 20% of the recommended daily fibre intake (25 g/d) for women, and this amount was used in our previous experimental studies showing negative health effects in pups when they become adults\(^8\)–\(^{10}\). At adulthood, male offspring whose mothers received dietary flaxseed (25%) exhibited higher body weight despite lower body visceral fat. These rats also exhibited lower total serum cholesterol and TAG concentrations. Thus, we can only suggest that serum catecholamine production or higher secretion. In the liver, the lower glyco- gen content can be due to higher catecholamine action, as corticosterone is known to increase liver glycogen concentra- tion\(^{18}\). No change was observed in the expression of ADR\(\beta_2\) in the liver. One limitation of the present study was that we did not measure serum catecholamine concentrations. Thus, we can only suggest that serum catecholamine concentration could be higher due to the observed reduced concentration of liver glyco- gen.

Regarding the HPA axis, the negative feedback is disrupted at least in the hypothalamus, as high corticosterone levels suppress CRH; on the contrary, we found that despite the higher expression of GR-\(\alpha\), CRH was normal. In the pituitary gland, high serum corticosterone concentrations decreased the expression of ACTH, albeit GR-\(\alpha\) expression was normal.

The regulation of GR-\(\alpha\) by glucocorticoids is not straightforward. The pioneer study of Burnstein et al.\(^\text{19}\) reported a down-regulation in cultured lymphocytic cells. However, another study\(^\text{20}\) showed exactly the opposite, i.e. an up-regulation of the promoter of the human GR-\(\alpha\) gene in immature T-lymphocytes. Furthermore, a more recent study\(^\text{21}\) showed that corticosterone up-regulates both the nuclear content of GR-\(\alpha\) (translocation) and DNA binding in the hypothalamus, but not in the pituitary gland. Thus, the fact that we showed higher hypothalamic GR-\(\alpha\) expression in the offspring from FLAX mothers could be consistent with these previous reports. However, it is puzzling how corticosterone did not decrease the levels of CRH, suggesting hypothalamic corticosterone resistance or the action of other

Fig. 4. (a) 11-\(\beta\)-Hydroxysteroid dehydrogenase type 1 (11\(\beta\)-HSD1), (b) adrenaline \(\beta_2\) receptor (ADR\(\beta_2\)) and (c) glucocorticoid receptor-\(\alpha\) (GR-\(\alpha\)) protein expression in the liver of the offspring at 180 d of age whose mothers were fed either a control (C) or flaxseed (F) diet during lactation. The analysis was con- ducted by Western blot and expressed as arbitrary units (a.u). \(\beta\)-Actin was loaded as a control, and data were normalised to \(\beta\)-actin by densitometry. (d) Repre- sentative bands are shown. Values are means of eight rats per group, with standard errors represented by vertical bars. * Mean value was significantly d ifferent from that of the control group (\(P<0.05\)).

These data also help to explain why these animals exhibited hyperinsulinaemia and increased insulin resistance\(^8\).

Maternal flaxseed intake during lactation results in small differences in body weight and food intake in the adult off- spring. The maternal flaxseed diet induces long-lasting changes in adrenal function. We observed higher serum corticosterone levels in the offspring from FLAX mothers, which can be due, at least in part, to an increase in hepatic 11\(\beta\)-HSD1 expression. The liver constitutes an important site for the intracellular production of glucocorticoids due to the conversion of 11-dehydrocorticosterone into the active hormone corticosterone to supply local demand\(^\text{11}\). 11\(\beta\)-HSD has a lipogenic activity. Chapagain et al.\(^\text{17}\) showed that high 11\(\beta\)HSD content and activity are associated with increased hepatic expression of lipogenic genes as well as increased insulin resistance. This helps to explain the association of higher hepatic TAG and cholesterol contents with increased expression of 11\(\beta\)-HSD1 in adult FLAX offspring\(^\text{17}\).
stimulatory factors on CRH, such as catecholamines, serotonin, acetylcholine and the cytokines IL-1 and IL-6\(^{22}\). However, we cannot overemphasise the measurement of GR-\(\alpha\), as it was evaluated in the total pituitary gland and hypothalamus, which may not reflect the expression in corticotroph cells and in the paraventricular nucleus, respectively.

Proulx et al.\(^{23}\) showed that rats exposed to high leptin concentrations during the first 10 d of life exhibited a high ACTH response to dexamethasone and higher GR-\(\alpha\) expression in the brain. Previously, we demonstrated that pups whose mothers received a flaxseed diet during lactation had hyperleptinaemia at weaning\(^{8}\). According to that study, we expect that GR-\(\alpha\) expression remained elevated at least in the hypothalamus. Other factors, such as somatostatin\(^{24}\), dopamine\(^{25}\) and oxytocin\(^{26}\), may suppress the levels of ACTH and could be increased in these programmed animals; however, we did not evaluate these neurohormones. Interestingly, for example, the level of dopamine can be increased in depressive patients\(^{27}\). This situation of intense stress has been associated with glucocorticoid resistance in the hypothalamus that could explain feedback disruption.

It is known that intra-pituitary and intra-hypothalamic corticotrophin-binding globulin (CBG) (transcortin) is important for genomic and non-genomic glucocorticoid effects. Since flaxseed contains phyto-oestrogens that may permanently affect CBG synthesis, it is possible that even at high corticosterone levels, the amount of free corticosterone at the cellular level may be differentially affected in the hypothalamus and pituitary gland, helping to explain why corticosterone seems to better regulate the expression levels of pituitary ACTH but not CRH\(^{28–30}\).

Some substances, such as arachidonate, could have a negative impact on GR-\(\alpha\) through a possibly direct but weak binding at sites different from steroid binding sites on receptor

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**Fig. 5.** (a) Corticotropin-releasing hormone (CRH) and (b) glucocorticoid receptor-\(\alpha\) (GR-\(\alpha\)) protein expression in the hypothalamus of the offspring at 180 d of age whose mothers were fed either a control (C) or flaxseed (F) diet during lactation. The analysis was conducted by Western blot and expressed as arbitrary units (a.u.). \(\beta\)-Actin was loaded as a control, and data were normalised to \(\beta\)-actin by densitometry. (c) Representative bands are shown. Values are means of eight rats per group, with standard errors represented by vertical bars. * Mean value was significantly different from that of the control group (\(P<0.05\)).

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**Fig. 6.** (a) Adrenocorticotropic hormone (ACTH) and (b) glucocorticoid receptor-\(\alpha\) (GR-\(\alpha\)) protein expression in the pituitary gland of the offspring at 180 d of age whose mothers were fed either a control (C) or flaxseed (F) diet during lactation. The analysis was conducted by Western blot and expressed as arbitrary units (a.u.). \(\beta\)-Actin was loaded as a control, and data were normalised to \(\beta\)-actin by densitometry. (c) Representative bands are shown. Values are means of eight rats per group, with standard errors represented by vertical bars. * Mean value was significantly different from that of the control group (\(P<0.05\)).
molecules. Gomez-Pinilla & Ying demonstrated that dietary DHA supplementation has differential effects on several of these class proteins. In these experiments, the DHA diet reduced the levels of glucocorticoid receptors in the hypothalamus. However, in the present study, whole flaxseed had a higher amount of n-3 in the form of α-linolenic acid, which can convert into EPA and DHA; however, this increase in DHA levels did not decrease the GR-α content in the hypothalamus. Previously, Ma et al. demonstrated that ovarioctomised mice treated with SDG for 21 d prevented the increase in the HPA axis response to chronic mild stress. Therefore, in the present study, SDG suppressed only the increase in the HPA axis response to chronic mild stress.

The present findings suggest that the maternal intake of flaxseed through the diet during lactation leads to changes in adrenal function in the adult life of the offspring. Nutritional imprinting factors could programme the offspring for hypercorticosteronaemia and perhaps for higher adrenal catecholamine secretion. Thus, during the critical period of lactation, it is advised that women restrict their intake of flaxseed to prevent future modification of adrenal function in their progeny.

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The authors’ contributions are as follows: E. G. d. M. and M. S. F. designed the study and wrote the manuscript; P. C. L. and E. d. O. revised the manuscript; M. S. F. and E. P. S. d. C. were responsible for animal programming, and biochemical and hormonal analysis. All authors contributed to and approved the final manuscript.

The authors declare that they have no conflicts of interest.

References


