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Mouse Plasma Progesterone Levels are Affected by Different Dietary ω6/ω3 Ratios

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Key words
- reproduction
- hormones
- oocyte
- dietary lipids
- polyunsaturated fatty acids

Abstract

An imbalance in the dietary polyunsaturated fatty acids (PUFAs) ω6/ω3 ratio, could influence negatively the reproductive performance. The aim of the study was to assess the effects of chronic administration of diets enriched with soybean or sunflower oils with different ω6/ω3 ratios on the reproductive parameters of adult female mice. Mice were fed different diets for 90 days: a commercial diet (CD), a 5 or 10% soy oil-enriched diet (SOD5 and SOD10, respectively), and a 5 or 10% sunflower oil-enriched diet (SFOD5 and SFOD10, respectively). The parameters evaluated were: body weight and food intake, estrous cycle, plasma progesterone concentration, ovulation rate, and oocyte quality. Progesterone concentrations (ng/ml) were significantly higher in the SFOD10: 14.9±2.8 vs CD: 5.4±1.2; SOD5: 5.6±1.1 and SFOD5: 4.6±1.4. Additional parameters evaluated were not affected. However, metestrous and luteal phases were shorter in subjects receiving SOD and longer in those under SFOD diets. In SFOD, there was a trend towards a smaller number of recruited oocytes compared to CD and SOD and a higher percentage of cleaved oocytes were quantified in SOD diets. A 3-month supply of a diet with elevated LA ω6/ALA ω3 ratio to adult female mice affects their reproductive physiology, modifying progesterone production, ovulation rate, and/or oocyte quality. Although some differences in the response to diets have been observed in several mammalian species, the present findings must be taken into consideration when a diet for optimizing reproductive capability is indicated.

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Introduction

There are no doubts about the potential impact of nutritional factors on the reproductive ability of males and females, both in humans as in other mammal species [1–6]. Close links were described among energy metabolism, nutritional status, and reproductive physiology [7]. Linoleic acid (LA ω6) and α-linolenic acid (ALA ω3) are essential fatty acids that can only be incorporated through the diet. LA is abundant in vegetable oils such as safflower, sunflower, corn, and soybean. However, only some vegetable oils such as chia and flaxseed contain appreciable amounts of ALA (50–60% or higher), whereas canola and soybean oils have lower contents (no more than 10%) [8,9].

Some metabolites derived from LA and ALA influence reproductive processes. The proportion of different polyunsaturated fatty acids (PUFAs) in the cell membranes reflects the amount in which they are consumed in the diet; therefore, its manipulation may affect the composition of these membranes and modify some processes involved in reproduction, such as the biosynthesis of eicosanoids and steroids [7,10,11].

The Joint Expert Consultation FAO/OMS recommends that the LA ω6/ALA ω3 ratio in the diet should be between 5:1 and 10:1 in humans [12]. Other authors suggest that humans evolved on a diet with a ω6/ω3 FA ratio of 1:1; however, this ratio is currently 10:1 to 20–25:1 in Western diets, indicating that they are deficient in ω3 fatty acids [11,13,14].

The eicosanoids synthesized by the cells depends on the diet LA ω6/ALA ω3 ratio and have different biological functions, being affected by the LA/ALA ratio of each diet [7,8,10,11,15,16].

Arachidonic acid (AA) acts as precursor of some eicosanoids such as prostaglandins E2 and $F_2\alpha$ (PGE_2, $PGF_{2\alpha}$), which are highly concentrated in the preovulatory follicular fluid and have significant implications on ovulation and regulation of luteolysis. It is well known that the luteinizing hormone surge triggers some reactions involved

Nutrient/Diet	CD	SOD5	SFOD5	SOD10	SFOD10
Carbohydrates (g/100 g)	42.00*	39.90	39.90	37.80	37.80
Proteins (g/100 g)	18.00*	17.10	17.10	16.20	16.20
Fats (g/100 g)	3.90*	8.59	8.68	13.28	13.45
Saturated fatty acids**	0.91	1.71	1.44	2.49	1.93
Monounsaturated fatty acids**	1.29	2.31	2.52	3.33	3.74
Polyunsaturated fatty acids**	1.67	4.57	4.73	7.46	7.77
Linoleic acid 18:2 ω6	1.59	4.14	4.64	6.68	7.68
Linolenic acid 18:3 ω3	0.08	0.43	0.09	0.78	0.09
ω6/ω3 ratio	19.10	9.55	54.06	8.53	87.17

Table 1 Diet composition.

CD: Control diet; SOD5, SOD10: Diets enriched with 5–10% soybean oil, respectively; SFOD5, SFOD10: Diets enriched with 5–10% sunflower oil, respectively.

Source: * GEPSA Feeds, Grupo Pilar SA. Mouse-rat autoclavable. ** The quantifications were performed by gas chromatography in the Departmento de Química Biológica, Facultad de Ciencias Agropecuarias, Universidad Nacional de Córdoba.

in ovulation, such as stimulation of cyclooxygenase-2, which increases the synthesis of PGE_2 and $PGF_{2\alpha}$ resulting in different and complementary effects on ovulation.

PGE_2 causes collagenolysis, which in turn, induces follicular rupture. In female mice, it increases the synthesis of hyaluronic acid, the expansion of the cumulus oophorus, and oocyte maturation. On the other hand, $PGF_{2\alpha}$ stimulates the smooth muscle fibers of the ovary, which, activated by oxytocin, promote follicle breakdown [7,17–21].

While more than 2000 papers have been published focusing on how essential fatty acids and their metabolites could affect the reproductive function, the evidences that ω3 and ω6 PUFAs have differential effects on ovarian function, oocytes, and embryo quality are inconsistent [7,11,22,23]. Preliminary results obtained in our laboratory showed that neither corpora lutea nor fetuses were found at gestational day 17 in female mice fed with control diet enriched with 10% soybean oil and fertilized "in vivo" by untreated males; therefore, we suggested that these diets would modify the luteal phase (equivalent to metestrous in rodents), whose main purpose is to prepare the uterus to receive fertilized oocytes. In addition, the longest surge of progesterone occurs in the metaestrus morning [24]. Based on those preliminary results, we can predict that the intake of diets enriched with sunflower or soybean oils, with different ω6/ω3 ratios, are capable to modify the reproductive function of female mice due to alterations in plasmatic progesterone concentrations and/or in gamete functional quality.

Materials and Methods

All experiments were conducted in accordance with the Animal Care and Use Guidelines of the Medical School – National University of Cordoba, Argentina.

Adult *Swiss albino* female mice (60–70 days old) were used (n=79). Animals were maintained under a 14:00/10:00 light/dark cycle at 22±3 °C with food and water provided *ad libitum*. The animals were housed in groups of 5 mice, in cages of 22 w×30 l×9 h cm, with wood shavings as bedding material.

Diets

The base of the different diets used in this study was a commercial mice pelleted food (Gepsa Feeds, Grupo Pilar, Córdoba, Argentina). Concentrations of daidzein (D) and genistein (G) were 6.5±1.3 (mg/kg) and 20.3±1 (mg/kg), respectively (compounds concentrations were calculated in triplicate and the mean value calculated in each case); isoflavones were identified and quantified by HPLC-ESI-MS/MS [25,26]. Considering the possibility that dietary phytoestrogens could potentially have an interaction and modify results, careful attention should be given to the content of these compounds provided in the diets. In the diets used in the present study, the levels of phytoestrogens were considerably lower than those used by other authors [27] where the lowest proportion of G+D in 5 g of diet/day/animal was 0.4 mg, whereas in ours it reaches a value of 0.134 mg.

Mice were randomly allocated in 5 groups of 15–17 animals and fed for a period of 90 days with the following diets: control (CD), CD enriched with 5 or 10% soybean oil (SOD5 and SOD10, respectively), and CD enriched with 5 or 10% sunflower oil (SFOD5 and SFOD10, respectively).

SOD 5–10 and SFOD 5–10 were prepared with the addition of 5–10 g of commercial soy oil (Sojola, 100% pure soy oil, Aceitera General Deheza, Córdoba, Argentina) or sunflower oil (Natura, 100% pure sunflower oil, Aceitera General Deheza, Córdoba, Argentina) to 90–95 g of pelleted CD, respectively. To prevent oxidation, oils were mixed with butylhydroxytoluene (2 g/l) [28]; diets were prepared weekly and stored at 4 °C. A fresh amount of diet was provided to the animals on a daily basis to protect against oxidation. The composition of the diets is shown in **Table 1**.

Food intake and body weight

Throughout the experimental period, food consumed by the animals in each cage was daily weighed and the individual average consumption per treatment was calculated by dividing the total amount of food consumed by the number of animals in each cage. Animals were weighed twice a week using an electronic balance with an accuracy of ±1 mg (Mettler Electronics Corp., USA).

Reproductive parameters

Characteristics of the estrous cycle

After a 2-month treatment, vaginal smears were performed daily for 30 days by means of vaginal exfoliative cytology. The reproductive status was classified in the following phases: proestrous, estrous, metestrous, and diestrous [29,30].

Plasma progesterone concentration

At the end of the treatment, 40 animals were sacrificed by decapitation in the metestrous phase; the blood was collected in heparinized tubes and centrifuged at 420 g for 30 min. The supernatant was separated and stored at −20 °C until processing [31]. Progesterone determinations were performed using a commercial ^{125}I-progesterone radioimmunoassay kit (Progesterone, Sie-

mens, CA, USA). The antiserum for progesterone had less than 4% cross-reactivity compared to other steroids, except for 5α-pregnan-3,20-dione (9%) in serum, plasma or urine (data provided by the company). The kit was provided with human serum-based calibrators and the assay sensitivity was 0.1–40 ng/ml. Intra-assay coefficient of variation was 7.82±0.98%. All samples were assayed on the same day and therefore, there was no inter-assay coefficient of variation.

Oocyte quality

At the end of the treatment, superovulation was induced with an intraperitoneal injection of 5 IU of PMS (Sigma Chemical Co., USA), followed 48 h later by 10 IU of hCG, (Endocorion, ELEA, Argentina). The animals were sacrificed by cervical dislocation 17–18 h after the hCG injection (n=39).The oviducts were then extracted with wide laparotomy and placed in Tyrode's solution [32,33].

The cumulus-oocyte complexes were collected from the oviducts by puncture of the swollen ampulla. Ovulation rate was expressed as the number of oocytes collected from each ampulla/animal. After removal of the cumulus oophorus with hyaluronidase, the percentage of oocytes without a visible germinal vesicle (mature) was evaluated under inverted microscope at 400× magnification [34]; results were expressed as percentage of mature oocytes.

For evaluation of spontaneous activation (parthenogenesis), other oocytes obtained with the same technique were suspended in 1 ml of Tyrode's medium and incubated at 37 °C, with 95% air and 5% CO_2 for 24 h. The analysis was performed using an inverted microscope at 400× magnification and the results were expressed as percentages of nonactivated or activated ova in each group; activated ova were classified as pronuclear stage, 2 cells or more than 2 cells. The results were expressed as percentages of activated oocytes [35].

Statistical analysis

Values were expressed as mean±standard error of the mean. Results were analyzed using a one-way analysis of variance or Kruskal-Wallis test, as appropriate. The Tukey's test was used for the post hoc comparisons, statistical significance was set at $p<0.05$. Correlation analysis was performed to determine the degree of association between the most relevant variables. Pearson's correlation coefficient was used when variables were normal and Spearman when they were not. In addition, we applied a linear regression analysis either by treatment or by group, to observe the trend slope of the weight curves, which were subsequently compared using ANOVA. Statistical analysis was performed using the Infostat 1.1 software program (Grupo Infostat, Facultad de Ciencias Agropecuarias, Universidad Nacional de Córdoba, Argentina).

Results

Body weight and food intake

Initial body weight was 29.29±0.45 g (n=79). The differences between final and initial weight of each group were CD=1.28±0.57 g (n=17); SOD5=2.10±0.65 g (n=16); SFOD5=1.70±0.57 g (n=15); SOD10=2.38±0.66 g (n=16); SFOD10=1.35±0.42 g (n=15). As regards to food intake, differences between weeks 14 and 1 in each group were: CD=−0.01±0.64 g; SOD5=−0.13±0.49 g; SFOD5=0.06±0.12 g; SOD10=−0.24±0.38 g; SFOD10=0.03±0.44 g. No significant differences were found in these parameters.

Characteristics of the estrous cycles

The length and number of estrous cycles in the different experimental conditions were not significantly different (**Table 2**). When all stages of the estrous cycle were analyzed, the metestrous or the luteal phases were shorter in SOD5 and SOD10 (0.94±0.13 and 0.90±0.17 days, respectively) and longer in SFOD5 and SFOD10 (1.29±0.33 and 1.28±0.27 days, respectively).

Plasma progesterone concentrations (ng/ml)

Plasma progesterone concentrations were significantly higher in SFOD10 when compared to CD, SFOD5, and SOD5 (**Fig. 1**). A positive correlation (rs=0.72, p=0.04) between progesterone concentrations and cycle length was detected in SOD10.

Ovulation rate and oocyte quality

In animals fed SFOD (5 or 10%), a trend was detected towards a smaller number of recruited oocytes compared to CD and SOD (5 or 10%); on the other hand, a higher percentage of cleaved oocytes was quantified in mice fed with SOD diets (**Table 3**). Oocyte

Table 2 Characteristics of the estrous cycles in *Swiss albino* mice (n=79) evaluated after receiving different diets for 2 months.

	n	Cycle length (days)	Number of cycles
CD	17	9.32±0.59	4.12±0.35
SOD 5	16	8.30±0.52	4.50±0.41
SFOD5	15	9.84±0.66	4.07±0.34
SOD10	16	9.26±0.63	4.56±0.34
SFOD10	15	9.83±0.69	3.87±0.32

CD: Control diet; SOD5, SOD10: Diets enriched with 5–10% soybean oil, respectively; SFOD5, SFOD10: Diets enriched with 5–10% sunflower oil, respectively. Mean values±SEM were obtained over a period of 41±8 days. n=number of animals.

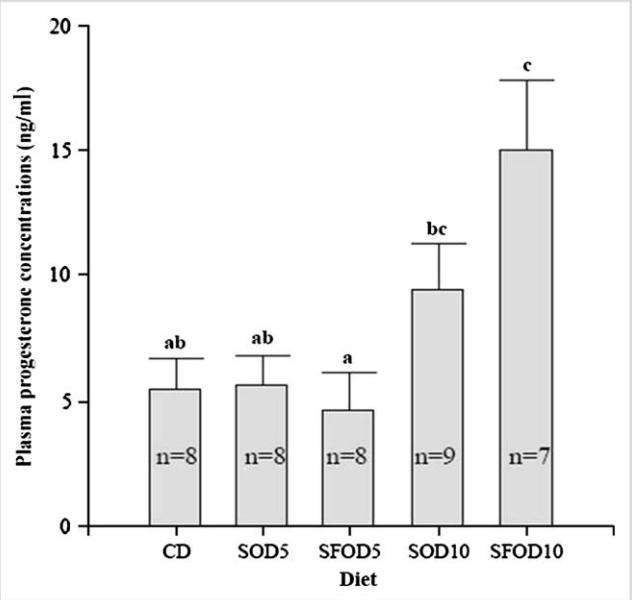

Fig. 1 Plasma progesterone concentrations quantified at metestrous in adult female *Swiss albino* mice fed different diets for 90 days. CD: control diet; SOD5, SOD10: Diets enriched with 5–10% soybean oil, respectively; SFOD5, SFOD10: Diets enriched with 5–10% sunflower oil, respectively. Results are expressed as mean values±SEM. n=number of animals. Different letters indicate significant differences ($p<0.05$).

Table 3 Ovulation rate and percentage of cleaved oocytes in superovulated adult Swiss albino mice fed different diets for a period of 90 days.

	n	Ovulation rate	n	Cleaved oocytes (%)
CD	9	20.67 ± 4.22	154	8.79 ± 4.77
SOD5	8	22.63 ± 2.82	140	26.68 ± 8.32
SFOD5	7	16.29 ± 2.35	94	8.57 ± 5.95
SOD10	7	22.71 ± 3.94	123	21.53 ± 6.13
SFOD10	8	17.75 ± 3.16	113	10.77 ± 6.37

CD: Control diet; SOD5, SOD10: Diets enriched with 5–10% soybean oil, respectively; SFOD5, SFOD10: Diets enriched with 5–10% sunflower oil, respectively. Results are expressed as mean ± SEM. n = number of animals for ovulation rate and total number of cleaved oocytes.

maturation, estimated by the absence of germinal vesicles, was not affected by the treatments used herein (results not shown).

Discussion

The benefits of ω3 PUFAs consumption in human and animal models have been demonstrated, especially for the treatment of cardiovascular diseases and other chronic diseases [8,11,16,35–37]. In our experimental model, the effects of chronic administration of diets enriched with soybean or sunflower oils at different proportions were analyzed in adult female mice. In accordance with previous reports, no significant differences were detected in body weight and food intake [5]. Kesey and Hirvonen [38] have proposed the existence of a "set point" for body weight in rodents and humans, through which any rise or reduction of this parameter is corrected by modifying intake and/or energy expenditure.

Studies conducted in cattle, sheep and other mammals have shown that the composition and content of PUFAs in the diet can modify the number and size of ovarian follicles, ovulation rate, oocyte maturation, permanence of the corpora lutea and progesterone production, as well as gestation length [7,10,18,39]. The dietary supplementation of fish oil [source of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)] has been proposed, not only to improve fertility in cattle and sheep, but also to reduce the rate of premature births in women; however, the results are controversial [7,18,24] because it has also been reported that PUFAs can adversely affect oocyte mitochondrial metabolism and embryo development in mice [40]. In our study, we quantified progesterone levels considering previous preliminary results where female mice fed with diets enriched with sunflower or corn oil (10%) were infertile, as it was described in the Introduction. For this reason, we can suggest that these diets would modify the luteal phase, equivalent to the rodent metestrous, in which the main function is to prepare the uterus to receive fertilized oocytes.

In addition, we based our analysis on previous reports suggesting that the pattern of progesterone secretion consists of 2 major surges, one that occurs during late afternoon of proestrus and the other in the morning of metestrus, the second one being a prolonged version [24].

Animals fed SOD10 and SFOD10 exhibited the highest concentration of plasma progesterone in the metestrous phase, this rise being even higher in SFOD10 and yielding the highest LA ω6 contents. Smaller increases in progesterone concentrations were found in SOD10, compared with SFOD10. This could be due to the enzymatic competition (desaturases and elongases) that arises from the presence of ALA in SOD10 [8,11,14,41]. In CD, SFOD5 and SOD5, our results were in accordance to the data previously reported [42].

The increase of progesterone in both 10% enriched diets was in agreement with several previous investigations, most of them in cattle and sheep fed with diets containing high amounts of fat provided by PUFAs, mainly LA ω6. High concentrations of plasma progesterone have been reported for the luteal phase of the estrous cycle or in the follicular fluid, with a simultaneous increase of total cholesterol and/or high density lipoprotein (HDL) in plasma of cows, holstein heifers, and ewes [43–48]. Cattle and sheep, like rodents, have higher amounts of circulating HDL, which provide cholesterol to steroidogenic tissues [49–52]. The increase of plasmatic progesterone concentrations in our experimental animals could be the result of an increase of cholesterol as substrate for ovarian steroidal synthesis; this mechanism has been reported in cattle and sheep, related to a reduced clearance of progesterone [10,18,43,45,52–54] or to several intracellular mechanisms that modify steroidogenesis [9,47,49]. Moreover, it is well known that diets rich in AL ω6 produce an increase of AA in membrane phospholipids. Some evidences support that an increase of AA can modify steroidogenesis by stimulating the transcription of factors for steroidogenic acute regulatory protein (StAR) expression at nuclear level, with a critical role in progesterone synthesis [7,55–57]. The absolute amounts of LA ω6 contained in each of the 10% enriched diets could be responsible for the increase of plasmatic progesterone concentration.

It is well known that PGE_2 is the major eicosanoid involved in follicular development and the mechanisms that trigger ovulation [17,18,20,21]. Rats fed on diets enriched with ω6 FAs had an excessive production of prostaglandin with an inhibitory effect on ovulation rates [58].This could be due to a downregulation mechanism [10,59] and may explain the trends observed in this study. The SFOD5 and SFOD10 groups (both with higher ω6/ω3 ratios) had the lowest, although not significant, ovulation rates. By the same mechanism, the action of $PGF_{2\alpha}$ could be reduced and may delay luteolysis, thereby contributing to the high plasmatic progesterone concentrations found mainly in SFOD10, and probably to an extension of the estrous cycle because of a prolonged luteal phase.

In animals fed SOD5 or SOD10 diets, the estrous cycles were slightly shorter, which could be attributed to a possible accelerated recruitment of oocytes. This is consistent with the higher percentage of oocytes cleaved spontaneously. It is possible that the amount of ALA in SOD-fed animals is enough to increase oocyte recruitment. It should also be noticed that these diets have a ω6/ω3 ratio, closer to values considered optimal. It could be inferred that these diets would predispose to a better reproductive performance since in species in which mating depends on female endocrine state, shorter cycles ensure better chances of reproductive success. However, this feature could affect oocyte quality, as reflected in an increased number of spontaneous cleavages. As these differences were not statistically significant, further experiments are required to confirm these trends.

Soybean meal is an ingredient common to standard natural diets, which contains a class of phytoestrogens called isoflavones. The 2 primary isoflavones are genistein and daidzein and they are present in the original pellets (control diet) which are provided to all animals in every experimental group. According to the literature, vegetable oils are not a source of isoflavones [60,61]. We should acknowledge that with the present experi-

mental design, we cannot clearly determine if the isoflavones may or may not have interacting effects with the fatty acids added to the diets. In this aspect, the available literature describes contradictory results depending on the proportion of PUFA added to the diets, the target tissue and the experimental model [62–64]. The amounts of these compounds in both enriched diets used in the present study were clearly similar to those in the control diet, or even lower because of the proportion of pelleted food used to prepare the SOD and SFOD diets. The content of these compounds in our diets was lower than those reported in other standard diets [27]. Therefore, we consider that the effects found in our study could not be attributed to the presence of isoflavones which were present in all the employed diets.

Finally, in Western societies the reduced consumption of ω3 fatty acids sources and the high intake of vegetable oils rich in ω6 are responsible for the ω6/ω3 altered ratio. Fatty acids must be supplied in adequate amounts and their natural sources should be carefully assessed in order to achieve balance, according to current recommendations.

Conclusions

A 3-month supply of LA enriched diets with reduced ALA content and elevated LA ω6/ALA ω3 ratio to adult female mice, affects reproductive physiology modifying progesterone production, ovulation rate, and/or oocyte quality. Although different mammal species respond differently to diets, these findings must be born in mind when diets are designed to optimize reproductive capability.

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Conflict of Interest

The authors declare that they have no conflicts of interest in the authorship or publication of this contribution.

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