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# Effect of Duration of Exposure to Fluoride and Type of Diet on Lipid Parameters and De Novo Lipogenesis

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## Abstract

The effect of duration of chronic treatment with fluoride (F, 50 mg/L as NaF) on the lipid profile, lipid droplets and triglycerides (TG) in liver was evaluated in mice with nonalcoholic fatty liver disease (NAFLD) previously induced by hyperlipidic diet and in animals fed normocaloric diet. In addition, the effect of F administered for a short period (20 days) was evaluated on de novo lipogenesis, by nuclear magnetic resonance. GRP78, Apo-E, and sterol regulatory element-binding protein (SREBP) were quantified by Western blotting. Our data indicate that F interferes in lipid metabolism and lipid droplets, having a different action depending on the exposure time and type of diet administered. F improved lipid parameters and reduced steatosis only when administered for a short period of time (up to 20 days) to animals fed normocaloric diet. However, when NAFLD was already installed, lipid parameters were only slightly improved at 20 days of treatment, but no effect was observed on the degree of steatosis. In addition, lipid profile was in general impaired when the animals were treated with F for 30 days, regardless of the diet. Moreover, F did not alter de novo lipogenesis in animals with installed NAFLD. Furthermore, hyperlipidic diet increased F accumulation in the body. GRP78 increased, while Apo-E and SREBP decreased in the F-treated groups. Our results provide new insights on how F affects lipid metabolism depending on the available energy source.

**Keywords** Fluoride · High-fat diet · Nonalcoholic fatty liver disease · Lipogenesis · NMR

## Introduction

Fluoride (F) is an element naturally found in water. In some sites the concentration is above the recommended limits, which can cause deleterious effects among which the most known is fluorosis that can be dental or skeletal [1, 2].

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Experimental studies where F is chronically administered to animals in high doses have reported alterations involving different tissues such as liver, kidney, muscle, and heart and affecting distinct proteins and enzymes involved in various molecular processes such as oxidative stress [3–11].

Liver is an important target of xenobiotics like F, since it is the main organ responsible for detoxifying the body and has a high metabolic activity, besides neutralizing and eliminating toxic substances [12, 13]. In addition, liver is responsible for lipid metabolism. Problems in the homeostasis of lipids, arising from an instability in external lipid absorption or biosynthesis of internal lipids, result in the generation of lipid inclusions in the liver [14].

Recent studies of our research group showed that male Wistar rats fed hypercaloric diet and treated with water containing 50 mg/L F presented changes in the proteomic profile in liver [5] and had reduction in plasma triglycerides and lipid droplets in the liver [15]. These lipid droplets are a histological spectrum of nonalcoholic fatty liver disease (NAFLD), which includes several forms of macrovesicular steatosis in the form of small and large drops, with or without portal triad inflammation, followed by steatohepatitis, characterized by steatosis,

inflammation and cellular injury, or nonalcoholic steatohepatitis [16–19]. This alteration is inwardly related with disturbances in metabolism, particularly those involving oxidative stress and, consequently, lipid peroxidation [20, 21], which, interestingly, are also affected by F [15, 22–24]. A recent study revealed an increase in 78 kDa glucose-regulated protein (GRP78—marker of oxidative stress) and a decrease in apolipoprotein-E (Apo-E—involved in transport and delivery of lipids to the body) in the liver of rats treated with 50 mg/L F and hypercaloric diet [15]. This was possibly related to the reduction in lipid droplets in the liver of animals treated with F and hypercaloric diet [5, 15], since increase in GRP78 inhibits endoplasmic reticulum stress, thus reducing hepatic steatosis [25]. In addition, decrease in Apo-E induced by F reduces the availability of lipoproteins to deliver fat to liver, which also reduces steatosis [26]. The study by Pereira et al. [5] was the first one to report beneficial effects (reduction in lipid droplets in the liver due to alterations in the expression of proteins involved in steatosis) of high dose of F in the liver of animals fed hypercaloric diet. In that study, the animals were treated with F for 60 days. It would be interesting to see if these beneficial effects also occur when the animals are treated with F for shorter periods, since the effects of F have been reported to be time-dependent. In addition, it is important to evaluate if these effects also occur when the animals are fed a normocaloric diet, since some studies report increase in total cholesterol, triglycerides (TG), very low-density lipoprotein (VLDL), or low-density lipoprotein (LDL) when animals are exposed to F and fed a normocaloric diet [27–29].

Thus, our primary aim was to evaluate the effect of duration of chronic treatment with F on the lipid profile, lipid droplets, and TG in liver, in mice with NAFLD previously induced by hyperlipidic diet and in animals fed normocaloric diet. In order to elucidate the mechanisms involved in the alterations induced by F in animals with installed NAFLD or not, de novo lipogenesis (LDN) was evaluated using nuclear magnetic resonance (NMR) and GRP78; Apo-E and sterol regulatory element-binding protein (SREBP) were evaluated by Western blotting.

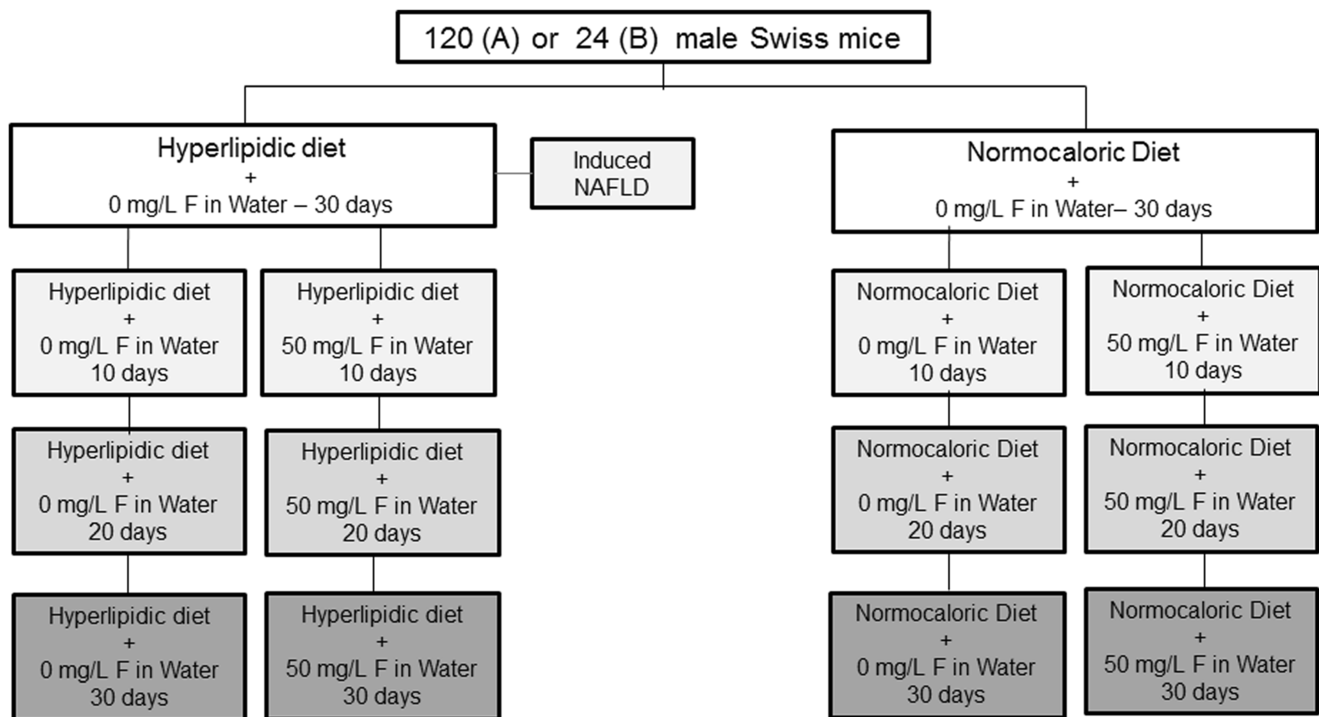
## Materials and Methods

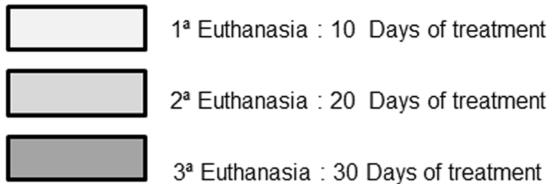
All experimental protocols were approved by the Ethics Committee for Animal Experiments of Bauru Dental School, University of São Paulo (protocol: 001/2014; 008/2015).

The first study, involving different durations of exposure to F, was conducted using Swiss male mice (26–44 g) that were obtained from São Paulo State University (UNESP, Bauru). The animals were housed in the Central Vivarium of Bauru School of Dentistry, University of São Paulo. After an adaptation period of 7 days, the animals were randomly distributed into two groups ($n = 60$/group). One of them received for 30 days an in-house prepared high-fat diet (hyperlipidic semi-purified unbalanced diet with 5350 kcal/kg) [30, 31] for induction of hepatic steatosis (hyperlipidic). The other group received, for the same period, normocaloric diet (Presence®, Purina, 3028 kcal/kg [32] (normocaloric)). After this period, for each type of diet, the animals were divided into two groups ($n = 30$/group), based on the type of drinking water to which the animals had free access: deionized water or water containing 50 mg/L F (as sodium fluoride). In both cases, the diet initially administered was maintained. Furthermore, treatment with F was done during three distinct periods: 10, 20, or 30 days, for each condition (Fig. 1(A)). At the end of the study, the animals were weighed and then received an intramuscular injection of anesthetic and muscle relaxant (ketamine chlorhydrate and xylazine chlorhydrate, respectively). Blood was collected, and plasma was obtained and frozen at − 20 °C until the analysis of TG, HDL, total cholesterol, LDL, VLDL, and F. Liver was also collected. The right lobe was used for histopathological analysis, while the remaining was employed for TG, F, and Western blotting analysis.

The second study, involving LDN and Western blotting analyses, weanling Swiss male mice (body weight ranging between 18 and 27 g) were obtained from Multidisciplinary Center for Biological Research in the Area of Science in Laboratory Animals (CEMIB)-UNICAMP. They were housed in the Central Vivarium of Bauru School of Dentistry, University of São Paulo, and randomly distributed into two groups ($n = 12$/group) that received, for 30 days, high-fat diet (hyperlipidic semi-purified unbalanced diet with 5350 kcal/kg) [30, 32] for induction of NAFLD or normocaloric diet (Presence®, 3.028 kcal/kg) [32]. All the animals were housed in standard cages (three per cage) with chow and water ad libitum. During this period, all animals had free access to deionized water. After this period, for each type of diet, the animals were further divided into two groups ($n = 6$/group), based on the type of drinking water to which they had free access for 20 days: deionized water or water containing 50 mg/L F (as sodium fluoride) (Fig. 1(B)). In both cases, the diet initially administered was maintained. Three days before euthanasia, the animals received by gastro gavage a volume of $^2H_2O$ in order to enrich body water in deuterium around 3% (assuming 75% of body water). For the remainder of the study, 3% $^2H_2O$ was added to the drinking water to keep the body water enrichment throughout the study. At the end of 20 days, the animals were weighed and then received an intramuscular injection of anesthetic and muscle relaxant (ketamine chlorhydrate and xylazine chlorhydrate, respectively). Liver was also analyzed by NMR.

In both studies, the temperature and humidity in the climate-controlled room, which had a 12-h light/dark cycle, were 23 ± 1 °C and 40–80%, respectively. These diets had a low F content (< 2 mg/kg). The administration of drinking water containing 50 mg/L F to mice leads to plasma F levels correspondent to those found in humans consuming water



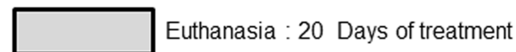




**Fig. 1** Experimental design of the first study (A) and second study (B)

containing ~ 10 mg/L F [31], which occurs in areas of endemic fluorosis [33].

## F Analysis

Liver and plasma were determined after overnight hexamethyldisiloxane (HMDS)-facilitated diffusion [1, 34] using the ion-specific electrode (Orion Research, Model 9409) and a miniature calomel electrode (Accumet #13-620-79) both coupled to a potentiometer (Orion Research, Model EA 940) ($n = 10$) as previously described [15].

## Histological Analysis

### *Histological Processing and Analysis*

The right liver lobules ($n = 10$) were fixed in 10% formalin in phosphate buffer for a week and processed for histology (paraffin embedded). Semi-serial sections were performed at 5 μm using a microtome (Microm, model HM 340 E, Germany). Sections were included in slides and stained with hematoxylin and eosin (HE) using routine histological protocols [35]. An Olympus Upright BX43 binocular microscope was used (Olympus Corporation Microscopes, Tokyo, Japan) to examine and evaluate the histological sections, together with a photographic camera Olympus SC30 (Olympus Corporation Microscopes, Tokyo, Japan) and the Olympus CellSens 1.14 software (Olympus Corporation Microscopes, Tokyo, Japan).

### *Morphometric Analysis*

It was performed blindly, using a Zeiss microscope in 40X magnification with reticular integration (10mm × 10mm). Ten fields were selected by systematic sampling [15, 35] in each lobule ($n = 3$). Nonalcoholic steatosis was classified according to the percentage of lipid droplets, as follows: normal (≤ 12.9%), mild (13.0–37.9%), moderate (38.0–61.3%), or

severe (≥ 61.4%) (adapted from Brunt, Tiniakos [17], with modifications Pereira et al. [15]).

#### *Morphological Analysis*

The morphological analysis comprised evaluation of steatosis per graded score. For this, three blind examiners performed the evaluations and was used a Zeiss microscope in 10X magnification. The qualitative classification of liver lipid content was based in the amount (0 to 5) and size (macro- or microdroplets), as follows: none (0), few microdroplets (1), moderate amount of microdroplets and few macrodroplets (2), many microdroplets and moderate amount of macrodroplets (3), microdroplets and agglomerate macrodroplets (4), and many macrodroplets throughout the tissue (5) [15].

This analysis needs calibration evaluated by kappa ($k$) coefficient. For this, 33% of the samples were evaluated and this was repeated after 15 days for the purpose of intra-examiner calibration. The $k$ coefficients obtained were > 0.8.

### Lipid Profile Analysis

TG quantitation in plasma and liver was determined using a commercial kit (Doles, Belo Horizonte, MG, Brazil). For this, 200 mg of liver tissue from each animal was homogenized in 0.5 mL 0.1% TritonX-100, sonicated for 45 s and centrifuged at 4000 rpm for 10 min, obtaining the supernatant for analysis.

HDL and total cholesterol analysis in plasma were quantified by commercial kit (Doles, Belo Horizonte, MG, Brazil). Determinations of LDL and VLDL were derived using the Friedewald formula [36].

### Extraction of Liver Lipids

We used the method of Folch et al. [37] with some modifications proposed by Christie [38]. The remaining liver was initially weighed and added to 5 mL of methanol per gram of liver. The homogenization was performed for about 5 min (LHNH Uniscience®) under vigorous stirring. After this step, 10 mL of chloroform per gram tissue was added and kept under stirring for 3 min. The mixture was centrifuged, the supernatant separated, and the solid residue subjected to new extraction by addition of 6 mL of 2:1 ($v/v$) chloroform-methanol solution, followed by vigorous homogenization for 5 min. The lipid present in chloroform was obtained by drying (without heating) and preserved for further analysis by NMR.

### Analysis of De Novo Lipogenesis by NMR

The de novo lipogenesis by NMR analysis was conducted in the Department of Life Sciences at the Faculty of Science and Technology, University of Coimbra, Portugal. The lipid samples were dissolved in 525 μL chloroform not deuterated ($CHCl_3$) together with 25 μL of standard deuterated and not deuterated pyrazine (internal standard) in $CHCl_3$ and 50 μL hexafluorobenzene ($C_6F_6$) to capture fluorine lock. For each sample, proton and deuterium spectra were obtained. These spectra allowed the quantification of the total amount of lipids isolated from each liver and also the percentage of deuteration of the same lipid for the purpose of quantifying the de novo lipogenesis process. The spectra were acquired on a Bruker 500 MHz NMR spectrometer using a SE-$^2$H-X probe ($^1$H) 5 mm (type: PH SEX 500S2 $^2$H-H-F-05 Z). For protons, the acquisition of a single transient was enough but for deuterium, accumulation of multiple transients had to be performed to obtain the ratio signal/noise suitable for quantitative analysis.

Typical parameters of acquisition included a pulse reading radio frequency of 45° and a time of interpulse repetition of 5 s to allow relaxation all deuterium both of the lipid sample and of the internal reference (pyrazine). This internal standard, whose amount is known, allowed to quantify absolutely both the total amount of lipid (proton spectrum) as the percentage of de novo lipogenesis, by assessing the percentage of incorporation of deuterium from body water into the units of acetyl-CoA used in the de novo synthesis. Processing of the spectra was done using the NUTSproTM software and deconvolution of the resonances necessary for the quantitative analysis was performed using a subroutine of the same software, which allows a successive iteration between calculated and experimental spectrum, reducing at each step the difference and allowing quantitation of the areas of each resonance and evaluation of multiple spectral parameters, namely widths at half height [39–41].

### Western Blotting Analysis

The Western blotting was performed as previously described [42]. Liver protein extracts were obtained by lysing homogenized tissue in Ripa buffer (0.5% sodium deoxycholate, 0.1% SDS, 1% NP-40) supplemented with protease inhibitors (Roche Diagnostics, Mannheim, Germany). Protein samples (40 μg) were resolved in 10% Tris-HCl polyacrylamide gels and subsequently transferred to a polyvinylidene difluoride (PVDF) membrane. Membranes were probed with commercially available rabbit polyclonal anti-Apo-E (1:500 dilution) (Abcam, Cambridge, MA, USA), anti-GRP-78 (1:500) (Abcam, Cambridge, MA, USA), anti-SREBP-1c (1:500) (Abcam, Cambridge, MA, USA), and anti-α-tubulin (1:2000) (Abcam, Cambridge, MA, USA), followed by HRP-conjugated anti-rabbit antibody (1:10000) for GRP-78, Apo-E, and α-tubulin and for SREBP-1c anti-mouse antibody (1:10000) and ECL Plus detection reagents (GE Biosciences, Piscataway, NJ, USA). Relative Apo-E, GRP-78, α-tubulin, and SREBP band densities were determined by densitometrical analysis using the Image Studio Lite software from LI-COR Corporate Offices-US (Lincoln, NE, USA). In

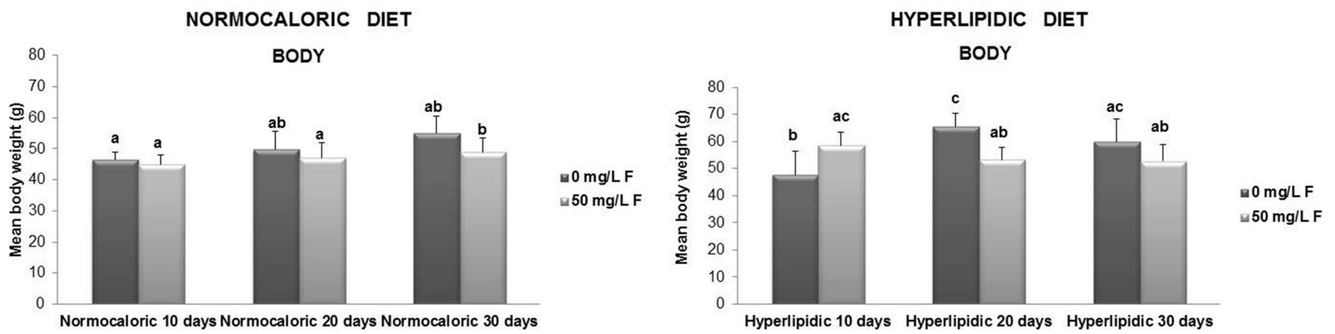



**Fig. 2** Mean body weight of mice fed normocaloric (A) or hyperlipidic (B) diets that received drinking water containing 0 mg/L or 50 mg/L fluoride for 10, 20, or 30 days. For each type of diet, distinct lower case letters indicate significant differences among the groups (two-way ANOVA and Tukey's test, $p < 0.05$). Bars indicate SD. $n = 10$

all instances, density values of bands were corrected by subtraction of the background values. The results were expressed as the ratio of GRP-78, Apo-E, and SREBP-1 to that of α-tubulin.

## Statistical Analysis

The software Statistica (version 10.0 for Windows, StatSoft, Inc., Tulsa, USA, 2011) was used. After checking normality and homogeneity, data were evaluated by two-way ANOVA (experimental time and F concentration as criteria) and Tukey's test, for each type of diet separately. For Western blotting data, the software GraphPad Prism (version 7.0 for Windows, La Jolla, CA, USA) was used. Data were evaluated by unpaired *t* test. The level of significance, in all cases, was set to 5%.

## Results

For the animals fed normocaloric diet, body weights tended to be more homogeneous, despite the animals treated with F for 30 days had significantly higher body weight when compared with animals treated with F or not for 10 days and animals treated with F for 20 days (Fig. 2(A)). On the other hand, for the hyperlipidic diet, treatment with F for 10 and 20 days significantly increased and reduced the body weight,

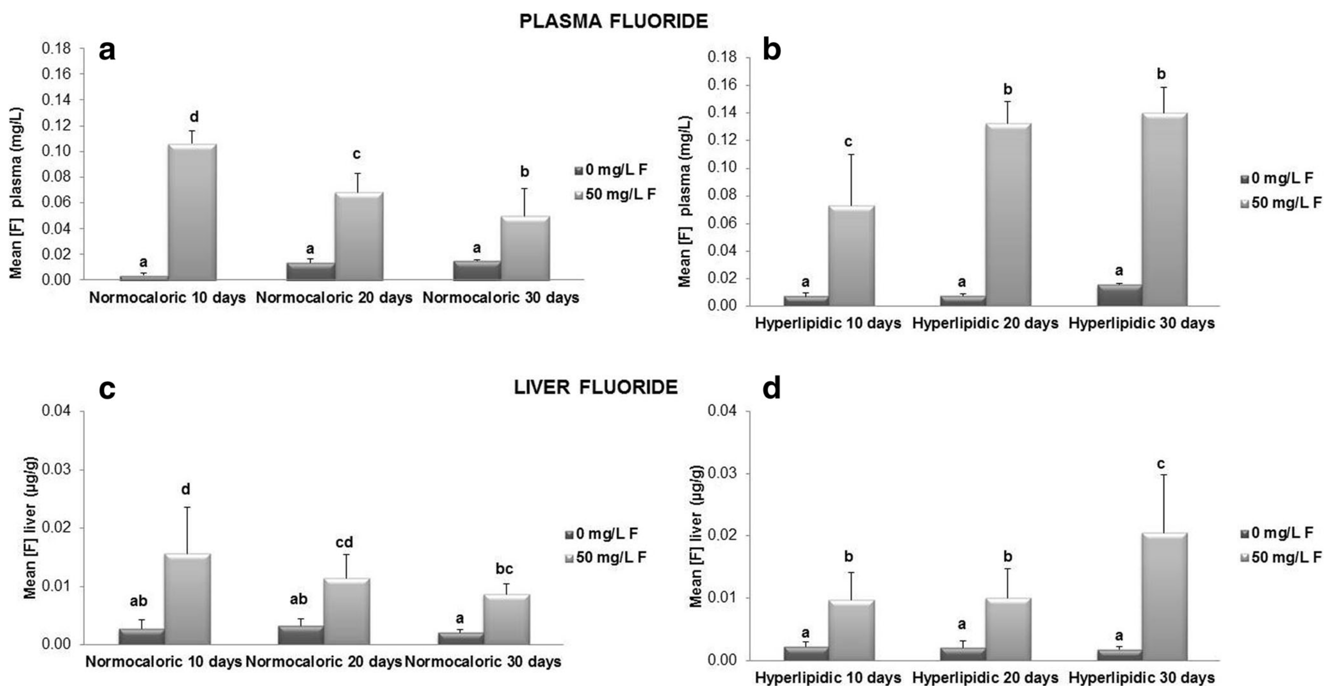



**Fig. 3** Mean fluoride concentrations in plasma (A, B) and liver (C, D) of mice fed normocaloric or hyperlipidic diets that received drinking water containing 0 mg/L or 50 mg/L fluoride for 10, 20, or 30 days. (A) Plasma concentration, normocaloric diet. For each type of diet, distinct lower case letters indicate significant differences among the groups (two-way ANOVA and Tukey's test, $p < 0.05$). Bars indicate SD. $n = 10$

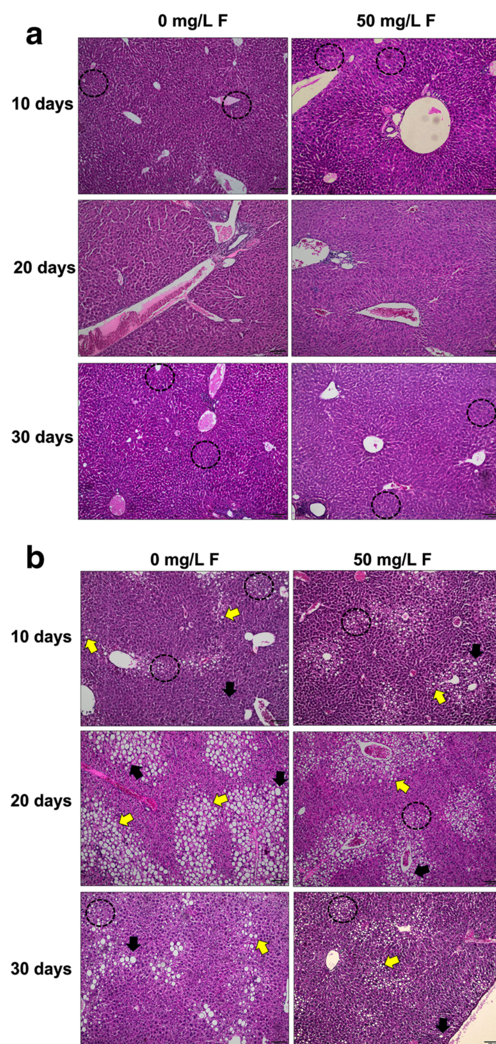



**Fig. 4** HE staining of liver of mice fed normocaloric (A) or hyperlipidic (B) diets that received drinking water containing 0 mg/L or 50 mg/L fluoride for 10, 20, or 30 days. The lipid droplets are represented by dashed circles (diameter $\leq 3$ μm), yellow arrows (diameter $> 3$ μm), or black arrow (diameter around 20 μm). The bar corresponds to 100 μm

respectively, while at 30 days, F did not interfere in the body weight (Fig. 2(B)).

F administration significantly increased plasma F concentrations for both types of diet (Fig. 3(A, B)). Animals fed normocaloric diet had a significant decrease in plasma F concentration along time (Fig. 3(A)), while those fed hyperlipidic diet treated for 20 and 30 days had significantly higher plasma F concentrations when compared to those treated for 10 days, but without significant difference between 20 and 30 days (Fig. 3(B)).

In addition, F administration also significantly increased liver F concentrations for both types of diet (Fig. 3(C, D)). For the normocaloric diet, groups treated with F presented a reduction in liver F concentrations along time. However, the difference was significant only between animals treated for 10 days when compared with those treated for 30 days (Fig. 3(C)). On the other hand, liver F concentrations were significantly higher at 30 days, when compared with 10 and 20 days, for animals fed hyperlipidic diet (Fig. 3(D)).

Histological analysis (Fig. 4) revealed that animals fed with normocaloric diet had a low amount of small lipid droplets (Fig. 4(A)), while those fed with hyperlipidic diet presented a high amount of intermediate and big lipid droplets (Fig. 4(B)). In order to quantify these lipid inclusions, morphometric (Fig. 5(A, B)) and morphological analyses (Fig. 5(C, D)) were applied. The morphometric analysis revealed that for the animals treated with the normocaloric diet, administration of F for 20 days significantly reduced the percentage of lipid droplets when compared to all the other groups (Fig. 5(A)). The percentage of lipid droplets was higher for the animals fed with hyperlipidic diet, but a tendency for reduction was found for the animals treated with F for 20 days (Fig. 5(B)).

Regarding the morphological analysis (Fig. 5(C, D)), the average scores found for the normocaloric diet were much lower than those found for the animals fed hyperlipidic diet. In addition, they were significantly lower for the animals treated for 20 days, regardless of the F administration, in comparison to the other periods (Fig. 5(C)). For hyperlipidic diet, data were more homogeneous, without noticeable differences among the groups, despite a tendency for reduction was also found at 20 days, but only for the animals treated with F (Fig. 5(D)).

In order to investigate the effect of F in lipid metabolism in animals with NAFLD and normal animals, as a function of the type of diet and duration of treatment, lipid parameters were evaluated in plasma and TG in the liver (Figs. 6 and 7). For the animals fed normocaloric diet, treatment with F for 10 days significantly reduced liver TG, plasma TG, and VLDL in comparison to the animals that received deionized water (Fig. 6(A–C)). When the animals were treated for 20 days, a significant decrease was observed in liver TG, total cholesterol, and LDL (Fig. 6(A, D, F)) in the animals treated with F when compared to those receiving deionized water. However, treatment with F for 30 days significantly increased total cholesterol and HDL (Fig. 6(D, E)) in comparison to the animals receiving deionized water.

For the groups that received the hyperlipidic diet, treatment with F for 10 days did not provoke any alterations in the lipid parameters. However, treatment with F for 20 days significantly reduced total cholesterol and LDL (Fig. 7(D, F)), but treatment for 30 days significantly reduced HDL (Fig. 7(E)), in comparison to the animals receiving deionized water. When the animals fed hyperlipidic diet were compared to those receiving normocaloric diet and deionized water, for each time period, treatment for 10 days reduced plasma TG and increased LDL, while treatment for 20 days reduced total cholesterol and LDL. Treatment for 30 days, however, increased total cholesterol, LDL, HDL, and liver TG (three-way ANOVA, data not shown).

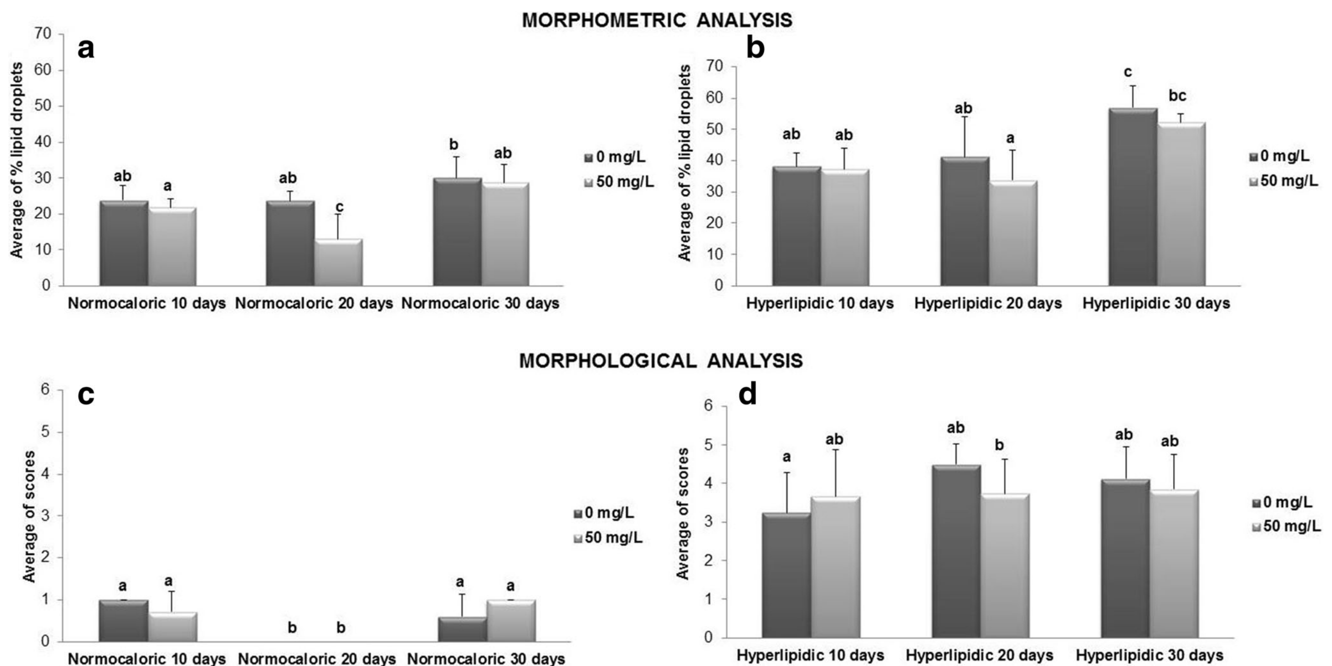



**Fig. 5** Morphometric (A, B) and morphological (C, D) analyses of lipid droplets in the liver of mice fed normocaloric or hyperlipidic diets that received drinking water containing 0 mg/L or 50 mg/L fluoride for 10, 20 or 30 days. (A) Mean percentage of lipid droplets for animals fed normocaloric diet; (B) mean percentage of lipid droplets for animals fed with hyperlipidic diet; (C) average of scores, based on morphological analysis, for animals fed normocaloric diet; (D) average of scores, based on morphological analysis, for animals fed hyperlipidic diet. For each type of diet, distinct lower case letters indicate significant differences among the groups (two-way ANOVA and Tukey's test, $p < 0.05$). Bars indicate SD. $n = 10$

The calculations of de novo lipogenesis were performed using the NMR spectra. For the analysis of $^{1}H$, the group calculated is the methyl group ($CH_3$) and for the analysis of $^{2}H$, the methyl group labeled with deuterium was used ($C^2H^1H_2$) (Supplementary Fig. A1). Animals fed hyperlipidic diet had less de novo lipogenesis when compared with those fed normocaloric diet. The treatment with F did not alter de novo lipogenesis in animals with NAFLD. For the animals fed normocaloric diet, there was a trend for reduction in de novo lipogenesis upon treatment with F (Fig. 8).

To investigate the mechanism by which F alters the lipid profile, key proteins related to lipid metabolism were identified and quantified by Western blotting. GRP-78 is related to de novo lipogenesis, Apo-E is involved in lipid transport and delivery to the liver [43], and SREBP participates in the activation of TG synthesis [25]. Regardless of the type of diet, GRP-78 was significantly increased, while Apo-E and SREBP were significantly decreased upon exposure to F (Fig. 9(A, B)).

## Discussion

Recently, the effect of F in lipid metabolism has been reported in many studies [5, 15, 27–29, 44]. However, only a few studies evaluated how this effect is affected by the type of diet and duration of treatment with F [5, 15]. In our study, in general, F had a beneficial effect on lipid parameters, which could suggest a preventive action of F against lipid disorders. To the best of our knowledge, this is the first study that evaluated the effect of F on lipid metabolism and on de novo lipogenesis in animals with installed NAFLD. This evaluation is important in order to see if a therapeutic effect of F could also be envisioned in these cases. Regarding the dose of F evaluated, lower doses of F, such as 10–25 mg/L, would have been be more realistic. However, we chose to treat the animals with 50 mg/L F because this concentration has been reported to cause more alterations in lipid metabolism in comparison to lower doses [5, 15]. Considering that rodents metabolize F five times faster than humans [31], this dose corresponds to ~ 10 mg/L F in the drinking water for humans. It is a high concentration, but it can be found naturally in the water in areas of endemic fluorosis worldwide [33].

In the present study, one of the variables was the duration of the treatment with F. In a previous study, it was observed that treatment of rats with 50 mg/L F in the drinking water for 20 days reduced lipid droplets in the liver and also decreased plasma TG, while no alterations were detected when the treatment with F lasted 60 days [15]. For this reason, we decided to evaluate the periods of 10, 20, and 30 days of treatment. This was proven to be important, since considerable variation in the results was observed depending on the duration of the treatment. For LDN analysis, the treatment was conducted for 20 days because treatment of rats with 50 mg/L F in the

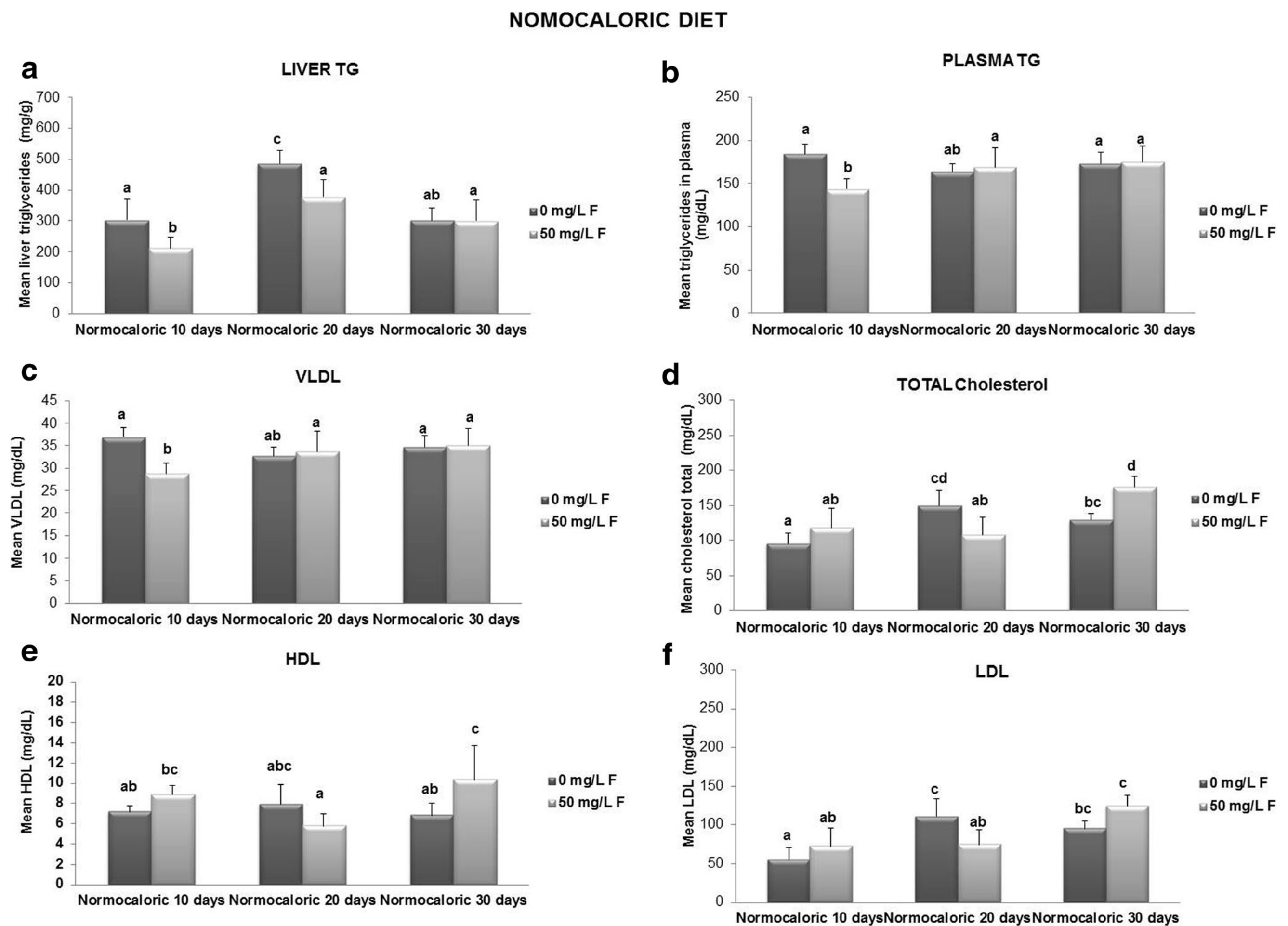



**Fig. 6** Means of the lipid profiles in plasma and liver of mice fed normocaloric diet receiving 0 mg/L or 50 mg/L fluoride in the drinking water for 10, 20, or 30 days. (A) Liver TG. (B) Plasma TG. (C) VLDL. (D) Total cholesterol. (E) HDL. (F) LDL. For each variable, distinct lower case letters indicate significant differences among the groups (two-way ANOVA and Tukey's test, $p < 0.05$). Bars indicate SD. $n = 10$

drinking water for the same period reduces lipid droplets in the liver and improves the lipid profile, while treatments for 10, 30 (this study), or 60 [15] days do not provoke any alterations.

We found only slight differences in body weight and liver weight (Fig. 2). In most of the studies, when administered at the concentration employed in the present study, F does not alter body weight [5, 24]. However, in these studies, F was administered for a longer time (usually more than 42 days). In the present study, it was noticeable that F administration for 20 days in animals with installed NAFLD significantly reduced body weight (Fig. 2(B)).

One interesting finding of the present study was the fact that when F was administered in conjunction with the normocaloric diet, F concentrations in liver and plasma reduced along time, while they increased in the group with installed NAFLD (Fig. 3). The main routes of F elimination from plasma are urinary excretion or uptake in the skeleton [2]. Thus, the reduction in plasma F concentrations along time when the animals were fed the normocaloric diet might be due to one of these events. Studies conducted in the 1950's report an increase in the absorption of F by dietary fat [45, 46], since high levels of fat in the duodenum reduce the rate of gastric emptying, which in turn increases the degree of gastric absorption of F [1], thus increasing plasma F levels [45, 46]. Additional studies on this topic are necessary to provide the basis for understanding these events, as well as to clarify why plasma and tissue F concentrations increase along time with consumption of a hyperlipidic diet. The increase in the retention of F in the organism when the hyperlipidic diet is consumed is relevant, since this might increase F toxicity, thus increasing the risk of occurrence of dental or skeletal fluorosis. There is only one study that associated body mass index with dental fluorosis in children, but no correlation was found [47]. However, it should be evaluated if children that ingest a diet with high fat content have a higher risk to develop dental fluorosis. On the other hand, a recent study reported that overweight and obesity are risk factors for skeletal fluorosis in adults [48].

It is well known that F induces oxidative stress, which could lead to an increase in steatosis [4, 11, 22–24, 44,

**Fig. 7** Means of the lipid profiles in plasma and liver of mice fed hyperlipidic diet receiving 0 mg/L or 50 mg/L fluoride in the drinking water for 10, 20, or 30 days. (A) Liver TG. (B) Plasma TG. (C) VLDL. (D) Total cholesterol. (E) HDL. (F) LDL. For each variable, distinct lower case letters indicate significant differences among the groups (two-way ANOVA and Tukey's test, $p < 0.05$). Bars indicate SD. $n = 10$

49–51]. However, in the present study, the animals treated with F for 20 days and fed with normocaloric diet presented a reduction of lipid droplets in the liver (Fig. 5(A)). In the meantime, for the animals that had installed NAFLD, F did not interfere in the degree of steatosis, which might be related to a possible adaptation of the animals to the hyperlipidic diet [52]. As expected, the severity of steatosis, as evaluated by

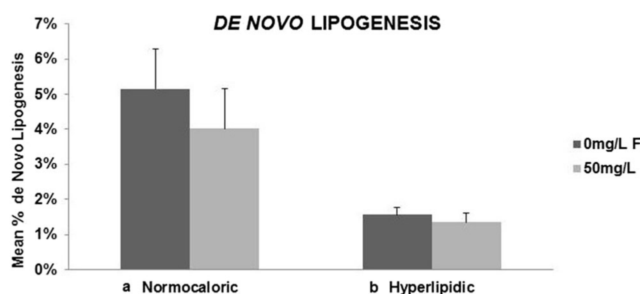



**Fig. 8** Average of percentage of de novo lipogenesis in mice fed normocaloric or hyperlipidic diets that received 0 mg/L or 50 mg/L fluoride in the drinking water for 20 days. Distinct lower case letters indicate significant differences between the types of diet (two-way ANOVA and Tukey's test, $p < 0.05$). Bars indicate SD. $n = 6$

scores, was much higher in the animals fed hyperlipidic diet [15, 53].

Our results for the lipid profile show that F can exert a distinct effect, depending on the duration of exposure and type of diet consumed. For the animals fed normocaloric diet, a significant improvement in lipid profile was seen at 10 and 20 days of treatment with F. However, animals treated for 30 days had increased total cholesterol levels, which was due to increase in HDL levels (Fig. 6). In the case of the hyperlipidic diet, however, a beneficial effect of F was only observed at 20 days (Fig. 7), even when we compared the fluoride-treated animals wen the animals receiving normocaloric diet and fluoride-free water (that could be regarded as "control"). In general, the studies available in the literature report an impairment in lipid parameters upon exposure to F in doses similar to the ones employed in the present study [27–29, 44]. However, the duration of the treatment with F in these studies is longer, which is consistent with the tendency of deleterious effect of F found for the period of 30 days in the present study. In other words, F seems to be able to improve the lipid parameters only when administered in the

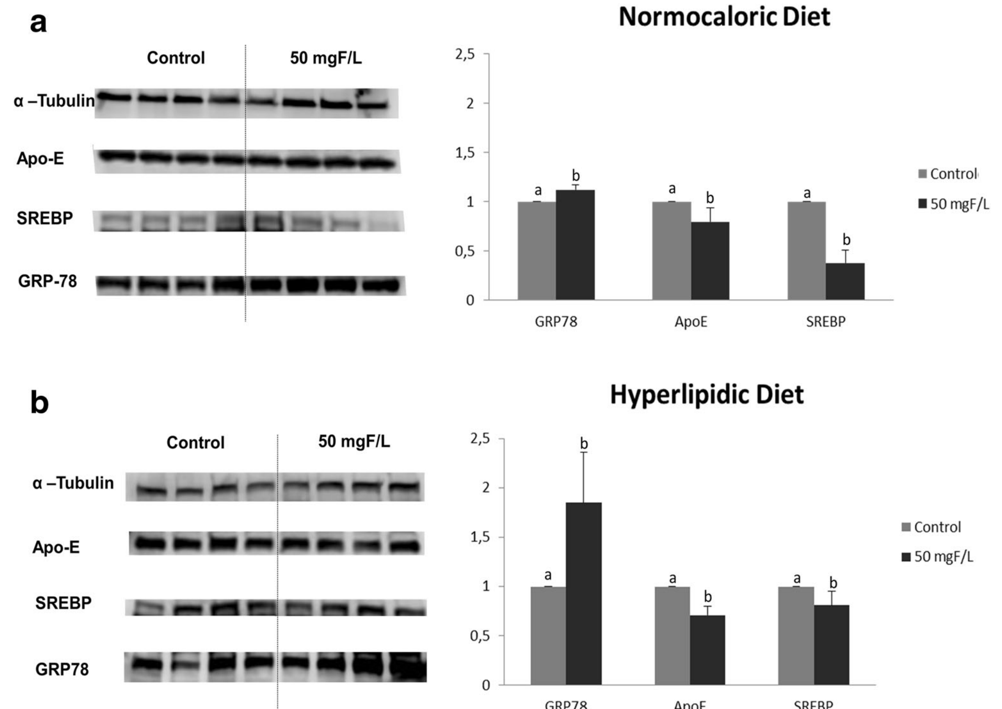



**Fig. 9** Representative expression of proteins GRP-78, Apo-E, SREBP, and of the constitutive protein α-tubulin in samples of individual animals ($n = 4$) from each group. Densitometric analysis was performed for four animals per group. (A) Protein expression in the liver of mice in the group fed normocaloric diet for 20 days; (B) protein expression in the liver of mice fed hyperlipic diet for 20 days. Densitometry was analyzed using the software Image Studio Lite. For each type of diet, distinct letters denote significant differences between animals treated with F or not (unpaired $t$ test, $p < 0.05$) Bars indicate SD. $n = 4$

short time (up to 20 days according to our model). One possibility for this dual effect of F depending on the duration of the treatment might be related to changes in calcium metabolism induced by F, due to the high affinity that these elements have to one another [54]. It was recently shown that lipid membranes have substantial calcium-binding capacity, with potential implications in calcium signaling [55]. It is also known that lipolysis is affected by calcium [56]. Additional studies should be conducted in attempt to clarify this dual effect of F on lipid parameters, especially what shifts its effect from a beneficial to a harmful action.

The significantly lower de novo lipogenesis (Fig. 8) found for the animals fed hyperlipidic diet was expected, because there are many free fatty acids circulating in animals fed with this diet. Thus, inhibitory mechanisms that prevent lipid synthesis from non-lipid sources (de novo lipogenesis) will be activated, as has been previously shown [57]. In addition, experimental models used to induce obesity can have influence de novo lipogenesis. In other words, the nutritional composition of the diet interferes on de novo lipogenesis [41, 58], which might help to explain the differences found in the studies that use "high-fat" or normocaloric diets [58]. Several studies where animals had NAFLD revealed a decrease in lipogenesis [58–61], in agreement with our results. This can be related to the composition of the hyperlipidic diet used in our study, which contains lard [58–61]. Moreover, the increase in de novo lipogenesis found for the animals fed normocaloric diet can be due to the high carbohydrate content (43.7%) of this diet [58, 62]. Regarding the influence of treatment with F on de novo lipogenesis according to the type of diet, those fed with normocaloric diet had a trend for reduction in de novo lipogenesis upon treatment with F.

Based on these results and on the current literature, a mechanism can be proposed to try to explain how F interferes on lipid metabolism and de novo lipogenesis, depending on the type of diet consumed, when the animals are treated with 50 mg/L F in the drinking water for 20 days (Fig. 10). The normocaloric diet used in this study contains 43.7% carbohydrates, while the hyperlipidic diet has 35% fat (31% from animal source (39% saturated fat) and 4% from vegetal source (soybean oil)) [32]. This causes an increase in the availability of carbohydrates and lipids in the organism. Upon treatment with normocaloric diet in the absence of F, the increase in the ingestion of carbohydrates leads to an increase in acetyl-CoA, which in turn increases pathways related to cholesterol, thus increasing low-density lipoprotein (LDL) and de novo lipogenesis. Increase in these precursors might, in turn, increase liver lipid droplets and triglycerides (TG) (Fig. 10(A)) [63]. When the animals are concomitantly treated with F, the increase in carbohydrates from the diet increases acetyl-CoA, as mentioned above, but F induces endoplasmic reticulum oxidative stress, which increases GRP-78 and this, in turn, reduces SREBP that deactivates de novo lipogenesis (Fig. 9) [5, 15, 25]. This deactivation reduces the amount of available TG, thus diminishing lipid accumulation in the liver (both as lipid droplets and TG). F also affects the cholesterol pathway, through the inhibition of Apo-E [5, 15], which diminishes the transport of cholesterol, thus reducing its accumulation in the organism, as well as the LDL levels (Figs. 9 and 10(B)). The consumption of hyperlipidic diet, in the absence of treatment

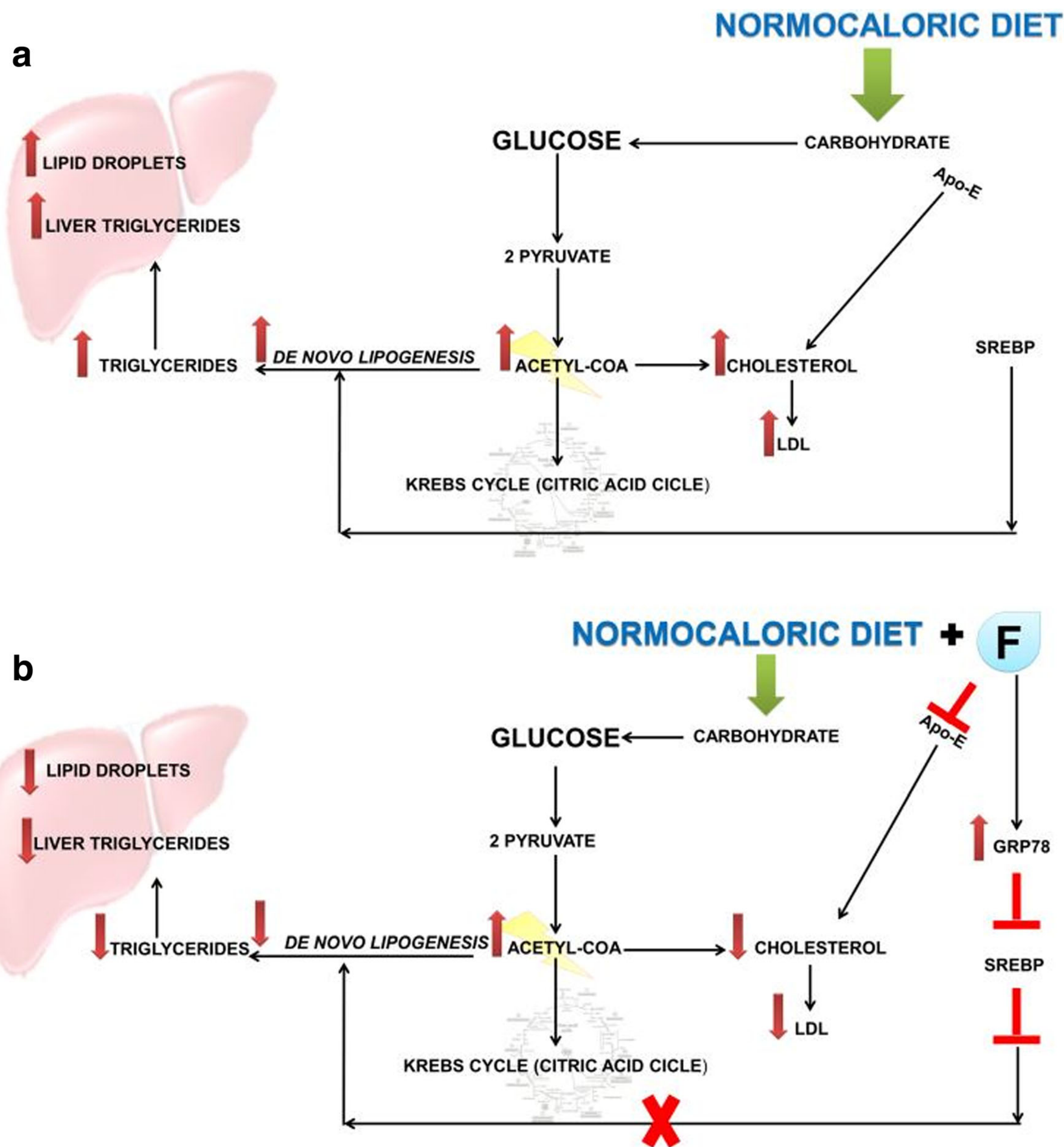



**Fig. 10** Proposed mechanism of action of fluoride (F) on lipid metabolism and de novo lipogenesis. The animals were fed normocaloric diet (increasing acetyl-CoA due to the ingestion of carbohydrates) and treated with 0 or 50 mg/L F in the drinking water for 20 days. (A) Normocaloric diet and 0 mg/L F: increase in acetyl-CoA increases cholesterol pathways (increase in total cholesterol and LDL) and de novo lipogenesis, augmenting lipid droplets and TG in the liver. (B) Normocaloric diet and 50 mg/L F: F causes endoplasmic reticulum oxidative stress, increasing GPR78, which in turn reduces SREBP, reducing de novo lipogenesis and available TG, which reduces lipid droplets and TG in the liver. F also affects the cholesterol pathway, through inhibition of Apo-E, which reduces the transport and circulating levels of cholesterol, as well as of LDL

with F, increases the availability of lipids in the organism, thus augmenting the levels of circulating lipids, as well as of acetyl-CoA. This increases the transport of lipids to the tissues, thus increasing circulating cholesterol and LDL as well as lipid droplets in the liver. On the other hand, due to the increase in the levels of circulating lipids, there is no need to form TG and de novo lipogenesis is reduced (Fig. 11(A)). When the animals are concomitantly treated with F, the great availability of lipids from the diet reduces de novo lipogenesis and increases lipid droplets in the liver. However, since F inhibits Apo-E (Fig. 9) [5, 15], the transport of cholesterol is diminished, thus reducing cholesterol and LDL levels (Fig. 11(B)) [5, 15, 63, 64].

The data obtained in the present study in conjunction with the available literature provide new insights on how F affects lipid metabolism depending on the available energy source. Taken together, our results show that F is able to improve lipid parameters and reduce steatosis only when administered for a short period of time (up to 20 days) to animals fed normocaloric diet. However, when NAFLD is already

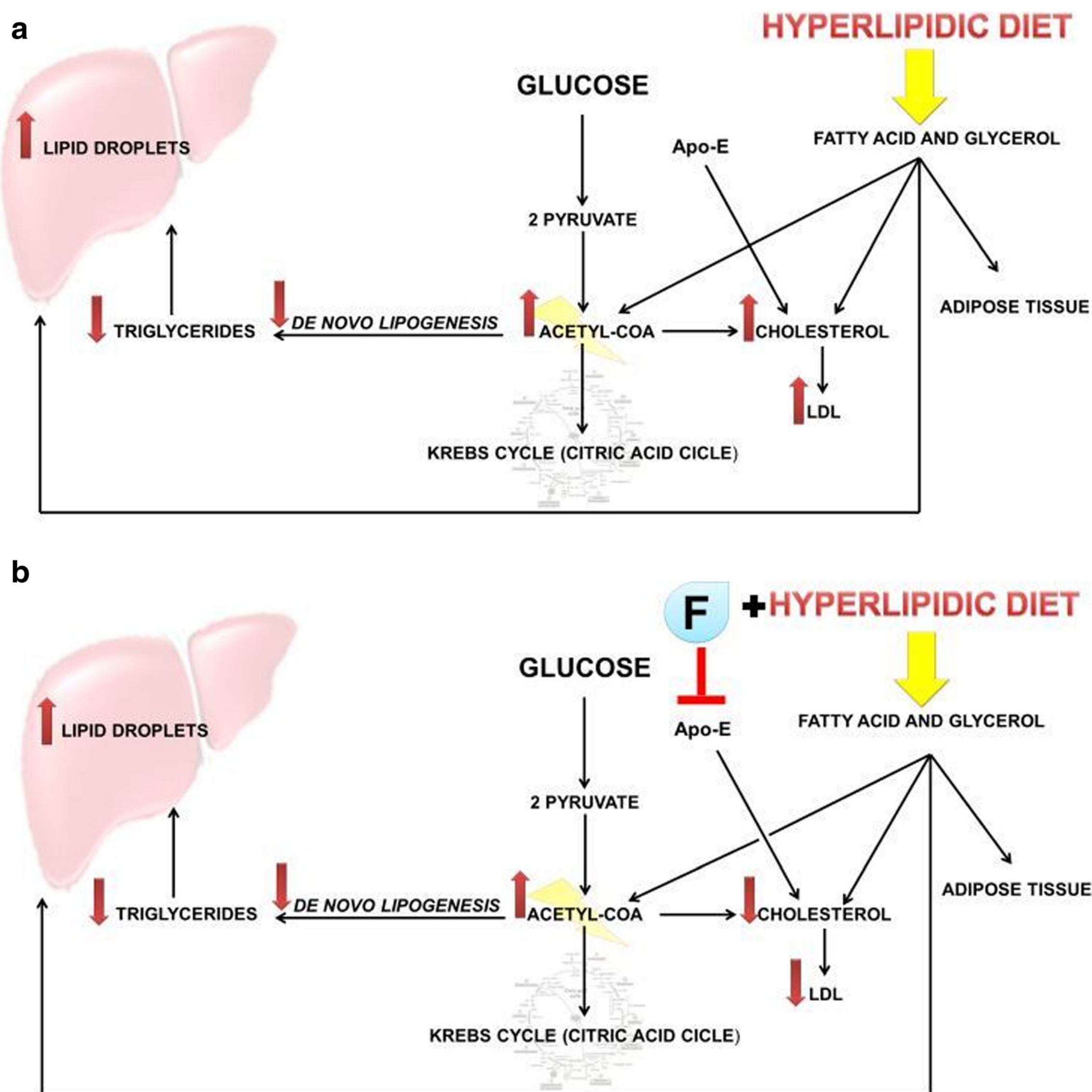



**Fig. 11** Proposed mechanism of action of fluoride (F) on lipid metabolism and de novo lipogenesis. The animals were fed hyperlipidic diets (increasing acetyl-CoA due to the ingestion of lipids) and treated with 0 or 50 mg/L F in the drinking water for 20 days. (A) Hyperlipidic diet and 0 mg/L F: increase in circulating lipids and acetyl-CoA augments the transport of lipids to the tissues and reduces the formation of TG through de novo lipogenesis. (B) Hyperlipidic diet and 50 mg/L F: increase in circulating lipids increases acetyl-CoA and reduces de novo lipogenesis, besides increasing lipid droplets in the liver. F inhibits Apo-E, reducing the transport and concentration of cholesterol, as well as of LDL

installed, lipid parameters are only slightly improved at 20 days of treatment, but no effect can be seen on the degree of steatosis (Supplementary Fig. A2). In addition, lipid profile was in general impaired when the animals were treated with F for 30 days, regardless of the diet. Moreover, it was observed that the retention of F in the organism is higher when hyperlipidic diet is consumed, which can have important implications in F toxicity and should be better investigated. In summary, when the diet contains a high fat content, F does not alter de novo lipogenesis, but when the main source of energy in the diet is derived from carbohydrates, F tends to reduce de novo lipogenesis. It should be noted that the aim of this study was to better understand the mechanisms by which F alters the lipid metabolism. Despite beneficial effects observed when the animals were treated with F up to 20 days, there was impairment in the longer treatment period. For this reason, our results do not support the consumption of F in the long term as a preventive method against lipid disorders. Additional studies should be conducted in order to investigate if the beneficial effects are maintained if the consumption of F is interrupted.

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**Author Contributions** Dionizio A, Pereira HABS, and Buzalaf MAR conceived the experiments. Dionizio A, Pereira HABS, Nogueira FN, and Carvalho, RA conducted the experiments. Dionizio A, Pereira HABS, Araujo TT, Sabino-Arias IT. Fernandes MS. Oliveira KA. Nogueira FN, Carvalho, RA, Raymundo FS, and Cestari TM participated in the research experiments. Dionizio A, Pereira HABS, and Buzalaf MAR drafted the article and analyzed and interpreted the results. All authors reviewed and approved the manuscript.

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## Compliance with Ethical Standards

All experimental protocols were approved by the Ethics Committee for Animal Experiments of Bauru Dental School, University of São Paulo (protocol: 001/2014; 008/2015).

**Conflict of Interest** The authors declare that they have no conflict of interest.

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