UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ODONTOLOGIA DE BAURU

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Fluoride effect on parameters related to glucose homeostasis in NOD mice: using proteomic analysis of liver and gastrocnemius muscle to unravel the underlying mechanisms

Efeito do fluoreto em parâmetros relacionados à homeostasia da glicose em camundongos NOD: uso da análise proteômica do fígado e músculo gastrocnêmio para revelar os mecanismos envolvidos

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Supervisor: Prof. Drª Marília Afonso Rabelo Buzalaf

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ABSTRACT

Fluoride effect on parameters related to glucose homeostasis in NOD mice: using proteomic analysis of liver and gastrocnemius muscle to unravel the underlying mechanisms

Water fluoridation is an important public health measure for the control of dental caries. Recent animal studies have shown that low doses of fluoride (F) in the drinking water, similar to those found in public water supplies, increase insulin sensitivity and reduce blood glucose. In the present study we evaluated the effects of low-level F exposure through the drinking water on glucose homeostasis in female NOD mice. Seventy-two 6-week mice were randomly divided into 2 groups according to the concentration of F in the drinking water (0-control, or 10 mg/L) they received for 14 weeks. After the experimental period the blood was collected for analyses of plasma F, glucose and insulin. Liver and gastrocnemius muscle were collected for proteomic analysis. Plasma F concentrations were significantly higher in the F-treated than in the control group. Despite treatment with fluoridated water reduced plasma levels glucose by 20% compared to control, no significant differences were found between the groups for plasma glucose and insulin. In the muscle, treatment with fluoridated water increased the expression of proteins related to muscle contraction, while in the liver, there was an increase in expression of antioxidant proteins and in proteins related to carboxylic acid metabolic process. Remarkably, phosphoenolpyruvate carboxykinase (PEPCK) was found exclusively in the liver of control mice. The reduction in PEPCK, a positive regulator of gluconeogenesis, thus increasing glucose uptake, might be a probable mechanism to explain the anti-diabetic effects of low doses of F, which should be evaluated in further studies.

Keywords: Diabetes Mellitus, Type 1.Sodium Fluoride. Proteomics

RESUMO

Efeito do fluoreto em parâmetros relacionados à homeostasia da glicose em camundongos NOD: uso da análise proteômica do fígado e músculo gastrocnêmio para revelar os mecanismos envolvidos

A fluoretação da água é uma importante medida de saúde pública para o controle da cárie dentária. Estudos recentes em animais mostraram que baixas doses de flúor (F) na água potável, semelhantes às encontradas no abastecimento público de água, aumentam a sensibilidade à insulina e reduzem a glicose no sangue. No presente estudo, avaliamos os efeitos da exposição de baixo nível de F através da água potável na homeostase da glicose em camundongas NOD fêmeas. Setenta e dois ratos de 6 semanas foram divididos aleatoriamente em 2 grupos, de acordo com a concentração de F na água potável (controle 0 ou 10 mg / L) que receberam por 14 semanas. Após o período experimental, o sangue foi coletado para análises de plasma F, glicose e insulina. Fígado e músculo gastrocnêmio foram coletados para análise proteômica. As concentrações plasmáticas de F foram significativamente maiores no grupo tratado com F do que no grupo controle. Apesar do tratamento com água fluoretada ter reduzido os níveis plasmáticos de glicose em 20% em comparação ao controle, não foram encontradas diferenças significativas entre os grupos para glicose plasmática e insulina. No músculo, o tratamento com água fluoretada aumentou a expressão de proteínas relacionadas à contração muscular, enquanto no fígado houve aumento na expressão de proteínas antioxidantes e de proteínas relacionadas ao processo metabólico do ácido carboxílico. Notavelmente, a fosfoenolpiruvato carboxiquinase (PEPCK) foi encontrada exclusivamente no fígado de camundongos controle. A redução do PEPCK, um regulador positivo da gliconeogênese, aumentando assim a captação de glicose, pode ser um mecanismo provável para explicar os efeitos antidiabéticos de baixas doses de F, que devem ser avaliadas em estudos futuros.

Palavras-chave: Diabetes Mellitus Tipo 1. Fluoreto de Sódio. Proteômica.

SUMÁRIO

INTRODUCTION

1 INTRODUCTION

Fluoride (F) has a fundamental role in maintaining the oral health, because of its potential to control the development of dental caries lesions (Fukushima *et al.*, 2011). Dental caries is considered a chronic worldwide prevalent multifactorial disease, becoming a serious public health problem in developed countries, affecting 2.4 billion individuals, about 35.5% of the total population (Barrington *et al.*, 2019), including around 60- 90% of school-age children (Iheozor-Ejiofor *et al.*, 2015). In addition, one of the most important public health measures aimed at controlling tooth decay is the use of F, mainly through water and fluoridated toothpaste, which has resulted in dramatic reductions in tooth decay over the years (Bratthall, 1996; Mcdonagh *et al.*, 2000; Iheozor-Ejiofor *et al.*, 2015).

When ingested, F is absorbed by the gastrointestinal system. It is mainly deposited in calcified tissues, such as bones and teeth, and what is not retained in the body is excreted through the urine. Several factors affect the metabolism of F, such as acid-base status, physical exercise, nutritional status, age, altitude and genetic *background*. These factors alter the retention of F in the body, and, consequently, its toxicity (Buzalaf e Whitford, 2011). As a result of the avidity of F for calcified tissues, the most common manifestation due to chronic excessive intake of F is dental fluorosis, whose prevalence has increased in recent years (Khan *et al.*, 2005; Ramires *et al.*, 2007; Amaral *et al.*, 2018).

Although, when excessive or chronic exposure to F occurs, this ion can interfere with important metabolic pathways in the biological systems, functioning as a potent inhibitor of many enzymes, including some of the glycolytic pathway (Barbier *et al.*, 2010). *In vivo* studies with animal conducted by our group have shown that chronic intake of F through drinking water can alter protein expression in the kidneys, liver, muscle and intestin of rats or mice (Kobayashi *et al.*, 2009; Carvalho *et al.*, 2013; Pereira *et al.*, 2013; Lima Leite *et al.*, 2014; Melo *et al.*, 2017; Dionizio *et al.*, 2018; Araujo, T. T. *et al.*, 2019). Among the effects of chronic ingestion of excessive levels of F in soft tissues, both increased glucose intolerance and impaired insulin secretion have been reported (Rigalli *et al.*, 1990). The insulin resistance induced by F may be caused by a decrease in tyrosine phosphorylation, and an increase in phosphorylation

of pp185 serine (IRS-1 / IRS-2) in the substrates of the insulin receptor in adipocytes, which results in reduced insulin signaling (Chiba *et al.*, 2010; Chiba *et al.*, 2012). However, it has been reported by our group that the chronic treatment of rats with diabetes previously induced by streptozotocin with a low concentration of F (10 ppm, which is equivalent to the concentration of 1 ppm for humans, which is similar to artificially fluoridated water (Dunipace *et al.*, 1995)) increases insulin sensitivity, possibly by increasing the expression of liver proteins that form a complex with GRP-78 (Nakatsuka *et al.*, 2012), present in the endoplasmic reticulum, thus manifesting favorable effects on the signs of glucose intolerance (Lobo, J. G. V. M. *et al.*, 2015). These results are extremely important, as they open a new perspective for the investigation of the beneficial effects of naturally or artificially fluoridated water to control the development of type 1 diabetes (T1D). Moreover, a recent systematic review and meta-analysis showed that the prevalence of dental caries in children and adolescents with TD1 was highest in South America (84%) (Wang *et al.*, 2019). Due to this link between T1D and dental caries, it would be of high interest the implementation of a public health measure that could control both diseases at the same time.

DM1 is one of the most important public health problems, representing 10% of diabetes cases worldwide. It is an autoimmune disease characterized by the destruction of the β cells in the pancreas, reducing its ability to secrete insulin, which may lead to ketoacidosis in the absence of exogenous insulin (Dumont-Driscoll, 2012; Acharjee *et al.*, 2013). Although insulin administration can correct hyperglycemia, this treatment is still insufficient to prevent complications that can occur in the long term (Li *et al.*, 2016), such as retinopathies, nephropathies, vasculopathies and neuropathies that reduce life expectancy in 12 years (Nathan *et al.*, 2005; De Ferranti *et al.*, 2014; Harding *et al.*, 2016).

Several animal models have been used to understand the mechanisms involved in the development of DT1, providing tools to understand the autoimmune damage to islets, facilitating the early detection, prevention and treatment of DT1. These models have contributed to the understanding of important advances in TD1 therapy. The most used models involve the induction of diabetes by the administration of streptozotocin, as well as rodents that develop diabetes spontaneously, such as the Non Obese Diabetic (NOD) mice and the Bio-Breeding (BB) mice (Acharjee *et al.*, 2013; Mullen,

2017). The NOD and BB models better mimic the development of diabetes in humans than the models that involve the use of streptozotocin, as they develop diabetes spontaneously through an autoimmune disorder, similar to what occurs in humans, although they still show some differences in development of insulitis, diabetes, characteristics of insulitis and immune response, as well as influence of different environmental factors between rodent and human models (Table 1) (Mullen, 2017).

Table 1. Characteristics of type 1 diabetes and insulitis in humans, NOD mice and BB rats (Mullen, 2017)

	Humans	**NOD mice**	**BB mice**
Development of insulits and diabetes	- Slighty higher in males - more commom in young	- Higher in female - Insulitis starts at 2-4 week in female and at 5-7 week in males - Hyperglyciemia develops at 12-30 week - At 30 weeks, 90-100% of females and 50-80% of males become diabetic	- No sex difference - insulitis is detected in the fifth week - Hyperglycemia and ketoacidosis develop from 8-17 weeks
Features of insulitis	- Contains fewer infiltrates, predominantly $CD8^+$ T cells - Common infiltrated inside the islet - Number of infiltrated cells is not large	- Often starts with peri-insulitis and then the cells infiltrate the islets - Various islet infiltrates consist of dendritic cells, macrophages, $CD4^+$ and CD8 + T cells, B cells and NK cells	- Immune cells infiltrate the islets, but to a lesser extent on their peripheries - Peri-insulitis is not severe - infiltrating cells are similar to those of NOD mice
Immune characteristics	- Reduced suppression of autologous CD^+ T cells by $CD4^+$ $CD25^{high}$ Fox^{P3+} cells in newly diagnosed patients with long-standing DT1	- Imbalance between autoreactive T cells and Fox^{P3+} Tred cell - Reduced ability of Treg cells to suppress T cell proliferation	- Specific T-cell lymphopenia - Impaired Treg cell function
Autoantibodies	- Present in 70% of recently diagnosed TD1 cases, including IAA, GAD-65A, IA-2nd	- Presence of IAA, GAD76A, absence of IA-2A	- Presence of IAA, GAD-65A, but levels are lower than in humans, absence of IA-2A
Environmental factors	- The incidence of viral infection - influence of environmental factors	- Increased incidence in Specific Pathogen Free conditions and is reduced by viral infection	- excellent model for testing various environmental factors

The NOD mice are widely used in studies involving DT1 due to the excellent genetic characterization, development of monoclonal antibodies and congenic strains (Kachapati *et al.*, 2012; Pearson *et al.*, 2016; Mullen, 2017).The NOD mouse model was developed in Japan in the 1970s, becoming well established in the 1980s (Makino *et al.*, 1980). Diabetes is caused by the selective destruction of β cells in the pancreatic islets after the infiltration of autoreactive immune cells. In NOD mice, the cellular infiltrate begins with macrophages and dendritic cells invading the outside of the islets. CD45 + cells are present around the islets in 4 weeks and expand into the islets quickly between 8 and 14 weeks. When insulitis is well established (12-14 weeks), lymphocytes become the dominant cells, while macrophages $CD11b^{+}$ and $F4^{80+}$ are found in fewer numbers (Magnuson *et al.*, 2015). Cytokines and soluble mediators produced by activated macrophages accelerate the recruitment of dendritic cells, being essential for the activation of $CD4^{+}$ and $CD8^{+}$ T cells (Young *et al.*, 1989; Jun *et al.*, 1999; Yoon e Jun, 2001). Natural killer cells (NK), which are normally associated with defenses against viruses and cells affected by intracellular pathogens, also infiltrate the islets and contribute to β cell apoptosis, both directly through perforin and granzyme-mediated cytotoxicity, and indirectly through the release of pro-inflammatory cytokines (Pearson *et al.*, 2016; Mullen, 2017).

In comparison to rodents, insulitis in humans is detected less frequently and in a relatively lower percentage (about 10%) in the islets of patients with T1D. The number of infiltrating cells is also lower. Insulitis appears more frequently in individuals who early develop T1D and can be seen soon after the onset of DT1 (Imagawa *et al.*, 2001; Willcox *et al.*, 2009). Positive insulitis in humans is defined as more than 3 islets containing more than 15 lymphocytes in the islet or peri-islet (Morgan *et al.*, 2014). This small number of lymphocyte infiltrates is significant, because the islets of non-diabetic individuals rarely contain more than 5 lymphoid cells in a single section (Morgan *et al.*, 2014). The infiltrates are generally dominated by CD8 + and CD4 + cells with a variable frequency of B lymphocytes (Willcox *et al.*, 2009; Coppieters *et al.*, 2012; Arif *et al.*, 2014). The differences between human and rodent insulitis may reflect fluctuations over time or merely the much faster progression of diabetes in rodents (Morgan *et al.*, 2014).

Thus, studies using an animal model of diabetes more similar to what occurs in humans (Kachapati *et al.*, 2012; Mullen, 2017) are important to provide additional

subsidies to our previous findings (Lobo, J. G. V. M. *et al.*, 2015), as well as to clarify the mechanisms involved. Our group recently completed a study in which NOD mice aged 35 to 60 days were treated for 21 days with water containing 0, 10 or 50 mgF/L. It was observed that the animals that received water containing 10 mgF/L had a significantly lower glycemia and a significantly higher %β cell function in relation to the control animals. The exposure of the animals to the low concentration of F (10 mgF/L) significantly altered the expression of proteins in the liver and gastrocnemius muscle of the animals. Among the proteins with altered expression in the liver of animals treated with 10 mgF/L, the presence of glutathione S-transferase P2 exclusively in this group was highlighted, as well as of heat shock-related 70 kDa protein 2, which are proteins involved in the antioxidant defense, in the liver (Malvezzi *et al.*, 2019). These results indicate that the exposure to water containing 10 mgF/L would increase the antioxidant defense in the liver of mice, which had already been described by our group in rats treated with 15 mgF/L (Iano *et al.*, 2014). It has been reported that the lower expression of genes associated with antioxidant defense mechanisms contributes to damage to β cells and to the development of diabetes in BB rats, which show an underexpression in the islets of several genes involved in the metabolism of reactive species of oxygen, including members of the glutathione S-transferase (GST) family, superoxide dismutases, peroxidases and peroxiredoxins. BB rats still have significantly lower plasma GST activity. In addition, systemic administration of the antioxidant N-acetylcysteine to BB rats reduces the severity of insulitis and delays (but does not prevent) the onset of diabetes (Bogdani *et al.*, 2013). Thus, it was hypothesized that the increased antioxidant defense induced by F, thus delaying of preventing T1D, could possibly help to explain the lower plasma glucose levels observed in the mice trated with water containing 10 mgF/L, which should be better investigated.

However, in our recent study (Malvezzi *et al.*, 2019), male NOD mice were used because they are more easily available. In addition, the animals were killed between 9 and 12 weeks of age. However, it is known that female NOD mice develop insulitis earlier (between 2-4 weeks) and become 90-100% diabetic with 30 weeks of ages, while male NOD mice develop insulitis between 5-7 weeks and only 50-80% become diabetic (Mullen, 2017). Therefore, further studies using female NOD mice and longer treatment times are needed to provide more evidence about the potential of fluoridated water to prevent or delay the onset of diabetes. These data, if confirmed in humans,

are relevant from the point of view of Public Health, as water fluoridation is considered one of the 10 best Public Health measures of the 20th century in the control of dental caries (Centers for Disease e Prevention, 1999) and has excellent cost-benefit ratio (Ran *et al.*, 2016), in addition to being an important agent in the prevention of dental caries in patients with diabetes (Wang *et al.*, 2019).

This dissertation is presented in the form of an article, in which we evaluated in female NOD mice the effect of exposure to F through drinking water on parameters related to glucose homeostasis. Proteomic analysis of the liver and gastrocnemius muscle was employed as a tool to unravel the possible mechanisms involved in this effect.

ARTICLE

2 ARTICLE

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Effects of low-level fluoride exposure through the drinking water on glucose homeostasis in female NOD mice

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Abstract

Water fluoridation is an important public health measure for the control of dental caries. Recent animal studies have shown that low doses of fluoride (F) in the drinking water, similar to those found in public water supplies, increase insulin sensitivity and reduce blood glucose. In the present study we evaluated the effects of low-level F exposure through the drinking water on glucose homeostasis in female NOD mice. Seventy-two 6-week mice were randomly divided into 2 groups according to the concentration of F in the drinking water (0-control, or 10 mg/L) they received for 14 weeks. After the experimental period the blood was collected for analyses of plasma F, glucose and insulin. Liver and gastrocnemius muscle were collected for proteomic analysis. Plasma F concentrations were significantly higher in the F-treated than in the control group. Despite treatment with fluoridated water reduced plasma levels glucose by 20% compared to control, no significant differences were found between the groups for plasma glucose and insulin. In the muscle, treatment with fluoridated water increased the expression of proteins related to muscle contraction, while in the liver, there was an increase in expression of antioxidant proteins and in proteins related to carboxylic acid metabolic process. Remarkably, phosphoenolpyruvate carboxykinase (PEPCK) was found exclusively in the liver of control mice. The reduction in PEPCK, a positive regulator of gluconeogenesis, thus increasing glucose uptake, might be a probable mechanism to explain the anti-diabetic effects of low doses of F, which should be evaluated in further studies.

Keywords: Fluoride, sensitivity in insulin, glucose, mice NOD.

Introduction

Fluoridation of public water is recognized among the top ten public health achievements of the last century (Centers for Disease, 1999), due to its safety (McDonagh et al., 2000) and excellent cost-benefit ratio for the control of dental caries (Ran et al., 2016). In addition to its well-known effect to control caries, an animal study showed that treatment of diabetic rats (induced by streptozotocin) with low dose of fluoride (F) in the drinking water (10 mg/L, similar to 1 mg/L for humans) for 22 days increases insulin sensitivity (Lobo et al., 2015b) Moreover, treatment of non-obese diabetic (NOD) male mice with water containing 10 mg/L F for 21 days significantly decreased blood glucose levels and increased the antioxidant defense in the liver (Malvezzi et al., 2019b). These findings opened a new perspective on the possible use of fluoridated water for the prophylaxis of diabetes. This is very relevant, since diabetes affects 425 million people worldwide (Cho et al., 2018).

NOD mice have been used for more than 35 years to investigate various agents and protocols that prevent or reverse type 1 diabetes (T1D) (Morgan, 2017). In our previous study we found reduced plasma glucose levels in male NOD mice treated with water containing 10 mg/L F for 21 days (Malvezzi et al., 2019a). However, female NOD mice develop insulitis to a higher degree than male ones. Moreover, in NOD mice insulitis starts at 2-4 weeks in females and in 5-7 weeks in males, while hyperglycemia develops at 12-30 weeks. By 30 weeks, it is expected that 90-100% of females and 50-80% of males become diabetic (Mullen, 2017). In addition, the effects of F in the organism are not only dose- but also time-dependent (Pereira et al., 2018; Araujo et al., 2019). Taking these considerations into account, the present study was designed to refine the results obtained in our previous one (Malvezzi et al., 2019a), by evaluating the effect of F on glucose homeostasis of female NOD mice treated with low dose of F in the drinking water for 14 weeks. In order to provide mechanistic insights, proteomic analysis of gastrocnemius muscle and liver was also conducted.

Material and Methods

Animals, treatment and samples collection

The Animals Ethics Committee of Bauru School of Dentistry, University of São Paulo, approved all the experimental protocols (CEUA-Proc. 013/2017). Seventy-two 6-week female NOD mice were randomly divided into 2 groups, according with the F concentration (as NaF) in the drinking water to which the animals had free access for 14 weeks: 0 (control) or 10 mg/L. The concentration of 10 mgF/L was chosen to simulate the ingestion of artificially fluoridated water by humans, taken into account that rodents metabolize F 10 times faster than humans (Dunipace et al., 1995).

During the experimental period, the animals were housed in pairs in standard cages with chow *ad libitum*. 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.

At the end of the experimental period, after fasting for 14 h, the animals were euthanized (exposure to CO_2 followed by decapitation). Blood was collected and stored at -20°C until analysis of F, glucose and insulin. Liver and gastrocnemius muscle were collected and stored at -80°C for proteomic analysis.

Fluoride analysis in plasma

After hexamethyldisiloxane-facilitated diffusion (Taves, 1968), F in plasma was analyzed by potentiometry, using an ion-specific electrode (Orion Research, model 9409) and a miniature calomel electrode (Accumet, #13-620-79). All the analyses were done in duplicate, after proper calibration, exactly as previously described (Pereira et al., 2013).

Analysis of plasma glucose and insulin levels and calculation of the HOMA2-IR

Fasting plasma glucose and insulin were analyzed in duplicate, by the glucose oxidase (commercial kit, Katal Biotecnologia, São Paulo, Brazil) and ELISA

(Mouse Insulin kit, #80-INSMN-E01, ALPCO Diagnostics, Salem, USA), respectively, according to manufacturer´s instructions.

Proteomics and Bioinformatics Analyses

Muscle and liver samples were prepared for proteomic analysis exactly as previously described (Lobo et al., 2015a). The identification of the peptides was done on a nanoAcquity UPLC-Xevo QTof MS system (Waters, Manchester, UK), as previously described (Leite et al., 2014). Difference in expression between the groups was obtained using the Protein Lynx Global Service (PLGS) software and expressed as $p<0.05$ for down-regulated proteins 1-$p>0.95$ for up-regulated proteins. Bioinformatics analysis was performed for comparison between the groups (10 mg/L F *vs*. control; Tables S1 and S2 for liver and muscle, respectively), as reported earlier (Orchard, 2012; Bauer-Mehren, 2013; Millan, 2013; Leite et al., 2014). The software CYTOSCAPE 3.7.2 (JAVA) was used to build networks of molecular interaction among the identified proteins, with the aid of ClueGo and ClusterMarker2.

Statistical Analysis

The software Graph Pad InStat version 3.0 for Windows for Windows (GraphPad Software Inc., La Jolla, CA, USA) was used. After checking for normality (Kolmogorov-Smirnov test) and homogeneity (Bartlett test), the appropriate statistical test was selected. Mann Whitney test was used for plasma fluoride levels and *t* test was used for plasma glucose and insulin. The significance level was set at 5%.

Results

Plasma F concentrations were significantly higher for the group treated with 10 mgF/L when compared with control ($U = 19.500$, $p = 0.0092$). There were no significant differences between control and treated groups in respect to plasma glucose ($t = 0.901$, $p = 0.377$) and plasma insulin ($t = 0.121$, $p = 0.905$), despite treatment with water containing 10 mgF/L reduced plasma glucose levels in 20% when compared with control (Table 1).

Table 1. Median (95% CI) plasma fluoride levels, as well as mean (SD) plasma glucose and plasma insulin of NOD female mice with intake water drinking containing 0 (control) or 10 mgF/L for 14 weeks. For each variable, distinct superscripts in the same lines denote significant differences among the groups (for Plasma fluoride and %B, Mann Whitney test and for other variables *t*-test, $p<0.05$). n=12-13.

Analysis	**Control**	**10 mgF/L**
Plasma fluoride (µg/mL)	0.050 (0.034-0.066)[a]	0.077 (0.066-0.089)[b]
Plasma glucose (mg/dL)	424 ± 280	339 ± 163
Plasma insulin (ng/mL)	0.49 ± 0.08	0.50 ± 0.07

Distinct letters in the same line indicate significant difference between the groups. Mann-Whitney test for plasma and unpaired *t* test for plasma glucose and insulin ($p<0.05$). n=12-13.

Supplementary table 1 shows the differentially expressed, as well as the unique proteins found in the gastrocnemius muscle of NOD mice treated with 10 mgF/L, when compared with control. Treatment with F increased 8 proteins and decreased 26 proteins in comparison with the control group. The numbers of proteins uniquely identified in treated or control groups were 2 and 27, respectively. Functional classification in relation to the biological processes most affected by F revealed mainly *muscle contraction* (32%) and *actomyosin structure organization* (27%) (Figure 1).

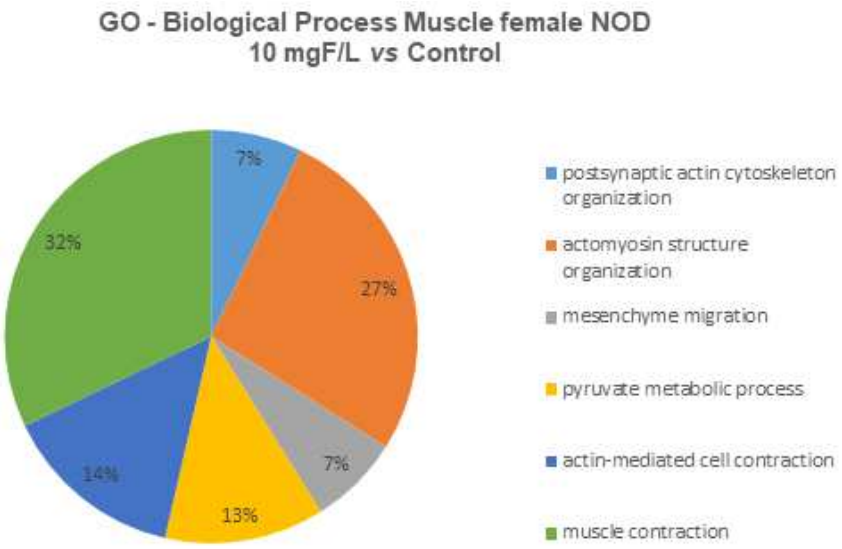

Figure 1. Functional distribution of proteins identified with differential expression in the liver of female non-obese diabetic (NOD) mice treated for 14 weeks with drinking water containing 10 mgF/L fluoride or not (control). Categories of proteins based on GO annotation Biological Process. Terms significant (Kappa=0.03) and distribution according to percentage of number of genes association.

Figure 2 shows the subnetworks generated by ClusterMarker2 for the comparison of proteins with change in expression for the comparison 10 mgF/L *vs.* control. Most of the proteins with altered expression interacted with *Fanconi anemia group D2 protein homolog* (Q80V62), *Polycomb protein EED* (Q921E6) and *14-3-3 protein beta/alpha* (Q9CQV8) (Figure 2A) or *14-3-3 protein épsilon* (P62259) e *F-box only protein 32* (Q9CPU7) (Figure 2B).

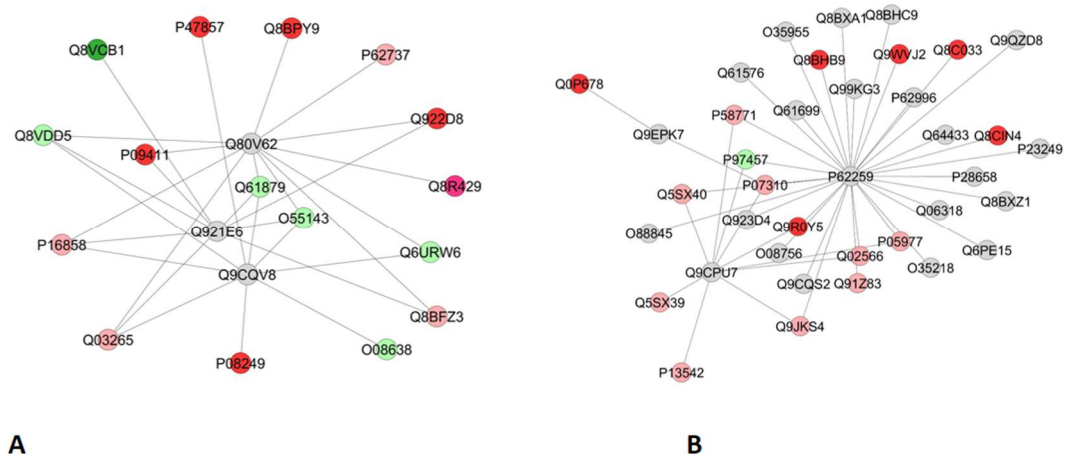

Figure 2. Subnetworks created by ClusterMarker to establish the relationship among proteins identified with differential expression in the gastrocnemius muscle of female non-obese diabetic (NOD) mice treated with water containing 10 mgF/L or not (control) for 14 weeks. The color of the nodes indicates difference in expression of the respective protein defined by its access code (UNIPROT ID). The dark red and dark green nodes indicate proteins unique to control and fluoride-treated groups, respectively. Light red and light green nodes indicate down and upregulation, respectively. The grey nodes indicate interacting proteins that were offered by CYTOSCAPE but were not identified in the present study.

Proteomic analysis of liver revealed 18 increased and 17 decreased proteins in the group treated with 10 mgF/L when compared with control. The numbers of proteins uniquely identified in treated or control groups were 13 and 93, respectively (Supplementary table 2). Functional classification in relation to the biological processes mostaffected by F revealed mainly *carboxylic acid metabolic process* (21%), *carboxylic acid catabolic process* (9%),

cellular amino acid metabolic process (7%) and *alpha-amino acid metabolic process* (7%) (Figure 3).

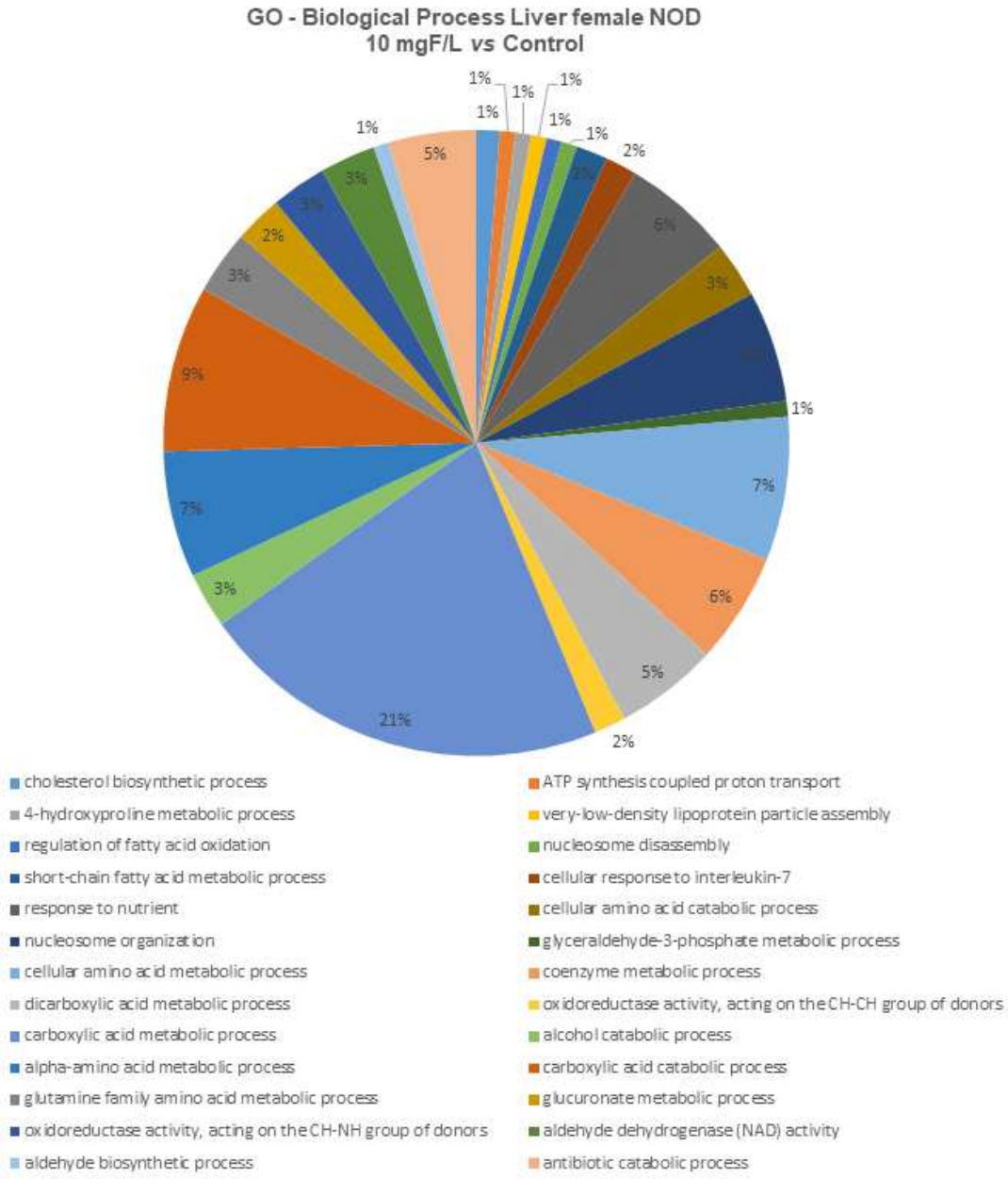

Figure 3. Functional distribution of proteins identified with differential expression in the gastrocnemius muscle of female non-obese diabetic (NOD) mice treated for 14 weeks with drinking water containing 10 mgF/L fluoride or not (control). Categories of proteins based on GO annotation Biological Process. Terms significant (Kappa=0.03) and distribution according to percentage of number of genes association.

Figure 4 shows the subnetworks generated by ClusterMarker2 for the comparison of proteins with change in expression for the comparison 10 mgF/L *vs.* control. Most of the proteins with altered expression interacted with *14-3-3 protein épsilon* (P62259) and *6-phosphogluconolactonase* (Q9CQ60) (Figure 4A) or with *F-box only protein 32* (Q9CPU7) and *Fanconi anemia group D2 protein homolog* (Q9CPU7) (Figure 4B).

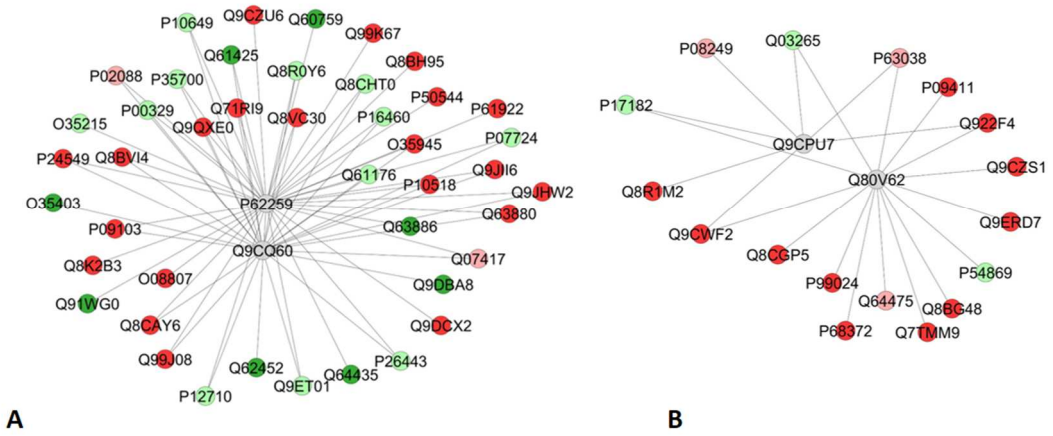

Figure 4. Subnetworks created by ClusterMarker to establish the relationship among proteins identified with differential expression in the liver of female non-obese diabetic (NOD) mice treated with water containing 10 mgF/L or not (control) for 14 weeks. The color of the nodes indicates difference in expression of the respective protein defined by its access code (UNIPROT ID). The dark red and dark green nodes indicate proteins unique to control and fluoride-treated groups, respectively. Light red and light green nodes indicate down and upregulation, respectively. The grey nodes indicate interacting proteins that were offered by CYTOSCAPE but were not identified in the present study.

Discussion

The present study was designed to refine the results obtained in our previous study, where 35-60-day-old male NOD treated with water containing 10 mgF/L for 21 days had significant reduction in plasma glucose levels when compared to those ingesting deionized water (Malvezzi et al., 2018). In the present study, considering the characteristics of the development of insulitis and diabetes in NOD mice, we included female mice and the treatment

was extended to 14 weeks. This was done because the degree of development of insulitis and diabetes in the female mice is higher than in the male ones (Mullen, 2017) that were evaluated in our previous study (Malvezzi et al., 2018). Moreover, in our previous study, the animals were euthanized when they were 9-13 weeks old (Malvezzi et al., 2019a) but it is known that in NOD mice insulitis starts at 2-4 weeks in females and in 5-7 weeks in males, while hyperglycemia develops at 12-30 weeks. By 30 weeks, it is expected that 90-100% of females and 50-80% of males become diabetic (Mullen, 2017). Taking this into consideration, as well as the fact that the effects of F in the organism are time-dependent (Pereira et al., 2018; Araujo et al., 2019), in the present study we extended the treatment period to 14 weeks, which means that the animals were euthanized when they were 20 weeks old.

In the present study, the treatment of female NOD mice with water containing 10 mgF/L, which is expected to lead to plasma F levels similar to the ones found in humans (Dunipace et al., 1995), reduced plasma glucose levels in 20% in respect to control, despite the difference was not significant. These results are in-line with our previous study with male NOD mice, where a 26% reduction in plasma glucose levels was observed upon treatment with 10 mgF/L water (Malvezzi et al., 2019a). However, the reduction observed by Malvezzi et al. (Malvezzi et al., 2019a) was significant. The lack of significant difference found in the present study might be due to the use of female mice, since it is expected a larger variability in the data due to hormonal fluctuations (Hughes, 2007; Smarr et al., 2017), which has been described also for the NOD mice (Leiter et al., 1987). We considered important to work with female NOD mice in this study because they develop insulitis and diabetes in a higher and faster fashion when compared with their male counterparts (Leiter et al., 1987; Mullen, 2017), as mentioned above. Thus, it is necessary to have studies with female mice, since the results obtained wit male ones cannot always be easily extrapolated to females (Smarr et al., 2017). Another factor that deserves attention regarding the plasma glucose levels is the treatment period. In the present study the female NOD mice were treated for 14 weeks, which led to very high levels of mean plasma glucose (339 ± 163 and 424 ±280 mg/dL in treated and control mice, respectively). However, in our previous study, the male NOD mice were treated for 3 weeks only and they

did not become overtly hyperglycemic, since mean plasma glucose levels were 141 ± 21 and 191 ± 17 mg/dL for the treated and control mice, respectively (Malvezzi et al., 2019a). Thus, the results of the present study not only corroborate our previous findings (Malvezzi et al., 2019a) but also indicate that F has an effect also in later stages of diabetes development. Considering that the fluoride levels employed in our study mimic the levels found in the artificially fluoridated water for humans, these results are very important from the point of view of public health, since artificially fluoridated water is employed worldwide as an effective measure to control dental caries (Buzalaf, 2018). Thus, it is of great interest to unravel the mechanisms by which F is able to reduce or delay the onset of diabetes.

Since muscle and liver are involved in glucose homeostasis, we performed proteomic analysis of these tissues to better understand the mechanisms involved in the reduction of plasma glucose levels by F. When the gastrocnemius muscle proteins were evaluated, we found 8 increased and 26 decreased proteins upon treatment with 10 mgF/L, when compared with control. As for the liver, the respective numbers were 18 and 17 proteins, respectively. However, the *fold change* was always lower than 2, with maximum ratio of 1.42 for increased and if 0.74 for decreased proteins for the muscle and 1.51 and 0.63 for the liver. Despite there is not a standard threshold of protein fold change that is related to a significant biological effect, in proteomics studies we usually emphasize the proteins presenting fold change ≥ 2. These findings give additional support to the safety of water fluoridation, since the alterations found were not extensive.

F is known for its ability to alter energy metabolism, leading to oxidative stress (Barbier et al., 2010; Zuo et al., 2018; Araujo et al., 2019). Thus, it is not surprising that most of the proteins with altered expression in the liver and muscle upon exposure to F were related to energetic pathways, as well as with antioxidant defense (Tables 2 and 3). Some of the metabolic effects of F on energy metabolism are related to its ability to inhibit enolase, which was firstly described in the 1940´s (Warburg and Christian, 1942). In the present study, Alpha enolase was increased in the liver upon exposure to F, in a possible attempt to maintain the energy flux (Table 3; Figure 4B). However, other glycolytic enzymes, such as

Phosphoglycerate kinase 1 and Triosephosphate isomerase were absent in the F-treated groups both in the liver (Table 3; Figure 4B) and in the muscle (ATP-dependent 6-phosphofructokinase and Phosphoglycerate kinase 1; Table 2, Figura 3A). Reduction in glycolytic enzymes upon exposure to low doses of F has also been described in other studies conducted in rats (Araujo et al., 2019) and NOD mice (Malvezzi et al., 2018). The impairment of glycolysis in the presence of F is expected to activate alternative pathways for glucose production, such as gluconeogenesis (increase in Fructose-1_6-bisphosphatase) and glycogenolysis (increase in Glycogen phosphorylase) (Table 3). Moreover, exposure to F also reduced β-oxidation, since Carnitine O-palmitoyltransferase 2_ mitochondrial (CPT), required for fatty acids mitochondrial uptake was absent, while Acylcarnitine hydrolase was increased (thus hydrolyzing acylcarnitine) upon exposure to F. Moreover, very long-chain acyl-CoA dehydrogenase was absent, while short-chain acyl-CoA dehydrogenase was reduced upon exposure to F. However, Hydroxyacyl-CoA dehydrogenase_ mitochondrial was exclusively found in the F-treated group (Table 3, Figure 4A). Acyl-CoA dehydrogenases are involved in the first reaction in β-oxidation. It is also important to highlight that Fatty acid-binding protein (FABP) that plays a role in lipoprotein-mediated cholesterol uptake in hepatocytes was increased upon exposure to F. These data, in conjunction, suggest preferential oxidation of short-chain fatty acids upon treatment with F.

The common fate of the metabolic fuels is the Tricarboxylic acids (TCA) cycle, which connects all pathways implicated in energy production. In the present study, some enzymes of the cycle were altered upon exposure to F. Citrate synthase, the first enzyme that catalyzes the condensation between acetyl-CoA and oxaloacetate to form citrate was absent in the presence of F, which *per se* impairs the cycle. This means that acetyl-CoA, instead of entering into the TCA cycle, might get condensed with acetoacetyl-CoA through the action of Hydroxymethylglutaryl-CoA synthase (HMG-CoA synthase), which was also increased in the presence of F (Table 3, Figure 4B). This enzyme was also increased by F in another study of our group (Araujo et al., 2019). It catalyzes the first reaction in the formation of ketone bodies.

The reduced flow of the TCA cycle in the presence of F is consistent also with the reduction in malate dehydrogenase, thus decreasing the formation of oxaloacetate.

When excessive amounts of amino acids are present and cannot be stored, they are therefore degraded through the urea cycle (Devlin, 2011). In the present study, several enzymes of the urea cycle were increased by F, such as Arginase-1, Argininosuccinate synthase and Glutaryl-CoA dehydrogenase (Figure 4A), as reported in previous studies (Lobo et al., 2015a; Araujo et al., 2019). Moreover, in the present study, consistently to what we found in a previous study (Araujo et al., 2019), Glutamate dehydrogenase (GLUD 1) was increased by F, which indicates a vigorous catabolism of amino acids.

The most remarkable finding of the present study was the fact that Phosphoenolpyruvate carboxykinase (PEPCK) was found exclusively in the liver of control mice. This enzyme has received considerable attention in the last decade, due to its link to enolase. Despite classically recognized as a glycolytic enzyme, enolase is now known to participate in several “moonlighting” functions not related to the glycolytic pathway (Jung et al., 2014). One of them is glucose homoeostasis via regulation of PEPCK expression. This function was first reported *in vivo* in 2013 using ENOblock, a nonsubstrate analogue that directly binds to enolase and inhibits its activity (Jung et al., 2013). This leads to down-regulation in the expression of PEPCK, a positive regulator of gluconeogenesis, thus increasing glucose uptake. Curiously, one of the most known inhibitors of enolase is F. This inhibition has been described since the 1940´s (Warburg and Christian, 1942). These observations strongly suggest that the reduction in plasma glucose levels in the present study (around 20%) is due to the inhibitory effect of F on enolase, which leads to down-regulation of PEPCK (Table 3). In addition, F was also reported to reduce Sterol regulatory element-binding protein (SREBP) (Pereira et al., 2016) similarly to ENOblock (Cho et al., 2019), which, in-turn, is also associated with anti-diabetic effects (Cho et al., 2017), since reduction of SREBP deactivates the triglycerides synthesis (Kammoun et al., 2009).

In conclusion, our results suggest, for the first time, a probable mechanism for the anti-diabetic effects of low F doses, reinforcing the importance of the dose and time of exposure in

these effects. Future mechanistic studies should be done to refine our findings, which is very important for public health because a safe and cost-effective measure to control dental caries might also control diabetes, a growing epidemy worldwide.

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Competing interests

The authors have declared no conflict of interest.

Authors contributions

J.S.T, M.B and J.R.B conceived the experiments. J.S.T, A.D, A.Q.D, A.C.M, M.B and J.R.B conducted the experiments. J.S.T, A.D, A.Q.D, T.M.C, J.R.B and N.R.B participated in the research experiments. J.S.T, A.D, A.Q.D, T.M.C, J.R.B, M.B and N.R.B. participated in the experiments analysis. J.S.T, A.C.M. and M.B. drafted the article; analyzed and interpreted the results. All authors reviewed and approved the manuscript.

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SUPPLEMENTARY TABLES

Supplementary Table 1. Proteins differentially expressed or exclusive in the gastrocnemius muscle of non-obese diabetic (NOD) female mice treated for 14 weeks with drinking water containing 10 mgF/L or not (control).

[a]Access Number	Protein name	PLGS score	[b]*Ratio* 10 mgF/L *vs* control
O55143	Sarcoplasmic/endoplasmic reticulum calcium ATPase 2	24	1.42
Q8R429	Sarcoplasmic/endoplasmic reticulum calcium ATPase 1	76	1.28
Q6URW6	Myosin-14	58	1.16
O08638	Myosin-11	58	1.16
Q61879	Myosin-10	58	1.15
Q8VDD5	Myosin-9	58	1.15
A2AQP0	Myosin-7B	223	1.14
P97457	Myosin regulatory light chain 2_ skeletal muscle isoform	344	1.13
Q5SX39	Myosin-4	2711	0.98
P13542	Myosin-8	2413	0.97
Q5SX40	Myosin-1 OS=Mus musculus	1837	0.96
Q91Z83	Myosin-7	1137	0.96
Q02566	Myosin-6 OS=Mus musculus	1113	0.95
Q9WUB3	Glycogen phosphorylase_ muscle form	238	0.94
P58771	Tropomyosin alpha-1 chain	314	0.94
O88990	Alpha-actinin-3	59	0.93
P07310	Creatine kinase M-type	4274	0.93
P05977	Myosin light chain 1/3_ skeletal muscle isoform	1416	0.90
P62737	Actin_ aortic smooth muscle	22243	0.90
P63268	Actin_ gamma-enteric smooth muscle	22243	0.90
P68033	Actin_ alpha cardiac muscle 1	22243	0.90
P68134	Actin_ alpha skeletal muscle	24734	0.90
Q9JKS4	LIM domain-binding protein 3	101	0.90
P05064	Fructose-bisphosphate aldolase A	4554	0.89
P19426	Negative elongation factor E	156	0.86
P56480	ATP synthase subunit beta_ mitochondrial	143	0.85
P60710	Actin_ cytoplasmic 1	17074	0.85
P63260	Actin_ cytoplasmic 2	17074	0.85
Q8BFZ3	Beta-actin-like protein 2	2985	0.85
Q03265	ATP synthase subunit alpha_ mitochondrial	102	0.82
P16858	Glyceraldehyde-3-phosphate dehydrogenase	2564	0.80
O09165	Calsequestrin-1	224	0.79
E9Q784	Zinc finger CCCH domain-containing protein 13	44	0.74
Q5SSE9	ATP-binding cassette sub-family A member 13	21	*10 mgF/L
Q8VCB1	Nucleoporin NDC1	36	10 mgF/L
Q9WVJ2	26S proteasome non-ATPase regulatory subunit 13	151	control
O08603	26S proteasome non-ATPase regulatory subunit 13	68	control
Q9R0Y5	Adenylate kinase isoenzyme 1	58	control
Q9JI91	Alpha-actinin-2	33	control
P05202	Aspartate aminotransferase, mitochondrial	33	control

P47857	ATP-dependent 6-phosphofructokinase, muscle type	27	control
Q922D8	C-1-tetrahydrofolate synthase, cytoplasmic	57	control
Q8BHB9	Chloride intracellular channel protein 6	55	control
Q3U1Y4	DENN domain-containing protein 4B	122	control
Q7TMY8	E3 ubiquitin-protein ligase HUWE1	10	control
Q8K2D3	Enhancer of mRNA-decapping protein 3	64	control
Q8BPY9	Fidgetin-like protein 1	31	control
P56916	Homeobox protein goosecoid-2	50	control
Q9D2I5	LisH domain-containing protein ARMC9	29	control
P06151	L-lactate dehydrogenase A chain	205	control
P16125	L-lactate dehydrogenase B chain	83	control
P00342	L-lactate dehydrogenase C chain	102	control
P08249	Malate dehydrogenase, mitochondrial	89	control
Q9JK37	Myozenin-1	80	control
P09411	Phosphoglycerate kinase 1	26	control
P70458	Plasma serine protease inhibitor	57	control
Q9DD19	Ras association domain-containing protein 7	26	control
Q8C033	Rho guanine nucleotide exchange factor 10	29	control
Q61062	Segment polarity protein dishevelled homolog DVL-3	41	control
Q8CIN4	Serine/threonine-protein kinase PAK 2	59	control
Q0P678	Zinc finger CCCH domain-containing protein 18	101	control

[a] Identification is based on proteins ID from UniProt protein databases, reviewed only (http://www.uniprot.org/).
[b] Protein with expression significantly altered are organized according to the ratio
* Indicates unique proteins in alphabetical order

Supplementary Table 2. Proteins differentially expressed or exclusive in the liver of non-obese diabetic (NOD) female mice treated for 14 weeks with drinking water containing 10 mgF/L or not (control).

[a]**Access Number**	**Protein name**	**PLGS score**	[b]***Ratio* 10 mgF/L *vs* control**
Q9ET01	Glycogen phosphorylase_ liver form	347	1.51
Q8CHT0	Delta-1-pyrroline-5-carboxylate dehydrogenase_ mitochondrial	201	1.48
P35700	Peroxiredoxin-1	1361	1.43
P54869	Hydroxymethylglutaryl-CoA synthase_ mitochondrial	719	1.34
P17182	Alpha-enolase	737	1.31
P10126	Elongation factor 1-alpha 1	1104	1.25
P12710	Fatty acid-binding protein_ liver	7108	1.22
P26443	Glutamate dehydrogenase 1_ mitochondrial	3426	1.20
O35215	D-dopachrome decarboxylase	2175	1.20
Q61176	Arginase-1	4109	1.17
Q9QXD6	Fructose-1_6-bisphosphatase 1	2642	1.16
P47738	Aldehyde dehydrogenase_ mitochondrial	2905	1.14
Q03265	ATP synthase subunit alpha_ mitochondrial	2326	1.13
P07724	Serum albumin	669	1.12

P00329	Alcohol dehydrogenase 1	352	1.11
P10649	Glutathione S-transferase Mu 1	2625	1.11
P16460	Argininosuccinate synthase	1344	1.08
Q8R0Y6	Cytosolic 10-formyltetrahydrofolate dehydrogenase	6743	1.07
P08249	Malate dehydrogenase_ mitochondrial	1725	0.89
P02088	Hemoglobin subunit beta-1	54841	0.88
P63038	60 kDa heat shock protein_ mitochondrial	1407	0.88
Q8CGP2	Histone H2B type 1-P	5593	0.87
P10853	Histone H2B type 1-F/J/L	5593	0.87
Q6ZWY9	Histone H2B type 1-C/E/G	5593	0.87
P70696	Histone H2B type 1-A	3711	0.86
Q64475	Histone H2B type 1-B	5593	0.86
Q64478	Histone H2B type 1-H	5593	0.86
Q64524	Histone H2B type 2-E	5593	0.86
Q8CGP0	Histone H2B type 3-B	5593	0.86
Q8CGP1	Histone H2B type 1-K	5593	0.86
Q9D2U9	Histone H2B type 3-A	5593	0.86
Q64525	Histone H2B type 2-B	5593	0.85
P10854	Histone H2B type 1-M	5593	0.85
P11679	Keratin_ type II cytoskeletal 8	381	0.63
Q07417	Short-chain specific acyl-CoA dehydrogenase_ mitochondrial	332	0.63
Q91WG0	Acylcarnitine hydrolase	21	*10 mgF/L
O35403	Amine sulfotransferase	95	10 mgF/L
Q60759	Glutaryl-CoA dehydrogenase_ mitochondrial	202	10 mgF/L
P62806	Histone H4	182	10 mgF/L
Q61425	Hydroxyacyl-coenzyme A dehydrogenase_ mitochondrial	260	10 mgF/L
Q9DBA8	Probable imidazolonepropionase	90	10 mgF/L
O88451	Retinol dehydrogenase 7	98	10 mgF/L
P08032	Spectrin alpha chain_ erythrocytic 1	16	10 mgF/L
Q63886	UDP-glucuronosyltransferase 1-1	160	10 mgF/L
P70691	UDP-glucuronosyltransferase 1-2	160	10 mgF/L
Q64435	UDP-glucuronosyltransferase 1-6	160	10 mgF/L
Q6ZQM8	UDP-glucuronosyltransferase 1-7C	160	10 mgF/L
Q62452	UDP-glucuronosyltransferase 1-9	169	10 mgF/L
Q9QXE0	2-hydroxyacyl-CoA lyase 1	132	control
P61922	4-aminobutyrate aminotransferase_ mitochondrial	275	control
P47955	60S acidic ribosomal protein P1	1460	control
Q8CAY6	Acetyl-CoA acetyltransferase_ cytosolic	366	control
Q5SWU9	Acetyl-CoA carboxylase 1	85	control
Q9QXG4	Acetyl-coenzyme A synthetase_ cytoplasmic	258	control
Q8R2S9	Actin-related protein 8	56	control
A2AS89	Agmatinase_ mitochondrial	544	control
Q9JII6	Alcohol dehydrogenase [NADP(+)]	172	control
Q9CZS1	Aldehyde dehydrogenase X_ mitochondrial	80	control
O35945	Aldehyde dehydrogenase_ cytosolic 1	97	control

Q99K67	Alpha-aminoadipic semialdehyde synthase_ mitochondrial	207	control
Q9Z0X1	Apoptosis-inducing factor 1_ mitochondrial	79	control
Q9DCX2	ATP synthase subunit d_ mitochondrial	425	control
Q562E2	BTB/POZ domain-containing protein KCTD19	123	control
B2RQC6	CAD protein	55	control
Q63880	Carboxylesterase 3A	242	control
P52825	Carnitine O-palmitoyltransferase 2_ mitochondrial	102	control
Q9CZU6	Citrate synthase_ mitochondrial	218	control
Q68FD5	Clathrin heavy chain 1	45	control
Q8R3I3	Conserved oligomeric Golgi complex subunit 6	140	control
P43024	Cytochrome c oxidase subunit 6A1_ mitochondrial	561	control
P28271	Cytoplasmic aconitate hydratase	124	control
Q8CDP0	Cytosolic carboxypeptidase 3	138	control
Q80XN0	D-beta-hydroxybutyrate dehydrogenase_ mitochondrial	82	control
P10518	Delta-aminolevulinic acid dehydratase	553	control
Q8BVI4	Dihydropteridine reductase	368	control
P62631	Elongation factor 1-alpha 2	147	control
P58252	Elongation factor 2	41	control
Q8BH95	Enoyl-CoA hydratase_ mitochondrial	228	control
Q8CI94	Glycogen phosphorylase_ brain form	44	control
Q9WUB3	Glycogen phosphorylase_ muscle form	54	control
P07901	Heat shock protein HSP 90-alpha	50	control
C0HKE1	Histone H2A type 1-B	1295	control
C0HKE2	Histone H2A type 1-C	1295	control
C0HKE3	Histone H2A type 1-D	1295	control
C0HKE4	Histone H2A type 1-E	1295	control
Q8CGP5	Histone H2A type 1-F	1295	control
C0HKE5	Histone H2A type 1-G	1295	control
Q8CGP6	Histone H2A type 1-H	1295	control
C0HKE6	Histone H2A type 1-I	1295	control
Q8CGP7	Histone H2A type 1-K	1295	control
C0HKE7	Histone H2A type 1-N	1295	control
C0HKE8	Histone H2A type 1-O	1295	control
C0HKE9	Histone H2A type 1-P	1295	control
Q6GSS7	Histone H2A type 2-A	1295	control
Q64522	Histone H2A type 2-B	1008	control
Q64523	Histone H2A type 2-C	1295	control
Q8BFU2	Histone H2A type 3	1295	control
Q8R1M2	Histone H2A.J	1295	control
Q3THW5	Histone H2A.V	1008	control
P0C0S6	Histone H2A.Z	1008	control
P27661	Histone H2AX	1008	control
P85094	Isochorismatase domain-containing protein 2A	147	control
P05784	Keratin_ type I cytoskeletal 18	83	control
P19001	Keratin_ type I cytoskeletal 19	60	control

Q71RI9	Kynurenine--oxoglutarate transaminase 3	937	control
P41216	Long-chain-fatty-acid--CoA ligase 1	101	control
P97772	Metabotropic glutamate receptor 1	56	control
Q99J09	Methylosome protein 50	84	control
Q8K009	Mitochondrial 10-formyltetrahydrofolate dehydrogenase	82	control
Q9JHW2	Omega-amidase NIT2	220	control
Q60862	Origin recognition complex subunit 2	48	control
P17742	Peptidyl-prolyl cis-trans isomerase A	985	control
O08807	Peroxiredoxin-4	681	control
Q9Z2V4	Phosphoenolpyruvate carboxykinase_ cytosolic [GTP]	206	control
P09411	Phosphoglycerate kinase 1	415	control
Q61838	Pregnancy zone protein	58	control
Q8BGV0	Probable asparagine--tRNA ligase_ mitochondrial	107	control
P67778	Prohibitin	179	control
P27773	Protein disulfide-isomerase A3	86	control
Q922R8	Protein disulfide-isomerase A6	61	control
P09103	Protein disulfide-isomerase	73	control
Q99LX0	Protein DJ-1	971	control
P24549	Retinal dehydrogenase 1	98	control
Q99J08	SEC14-like protein 2	364	control
Q8BG48	Serine/threonine-protein kinase 17B	320	control
Q91WS4	S-methylmethionine--homocysteine S-methyltransferase BHMT2	135	control
Q8K2B3	Succinate dehydrogenase [ubiquinone] flavoprotein subunit_ mitochondrial	84	control
Q8VC30	Triokinase/FMN cyclase	102	control
P17751	Triosephosphate isomerase	2645	control
A2AQ07	Tubulin beta-1 chain	67	control
Q7TMM9	Tubulin beta-2A chain	256	control
Q9CWF2	Tubulin beta-2B chain	162	control
Q9ERD7	Tubulin beta-3 chain	98	control
Q9D6F9	Tubulin beta-4A chain	64	control
P68372	Tubulin beta-4B chain	74	control
P99024	Tubulin beta-5 chain	162	control
Q922F4	Tubulin beta-6 chain	118	control
E9Q3T0	Uncharacterized protein	1374	control
P50544	Very long-chain specific acyl-CoA dehydrogenase_ mitochondrial	63	control
Q9WUK6	Zinc finger and BTB domain-containing protein 18	97	control
O88532	Zinc finger RNA-binding protein	239	control

[a] Identification is based on proteins ID from UniProt protein databases, reviewed only (http://www.uniprot.org/).

[b] Protein with expression significantly altered are organized according to the ratio

* Indicates unique proteins in alphabetical order

DISCUSSION

3 DISCUSSION

The present study was designed to shed light onto the mechanisms underlying the effects of F in glucose homeostasis. The F concentration in the drinking water employed in the present study (10 mg/L) mimicks the one present in articially fluoridated water (around 1 mg/L), since rodents metabolize F 5-10 faster than humans. Thus, it is expected that when rodents ingest water containing 10 mgF/L, their plasma F levels correspond to those found in humans that drink water containing 1 mgF/L (Dunipace *et al.*, 1995).

We chose the NOD mice model, since these mice develop diabetes spontaneously through an autoimmune disorder, similarly to what occurs in humans (Mullen, 2017). In our previous study using the same strain we treated, for 21 days, male NOD mice with water containing 10 mgF/L (Malvezzi *et al.*, 2019). However, in the present study we worked with female NOD mice, since they start developing insulitis earlier and develop diabetes in a greater proportion than the male ones (Malvezzi *et al.*, 2019). Regarding the duration of the treatment with fluoridated water, in the present study the treatment was extended to 14 weeks (versus 3 weeks our previous study (Malvezzi *et al.*, 2019)). This was done considering that the timing of T1D development in female NOD mice. In these mice insulitis starts at 2-4 weeks in females and, by 30 weeks of age, it is expected that 90-100% of them become diabetic (Mullen, 2017). We conducted a pilot study (data not shown) to refine the duration of the treatment, since the development of T1D by the NOD mice varies. In this pilot study, the blood of the mice was collected weekly for up to 14 weeks. In this period the rats received water containing 10 mgF/L. It was observed that at 14 weeks of treatment (20 weeks of age), most of the rats already had plasma glucose levels higher than at baseline. Moreover, the period of treatment was not extended to more than 14 weeks because this would increase the chance of death. Thus, our intention was to evaluate the effect of F to delay of prevent the onset of T1D, which was evaluated by analysis of plasma glucose levels. In fact, the mice treated with F had plasma glucose levels 20% lower when compared with the control mice that received deionized water, although the difference did not reach statistical significance. In our previous study, we observed 26% reduction in plasma glucose levels of male NOD mice treated with water

10 mgF/L for 3 weeks only and killed at 9-13 weeks of age. And this reduction was significant (Malvezzi *et al.*, 2019). We believe that the reasons for the lack lack of significance in the present study compared to the previous one (Malvezzi *et al.*, 2019) might be explained by several factors: 1) The use of female mice, which are subject to hormonal fluctuations (Hughes, 2007; Smarr *et al.*, 2017) that have already been shown to occur in NOD mice (Leiter *et al.*, 1987); 2) The period of treatment. In the present study the treatment was performed over 14 weeks and this led to higher plasma glucose levels (339 ± 163 and 424 ±280 mg/dL in treated and control mice, respectively) than in our previous study, in which the treatment was performed over 3 weeks only (141 ± 21 and 191 ± 17 mg/dL for the treated and control mice, respectively (Malvezzi *et al.*, 2019). Moreover, the effects of F in the organism are known to be not only dose- but also time-dependent. It has been reported that lower chronic doses of F, such as those employed in the present, study incitate a delayed adaptative response of the organism (Dabrowska *et al.*, 2006; Pereira *et al.*, 2018; Araujo, T.T. *et al.*, 2019). Thus, our results indicate that F has an effect also in the later stages of T1D development, which is quite important from the public health point-of-view. This importance relays on the fact that fluoridation of the public water supply is a recognized measure to control caries (Buzalaf, 2018) and, when implemented, tipically lasts for the lifetime.

This is the third study that reported a beneficial effect of fluoridated water on glucose homeostasis. The other first two studies, despite showing the beneficial effects of F (Lobo, J. G. *et al.*, 2015; Malvezzi *et al.*, 2018), provided only limited information on the possible mechanisms involved. Thus, in the present study we attempted to better investigate the mechanistic aspects, employing proteomic analysis of the liver and gastrocnemius muscle, important organs/systems involved in glucose homeostasis.

Some important findings arose from our proteomic data: 1) the *fold change* when comparing treated *vs.* control groups was, in all cases, lower 2, which indicates that the F-induced protein alterations were mild, giving additional support to public water fluoridation; 2) Most of the proteins with altered expression were related to energy metabolism and antoxidant defense, which is not a surprise since F is known for its ability to alter energy metabolism, leading to oxidative stress (Barbier *et al.*, 2010;

Zuo *et al.*, 2018; Araujo, T.T. *et al.*, 2019) and diabetes also provokes oxidative stress (Reus *et al.*, 2019).

Among the proteins related to energy metabolism that were differentialy expressed upon treatment with F, the most remarkable alteration was found for Phosphoenolpyruvate carboxykinase (PEPCK), exclusively identified in the liver of control mice. This enzyme is linked to enolase that despite classically described as a glycolytic enzyme, participates in several “moonlighting” functions not related to the glycolytic pathway (Jung *et al.*, 2014). One of these “moonlighting” functions is glucose homoeostasis via regulation of PEPCK. F is known to inhibit enolase since the 1940´s (Warburg e Christian, 1942) and inhibition of enolase reduces the expression of PEPCK, a positive regulator of gluconeogenesis, thus increasing glucose uptake (Jung *et al.*, 2013), which consequently removes glucose from the blood. Thus, the increase in cell glucose uptake in the presence of F might help to explain the lower plasma glucose levels found in the present study (Fig. 1).

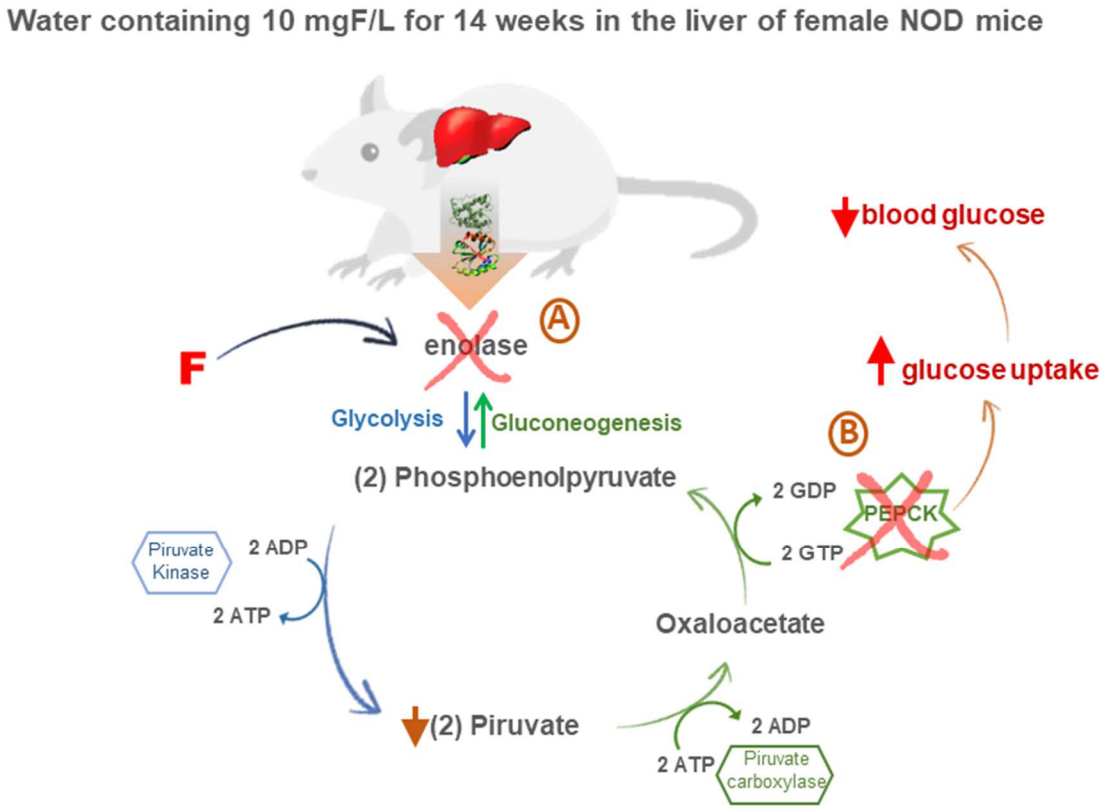

Figure 1. Scheme of the action of F in the liver of NOD mice. A In the glycolytic , fluoride (F) inhibits enolase. This enzyme forms phosphoenolpyruvate, which is transformed into pyruvate through the action of the enzyme. Thus, the formation of piruvite is reduced by F. **B** On the other hand, in the gluconeogenesis pathway, phosphoenolpiruvate carboxinase (PEPCK) converts oxaloacetate to phosphoenolpyruvate. Since F reduces the expression of PEPCK, an increase in glucose uptake occurs, thus reducing blood glucose.

Thus, the present study is the pioneer in suggesting a probable mechanism for the anti-diabetic effects of low F doses, similar to the ones typically found in artificially fluoridated water. Additional proof-of-concept studies must be conducted to confirm this proposed mechanism that is of great relevance for public health, since a safe and cost-effective measure to control caries might also delay or prevent the onset of diabetes.

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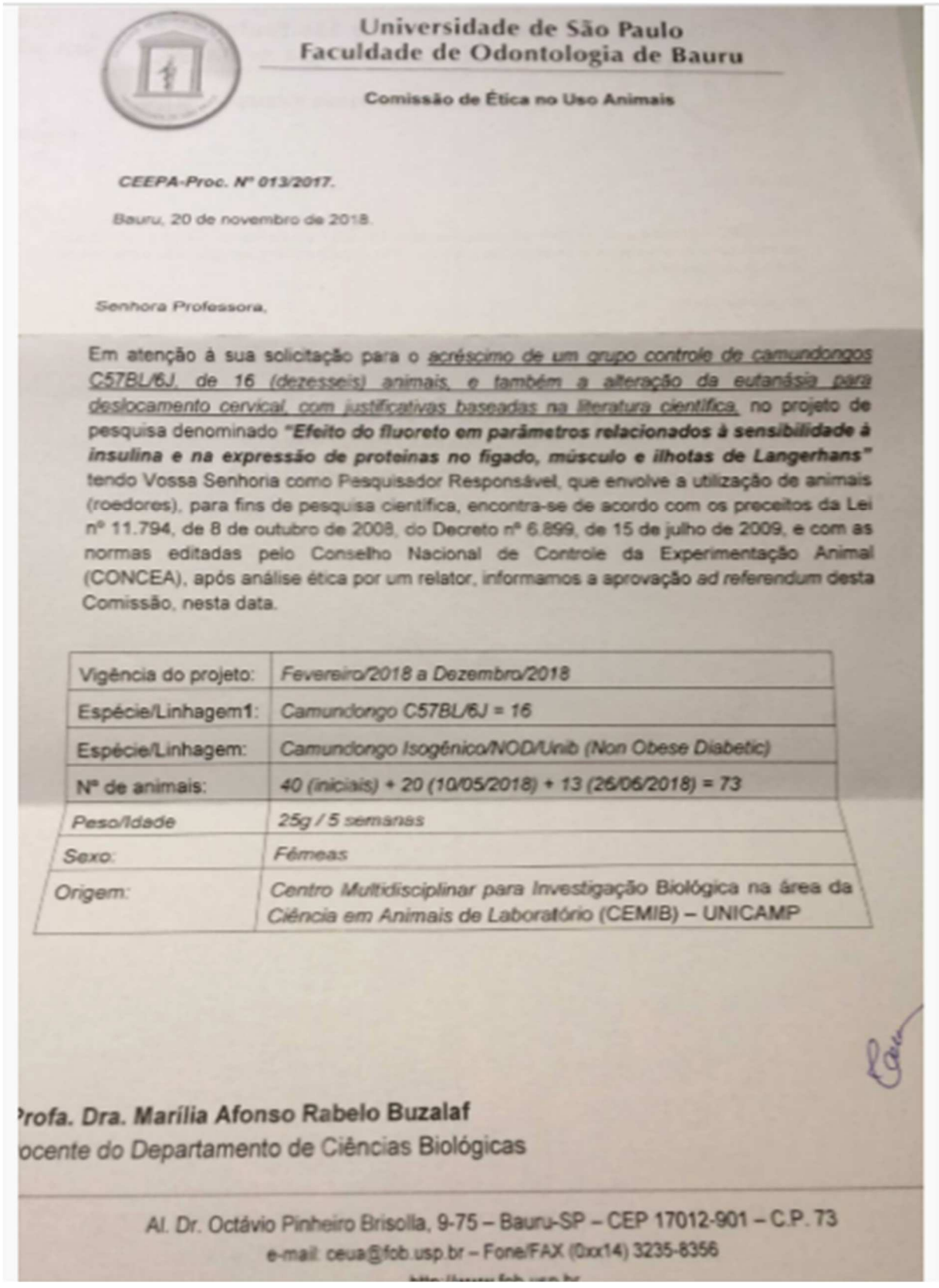
ANNEX

ANNEX 1



Universidade de São Paulo
Faculdade de Odontologia de Bauru

Comissão de Ética no Uso Animais

CEEPA-Proc. Nº 013/2017.

Bauru, 20 de novembro de 2018.

Senhora Professora,

Em atenção à sua solicitação para o *acréscimo de um grupo controle de camundongos C57BL/6J, de 16 (dezesseis) animais, e também a alteração da eutanásia para deslocamento cervical, com justificativas baseadas na literatura científica,* no projeto de pesquisa denominado ***"Efeito do fluoreto em parâmetros relacionados à sensibilidade à insulina e na expressão de proteínas no fígado, músculo e ilhotas de Langerhans"*** tendo Vossa Senhoria como Pesquisador Responsável, que envolve a utilização de animais (roedores), para fins de pesquisa científica, encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), após análise ética por um relator, informamos a aprovação *ad referendum* desta Comissão, nesta data.

Vigência do projeto:	*Fevereiro/2018 a Dezembro/2018*
Espécie/Linhagem1:	*Camundongo C57BL/6J = 16*
Espécie/Linhagem:	*Camundongo Isogênico/NOD/Unib (Non Obese Diabetic)*
Nº de animais:	*40 (iniciais) + 20 (10/05/2018) + 13 (26/06/2018) = 73*
Peso/Idade	*25g / 5 semanas*
Sexo:	*Fêmeas*
Origem:	*Centro Multidisciplinar para Investigação Biológica na área da Ciência em Animais de Laboratório (CEMIB) – UNICAMP*

Profa. Dra. Marília Afonso Rabelo Buzalaf
Docente do Departamento de Ciências Biológicas

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Universidade de São Paulo
Faculdade de Odontologia de Bauru

Comissão de Ética no Uso Animais

(Cont 2/2)

Esta CEUA solicita que ao final da pesquisa seja enviado um Relatório com os resultados obtidos para análise ética e emissão de parecer final, o qual poderá ser utilizado para fins de publicação científica.

Atenciosamente,

Cassia Maria Fischer Rubira

Profª Drª Cássia Maria Fischer Rubira

Vice-Presidente no exercício da Comissão de Ética no Uso de Animais

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