UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ODONTOLOGIA DE BAURU

ZOHAIB NISAR KHAN

Proteomic analysis of Liver in mice with different susceptibilities to Fluorosis

Análise proteômica do fígado de camundongos com diferentes suscetibilidades à fluorose

BAURU
2017

ZOHAIB NISAR KHAN

Proteomic analysis of Liver in mice with different susceptibilities to Fluorosis

Análise proteômica do fígado de camundongos com diferentes suscetibilidades à fluorose

Thesis presented to the Bauru School of Dentistry of the University of São Paulo to obtain the PhD degree in Sciences in the program of Applied Oral Sciences, Stomatology and Oral Biology concentration area.

Supervisor: Prof. Drª Marília Afonso Rabelo Buzalaf

Tese apresentada à Faculdade de Odontologia de Bauru da Universidade de São Paulo para obtenção do título de Doutor em Ciências no Programa de Ciências Odontológicas Aplicadas, área de concentração Estomatologia e Biologia Oral.

Orientadora: Prof. Drª Marília Afonso Rabelo Buzalaf

Versão Corrigida

BAURU

2017

Khan, Zohaib Nisar

K527p Proteomic analysis of Liver in mice with different susceptibilities to Fluorosis / Zohaib Nisar Khan – Bauru, 2017.

97 p. : il. ; 31cm.

Tese (Doutorado) – Faculdade de Odontologia de Bauru. Universidade de São Paulo

Orientadora: Prof. Drª Marília Afonso Rabelo Buzalaf

Nota: A versão original desta tese encontra-se disponível no Serviço de Biblioteca e Documentação da Faculdade de Odontologia de Bauru – FOB/USP.

Comitê de Ética em Animais da FOB-USP

Protocolo nº: 031/2013

Data: 20/08/2013

FOLHA DE APROVAÇÃO

DEDICATÓRIA

Todo trabalho desafiador necessita de esforços, bem como da orientação dos mais experientes, especialmente daqueles que estão em nossos corações. Este trabalho e todas as minhas conquistas acadêmicas são dedicadas aos meus amados pais:

Rukhsana & Nisar.

AGRADECIMENTOS

Primeiramente e o mais importante, eu gostaria de agradecer a *"ALLAH, Glória a Ele, O Exaltado."*

Eu nunca imaginaria um único dia sem ELE, pois ELE é tudo para mim; eu sou o que sou somente e tão somente por causa DELE. Eu sou grato a ELE por me dar forças para continuar este projeto e por me abençoar com ótimas pessoas que foram meus maiores apoios na vida profissional e pessoal.

Aos meus amados pais **Rukhsana** & **Nisar**,

Cujo afeto, muito amor, encorajamento e orações, me carregaram e inspiraram nos "bons e maus momentos", desde o início da minha carreira acadêmica e me possibilitaram alcançar o "sucesso e honra" que estou sentindo enquanto escrevo esta tese. Eu não posso devolver, restituir ou reembolsar os esforços que vocês fizeram por mim; eu não posso nem expressar meus sentimentos ao pensar nestes momentos, eu realmente tenho calafrios neste momento. Eu amo ser um menino da mamãe. Mãe, não sou eu quem está concluindo a pós-graduação, é na verdade VOCÊ. Papai, eu não tenho palavras para expressar o que eu sinto em meu coração por você. Meus olhos ficam molhados quando me lembro de você, pois faz falta em nosso quadro familiar. Eu nunca gostei do seu perfil rigoroso, mas eu estava tão bem adaptado ao seu rigor que eu estou sentindo muita falta disto agora. Eu ainda não tenho coragem para expressar meus sentimentos, então, vou deixá-los fluir em algum lugar dentro de mim. Que a sua alma descanse no céu (Amém).

À minha irmã **Hina**,

Que sempre teve um “olhar austero” para comigo, o qual me manteve focado em meus objetivos e, sobretudo, eu sempre serei agradecido pela ajuda (financeira e moral) que você me deu para que eu perseguisse meus objetivos. Nós tivemos muitas guerras, sim! Você me irritava de todos os modos possíveis, você foi a crítica que me rasgava, não para me colocar para baixo, mas para me ajudar a voar mais alto. Eu devo a você um grande “Obrigado”. Não importa aonde eu vá, ou o quanto eu envelhecer, eu nunca me esquecerei da minha infância. Eu verdadeiramente adorei e adoro ser seu “Mano”.

À minha irmã **Sana,**

Que é mais uma amiga, minha “guardadora de segredos”, minha terapeuta e a pessoa que eu sempre desejei ser. Eu não sei da existência de super-heróis, mas considerando você, eu posso confirmar a existência de super-heroínas. Todos os meus amigos são mágicos, mas eles somente desaparecem quando eu estou com problemas. Obrigado por ser a única amiga real que tenho. Eu nunca olharei para uma celebridade ou para uma personalidade famosa como um modelo a seguir enquanto eu tiver uma irmã como você. A melhor carta de recomendação que você pode ter é de mim dizendo que você fez um trabalho excelente sendo minha irmã.

À minha irmã **Komal,**

Oh sim, nós temos características tão próximas como “gêmeos siameses”, mas eu não posso competir com você em sua beleza, pureza, afeto e sobretudo o gesto real de carinho que você tem; de algum modo, você é uma porcentagem grande de minha inspiração. Quem precisa de wikiHow ou Wikipedia quando eu tenho uma “mana” legal como você? Você é simplesmente surpreendente.

Ao meu primeiro e único irmão **Uzair,**

Embora nós não conversemos muito, nem saiamos muito, você é protetor sem sufocar, liberal sem ser descuidado e vigilante sem ser constrangedor, você sempre se levantou alto para me defender. E apesar de eu ter ciúme de você considerando sua “altura”, eu sei do fundo do meu coração que você sempre estará lá por mim quando eu gritar. Eu te amo!

À minha Sobrinha **Zartashia,**

Você é a real beleza, você é alegria para meu coração. Você é um presente precioso para nossa família inteira. O que eu sei é que seu reflexo sempre ajuda a acalmar minha mente, quero dizer que toda vez que eu me sinto estressado, eu só penso em seus gestos e desejos infantis e estes são elementos finais, que me levam a uma zona de saciedade. Você só tem 5 anos agora, mas eu tenho certeza de que o dia em que você crescer o suficiente para ler toda esta admiração verbal, (claro que seu tio estará bem mais velho até lá) você certamente se sentirá orgulhosa o suficiente para dividir isto como uma "fofoca elegante" com seu grupo de amigas. Te amo.

Aos meus pequenos, **Eshal** e **Araiz**,

Vocês vieram como um negócio "compre um e receba outro de graça" mas vocês dois são uma completa fonte de "destruição" pois vocês nunca deixam ninguém trabalhar. Estas palavras são boas o suficiente para justificar o meu resto de amor por vocês. Sejam abençoados!

Aos meus colegas do **laboratório de bioquímica.**

Eu gostaria de agradecer meus colegas Larissa e Thelma, vocês não somente me ajudaram em meu trabalho mas também foram apoiadoras através de minha jornada que valem ser mencionadas aqui. O conhecimento que eu aprendi com vocês certamente me ajudará no futuro. O mínimo que eu poderia dizer é que bons colegas são aqueles fáceis de se aproximar e difíceis de culpar. Obrigado por serem assim.

À Especialista Técnica **Aline,**

Aline é uma pessoa do laboratório que eu gostaria de agradecer especialmente. Eu me lembro do meu primeiro encontro com você, em que eu fui informado que você era a "moça da Espectrometria de Massas". As regras, regulamentos, as habilidades, as técnicas que você me ensinou, certamente um dia eu implementarei no meu "laboratório em potencial" onde eu me sentirei como você. Você, uma pessoa rigorosa, mas de coração puro e sim, minha grande inspiração para seguir uma dieta de baixo teor de carboidratos. Além disso, você foi também minha porfessora de línguas (lembre-se do cara ignorante de Português). Eu não sei como demonstrar tamanha apreciação a uma colega de trabalho que me deu tanta motivação. Obrigado por ser tal inspiração.

À minha colega **Heloisa**.

Helo, obrigado pela sua ajuda ao longo do meu trabalho. Eu desejo a você tudo de melhor na vida.

Aos professores **Rodrigo** e **Ana Carolina.**

Obrigado por me fornecerem todo o conhecimento e apoio moral que eu preciso para enfrentar meus desafios futuros. Vocês foram sempre mentores inspiradores.

Aos **funcionários do Biotério**

Obrigado pela ajuda e orientação que eu tive para tartar meus animais da melhor forma.

À **Faculdade de Odontologia de Bauru – USP** e **CNPq/TWAS.**

Obrigado às minhas agências de financiamento, **CNPq/TWAS** pelo apoio financeiro através de minha jornada de pesquisa, e claro para a USP, pelo apoio institucional que, de modo contrário, não seria possível.

AGRADECIMENTOS ESPECIAIS

À professora ***Dra. Marília Afonso Rabelo Buzalaf***, da Faculdade de Odontologia de Bauru, que é academicamente minha orientadora, oficialmente minha *CHEFE*, mas pessoalmente, é muito mais do que estes termos gigantes que eu utilizei. Com respeito a ela eu me sentia e me sinto em uma super zona de conforto; ela é uma pessoa extraordinária e isto eu digo do fundo do meu coração; eu nunca tive senti medo dela, já que eu sou de uma cultura diferente onde sempre estamos numa zona de medo enquanto conversamos com professores de fora. Marília é a pessoa que mudou completamente a definição de "professor" que eu tinha. Eu orgulhosamente a idealizo como um modelo.

Como dizem "ninguém é perfeito", mas ela esta próxima da perfeição. Eu a conheci há 4 anos quando eu a enviei o meu primeiro e-mail solicitando uma carta de aceitação. Eu não tinha um currículo muito forte e considerando este fato, eu não esperava uma resposta dela. No entanto, para minha surpresa, ela me respondeu rapidamente e marcou uma reunião por Skype. Foi uma chamada por áudio e no momento que tocou eu estava tremendo ao atendê-la. Ela fez algumas poucas perguntas sobre mim e no outro dia, eu recebi uma carta para começar minha carreira sob sua supervisão.

O dia em que eu aterrisei pela primeira vez no Brasil, eu não sabia Português; foi somente Marília, que me ajudou mais do que eu esperava. Eu tenho arrepios de pensar naquele tempo. Não há meios de agradecê-la por todos os momentos de apoio. Eu aprendi muito com você. No momento que eu tive minha primeira publicação com você, eu senti que algo grande foi adicionado à minha carreira e sim, foi VERDADEIRO!

Eu não tenho palavras "MA", para expresser minha gratidão a você. Eu quero ser tão decente, tão criativo, tão bem sucedido quanto você. O futuro da humanidade não está nas novas invenções tecnológicas e avanços científicos. Está nas mãos de professores ambiciosos como você… porque é onde tudo começa. Eu não devo o meu sucesso professional ao meu destino, coragem, sorte, crença, confiança ou fortuna. Eu devo a uma professora maravilhosa como você. Não há meios de dizer obrigado por tudo que você fez por mim.

Que DEUS a abençoe sempre em cada passo da sua vida, Amém!

ABSTRACT

Proteomic analysis of liver in mice with different susceptibilities to Fluorosis.

Fluoride (F) is a potent anti-cariogenic element, but only an appropriate dose is effective to have therapeutic action, else systemic toxicity may be observed. Additionally, two factors, amount of F and time of exposure, drive its action. Surprisingly, the susceptibility to toxic effects of F is genetically determined. The present study identified the effects of F on the liver proteome of mice susceptible (A/J) or resistant (129P3/J) to the effects of F. Weanling male A/J (n=6) and 129P3/J mice (n=6) were housed in pairs and assigned to three groups given low-F diet and drinking water containing 0, 15 or 50 ppm F for 7 weeks. Liver proteome profiles were examined using nano-LC-ESI-MS/MS. Protein function was classified by GO biological process (Cluego v2.0.7 + Clupedia v1.0.8). Difference in expression among the groups was determined using the PLGS software. In the control group (0 ppm F), most proteins with fold change were increased in A/J mice. Precisely the proteins related to energy flux and oxidative stress were quite significant in this context, suggesting the high susceptibility of these mice to the effects of F, since the exposure also induces oxidative stress. Treatment with the lower F concentration provoked more pronounced alterations in fold change in liver proteins in comparison to the treatment with the higher F concentration. Strikingly, most of the proteins with fold change upon following 15 ppm F treatment, were increased in the A/J mice compared with their 129P3/J counterparts, thus suggesting attempt of the former to fight against the toxic effects of F. With respect to 50 ppm F, most proteins with fold change were decreased in the A/J mice compared with their 129P3/J counterparts, especially proteins related to oxidative stress and protein folding, which might be related to the higher susceptibility of the A/J animals to the deleterious effects of F. Our findings can provide new insights into the molecular mechanisms underlying genetic susceptibility to fluorosis by indicating key protein players which need to be better addressed in future experimental studies.

Keywords: Liver; Fluoride; Fluorosis; Proteomics; Oxidative stress; Genetic susceptibility.

RESUMO

O Fluoreto (F) é um potente elemento anti-cariogênico, mas é somente efetivo terapeuticamente em uma dose apropriada. Por outro lado, doses acima das recomendadas levam a toxicidade sistêmica. Em adição, dois fatores decidem sua efetividade de ação: quantidade de F e tempo de exposição. A suscetibilidade aos efeitos tóxicos do F é determinada geneticamente. O presente estudo avaliou os efeitos do F no proteoma do fígado de camundongos suscetíveis (A/J) ou resistentes (129P3/J) aos efeitos do F. Camundongos machos desmamados A/J (n=6) e 129P3/J (n=6) foram alojados em pares e divididos em três grupos tratados com ração com baixo teor de F e água contendo 0, 15, ou 50 ppm de F por 7 semanas. Perfis proteômicos do fígado foram examinados usando nano-LC-ESI-MS/MS. A função de proteínas foi classificada pelo processamento biológico GO (Cluego v2.0.7 + Clupedia v1.0.8). A diferença de expressão entre os grupos foi determinada usando o *software* PLGS. No grupo controle (0 ppm F), a expressão da maioria das proteínas foi aumentada nos camundongos A/J e precisamente as proteínas relacionadas ao fluxo de energia e estresse oxidativo foram significativas neste contexto, sugerindo portanto, a alta sucetibilidade destes camundongos aos efeitos do F, já que a exposição também induz o estresse oxidativo. O tratamento com baixa concentração de F provocou alterações mais pronunciadas em proteínas do fígado comparado ao tratamento com alta concentração de F. Notadamente, a maioria das proteínas encontradas no fígado dos animais tratados com 15 ppm de F foi aumentada em camundongos A/J comparados aos camundongos 129P3/J, demonstrando portanto, uma tentativa dos A/J de neutralizar os efeitos tóxicos do F. Já nos animais tratados com 50 ppm de F, a maioria das proteínas foi diminuída nos camundongos comparados aos seus pares 129P3/J, especialmente proteínas relacionadas ao estresse oxidativo e enovelamento de proteínas, o que pode estar relacionado à alta suscetibilidade dos animais A/J aos efeitos deletérios do F. Nossos achados podem fornecer novos *insights* que podem contribuir para a interpretação os mecanismos moleculares relacionados à suscetibilidade genética à fluorose, indicando proteínas chaves que precisam ser melhor estudadas em estudos futuros.

Palavras-Chave: Fígado; Fluoreto; Fluorose; Proteômica; Estresse oxidativo; Suscetibilidade genética.

TABLE OF CONTENTS

1 INTRODUCTION

1 INTRODUCTION

Fluorine is a widely distributed natural chemical element in the nature and because of its strong binding affinity, it mostly exists only in combined as the main component of minerals in soil and rocks; moreover, being the most electronegative element it also reacts with other elements to form fluorides (F) (COPLAN et al., 2007), besides being the thirteenth most abundant element in the earth's crust (SHANTAKUMARI; SRINIVASALU; SUBRAMANIAN, 2004). Fluoride salts are credited to play an important role in our daily lives e.g., sodium fluoride (NaF) is a widely used component of toothpaste and mouth rinse products (DANG; MAILANG; LALIC, 2014; VOGEL et al., 2015), potassium fluoride (KF) is one of the known fluoridation agent in chemical synthesis, whereas zinc fluoride tetra-hydrate (ZnF_2) is used in the preparation of thin-film optical coatings (WANG; YANG; LEE, 2014).

Multi-generational studies have confirmed the presence of F in different tissues of humans and animals, where F in certain amount is considered as an effective prophylactic agent necessary for dental health and bone development (ZHOU et al., 2015). On contrary, F has been reported to be cytotoxic in a concentration-dependent manner (ZARGAR et al., 2015; DUBEY; KHAN; RAINA, 2013; CHRISTEN et al., 1994). Additionally, the extent of its toxicity effect seems to be dependent on the duration of F administration and the age of the animals because of their adaptation to F at younger and older ages (DABROWSKA; LETKO; BALUNOWSKA, 2006).

Endemic chronic fluorosis has been reported in more than 50 countries where source of drinking water may be the causal factor (WU et al., 2002). According to WHO standards, the upper intake level of F around 1.5 mg/l is considered beneficial to human health. However, among the 25 countries that have high F concentration (>1.5 mg/L) in the water, such as China, India, México and Argentina, more than 200 million people suffer from endemic fluorosis (ZHOU et al; 2015; AMINI et al., 2008). High concentrations of F have been proven quite harmful to various dental and biochemical aspects of experimental animals, which thus highlights its toxicity to human health (MANDINIC et al., 2010; PERUMAL et al., 2013; ZHOU et al., 2013).

Living organisms face a great exposure to F through food, drinking water, fluoride additives, toothpastes, fluoride gel (ROHR et al., 1996) and inhalation of hydrofluoric gases through smoke, vapors, dust from burning coal, industrial manufacture of phosphate fertilizers, and volcanoes (PECKHAN; AWOFESO, 2014).Generally, a very small amount of F is desirable otherwise high levels are difficult to cope with and thus cannot be metabolized effectively (YANG et al., 2017). On crossing the threshold concentration, F accumulation leads to cascading effects resulting in altered physiological functions in humans (SAMANTA et al., 2016).The chronic F exposure is well documented and correlated to have various effects using animal models. Many proteins and enzymatic systems have been shown to suffer changes upon exposure to high F levels (BARBIER; ARREOLA-MENDOZA; DEL RAZO, 2010)**.** After being absorbed by the gastric-intestinal system, F is distributed to all soft and mineralized tissues by simple diffusion and upon high levels of exposure damage to various biological systems can occur (WHITFORD, 1996; MITTAL; FLORA, 2006). Significantly, high amounts of fluoride are known to cause configurationally altered enzyme activity in cells, and to influence lipid metabolism (SANA et al., 2017) including progressive degeneration of the structure and functions of the skeletal muscles, brain, and spine (MULLENIX et al., 1995). Furthermore, it increases the aerobic metabolism and promotes alterations in the metabolism of cellular free radicals in various organs such as liver, kidneys, and heart (SHANTAKUMARI; SRINIVASALU; SUBRAMANIAN, 2004; CIMASONI, 1972; JENKINS; VENKATESWARLU; ZIPKIN, 1970).

F is a potent inhibitor of many enzymes (WHITFORD, 1996) and even at micromolar concentrations, it can induce apoptosis and affect the regulation of immune response (ANURADHA; KANNO; HIRANO, 2001; REFSNES et al., 2003, 2001). Many reports have demonstrated that fluoride induces a high amount of free oxygen radical generation (PRZYBYSZEWSKI et al., 2005) and causes a down regulation of the antioxidant enzymes such as SOD and CAT (SAMANTA et al., 2016; ZHOU et al; 2015; ZHANG et al., 2007; DOGAN et al., 2011; LIU et al., 2014; MALIK et al., 2014) leading to oxidative stress. This is one of the accepted mechanisms of F toxicity, mainly triggered by the imbalance between production and elimination of free radicals (HASSAN; YOUSEF, 2009; LOGANAYAKI; SIDDHURAJU; MANIAN, 2013; AGLAKOVA; GUSEV, 2012). It is mainly the disturbance of body's antioxidant capacity as when it can no longer protect the cell from oxidative damage, free radicals such as

reactive oxygen species (ROS) and reactive nitrogen species (RNS) start to accumulate and exert detrimental effects including lipid peroxidation (LPO), protein oxidation, and DNA damage (FERREIRA et al., 2010; KRYSTON et al., 2011; KUBRAK et al., 2011). F is also known to have an adverse effect on hematopoietic organs (EREN; OZTURK; CANATAN, 2005) that suppress the ability of white blood cells (CURNETTE; BABIOR; KARNOVSKY, 1979). Aging and bone cancers are also studied as the severe known effects of F, despite this is quite controversial (MAURER et al., 1990; LEONE et al., 1954).

In the last decade, A/J and 129P3/J mice strains have been widely considered, since they respond differently to fluoride (F) exposure in the mineralized and soft tissues. Upon having an exposure to the same doses of F, the A/J strain reflects its "susceptibility" with rapid onset and severe development of fluorosis, while the 129P3/J is "resistant", with minimum development of fluorosis at the same time (BAYKOV; ALEXANDROV; SMIRNOVA, 1992). Further studies confirmed the fact that these strains also differ with respect to their susceptibilities to the effect of F in bone (MOUSNY et al., 2006, 2008) and kidney (CARVALHO et al., 2009) whereas one of the metabolic study also showed that the 129P3/J mice excrete less F in urine, have higher circulating F levels and, consequently, higher bone F levels; however, they still are remarkably resistant to the development of dental fluorosis (CARVALHO et al., 2009). Realistically, some of the differences between respective strains are intrinsic to themselves and do not depend on the F exposure, for instance the A/J mice were shown to drink significantly higher volumes of water than their 129P3/J counterparts (CARVALHO et al., 2013).

Liver represents the main detoxifying tissue by processing, neutralizing, and eliminating toxins from the digestive tract through hepatocyte-mediated enzymatic detoxification systems. Although a significant number of studies demonstrated the fact that excessive intake of F can cause serious liver damage (PAIZIS et al., 2005; ZHANG et al., 2013; GU; MANAUTOU, 2012; ATMACA et al., 2014; ARGUELLES et al., 2004; INKIELEWICZ-STEPNIAK; CZARNOWSKI, 2010; SARKAR et al., 2014) the exact molecular mechanisms underlying the effects of chronic F-induced damage in this organ remain unclear. This has led to the hypothesis of a systematic approach which was assumed to narrow down the study, in order to identify the new toxicity biomarkers in the liver. Additionally, it would be interesting to investigate if the effects

of F on the profile of protein expression in the liver is also influenced by genetics, as has been shown to occur for the kidney (CARVALHO et al., 2009). In order to address our aims, liver proteomic analysis seems to be the most suitable analytical method, which thus enabled us to explore and identify large number of proteins in the liver samples chronically exposed to the respective concentrations of F under study.

2 ARTICLE I

2 ARTICLE I

Article formatted according to Journal of Applied Oral Science.

Liver proteome of mice with different genetic susceptibilities to the effects of fluoride

Zohaib Nisar Khan[1]

Aline de Lima Leite[1,2]

Senda Charone[1]

Isabela Tomazini Sabino[1]

Tatiana Martini[1]

Heloísa Aparecida Barbosa da Silva Pereira[2]

Rodrigo Cardoso de Oliveira[1]

*Marília Afonso Rabelo Buzalaf[1]

[1]Department of Biological Sciences, Bauru School of Dentistry, University of São Paulo, Al. Octávio Pinheiro Brisolla, 9-75, 17012-901, Bauru, São Paulo, Brazil.

[2]Department of Genetics and Evolution, Center of Biological Sciences and the Health, Federal University of São Carlos, Washington Luis, km 235,13560-970, São Carlos, São Paulo, Brazil

*Corresponding author:

Marília Afonso Rabelo Buzalaf

Department of Biological Sciences, Bauru Dental School, University of São Paulo, Bauru, SP. Al. Octávio Pinheiro Brisolla, 9-75. Bauru-SP, 17012-901, Brazil. Tel. + 55 14 32358346 Fax + 55 14 32271486 e-mail: mbuzalaf@fob.usp.br

ABSTRACT

A/J and 129P3/J mice strains have been widely studied in the last few years because they respond quite differently to fluoride (F) exposure. 129P3/J mice are remarkably resistant to dental fluorosis development, despite excreting less F in urine and having higher circulating F levels. These two strains also present different characteristics regardless F exposure. In the present study, we investigated the differential pattern of protein expression in the liver of these mice in order to provide insights on why they have different responses to F. Weanling male A/J and 129P3/J mice (n=10 from each strain) were housed in pairs in metabolic cages with *ad libitum* access to low-F food and deionized water for 42 days. Liver proteome profiles were examined using *n*LC-MS/MS. Protein function was classified by GO biological process (Cluego v2.0.7 + Clupedia v1.0.8) and protein-protein interaction networking was constructed (PSICQUIC, Cytoscape). Most of the proteins with fold change were increased in A/J mice. The functional category with the highest percentage of number of altered genes was oxidation-reduction process (20%). The analysis of the subnetwork revealed that proteins with fold change interacted with Disks large homolog 4 and Calcium-activated potassium channel subunit alpha-1. A/J mice had an increase in proteins related to energy flux and oxidative stress. This could be a possible explanation for the high susceptibility of these mice to the effects of F, since exposure to F also induces oxidative stress.

Keywords: Proteomics; fluoride; liver; oxidative stress

1. INTRODUCTION

A/J and 129P3/J mice strains have been widely studied in the last few years because they respond quite differently to fluoride (F) exposure. When given the same dose of F, the A/J strain responds with a rapid onset and severe development of dental fluorosis, while the 129P3/J strain develops minimal fluorosis[8]. It was believed that this could be due to faster excretion of F by the 129P3/J strain. Surprisingly, a metabolic study showed that the 129P3/J mice excrete less F in urine, have higher circulating F levels and consequently higher bone F levels and even so are remarkably resistant to the development of dental fluorosis[5].

Some differences between these strains are intrinsic to themselves and do not depend on the F exposure. For example, the A/J mice drink significantly higher volumes of water than their 129P3/J counterparts[4], what can be explained by the increased expression of Alpha-aminoadipic semialdehyde dehydrogenase in the kidney of 129P3/J mice, regardless exposure to F. This enzyme metabolyzes irreversibly betaine aldehyde to betaine that is the most effective osmoprotectant accumulated by eukaryotic organisms to cope with osmotic stress[4]. In addition, exclusive proteins expressed in the kidney of A/J or 129P3/J mice exhibited the

same profile, regardless exposure to F. This suggests that the genetic background *per se* accounts for such differences between these two strains of mice.

Liver represents the main detoxifying tissue in the body by processing, neutralizing, and eliminating toxins from the digestive tract through hepatocyte-mediated enzymatic detoxification systems. Due to these important functions, liver is one of the organs most subject to injury in the organism. Thus, it is believed that the differential pattern of protein expression in the liver of A/J and 129P3/J mice can provide new insights that could help to explain why they respond differently when exposed to F. To achieve this, state-of-the-art shotgun proteomics combined to bioinformatics approaches were used.

2. EXPERIMENTAL SECTION

2.1. Animals and Samples Collection

Weanling male mice from the A/J and 129P3/J inbred strains (3-week-old; n=10 from each strain) were housed in pairs in metabolic cages with *ad libitum* access to low-F food (AIN76A, PMI Nutrition, Richmond, IN, USA, 0.95 mg/Kg F) and deionized water for 42 days. 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. All experimental protocols were approved by the Ethics Committee for Animal Experiments of Bauru Dental School, University of São Paulo (Protocol # 031/2013). At the end of the study, the mice were anesthetized with ketamine/xylazine and livers were collected. Samples designated for proteomic analysis were stored at -80°C, while those designated for F analysis were stored at -20°C.

2.2 Fluoride analysis in liver

Fluoride analysis was done with the ion-sensitive electrode, after hexamethyldisiloxane-facilitated diffusion[22], exactly as previously described[20].

2.3 Statistical Analysis

For liver F concentration, the software GraphPad InStat version 4.0 for Windows (GraphPad software Inc., La Jolla, USA) was used. Data were analysed by unpaired *t test* ($p<0.05$).

2.4 Sample Preparation for Proteomic Analysis

Samples were prepared for analysis as previously described[17]. The frozen tissue was homogenized in a cryogenic mill (model 6770, Spex, Metuchen, NJ, EUA). For protein extraction, liver homogenate was incubated in lysis buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 1% IPG buffer pH 3-10, 40 mM DTT for 1h at 4ºC with occasional shaking. After

this period, the homogenate was centrifuged at 15,000 rpm for 30 min at 4ºC and the supernatant containing soluble proteins was recovered. The proteins were precipitated using the kit *PlusOne 2D Cleanup* (GE Healthcare, Uppsala, Sweden), as recommended by the manufacturer. Pellets were resuspended in rehydration buffer (7 M urea, 2 M thiourea, 0.5% CHAPS, 0.5% IPG buffer pH 3–10, 18 mM DTT, 0.002% bromophenol blue). Twenty-five µL of liver proteins from each animal of the same group were combined to constitute a pool that was centrifuged for clarification. To each pool, 50 mM AMBIC containing 3 M urea was added. Each sample was filtered twice in 3 kDa AMICON (Millipore, St Charles, MO, USA). Protein quantification was measured in the pooled samples by Bradford protein assay[3]. To each sample (50 µg of total protein for each pool in a volume of 50 µL) 10 µL of 50 mM AMBIC were added. In sequence, 25 µL of 0.2% *Rapi*GEST™ (Waters Co., Manchester, UK) were added and incubated at 80°C for 15 min. Following, 2.5 µL of 100 mM DTT were added and incubated at 60°C for 30 min. 2.5 µL of 300 mM IAA were added and incubated for 30 min at room temperature (under dark). Then 10 µL of trypsin (100 ng; Trypsin Gold Mass Spectrometry, Promega, Madison, USA) was added and digestion was allowed to occur for 14 h at 37°C. After digestion, 10 µl of 5 % TFA was added, incubated for 90 min at 37ºC and sample was centrifuged (14000rpm for 30min). The supernatant was collected and 5 µL of ADH (1 pmol/µL) plus 85 µL 3% ACN were added.

2.5. LC-MS/MS and bioinformatics analyses

The peptides separation and identification was performed on a nanoAcquity UPLC-Xevo QTof MS system (Waters, Manchester, UK), exactly as previously described[15]. Difference in expression among the groups was obtained using PLGS software and expressed as $p<0.05$ for down-regulated proteins $1\text{-}p>0.95$ for up-regulated proteins (Table1). Bioinformatics analysis was performed, as reported earlier[1,15,17-19]. Briefly, Uniprot protein ID accession numbers were mapped back to their associated encoding Uniprot gene entries for the comparison A/J X 129P3/J. Gene Ontology annotation of Broad Biological Process was performed using Cluego v2.0.7 + Clupedia v1.0.8, a Cytoscape plugin. Uniprot IDs were uploaded from Table 1 and analyzed with default parameters, which specify a Enrichment (Right-sided hypergeometric test) correction method using Bonferroni step down, analysis mode “Function” and load gene cluster list for *Mus Musculus*, Evidence Codes “All”, set networking specificity “medium” (GO levels 3 to 8) and KappaScoreThreshold 0.03. The protein-protein interaction networking was downloaded from PSICQUIC, built in Cytoscape version 3.0.2 and constructed as proposed by Millan[18]. A network was then constructed, providing global view of potentially relevant interacting partners of proteins whose abundances change.

3.RESULTS

3.1. Liver F analysis

Mean±SD liver F concentrations found in 129P3/J mice (0.022±0.003 µg/g) were significantly higher than those found in A/J mice (0.015±0.002 µg/g) (t = 4.929, p = 0.0006).

3.2. Liver Proteome Profile and Identification of Differentially Expressed Proteins

Table 1 shows proteins with changes expression in A/J and 129P3/J mice. In general, most of the proteins with fold change were increased in A/J mice.

3.3 Gene Ontology Annotation

Figure 1 shows the functional classification according to the biological process with most significant term. Twelve categories were observed. Among them, the category with the highest percentage of number of genes was oxidation-reduction process (20%), followed by cellular amino acid metabolic process (16%) and response to oxidative stress (12%).

3.4 Protein-protein interaction network

For the comparison displayed above, a network was created, employing all the interactions found in the search conducted using PSICQUIC. After the global network was created, nodes and edges were filtered using the specification for *Mus musculus* taxonomy (10090). The value of fold change and also the p-value were added in new columns. The ActiveModules 1.8 plug-in to Cytoscape was used to make active modules connected subnetworks within the molecular interaction network whose genes presented significant coordinated changes in fold changes and p-value, as shown in the original proteomic analysis. Figure 2 shows the subnetwork generated by VizMapper. As can be seen, most of proteins with fold change present interaction with Disks large homolog 4 (Q62108; 11 proteins) and Calcium-activated potassium channel subunit alpha-1 (Q08460; 18 proteins).

4. Discussion

129P3/J mice interestingly have been reported to excrete less F and as consequence to have higher circulating F levels, bone and enamel F levels and even so they are remarkably resistant to the development of dental fluorosis[5,7-8,13]. In the present study, even without administration of F through the drinking water and with consumption of a low-F diet, 129P3/J mice had significantly higher liver F concentrations, which might have been due to the residual amounts of F present in their diets and is in-line with the metabolic characteristics of this strain with respect to F [4-5].

In the present study, proteomic analysis of liver of 129P3/J and A/J mice was employed to provide insights into the possible mechanisms that could help to explain the differential metabolic handling and effects of F in these two strains. It has been shown that even without exposure to F, A/J mice present a higher retention of proteins in the maturing enamel[9]. For this reason, the mice were not treated with F, because we wanted to see differences in the liver proteome profile that were intrinsic to the strains. Most of the proteins with fold change were increased in the A/J mice (Table 1), with fold changes ranging between 1 and 2. *Formimidoyltransferase-cyclodeaminase*, however, was increased 3.82 times in A/J mice. This enzyme is a liver-specific antigen recognized by sera of patients with autoimmune hepatitis[14] and is found down-regulated in hepatocellular carcinoma[16]. *Formimidoyltransferase-cyclodeaminase* has two enzymatic functions. In one of them, formiminotetrahydrofolate and glutamate are produced. Through its cyclodeaminase function, the enzyme breaks down formiminotetrahydrofolate, involved in the synthesis of purines and pirimidines and aminoacids (UNIPROT). Thus, increase in this enzyme might help to explain the increased expression of other liver proteins in A/J mice due to the higher supply of nucleotides and aminoacids.

Remarkably, the functional category with the highest percentage of number of altered genes was oxidation-reduction process. The increase of proteins such as ATP synthase subunit alpha, mitochondrial, Heat shock cognate 71 kDa protein, Electron transfer flavoprotein subunit beta, Alpha-enolase, Beta-enolase, Gamma-enolase and, Malate dehydrogenase in the A/J mice indicate an increased energy flux in this strain, which might generate oxidative stress. This can be confirmed by the concomitant increase in GRP78, which suggests endoplasmic reticulum (ER) stress[20]. ER stress occurs when nascent proteins are not folded properly or are misfolded, leading to the initiation of the unfolded protein response, as the unfolded proteins accumulate in the ER[12]. It has been demonstrated that F is able to induce an ER stress response in the ameloblast derived LS8 cell line, which could be implicated in the pathogenesis of dental fluorosis[12]. In addition, administration of F through the drinking water is able to increase the expression of GRP78 in the liver of rats[20]. Thus, considering that A/J mice present an increased energy flux and tendency to oxidative stress even without exposure to F, that exposure to F has been shown to worsen oxidative stress[20], which this can be implicated in the pathogenesis of dental fluorosis[8], this can be a possible explanation for the high susceptibility of the A/J to the effects of F.

The proteins in the center of the protein-protein interaction network are related to potassium channels. One of them (Calcium-activated potassium channel subunit alpha-1) is a potassium channel activated by both membrane depolarization or increase in cytosolic Ca^{2+} that mediates export of K^{+}. It is also activated by the concentration of cytosolic Mg^{2+}. Its activation dampens the excitatory events that elevate the cytosolic Ca^{2+} concentration and/or

depolarize the cell membrane. It therefore contributes to repolarization of the membrane potential and play a key role in controlling excitability in a number of systems, such as regulation of the contraction of smooth muscle[21], the tuning of hair cells in the cochlea[6], regulation of transmitter release[6] and innate immunity[2]. The other one is Disks large homolog 4 that is required for synaptic plasticity associated with NMDA (N-methyl-D-aspartate) receptor signaling[11]. It interacts with shaker-type potassium channels and the cytoplasmic tail of NMDA receptor subunits. At first glance, the presence of a protein associated with the nervous system in the center of the network in the present study seems to be odd. However, it must be considered that liver failure leads to the accumulation of ammonia that affects the cerebral function[10]. As mentioned above, A/J mice presented several proteins related to the energy flux increased in the liver, which might have caused oxidative stress and contributed to liver damage, which in turn might have provoked cerebral alterations. Since this was a preliminary exploratory work, future studies comparing the proteomic profile of the brain of these mice strains should be conducted to add new light into this topic. Also additional studies should be done in order to quantify, by other techniques, the proteins with changing expression in the present work. Despite this was an exploratory study, the lack of additional techniques to confirm the proteins with altered expression identified by nLC-MS/MS might be considered a limitation of the present work.

In conclusion, A/J mice had an increase in proteins related to energy flux and oxidative stress. This could be a possible explanation for the high susceptibility of these mice to the effects of F, since exposure to F also induces oxidative stress.

Acknowledgments: The authors thank CNPq/TWAS for the concession of scholarship to the first author.

Conflict of interest statement: The authors have declared no conflict of interest.

References

1. Bauer-Mehren A. Integration of genomic information with biological networks using Cytoscape. Methods Mol Biol. 2013; 1021: 37-61.
2. Butler A, Tsunoda S, McCobb DP, Wei A, Salkoff L. mSlo, a complex mouse gene encoding "maxi" calcium-activated potassium channels. Science. 1993; 9;261(5118):221-4.
3. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976; 72: 248-254.
4. Carvalho JG, Leite lAde, Peres-Buzalaf C, Salvato F, Labate CA, Everett ET, Whitford GM, Buzalaf MAR. Renal proteome in mice with different susceptibilities to fluorosis. PLoS One .2013; 8(1): e53261.
5. Carvalho JG, Leite AL, Yan D, Everett ET, Whitford GM, Buzalaf MAR. Influence of genetic background on fluoride metabolism in mice. J Dent Res. 2009; 88(11): 1054-1058.
6. Cabo R, Zichichi R, Viña E, Guerrera MC, Vázquez G, García-Suárez O, Vega JA, Germanà A. Calcium-activated potassium channel SK1 is widely expressed in the peripheral nervous system and sensory organs of adult zebrafish . Neurosci Lett. 2013; 25;555:62-7.
7. Charone S, Leite AL, Peres-Buzalaf C, Fernandes MS, Almeida LF, Graeff MSF, Oliveira RC, Campanelli AP, Groisman S, Whitford GM, Everett ET, Buzalaf MAR. Proteomics of secretory and maturation stage enamel of genetically distinct mice. Caries Res (in press).
8. Everett ET, McHenry MA, Reynolds N, Eggertsson H, Sullivan J, Kantmann C, Martinez-Mier CA, Warrick JM, Stookey GK. Dental fluorosis: variability among different inbred mouse strains. J Dent Res. 2002; 81(11): 794-798.
9. Everett ET, Yan D, Weaver M, Liu L, Foroud T, Martinez-Mier EA. Detection of dental fluorosis-associated quantitative trait Loci on mouse chromosomes 2 and 11. Cells Tissues Organs. 2009; 189(1-4): 212-218.
10. Felipo V. Hepatic encephalopathy: effects of liver failure on brain function. Nat Rev Neurosci. 2013; 14(12): 851-858.
11. Halff AW, Gómez-Varela D, John D, Berg DK. A novel mechanism for nicotinic potentiation of glutamatergic synapses. J Neurosci. 2014; 5;34(6):2051-64.
12. Kubota K, Lee DH, Tsuchiya M, Young CS, Everett ET, Martinez-Mier EA, Snead ML, Nguyen L, Urano F, Bartlett JD. Fluoride induces endoplasmic reticulum stress in ameloblasts responsible for dental enamel formation. J Biol Chem. 2005; 280(24): 23194-23202.

13. Kobayashi CA, Leite AL, Peres-Buzalaf C, Carvalho JG, Whitford GM, Everett ET, Siqueira WL, Buzalaf MA. Bone response to fluoride exposure is influenced by genetics. PLoS One. 2014; 11;9(12):e114343.

14. Lapierre P, Hajoui O, Homberg JC, and Alvarez, F. (1999) Formimino-transferase cyclodeaminase is an organ-specific autoantigen recognized by sera of patients with autoimmune hepatitis. Gastroenterology 116, 643– 649

15. Leite AL, Gualiume JVML, Barbosa da Silva Pereira HA, Silva Fernandes M, Martini T, Zucki F, Sumida DH, Rigalli A, Buzalaf MAR. Proteomic analysis of gastrocnemius muscle in rats with streptozotocin-induced diabetes and chronically exposed to fluoride. PLoS One. 2014; 9(9): e106646.

16. Liang CR, Leow CK, Neo JC, Tan GS, Lo SL, Lim JW, Seow TK, Lai PB, Chung MC. Proteome analysis of human hepatocellular carcinoma tissues by two-dimensional difference gel electrophoresis and mass spectrometry. Proteomics. 2005; 5 (8) 2258–2271.

17. Lobo JG, Leite AL, Pereira HA, Fernandes MS, Peres-Buzalaf C, Sumida DH, Rigalli A, Buzalaf MAR. Low-Level Fluoride Exposure Increases Insulin Sensitivity in Experimental Diabetes. J Dent Res. 2015.

18. Millan, P. P. (2013). Visualization and analysis of biological networks. Methods Mol Biol 1021: 63-88.

19. Orchard S. Molecular interaction databases. Proteomics. 2012; 12(10): 1656-1662.

20. Pereira HA, Leite lAde, Charone S, Lobo JG, Cestari TM, Peres-Buzalaf C, Buzalaf MAR. "Proteomic analysis of liver in rats chronically exposed to fluoride." PLoS One. 2013; 8(9): e75343.

21. Sánchez-Pastor E, Andrade F, Sánchez-Pastor JM, Elizalde A, Huerta M, Virgen-Ortiz A, Trujillo X, Rodríguez-Hernández A. Cannabinoid receptor type 1 activation by arachidonylcyclopropylamide in rat aortic rings causes vasorelaxation involving calcium-activated potassium channel subunit alpha-1 and calcium channel, voltage-dependent, L type, alpha 1C subunit. Eur J Pharmacol. 2014; 15(729):100-6.

22. Taves DR. Separation of fluoride by rapid diffusion using hexamethyldisiloxane. Talanta. 1968; 15(9): 969-974.

Figures Legends

Fig1. Functional distribution of proteins identified with differential expression in liver of mice belonging to Control Group A/J vs. 129p3/J strains. Categories of proteins based on GO annotation Biological Process. Terms significant (Kappa=0.03) and distribution according to percentage of number of genes association.

Fig2. Subnetworks generated by VizMapper for each comparison – **A** Group A/J vs. 129p3/J (Control) Color of node and * indicate the differential expression of the respective protein, for each comparison. Red and green nodes indicate protein down-regulation and up-regulation, respectively, while * and ** indicate presence and absence of protein, respectively, in the respective group. Purple node indicates proteins presenting interaction but that were not identified in the present study. The access numbers in nodes correspond to: P68134- (Acta1)Actin, alpha skeletal muscle; P10518- (Alad) Delta-aminolevulinic acid dehydratase; Q9DCW4- (Etfb) Electron transfer flavoprotein subunit beta; P60710- (Actb) Actin, cytoplasmic 1; P17182- (Eno1) Alpha-enolase; P20029- (Hspa5) 78 kDa glucose-regulated protein; P10649- (Gstm1) Glutathione S-transferase Mu 1; P17751- (Tpi1) Triosephosphate isomerase; Q8VCT3- (Rnpep) Aminopeptidase B; Q9CPU0- (Glo1) Lactoylglutathionelyase; P21550- (Eno3) Beta-enolase; P17183- (Eno2) Gamma-enolase; P08249- (Mdh2) Malate dehydrogenase; P63017- (Hspa8) Heat shock cognate; P38647- (Hspa9) Stress-70 protein; Q03265- (Atp5a1) ATP synthase subunit alpha; P09411- (Pgk1) Phosphoglycerate kinase 1; P26443- (Glud1) Glutamate dehydrogenase 1; P47738- (Aldh2) Aldehyde dehydrogenase; P10126- (Eef1a1) Elongation factor 1-alpha 1; P19157- (Gstp1) Glutathione S-transferase P 1; P52760- (Hrsp12) Ribonuclease; Q8K2B3- (Sdha) Succinate dehydrogenase; P11499- (Hsp90ab1) Heat shock protein; Q62108- (Dlg4) Disks large homolog 4; Q08460- (Kcnma1) Calcium-activated potassium channel subunit alpha-1.

Fig1

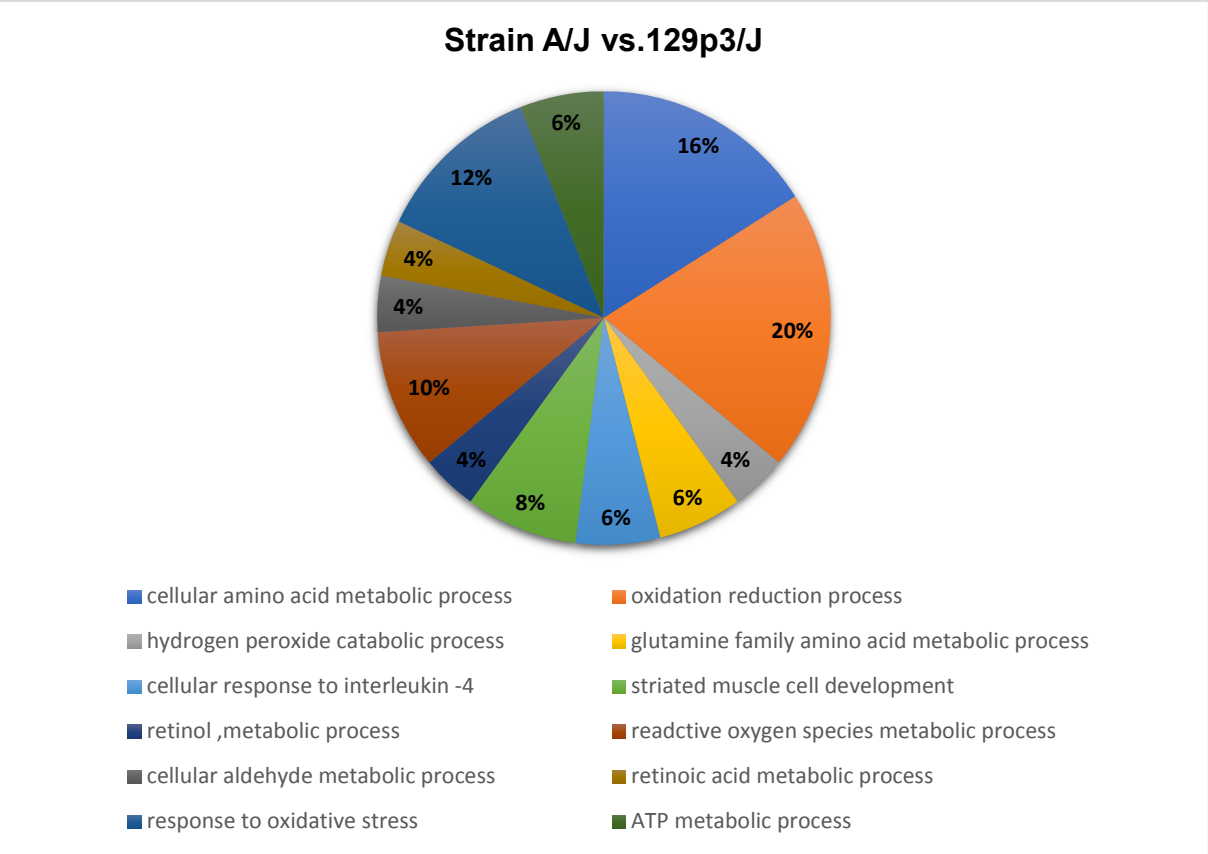

Strain A/J vs.129p3/J
16%
20%
4%
6%
6%
8%
4%
10%
4%
4%
12%
6%
cellular amino acid metabolic process
oxidation reduction process
hydrogen peroxide catabolic process
glutamine family amino acid metabolic process
cellular response to interleukin -4
striated muscle cell development
retinol ,metabolic process
readctive oxygen species metabolic process
cellular aldehyde metabolic process
retinoic acid metabolic process
response to oxidative stress
ATP metabolic process

Fig2.

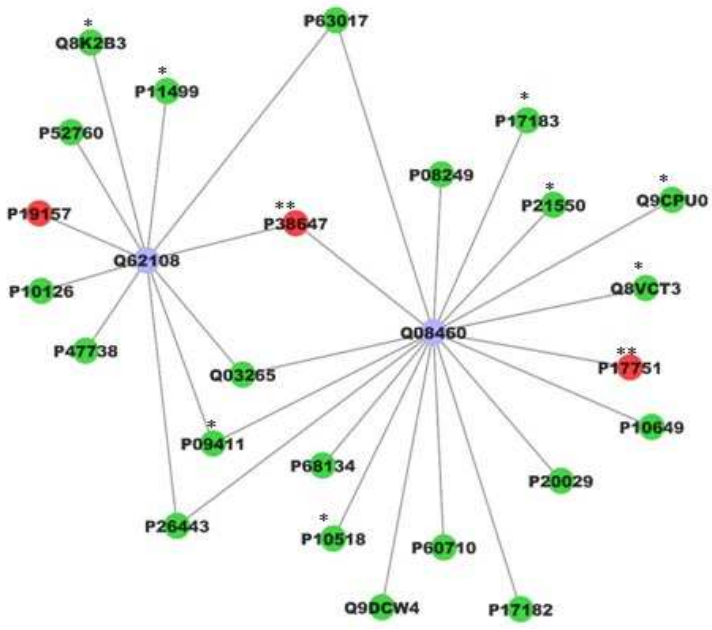

P63017
Q8K2B3
P11499
P52760
P17183
P08249
P21550
Q9CPU0
P19157
P38647
Q62108
P10126
Q8VCT3
P47738
Q08460
Q03265
P17751
P10649
P09411
P68134
P20029
P26443
P10518
P60710
Q9DCW4
P17182

Table1.

Identified proteins with expression significantly altered in the liver of mice of group A/J (0 ppm F) vs. 129 (0 ppm F)

				Fold change	
[a]Access Number	Gene name	Protein name description	PLGS score	A/J 0ppm	129P3/J 0ppm
Q921H8	Acaa1a	3-ketoacyl-CoA thiolase A, peroxisomal	195.3	1.65	-1.65
Q8VCH0	Acaa1b	3-ketoacyl-CoA thiolase B, peroxisomal	195.3	1.70	-1.70
Q8BWT1	Acaa2	3-ketoacyl-CoA thiolase, mitochondrial	189.2	1,42	-1,42
P63038	Hspd1	60 kDa heat shock protein, mitochondrial	153.6	1.55	-1.55
P20029	Hspa5	78 kDa glucose-regulatedprotein	254.4	1.43	-1.43
P68033	Actc1	Actin, alpha cardiacmuscle 1	630.1	1.28	-1.28
P68134	Acta1	Actin, alpha skeletalmuscle	630.1	1.28	-1.28
P62737	Acta2	Actin, aorticsmoothmuscle	60.2	1.35	-1.35
P60710	Actb	Actin, cytoplasmic 1	62.4	1.25	-1.25
P63260	Actg1	Actin, cytoplasmic 2	62.4	1.26	-1.26
P63268	Actg2	Actin, gamma-enteric smooth muscle	60.2	1.34	-1.34
P47738	Aldh2	Aldehydededehydrogenase, mitochondrial	72.6	1.67	-1.67
P17182	Eno1	Alpha-enolase OS=Mus musculus	129.4	1.46	-1.46
P16460	Ass1	Argininosuccinatesynthase	58.6	1.28	-1.28
P05202	Got2	Aspartateaminotransferase, mitochondrial	79.3	1.34	-1.34
Q03265	Atp5a1	ATP synthase subunit alpha, mitochondrial	74.7	1.43	-1.43
P56480	Atp5b	ATP synthasesubunit beta, mitochondrial	138.6	1.35	-1.35
O35490	Bhmt	Betaine--homocysteine S-methyltransferase 1	40.6	1.23	-1.23
Q8C196	Cps1	Carbamoyl-phosphate synthase [ammonia], mitochondrial	269.2	1.39	-1.39
Q63880	Ces3a	Carboxylesterase 3A	336.9	1.46	-1.46
Q8VCU1	Ces3b	Carboxylesterase 3B	139.1	1.65	-1.65
P24270	Cat	Catalase	260.8	1.62	-1.62
Q8R0Y6	Aldh1l1	Cytosolic 10-formyltetrahydrofolate dehydrogenase	53.1	1.55	-1.55
Q9DCW4	Etfb	Electron transfer flavoprotein subunit beta	174.4	1.48	-1.48
P10126	Eef1a1	Elongationfactor 1-alpha 1	245.5	1.39	-1.39
P70694	Akr1c6	Estradiol 17 beta-dehydrogenase 5	207.5	1.48	-1.48
Q91XD4	Ftcd	Formimidoyltransferase-cyclodeaminase	121.1	3.82	-3.82
Q91Y97	Aldob	Fructose-bisphosphatealdolase B	96.1	1.62	-1.62
P35505	Fah	Fumarylacetoacetase	136.0	1.46	-1.46
P26443	Glud1	Glutamatedehydrogenase 1, mitochondrial	467.9	1.84	-1.84

P10649	Gstm1	Glutathione S-transferase Mu 1	129.1	1.26	-1.26
P15626	Gstm2	Glutathione S-transferase Mu 2	109.8	1.32	-1.32
P48774	Gstm5	Glutathione S-transferase Mu 5	109.8	1.32	-1.32
P19157	Gstp1	Glutathione S-transferase P 1	317.2	-0.66	0.66
P63017	Hspa8	Heat shock cognate 71 kDa protein	275.2	1.36	-1.36
P01942	Hba	Hemoglobinsubunit alpha	1252.1	-0.85	0.85
P02104	Hbb-y	Hemoglobinsubunit epsilon-Y2	854.2	-0.48	0.48
Q8CGP6	Hist1h2ah	Histone H2A type 1-H	193.0	1.22	-1.22
Q64522	Hist2h2ab	Histone H2A type 2-B	241.3	1.51	-1.51
P62806	Hist1h4a	Histone H4	88.1	1.54	-1.54
P54869	Hmgcs2	Hydroxymethylglutaryl-CoAsynthase, mitochondrial	292.1	1.22	-1.22
P11588	Mup1	Major urinaryprotein 1	815.0	-0.53	0.53
B5X0G2	Mup17	Major urinaryprotein 17	824.6	-0.54	0.54
P11589	Mup2	Major urinaryprotein 2	815.0	-0.54	0.54
P11591	Mup5	Major urinaryprotein 5	389.7	-0.57	0.57
P02762	Mup6	Major urinaryprotein 6	815.0	-0.53	0.53
P04938	Mup8	Major urinary proteins 11 and 8 (Fragment)	815.0	-0.54	0.54
P08249	Mdh2	Malatedehydrogenase, mitochondrial	247.9	1.45	-1.45
Q64374	Rgn	Regucalcin	107.2	1.36	-1.36
P24549	Aldh1a1	Retinaldehydrogenase 1	208.9	1.49	-1.49
P52760	Hrsp12	Ribonuclease UK114	261.2	1.57	-1.57
Q99LB7	Sardh	Sarcosinedehydrogenase, mitochondrial	104.7	1.34	-1.34
P07724	Alb	Serumalbumin	108.5	1.34	-1.34

Q8R519	Acmsd	2-amino-3-carboxymuconate-6-semialdehyde decarboxylase	89.6	+	-
O08756	Hsd17b10	3-hydroxyacyl-CoA dehydrogenase type-2	114.0	+	-
P00329	Adh1	Alcoholdehydrogenase 1	163.3	+	-
Q61234	Snta1	Alpha-1-syntrophin	77.6	+	-
Q8VCT3	Rnpep	Aminopeptidase B	73.8	+	-
Q9D3D9	Atp5d	ATP synthasesubunit delta, mitochondrial	183.6	+	-
Q62210	Birc2	Baculoviral IAP repeat-containing protein 2	65.9	+	-
Bad	Q61337	Bcl2 antagonist of cell death	116.2	-	+
P21550	Eno3	Beta-enolase	161.0	+	-
P34914	Ephx2	Bifunctionalepoxidehydrolase 2	441.9	+	-
Q8R1G2	Cmbl	Carboxymethylenebutenolidasehomolog	73.2	+	-

Q61686	Cbx5	Chromoboxproteinhomolog 5	96.9	+	-
Q3V079	Ccdc176	Coiled-coil domain-containing protein 176	66.5	+	-
P50172	Hsd11b1	Corticosteroid 11-beta-dehydrogenase isozyme 1	100.4	+	-
Cth	Q8VCN5	Cystathioninegamma-lyase	100.5	-	+
P48771	Cox7a2	Cytochrome c oxidase subunit 7A2, mitochondrial	185.6	+	-
P10518	Alad	Delta-aminolevulinicaciddehydratase	316.8	+	-
Q9DBT9	Dmgdh	Dimethylglycinedehydrogenase, mitochondrial	89.4	+	-
Q99LC5	Etfa	Electron transfer flavoprotein subunit alpha, mitochondrial	77.6	+	-
Q9ER73	Elp4	Elongatorcomplexprotein 4	103.4	+	-
P63242	Eif5a	Eukaryotic translation initiation factor 5A-1	104.8	+	-
Q9QXD6	Fbp1	Fructose-1,6-bisphosphatase 1	154.4	+	-
P17183	Eno2	Gamma-enolase	159.3	+	-
Q3UHD2	Gfod1	Glucose-fructose oxidoreductase domain-containing protein 1	83.6	+	-
P11352	Gpx1	Glutathioneperoxidase 1	419.0	+	-
P24472	Gsta4	Glutathione S-transferase A4	127.0	+	-
Q9QYE6	Golga5	Golginsubfamily A member 5	103.4	+	-
P07901	Hsp90aa1	Heat shock protein HSP 90-alpha	67.4	+	-
P11499	Hsp90ab1	Heat shock protein HSP 90-beta	107.9	+	-
P68433	Hist1h3a	Histone H3.1	163.6	+	-
P84228	Hist1h3b	Histone H3.2	163.6	+	-
P84244	H3f3a	Histone H3.3	163.6	+	-
P02301	H3f3c	Histone H3.3C	163.6	+	-
Hgd	O09173	Homogentisate 1,2-dioxygenase	95.6	-	+
Hadh	Q61425	Hydroxyacyl-coenzyme A dehydrogenase, mitochondrial	183.9	-	+
Q5U5V2	Hykk	Hydroxylysinekinase	78.0	+	-
Q8BLR9	Hif1an	Hypoxia-inducible factor 1-alpha inhibitor	96.3	+	-
O88844	Idh1	Isocitratedehydrogenase [NADP] cytoplasmic	69.5	+	-
Q9CPU0	Glo1	Lactoylglutathionelyase	203.5	+	-
P06151	Ldha	L-lactatedehydrogenase A chain	153.0	+	-
Acsl1	P41216	Long-chain-fatty-acid--CoA ligase 1	48.0	-	+
Q9DB40	Med27	Mediator of RNA polymerase II transcription subunit 27	68.9	+	-
Q8BPT6	Immp2l	Mitochondrial inner membrane protease subunit 2	65.7	+	-
Myef2	Q8C854	Myelinexpressionfactor 2	44.9	-	+
Q9DC69	Ndufa9	NADH dehydrogenase [ubiquinone] 1 alpha subcomplexsubunit 9, mitochondrial	79.2	+	-
Ncoa5	Q91W39	Nuclear receptor coactivator 5	67.7	-	+
P11725	Otc	Ornithinecarbamoyltransferase, mitochondrial	217.0	+	-
O08807	Prdx4	Peroxiredoxin-4	391.3	+	-

Prdx5	P99029	Peroxiredoxin-5, mitochondrial	174.7	-	+
O08709	Prdx6	Peroxiredoxin-6	321.1	+	-
P09411	Pgk1	Phosphoglyceratekinase 1	106.8	+	-
Pgap2	Q3TQR0	Post-GPI attachment to proteins factor 2	60.0	-	+
Prdm12	A2AJ77	PR domainzincfingerprotein 12	43.7	-	+
Q9D6Y4	Mef2bnb	Protein MEF2BNB	101.0	+	-
O55125	Nipsnap1	ProteinNipSnaphomolog 1	169.9	+	-
P61458	Pcbd1	Pterin-4-alpha-carbinolamine dehydratase	171.8	+	-
Qtrt1	Q9JMA2	QueuinetRNA-ribosyltransferase	53.4	-	+
Q80U40	Rimbp2	RIMS-bindingprotein 2	74.3	+	-
B2RY56	Rbm25	RNA-bindingprotein 25	80.8	+	-
Q91X83	Mat1a	S-adenosylmethionine synthase isoform type-1	177.4	+	-
Q99J08	Sec14l2	SEC14-like protein 2	106.4	+	-
P47758	Srprb	Signal recognition particle receptor subunit beta	68.7	+	-
Hspa9	P38647	Stress-70 protein, mitochondrial	119.8	-	+
Q8K2B3	Sdha	Succinatedehydrogenase [ubiquinone] flavoproteinsubunit, mitochondrial	74.3	+	-
Q62264	Thrsp	Thyroid hormone-inducible hepatic protein	180.0	+	-
P97360	Etv6	Transcriptionfactor ETV6	64.7	+	-
Tmem42	Q9CR22	Transmembraneprotein 42	110.6	-	+
Tpi1	P17751	Triosephosphateisomerase	149.7	-	+
Q9D6F9	Tubb4a	Tubulin beta-4A chain	101.3	+	-
P68372	Tubb4b	Tubulin beta-4B chain	109.0	+	-
Ube2w	Q8VDW4	Ubiquitin-conjugatingenzyme E2 W	102.0	-	+
Q5QNV8	Heatr9	Uncharacterizedprotein C17orf66 homolog	91.1	+	-
N/A	Q8C4X7	UPF0258 protein KIAA1024-like homolog	38.4	-	+
P25688	Uox	Uricase	92.7	+	-

The identified proteins are organized according to alphabetical order. Relative differential is indicated by + sign, when the protein is up-regulated and by - sign, when the protein is down-regulated in the respective comparison. [a]Identification is based on protein ID from UniProt protein database (http://www.uniprot.org/).

2 ARTICLE II

3 ARTICLE II

Article formatted according to Biological Trace Element Research.

Liver proteome of mice with different genetic susceptibilities to fluorosis treated with different concentrations of F in the drinking water

Zohaib Nisar Khan[1]

Isabela Tomazini Sabino[1]

Carina Guimarães de Souza Melo[1]

Tatiana Martini[1]

Heloísa Aparecida Barbosa da Silva Pereira[1,2]

*Marília Afonso Rabelo Buzalaf[1]

[1]Department of Biological Sciences, Bauru School of Dentistry, University of São Paulo, Al. Octávio Pinheiro Brisolla, 9-75, 17012-901, Bauru, São Paulo, Brazil.

[2]Department of Genetics and Evolution, Center of Biological Sciences and the Health, Federal University of São Carlos, Washington Luis, km 235,13560-970, São Carlos, São Paulo, Brazil

*Corresponding author:

Marília Afonso Rabelo Buzalaf

Department of Biological Sciences, Bauru Dental School, University of São Paulo, Bauru, SP. Al. Octávio Pinheiro Brisolla, 9-75. Bauru-SP, 17012-901, Brazil. Tel. + 55 14 32358346 Fax + 55 14 32271486 e-mail: mbuzalaf@fob.usp.br

Short title: Liver proteome of mice with distinct genetic susceptibilities to F.

Abstract

Appropriate doses of fluoride (F) have therapeutic action against dental caries but higher levels can cause disturbances in soft and mineralized tissues. Interestingly, the susceptibility to the toxic effects of F is genetically determined. This study evaluated the effects of F on the liver proteome of mice susceptible (A/J) or resistant (129P3/J) to the effects of F. Weanling male A/J (n=6) and 129P3/J mice (n=6) were housed in pairs and assigned to two groups given low-F food and drinking water containing 15 or 50 ppm F for 7 weeks. Liver proteome profiles were examined using nano-LC-ESI-MS/MS. Difference in expression among the groups was determined using the PLGS software. Treatment with the lower F concentration provoked more pronounced alterations in fold change in liver proteins in comparison to the treatment with the higher F concentration. Interestingly, most of the proteins with fold change upon treatment with 15 ppm F were increased in the A/J mice compared with their 129P3/J counterparts, suggesting an attempt of the former to fight the deleterious effects of F. However, upon treatment with 50 ppm F, most proteins with fold change were decreased in the A/J mice compared with their 129P3/J counterparts, especially proteins related to oxidative stress and protein folding, which might be related to the higher susceptibility of the A/J animals to the deleterious effects of F. Our findings add light into the mechanisms underlying genetic susceptibility to fluorosis.

Keywords: Liver; Fluoride; Fluorosis; Proteomics; Genetic susceptibility.

1. INTRODUCTION

Fluorine is not only a common element present in the earth crust but it is also found in the form of fluoride (F) in the soils, rocks and water throughout the world. Higher concentrations are found in the areas where there have been recent/past pyroclastic activities or geologic uplift. In addition, fluoride is broadly employed in many industrial processes nowadays. The major sources of systemic fluoride exposure are the diet (food and water) and dental products, especially toothpastes (1).

Mild doses of fluoride have therapeutic action against dental caries while elevated levels will increase the body burden through intake of water, toothpastes and diets

containing high F levels. The therapeutic window is very narrow and there has been a lot of discussion concerning the appropriate levels of F intake to provide the maximum benefit (caries prevention) with minimum risk to cause disbenefits (2).

Once absorbed by the gastric intestinal system, F is distributed to all soft and mineralized tissues via blood stream (3). Despite the most common adverse effects of excessive F intake are seen in the mineralized tissues (teeth and bones; dental and skeletal fluorosis, respectively) (4, 5), the damage caused to soft tissues by F has been extensively investigated. Studies involving the effects of F in different tissues, such as testis (6, 7), thyroid gland (8, 9) spleen (10, 11), liver (12), kidney (13, 14) and brain (15, 16), have been published.

Interestingly, the susceptibility to the toxic effects of F appears to be genetically determined. There are reports in the literature of populations that tend to develop higher levels of dental fluorosis than it would be expected from their background exposure to F (17-19). Moreover, it was reported that inbred mice strains have different susceptibilities to dental fluorosis. The A/J strain is susceptible, with rapid onset and more severe development of dental fluorosis, while the 129P3/J strain is resistant, developing minimal dental fluorosis even with high exposure to F (20). Despite the 129P3/J mice are resistant to development of dental fluorosis, they excrete less F in urine and have higher circulating levels of F (21), which is very intriguing. Moreover, A/J mice drink higher volume of water than 129P3/J mice (21), which might be explained by the higher expression of α-AASA dehydrogenase (Alpha-amino adipic semialdehyde dehydrogenase in the latter (14), since this protein is an effective osmoprotectant.

Since the liver is a central organ in the metabolism, the metabolic differences shown by these animals in F handling prompted us recently to investigate the differential pattern of protein expression in the liver of these mice. We observed that A/J mice had many proteins increased when compared with their 129P3/J counterparts, most of them related to energy flux and oxidative stress, which could be implicated in their higher susceptibility to the development of dental fluorosis (22). Thus, it is quite interesting to evaluate the effect of F in the pattern of protein expression in the liver of A/J mice in comparison with 129P3/J mice, which was the aim of the present study.

2. EXPERIMENTAL SECTION

2.1. Animals and Samples Collection

Weanling mice pairs from two different inbred strains, A/J and 129P3/J (3-week-old; n=6 from each strain) were kept in metabolic cages with *ad libitum* access to low-F food (AIN76A, PMI Nutrition, Richmond, IN, USA, 0.95 mg/Kg F) and water containing 15 or 50 ppm F (as NaF) for a period of 7 weeks. During the course of treatment, the humidity and temperature in the climate-controlled room, were 23±1°C and 40%-80% respectively.

The experimental protocols were approved by the Ethics Committee for Animal Experiments of Bauru Dental School, University of São Paulo (Protocol # 031/2013). At the end of the study, the mice were anesthetized with ketamine/xylazine and livers were collected for the study. Samples to be used for proteomic analysis were stored at -80°C, while those designated for F analysis, were stored at -20°C.

2.2 Fluoride analysis in liver

Fluoride analysis data was obtained with the ion-sensitive electrode, after hexamethyldisiloxane-facilitated diffusion (23), as previously described (12).

2.3 Statistical Analysis

The software GraphPad Prism version 6.0 for Windows (GraphPad software Inc., La Jolla, USA) was used to analyze differences in liver F concentration. Data passed normality (Kolmogorov-Smirnov test) and homogeneity (Bartlett test) and were then analyzed by 2-way ANOVA followed by Sidak´s multiple comparison test. The significance level was set at 5%.

2.4 Sample Preparation for Proteomic Analysis

Samples were prepared for proteomic analysis as previously described (24). Cryogenic mill (model 6770, Spex, Metuchen, NJ, EUA) was used for the homogenization of frozen tissue. In order to extract proteins, liver homogenate was incubated in lysis buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 1% IPG buffer pH 3-10, 40 mM DTT for 1h at 4ºC with infrequent shaking. Afterwards, the

homogenate was centrifuged at 15,000 rpm for 30 min at 4ºC and the supernatant having soluble proteins was collected. For the precipitation of proteins, *PlusOne 2D Cleanup* (GE Healthcare, Uppsala, Sweden) kit was used as recommended by the manufacturer. The pellets thus obtained were resuspended in rehydration buffer (7 M urea, 2 M thiourea, 0.5% CHAPS, 0.5% IPG buffer pH 3–10, 18 mM dithiothreitol (DTT), 0.002% bromophenol blue). Later on a protein pool was constituted while having twenty-five µL of liver proteins from each animal of the same group; that was centrifuged for clarification. To each pool, 50 mM AMBIC containing 3 M urea was added. Each sample was filtered twice in 3 kDa AMICON (Millipore, St Charles, MO, USA). Bradford protein assay (25) was done in order to quantify the proteins present in the pooled samples. To each sample (50 µg of total protein for each pool in a volume of 50 µL), 10 µL of 50 mM AMBIC was added. In sequence, 25 µL of 0.2% *Rapi*GEST™ (Waters Co., Manchester, UK) was added and incubated at 80°C for 15 min. Afterwards, 2.5 µL of 100 mM DTT was added and incubated at 60°C for 30 min. 2.5 µL of 300 mM iodoacetamide (IAA) was added and incubated for 30 min at room temperature (under dark). Then 10 µL of trypsin (100 ng; Trypsin Gold Mass Spectrometry, Promega, Madison, USA) was added and digestion was allowed to occur for 14 h at 37°C. After digestion, 10 µl of 5 % TFA was added, and sample was left in an incubation phase for 90 min at 37ºC. It was then centrifuged (14000rpm for 30min). Finally, the supernatant was collected and 5 µL of ADH (1 pmol/µL) plus 85 µL 3% ACN were added to it.

2.5. LC-MS/MS and bioinformatics analyses

The nanoAcquity UPLC-Xevo QTof MS system (Waters, Manchester, UK), was used for the separation and identification of peptides, exactly as previously described (26). In order to notify the differences in the expression among the respective groups, Protein Lynx Global Service (PLGS) software was used where $p<0.05$ and $1\text{-}p>0.95$ thus showed the down-regulation or up-regulation of proteins, respectively (Table1*)*. Bioinformatics analysis was performed, as reported earlier (24, 26-28). For the comparison A/J X 129P3/J, Uniprot protein ID accession numbers were mapped back to their associated encoding Uniprot gene entries. Furthermore gene Ontology annotation of Broad Biological Process was performed using Cluego v2.0.7 + Clupedia v1.0.8, a Cytoscape plugin. Uniprot IDs were uploaded from Table 1 and analyzed with default parameters, which specify a Enrichment (Right-sided hypergeometric test)

correction method using Bonferroni step down, analysis mode “Function” and load gene cluster list for *Mus Musculus*, Evidence Codes “All”, set networking specificity “medium” (GO levels 3 to 8) and KappaScoreThreshold 0.03. The protein-protein interaction networking was downloaded from PSICQUIC, built in Cytoscape version 3.0.2 and constructed as proposed by Millan (29). Ultimately a network was constructed, providing global view of potentially relevant interacting partners of proteins whose abundances change.

3. RESULTS

3.1. Liver Fluoride Analysis

Liver F concentrations presented significant differences between the strains ($F = 12.68$, $p = .002$) and F concentrations in the drinking water ($F = 36.55$, $p<0.0001$), without interaction between these criteria ($F = 1.79$, $p = 0.196$). A dose-response pattern was observed for liver F concentrations. However, when the strains were compared, only the 129P3/J mice the animals treated with 50 ppm F in the drinking water had liver F concentrations significantly higher than their A/J counterparts (Figure 1).

3.2. Functional Distribution of Identified Proteins

Figure 2 shows, for each comparison, the functional classification according to the biological process with most significant term. For the 15 ppm F (*Figure 2a*) and 50 ppm F (Figure 2b) groups, the identified proteins were divided into 14 and 12 functional categories, respectively. For both groups, the category with the highest percentage of number of genes was carboxylic acid metabolic process (24% and 31 % for 15 and 50 ppm F, respectively), followed by cellular amino acid metabolic process (13% and 22% for 15 and 50 ppm F, respectively).

3.3. Liver Proteome Profile and Identification of Differentially Expressed Proteins

Tables 1 and 2 show the proteins with changes in expression when A/J mice are compared with their 129P3/J counterparts for the groups treated with 15 and 50 ppm F, respectively. Treatment with the lower F concentration (15 ppm) provoked more

pronounced alterations in fold change in comparison to treatment with the higher F concentration (50 ppm). Remarkably, all proteins with fold change upon treatment with 15 ppm F were increased in the A/J mice compared with their 129P3/J counterparts. Among them are Delta-amino levulinic acid dehydratase (P10518) and 3-ketoacyl-CoA thiolase A, peroxisomal (Q921H8) that were increased 5.58 and 4.18 fold in the A/J mice, in comparison with 129P3/J mice. In addition, most of the unique proteins were identified in the A/J mice. Treatment with 50 ppm F caused less fold change in comparison with the treatment with 15 ppm F, with some proteins increased and others decreased in the A/J mice in comparison to their 129P3/J counterparts.

3.4. Protein interaction networks

A network was created for each of the comparisons displayed above, employing all the interactions found in the search conducted using PSICQUIC. After the global network was created, nodes and edges were filtered using the specification for *Mus musculus* taxonomy (10090). The value of fold change and also the p-value were added in new columns. The ActiveModules 1.8 plug-in to Cytoscape was used to make active modules connected subnetworks within the molecular interaction network whose genes presented significant coordinated changes in fold changes and p-value, as shown in the original proteomic analysis. Figure 3 (a and b) shows the subnetwork generated by VizMapper for each comparison. Regardless of the F concentration, most of the proteins with fold change when the two strains were compared presented interaction with Disks large homolog 4 (Q62108) and Calcium-activated potassium channel subunit alpha-1 (Q08460).

When the animals were treated with 15 ppm F, 7 proteins with fold change between the two strains interacted with Disks large homolog 4 (Q62108), while 14 proteins with fold change presented interaction with Calcium-activated potassium channel subunit alpha-1 (Q08460). Moreover, 11 proteins were upregulated and 5 proteins were downregulated in A/J mice, while 5 proteins were present only in A/J mice and another 5 proteins were present only in 129P3/J mice (*Figure 3a).*

For the animals treated with 50 ppm F, 9 proteins with fold change interacted with Disks large homolog 4 (Q62108), while 16 proteins with fold change interacted with Calcium-activated potassium channel subunit alpha-1 (Q08460). Moreover, 7 proteins were upregulated and 13 proteins were downregulated in A/J mice, while 5

proteins were present only in A/J mice and another 7 proteins were present only in 129P3/J mice (*Figure 3b*).

4. Discussion

Despite there are many reports highlighting the toxic effects of excessive F ingestion in different organs (30) (14, 31-33), information related to liver, especially to proteomics, is quite scarce and limited to two studies conducted with rats (34, 35). The liver is an important organ in the body, which secrets bile and efficiently processes nutrients (36). Moreover, the majority of toxicants are bio-transformed into metabolites by liver through various enzyme systems; consequently, liver undergoes different levels of damage and alterations. These damage/alterations are often associated with a degenerative-necrotic condition (37). Various reports have shown that drugs and other chemical substances can damage hepatocytes, thus leading to hepatic dysfunction (38) (39) (40). In addition, excessive F intake can damage liver and the degree of damage is related to the quantity of F ingested (41) (42) (43). However, the mechanism of F-induced liver dysfunction remains unclear, thus leaving a window open to investigate it in depth.

A/J and 129P3/J mice strains have been widely studied over the last few years because they respond quite differently to F exposure (14, 20, 44-46). Thus, it is interesting to know if the distinct responses to F by these two strains are related to events occurring in the liver and what are the mechanisms involved. Recently, we observed that even without exposure to F, A/J mice had an increase in proteins related to energy flux and oxidative stress when compared to their 129P3/J counterparts (22), which seems to be a good explanation for the high susceptibility of these mice to the effects of F, since the exposure to this ion also induces oxidative stress (47) (48) (49) (50). Thus, it is of great interest to investigate the effects of both genetic background and F exposure on the liver proteomic profile, which was the main aim of the present study.

In the present study, a dose-response relationship was observed in liver F concentrations, which confirms that the treatment with F was effective. Regarding the differences between the strains, despite 129P3/J mice had higher liver F concentrations when compared with A/J counterparts, this difference was only significant for the highest F dose, what could be explained by F uptake into mineralized

tissues, while higher doses of F could saturate the effect of its uptake into mineralized tissues (51). The higher F concentrations found in the liver of 129P3/J mice is in-line with previous findings showing that these mice excrete less F and thus have higher circulating F levels than A/J mice, which is reflected in the F concentrations found in bone, enamel and different organs of these mice (44) (46) (52). Histological analyses of liver could be instructive to see alterations in the tissue structure after exposure to different doses of F.

One remarkable finding of the present study was the fact that treatment with the lower F concentration (15 ppm) provoked more pronounced alterations in fold change in liver proteins in comparison to the treatment with the higher F concentration (50 ppm). This is in-line with previous studies showing that treatment with 15 ppm F causes more alterations in liver and kidney than treatment with 50 ppm F (53, 34, 54). The reason for this finding is attributed to adaptive mechanisms of the organism to F (55) that are triggered by higher doses of this ion, but not by lower ones (54). It is possible that if the treatment with 50 ppm F had been conducted for a shorter time, it could have led to more pronounced alterations, similar to the ones seen in the 15 ppm F group in the present study. In fact, F can have a dual effect (protective or toxic), depending on the dose and time of exposure (56) (55) (53) (57) (35). Interestingly, all proteins with fold change upon treatment with 15 ppm F were increased in the A/J mice compared with their 129P3/J counterparts (*Table 1*), which suggests an attempt of the organism to fight the deleterious effects of F, since they seem to be dependent on the F dose and duration of the treatment (55). Treatment with the lower F concentration also led to increase in protein in the duodenum of rats after chronic exposure (58). Among the increased proteins, the ones with the highest fold changes are related to metabolism Delta-aminolevulinic acid dehydratase (δ-ALA-D) and 3-ketoacyl-CoA thiolase A, peroxisomal with fold changes of 5.58 and 4.18, respectively. δ-ALA-D is a sulphydryl-containing enzyme very sensitive to oxidizing agents (59). It is essential in the heme biosynthesis (60), since it catalyzes the asymmetric condensation of two molecules of δ-ALA to porphobilinogen, which, in subsequent steps is assembled into tetrapyrole molecules that constitute the prosthetic groups of proteins and enzymes, such as catalase (CAT) (61). CAT is an important antioxidant enzyme that is inhibited by F (62). In fact, treatment with water containing 15 ppm F led to the lowest CAT activity in the liver of rats after treatment for 60 days (53), which is similar to the protocol employed

in the present study. Thus, the increase in δ-ALA-D in our study might be an attempt to increase the activity of CAT, which is expected to be decreased upon treatment with F (53). It is also important to highlight that in our previous study where the animals were not treated with F, this enzyme was present only in the liver of A/J animals but not in their 129P3/J counterparts (22), which could be an attempt to fight against the oxidative stress in the first. Another protein, 3-ketoacyl-CoA thiolase A, peroxisomal had an increase of 4.18 times in A/J mice treated with 15 ppm F, in comparison to their 129P3/J counterparts. This protein is involved in the beta-oxidation of fatty acids, which is a multi-step process by which fatty acids are broken down to produce energy. These events take place in mitochondria and peroxisomes, by mechanisms involving dehydrogenation, hydration, redehydrogenation, and thiolytic cleavage (63). Increase in enzymes related to fatty acids beta oxidation in the presence of F have been previously reported in kidney (64) (33) and liver (34) suggesting a vigorous state of fatty acids metabolism, in attempt to counteract the inhibitory effect of F in the glycolytic pathway that has been known for a long time (65).

On the other hand, differently to what was seen for the animals treated with 15 ppm F, most of the proteins with fold change upon treatment with 50 ppm F were decreased in the A/J mice compared with their 129P3/J counterparts. The protein that presented the highest level of downregulation was *Stress-70 protein, mitochondrial* that is a heat-shock protein related to protein folding (UNIPROT). The downregulation means a lower degree of protein folding with consequent reduced toleration to F-induced stress in A/J mice (26, 34). The other two proteins with the highest degree of downregulation in the A/J mice in the group treated with 50 ppm F were two isoforms of Glutathione S-transferase (GST). GSTs are a multigene family of isozymes responsible for the detoxification of electrophiles by conjugation with the nucleophilic thiol reduced GSH (66). These enzymes play a crucial role in cellular detoxification of endogenous and xenobiotic substrates and protection against oxidative stress (67) and their downregulation means a difficulty of A/J mice treated with 50 ppm F to fight oxidative stress. Despite most of the proteins with fold change in the A/J mice treated with 50 ppm F were downregulated in comparison with their 129P3/J counterparts, one of the upregulated protein should be highlighted. This is the case of Retinal dehydrogenase 1 (ALDH1A1). These enzymes are known to have an antioxidant role by producing NAD(P)H (68) (69) and the upregulation of ALDH1A1 might have been

induced by the high dosage of F.

The two proteins in the center of the protein-protein interaction networks (Calcium-activated potassium channel subunit alpha-1 and Disks large homolog 4), regardless the F concentration, are related to potassium channels. Curiously, the same proteins were found in the center of the interaction networks in a previous study where A/J mice were compared with their 129P3/J-mice without exposure to F (22), which suggests that the pattern of the networks is driven mainly by the type of strain than by the exposure to F. In our previous study, the presence of these two interacting proteins in the center of the network was suggested to be related to alteration in the brain function of A/J mice (22) induced by accumulation of ammonia due to liver failure (70) and this needs to be investigated in further studies. Among the proteins identified with altered expression in the present study in the interaction networks, some of them were upregulated in the A/J mice, regardless the F concentration in the drinking water. One of them was Glutamate dehydrogenase 1, mitochondrial (GLUD1), a sensitive marker of hepatotoxicity. This enzyme is highly expressed in the hepatic mitochondria and its upregulation supports the occurrence of mitochondria dysfunction (71) (72), which is described to be induced upon exposure to F (73) (34). Another upregulated protein in A/J mice, regardless exposure to F, was Serum Albumin. The main function of this protein is the regulation of the colloidal osmotic pressure of blood, since it has a good binding capacity for water, Ca2+, Na+, K+ (UNIPROT). Its upregulation in A/J mice might be related to the higher volume of water ingested by this strain, regardless exposure to F (44).

In the network generated for the groups treated with 15 ppm F, most of the proteins with altered expression are associated with energy flux. The increase of the glycolytic enzyme Beta-enolase, the reduction of L-lactate dehydrogenase as well as the increase in subunits of ATP synthase involved in the oxidative phosphorylation indicate an increased energy flux in the A/J strain and this was also observed in our previous study that did not include exposure to F (22). This increased energy flux might produce oxidative stress, which is reflected in the increased levels of SOD and ALAD.

Despite the network generated for the groups treated with 50 ppm F had the same proteins in the center as the one generated for the groups treated with 15 ppm F, some of the interacting partners were different. One of the downregulated proteins

was Histone H2B type 1-P. Despite not appearing in the network, many other isoforms of Histones were downregulated in the A/J mice treated with 50 ppm F, as compared with their 129P3/J counterparts (*Table 2*). Histones are quite known as core components of nucleosome and their reduction leads to disorganized and ineffectively structured genomic DNA, which might impair gene transcription (74). This might help to explain the fact that most of the proteins with fold change upon treatment with 50 ppm F were decreased in the A/J mice compared with their 129P3/J counterparts.

In conclusion, treatment with the lower F concentration (15 ppm) provoked more pronounced alterations in fold change in liver proteins in comparison to the treatment with the higher F concentration (50 ppm), which is in-line with previous findings (34, 54, 75) and is possibly related to the duration of the treatment with F (55). Interestingly, most of the proteins with fold change upon treatment with 15 ppm F were increased in the A/J mice compared with their 129P3/J counterparts, suggesting an attempt of the former to fight the deleterious effects of F. However, upon treatment with 50 ppm F, most proteins with fold change were decreased in the A/J mice compared with their 129P3/J counterparts, especially proteins related to oxidative stress and protein folding, which might be related to the higher susceptibility of the A/J animals to the deleterious effects of F. Our findings add light into the mechanisms underlying genetic susceptibility to fluorosis.

Acknowledgements: The authors thank CNPq/TWAS for providing a scholarship to the first author (190145/2013-7).

References

1. Buzalaf MA and SM Levy (2011) Fluoride intake of children: considerations for dental caries and dental fluorosis. Monogr Oral Sci 22: 1-19.
2. Rugg-Gunn AJ, AE Villa, and MR Buzalaf (2011) Contemporary biological markers of exposure to fluoride. Monogr Oral Sci 22: 37-51.
3. Buzalaf MA and GM Whitford (2011) Fluoride metabolism. Monogr Oral Sci 22: 20-36.
4. Aoba T and O Fejerskov (2002) Dental fluorosis: chemistry and biology. Crit Rev Oral Biol Med 13: 155-70.

5. Krishnamachari KA (1986) Skeletal fluorosis in humans: a review of recent progress in the understanding of the disease. Prog Food Nutr Sci 10: 279-314.
6. Pushpalatha T, M Srinivas, and P Sreenivasula Reddy (2005) Exposure to high fluoride concentration in drinking water will affect spermatogenesis and steroidogenesis in male albino rats. Biometals 18: 207-12.
7. Zhang S, C Jiang, H Liu, Z Guan, Q Zeng, C Zhang, et al. (2013) Fluoride-elicited developmental testicular toxicity in rats: roles of endoplasmic reticulum stress and inflammatory response. Toxicol Appl Pharmacol 271: 206-15.
8. Ge YM, HM Ning, SL Wang, and JD Wang (2005) DNA damage in thyroid gland cells of rats exposed to longterm intake of high fluoride and low iodine. Fluoride 38: 318-323.
9. Susheela AK, M Bhatnagar, K Vig, and NK Mondal (2005) Excess fluoride ingestion and thyroid hormone derangements in children living in Delhi, India. Fluoride 38: 98-108.
10. Chen T, HM Cui, Y Cui, CM Bai, and T Gong (2011) Decreased antioxidase activities and oxidative stress in the spleen of chickens fed on high-fluorine diets. Human & Experimental Toxicology 30: 1282-1286.
11. Podder S, A Chattopadhyay, S Bhattacharya, and MR Ray (2010) Histopathology and Cell Cycle Alteration in the Spleen of Mice from Low and High Doses of Sodium Fluoride. Fluoride 43: 237-245.
12. Pereira HABD, AD Leite, S Charone, JGVM Lobo, TM Cestari, C Peres-Buzalaf, et al. (2013) Proteomic Analysis of Liver in Rats Chronically Exposed to Fluoride. Plos One 8.
13. Kobayashi CAN, AL Leite, TL Silva, LD Santos, FCS Nogueira, RC Oliveiraa, et al. (2009) Proteomic analysis of kidney in rats chronically exposed to fluoride. Chemico-Biological Interactions 180: 305-311.
14. Carvalho JG, AD Leite, C Peres-Buzalaf, F Salvato, CA Labate, ET Everett, et al. (2013) Renal Proteome in Mice with Different Susceptibilities to Fluorosis. Plos One 8.
15. Mullenix PJ, PK Denbesten, A Schunior, and WJ Kernan (1995) Neurotoxicity of Sodium-Fluoride in Rats. Neurotoxicology and Teratology 17: 169-177.
16. Niu RY, YL Zhang, SL Liu, FY Liu, ZL Sun, and JD Wang (2015) Proteome Alterations in Cortex of Mice Exposed to Fluoride and Lead. Biological Trace Element Research 164: 99-105.
17. Manji F, V Baelum, and O Fejerskov (1986) Dental Fluorosis in an Area of Kenya with 2 Ppm Fluoride in the Drinking-Water. Journal of Dental Research 65: 659-662.
18. Manji F, V Baelum, O Fejerskov, and W Gemert (1986) Enamel Changes in 2 Low-Fluoride Areas of Kenya. Caries Research 20: 371-380.
19. Yoder KM, L Mabelya, VA Robison, AJ Dunipace, EJ Brizendine, and GK Stookey (1998) Severe dental fluorosis in a Tanzanian population consuming water with negligible fluoride concentration. Community Dentistry and Oral Epidemiology 26: 382-393.

20. Everett ET, MAK McHenry, N Reynolds, H Eggertsson, J Sullivan, C Kantmann, et al. (2002) Dental fluorosis: Variability among different inbred mouse strains. Journal of Dental Research 81: 794-798.
21. Carvalho JG, AL Leite, D Yan, ET Everett, GM Whitford, and MAR Buzalaf (2009) Influence of Genetic Background on Fluoride Metabolism in Mice. Journal of Dental Research 88: 1054-1058.
22. Khan ZN, AL Leite, S Charone, IT Sabino, T Martini, HABS Pereira, et al. (2016) Liver proteome of mice with different genetic susceptibilities to the effects of fluoride. Journal of Applied Oral Science 250-257.
23. Taves DR (1968) Separation of Fluoride by Rapid Diffusion Using Hexamethyldisiloxane. Talanta 15: 969-&.
24. Lobo JGVM, AL Leite, HABS Pereira, MS Fernandes, C Peres-Buzalaf, DH Sumida, et al. (2015) Low-Level Fluoride Exposure Increases Insulin Sensitivity in Experimental Diabetes. Journal of Dental Research 94: 990-997.
25. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248-54.
26. Leite AL, JGVM Lobo, HABD Pereira, MS Fernandes, T Martini, F Zucki, et al. (2014) Proteomic Analysis of Gastrocnemius Muscle in Rats with Streptozotocin-Induced Diabetes and Chronically Exposed to Fluoride. Plos One 9.
27. Bauer-Mehren A (2013) Integration of genomic information with biological networks using Cytoscape. Methods Mol Biol 1021: 37-61.
28. Orchard S (2012) Molecular interaction databases. Proteomics 12: 1656-62.
29. Millan PP (2013) Visualization and analysis of biological networks. Methods Mol Biol 1021: 63-88.
30. Lima Leite A, J Gualiume Vaz Madureira Lobo, HA Barbosa da Silva Pereira, M Silva Fernandes, T Martini, F Zucki, et al. (2014) Proteomic analysis of gastrocnemius muscle in rats with streptozotocin-induced diabetes and chronically exposed to fluoride. PLoS One 9: e106646.
31. Buzalaf MA, EE Caroselli, R Cardoso de Oliveira, JM Granjeiro, and GM Whitford (2004) Nail and bone surface as biomarkers for acute fluoride exposure in rats. J Anal Toxicol 28: 249-52.
32. Buzalaf MA, EE Caroselli, JG de Carvalho, RC de Oliveira, VE da Silva Cardoso, and GM Whitford (2005) Bone surface and whole bone as biomarkers for acute fluoride exposure. J Anal Toxicol 29: 810-3.
33. Kobayashi CA, AL Leite, TL Silva, LD Santos, FC Nogueira, RC Oliveira, et al. (2009) Proteomic analysis of kidney in rats chronically exposed to fluoride. Chem Biol Interact 180: 305-11.
34. Pereira HA, L Leite Ade, S Charone, JG Lobo, TM Cestari, C Peres-Buzalaf, et al. (2013) Proteomic analysis of liver in rats chronically exposed to fluoride. PLoS One 8: e75343.

35. Lobo JG, AL Leite, HA Pereira, MS Fernandes, C Peres-Buzalaf, DH Sumida, et al. (2015) Low-Level Fluoride Exposure Increases Insulin Sensitivity in Experimental Diabetes. J Dent Res 94: 990-7.
36. Zhou BH, J Zhao, J Liu, JL Zhang, J Li, and HW Wang (2015) Fluoride-induced oxidative stress is involved in the morphological damage and dysfunction of liver in female mice. Chemosphere 139: 504-11.
37. Mukhopadhyay D and A Chattopadhyay (2014) Induction of oxidative stress and related transcriptional effects of sodium fluoride in female zebrafish liver. Bull Environ Contam Toxicol 93: 64-70.
38. Sun K, SE Eriksson, Y Tan, L Zhang, ES Arner, and J Zhang (2014) Serum thioredoxin reductase levels increase in response to chemically induced acute liver injury. Biochim Biophys Acta 1840: 2105-11.
39. Wang X, Z Jiang, M Xing, J Fu, Y Su, L Sun, et al. (2014) Interleukin-17 mediates triptolide-induced liver injury in mice. Food Chem Toxicol 71: 33-41.
40. Wang ZJ, J Lee, YX Si, W Wang, JM Yang, SJ Yin, et al. (2014) A folding study of Antarctic krill (Euphausia superba) alkaline phosphatase using denaturants. Int J Biol Macromol 70: 266-74.
41. Xiong X, J Liu, W He, T Xia, P He, X Chen, et al. (2007) Dose-effect relationship between drinking water fluoride levels and damage to liver and kidney functions in children. Environ Res 103: 112-6.
42. Cao J, J Chen, J Wang, R Jia, W Xue, Y Luo, et al. (2013) Effects of fluoride on liver apoptosis and Bcl-2, Bax protein expression in freshwater teleost, Cyprinus carpio. Chemosphere 91: 1203-12.
43. Zlatkovic J, N Todorovic, N Tomanovic, M Boskovic, S Djordjevic, T Lazarevic-Pasti, et al. (2014) Chronic administration of fluoxetine or clozapine induces oxidative stress in rat liver: a histopathological study. Eur J Pharm Sci 59: 20-30.
44. Carvalho JG, AL Leite, D Yan, ET Everett, GM Whitford, and MA Buzalaf (2009) Influence of genetic background on fluoride metabolism in mice. J Dent Res 88: 1054-8.
45. Kobayashi CA, AL Leite, C Peres-Buzalaf, JG Carvalho, GM Whitford, ET Everett, et al. (2014) Bone response to fluoride exposure is influenced by genetics. PLoS One 9: e114343.
46. Charone S, A De Lima Leite, C Peres-Buzalaf, M Silva Fernandes, L Ferreira de Almeida, MS Zardin Graeff, et al. (2016) Proteomics of Secretory-Stage and Maturation-Stage Enamel of Genetically Distinct Mice. Caries Res 50: 24-31.
47. Arguelles S, S Garcia, M Maldonado, A Machado, and A Ayala (2004) Do the serum oxidative stress biomarkers provide a reasonable index of the general oxidative stress status? Biochim Biophys Acta 1674: 251-9.
48. Inkielewicz-Stepniak I and W Czarnowski (2010) Oxidative stress parameters in rats exposed to fluoride and caffeine. Food Chem Toxicol 48: 1607-11.
49. Nabavia SM, A Suredac, SF Nabavia, AM Latifia, AH Moghaddam, and C Hellioe (2012) Neuroprotective effects of silymarin on sodium fluoride-induced oxidative stress. Journal of Fluorine Chemistry 1425: 79–82.

50. Atmaca N, HT Atmaca, A Kanici, and T Anteplioglu (2014) Protective effect of resveratrol on sodium fluoride-induced oxidative stress, hepatotoxicity and neurotoxicity in rats. Food Chem Toxicol 70: 191-7.
51. Ekstrand J, Y Ericsson, and S Rosell (1977) Absence of protein-bound fluoride from human and blood plasma. Arch Oral Biol 22: 229-32.
52. Everett ET, MA McHenry, N Reynolds, H Eggertsson, J Sullivan, C Kantmann, et al. (2002) Dental fluorosis: variability among different inbred mouse strains. J Dent Res 81: 794-8.
53. Iano FG, MC Ferreira, GB Quaggio, MS Mileni Silva Fernandes, RC Oliveira, VF Ximenes, et al. (2014) Effects of chronic fluoride intake on the antioxidant systems of the liver and kidney in rats. Journal of Fluorine Chemistry 168: 212–217.
54. Pereira HA, AS Dionizio, MS Fernandes, TT Araujo, TM Cestari, CP Buzalaf, et al. (2016) Fluoride Intensifies Hypercaloric Diet-Induced ER Oxidative Stress and Alters Lipid Metabolism. PLoS One 11: e0158121.
55. Dabrowska E, R Letko, and M Balunowska (2006) Effect of sodium fluoride on the morphological picture of the rat liver exposed to NaF in drinking water. Adv Med Sci 51 Suppl 1: 91-5.
56. Barbier O, L Arreola-Mendoza, and LM Del Razo (2010) Molecular mechanisms of fluoride toxicity. Chem Biol Interact 188: 319-33.
57. Dunipace AJ, EJ Brizendine, W Zhang, ME Wilson, LL Miller, BP Katz, et al. (1995) Effect of aging on animal response to chronic fluoride exposure. J Dent Res 74: 358-68.
58. Melo CGS, J Perles, JN Zanoni, SRG Souza, EX Santos, AL Leite, et al. (2017) Enteric innervation combined with proteomics for the evaluation of the effects of chronic fluoride exposure on the duodenum of rats. Sci Rep 7: 1070.
59. Bottari NB, RE Mendes, MD Baldissera, GV Bochi, RN Moresco, ML Leal, et al. (2016) Relation between iron metabolism and antioxidants enzymes and delta-ALA-D activity in rats experimentally infected by Fasciola hepatica. Exp Parasitol 165: 58-63.
60. Sassa S (1982) Delta-aminolevulinic acid dehydratase assay. Enzyme 28: 133-45.
61. Sassa S (1998) ALAD porphyria. Semin Liver Dis 18: 95-101.
62. Shanthakumari D, S Srinivasalu, and S Subramanian (2004) Effect of fluoride intoxication on lipidperoxidation and antioxidant status in experimental rats. Toxicology 204: 219-28.
63. Wanders RJ and HR Waterham (2006) Biochemistry of mammalian peroxisomes revisited. Annu Rev Biochem 75: 295-332.
64. Xu H, LS Hu, M Chang, L Jing, XY Zhang, and GS Li (2005) Proteomic analysis of kidney in fluoride-treated rat. Toxicol Lett 160: 69-75.
65. Warburg O and W Chistian (1941) Isohering und kristallisation des görungs ferments enolase. Biochem. Zool. 310: 384-421.

66. Hayes JD and DJ Pulford (1995) The glutathione S-transferase supergene family: regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. Crit Rev Biochem Mol Biol 30: 445-600.
67. Schroer KT, AM Gibson, U Sivaprasad, SA Bass, MB Ericksen, M Wills-Karp, et al. (2011) Downregulation of glutathione S-transferase pi in asthma contributes to enhanced oxidative stress. J Allergy Clin Immunol 128: 539-48.
68. Pappa A, C Chen, Y Koutalos, AJ Townsend, and V Vasiliou (2003) Aldh3a1 protects human corneal epithelial cells from ultraviolet- and 4-hydroxy-2-nonenal-induced oxidative damage. Free Radic Biol Med 34: 1178-89.
69. Lassen N, A Pappa, WJ Black, JV Jester, BJ Day, E Min, et al. (2006) Antioxidant function of corneal ALDH3A1 in cultured stromal fibroblasts. Free Radic Biol Med 41: 1459-69.
70. Felipo V (2013) Hepatic encephalopathy: effects of liver failure on brain function. Nat Rev Neurosci 14: 851-8.
71. Stanley CA (2009) Regulation of glutamate metabolism and insulin secretion by glutamate dehydrogenase in hypoglycemic children. Am J Clin Nutr 90: 862S-866S.
72. McGill MR, MR Sharpe, CD Williams, M Taha, SC Curry, and H Jaeschke (2012) The mechanism underlying acetaminophen-induced hepatotoxicity in humans and mice involves mitochondrial damage and nuclear DNA fragmentation. J Clin Invest 122: 1574-83.
73. Anuradha CD, Kanno S, and H S. (2001) Oxidative damage to mitochondria is a preliminary step to caspase-3 activation in fluoride-induced apoptosis in HL-60 cells. Free Radical Biology and Medicine 31: 367-73.
74. Chen R, R Kang, XG Fan, and D Tang (2014) Release and activity of histone in diseases. Cell Death Dis 5: e1370.
75. Jakubowski H (2006) Pathophysiological consequences of homocysteine excess. J Nutr 136: 1741S-1749S.

Figure Legends

Fig 1 Liver fluoride concentrations in A/J and 129P3/J mice treated with water containing 15 or 50 ppm fluoride in the drinking water for 42 days. For each fluoride concentration, distinct lower case letters indicate significant differences between the strains. Distinct upper case letters denote significant differences between the fluoride concentrations. Two-way ANOVA and Sidak´s test ($p<0.05$). n=6. Bars indicate SD.

Fig 2 Functional distribution of proteins identified with differential expression in the liver of mice belonging to Group A/J vs. 129p3/J treated with fluoride (15 ppm-A and 50 ppm-B). Categories of proteins based on GO annotation Biological Process. Terms significant (Kappa=0.03) and distribution according to percentage of number of genes association.

Fig 3a Subnetworks generated by VizMapper for each comparison – Group A/J vs. 129p3/J treated with Fluoride (15 ppm); Color of node and * indicate the differential expression of the respective protein, for each comparison. Red and green nodes indicate protein down-regulation **OR** absence and up-regulation **OR** presence respectively, while * and ** indicate presence and absence of protein respectively, in the first group of each comparison respectively. Purple node indicates proteins presenting interaction but that were not identified in the present study. The access numbers in nodes correspond to: Q811D0- (Dlg1) Disks large homolog 1; Q9DB20- (Atp5o) ATP synthase subunit O, mitochondrial; P08228-(Sod1**)** Superoxide dismutase [Cu-Zn]; P16858- (Gapdh) Glyceraldehyde-3-phosphate dehydrogenase; P27773- (Pdia3) Protein disulfide-isomerase A3; P56480- (Atp5b) ATP synthase subunit beta, mitochondrial; P26443- (Glud1) Glutamate dehydrogenase 1, mitochondrial; P21550- (Eno3) Beta-enolase; Q9CPU0- (Glo1) Lactoylglutathione lyase; P10518- (Alad) Delta-aminolevulinic acid dehydratase; P60710- (Actb) Actin, cytoplasmic 1; P63260- (Actg1) Actin, cytoplasmic 2; P16627- (Hspa1l) Heat shock 70 kDa protein 1-like; P06151-(Ldha) L-lactate dehydrogenase A chain; P07724- (Alb) Serum albumin; P63101- (Ywhaz) 14-3-3 protein zeta/delta; Q62108- (Dlg4) Disks large homolog 4; Q08460- (Kcnma1) Calcium-activated potassium channel subunit alpha-1.

Fig 3b Subnetworks generated by VizMapper for each comparison – Group A/J vs. 129p3/J treated with Fluoride (50 ppm); Color of node and * indicate the differential

expression of the respective protein, for each comparison. Red and green nodes indicate protein down-regulation and up-regulation, respectively, while * and ** indicate presence and absence of protein, respectively, in the first group of each comparison. Gray node indicates proteins presenting interaction but that were not identified in the present study. The access numbers in nodes correspond to: Q8CGP2- (Hist1h2bp) Histone H2B type 1-P; P11499- (Hsp90ab1) Heat shock protein HSP 90-beta; P19157- (Gstp1) Glutathione S-transferase P 1; P52760- (Hrsp12) Ribonuclease UK114; P56480- (Atp5b) ATP synthase subunit beta, mitochondrial; P26443- (Glud1) Glutamate dehydrogenase 1, mitochondrial; P38647- (Hspa9) Stress-70 protein, mitochondrial; P27773- (Pdia3) Protein disulfide-isomerase A3; P16858- (Gapdh) Glyceraldehyde-3-phosphate dehydrogenase; P10649- (Gstm1) Glutathione S-transferase Mu 1; P05064- (Aldoa) Fructose-bisphosphate aldolase A; P10518- (Alad) Delta-aminolevulinic acid dehydratase; P06151- (Ldha) L-lactate dehydrogenase A chain; P70296- (Pebp1) Phosphatidylethanolamine-binding protein 1; P05201- (Got1) Aspartate aminotransferase, cytoplasmic; P17182- (Eno1) Alpha-enolase; P17742- (Ppia) Peptidyl-prolyl cis-trans isomerase A ; Q00623- (Apoa1) Apolipoprotein A-I ; Q9CPU0- (Glo1) Lactoylglutathionelyase; P07724- (Alb) Serum albumin; Q08460- (Kcnma1) Calcium-activated potassium channel subunit alpha-1; Q62108- (Dlg4) Disks large homolog 4.

Fig1.

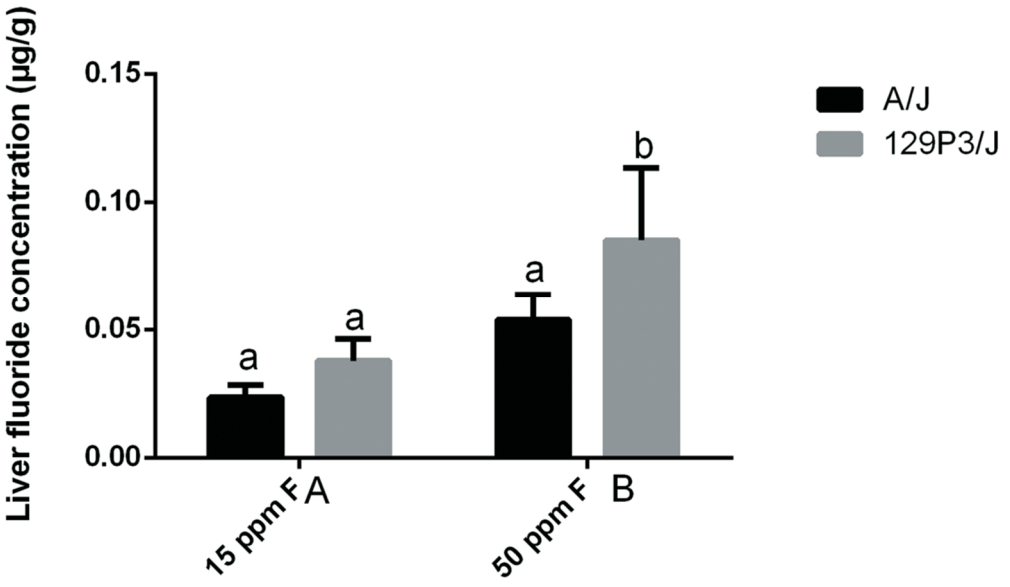

Liver fluoride concentration (µg/g)
0.15
0.10
0.05
0.00
a
a
a
b
A/J
129P3/J
15 ppm F
A
50 ppm F
B

Fig2.

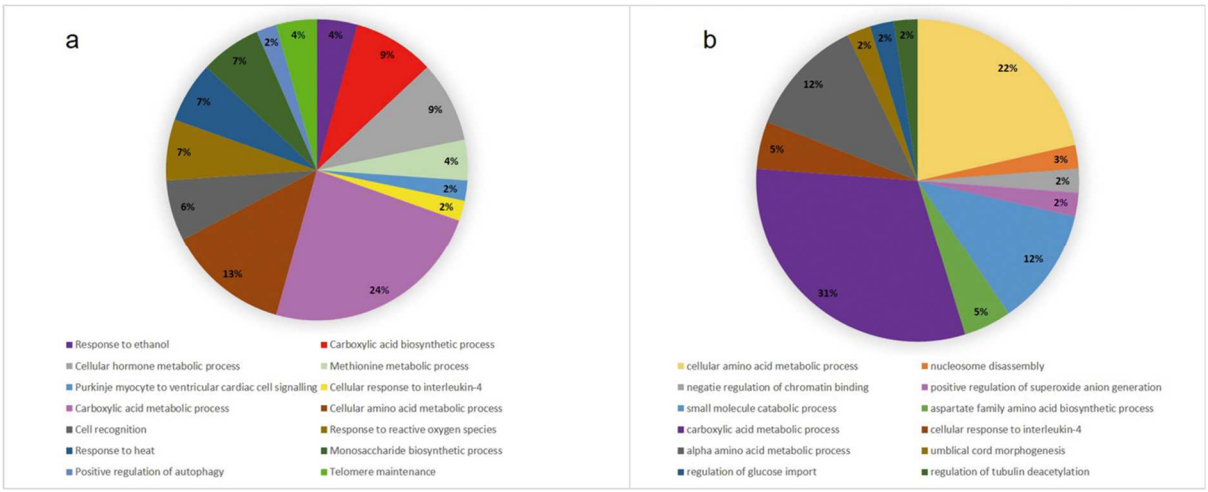

a
4%
9%
9%
4%
2%
2%
24%
13%
6%
7%
7%
7%
2%
4%
Response to ethanol
Carboxylic acid biosynthetic process
Cellular hormone metabolic process
Methionine metabolic process
Purkinje myocyte to ventricular cardiac cell signalling
Cellular response to interleukin-4
Carboxylic acid metabolic process
Cellular amino acid metabolic process
Cell recognition
Response to reactive oxygen species
Response to heat
Monosaccharide biosynthetic process
Positive regulation of autophagy
Telomere maintenance
b
22%
3%
2%
2%
12%
5%
31%
5%
12%
2%
2%
2%
cellular amino acid metabolic process
nucleosome disassembly
negatie regulation of chromatin binding
positive regulation of superoxide anion generation
small molecule catabolic process
aspartate family amino acid biosynthetic process
carboxylic acid metabolic process
cellular response to interleukin-4
alpha amino acid metabolic process
umblical cord morphogenesis
regulation of glucose import
regulation of tubulin deacetylation

Fig3.

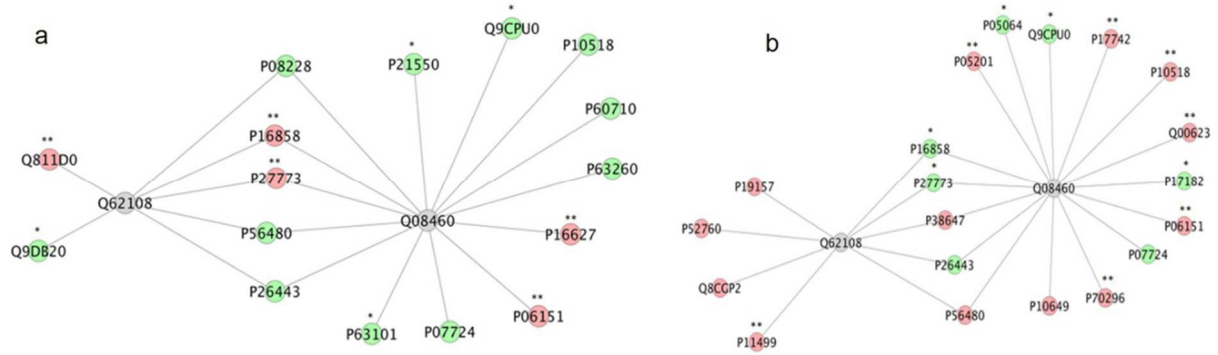

a
Q9CPU0
P10518
P08228
P21550
P60710
P16858
Q811D0
P27773
P63260
Q62108
Q08460
P56480
P16627
Q9DB20
P26443
P06151
P63101
P07724
b
P05064
Q9CPU0
P17742
P05201
P10518
Q00623
P16858
P19157
P27773
Q08460
P17182
P38647
P52760
P06151
Q62108
P07724
P26443
Q8CCP2
P70296
P10649
P56480
P11499

Table 1.

Table 1. Proteins with expression significantly altered in the liver of A/J and 129P3/j mice treated with 15 ppm F.

				Fold change	
[a]Access Number	Gene name	Protein name description	PLGS score	A/J 15ppm	129P3/J 15ppm
P10518	Alad	Delta-aminolevulinicaciddehydratase	113.6	5.58	-5.58
Q921H8	Acaa1a	3-ketoacyl-CoA thiolase A, peroxisomal	88.9	4.18	-4.18
P24549	Aldh1a1	Retinaldehydrogenase 1	141.7	2.80	-2.80
Q64442	Sord	Sorbitoldehydrogenase	277.3	2.29	-2.29
Q8R0Y6	Aldh1l1	Cytosolic 10-formyltetrahydrofolate dehydrogenase	67.5	2.27	-2.27
P24270	Cat	Catalase	183.6	1.75	-1.75
Q64374	Rgn	Regucalcin	47.7	1.63	-1.63
P26443	Glud1	Glutamatedehydrogenase 1, mitochondrial	279.4	1.62	-1.62
Q9QXF8	Gnmt	Glycine N-methyltransferase	287.8	1.62	-1.62
P62806	Hist1h4a	Histone H4	50.0	1.60	-1.60
P62737	Acta2	Actin, aorticsmoothmuscle	139.1	1.51	-1.51
P16015	Ca3	Carbonicanhydrase 3	308.3	1.49	-1.49
P08228	Sod1	Superoxidedismutase [Cu-Zn]	105.8	1.49	-1.49
P11725	Otc	Ornithinecarbamoyltransferase, mitochondrial	150.3	1.46	-1.46
P68134	Acta1	Actin, alpha skeletalmuscle	195.6	1.43	-1.43
P68033	Actc1	Actin, alpha cardiacmuscle 1	195.6	1.42	-1.42
O35490	Bhmt	Betaine--homocysteine S-methyltransferase 1	494.2	1.39	-1.39
P60710	Actb	Actin, cytoplasmic 1	310.5	1.38	-1.38
P10649	Gstm1	Glutathione S-transferase Mu 1	44.2	1.38	-1.38
P07724	Alb	Serumalbumin	285.4	1.36	-1.36
P63260	Actg1	Actin, cytoplasmic 2	310.5	1.31	-1.31
P56480	Atp5b	ATP synthasesubunit beta, mitochondrial	33.7	1.28	-1.28
O70456	Sfn	14-3-3 protein sigma	126.3	+	-
P63101	Ywhaz	14-3-3 proteinzeta/delta	93.1	+	-
P49429	Hpd	4-hydroxyphenylpyruvate dioxygenase	189.9	+	-

Q9JII6	Akr1a1	Alcoholdehydrogenase [NADP(+)]	60.2	+	-
P00329	Adh1	Alcoholdehydrogenase 1	184.8	+	-
P00330	ADH1	Alcoholdehydrogenase 1	68.6	+	-
O35945	Aldh1a7	Aldehydedehydrogenase, cytosolic 1	105.0	+	-
Q8K023	Akr1c18	Aldo-keto reductase family 1 member C18	76.8	+	-
Q91ZU0	Asb7	Ankyrin repeat and SOCS box protein 7	99.9	+	-
Q61176	Arg1	Arginase-1	252.3	+	-
Q91YI0	Asl	Argininosuccinatelyase	71.8	+	-
Q9CQQ7	Atp5f1	ATP synthase subunit b, mitochondrial	55.9	+	-
Q9DB20	Atp5o	ATP synthasesubunit O, mitochondrial	68.4	+	-
P21550	Eno3	Beta-enolase	194.7	+	-
P50172	Hsd11b1	Corticosteroid 11-beta-dehydrogenase isozyme 1	97.3	+	-
Q60773	Cdkn2d	Cyclin-dependent kinase 4 inhibitor D	71.4	+	-
Q9D8U7	Dtwd1	DTW domain-containingprotein 1	64.2	+	-
O88513	Gmnn	Geminin	72.8	+	-
P24472	Gsta4	Glutathione S-transferase A4	160.1	+	-
P62827	Ran	GTP-binding nuclear protein Ran	74.8	+	-
P68433	Hist1h3a	Histone H3.1	490.5	+	-
P84228	Hist1h3b	Histone H3.2	490.5	+	-
P84244	H3f3a	Histone H3.3	461.5	+	-
P02301	H3f3c	Histone H3.3C	461.5	+	-
Q9CPU0	Glo1	Lactoylglutathionelyase	436.0	+	-
Q5S006	Lrrk2	Leucine-rich repeat serine/threonine-protein kinase 2	11.4	+	-
Q8BPT6	Immp2l	Mitochondrial inner membrane protease subunit 2	73.9	+	-
Q9DC69	Ndufa9	NADH dehydrogenase [ubiquinone] 1 alpha subcomplexsubunit 9, mitochondrial	88.0	+	-
O08807	Prdx4	Peroxiredoxin-4	160.8	+	-
P99029	Prdx5	Peroxiredoxin-5, mitochondrial	70.0	+	-
Q6PDH0	Phldb1	Pleckstrin homology-like domain family B member 1	49.6	+	-
Q8CD94	Lin52	Protein lin-52 homolog	92.8	+	-
E9Q401	Ryr2	Ryanodine receptor 2	12.3	+	-
Q91X83	Mat1a	S-adenosylmethionine synthase isoform type-1	288.0	+	-

P42209	sept1.	Septin-1	67.0	+	-
Q8BR65	Suds3	Sin3 histonedeacetylasecorepressorcomplexcomponent SDS3	68.2	+	-
O55060	Tpmt	Thiopurine S-methyltransferase	63.5	+	-
P34914	Ephx2	Bifunctionalepoxidehydrolase 2	170.6	-	+
Q8VCU1	Ces3b	Carboxylesterase 3B	84.7	-	+
Q0VEJ0	Cep76	Centrosomalproteinof 76 kDa	96.0	-	+
Q80YP0	Cdk3	Cyclin-dependentkinase 3	77.3	-	+
Q80XN0	Bdh1	D-beta-hydroxybutyratedehydrogenase, mitochondrial	127.0	-	+
Q811D0	Dlg1	Disks largehomolog 1	48.8	-	+
O54734	Ddost	Dolichyl-diphosphooligosaccharide--proteinglycosyltransferase 48 kDasubunit	42.7	-	+
P16858	Gapdh	Glyceraldehyde-3-phosphate dehydrogenase	211.8	-	+
Q61696	Hspa1a	Heat shock 70 kDa protein 1A	75.2	-	+
P17879	Hspa1b	Heat shock 70 kDa protein 1B	75.2	-	+
P16627	Hspa1l	Heat shock 70 kDa protein 1-like	75.2	-	+
P01868	Ighg1	Ig gamma-1 chain C region secreted form	89.2	-	+
Q9QZ29	Igbp1b	Immunoglobulin-bindingprotein 1b	84.8	-	+
P06151	Ldha	L-lactatedehydrogenase A chain	158.5	-	+
P34884	Mif	Macrophagemigrationinhibitoryfactor	122.3	-	+
P14152	Mdh1	Malatedehydrogenase, cytoplasmic	189.8	-	+
P27773	Pdia3	Proteindisulfide-isomerase A3	57.9	-	+
Q9DB54	Fam216a	Protein FAM216A	116.4	-	+
Q05920	Pc	Pyruvatecarboxylase, mitochondrial	113.3	-	+
P17563	Selenbp1	Selenium-bindingprotein 1	101.1	-	+
Q63836	Selenbp2	Selenium-bindingprotein 2	101.1	-	+
Q9R0P3	Esd	S-formylglutathionehydrolase	57.7	-	+

The identified proteins are organized according to highest to lowest fold change. + sign indicates presence of the protein, while – sign indicates absence of the protein in the respective groups. [a]Identification is based on protein ID from UniProt protein database(http://www.uniprot.org/)

Table 2. Proteins with expression significantly altered in the liver of A/J and 129P3/j mice treated with 50 ppm F.

				Fold change	
[a]Access Number	Gene name	Protein name description	PLGS score	A/J 50ppm	129P3/J 50ppm
Q91VS7	Mgst1	Microsomalglutathione S-transferase 1	239.2	1.31	-1.31
P11725	Otc	Ornithinecarbamoyltransferase, mitochondrial	123.3	1.31	-1.31
P26443	Glud1	Glutamatedehydrogenase 1, mitochondrial	269.9	1.28	-1.28
P24549	Aldh1a1	Retinaldehydrogenase 1	385.7	1.28	-1.28
P16015	Ca3	Carbonicanhydrase 3	340.1	1.25	-1.25
P07724	Alb	Serumalbumin	266.0	1.23	-1.23
Q8C196	Cps1	Carbamoyl-phosphate synthase [ammonia], mitochondrial	1282.5	1.20	-1.20
Q8VCU1	Ces3b	Carboxylesterase 3B	105.6	1.20	-1.20
O35490	Bhmt	Betaine--homocysteine S-methyltransferase 1	561.6	1.18	-1.18
Q9DCW4	Etfb	Electron transfer flavoprotein subunit beta	240.8	1.17	-1.17
P54869	Hmgcs2	Hydroxymethylglutaryl-CoAsynthase, mitochondrial	383.1	1.17	-1.17
Q8BWT1	Acaa2	3-ketoacyl-CoA thiolase, mitochondrial	1934.3	1.14	-1.14
P02104	Hbb-y	Hemoglobinsubunit epsilon-Y2	2492.3	1.06	-1.06
P38647	Hspa9	Stress-70 protein, mitochondrial	115.4	-0.55	0.55
P19157	Gstp1	Glutathione S-transferase P 1	4570.8	-0.61	0.61
P46425	Gstp2	Glutathione S-transferase P 2	2746.8	-0.61	0.61
P10853	Hist1h2bf	Histone H2B type 1-F/J/L	997.5	-0.74	0.74
P10854	Hist1h2bm	Histone H2B type 1-M	997.5	-0.74	0.74
Q6ZWY9	Hist1h2bc	Histone H2B type 1-C/E/G	997.5	-0.75	0.75
Q8CGP2	Hist1h2bp	Histone H2B type 1-P	996.3	-0.75	0.75
Q64525	Hist2h2bb	Histone H2B type 2-B	997.5	-0.75	0.75
Q64524	Hist2h2be	Histone H2B type 2-E	964.4	-0.75	0.75
Q9D2U9	Hist3h2ba	Histone H2B type 3-A	963.1	-0.75	0.75
P02762	Mup6	Major urinaryprotein 6	1965.1	-0.75	0.75
Q64475	Hist1h2bb	Histone H2B type 1-B	997.5	-0.76	0.76
Q8CGP1	Hist1h2bk	Histone H2B type 1-K	997.5	-0.76	0.76

Q8CGP0	Hist3h2bb	Histone H2B type 3-B	964.4	-0.76	0.76
P11588	Mup1	Major urinaryprotein 1	1965.1	-0.76	0.76
P11589	Mup2	Major urinaryprotein 2	1965.1	-0.76	0.76
P04938	Mup8	Major urinary proteins 11 and 8 (Fragment)	1965.1	-0.76	0.76
Q64478	Hist1h2bh	Histone H2B type 1-H	997.5	-0.77	0.77
B5X0G2	Mup17	Major urinaryprotein 17	1845.7	-0.81	0.81
P52760	Hrsp12	Ribonuclease UK114	1703.5	-0.84	0.84
P01942	Hba	Hemoglobinsubunit alpha	6669.6	-0.86	0.86
P56480	Atp5b	ATP synthasesubunit beta, mitochondrial	1282.4	-0.89	0.89
P10649	Gstm1	Glutathione S-transferase Mu 1	949.1	-0.90	0.90

O35972	Mrpl23	39S ribosomalprotein L23, mitochondrial	111.7	+	-
O08756	Hsd17b10	3-hydroxyacyl-CoA dehydrogenase type-2	64.4	+	-
P14115	Rpl27a	60S ribosomalprotein L27a	61.2	+	-
Q9CQJ0	Them5	Acyl-coenzyme A thioesterase THEM5	82.9	+	-
P17182	Eno1	Alpha-enolase	179.3	+	-
Q922J3	Clip1	CAP-Gly domain-containing linker protein 1	74.2	+	-
Q9D7F7	Chmp4c	Charged multivesicular body protein 4c	282.4	+	-
Q99LI2	Clcc1	Chloride channel CLIC-like protein 1	63.4	+	-
P50172	Hsd11b1	Corticosteroid 11-beta-dehydrogenase isozyme 1	99.7	+	-
Q60772	Cdkn2c	Cyclin-dependent kinase 4 inhibitor C	227.8	+	-
P00405	Mtco2	Cytochrome c oxidase subunit 2	108.3	+	-
O35215	Ddt	D-dopachromedecarboxylase OS=Mus musculus GN=Ddt PE=1 SV=3	200.8	+	-
P58252	Eef2	Elongationfactor 2	66.3	+	-
Q91XD4	Ftcd	Formimidoyltransferase-cyclodeaminase	156.9	+	-
P05064	Aldoa	Fructose-bisphosphatealdolase A	88.4	+	-
P16858	Gapdh	Glyceraldehyde-3-phosphate dehydrogenase	104.7	+	-
Q9WV93	Hey1	Hairy/enhancer-of-split related with YRPW motif protein 1	81.6	+	-
P43276	Hist1h1b	Histone H1.5	58.4	+	-
Q64522	Hist2h2ab	Histone H2A type 2-B	411.1	+	-
P08730	Krt13	Keratin, type I cytoskeletal 13	64.6	+	-

Q9R0H5	Krt71	Keratin, type II cytoskeletal 71	63.3	+	-
Q8BGZ7	Krt75	Keratin, type II cytoskeletal 75	79.2	+	-
Q9CPU0	Glo1	Lactoylglutathionelyase	462.7	+	-
Q05CL8	Larp7	La-relatedprotein 7	57.8	+	-
P41216	Acsl1	Long-chain-fatty-acid--CoA ligase 1	105.5	+	-
Q9D7Q0	Lyg1	Lysozyme g-likeprotein 1	70.8	+	-
Q9JLB0	Mpp6	MAGUK p55 subfamilymember 6	66.5	+	-
Q99NA9	Pcgf6	Polycomb group RING finger protein 6	81.7	+	-
P27773	Pdia3	Proteindisulfide-isomerase A3	133.8	+	-
O55125	Nipsnap1	ProteinNipSnaphomolog 1	98.8	+	-
P61458	Pcbd1	Pterin-4-alpha-carbinolamine dehydratase	133.6	+	-
Q05920	Pc	Pyruvatecarboxylase, mitochondrial	61.1	+	-
Q3THS6	Mat2a	S-adenosylmethionine synthase isoform type-2	81.6	+	-
Q07417	Acads	Short-chain specific acyl-CoA dehydrogenase, mitochondrial	83.5	+	-
Q9D9R3	Spata9	Spermatogenesis-associatedprotein 9	120.2	+	-
Q8VE22	Mrps23	28S ribosomalprotein S23, mitochondrial	96.5	-	+
Q78JT3	Haao	3-hydroxyanthranilate 3,4-dioxygenase	172.7	-	+
P49429	Hpd	4-hydroxyphenylpyruvate dioxygenase	129.3	-	+
P50247	Ahcy	Adenosylhomocysteinase	137.7	-	+
P00329	Adh1	Alcoholdehydrogenase 1	202.1	-	+
O35945	Aldh1a7	Aldehydedehydrogenase, cytosolic 1	113.9	-	+
Q00623	Apoa1	Apolipoprotein A-I	95.3	-	+
P05201	Got1	Aspartateaminotransferase, cytoplasmic	110.3	-	+
Q8CHT0	Aldh4a1	Delta-1-pyrroline-5-carboxylate dehydrogenase, mitochondrial	93.9	-	+
P10518	Alad	Delta-aminolevulinicaciddehydratase	274.5	-	+
P19639	Gstm3	Glutathione S-transferase Mu 3	220.8	-	+
P07901	Hsp90aa1	Heat shock protein HSP 90-alpha	109.4	-	+
P11499	Hsp90ab1	Heat shock protein HSP 90-beta	226.1	-	+
P70696	Hist1h2ba	Histone H2B type 1-A	101.1	-	+
Q61425	Hadh	Hydroxyacyl-coenzyme A dehydrogenase, mitochondrial	86.2	-	+

P05784	Krt18	Keratin, type I cytoskeletal 18	137.8	-	+
P06151	Ldha	L-lactatedehydrogenase A chain	168.9	-	+
Q9DAT2	Mrgbp	MRG/MORF4L-binding protein	163.6	-	+
Q80VA0	Galnt7	N-acetylgalactosaminyltransferase 7	84.2	-	+
Q9CR61	Ndufb7	NADH dehydrogenase [ubiquinone] 1 beta subcomplexsubunit 7	125.5	-	+
P17742	Ppia	Peptidyl-prolylcis-transisomerase A	258.1	-	+
P70296	Pebp1	Phosphatidylethanolamine-bindingprotein 1	273.3	-	+
P58659	Eva1c	Protein eva-1 homolog C	92.8	-	+
O70622	Rtn2	Reticulon-2	109.8	-	+
B2RY56	Rbm25	RNA-bindingprotein 25	93.0	-	+
Q8BG73	Sh3bgrl2	SH3 domain-binding glutamic acid-rich-like protein 2	90.5	-	+
P59096	Stard6	StAR-related lipid transfer protein 6	135.8	-	+
Q8CHV6	Tada2a	Transcriptionaladapter 2-alpha	85.2	-	+
Q8BMS1	Hadha	Trifunctional enzyme subunit alpha, mitochondrial	107	-	+
Q9CWR1	Wdr73	WD repeat-containingprotein 73	93.7	-	+

The identified proteins are organized according to highest to lowest fold change. + sign indicates presence of the protein, while – sign indicates absence of the protein in the respective groups. [a]Identification is based on protein ID from UniProt protein database (http://www.uniprot.org/)

4 DISCUSSION

4 DISCUSSION

Although there is a significant number of studies addressing F toxicity in various organs (LIMA LEITE et al., 2014; CARVALHO et al., 2013; BUZALAF et al., 2004, 2005; KOBAYASHI et al., 2009), only a few studies focus on liver (PEREIRA et al., 2013; LOBO et al., 2015). Liver is not only the largest gland in the body (ZANNETTI et al., 2016; SUSNEA; WEISKIRCHEN, 2016) but it is also a “hub” where most of the toxicants are bio-transformed into metabolites making it prone to damage (MUKHOPADHYAY; CHATTOPADHYAY, 2014). Hepatic dysfunction is one of the serious drawbacks of excessive drugs and chemical exposure (SUN et al., 2014; WANG et al., 2014a; WANG et al., 2014b). Coming to F intake, the degree of liver damage is related to the quantity of F ingested (XIONG et al., 2007; CAO et al., 2013; ZLATKOVIC et al., 2014). However, the mechanism of F-induced liver dysfunction still deserves investigation. The present study reflects a comprehensive scenario where we have tried our best to address and identify the proteins potentially involved in hepatic F metabolism that are either exclusively or differentially expressed in the two mice strains (A/J & 129P3/J) having quite contrasting behaviors (CARVALHO et al., 2009; CHARONE et al., 2016; KOBAYASHI et al., 2014). Over the last few years, these two strains have been studied widely as they respond quite differently when submitted to F (CARVALHO et al., 2009, 2013; EVERETT et al., 2002; KOBAYASHI et al., 2014; CHARONE et al., 2016). A/J and 129P3/J are reported to have genomic background which is considered as an important factor responsible for the differential handling of F by them (YAN et al., 2007, 2011) moreover, even without having an exposure to F, A/J mice present a higher retention of proteins in the maturing enamel (EVERETT et al., 2009).

In order to explore this distinct response and the mechanisms responsible for this diverse pattern in liver, the first part of our study was confined strictly to the control groups (0 ppm F), and we came up with the striking finding that even following low-F diet, and without having the administration of F through the drinking water,129P3/J mice had significantly higher liver F concentrations, and it is possibly because of the residual amounts of F present in their diets and is in-line with the metabolic characteristics of this strain regarding F metabolism (greater retention of F by 129P3/J

mice), as supported by previous studies (CARVALHO et al., 2009, 2013; CHARONE et al., 2016; EVERETT et al., 2002) whereas in the F treated groups (15 and 50 ppm) a dose-response pattern was found in the liver F concentrations confirming the efficacy of the treatment followed. Although 129P3/J mice had higher liver F concentrations when compared with A/J counterparts, this difference was only significant for 50 ppm F dose. This could be explained by the incorporation of part of F into the mineralized tissues, while higher doses of F saturate the effect of its uptake into mineralized tissues (EKSTRAND; ERICSSON; ROSELL, 1977).

To dig it further down, proteomic approach of the liver was employed. The highest difference was noted in the control group. A total of 119 proteins were identified in general, while 53 were exclusive for A/J and 15 were exclusive for 129P3/J respectively. In addition, 42 of the identified proteins had an upregulated expression in A/J mice while 9 of the proteins, expressed a down regulated pattern compared with their 129P3/J counterparts. Furthermore, most of the proteins with fold change were increased in A/J mice, ranging between 1 and 2. One of the enzymes, Formimidoyl transferase-cyclodeaminase was found to have the highest fold change as 3.82. An important function of this enzyme is to breakdown formimino tetra-hydrofolate, which is thus involved in the synthesis of purines and pyrimidines, and amino acids (*UNIPROT*). The indirect increase in the supply of nucleotides and amino acids, thus suggesting the increased expression of other liver proteins noted in A/J mice.

Another enzyme, Glutamate dehydrogenase 1, mitochondrial (GDH) was found to have the second highest fold change value as 1.84. GDH, is a metabolic enzyme, that catalyzes the reversible reaction of *L-glutamate to α-ketoglutarate* (α-KG), with the concomitant reduction of NAD(P)+ to NAD(P)H or vice versa (WANG et al., 2014a; WANG et al., 2014b; WARBURG; CHISTIAN, 1941). Increased expression of GDH was also observed in various brain pathologies including Parkinson's disease, schizophrenia and brain cancer (PLAITAKIS et al., 2010; BAO et al., 2009; YANG et al., 2009; BURBAEVA et al., 2003). The increased GDH activity is found to increase the production of ROS through glutamate oxidation (WHITFORD, 1996) which thus adds more to enrich the oxidative stress, as already faced by the hepatocytes, since F is equally known to cause oxidative stress (LUO et al., 2017).

The functional category represented the highest percentage (20%) of the altered genes was oxidation-reduction process. In A/J mice, some of the identified proteins had significant increase in their expression including Heat shock cognate 71 kDa protein, Electron transfer flavo-protein subunit beta, Aalpha-enolase, Beta-enolase, Gamma-enolase, ATP synthase subunit alpha, mitochondrial, Malate dehydrogenase, indicating an increased energy flux which thus might be suggestive of the observed oxidative stress and contributed to liver damage in the respective strain. In summary, A/J mice had increased energy flux and have an intrinsic tendency to oxidative stress even without exposure to F, so coming to F exposure, the oxidative stress will worse out (PEREIRA et al., 2013) leaving animals in challenging environment.

The second part of our study was focused on the effect of exposure to F (15 and 50 ppm) on the liver proteome of animals with different genetic backgrounds(A/J and 129P3/J). It is worth mentioning that the F concentrations (15 and 50 ppm) added to the drinking water in the present study were chosen to mimic the concentrations of 3 and 10 mg/L respectively in the drinking water ingested by humans (DUNIPACE et al., 1995).These doses correspond to the fluoride concentration that is naturally found in endemic regions of dental and skeletal fluorosis, respectively .

In the 15 ppm F treated group, a total of 81 proteins were identified in general while 37 were exclusive for A/J and 22 were exclusive for 129P3/J, respectively. In addition, 22 of the identified proteins had an upregulated expression in A/J mice compared to their 129P3/J counterparts. One of the striking finding in the present study was that the lower F concentration (15 ppm) reflected more fold changes in liver proteome as compared to the 50 ppm F treated group which is pretty much in accordance with previous studies conducted in liver and kidney (IANO et al., 2014; PEREIRA et al., 2013, 2016). A possible reason is inclined to the adaptive mechanism of the organisms to F (DABROWSKA; LETKO; BALUNOWSKA, 2006) that was triggered by higher doses, but not by lower ones (PEREIRA et al., 2016). It is quite probable if the treatment with 50 ppm F was conducted for a shorter time, it could have led to more sharp alterations, as those seen in the 15 ppm F group. One of the recently published work also reported the same findings where treatment with low F dose turned out to be more harmful in the duodenum of rats (MELO et al., 2017).

Furthermore, two of the identified proteins, Delta-aminolevulinic acid dehydratase (δ-ALA-D) and 3-ketoacyl-CoA thiolase A, peroxisomal have the highest fold changes as 5.58 and 4.18. δ-ALA-D is an enzyme required for" heme" biosynthesis (SASSA, 1982) and also reportedly sensitive to oxidizing agents (BOTTARI et al., 2016). It also has an indirect role in the assembling of tetrapyrole molecules that constitute the prosthetic groups of enzymes, such as catalase (CAT) (SASSA, 1982) whereas catalase is an important member of antioxidant system and found sensitive to F (SHANTAKUMARI; SRINIVASALU; SUBRAMANIAN, 2004). Conclusively, the significant increase noted in the expression of δ-ALA-D in our study might be an attempt to increase the activity of CAT, which is expected to be decreased upon treatment with F (IANO et al., 2014) whereas in control groups (0 ppm F), this enzyme was found identified only in the liver of A/J animals but not in their 129P3/J counterparts (KHAN et al., 2016) which is suggestive of a probable fight against the oxidative stress in the former. Concisely, in the15 ppm F treated group, all proteins with fold change showed an increase in A/J mice compared to their 129P3/J counterparts along with a significant increase in δ-ALA-D thus supporting the same hypothetical view we had in control groups.

The increase of enzyme ketoacyl-CoA thiolase A, peroxisomal was lastly reported in kidneys and liver upon having an exposure to F. It is involved in fatty acids beta oxidation (KOBAYASHI et al., 2009; PEREIRA et al., 2013; XU et al., 2005). The observed fold change, thus signifying a vigorous state of fatty acids metabolism, alternatively an attempt to withstand the inhibitory effect of F in the glycolytic pathway (WARBURG; CHISTIAN, 1941). Remarkably, most of the proteins upregulated in A/J mice including Beta-enolase, L-lactate dehydrogenase as well as the subunits of ATP synthase, are involved in the oxidative phosphorylation thus indicating a state of an increased energy flux in the A/J mice strain and adding more to the oxidative stress as reflected by the altered expressions of SOD and ALAD enzymes.

Coming to the 50 ppm F treated group, a total of 101 proteins were identified in general while 35 of them were exclusive for A/J mice and 30 were exclusive for 129P3/J mice. In addition, 13 of the identified proteins had an upregulated expression in A/J mice while 23 of the proteins expressed a down regulated pattern compared to their 129P3/J counterparts. Precisely, A/J mice in 50 ppm F treated group showed a pattern contrary to what was seen in the 15 ppm F group. A significant number of

proteins were decreased in A/J mice compared to their 129P3/J counterparts. Few of them are worth mentioning here including the heat-shock protein (Stress-70 protein, mitochondrial*)* and two isoforms of Glutathione S-transferase (GST).

Heat-shock proteins are frequently reported to be involved in proper protein folding (FAN et al., 2017; SOTTILE; NADIN, 2017; LIY et al., 2017) which is a quite important for their proper functioning (CYMER; VON HEIJNE; WHITE, 2015; O´BRIEN et al., 2014). The down regulation of heat-shock proteins depicting a disturbance in the protein folding which thus surely would affect their regular functioning, consequently it would cause a reduced tolerance to F-induced stress in the A/J mice (CIEPLAK, 2017; LIMA LEITE et al., 2014; PEREIRA et al., 2013). GSTs isozymes are responsible for the detoxification of electrophiles (HAYES; PULFORD, 1995) and well documented to play a crucial role in cellular detoxification of endogenous and xenobiotic substrates and protection against oxidative stress (SCHROER et al., 2011). The observed downregulation of GSTs in A/J mice might be an attempt to fight the oxidative stress in 50 ppm F treated group.

Furthermore, many of the isoforms of histones such as: H2B type 1-P, H2B type 1-M, H2B type 2-B, H2B type 2-E, H2B type 3-A*,* were downregulated in the A/J mice. Histones are functionally involved in the assembly of nucleosomes and any disturbance in their pattern leads to disorganization in genomic DNA, which might impair gene transcription (CHEN et al., 2014). This fact is somehow suggestive of the decreased expression of most of the proteins in the 50 ppm F treated group. Although most of the proteins in A/J mice had a sharp decrease in their expression in 50 ppm F treated group but one of the upregulated enzymes, Retinal dehydrogenase 1 (ALDH1A1) is worth noticing here, as it has been frequently reported to have an antioxidant activity (PAPPA et al., 2003; LASSEN et al., 2006) whereas in our study its upregulated expression might be credited to the F dose employed.

Apart from all these F-oriented alterations, couple of the identified proteins were found upregulated solely in A/J mice irrespective of the F concentration in drinking water. One of them is Glutamate dehydrogenase 1, mitochondrial (GLUD1), which is frequently reported as one of the sensitive biomarkers of hepatotoxicity and upregulation in its pattern highlights the occurrence of mitochondria dysfunction (STANLEY, 2009; MCGILL et al., 2012). Serum Albumin is another candidate protein,

which manifested F-independent upregulated pattern in A/J mice. It is mainly involved in the regulation of the colloidal osmotic pressure of blood (UNIPROT). Its upregulation is somehow suggestive of the high ingestion of water by A/J mice (CARVALHO et al., 2009).

The proteins found in the center of the network (Figure 2 in Article II) are Calcium-activated potassium channel subunit alpha-1 (Kcnma1) and Disks large homolog 4 (Dlg4). Kcnma 1, is mainly driven by membrane depolarization or increase in cytosolic Ca^{2+} ions, which controls flow of K^{+}. It is also sensitive to the concentration of cytosolic Mg^{2+} ions (UNIPROT). As reported, once activated, they play an important role in various systems including regulation of the contraction of smooth muscle (SANCHEZ-PASTOR et al., 2014), the tuning of hair cells in the cochlea including regulation of transmitter release (CABO et al., 2013).

The other protein found in the center of the network is Disks large homolog 4 (Dlg4). Dlg4 (disks large homolog 4, also known as postsynaptic density protein 95) is a member of the membrane-associated guanylate kinase family (KIM et al., 2013). Moreover, it also interacts with potassium channels and was found to be involved in NMDA (N-methyl-D-aspartate) receptor signaling (HALFF et al., 2014). Being a player of nervous system, the presence of Dlg4 in the center of network was a bit unusual. However, the reported fact cannot be neglected here, since liver failure-damage causes the accumulation of ammonia, leading to neurological and psychiatric alterations; referred as hepatic encephalopathy (HE), which thus affects the cerebral function (FELIPO, 2013). Furthermore, oxidative stress is one of the important mechanisms underlying the pathogenesis of ischemic stroke (FANG et al., 2017). Since the same proteins were found residing in the center of networks irrespective of F exposure (KHAN et al., 2016), the fact strongly suggests that it is something related to intrinsic behavior of the strains where the pattern of the networks is mainly driven by them. This finding is leaving a space in our conclusive remarks, which needs to be investigated in way that is more appropriate and for that, much-streamlined experiments are required. A brief hypothetical cascade thus justifying our findings goes in way, where A/J mice had increased expression of protein related to energy flux, which in turn might have played a role to further enrich the oxidative stress, thus contributing to liver damage (KHAN et al., 2016).

5 CONCLUDING REMARKS

5 CONCLUDING REMARKS

A/J mice had an increase in proteins related to energy flux and oxidative stress as observed in the first study where no F was administered. This could be one of the possible explanations for the high susceptibility of A/J mice to the effects of F, since exposure to F is also known to induce oxidative stress. Treatment with lower F concentration (15 ppm) provoked more pronounced alterations in fold change in liver proteins in comparison to the treatment with the higher F concentration (50 ppm). Strikingly, most of the proteins with fold change upon following 15 ppm F treatment, were increased in the A/J mice compared with their 129P3/J counterparts, thus depicting attempt of the former to fight against the toxic effects of F. With respect to 50 ppm F, most proteins with fold change were decreased in the A/J mice compared with their 129P3/J counterparts, especially proteins related to oxidative stress and protein folding, which might be related to the higher susceptibility of the A/J animals to the deleterious effects of F.

Our findings can contribute to interpret the molecular mechanisms underlying genetic susceptibility to fluorosis by indicating key protein players which need to be better addressed in future experimental studies.

REFERENCES

REFERENCES

Agalakova NI, Gusev GP. Fluoride induces oxidative stress and ATP depletion in the rat erythrocytes in vitro. Environ Toxicol Pharmacol. 2012;34(2):334-7.

Amini M, Mueller K, Abbaspour KC, Rosenberg T, Afyuni M, Moller KN, et al. Statistical modeling of global geogenic fluoride contamination in groundwaters. Environ Sci Technol. 2008;42(10):3662-8.

Anuradha CD, Kanno S, Hirano S. Oxidative damage to mitochondria is a preliminary step to caspase-3 activation in fluoride-induced apoptosis in HL-60 cells. Free Radic Biol Med. 2001;31(3):367-73.

Arguelles S, Garcia S, Maldonado M, Machado A, Ayala A. Do the serum oxidative stress biomarkers provide a reasonable index of the general oxidative stress status? Biochim Biophys Acta. 2004;1674(3):251-9.

Atmaca N, Atmaca HT, Kanici A, Anteplioglu T. Protective effect of resveratrol on sodium fluoride-induced oxidative stress, hepatotoxicity and neurotoxicity in rats. Food Chem Toxicol. 2014;70:191-7.

Bao X, Pal R, Hascup KN, Wang Y, Wang WT, Xu W, et al. Transgenic expression of Glud1 (glutamate dehydrogenase 1) in neurons: in vivo model of enhanced glutamate release, altered synaptic plasticity, and selective neuronal vulnerability. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2009;29(44):13929-44.

Barbier O, Arreola-Mendoza L, Del Razo LM. Molecular mechanisms of fluoride toxicity. Chem Biol Interact. 2010;188(2):319-33.

Baykov AA, Alexandrov AP, Smirnova IN. A two-step mechanism of fluoride inhibition of rat liver inorganic pyrophosphatase. Arch Biochem Biophys. 1992;294(1):238-43.

Bottari NB, Mendes RE, Baldissera MD, Bochi GV, Moresco RN, Leal ML, et al. Relation between iron metabolism and antioxidants enzymes and delta-ALA-D activity in rats experimentally infected by Fasciola hepatica. Experimental parasitology. 2016;165:58-63.

Burbaeva G, Boksha IS, Turishcheva MS, Vorobyeva EA, Savushkina OK, Tereshkina EB. Glutamine synthetase and glutamate dehydrogenase in the prefrontal cortex of

patients with schizophrenia. Progress in neuro-psychopharmacology & biological psychiatry. 2003;27(4):675-80.

Buzalaf MA, Caroselli EE, Cardoso de Oliveira R, Granjeiro JM, Whitford GM. Nail and bone surface as biomarkers for acute fluoride exposure in rats. Journal of analytical toxicology. 2004;28(4):249-52.

Buzalaf MA, Caroselli EE, de Carvalho JG, de Oliveira RC, da Silva Cardoso VE, Whitford GM. Bone surface and whole bone as biomarkers for acute fluoride exposure. Journal of analytical toxicology. 2005;29(8):810-3.

Cabo R, Zichichi R, Vina E, Guerrera MC, Vazquez G, Garcia-Suarez O, et al. Calcium-activated potassium channel SK1 is widely expressed in the peripheral nervous system and sensory organs of adult zebrafish. Neurosci Lett. 2013;555:62-7.

Cao J, Chen J, Wang J, Jia R, Xue W, Luo Y, et al. Effects of fluoride on liver apoptosis and Bcl-2, Bax protein expression in freshwater teleost, Cyprinus carpio. Chemosphere. 2013;91(8):1203-12.

Carvalho JG, Leite Ade L, Peres-Buzalaf C, Salvato F, Labate CA, Everett ET, et al. Renal proteome in mice with different susceptibilities to fluorosis. PLoS One. 2013;8(1):e53261.

Carvalho JG, Leite AL, Yan D, Everett ET, Whitford GM, Buzalaf MA. Influence of genetic background on fluoride metabolism in mice. J Dent Res. 2009;88(11):1054-8.

Charone S, De Lima Leite A, Peres-Buzalaf C, Silva Fernandes M, Ferreira de Almeida L, Zardin Graeff MS, et al. Proteomics of Secretory-Stage and Maturation-Stage Enamel of Genetically Distinct Mice. Caries Res. 2016;50(1):24-31.

Chen R, Kang R, Fan XG, Tang D. Release and activity of histone in diseases. Cell death & disease. 2014;5:e1370.

Christen S, Thomas SR, Garner B, Stocker R. Inhibition by interferon-gamma of human mononuclear cell-mediated low density lipoprotein oxidation. Participation of tryptophan metabolism along the kynurenine pathway. J Clin Invest. 1994;93(5):2149-58.

Cieplak AS. Protein folding, misfolding and aggregation:The importance of two-electron stabilizing interactions. PLoS One. 2017;12(9):e0180905.

Cimasoni G. The inhibition of enolase by fluoride in vitro. Caries Res. 1972;6(2):93-102.

Coplan MJ, Patch SC, Masters RD, Bachman MS. Confirmation of and explanations for elevated blood lead and other disorders in children exposed to water disinfection and fluoridation chemicals. Neurotoxicology. 2007;28(5):1032-42.

Curnutte JT, Babior BM, Karnovsky ML. Fluoride-mediated activation of the respiratory burst in human neutrophils. A reversible process. J Clin Invest. 1979;63(4):637-47.

Cymer F, von Heijne G, White SH. Mechanisms of integral membrane protein insertion and folding. J Mol Biol. 2015;427(5):999-1022.

Dabrowska E, Letko R, Balunowska M. Effect of sodium fluoride on the morphological picture of the rat liver exposed to NaF in drinking water. Advances in medical sciences. 2006;51 Suppl 1:91-5.

Dang H, Mailig M, Lalic G. Mild copper-catalyzed fluorination of alkyl triflates with potassium fluoride. Angew Chem Int Ed Engl. 2014;53(25):6473-6.

Dogan D, Can C, Kocyigit A, Dikilitas M, Taskin A, Bilinc H. Dimethoate-induced oxidative stress and DNA damage in Oncorhynchus mykiss. Chemosphere. 2011;84(1):39-46.

Dubey N, Khan AM, Raina R. Sub-acute deltamethrin and fluoride toxicity induced hepatic oxidative stress and biochemical alterations in rats. Bull Environ Contam Toxicol. 2013;91(3):334-8.

Dunipace AJ, Brizendine EJ, Zhang W, Wilson ME, Miller LL, Katz BP, et al. Effect of aging on animal response to chronic fluoride exposure. J Dent Res. 1995;74(1):358-68.

Ekstrand J, Ericsson Y, Rosell S. Absence of protein-bound fluoride from human and blood plasma. Archives of oral biology. 1977;22(4):229-32.

Everett ET, McHenry MA, Reynolds N, Eggertsson H, Sullivan J, Kantmann C, et al. Dental fluorosis: variability among different inbred mouse strains. J Dent Res. 2002;81(11):794-8.

Everett ET, Yan D, Weaver M, Liu L, Foroud T, Martinez-Mier EA. Detection of dental fluorosis-associated quantitative trait Loci on mouse chromosomes 2 and 11. Cells Tissues Organs. 2009;189(1-4):212-8.

Fan W, Fan SS, Feng J, Xiao D, Fan S, Luo J. Elevated expression of HSP10 protein inhibits apoptosis and associates with poor prognosis of astrocytoma. PLoS One. 2017;12(10):e0185563.

Fang Y, Chu L, Li L, Wang J, Yang Y, Gu J, et al. Tetramethylpyrazine protects bone marrow-derived mesenchymal stem cells against hydrogen peroxide-induced apoptosis through PI3K/Akt and ERK1/2 pathways. Biol Pharm Bull. 2017.

Felipo V. Hepatic encephalopathy: effects of liver failure on brain function. Nat Rev Neurosci. 2013;14(12):851-8.

Ferreira D, da Motta AC, Kreutz LC, Toni C, Loro VL, Barcellos LJ. Assessment of oxidative stress in Rhamdia quelen exposed to agrichemicals. Chemosphere. 2010;79(9):914-21.

Gu X, Manautou JE. Molecular mechanisms underlying chemical liver injury. Expert Rev Mol Med. 2012;14:e4.

Halff AW, Gomez-Varela D, John D, Berg DK. A novel mechanism for nicotinic potentiation of glutamatergic synapses. J Neurosci. 2014;34(6):2051-64.

Hassan HA, Yousef MI. Mitigating effects of antioxidant properties of black berry juice on sodium fluoride induced hepatotoxicity and oxidative stress in rats. Food Chem Toxicol. 2009;47(9):2332-7.

Hayes JD, Pulford DJ. The glutathione S-transferase supergene family: regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. Critical reviews in biochemistry and molecular biology. 1995;30(6):445-600.

Iano FG, Ferreira MC, Quaggio GB, Mileni Silva Fernandes MS, Oliveira RC, Ximenes VF, et al. Effects of chronic fluoride intake on the antioxidant systems of the liver and kidney in rats. Journal of Fluorine Chemistry. 2014;168:212–7.

Inkielewicz-Stepniak I, Czarnowski W. Oxidative stress parameters in rats exposed to fluoride and caffeine. Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association. 2010;48(6):1607-11.

Jenkins GN, Venkateswarlu P, Zipkin I. Physiological effects of small doses of fluoride. Monogr Ser World Health Organ. 1970;59:163-223.

Khan ZN, Leite AL, Charone S, Sabino IT, Martini T, Pereira HABS, et al. Liver proteome of mice with different genetic susceptibilities to the effects of fluoride. Journal of Applied Oral Science. 2016(24):250-7.

Kim GH, Park EC, Yun SH, Hong Y, Lee DG, Shin EY, et al. Proteomic and bioinformatic analysis of membrane proteome in type 2 diabetic mouse liver. Proteomics. 2013;13(7):1164-79.

Kobayashi CA, Leite AL, Peres-Buzalaf C, Carvalho JG, Whitford GM, Everett ET, et al. Bone response to fluoride exposure is influenced by genetics. PLoS One. 2014;9(12):e114343.

Kobayashi CA, Leite AL, Silva TL, Santos LD, Nogueira FC, Oliveira RC, et al. Proteomic analysis of kidney in rats chronically exposed to fluoride. Chem Biol Interact. 2009;180(2):305-11.

Kryston TB, Georgiev AB, Pissis P, Georgakilas AG. Role of oxidative stress and DNA damage in human carcinogenesis. Mutat Res. 2011;711(1-2):193-201.

Kubrak OI, Husak VV, Rovenko BM, Storey JM, Storey KB, Lushchak VI. Cobalt-induced oxidative stress in brain, liver and kidney of goldfish Carassius auratus. Chemosphere. 2011;85(6):983-9.

Lassen N, Pappa A, Black WJ, Jester JV, Day BJ, Min E, et al. Antioxidant function of corneal ALDH3A1 in cultured stromal fibroblasts. Free radical biology & medicine. 2006;41(9):1459-69.

Leone NC, Shimkin MB, Arnold FA, Jr., Stevenson CA, Zimmermann ER, Geiser PB, et al. Medical aspects of excessive fluoride in a water supply. Public Health Rep. 1954;69(10):925-36.

Lima Leite A, Gualiume Vaz Madureira Lobo J, Barbosa da Silva Pereira HA, Silva Fernandes M, Martini T, Zucki F, et al. Proteomic analysis of gastrocnemius muscle in rats with streptozotocin-induced diabetes and chronically exposed to fluoride. PLoS One. 2014;9(9):e106646.

Liu C, Li M, Cao Y, Qu JP, Zhang ZW, Xu SW, et al. Effects of avermectin on immune function and oxidative stress in the pigeon spleen. Chem Biol Interact. 2014;210:43-50.

Loganayaki N, Siddhuraju P, Manian S. Antioxidant activity and free radical scavenging capacity of phenolic extracts from Helicteres isora L. and Ceiba pentandra L. J Food Sci Technol. 2013;50(4):687-95.

Lu Y, Luo Q, Cui H, Deng H, Kuang P, Liu H, et al. Sodium fluoride causes oxidative stress and apoptosis in the mouse liver. Aging (Albany NY). 2017;9(6):1623-39.

Luo Q, Cui H, Deng H, Kuang P, Liu H, Lu Y, et al. Histopathological findings of renal tissue induced by oxidative stress due to different concentrations of fluoride. Oncotarget. 2017;8(31):50430-46.

Malik UU, Siddiqui IA, Hashim Z, Zarina S. Measurement of serum paraoxonase activity and MDA concentrations in patients suffering with oral squamous cell carcinoma. Clin Chim Acta. 2014;430:38-42.

Mandinic Z, Curcic M, Antonijevic B, Carevic M, Mandic J, Djukic-Cosic D, et al. Fluoride in drinking water and dental fluorosis. Sci Total Environ. 2010;408(17):3507-12.

Maurer JK, Cheng MC, Boysen BG, Anderson RL. Two-year carcinogenicity study of sodium fluoride in rats. J Natl Cancer Inst. 1990;82(13):1118-26.

McGill MR, Sharpe MR, Williams CD, Taha M, Curry SC, Jaeschke H. The mechanism underlying acetaminophen-induced hepatotoxicity in humans and mice involves mitochondrial damage and nuclear DNA fragmentation. The Journal of clinical investigation. 2012;122(4):1574-83.

Melo CGS, Perles J, Zanoni JN, Souza SRG, Santos EX, Leite AL, et al. Enteric innervation combined with proteomics for the evaluation of the effects of chronic fluoride exposure on the duodenum of rats. Scientific reports. 2017;7(1):1070.

Mittal M, Flora SJ. Effects of individual and combined exposure to sodium arsenite and sodium fluoride on tissue oxidative stress, arsenic and fluoride levels in male mice. Chem Biol Interact. 2006;162(2):128-39.

Mousny M, Banse X, Wise L, Everett ET, Hancock R, Vieth R, et al. The genetic influence on bone susceptibility to fluoride. Bone. 2006;39(6):1283-9.

Mousny M, Omelon S, Wise L, Everett ET, Dumitriu M, Holmyard DP, et al. Fluoride effects on bone formation and mineralization are influenced by genetics. Bone. 2008;43(6):1067-74.

Mukhopadhyay D, Chattopadhyay A. Induction of oxidative stress and related transcriptional effects of sodium fluoride in female zebrafish liver. Bulletin of environmental contamination and toxicology. 2014;93(1):64-70.

Mullenix PJ, Denbesten PK, Schunior A, Kernan WJ. Neurotoxicity of sodium fluoride in rats. Neurotoxicol Teratol. 1995;17(2):169-77.

O'Brien EP, Ciryam P, Vendruscolo M, Dobson CM. Understanding the influence of codon translation rates on cotranslational protein folding. Acc Chem Res. 2014;47(5):1536-44.

Paizis G, Tikellis C, Cooper ME, Schembri JM, Lew RA, Smith AI, et al. Chronic liver injury in rats and humans upregulates the novel enzyme angiotensin converting enzyme 2. Gut. 2005;54(12):1790-6.

Pappa A, Chen C, Koutalos Y, Townsend AJ, Vasiliou V. Aldh3a1 protects human corneal epithelial cells from ultraviolet- and 4-hydroxy-2-nonenal-induced oxidative damage. Free radical biology & medicine. 2003;34(9):1178-89.

Peckham S, Awofeso N. Water fluoridation: a critical review of the physiological effects of ingested fluoride as a public health intervention. ScientificWorldJournal. 2014;2014:293019.

Pereira HA, Dionizio AS, Fernandes MS, Araujo TT, Cestari TM, Buzalaf CP, et al. Fluoride Intensifies Hypercaloric Diet-Induced ER Oxidative Stress and Alters Lipid Metabolism. Plos One. 2016;11(6):e0158121.

Pereira HA, Leite Ade L, Charone S, Lobo JG, Cestari TM, Peres-Buzalaf C, et al. Proteomic analysis of liver in rats chronically exposed to fluoride. Plos One. 2013;8(9):e75343.

Perumal E, Paul V, Govindarajan V, Panneerselvam L. A brief review on experimental fluorosis. Toxicol Lett. 2013;223(2):236-51.

Plaitakis A, Latsoudis H, Kanavouras K, Ritz B, Bronstein JM, Skoula I, et al. Gain-of-function variant in GLUD2 glutamate dehydrogenase modifies Parkinson's disease onset. European journal of human genetics : EJHG. 2010;18(3):336-41.

Przybyszewski WM, Kasperczyk J, Stoklosa K, Bkhiyan A. [DNA damage induced by products of lipid peroxidation]. Postepy Hig Med Dosw (Online). 2005;59:75-81.

Refsnes M, Schwarze PE, Holme JA, Lag M. Fluoride-induced apoptosis in human epithelial lung cells (A549 cells): role of different G protein-linked signal systems. Hum Exp Toxicol. 2003;22(3):111-23.

Refsnes M, Thrane EV, Lag M, Thoresen GH, Schwarze PE. Mechanisms in fluoride-induced interleukin-8 synthesis in human lung epithelial cells. Toxicology. 2001;167(2):145-58.

Rohr J, Kittner S, Feeser B, Hebel JR, Whyte MG, Weinstein A, et al. Traditional risk factors and ischemic stroke in young adults: the Baltimore-Washington Cooperative Young Stroke Study. Arch Neurol. 1996;53(7):603-7.

Samanta A, Chanda S, Bandyopadhyay B, Das N. Establishment of drug delivery system nanocapsulated with an antioxidant (+)-catechin hydrate and sodium meta borate chelator against sodium fluoride induced oxidative stress in rats. J Trace Elem Med Biol. 2016;33:54-67.

Sana S, Ghosh S, Das N, Sarkar S, Mandal AK. Vesicular melatonin efficiently downregulates sodium fluoride-induced rat hepato- and broncho-TNF-alpha, TGF-beta expressions, and associated oxidative injury: a comparative study of liposomal and nanoencapsulated forms. Int J Nanomedicine. 2017;12:4059-71.

Sanchez-Pastor E, Andrade F, Sanchez-Pastor JM, Elizalde A, Huerta M, Virgen-Ortiz A, et al. Cannabinoid receptor type 1 activation by arachidonylcyclopropylamide in rat aortic rings causes vasorelaxation involving calcium-activated potassium channel subunit alpha-1 and calcium channel, voltage-dependent, L type, alpha 1C subunit. Eur J Pharmacol. 2014;729:100-6.

Sarkar C, Pal S, Das N, Dinda B. Ameliorative effects of oleanolic acid on fluoride induced metabolic and oxidative dysfunctions in rat brain: Experimental and biochemical studies. Food Chem Toxicol. 2014;66:224-36.

Sassa S. Delta-aminolevulinic acid dehydratase assay. Enzyme. 1982;28(2-3):133-45.

Schroer KT, Gibson AM, Sivaprasad U, Bass SA, Ericksen MB, Wills-Karp M, et al. Downregulation of glutathione S-transferase pi in asthma contributes to enhanced oxidative stress. The Journal of allergy and clinical immunology. 2011;128(3):539-48.

Shanthakumari D, Srinivasalu S, Subramanian S. Effect of fluoride intoxication on lipidperoxidation and antioxidant status in experimental rats. Toxicology. 2004;204(2-3):219-28.

Sottile ML, Nadin SB. Heat shock proteins and DNA repair mechanisms: an updated overview. Cell Stress Chaperones. 2017.

Stanley CA. Regulation of glutamate metabolism and insulin secretion by glutamate dehydrogenase in hypoglycemic children. The American journal of clinical nutrition. 2009;90(3):862S-6S.

Sun K, Eriksson SE, Tan Y, Zhang L, Arner ES, Zhang J. Serum thioredoxin reductase levels increase in response to chemically induced acute liver injury. Biochimica et biophysica acta. 2014;1840(7):2105-11.

Susnea I, Weiskirchen R. Trace metal imaging in diagnostic of hepatic metal disease. Mass Spectrom Rev. 2016;35(6):666-86.

Vogel GL, Schumacher GE, Chow LC, Tenuta LM. Oral fluoride levels 1 h after use of a sodium fluoride rinse: effect of sodium lauryl sulfate. Caries Res. 2015;49(3):291-6.

Wang FH, Yang CF, Lee YH. Deposition of F-doped ZnO transparent thin films using ZnF2-doped ZnO target under different sputtering substrate temperatures. Nanoscale Res Lett. 2014;9(1):97.

Wang X, Jiang Z, Xing M, Fu J, Su Y, Sun L, et al. Interleukin-17 mediates triptolide-induced liver injury in mice. Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association. 2014a;71:33-41.

Wang ZJ, Lee J, Si YX, Wang W, Yang JM, Yin SJ, et al. A folding study of Antarctic krill (Euphausia superba) alkaline phosphatase using denaturants. International journal of biological macromolecules. 2014b;70:266-74.

Warburg O, Chistian W. Isohering und kristallisation des görungs ferments enolase. Biochem Zool. 1941;310:384-421.

Whitford GM. The metabolism and toxicity of fluoride. Monogr Oral Sci. 1996;16 Rev 2:1-153.

Wu W, Xie Z, Xu J, Hong Z, Liu C. [Characteristics of forms of fluorine in soils and influential factors]. Huan Jing Ke Xue. 2002;23(2):104-8.

Xiong X, Liu J, He W, Xia T, He P, Chen X, et al. Dose-effect relationship between drinking water fluoride levels and damage to liver and kidney functions in children. Environmental research. 2007;103(1):112-6.

Xu H, Hu LS, Chang M, Jing L, Zhang XY, Li GS. Proteomic analysis of kidney in fluoride-treated rat. Toxicology letters. 2005;160(1):69-75.

Yan D, Gurumurthy A, Wright M, Pfeiler TW, Loboa EG, Everett ET. Genetic background influences fluoride's effects on osteoclastogenesis. Bone. 2007;41(6):1036-44.

Yan D, Willett TL, Gu XM, Martinez-Mier EA, Sardone L, McShane L, et al. Phenotypic variation of fluoride responses between inbred strains of mice. Cells Tissues Organs. 2011;194(2-4):261-7.

Yang C, Sudderth J, Dang T, Bachoo RM, McDonald JG, DeBerardinis RJ. Glioblastoma cells require glutamate dehydrogenase to survive impairments of glucose metabolism or Akt signaling. Cancer research. 2009;69(20):7986-93.

Yang H, Xing R, Liu S, Yu H, Li P. Rescuing fluoride-induced damages in liver with gamma aminobutyric acid. Biochem Biophys Res Commun. 2017;491(1):19-24.

Zannetti C, Roblot G, Charrier E, Ainouze M, Tout I, Briat F, et al. Characterization of the Inflammasome in Human Kupffer Cells in Response to Synthetic Agonists and Pathogens. J Immunol. 2016;197(1):356-67.

Zargar S, Siddiqi NJ, Al Daihan SK, Wani TA. Protective effects of quercetin on cadmium fluoride induced oxidative stress at different intervals of time in mouse liver. Acta Biochim Pol. 2015;62(2):207-13.

Zhang J, Song J, Zhang J, Chen X, Zhou M, Cheng G, et al. Combined effects of fluoride and cadmium on liver and kidney function in male rats. Biol Trace Elem Res. 2013;155(3):396-402.

Zhang M, Wang A, He W, He P, Xu B, Xia T, et al. Effects of fluoride on the expression of NCAM, oxidative stress, and apoptosis in primary cultured hippocampal neurons. Toxicology. 2007;236(3):208-16.

Zhang S, Jiang C, Liu H, Guan Z, Zeng Q, Zhang C, et al. Fluoride-elicited developmental testicular toxicity in rats: roles of endoplasmic reticulum stress and inflammatory response. Toxicology and applied pharmacology. 2013;271(2):206-15.

Zhou BH, Zhao J, Liu J, Zhang JL, Li J, Wang HW. Fluoride-induced oxidative stress is involved in the morphological damage and dysfunction of liver in female mice. Chemosphere. 2015;139:504-11.

Zhou Y, Qiu Y, He J, Chen X, Ding Y, Wang Y, et al. The toxicity mechanism of sodium fluoride on fertility in female rats. Food Chem Toxicol. 2013;62:566-72.

Zlatkovic J, Todorovic N, Tomanovic N, Boskovic M, Djordjevic S, Lazarevic-Pasti T, et al. Chronic administration of fluoxetine or clozapine induces oxidative stress in rat liver: a histopathological study. European journal of pharmaceutical sciences : official journal of the European Federation for Pharmaceutical Sciences. 2014;59:20-30.

APPENDIX

APPENDIX A

DECLARATION OF EXCLUSIVE USE OF THE ARTICLE IN DISSERTATION/THESIS

We hereby declare that we are aware of the article (***Liver proteome of mice with different genetic susceptibilities to the effects of fluoride***) will be included in Dissertation/Thesis entitled ***Proteomic analysis of Liver in mice with different susceptibilities to fluorosis,*** of the student (***Zohaib Nisar Khan***) was not used and may not be used in other works of Graduate Programs at the Bauru School of Dentistry, University of São Paulo.

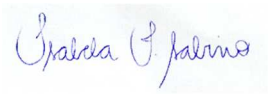
Bauru, 13 de novembro 2017.

Aline LEITE

Author

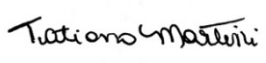
Senda CHARONE

Author



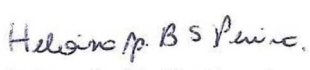
Isabela Tomazini SABINO

Author



Tatiana MARTINI

Author



Heloísa Ap. B. da Silva PEREIRA

Author

Rodrigo Cardoso OLIVEIRA

Author

Marília Afonso Rabelo Buzalaf

Author

APPENDIX B

DECLARATION OF EXCLUSIVE USE OF THE ARTICLE IN DISSERTATION/THESIS

We hereby declare that we are aware of the article (**Liver proteome of mice with different genetic susceptibilities to fluorosis treated with different concentrations of F in the drinking water** – submitted) will be included in Dissertation/Thesis entitled ***Proteomic analysis of Liver in mice with different susceptibilities to fluorosis,*** of the student (***Zohaib Nisar Khan***) was not used and may not be used in other works of Graduate Programs at the Bauru School of Dentistry, University of São Paulo.

Bauru, 13 de novembro 2017.

Carina Guimaraes de Souza Melo

Author

Isabela Tomazini SABINO

Author

Tatiana MARTINI

Author

Heloísa Ap. B. da Silva PEREIRA

Author

Marília Afonso Rabelo Buzalaf

Author

ANNEX

ANNEX 1

Universidade de São Paulo
Faculdade de Odontologia de Bauru

Comissão de Ética no Ensino e Pesquisa em Animais

50 ANOS 1962 - 2012 FOB USP

CEEPA-Proc. Nº 031/2013

Bauru, 20 de agosto de 2013.

Senhora Professora,

O projeto de pesquisa encaminhado a esta Comissão de Ética no Ensino e Pesquisa em Animais, denominado **Efeito dose-resposta do fluoreto em parâmetros relacionados com a resistência à insulina em linhagens de camundongos com diferentes susceptibilidades genéticas à fluorose**, de autoria de Isabela Tomazini Sabino e Janete Gualiume Vaz Madureira Lobo, sob sua orientação, foi enviado a um relator para avaliação e considerado APROVADO COM RECOMENDAÇÃO em reunião desta Comissão, realizada no dia **19 de agosto de 2013**.

Análise e parecer do relator:

Recomendações:

a) *Corrigir a idade prevista dos animais para o experimento. No protocolo consta 30 dias, página 5, e no projeto, 21 dias, nas páginas 25 e 26;*

b) *Especificar o número de animais por grupo experimental na tabela apresentada no "Protocolo para Uso de Animais em Experimentação", página 6 do processo. Sugestão: utilizar a tabela da página 26 do projeto.*

Diante do exposto, solicitamos as providências necessárias e retorno para nova análise desta Comissão.

Atenciosamente,

Prof. Dr. Gustavo Pompermaier Garlet
Presidente da Comissão de Ética no Ensino e Pesquisa em Animais

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

Al. Dr. Octávio Pinheiro Brisolla, 9-75 – Bauru-SP – CEP 17012-101 – C.P. 73
e-mail: mferrari@fob.usp.br – Fone/FAX (0xx14) 3235-8356
http://www.fob.usp.br