MILD CHRONIC NaF INTAKE PROMOTES INSULIN RESISTANCE AND INCREASE IN INFLAMMATORY SIGNALING IN THE WHITE ADIPOSE TISSUE OF RATS

Fernando Yamamoto Chiba,[a] Thaís Verônica Saori Tsosura,[b] Renato Felipe Pereira,[b] Maria Sara de Lima Coutinho Mattera,[b] Rodrigo Martins dos Santos,[b] Fernando Marani,[b] Cléa Adas Saliba Garbin,[a] Suzely Adas Saliba Moimaz,[a] Doris Hissako Sumida[b]

Araçatuba, Brazil

ABSTRACT: Excessive fluoride intake is associated with systemic metabolic alterations similar to those observed in type 2 diabetes such as decreased insulin secretion, impaired glycemic control, and insulin resistance. However, the underlying mechanisms for these changes are not fully understood. This study aimed to evaluate the effect of chronic NaF intake on insulin signaling and inflammatory pathways in the white adipose tissue (WAT) of rats. Seven-week-old castrated male Wistar rats were randomly distributed into 2 groups; a control group, which received 76.4 mg/L NaCl in their drinking water, and a fluoride group, which received 54.9 mg/L NaF in their drinking water and F present in their food pellets (total estimated fluoride intake = 4.0 mg/kg body weight/day). After 42 days, the WAT content of protein kinase B (PKB/Akt), c-Jun N-terminal kinase (JNK), inhibitor of kappa B kinase (IκKα/β), and tumor necrosis factor α (TNF-α); as well as the phosphorylation status of Akt serine, Akt threonine, JNK, and IκKα/β were evaluated by western blotting. The fluoride group showed a decrease in Akt serine phosphorylation status after insulin stimulation, and an increase in TNF-α content and IκKα/β phosphorylation compared to the control group. No alteration was observed in the content of Akt, JNK, and IκKα/β or in the phosphorylation status of JNK. Chronic NaF intake promoted attenuation of insulin signaling and activation of inflammatory signaling in the WAT of rats. These findings highlight the need for careful monitoring of fluoride intake to avoid its deleterious health effects.

Keywords: Diabetes mellitus; Inflammation; Insulin resistance.

INTRODUCTION

Excessive fluoride intake may induce changes in carbohydrate metabolism such as inhibition of glycolysis, reduction of insulin secretion, and hyperglycemia.[1] In this context, children are the focus of this concern since they are subjected to fluoride intake from several sources such as diet; fluoridated water; infant formulas, teas, and candies such as chocolate bars, cookies, morning cereals, and snacks; and, largely, fluoridated toothpastes.[2] Previous studies have suggested that chronic NaF intake could lead to insulin resistance (IR).[3-5] However, the underlying mechanisms involved in this change are not fully understood.

Insulin resistance is related to decreased insulin signaling.[3] Under normal conditions, binding of insulin to its receptor promotes tyrosine phosphorylation of substrates 1 and 2 of the insulin receptor (IRS-1/IRS-2), phosphatidylinositol 3-kinase (PI3K) activation, followed by phosphorylation of serine and threonine residues on protein kinase B (PKB/Akt).[6] Akt performs an important role in the

[a]São Paulo State University (Unesp), School of Dentistry, Araçatuba, Department of Infant and Social Dentistry, Rua José Bonifácio, 1193, zip code: 16015-050 Araçatuba, SP, Brazil; [b]São Paulo State University (Unesp), School of Dentistry, Araçatuba, Department of Basic Sciences, Rua José Bonifácio, 1193, zip code: 16015-050 Araçatuba, SP, Brazil; *For correspondence: Prof. Dr. Fernando Yamamoto Chiba - São Paulo State University (Unesp), School of Dentistry, Araçatuba, Brazil, R. José Bonifácio, 1193, CEP: 16015-050, Araçatuba-SP, Brazil. E-mail: fernandochiba@foa.unesp.br

glucose uptake process by stimulating the translocation of the glucose transporter type 4 (GLUT4) from the intracellular compartment to the plasma membrane.[7]

The adipose tissue is an endocrine organ that can influence glucose metabolism by releasing proinflammatory cytokines, which alter insulin signaling.[8] Chiba et al.[4] demonstrated that chronic treatment of rats with NaF (4.0 mg fluoride/kg body weight (BW)/day) impairs the initial steps of the insulin signaling pathway in the periepididymal white adipose tissue (WAT) by the decrease in the IRS-1/IRS-2 tyrosine phosphorylation status and increase in the IRS-1 serine phosphorylation status after insulin stimulation. Moreover, studies have shown that rats exposed to chronic fluoride treatment have elevated plasma concentrations of proinflammatory cytokines, such as tumor necrosis factor α (TNF-α),[4] which is associated with the development of IR, as it can activate inflammatory proteins, including the inhibitor of kappa B (IκB) kinase complex (IκK) and c-Jun N-terminal kinase (JNK), resulting in insulin signaling attenuation.[9] Thus, the IR observed in fluoride-treated animals could be mediated by the activation of inflammatory pathways.

In order to elucidate the mechanisms by which mild chronic NaF treatment elevates TNF-α plasma concentration and attenuates insulin signaling, this study was conducted to evaluate whether these alterations are caused by modifications in TNF-α expression and/or IκK and JNK phosphorylation or content in the WAT. Moreover, we investigated the Akt serine and threonine phosphorylation status as a downstream of insulin signaling.

MATERIAL AND METHODS

Experimental design: All experimental procedures were approved by the Institutional Committee on Animal Research and Ethics (Protocol No. 2006-08802). Four-week-old male Wistar rats were kept under a 12/12 hr light/dark cycle (lights on at 07:00) and at a room temperature of 23 ± 2 ºC, with free access to a standard laboratory rat diet (LABINA Indústria de rações do Brasil LTDA, Paulínia, Brasil) and water (containing NaF or NaCl). Rats were castrated because studies demonstrate that testosterone levels can influence insulin signaling.[10] After 21 days, 20 7-week-old castrated rats were randomly distributed into 2 groups: 1) control group (n = 10), rats received an average of 76.4 mg/L NaCl in their drinking water; 2) fluoride group (n = 10), rats received an average of 54.9 mg/L NaF in their drinking water and fluoride in their food pellets (total estimated fluoride intake = 4.0 mg/kg BW/day; 3.1 mg/kg BW/day from drinking water and 0.9 mg/kg BW/day from diet). During the next 42 days, body weight and daily volume of water consumed per rat were measured every 2 or 3 days. At the end of this period, control and fluoride group rats were fasted for 14 hr and deprived from the NaF solution for 4 hr before the day of the experimental measurements to avoid an acute fluoride effect. Rats from both groups were anesthetized by an intraperitoneal injection of sodium thiopental (Thiopental 3%, 5 mg/100 g BW), then, 10 control and 10 fluoride-treated rats were used to quantify the Akt, IκKα/β, and JNK expression and phosphorylation status, as well as the TNF-α expression in the WAT by western blotting.

Assessment of Akt, JNK, and IκKα/β content and phosphorylation status, and TNF-α content: Samples of WAT were collected from the animals before and 120 sec after intravenous administration of regular insulin (1.5 U). Tissue samples were prepared

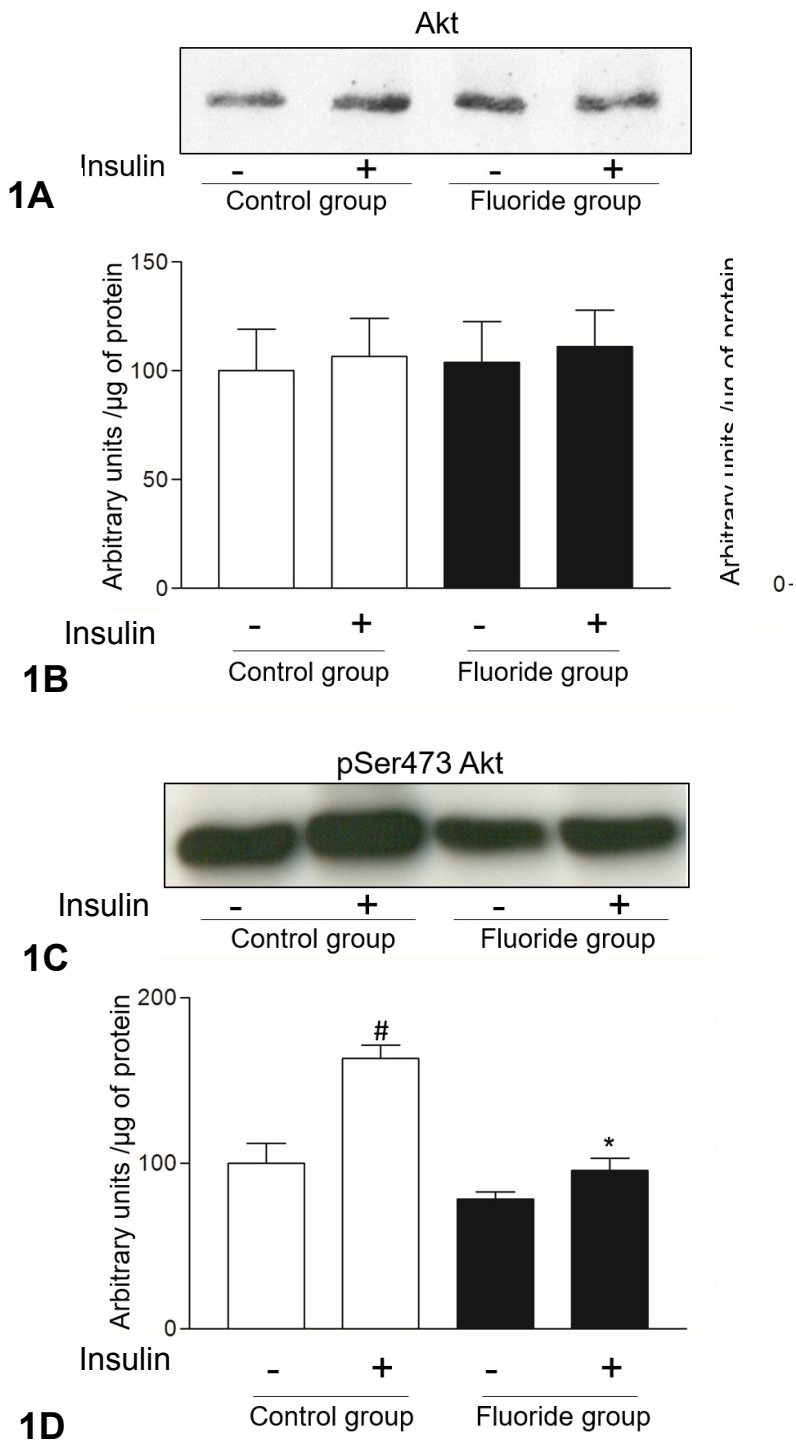
according to the method described by Carvalho et al.[11] and submitted to western blotting for quantification of: a) Akt content and Akt serine and threonine phosphorylation status using anti-Akt1/2/3, anti-phosphoserine (AktSer473), and anti-phosphothreonine (AktThr308) antibodies, respectively (Santa Cruz Biotechnology, CA,USA); b) IκKα/β and JNK content and phosphorylation status using antibodies against p-IκKα/β, p-JNK, IκKα/β, and JNK (Santa Cruz Biotechnology, CA,USA); c) TNF-α content using anti-TNF-α antibody (Santa Cruz Biotechnology, CA,USA). Proteins of the inflammatory pathway were analyzed in WAT samples collected before the insulin stimulus. Immunoreactive bands were detected by autoradiography using a chemiluminescent substrate commercial kit (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions. Quantitative analysis of the blots was carried out by Scion Image software (Scion Image, Release Beta 3b; National Institutes of Health).

Statistical analysis: The normality of the data set was verified and statistical analyses were performed: 1) Analysis of insulin signaling (intergroups), body weight, and volume of water consumed: Data were analyzed by repeated measures one-way analysis of variance, followed by Tukey's post hoc test. 2) Comparison of insulin signaling (intragroup) before (–) and after (+) insulin stimulation was performed by paired t-test. 3) Analysis of proteins of the inflammatory pathway: Data were analyzed by Student's t-test for non-paired samples. All numerical values are presented as the mean ± standard error of the mean (SEM), and differences among groups were considered significant at $p < 0.05$.

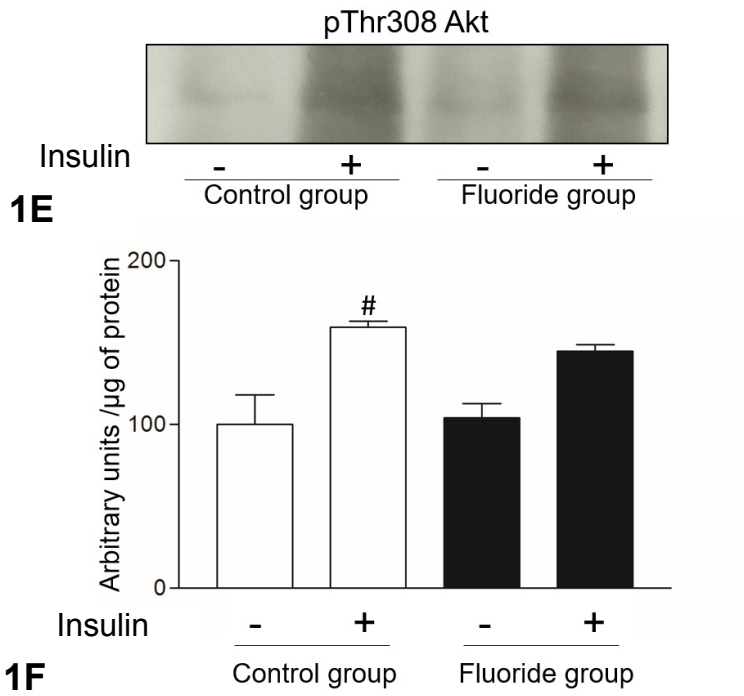
RESULTS

There was no significant difference in average body weights and daily volumes of consumed water per rat between control and fluoride groups throughout the study period. Typical autoradiographies of the total Akt content (Akt) and insulin-stimulated serine and threonine phosphorylation status of Akt (pSer473 Akt and pThr308 Akt, respectively) in the WAT are shown in Figures 1A, 1C, and 1E; the data expressed in arbitrary units per µg of protein are shown in Figures 1B, 1D, and 1F. Only in the control group, there was a significant intra-group difference ($p<0.05$) in Akt serine and threonine phosphorylation status after insulin stimulation. Fluoride group showed a decrease in Akt serine phosphorylation status after insulin stimulus compared to the control group. However, there was no difference in the total Akt content and threonine phosphorylation status of Akt between the groups.

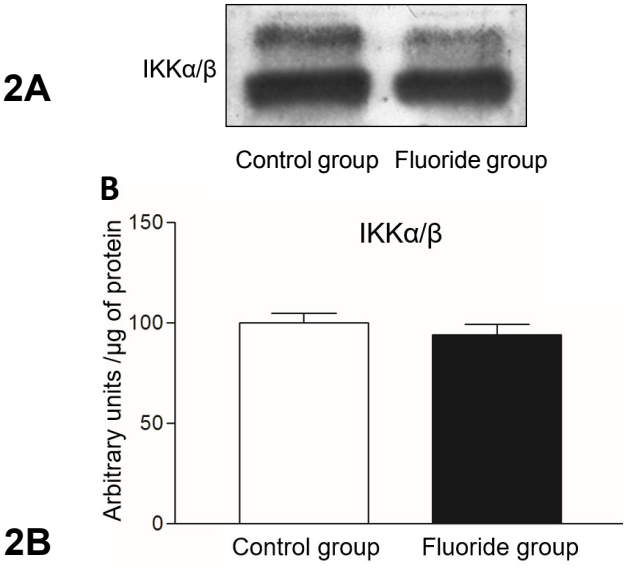
Typical autoradiographies of IκKα/β, JNK, and TNF-α contents, as well as IκKα/β and JNK phosphorylation status in the WAT are shown in Figures 2A, 2E, 2I, 2C and 2G; the data expressed in arbitrary units per µg of protein are shown in Figures 2B, 2F, 2J, 2D, and 2H. Results showed an increase in TNF-α content and IκKα/β phosphorylation status in the fluoride group compared to the control group, while there was no difference in IκKα/β content and in JNK content and phosphorylation status between the groups.



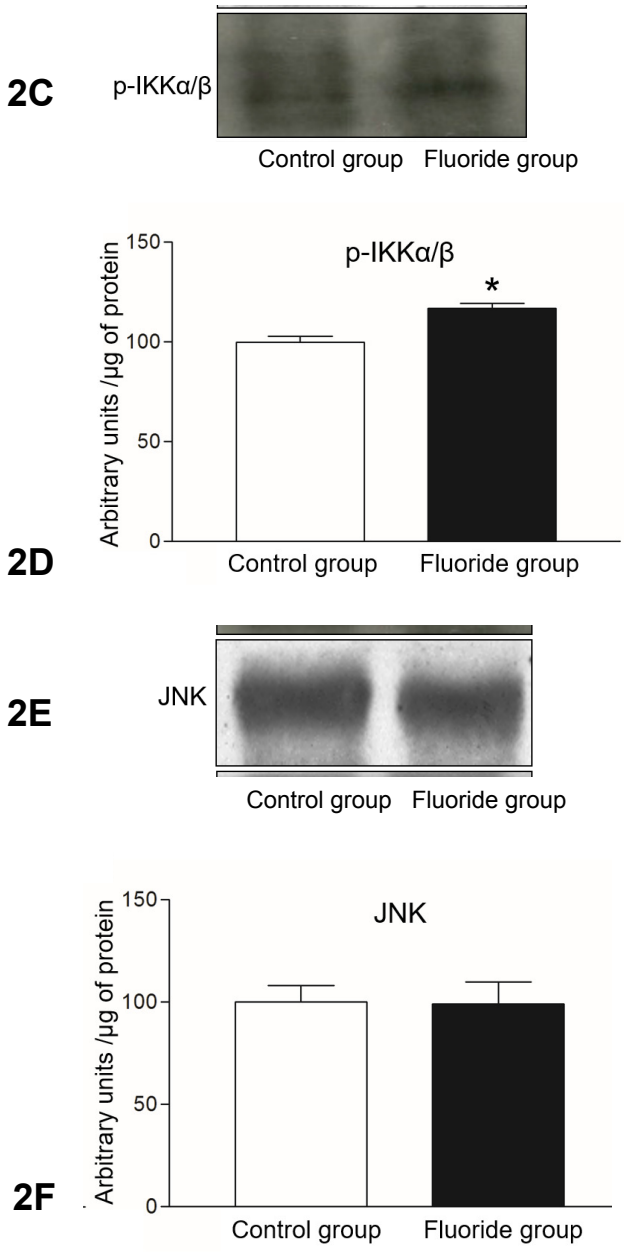
Figures 1A, 1B, 1C, and 1D. Evaluation of total Akt content and insulin-stimulated serine phosphorylation status of Akt (pAkt Ser473) in the white adipose tissue (WAT) of control and fluoride group rats, before (–) and after (+) insulin stimulation. 1A: Typical autoradiography of total Akt content; 1B: Total Akt content expressed in arbitrary units and presented as mean±SEM; 1C: Typical autoradiography of total insulin-stimulated serine phosphorylation status of Akt (pAkt Ser473); 1D: Total Akt serine phosphorylation status expressed in arbitrary units and presented as mean±SEM; n = 10. #: $p<0.05$ in insulin (–) vs. insulin (+) group; *: $p<0.05$ in control (+) vs. fluoride (+) group.



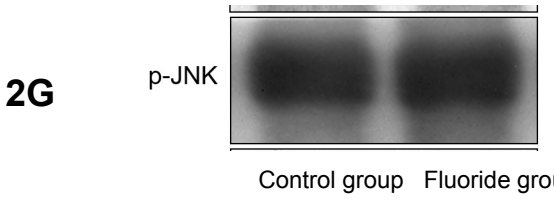
Figures 1E and 1F. Evaluation of total insulin-stimulated threonine phosphorylation status of Akt (pAkt Thr308) in the white adipose tissue (WAT) of control and fluoride group rats, before (–) and after (+) insulin stimulation. 1E: Typical autoradiography of insulin-stimulated threonine phosphorylation status of Akt (pAkt Thr308); 1F: Total Akt threonine phosphorylation status expressed in arbitrary units and presented as mean±SEM; n = 10. #: p<0.05 in insulin (–) vs. insulin (+) group; *: p<0.05 in control (+) vs. fluoride (+) group.

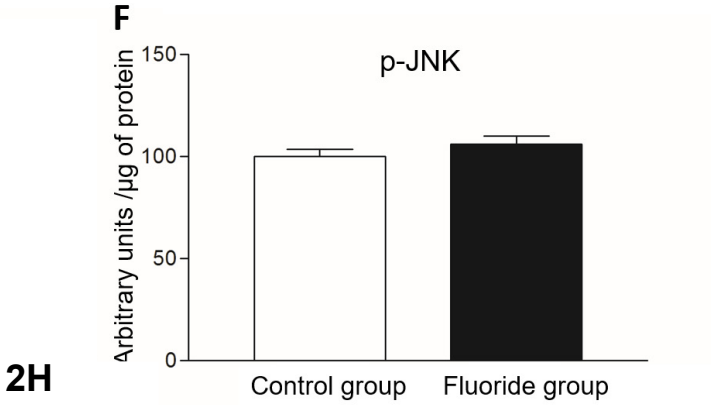


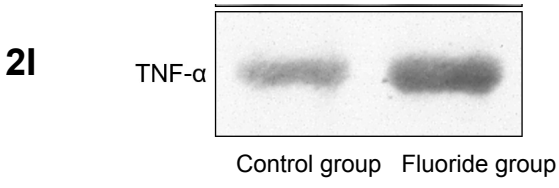
Figures 2A and 2B. Evaluation of IkKα/β content in the white adipose tissue (WAT) of rats. 2A: Typical autoradiography of IkKα/β content: equal protein amounts (185 µg) were subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis; 2B: Total IkKα/β content expressed in arbitrary units and presented as mean±SEM; n = 10.*: p<0.05 vs. control group.

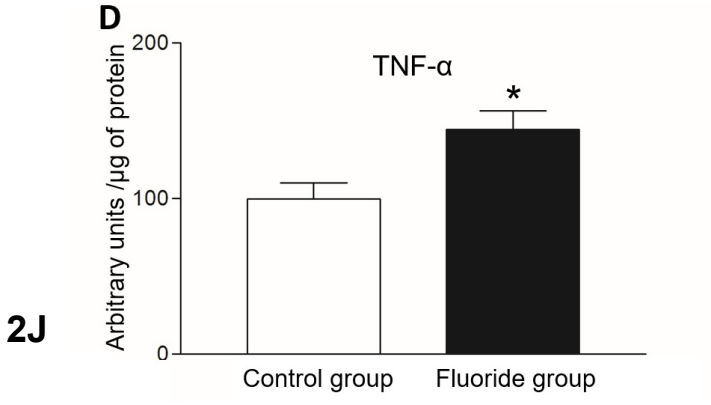


Figures 2C, 2D, 2E, and 2F. Evaluation of p-IkKα/β phosphorylation status and JNK content in the white adipose tissue (WAT) of rats. 2C: Typical autoradiography of p-IkKα/β content: equal protein amounts (185 µg) were subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis; 2D: Total p-IkKα/β phosphorylation status expressed in arbitrary units and presented as mean±SEM; 2E: Typical autoradiography of JNK content: equal protein amounts (185 µg) were subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis; 2F: Total JNK content expressed in arbitrary units and presented as mean±SEM; n = 10.*: $p<0.05$ vs. control group.









Figures 2G, 2H, 2I, and 2J. Evaluation of p-JNK phosphorylation status and TNF-α content in the white adipose tissue (WAT) of rats. 2G: Typical autoradiography of p-JNK content: equal protein amounts (185 µg) were subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis; 2H: Total p-JNK phosphorylation status expressed in arbitrary units and presented as mean±SEM; 2I: Typical autoradiography of TNF-α content: equal protein amounts (185 µg) were subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis; 2J: Total TNF-α content expressed in arbitrary units and presented as mean±SEM; n = 10.*: p<0.05 vs. control group.

DISCUSSION

In this study, chronic NaF treatment (4.0 mg/kg BW/day for 42 days) was found to induce a decrease in the insulin-stimulated Akt serine phosphorylation status and an increase in TNF-α content and IκKα/β phosphorylation status in the WAT, while it did not affect Akt, JNK, and IκKα/β contents, nor JNK phosphorylation status.

No significant differences in body weight and water intake were observed between the fluoride-treated and control rats during the study period, demonstrating that these parameters are not affected by changes in insulin signaling and activation of inflammatory pathways.

By investigation of inflammatory signaling, fluoride-treated rats were found to demonstrate a significant increase in the WAT TNF-α content compared to control rats (Figures 2I and 2J). Similar results were observed in a study carried out on isolated lymphocytes from the splenic tissue of healthy mice, in which the exposure to high fluoride levels (100, 200, and 400 mg/L) increased TNF-α levels in a dose-dependent manner.[12] In another study, rats subjected to the same NaF treatment model used in the present study (4.0 mg/kg BW/day) demonstrated an increase in TNF-α plasma concentrations in association with impairments in the initial steps of the insulin signaling pathway in the WAT.[4] Thus, it is possible to suggest that the decrease in the Akt serine phosphorylation status in the WAT may be related to the increase in the tissue expression and plasma concentration of TNF-α.

Moreover, studies have reported that insulin-resistant obese animals exhibit elevated TNF-α expression levels in the WAT,[13] and that the neutralization of this cytokine by treatment with anti-TNF-α antibodies or by mutation of the genes that encode for TNF-α and its receptors can improve insulin sensitivity.[14] In addition, Lumeng et al.[15] verified that TNF-α can block insulin action in adipocytes by reducing the expression of GLUT4 and IRS-1, thereby decreasing Akt phosphorylation and impairing the insulin-stimulated GLUT4 translocation to the plasma membrane. Taken together, these data reinforce the results of the present study, suggesting that TNF-α plays an important role in the development of IR related to excessive fluoride intake.

In order to investigate the association between the activation of inflammatory pathways and fluoride-mediated impairments in insulin signaling, the contents and phosphorylation status of IκKα/β and JNK were evaluated.

The results demonstrated an increase in the IκKα/β phosphorylation status in the fluoride group compared to the control group. Studies have suggested that TNF-α can activate the serine kinase S6K1 (p70S6k) through IκKβ, thereby promoting IRS-1 phosphorylation in multiple serine residues, resulting in inhibition of insulin signaling.[16]

It is known that proinflammatory cytokines such as TNF-α and IL-1β can activate the intracellular pathways of JNK and IκKβ/nuclear factor kappa B (IκKβ/NF-κB) through mechanisms mediated by plasma membrane receptors.[17] The IκK complex is composed of two catalytic subunits; IκKα and IκKβ, and by the regulatory unit IκKγ, known as NF-κB-essential modulator, which leads to the IκB phosphorylation.[18] After IκB degradation, NF-κB dimers are translocated to the nucleus, where they promote the transcription of a large number of genes, including genes encoding for inflammatory cytokines such as TNF-α and IL-6, which are associated with IR.[18-22]

Previous studies have shown that increased JNK activation is associated with insulin signaling attenuation.[10] Moreover, TNF-α is known to increase JNK phosphorylation; however, despite the increase in TNF-α content, no alteration in JNK phosphorylation was observed in our study (Figures 2G and 2H). Studies have

shown that JNK activation promotes cellular apoptosis. In contrast, the increase in IκKβ phosphorylation status consequently leads to an increase in the activity of the transcription factor NF-κB,[23] which plays a role in the inhibition of apoptosis.[24-26] Accordingly, the unaltered JNK phosphorylation status observed in our study can be explained by the increase in the activation of NF-κB. An important mechanism by which NF-κB inhibits cell death is the attenuation of TNF-α-induced JNK activation.[26] Moreover, NF-κB is known to regulate several factors that play a role in decreasing the JNK activity, like the transcription factors X-linked inhibitor of apoptosis and growth arrest and DNA damage inducible protein 45, as well as the antioxidant enzyme superoxide dismutase 2.[26]

Regarding insulin signaling, our results demonstrated that there was a decrease in the insulin-stimulated Akt serine phosphorylation status in the WAT of fluoride-treated rats compared to control rats (Figures 1C and 1D), while there was no difference in WAT Akt content between the groups, demonstrating that the reduction in the Akt serine phosphorylation status is not related to Akt expression (Figures 1A and 1B). These findings corroborate those of previous studies,[4] which demonstrated a decrease in IRS-1/IRS-2 tyrosine phosphorylation status and an increase in the IRS-1 serine phosphorylation status in the WAT of rats exposed to chronic NaF treatment, evidencing that the exposure to high fluoride levels could impair several steps in the insulin signaling pathway. Similar results were observed in the study carried out by Pereira et al.,[5] in which chronic treatment of ovariectomized rats with a high dose of NaF (50 mg/L in drinking water for 42 days) caused a decrease in the Akt serine phosphorylation status after insulin stimulation in gastrocnemius muscle. Moreover, Tan et al.[27] verified that the treatment of murine adipocytes from the 3T3-L1 lineage with Akt inhibitors significantly reduced the insulin-stimulated glucose uptake, which indicates the fundamental role of Akt in mediating insulin action.

As previously mentioned, Akt activation involves phosphorylation in serine and threonine residues. In order to better understand the effect of NaF on this step of insulin signaling, Akt threonine phosphorylation status after insulin stimulation was also evaluated; however, our results showed no difference in this parameter between the groups (Figures 1E and 1F). This can be explained by the complex participation of several different proteins in the process of Akt activation. Tsuchiya et al.[28] demonstrated that the 3-phosphoinositide-dependent protein kinase 1 pathway promotes the phosphorylation of Akt1 in Thr 308 and Akt2 in Thr 309 while the PI3K pathway phosphorylates Akt1 in Thr 308 and Ser 473 and Akt2 only in Ser 474. It can be concluded from these results that Akt activation is subject to extremely complex molecular regulation that is dependent on different proteins. From the results of this study, it is possible to suggest that chronic NaF treatment promoted alteration in PI3K pathway, thereby inducing greater changes in the Akt serine phosphorylation.

Our results suggest that the increase in TNF-α plasma concentration and the attenuation of the insulin signaling induced by chronic NaF treatment in previous studies may be associated with an increase in the IκKα/β phosphorylation status and TNF-α expression in the WAT, but not in the JNK content or phosphorylation. These changes may also be involved in the impairment of the downstream insulin signaling pathway as demonstrated by the decrease in the Akt serine phosphorylation status.

CONCLUSIONS

Based on the findings of the present study, we can propose that the chronic fluoride treatment-induced inhibition of insulin signaling in the WAT of rats is related to the increase in IκK phosphorylation status and TNF-α expression. These results indicate that IR induced by excessive fluoride intake may be mediated by activation of inflammatory signaling pathways. Therefore, consumption of fluoride-containing products, especially by children, requires careful control to avoid the deleterious effects caused by excessive fluoride intake.

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