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CHRONIC SODIUM FLUORIDE INTAKE PROMOTES CHANGES IN INSULIN SIGNALING AND INFLAMMATORY PATHWAYS IN THE SKELETAL MUSCLE OF RATS

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ABSTRACT: This study aimed to investigate the final step of insulin signaling and the relationship between the inflammatory pathway and insulin signal attenuation in the gastrocnemius muscle (GM) of rats chronically treated with NaF. Thirty-two seven-week-old male Wistar rats were randomly distributed into 2 groups: a control group, which was treated with drinking water without fluoride; and a fluoride group, which received drinking water with NaF and F present in their food pellets (total fluoride intake= 4.0 mg/kg body weight/day). After six weeks, the following were measured in the GM: content of protein kinase B (Akt), inhibitor of kappa B kinase (IκKα/β), tumor necrosis factorα (TNF-α), c-Jun N-terminal kinase (JNK), and glucose transporter type 4 (GLUT4) by western blotting; phosphorylation status of Akt threonine, Akt serine, IκKα/β, and JNK by western blotting; and expression of GLUT4 mRNA by real-time PCR. The fluoride-treated rats showed a decrease ($p<0.05$) in the insulin-stimulated Akt serine phosphorylation status, GLUT4 gene expression and its protein content in the plasma membrane fraction and translocation index; and increased ($p<0.05$) IκKα/β phosphorylation status and TNF-α protein content in GM. No significant differences in the Akt threonine and JNK phosphorylation status, and protein contents of Akt, IκKα/β, and JNK were observed between the fluoride-treated and control rats. Chronic NaF intake led to alterations in the final step of insulin signaling, and increased the IκKα/β phosphorylation status and TNF-α content in GM of rats. Insulin resistance induced by excessive fluoride intake might be related to the activation of inflammatory signaling pathways.

Keywords: Inflammation; Insulin resistance; Glucose transporter type 4.

INTRODUCTION

Fluoride controls and prevents dental caries; however, excessive ingestion is related to systemic toxicity.[1] Studies have shown that excessive sodium fluoride (NaF) intake in rats promotes insulin resistance (IR).[2] Changes in the insulin signal (IS) associated with increased proinflammatory cytokine levels have been suggested as contributing factors for IR.[3] However, the exact mechanisms of this association are not well elucidated.

Insulin exerts its effect by binding to the receptor that promotes tyrosine phosphorylation of insulin receptor substrates 1 and 2 (pp185), and stimulation of phosphatidylinositol 3-kinase (PI3K).[4] Finally, this pathway triggers serine and

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threonine phosphorylation of protein kinase B (Akt), which allows glucose uptake by cells translocating the glucose transporter type 4 (GLUT4) from the intracellular compartment to the plasma membrane.[5] However, excessive tumor necrosis factor-α (TNF-α) levels cause IR.[6] TNF-α can activate I kappa B kinase (IKK) and c-Jun N-terminal kinase (JNK), which promote attenuation of IS (Figure 1).[7]

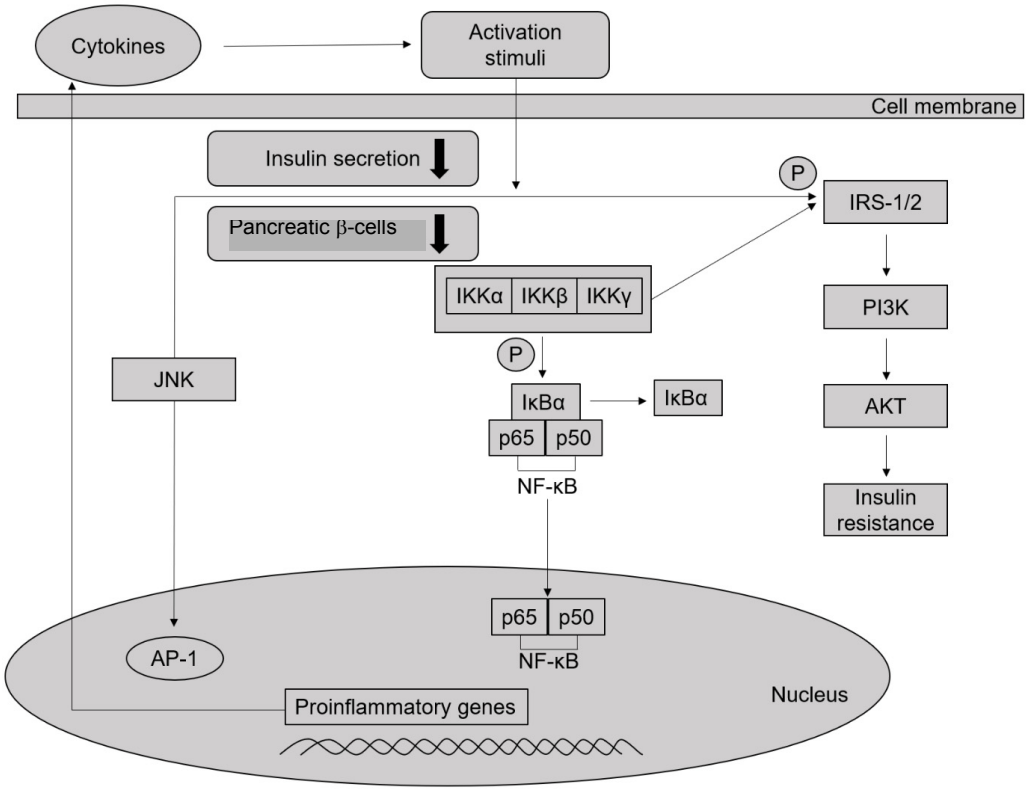

Figure 1. Inflammatory pathways linking inflammation to insulin resistance. Activation of JNK and NF-κB pathways causes serine kinase phosphorylation of IRS-1 or IRS-2, which may block insulin signaling and finally lead to the occurrence of IR. In addition, JNK and NF-κB pathways are involved in the production of proinflammatory cytokines which may in turn become activation stimuli of the pathways. Source: Chen et al.[8]

Chiba et al.[2,3] showed an increase in the TNF-α plasma concentration and decrease in the insulin-stimulated tyrosine phosphorylation status in the gastrocnemius muscle (GM) of rats subjected to NaF treatment (4.0 mg/kg body weight (BW)/day for 42 days). This study aimed to investigate the final step of IS and the role of the inflammatory pathway in IS attenuation in the GM of rats chronically treated with NaF.

MATERIAL AND METHODS

Experimental design: This study followed the Ethical Principles and Guidelines for Animal Experimentation and was approved by the local ethics committee of São Paulo State University (UNESP), School of Dentistry, Araçatuba, Brazil (protocol number 2006-08802). Four-week-old male Wistar rats were kept in an environment with controlled temperature (22 ± 2ºC), humidity (55% ± 10%), and luminosity (07:00–19:00), and 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.[9] After 21 days, 32 7-week-old castrated rats were randomly distributed into 2 groups: 1) control group (n = 16), rats received an average of 76.4 mg/L NaCl in their drinking water; and 2) fluoride group (n = 16), 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, the body weight and daily volume of water consumed per rat were measured every 2 or 3 days. At the end of this period, both control and fluoride group rats were subjected to fasting 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), after which 10 control and 10 fluoride-treated rats were used to quantify the Akt, IKKα/β, and JNK phosphorylation status and the protein content of Akt, IKKα/β, JNK, and TNF-α in GM by western blotting. From another group of animals (n = 6 per group), the right sided GM was dissected to measure GLUT4 gene expression by real time PCR method and left sided GM to evaluate the GLUT4 protein content and its plasma membrane translocation index by western blotting.

Assessment of Akt, JNK, and IKKα/β protein content and phosphorylation status, and TNF-α protein content: Samples of GM were collected from the animals before and 90 sec after intravenous administration of regular insulin (1.5 U). Tissue samples were prepared according to the method described by Carvalho et al.[10] and subjected 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) IKKα/β and JNK content and phosphorylation status using antibodies against p-IKKα/β, p-JNK, IKKα/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 GM 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 performed using Scion Image software (Scion Image, Release Beta 3b; National Institutes of Health).

Evaluation of GLUT4 protein content and its plasma membrane translocation index: Tissue samples were homogenized in sucrose buffer pH 7.4 (10 mmol/L Tris-HCl, 1 mmol/L EDTA, and 250 mmol/L sucrose) and subjected to differential centrifugations to obtain the plasma membrane (PM) and microsomal-enriched (M) fractions of the GM.[11] Equal amounts of the membrane and microsomal-enriched protein fractions were resolved in a 10% SDS gel. The blots were quantified by densitometry (ImageQuant TL, GE Healthcare UK Limited, Buckinghamshire, UK). In the GLUT4 protein analysis, the Ponceau stained membrane was used as a loading control. The translocation index was calculated as follows:[12]

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$$\text{Translocation index of GLUT4} = \text{PM} \times \frac{100}{\text{PM} + \text{M}}$$

Where:

GLUT4= glucose transporter type 4
PM = plasma membrane fraction of the gastrocnemius muscle
M = microsomal-enriched fraction of the gastrocnemius muscle

GLUT4 gene expression: Total RNA was extracted from GM using a reagent (Trizol LS, Invitrogen, Thermo Fisher Scientific; Carlsbad, CA) according to manufacturer's instructions. Samples were treated with an endonuclease (DNAse I, Sigma-Aldrich, St. Louis, Missouri) and the total RNA was quantified by spectrophotometry. The total RNA from each sample was reverse transcribed to complementary DNA using reverse transcriptase (SuperScriptTM II Reverse Transcriptase, Invitrogen) according to the manufacturer's instructions. Gene expression analysis of *Slc2a4* (Glut4) was performed by qRT-PCR, using a Real-Time PCR instrument (StepOne-PlusTM Real-Time PCR Systems, Applied Biosystems, Foster City) and kit (*Slc2a4* - Rn01752377_m1; FAM fluorophore reporter/non-fluorescent quencher MGB) (TaqManTM Gene Expression Assay, Applied Biosystems, ThemoFisher Scientific, Carlsbad, CA). The relative amount of transcripts was determined by the 2-(ffCT) method, with target expression normalization with Actb (Rn00667869_m1, Applied Biosystems), as the housekeeping gene, and control group as the calibrator.

Statistical analysis: The normality of the data set was verified and statistical analyses were performed: 1) Analysis of insulin signaling (intergroups), body weight, food intake, 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 the 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

Body weight and food and water intake: There was no difference in the body weight and food and water intake between the groups during the experimental period (Figures 2A–2C).

Evaluation of the Akt serine and threonine phosphorylation status and Akt protein content in GM: There was a decrease ($p<0.05$) in the Akt serine phosphorylation status after insulin stimulation in the Fluoride group compared to Control group (Figures 3A–3F). However, there was no difference in the Akt threonine phosphorylation status after insulin stimulation and in the protein content of Akt between the groups.

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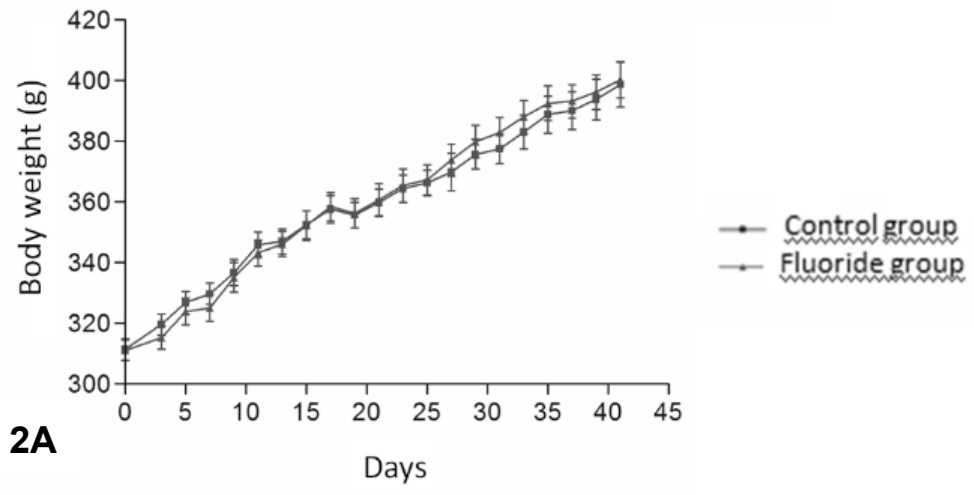
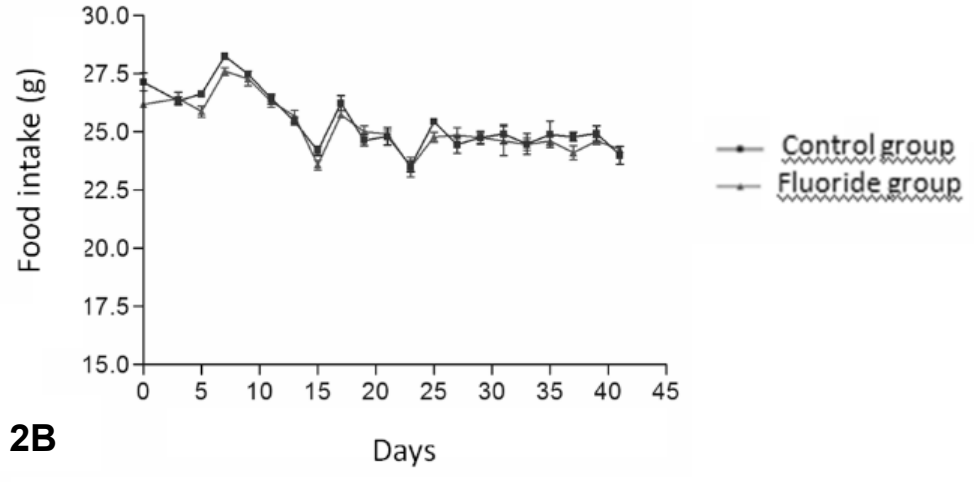
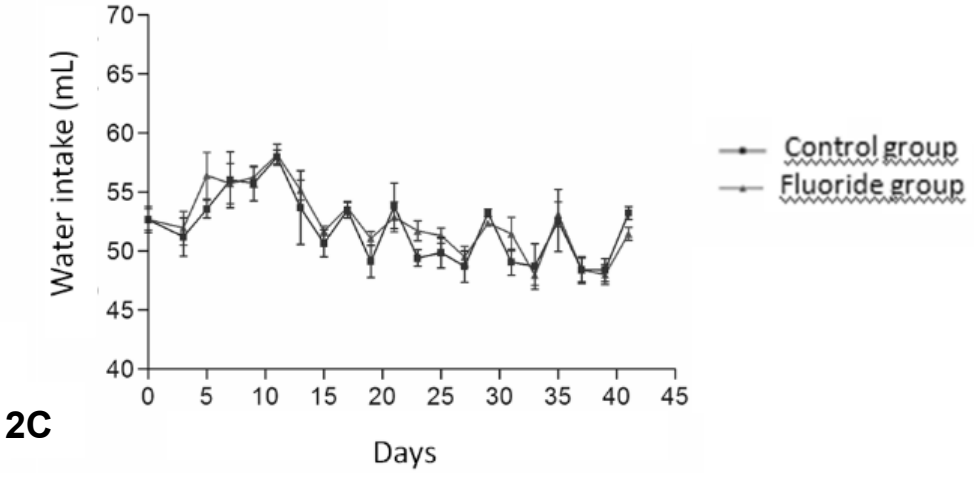


2A



2B



2C

Figures 2A–2C. 2A: Body weight (g) of the control and fluoride groups for 42 days; 2B: Food intake (g) of the control and fluoride groups for 42 days; and 2C: Water intake (mL) of the control and fluoride groups for 42 days. Values are presented as mean ± SEM; n = 10 per group.

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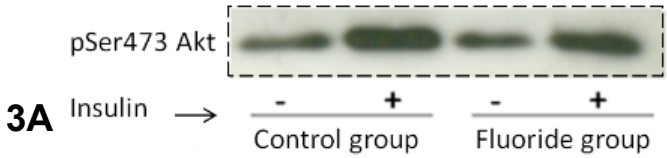
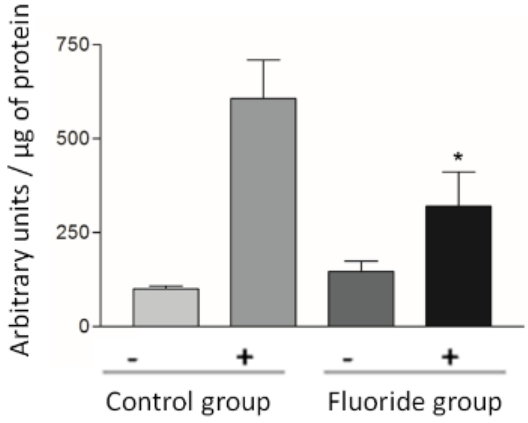


3A



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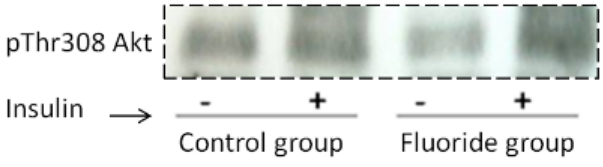

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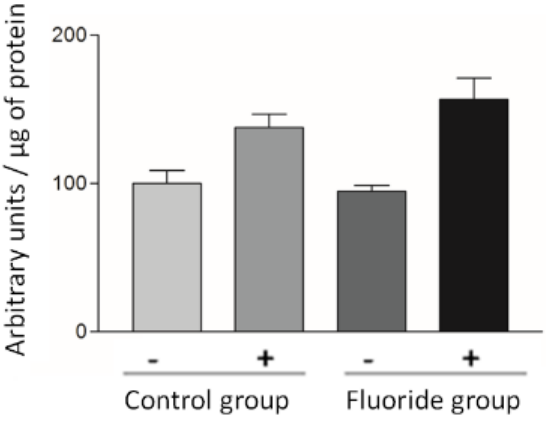

3D

Figures 3A–3D. 3A and 3B: Evaluation of Akt serine (Ser473) phosphorylation status before (–) and after (+) insulin stimulation in the gastrocnemius muscle of the control and fluoride groups; 3C and 3D: Evaluation of threonine (Thr308) phosphorylation status before (–) and after (+) insulin stimulation in the gastrocnemius muscle of the control and fluoride groups. Typical autoradiography: equal amounts of protein (185 µg) were subjected to sodium dodecylsulfate–polyacrylamide gel electrophoresis (3A and 3C). The values of Akt serine and threonine phosphorylation status (expressed in arbitrary units) are presented as mean ± SEM (3B and 3D), n = 10 animals per group. *: $p<0.05$ control group (+) vs. fluoride group (+).

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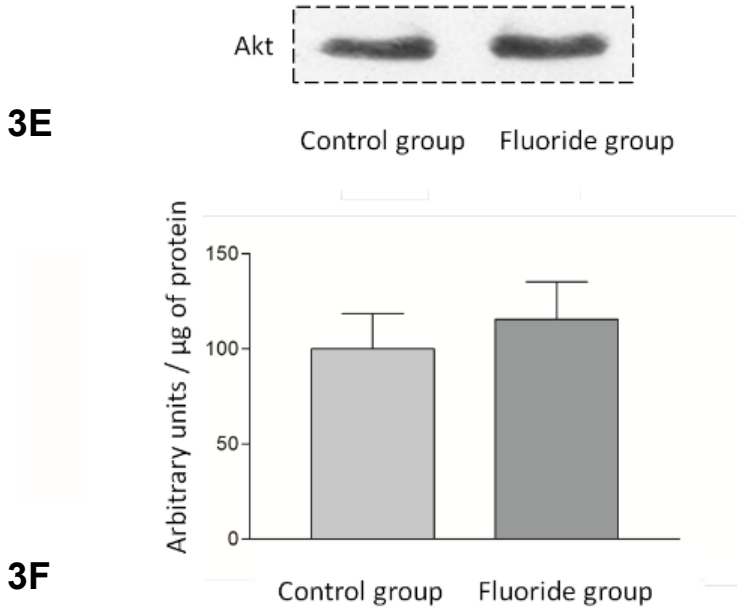
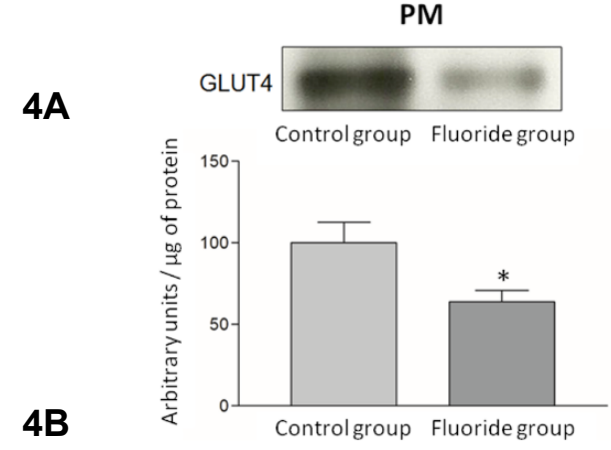


Figures 3E and 3F. 3E and 3F: Evaluation of Akt protein content in the gastrocnemius muscle of the control and fluoride groups. 3E: Typical autoradiography: equal amounts of protein (185 µg) were subjected to sodium dodecylsulfate–polyacrylamide gel electrophoresis. 3F: The values of the Akt protein content (expressed in arbitrary units) are presented as mean ± SEM, n = 10 animals per group. *: p<0.05 control group (+) vs. fluoride group (+).

Evaluation of GLUT4 protein content and its plasma membrane translocation index in GM: There was no significant difference in the microsomal fraction between the Control and Fluoride groups (Figures 4A and 4B); however, the plasma membrane fraction in the Fluoride group was lower (p<0.05) compared to that in the Control group (Figures 4C and 4D). In addition, a difference in the translocation index of GLUT4 to the plasma membrane was observed (Figures 4E and 4F).



Figures 4A and 4B. Evaluation of GLUT4 protein content in the plasma membrane (PM) in gastrocnemius muscle of control and fluoride groups. 4A: autoradiography; 4B: Result presented as mean ± SEM, n = 6 per group. *: p<0.05 control group vs. fluoride group.

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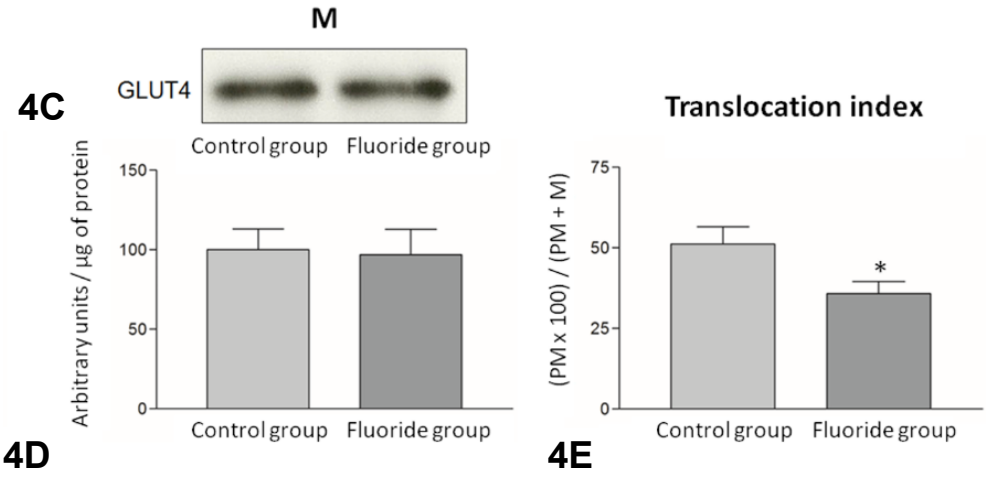
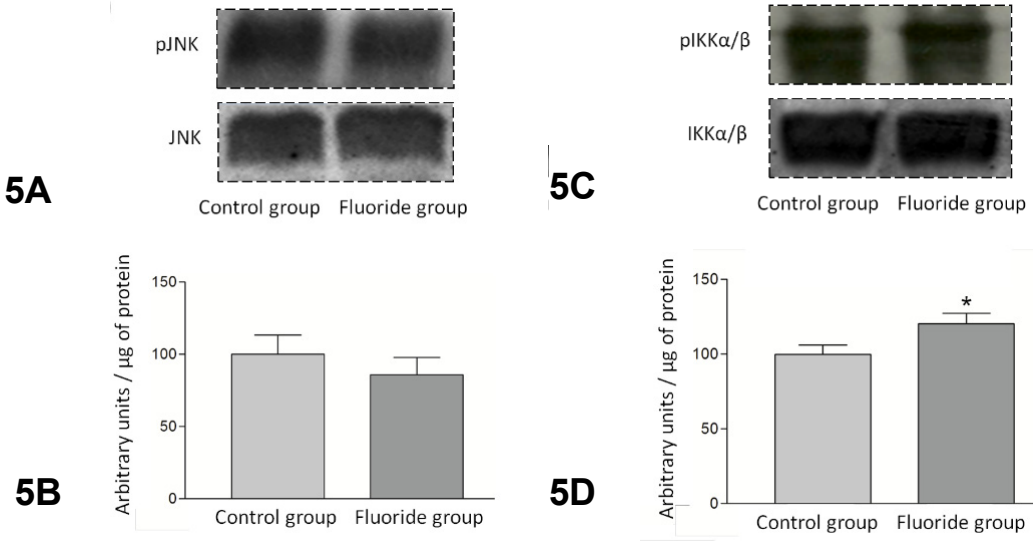
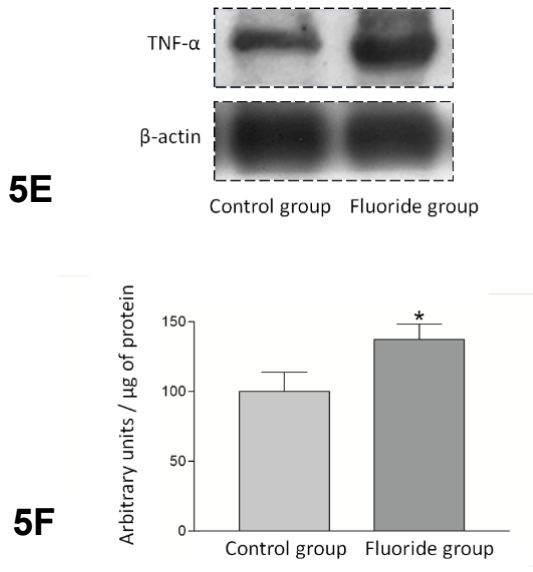


Figures 4C–4E. Evaluation of GLUT4 protein content and the GLUTA4 translocation index in the gastrocnemius muscle of control and fluoride groups. 4C and 4D: Evaluation of GLUT4 protein content in the microsomal-enriched fraction (M) in gastrocnemius muscle of control and fluoride groups. 4C: autoradiography; 4D: Result presented as mean ± SEM, n = 6 per group. *: p<0.05 control group vs. fluoride group. 4E: Evaluation of GLUT4 translocation index in gastrocnemius muscle of control and fluoride groups. Results are presented as mean ± SEM, n = 6 per group. *: p<0.05 control group vs. fluoride group.

Evaluation of JNK and IKKα/β phosphorylation status and their protein content and TNF-α protein content in GM: There was an increase (p<0.05) in the IKKα/β phosphorylation status and TNF-α protein content in GM in the Fluoride group compared to the Control group (Figures 5A–5F). However, there was no difference in the JNK phosphorylation status in this tissue between the groups.



Figures 5A–5D. Evaluation of JNK and IKKα/β phosphorylation status in the gastrocnemius muscle of the control and fluoride groups. 5A and 5C: Typical autoradiography: equal amounts of protein (185 µg) were subjected to sodium dodecylsulfate–polyacrylamide gel electrophoresis. JNK and IKKα/β protein content were used as the control. 5B and 5D: The values of JNK and IKKα/β phosphorylation status (expressed in arbitrary units) are presented as mean ± SEM, n = 10 animals per group. *: p<0.05 control group vs. fluoride group.



5E

5F

Figures 5E and 5F. Evaluation of TNF-α protein content in the gastrocnemius muscle of the control and fluoride groups. 5E: Typical autoradiography: equal amounts of protein (185 µg) were subjected to sodium dodecylsulfate–polyacrylamide gel electrophoresis. β-actin was used as the control. 5F: The value of TNF-α protein content (expressed in arbitrary units) is presented as mean ± SEM, n = 10 animals per group. *: $p<0.05$ control group vs. fluoride group.

Evaluation of GLUT4 gene expression in GM: The Fluoride group showed a significant reduction ($p<0.05$) in GLUT4 gene expression compared to the Control group (Figure 6).

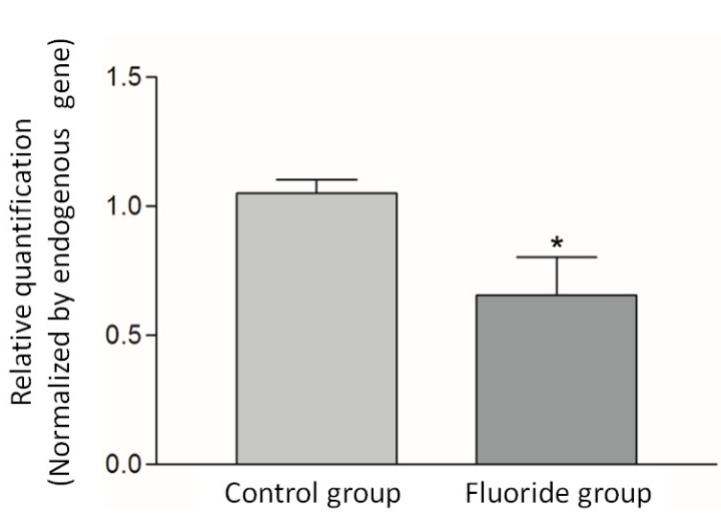


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Figure 6. Evaluation of GLUT4 gene expression in the gastrocnemius muscle of control and fluoride groups. Results are presented as mean ± SEM, n = 6 per group. *: $p<0.05$ control group vs. fluoride group.

DISCUSSION

In the present study, chronic treatment with NaF (4.0 mg/kg BW/day for 42 days) led to a decrease in the insulin-stimulated Akt serine phosphorylation status, GLUT4 gene expression and its protein content in the plasma membrane fraction and translocation index, and an increase in the IKKα/β phosphorylation status and TNF-α protein content in GM. There were no changes in the Akt threonine and JNK phosphorylation status, and Akt, IKKα/β, and JNK protein contents in this tissue.

Glucose uptake is regulated by several mechanisms, in which insulin plays a prominent role. GLUT4 is a glucose transporter predominantly expressed in the adipocytes and skeletal and cardiac muscle cells and is involved in the regulation of insulin-mediated glucose homeostasis.[13,14] In these tissues, most GLUT4 remains internalized in the cytoplasmic vesicles in the absence of insulin stimulation, and after insulin stimulation, these transporters are translocated to the plasma membrane, thereby promoting glucose uptake.[5] Thus, the failure of GLUT4 translocation to the plasma membrane in response to insulin is associated with IR.[14]

In this study, no difference was observed in the GLUT4 protein content in the microsomal fraction of the GM between the groups. However, there was a decrease in the GLUT4 protein content in the plasma membrane fraction in this tissue in rats treated with fluoride, compared to the control rats, suggesting impairment in the translocation of this glucose transporter. Indeed, it was observed that the GLUT4 translocation index was significantly lower in the Fluoride group compared to that in the Control group. Leite et al.[15] demonstrated through proteomic analysis, that diabetic rats subjected to fluoride intake show alterations in many proteins that interact with GLUT4, thereby impairing its function in the muscle tissue. It is known that Akt is one of the proteins that interacts with GLUT4 and plays a key role in insulin signaling.

Akt actively participates in the glucose uptake by stimulating GLUT4 translocation to the plasma membrane.[5] Akt phosphorylation is essential for the metabolism of glucose in the skeletal muscle. Hence, in the present study we evaluated the final step of the insulin signaling pathway, and results revealed that rats subjected to fluoride intake showed a decrease in the insulin-stimulated Akt serine phosphorylation status in the GM, independent of the content of this protein. This indicates that the impairment of GLUT4 translocation index observed in the GM might have been due to the decrease in Akt serine phosphorylation status. Our findings are in accordance with the study by Chiba et al.[16] that verified a reduction in the insulin-stimulated Akt serine phosphorylation status in the white adipose tissue of rats exposed to chronic NaF treatment compared to the control animals. Similar results were observed in a study by Pereira et al.,[17] 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 in the skeletal muscle after insulin stimulation.

It is known that Akt activation involves serine phosphorylation by the mammalian target of rapamycin complex 2 (mTORC2) and threonine phosphorylation by phosphoinositide-dependent kinase 1 (PDK1), conferring maximum Akt activity.[18,19] 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. This can be

explained by the complex participation of several different proteins in the process of Akt activation. Tsuchiya et al.[20] 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. In another study in rats chronically treated with fluoride, it was observed that the alteration of insulin signal in the white adipose tissue was also associated only with a decrease in the phosphorylation status of insulin-stimulated Akt serine.[16] From the results of this study, it can be inferred that chronic NaF treatment promoted alteration in the PI3K pathway, thereby inducing greater changes in Akt serine phosphorylation.

As previously mentioned, TNF-α might contribute significantly to the attenuation of insulin signaling and development of IR.[7] Our results demonstrated that chronic treatment with NaF promoted an increase in TNF-α protein content in the GM of rats. These findings are in agreement with the *in vitro* study by Shenoy et al.,[21] which reported a time-dependent increase in TNF-α gene expression in the C2C12 murine myoblasts cell line exposed to NaF at a concentration of 1.5 ppm.

Lumeng et al.[22] found that factors secreted by macrophages blocked the action of insulin in adipocytes via downregulation of GLUT4 and decrease in Akt phosphorylation. Interestingly, the treatment of these adipocytes with TNF-α blocking antibodies partially reversed these changes, demonstrating that TNF-α is a contributing factor for IR. In addition, Plomgaard et al.[6] demonstrated that infusion of TNF-α in humans increases phosphorylation of p70 S6 kinase (S6K), extracellular signal-regulated kinase (ERK: ERK1 and ERK2) and JNK, concomitant with decreased tyrosine phosphorylation of IRS-1 and Akt substrate AS-160 phosphorylation (responsible for the regulation of GLUT4 translocation), and increased serine phosphorylation of IRS-1. Considered 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 consumption.

Proinflammatory cytokines such as TNF-α and IL-1, can activate the intracellular pathways of JNK and IκB kinase β/nuclear factor κB (IKKβ / NF- κB) by means of membrane receptor-mediated mechanisms.[23] To verify the mechanisms by which TNF-α might impair insulin signal transduction due to a high amount of chronic fluoride intake, we investigated the inflammatory pathway. Our results demonstrated that rats treated with fluoride showed an increase in the IKKα/β phosphorylation status in the GM compared to that in control rats, suggesting a fluoride-induced muscle inflammatory response. These findings are in agreement with the study by Chen et al.,[24] which showed an increase in IKKβ protein content and gene expression in the liver of mice submitted to NaF (24 and 48 mg/kg NaF administered by gavage for 42 days).

The IKK kinase complex is the central element of the NF-κB cascade. It is essentially made of two kinases (IKKα and IKKβ) and a regulatory subunit, NEMO / IKKγ.[25] NF-κB increases the coding of pro-inflammatory mediators such as TNF-α, interleukins (IL) -6, and IL-1β.[24] In this context, it can be suggested that the increase

in TNF-α protein content observed in the present study might have been due to the increase in IKKα/β activity.

Furuya et al.[26] provided the first evidence that NF-κB can bind to the promoter of the *Slc2a4* gene (encoding the GLUT4 protein) and inhibit its transcription. Changes in GLUT4 expression in the adipose tissue and skeletal muscle correlate directly with increased or decreased insulin sensitivity.[27] Indeed, while knockout of the *Slc2a4* gene results in IR, its overexpression improves diabetes.[28,29] In this context, to investigate the mechanisms involved in the association between IR and inflammatory responses via activation of IKK signaling pathway due to chronic NaF intake, we investigated the GLUT4 gene expression in the GM. The results indicated that rats treated with fluoride showed a decrease in this parameter compared to that in control rats.

Our results revealed no difference in the JNK phosphorylation status between the groups. These data are in agreement with the study by Chiba et al.[16], which showed that rats subjected to NaF treatment showed no change in the JNK phosphorylation status in the white adipose tissue compared to that in control rats. Tang et al.[30] showed that IKK negatively modulates JNK activity, most likely through the induction of NF-κB target genes that encode proteins such as X-chromosome-linked inhibitor of apoptosis - XIAP, which interferes with TNF-α-mediated JNK activation. These findings reveal that the NF-κB and JNK signaling pathways are functionally interconnected, and that these signaling pathways interact in a complex network at several levels; the activation of one pathway often depends on the inactivation of another pathway, which suggests that cells are capable of directing TNF-α-induced signals towards the appropriate response.[31] This could be the reason that we found no difference in JNK phosphorylation, as the IKK signaling pathway overrode the JNK signaling pathway.

CONCLUSIONS

Based on our results it can be inferred that alterations in the final step of insulin signal in the skeletal muscle of rats exposed to chronic NaF treatment is associated with a greater IKKα/β phosphorylation status and increase in TNF-α protein content. These results indicate that IR induced by excessive fluoride intake might be mediated by activation of the inflammatory signaling pathways. Therefore, consumption of fluoride-containing products requires careful control to avoid the deleterious effects caused by excessive fluoride intake.

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