

INSULIN SIGNAL DECREASE IN MUSCLE BUT NOT IN THE LIVER OF CASTRATED MALE RATS FROM CHRONIC EXPOSURE TO FLUORIDE

Fernando Yamamoto Chiba,^{a,b} Natália Helena Colombo,^a Daisy Jaqueline Shirakashi,^a

Wendrew Douglas de Souza Gomes,^a Suzely Adas Saliba Moimaz,^b

Cléa Adas Saliba Garbin,^b Cristina Antoniali Silva,^a

Doris Hissako Sumida^a

Araçatuba, Brazil

SUMMARY: With half the animals as controls without fluoride (F) in their drinking water, 16 of 32 seven-week-old castrated male Wistar rats were administered NaF in their drinking water and F contained in food pellets (estimated total F intake: 4.0 mg F/kg bw/day). After 42 days, determinations were made of the insulin receptor substrate (pp185–IRS-1/IRS/2), tyrosine phosphorylation status, and the extent of glycemia and insulinemia. This chronic treatment with F promoted: 1) decrease in the pp185 tyrosine phosphorylation status in the muscle tissue but not in the liver; 2) increase in the plasma F level; 3) no alteration in glycemia and insulinemia; 4) an increase in insulin resistance.

Keywords: Fluoride; Insulin receptor substrate; Insulin sensitivity; Insulin signal; Rats and insulin.

INTRODUCTION

Excessive fluoride (F) intake can lead to dental fluorosis¹ and alterations in carbohydrate metabolism.²⁻⁴ In 1990, Rigalli et al. reported finding diminished insulin secretion in both humans and in rats from ingestion of high doses of F.² In 1995, he and co-authors showed that plasma F above 5 µmol/L affects glucose homeostasis.³ Two years earlier, Trivedi et al. found that 40% of patients with endemic fluorosis had impaired glucose tolerance, but this anomaly was reversed by changing to low-F drinking water.⁵ In 1997, De La Sota et al. showed that glucose tolerance tests on residents in an endemic fluorosis area indicated that plasma insulin levels as a function of time were inversely correlated with fluoremia.⁶ Three years later, in another study, higher plasma glucose levels and delay in the peak plasma insulin were observed after a glucose tolerance test in people with high F intake.⁷ Furthermore, elevated serum F is associated with glycolysis inhibition,⁸ hyperglycemia,⁴ and depletion of hepatic and muscular glycogen.^{8,9} Knowing therefore that F can interfere with carbohydrate metabolism, we felt it would be useful and important to examine the chronic effect of elevated F exposure in a rat model on insulin signal transduction.

MATERIALS AND METHODS

Animals: Four-week-old male Wistar rats, obtained from the Araçatuba Dental School Central Vivarium (São Paulo State University, Araçatuba – SP) were kept

^aFor correspondence: Dental School of Araçatuba, UNESP - Univ Estadual Paulista, Brazil, R. José Bonifácio, 1193, CEP: 16015-050, Araçatuba-SP, Brazil.

E-mail: dorishs@foa.unesp.br. ^aDepartment of Basic Sciences, Dental School of Araçatuba, UNESP - Univ Estadual Paulista, Brazil. ^bDepartment of Child and Social Dentistry, Dental School of Araçatuba, UNESP - Univ Estadual Paulista, Brazil.

under a 12/12 hr light/dark cycle (light on at 0700) and temperature of $23 \pm 2^\circ\text{C}$, with free access to a regular laboratory rat diet (LABINA Indústria de rações do Brasil LTDA, Paulínia, Brasil) and water (containing NaF or NaCl). The rats were castrated to avoid any influence of testosterone. After 21 days, 32 seven-week-old castrated rats were divided into two groups: the control group (n=16) received an average of 76.4 mg NaCl/L in their drinking water throughout the experiment; the F group (n=16) received an average of 54.9 mg NaF/L in their drinking water throughout the experiment plus F present in the food pellets (total estimated F intake = 4.0 mg F/kg bw/day, resulting from 3.1 mg F/kg bw/day from drinking water and 0.9 mg F/kg bw/day from the diet). During the next 42 days, the body weight and the daily volume of water consumed per rat in the F group were measured every 2 or 3 days (Table 1). At the end of this period, the F group rats were fasted for 14 hr from food and deprived for 4 hr from the NaF solution before the day of the experimental measurements to avoid an acute effect of F. The F group and the control group were then anesthetized with sodium thiopental (Thiopenta® 3%, 5 mg/100 g bw, i.p.). Each group was divided into two subgroups in order to measure glycemia, insulinemia, and plasma fluoride levels, and to determine the phosphorylation status of the insulin receptor substrate (pp185-IRS-1/IRS2). Median laparotomy was performed in 10 control and 10 F-treated rats, and blood was collected from the inferior cava vein. The plasma was stored at -20°C until the F was determined. Six control and 6 F-treated rats were used to quantify the pp185 tyrosine phosphorylation status in the gastrocnemius muscle and liver after stimulation by i.v. administration of 1.5 U of regular human insulin.

The design of this research conformed to the Ethical Principles in Animal Experimentation adopted by the Brazilian College of Animal Experimentation and was approved by the Ethics Commission on Animal Experimentation at a meeting on December 21, 2006 in accordance with Protocol No. 2006-08802.

Analysis methods: Samples from the plasma were used for determination of glycemia by the oxidase glucose method (Enzymatic glucose, ANALISA Diagnóstica, Belo Horizonte, MG, Brazil), and for insulinemia by the radioimmunity (RIA) method (Coat-A-Count, DPC, Diagnostic products, Los Angeles, CA, USA). The resistance for insulin was evaluated by index of HOMA-IR (homeostasis model assessment of insulin resistance), calculated by the formula: $\text{HOMA} - \text{IR} = \text{fasting glycemia (mmol/L)} \times \text{fasting insulinemia } (\mu\text{UI/mL})/22.5$.¹⁰

Determination of F ion concentration in blood plasma: For analysis of F in plasma, pre-diffusion with heated HMDS- H_2SO_4 was done to remove CO_2 . The F concentration in plasma was determined after 12 hr of diffusion facilitated by the hexamethyldisiloxane (HMDS)¹¹ as modified by Whitford¹² using a F ion selective electrode (Orion Research, Cambridge, Mass., USA, model 9409) and a calomel reference microelectrode (Accumet, #13-620-79) joined and coupled to a potentiometer (Orion Research, model EA 940), as described by Buzalaf et al.¹³

Assessment of the insulin receptor substrate (pp185–IRS-1/IRS-2) tyrosine phosphorylation status: These tests were performed according to the method described by Carvalho et al.¹⁴ Thirty seconds after i.v. injection with human insulin hormone for insulin stimulation, a sample of the liver was removed and, 60 s later, a sample of the gastrocnemius muscle was extracted. These tissue samples were then treated by the Western Blotting method to quantify the tyrosine phosphorylation status of pp185 using the anti-phosphotyrosine antibody (Santa Cruz Technology, Santa Cruz, CA).

Statistical Analysis: All numerical values are presented as means SEM. Statistical analysis was done by the Student t test for non-paired samples, and the differences between the two groups were considered significant when $p < 0.05$.

RESULTS

Table 1 shows the means of the F group of 16 rats: 1) the NaF and the F concentration in the drinking water; 2) the body weight; 3) the daily volume of water consumed per rat, on the first day, the twenty-first day and the forty-second day of F treatment.

Table 1. Parameters of fluoride group rats (mean + SEM; n=16)

Parameter	1st day	21st day	42nd day
NaF concentration in drinking water (mg/L)	46.1 ± 0.8	59.3 ± 0.8	66.6 ± 1.0
F concentration in drinking water (mg/L)	20.9 ± 0.4	26.8 ± 0.4	30.1 ± 0.4
Body weight (g)	236.1 ± 4.5	310.8 ± 4.7	365.1 ± 5.9
Daily volume of water consumed per rat (mL)	35.1 ± 0.4	35.9 ± 0.4	37.6 ± 0.7

As seen in Table 2, the F-treated rats presented a highly significant increase in plasma F in comparison with the control group.

Table 2. Plasma glucose, insulin, and serum F concentration, and HOMA-IR index in control and F groups of rats (mean ± SEM; n=10)

Parameter	Control group	Fluoride group
Glycemia (mmol/L)	6.6 ± 0.2	6.9 ± 0.1
Insulinemia (µU/mL)	5.5 ± 0.8	7.2 ± 0.8
Fluoremia (µg F ³ /mL)	0.05 ± 0.004	0.12 ± 0.008*
HOMA IR	1.53 ± 0.15	2.21 ± 0.26*

* $p < 0.05$ compared to control group.

On the other hand, the glycemia and insulinemia readings in the two groups show no significant differences. From these values, the HOMA-IR was calculated to verify the presence of insulin resistance. The HOMA-IR index was significantly ($p < 0.05$) higher in the F-treated group than in the control group.

In the Figure, A) and C) show a typical autoradiogram with reference to the insulin-stimulated tyrosine phosphorylation status of pp185 in the muscle and liver, respectively. B) and D) give the results, expressed in arbitrary units per μg of protein. In the muscle tissue, the F group showed a significantly lower phosphorylation status of this substrate compared with the control group. On the other hand, no alteration was found in the phosphorylation of this substrate in the liver between the F and the control groups.

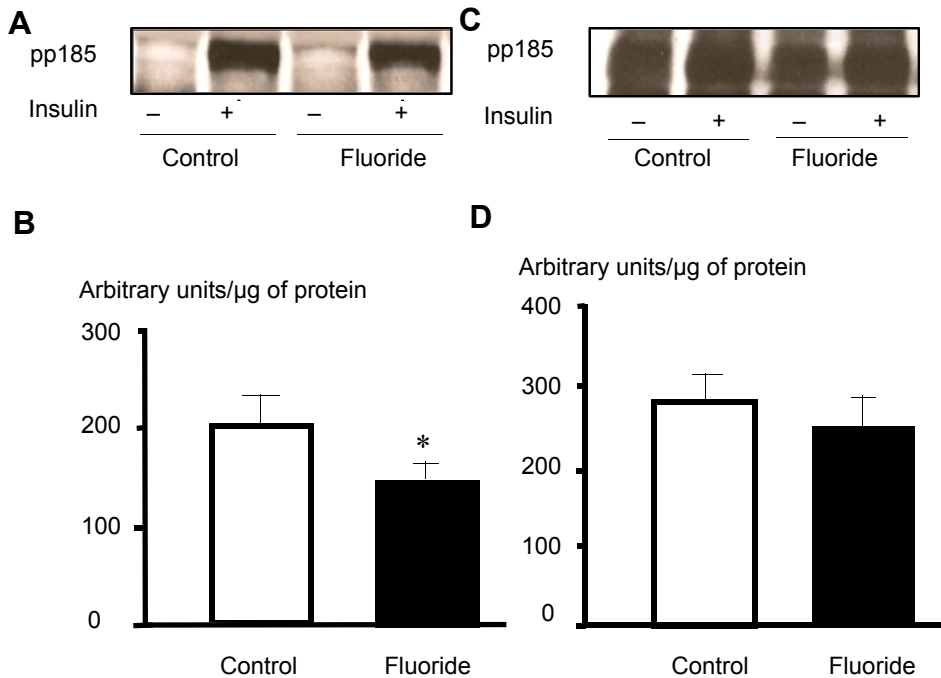


Figure. Insulin-stimulated tyrosine phosphorylation status of pp185 (IRS-1/IRS-2) in the muscle and in the liver of control ($n=6$) and F-treated rats ($n=6$). A) and C) are typical autoradiograms of muscle and liver, respectively. B) and D) depict the results of the insulin-stimulated tyrosine phosphorylation status of pp185 in the muscle and in the liver, respectively, expressed in arbitrary units per μg of protein (mean \pm SEM; $n=6$). * $p < 0.05$: F group (+) vs. Control (+).

DISCUSSION

Recently, Menoyo et al.¹⁵ demonstrated that acute treatment of rats with F (from a single dose of NaF (16.8 mg/kg bw) induces insulin resistance. Our results with chronic F intake in rats are in agreement as demonstrated by the increase in HOMA-IR seen in Table 2. In the other hand, Chehoud et al.⁴ found that treatment of rats with a single dose of NaF (1.0 mg F/kg bw) by gavage did not change insulin sensitivity. This divergence in results may be due to the difference in the dose of NaF used.

As noted in the Results, the values of glycemia and fasting insulinemia did not show alteration in the F group compared to the control group (Table 2). This fact may be due to the 4 hr the animals were without the NaF solution, a finding in agreement with the results of Rigalli et al.² and Menoyo et al.¹⁵ showing that, after 4 hr, the infusion of a solution containing 16.8 mg NaF/kg bw in rats), the baseline values of glycemia and insulinemia had returned to normal. This result may reflect a lowering of F in the blood by rapid uptake of F by the bones.

As found here, chronic treatment of rats with NaF induced a decrease in the pp185 tyrosine phosphorylation status in the muscle tissue after insulin stimulus. (Figure 1). These results in muscle tissue, are in line with the *in vitro* findings of Viñals et al.¹⁶ on insulin receptors isolated from rat muscle tissue and human placentas showing that F caused a reduction in the insulin-induced auto-phosphorylation and phosphorylation of the exogenous substrates used. However, no significant alteration was found in the pp185 tyrosine phosphorylation status in the liver. This result is in agreement with other models of insulin resistance, such as in transgenic rats with chronic excess of growth hormone (TRGH).¹⁷ In that TRGH study, Cho et al. found that insulin-induced IRS-1 tyrosine phosphorylation was decreased in the liver, but IRS-2 tyrosine phosphorylation was significantly increased.¹⁷ In our results, as the pp185 contains both insulin substrates IRS-1 and IRS-2, the absence of alteration in the phosphorylation status of pp185 may be a result of the balance between the increase and decrease of the tyrosine phosphorylation of IRS-2 and IRS-1, respectively. Cho et al. suggest that the liver can compensate the insulin resistance found in adipose or muscle tissue to prevent hyperglycemia.¹⁷

When analyzing the plasma F ion concentration, we noticed a statistically significant increase in the F group compared to the control group (Table 2), thereby confirming the relation between F ingestion and absorption by the body.

In the present study, the F dose used was 20 times higher than the proposed maximum allowable value a child can ingest through diet (0.045 mg F/kg bw/day) and during tooth brushing (0.155 mg F/kg bw/day) with a fluoridated dentifrice.¹⁸ This dose was used because Rigalli et al. carrying out studies in humans and in rats, observed that they needed to increase the fluoride dose by this proportion, administered in rats in relation to the dose in humans, to attain a similar plasmatic peak in the two models. This is probably due to the faster bone turnover rate in rats compared to humans.² In our study, the calculation of the F concentration in the drinking water was based on the body weight and the daily volume of water consumed per rat (Table1).

Based on the results obtained here, we can conclude that chronic treatment with NaF promoted: 1) decrease in the pp185 tyrosine phosphorylation status in the muscle tissue but not in the liver; 2) increase in plasma F level; 3) no alteration in glycemia and insulinemia; 4) an increase in insulin resistance.

Therefore, knowing that chronic NaF intake is capable of decreasing insulin signal and causing insulin resistance, we recommend the use of dentifrices with

lower F content, especially for diabetic children, for whom excessive F consumption may lead to worsening the health of these children.

ACKNOWLEDGEMENTS

This work was supported by grants (08/52688-8) from the São Paulo Foundation for Research (FAPESP), FUNDUNESP, and CNPq. We are also thanks Mrs. Noemia Akamine Kajimoto (English teacher) for her technical assistance in the preparation of this manuscript.

REFERENCES

- 1 Burt BA. The changing patterns of systemic fluoride intake. *J Dent Res* 1992;71(5):1228-37.
- 2 Rigalli A, Ballina JC, Roveri E, Puche RC. Inhibitory effect of fluoride on the secretion of insulin. *Calcif Tissue Int* 1990;46(5):333-8.
- 3 Rigalli A, Alloatti R, Menoyo I, Puche RC. Comparative study of effect of sodium fluoride and sodium monofluorophosphate on glucose homeostasis in the rat. *Arzneimittelforschung* 1995;45(3):189-92.
- 4 Chehoud KA, Garbin CAS, Sasaki KT, Chiba FY, Sumida DH. Effects of fluoride intake on insulin sensitivity and insulin signal transduction. *Fluoride* 2008;41(4):270-5.
- 5 Trivedi N, Mithal A, Gupta SK, Godbole MM. Reversible impairment of glucose tolerance in patients with endemic fluorosis. Fluoride Collaborative Study Group. *Diabetologia* 1993;36(9):826-8.
- 6 De La Sota M, Puche R, Rigalli A, Fernández LM, Benassati S, Boland R. Modificaciones en la massa osea y en la homeostasis de la glucosa en residentes de la zona de Bahia Blanca con alta ingesta espontanea de flúor. *Medicina (B Aires)* 1997;57(4):417-20.
- 7 Xie YP, Ge XJ, Jiang YT, Feng, MY, Fan YH, Wang FL, et al. Clinical study of the effect of high fluoride on the function of the pancreatic islet's B cells. *Chinese J Endemiol* 2000;19(2):84-6.
- 8 Dost FN, Knaus RM, Johnson DE, Wang CH. Fluoride impairment of glucose utilization: nature of effect in rats during and after continuous NaF infusion. *Toxicol Appl Pharmacol* 1977;41(3):451-8.
- 9 Shashi, JP, Singh, Thapar, SP. Changes in glycogen content in same tissues during fluorosis – an experimental study on rabbits. *Fluoride* 1988;21:82-6.
- 10 Bonora E, Targher G, Alberiche M, Bonadonna RC, Saggiani F, Zenere MB, et al. Homeostasis model assessment closely mirrors the glucose clamp technique in the assessment of insulin sensitivity: studies in subjects with various degrees of glucose tolerance and insulin sensitivity. *Diabetes Care* 2000;23:57-63.
- 11 Taves DR. Separation of fluoride by rapid diffusion using hexamethyldisiloxane. *Talanta* 1968;15(9):969-74
- 12 Whitford GM. The metabolism and toxicity of fluoride. New York: Karger; 1996.
- 13 Buzalaf MAR, Fuhushima R, Granjeiro JM, Cury JA. Correlation between plasma and nail fluoride concentrations in rats given different levels of fluoride in water. *Fluoride* 2002;35(3):185-92.
- 14 Carvalho CR, Brenelli SL, Silva AC, Nunes AL, Velloso LA, Saad MJ. Effect of aging on insulin receptor, insulin receptor substrate-1, and phosphatidylinositol 3-kinase in liver and muscle of rats. *Endocrinology* 1996;137(1):151-9.
- 15 Menoyo I, Puche RC, Rigalli A. Fluoride-induced resistance to insulin in the rat. *Fluoride* 2008;41(4):260-9.
- 16 Viñals F, Testar X, Palacin M, Zorzano, A. Inhibitory effect of fluoride on insulin receptor autophosphorylation and tyrosine kinase activity. *Biochem J* 1993;291:615-22.
- 17 Cho Y, Ariga M, Uchijima Y, Kimura K, Rho J, Furuhashi Y, et al. The novel roles of liver for compensation of insulin resistance in human growth hormone transgenic rats. *Endocrinology* 2006;147(11):5374-84.
- 18 Pessan JP, Silva SMB, Buzalaf MAR. Avaliação da ingestão total de flúor de crianças entre 4 e 7 anos de idade através da dieta e dentifício. *J Appl Oral Sci* 2003;11(2):150-6.