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DIFFERENTIAL EXPRESSION OF PROTEIN IN MUSCLE OF RATS TREATED WITH FLUORIDE AND EXERCISE

Mercedes Lombarte,[a] Brenda L Fina,[a] Aline Leite Lima,[b] Maela Lupo,[a] Marilia AR Buzalaf[b]

Rosario, Argentina, and Bauru, Brazil

ABSTRACT: Chronic ingestion of fluoride (F) has been reported to produce insulin resistance. Conversely, exercising (E) increases the peripheral response to insulin, enhancing the effect of the hormone on target tissues. Previous work has shown that E can reverse insulin resistance caused by ingesting F. Therefore, the objective of this work was: to study the pattern of protein expression in rats that reversed the insulin resistance induced by F when performing E. Healthy female Sprague-Dawley rats (50 days) were divided into 3 groups (n = 6 per group): Control: drinking water F 0 ppm, F: drinking water F 15ppm and FE: drinking water F 15ppm + E 30 min per day on a treadmill. The treatments were applied for 30 days, then the animals were euthanized and the gastrocnemius muscles were obtained for proteomic analysis. Proteins related to carbohydrate metabolism are less expressed in group F than in the control. In contrast, the decrease in carbohydrate metabolism proteins was not observed in rats treated with F and E. Conclusion: Physical exercise reverts the resistance to insulin induced by fluoride counteracting fluoride effects on proteins related to carbohydrate metabolism.

Keywords: Exercise; Muscle; Protein expression.

INTRODUCTION

Fluoride (F^-) enters the body spontaneously or as a therapeutic resource. Despite its anti-cariogenic activity, this ion can produce dental and/or skeletal fluorosis.[1–3] Deleterious effects of F^- are not limited to bones and teeth, several toxic effects on the endocrine system have been reported such as disorders of glucose homeostasis.[4] The administration of a dose of F^- causes a decrease of insulin levels, which was attributed to a decrease in insulin secretion through *in vitro* experiments.[5,6] On the other hand, the chronic intake of F^- generates hyperglycaemia with high plasma insulin levels.[7–9] Several countries like China,[10] India,[11] and Argentina[12–14] have drinking water with a high content of F^- that exceeds the limit recommended by the WHO (1.5mg/L).[15] The mechanism by which, F^- generates hyperglycaemia involves several factors, many of them were found in *in vitro* models using concentrations that are higher than physiological concentrations of fluoride in plasma.[4]

Previous works show that daily running on a treadmill for 60 min can reverse the insulin resistance in rats treated with 15 mg/L of F^- in drinking water for 30 days. The rats subjected to physical exercise also showed a decrease in plasma fluoride levels and an increase in the amount of fluoride in bone tissue. The authors concluded that the reduction of insulin resistance could be the consequence of the decrease in plasma F^- levels due to bone F^- uptake.[16] Moreover, exercise increase insulin-sensitive tissues increasing of glucose transporters GLUT4 expression in tissues.[17] There are, also, other works that reported a reduction of the toxic effect of F^- by doing moderate exercise.[18,19] Thus, the aim of this work was to evaluate the differential expression of proteins in muscle of rats treated with F^- that performed exercise.

[a]Bone Biology Laboratory, School of Medicine, Rosario National University. Santa Fe 3100, Rosario, Santa Fe, Argentina.

[b]Department of Biological Sciences, Bauru Dental School, University of São Paulo, Oct·vio Pinheiro Brisola, 9-75, Vila Universit·ria 17012-901 - Bauru, São Paulo, Brazil.

Corresponding author: Mercedes Lombarte. mercedeslombarte@gmail.com

MATERIALS AND METHODS

Animals: Experiments were carried out in 70-day-old female Sprague Dawley rats (body weight: 211±26 g). Rats were housed in collective cages with water and balanced food (Gepsa, Pilar, Córdoba, Argentina) *ad libitum*. During the experiments, rats were kept in a temperature-controlled environment of 23–25ºC, with a 12hr-12hr light-dark cycle and filtered airflow at scheduled time intervals. Rats were treated according to the accepted international standards for animal care.[20] This work has been approved by the ethical committee of the School of Medicine of Rosario National University. Rats were randomly divided into 3 groups (n=6 per group): Control: drinking water without F^-, F: drinking water with F^- 15 mg/L for 30 days and FE: daily running on a treadmill during 60 minutes at 2.25 m/min and drinking water with F^- 15 mg/L for 30 days.

At the end of the experiment, rats were euthanized 24 hr after the last exercise session and with 12 hr of fast. Rats were euthanized by CO_2 inhalation under profound anesthesia[21] and gastrocnemius muscles were extracted for proteomic analysis. Blood samples were obtained by heart puncture into heparinized tubes, which were centrifuged at 5000 rpm and plasma was immediately processed for glucose, insulin and fluoride measurement.[22]

Insulin resistance was evaluated by calculating HOMA-IR index (homeostasis model assessment-insulin resistance). This index is calculated as:

$$\text{HOMA-IR index} = \text{Fasting glucose level (mmol/L)} \times \frac{\text{Fasting insulin level (µUI/mL}}{22.5}$$

Where:

HOMA-IR index = Homeostasis model assessment-insulin resistance index

.The value of this index is interpreted as follows: when the value of HOMA-IR increases, insulin resistance augments, which means that insulin sensitivity, decreases.[23]

Biochemistry measurements: Plasma glucose levels were measured with a commercial kit (Wiener Laboratorios, Rosario, Argentina). Plasma insulin levels were measured by RIA (Ria kit Rat insulin, Millipore Corporation, Billerica, MA, USA). Plasma fluoride levels were measured by direct potentiometry using an ion selective electrode ORION 94-09, Orion Research (MS, USA) after isothermal distillation. Results are shown as Mean±Standard Error (SE) and they were compared with one-way ANOVA, and LSD post-test. Differences were considered significant when $p<0.05$. Statistical analyses were performed with R 2.14.1 software.[24]

Protein extraction/Sample preparation: The frozen tissue was homogenized in a cryogenic mill, model 6770 Freezer Mill (Spex, Metuchen, NJ, EUA). For the protein extraction, gastrocnemius muscle homogenate was processed according to previous protocols.[25] The protein concentration of each sample was measured by the Bradford protein assay.[26] After quantification, 1000 mg of muscle protein from each animal in a single test-group was pooled and submitted for proteomic analysis in triplicate, as described below.

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LC-MS/MS: The peptides identification was done on a nanoAcquity UPLC-Xevo QTof MS system (WATERS, Manchester, UK), using the PLGS software, as previously described.[25] Changes in relative protein abundance between the groups (A/B) were regarded as significant when the ratio was <0.5 for increase in abundance in B compared to A (B.A) or >1.5 for decrease in abundance in B compared to A (B.A), and a p-value<0.05. Identified muscle proteins were classified according to their biological processes using the software CYTOSCAPE 3.0.4 (JAVA) and Gene Ontology Consortium.

RESULTS

Plasma glucose, insulin, and fluoride level and HOMA-IR index are shown in Table 1. Fluoride increases the HOMA-IR index due to an increase in plasma insulin level; and exercise ameliorate this effect of fluoride.

Table 1. Effect of treatments on plasma variables. The table shows the mean±SE of groups and p value of one-way ANOVA for the studied variables. Different superscript letters in a row indicates significant differences, p<0.05

Variable/Factor	p	Control	Fluoride	Exercise
Plasma glucose level (mg/dL)	0.248	122.80±3.1^{a}	129.60±4.9^{a}	120.42.11±3.3^{a}
Plasma insulin level (pmol/L)	0.094	14.17±5.4^{b}	50.55±16.5^{a}	24.55±8.9^{ab}
Plasma fluoride level (µmol/L)	0.235	4.87±1.7^{a}	8.67±2.3^{a}	4.77±1.1^{a}
HOMA-IR index	0.069	0.59±0.2^{b}	2.28±0.7^{a}	0.99±0.4^{ab}

Quantitative proteomic analysis of muscle:

Two way differential analysis for relative protein abundance was performed with SIEVE software 1.3 (Thermo Fisher Scientific, San Jose, CA). Comparisons between control and F groups showed an increase in level of 24 proteins in F groups respect to control group, while 18 protein decreased in level in F group (Table 2). Comparisons of the fluoride treated groups showed an increase and decrease in the levels of 20 and 29 proteins, respectively, in the FE group compared to F group (Table 2).

Table 2. Comparison of relative protein abundance between different experimental groups

Comparison group A vs B	F vs Control	FE vs F	FE vs Control
Total number of protein identifier	348	358	368
Number of proteins with differential abundance	136	160	153
Number of proteins ratio A/B ≥ 1.5	24	20	18
Number of proteins ratio A/B ≤ 0.5	18	29	8

Proteins with differences in abundance in each comparison were classified according to their biological process. Proteins that increased their expression in F group than control group correspond mainly to proteins related to muscle morphogenesis and regulation of ATP synthase activity. On the other hand, proteins that decreased their expression due to fluoride treatment, correspond to proteins related to skeletal muscle contraction and carbohydrate catabolism.

When F group was compared with FE group proteins that increased their expression in FE group are proteins related to muscle contraction.

Finally, proteins that show a decrease in expression when F is combined with exercise (FE group) respect to treatment with only F belong to pyridine nucleotide metabolic process and ADP metabolic process; none of them related to the catabolism of carbohydrates. However, some proteins related to carbohydrates metabolism changed their expression (Table 3).

Treatment with fluoride increases the abundance of several proteins of glycolytic pathway such as glyceraldehyde-3-phosphate dehydrogenase and enolase (Table 4); and decrease the expression of others like glycogen phosphorylase. In other hand, when the treatment includes exercise glycogen phosphorylase and alpha-1,4 glucan phosphorylase increases their expression respect to fluoride treatment and pyruvate kinase, glyceraldehyde-3-phosphate dehydrogenase, fructose-bisphosphate aldolase, malate dehydrogenase, adenylate kinase and enolase decrease their abundance (Table 4). Finally, when expression of proteins of FE group is compared with control group any change in proteins of metabolism of carbohydrates are observed (data not shown).

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Table 3. Summary of identified proteins with differences in abundance in the comparison between FE group and F group. [a] Protein accession numbers from UniProtKB. [b] Protein name. [c]Ratio of the relative protein abundance between FE group and F group. Significant differences in protein abundance were considered when: a ratio ≤ 0.5 means increase in F group in relation to FE group, and a ratio ≥ 1 means increase in FE group in relation to F group.

Acession number[a]	Protein[b]	Ratio[c]
P09812	Glycogen phosphorylase, muscle form OS=*Rattus norvegicus* GN=Pygm PE=2 SV=5	1.4
P00489	Glycogen phosphorylase, muscle form OS=*Oryctolagus cuniculus* GN=PYGM PE=1 SV=3	1.6
G3V6Y6	Alpha-1,4 glucan phosphorylase OS=*Rattus norvegicus* GN=Pygb PE=3 SV=1	1.7
P53534	Glycogen phosphorylase, brain form (Fragment) OS=*Rattus norvegicus* GN=Pygb PE=1 SV=3	1.6
Q9ESV6	Glyceraldehyde-3-phosphate dehydrogenase, testis-specific OS=*Rattus norvegicus* GN=Gapdhs PE=1 SV=1	0.8
B1WBQ8	Glyceraldehyde-3-phosphate dehydrogenase OS=*Rattus norvegicus* GN=Gapdhs PE=2 SV=1	0.8
P05065	Fructose-bisphosphate aldolase A OS=*Rattus norvegicus* GN=Aldoa PE=1 SV=2	0.9
P04636	Malate dehydrogenase, mitochondrial OS=*Rattus norvegicus* GN=Mdh2 PE=1 SV=2	0.7
D3ZGY4	Glyceraldehyde-3-phosphate dehydrogenase OS=*Rattus norvegicus* GN=Gapdh-ps2 PE=3 SV=1	0.8
M0R660	Glyceraldehyde-3-phosphate dehydrogenase OS=*Rattus norvegicus* GN=RGD1565368 PE=3 SV=1	0.8
D4A3W5	Glyceraldehyde-3-phosphate dehydrogenase OS=*Rattus norvegicus* PE=3 SV=1	0.8
F1M9V3	Enolase (Fragment) OS=*Rattus norvegicus* GN=RGD1559534 PE=3 SV=1	0.6
M0R451	Glyceraldehyde-3-phosphate dehydrogenase OS=*Rattus norvegicus* PE=3 SV=1	0.8
F1M1E8	Glyceraldehyde-3-phosphate dehydrogenase (Fragment) OS=*Rattus norvegicus* PE=3 SV=2	0.7
M0R4B8	Pyruvate kinase OS=*Rattus norvegicus* PE=3 SV=1	0.7
D3ZYU0	Enolase OS=*Rattus norvegicus* PE=3 SV=1	0.7
P04797	Glyceraldehyde-3-phosphate dehydrogenase OS=*Rattus norvegicus* GN=Gapdh PE=1 SV=3	0.8
M0R590	Glyceraldehyde-3-phosphate dehydrogenase OS=*Rattus norvegicus* GN=LOC685186 PE=3 SV=1	0.8
E9PTN6	Glyceraldehyde-3-phosphate dehydrogenase OS=*Rattus norvegicus* GN=RGD1564688 PE=3 SV=1	0.8
M0RD14	Pyruvate kinase OS=*Rattus norvegicus* PE=3 SV=1	0.7
E9PTV9	Glyceraldehyde-3-phosphate dehydrogenase OS=*Rattus norvegicus* GN=RGD1562758 PE=3 SV=2	0.7
P11980	Pyruvate kinase PKM OS=*Rattus norvegicus* GN=Pkm PE=1 SV=3	0.7

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Table 4. Summary of identified proteins with differences in abundance in the comparison between F group and control group. [a]Protein accession numbers from UniProtKB. [b]Protein name. [c]Ratio of the relative protein abundance between F group and control group. Significant differences in protein abundance were considered when: a ratio ≤ 0.5 means increase in control group in relation to F group, and a ratio ≥ 1 means increase in F group in relation to control group.

Accession Number[a]	Protein[b]	Ratio[c]
D3ZYU0	Enolase OS=*Rattus norvegicus* PE=3 SV=1	1.2
E9PTN6	Glyceraldehyde-3-phosphate dehydrogenase OS=*Rattus norvegicus* GN=RGD1564688 PE=3 SV=1	1.3
Q9ESV6	Glyceraldehyde-3-phosphate dehydrogenase, testis-specific OS=*Rattus norvegicus* GN=Gapdhs PE=1 SV=1	1.3
F1M1E8	Glyceraldehyde-3-phosphate dehydrogenase (Fragment) OS=*Rattus norvegicus* PE=3 SV=2	1.3
M0R660	Glyceraldehyde-3-phosphate dehydrogenase OS=*Rattus norvegicus* GN=RGD1565368 PE=3 SV=1	1.3
M0R590	Glyceraldehyde-3-phosphate dehydrogenase OS=*Rattus norvegicus* GN=LOC685186 PE=3 SV=1	1.3
D3ZGY4	Glyceraldehyde-3-phosphate dehydrogenase OS=*Rattus norvegicus* GN=Gapdh-ps2 PE=3 SV=1	1.3
P04797	Glyceraldehyde-3-phosphate dehydrogenase OS=*Rattus norvegicus* GN=Gapdh PE=1 SV=3	1.33
M0R451	Glyceraldehyde-3-phosphate dehydrogenase OS=*Rattus norvegicus* PE=3 SV=1	1.43
F1M9V3	Enolase (Fragment) OS=*Rattus norvegicus* GN=RGD1559534 PE=3 SV=1	1.43
E9PTV9	Glyceraldehyde-3-phosphate dehydrogenase OS=*Rattus norvegicus* GN=RGD1562758 PE=3 SV=2	1.5
D4A3W5	Glyceraldehyde-3-phosphate dehydrogenase OS=*Rattus norvegicus* PE=3 SV=1	1.5
P09811	Glycogen phosphorylase, liver form OS=*Rattus norvegicus* GN=Pygl PE=1 SV=5	0.6
P53534	Glycogen phosphorylase, brain form (Fragment) OS=*Rattus norvegicus* GN=Pygb PE=1 SV=3	0.6
P00489	Glycogen phosphorylase, muscle form OS=*Oryctolagus cuniculus* GN=PYGM PE=1 SV=3	0.7
P09812	Glycogen phosphorylase, muscle form OS=*Rattus norvegicus* GN=Pygm PE=2 SV=5	0.7
P09811	Glycogen phosphorylase, liver form OS=*Rattus norvegicus* GN=Pygl PE=1 SV=5	0.6
P53534	Glycogen phosphorylase, brain form (Fragment) OS=*Rattus norvegicus* GN=Pygb PE=1 SV=3	0.6
P00489	Glycogen phosphorylase, muscle form OS=*Oryctolagus cuniculus* GN=PYGM PE=1 SV=3	0.7

DISCUSSION

Fluoride produces an increase in blood glucose because of the inhibition of insulin secretion when a dose is ingested; or resistance to the action of this hormone when it is ingested chronically.[4] Previous studies shown that physical exercise performance ameliorates the insulin resistance caused by fluoride,[16] because of improving muscle sensitivity to insulin.[27] Therefore, in this work, a proteomic analysis of the muscle of rats treated with fluoride and exercise was carried out, focusing this analysis on proteins related to carbohydrate metabolism. As presented in the results, it was observed that fluoride alters the expression of proteins related to the use of glucose, increasing the expression of glycolytic enzymes, and decreasing that of glycogen phosphorylase, the enzyme responsible for the degradation of glycogen. Fluoride inhibits enolase, increasing the requirement for 2-phosphoglycerate,[28,29] this effect joins to the secondary failure to regenerate NAD by pyruvate reduction and results in inhibition of the glyceraldehyde-3-phosphate dehydrogenase reaction with accumulation of fructose di-phosphate, dihydroxyacetone phosphate, and 3-phosphoglycerate, and no change in the concentration of 2,3-diphosphoglycerate. As consequence of this situation, one would expect glucose utilization to proceed up to the point at which the NAD depletion results in glycolytic obstruction and in stoichiometric depletion of ATP by hexokinase and phosphofructokinase.[30] In addition, it is known that fluoride is an inhibitor of the respiratory chain.[31] Ultimately, ATP becomes insufficient. This decrease in ATP levels could result in a stimulus for the synthesis of more glycolytic enzymes trying to compensate the inhibition of this pathway.[32] On the other hand, it is known that fluoride increases cAMP levels and thereby the degradation of glycogen is stimulated.[33] In this aspect the result at the level of the expression of this enzyme also seems to be contradictory. However it is possible that when the cell is stimulated this route saves energy by avoiding synthesizing more of this enzyme. This situation is in accordance to the increase in blood glucose observed because of fluoride treatment. On the other hand, in the case of the combined treatment of fluoride and exercise, the change in protein expression seems to indicate a normalization in the expression of the enzymes related to carbohydrate metabolism altered by the fluoride treatment. This statement is underscored by the lack of alteration in the expression of proteins related to carbohydrate metabolism in the FE group when compared to the control group. In this way we can conclude that physical exercise reverts the resistance to insulin induced by fluoride when counteracting the effects that this ion has on the metabolism of carbohydrates.

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