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IN POSTMENOPAUSAL OSTEOPOROSIS THE BONE INCREASING EFFECT OF MONOFLUOROPHOSPHATE IS NOT DEPENDENT ON SERUM FLUORIDE

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Abstract According to previous pharmacokinetic studies the bioavailability of fluorine (F) from sodium monofluorophosphate (MFP) doubles that of sodium fluoride (NaF). This paper reports a study designed to verify whether the vertebral bone mass increasing effect of NaF (30 mg F/day) was comparable to that of MFP (15 mg F/day), given for 18 months to osteoporotic postmenopausal women. The BMD of lumbar vertebrae of both groups showed significant increases (MFP: 60 ± 15 mg/cm^2, NaF: and 71 ± 12 mg/cm^2) over basal levels ($P < 0.001$). The difference between treatments was not significant ($P = 0.532$). The serum levels of ionic F (the mitogenic species on osteoblasts) were not related to the above mentioned effects. In NaF-treated patients, the fasting levels of total serum F increased significantly (6.7 ± 0.9 µM vs. Basal: 2.0 ± 0.8 µM; $P < 0.001$). This phenomenon was accounted for by ionic fluoride that increased over 20-fold (6.5 ± 1.9 µM vs. Basal: 0.3 ± 0.04 µM). In MFP-treated patients the fasting serum levels of total (7.0 ± 0.7 µM vs. Basal: 2.2 ± 0.9 M) and diffusible F (0.5 ± 0.02 µM vs. Basal 0.2 ± 0.02 µM) increased significantly ($P< 0.001$). The increase in the non diffussible F fraction is accounted for by protein-bound F, probably by the complexes formed between MFP and α2-macroglobulin and C3. Serum diffusible F was formed by two fractions: ionic F and F bound to low molecular weight macromolecule/s (2 200 ± 600 Da), in approximately equal amounts. The general information afforded by the present observations support the hypothesis that ionic F is released progressively during the metabolism of MFP bound to α2-macroglobulin and C3. These phenomena explain why comparable effects to those obtained with 30 mg F/d of NaF could by obtained with one half the dose of MFP.

Resumen ***El efecto del monofluorfosfato en la osteoporosis postmenopáusica no depende de la fluoremia.*** De acuerdo con estudios farmacocinéticos realizados previamente, el monofluorfosfato de sodio (MFP) tiene doble biodisponibilidad de flúor (F) que el fluoruro de sodio (NaF). Este trabajo describe un estudio destinado a comprobar si el aumento de la masa ósea de las vértebras lumbares L2-L4, obtenido con NaF (30 mg F/día) o MFP (15 mg F/día), después de 18 meses de tratamiento a mujeres osteoporóticas postmenopáusicas, eran comparables. La densidad mineral de las vértebras lumbares de ambos grupos mostraron aumentos significativos (MFP: 60 ± 15 mg/cm^2, NaF: and 71 ± 12 mg/cm^2) sobre los valores iniciales ($P < 0.001$). La diferencia entre tratamientos no fue significativa ($P = 0.532$). Las concentraciones de F iónico en el suero (la especie activa sobre los osteoblastos) no se encontraron relacionados con el efecto descripto mas arriba. En los pacientes tratados con NaF la fluoremia total en ayunas aumentó significativamente (6.7 ± 0.9 µM vs. Basal: 2.0 ± 0.8 µM; $P < 0.001$). Este aumento es producido por F iónico, cuya concentración aumentó más de 20 veces (6.5 ± 1.9 µM vs Basal: 0.3 ± 0.04 µM). En los pacientes tratados con MFP, las concentraciones de F sérico total (7.0 ± 0.7 µM vs Basal: 2.2 ± 0.9 µM) y difusible (0.5 ± 0.02 µM vs Basal 0.2 ± 0.02 µM) aumentaron significativamente ($P < 0.001$). El F sérico difusible está compuesto por dos fracciones: F iónico (fluoruro) y F ligado a péptidos de bajo peso molecular (2 200 ± 600 Da), en aproximadamente partes iguales. El aumento en la fracción de F sérico no difusible se explica por la aparición de F ligado a proteínas, probablemente los complejos formados entre MFP y α2-macroglobulina y C3. En conclusión, la información obtenida apoya la hipótesis que la degradación de los complejos MFP-α2-macroglobulina y MFPC3 suministra fluoruro iónico, sería la causa de la mayor biodisponibilidad de F del MFP respecto de la de NaF y posibilita la reducción de la dosis terapéutica de F.

Key words: monofluorophosphate, serum F (ionic, protein bound), bone mineral density, osteoporosis, α2-macroglobulin

Fluorine (F) is used for the treatment of idiopathic and postmenopausal osteoporosis. Sodium monofluoro-phosphate (MFP) and sodium fluoride (NaF) are the salts commonly employed by the pharmaceutical industry. With the latter drug, vertebral fracture rate decreased as bone mass density (BMD) increased, provided that patients with high (toxic) serum fluoride levels were not included in the comparison[1, 2]. On the other hand, a preliminary report of the FAVO Study[3] stated that a F+Ca+Vit.D regimen was

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not more effective than Ca+Vit.D supple-ments for the secondary prevention of vertebral fractures.

Recent work from this laboratory has shown that a fraction of each dose of MFP is absorbed without hydrolysis and binds to serum α2-macroglobulin and C3[4, 6]. This protein-bound F compartment is assumed responsible for the greater bioavailability of F of MFP, compared with NaF[6, 7]. The latter drug does not bind to serum proteins[4, 8].

The pharmacodynamic trial reported in this paper was designed to confirm, in human beings, the prediction of previous pharmacokinetic experiments, namely, that similar increases in vertebral bone mass would be obtained with 30 mg F/day of NaF or with 15 mg F/day of MFP. In addition, the presence of F species (diffusible and protein-bound) in the serum of treated subjects agreed with those observed in previous papers[4, 8].

Material and Methods

Subjects. This report contains information obtained during an investigation approved by the Ethics Committee of the Medical School of Rosario. The subjects for this study were women 49-64 years, attending the Menopause Clinic. Patients complaining of backache, with one or more vertebral osteoporotic fractures[10] were invited to participate in this study. Vertebrae were defined as fractured when they had a reduction of at least 25% in anterior height as compared with posterior height. The BMD at the lumbar spine was measured in vertebrae L2-4, anterior-posterior projection, using DXA with a Norland XR-26 ins-trument. Pre-cision of technique was 2%. Variance coefficient of measurements of the calibration standard during the study was 0.1%.

Additional inclusion criteria were: creatinine clearance greater than 80 ml/min, hematocrit greater than 40%, hemoglobin greater than 12 g/dL, absence of secondary osteoporosis, other metabolic diseases, gastric ulcer or treatment with drugs affecting bone metabolism.

Duration of this study was 18 months. Participants were trea-ted (according to a table of randomization) with sodium fluoride (2 x 15 mg F/day) or sodium monofluorophosphate (2 x 7.5 mg of F/day). Daily (spontaneous) calcium intake was 370-450 mg/day. A supplement of 600 mg Ca/day was indicated (see below).

The MFP tablets (CASASCO SAIC, plain,without enteric coating) contained 57 mg of the drug (7.5 mg of elemental F) and 625 mg of calcium carbonate (250 mg of elemental calcium). Patients were instructed to take a tablet with each lunch and dinner.

NaF tablets (Pharmacy of the University Hospital, plain, without enteric coating) contained 33 mg of the salt (15 mg of elemental F). Patients were instructed to take a tablet 2-3 hours before each lunch and dinner. A tablet containing 625 mg of calcium carbonate was taken with each lunch and dinner.

BMD at the lumbar spine was measured before and at 18 months of treatment. The levels of total and diffusible serum F (see below) and the daily urinary F excretions were measured before treatment and at three month intervals. Blood was drawn in the fasting state (ca. 10 hours after drug intake).

Chromatography of serum. Aliquots of serum (200 µl) from untreated, NaF or MFP-treated subjects (n = 6 per group) were chromatographed on Sephadex G-50 (Pharmacia, Uppsala, Sweden) suspended in PBS (saline containing 50 mM phosphate, pH 7.4). These experiments were done to figure out the serum F-containing species: high and low molecular weight polypeptides and ionic fluoride. Assay of fractions, calibration of the column and estimation of molecular weight were carried out as described elsewhere[5, 9].

Fluoride measurements. The F contents of whole serum aliquots, serum ultrafiltrates or chromatographic fractions were measured by isolation of F through the isothermal distillation technique of Taves[11]. A known volume of the sample (100-1 000 µl) was mixed with 100 µl of 6.0 N hydrochloric acid saturated with hexamethyldisiloxane, in the distillation chamber. The fluoride was distilled into the alkali trap (20 µl of 1.65 M NaHO) for five days at room temperature. A known amount (usually 20 µl) of 2.5 M acetic acid was then added to the dry trap container to dissolve the residue. The solution had a pH of 5.5. A standard curve was prepared with every run, distilling aliquots of NaF standards.

Fluoride was measured in the distillate with an ion-specific electrode (94-09, Orion Research Inc., Cambridge MA, USA). A millivoltimeter with gain setting of x 10 was employed ensuring a minimum reproducibility of ± 0.2 mV, recommended in USP XXII[12] for measurement of fluoride concentrations between 1 and 10 µM. Electrodes were assembled as stated by Hallsworth et al.[13] to measure small volume (20 µl) samples.

To measure diffusible F, the ultrafiltrate of serum was obtai-ned by centrifugation at 1 000 g for 15 minutes through Ultrafree-MC centrifugal filter units (Millipore Corp.) with a 30 000 molecular weight cut off. The ultrafiltrate was processed as indicated above.

Distillation of whole serum allows to measure its total F content. The difference between total and diffusible F is assumed to measure protein-bound F. As showed elsewhere[5, 7], in MFP-treated subjects or rats, this fraction most probably measures the complexes of MFP with α2-macroglobulin and C3.

Urinary F was measured directly in urine with the fluoride electrode according to manufacturer instructions. Data are expressed as µmoles of F per day.

Statistical methods. Student's "t" tests for paired data and one way variance analyses were used for the assessment of the data[14].

Results

Treatment with MFP (N = 16) or NaF (N = 12) increased BMD of lumbar vertebrae by 60 ± 15 and 71 ± 12 mg/cm^2 (8 and 9.5% over basal levels, respectively). Both responses differ significantly from zero ($P < 0.001$), but not between themselves ($P = 0.532$).

Two patients in each of the NaF or MFP groups had negative (final-initial) BMD L2-4 values and were excluded in the calculation of BMD response reported in Tables 1 and 2. They were regarded as "non responders" to F therapy. Their serum F levels were not different from those from patients that showed increased BMD at their lumbar spine. Mild gastrointestinal side effects were reported by two patients in each group in whom treatment was suspended for two weeks and reassumed afterwards.

As expected, fasting serum F levels increased in NaF and MFP treated subjects (Tables 1 and 2, $P < 0.001$ respect to basal levels). In MFP-treated subjects, the phenomenon was accounted for by the 3-fold increase in protein-bound and diffusible fractions. In the NaF treated group,

TABLE 1.– *Metabolic variables determined along 18 months of study in postmenopausal women treated with MFP (n = 16)*

	Basal	3-6 months	9-12 months	18 months
BMD L2-4 mg/cm^2	735 ± 50	n.m.	n.m.	815 ± 50
Total serum F, µM	2.2 ± 0.9	6.5 ± 1.3	6.6 ± 2.1	7.9 ± 1.9
Diffusible serum F, µM	0.2 ± 0.03	0.5 ± 0.03	0.6 ± 0.03	0.4 ± 0.03
Urinary F µmoles/day	20 ± 4	118 ± 30	124 ± 32	102 ± 30

The figures indicate mean ± SEM
n.m.: not measured

TABLE 2.– *Metabolic variables determined along 18 months of study in postmenopausal women treated with NaF (n = 12)*

	Basal	3-6 months	9-12 months	18 months
BMD L2-4 mg/cm^2	745 ± 60	n.m.	n.m.	789 ± 75
Total serum F, µM	2.2 ± 0.8	7.4 ± 1.2	6.1 ± 2.2	6.6 ± 3.0
Diffusible serum F, µM	0.3 ± 0.04	7.5 ± 3.2	5.5 ± 4.0	6.4 ± 2.6
Urinary F µmoles/day	25 ± 8	220 ± 41	180 ± 33	252 ± 45

The figures indicate mean ± SEM
n.m.: not measured

on the other hand, total and diffusible serum fluoride were not significantly different, showing that the increase in the former fraction was accounted for by the latter.

These findings were confirmed by chromatography of serum aliquots from untreated, and NaF or MFP-treated subjects (Figure 1). The serum obtained in the fasting state from subjects before treatment, revealed small amounts of F bound to high molecular weight proteins and even smaller amounts of ionic fluoride. In MFP treated subjects, F was found associated to high (peak at 1-2 ml) and low molecular weight macro-molecules (peak at 6-7 ml, 2 200 ± 600 Da.) as well as ionic fluoride (peak at 12 ml). The two latter fractions are below the molecular weight cut off of the ultrafiltration membrane used and are reported (summed) as "diffusible F" in Table 1. The pattern obtained with the sera from NaF-treated subjects confirmed that the increase in F concentration was due to ionic fluoride.

Urinary F was measured to check the adherence of patients to their treatment. All patients increased their basal urinary excretion of F ($P < 0.001$).

Discussion

Effect of F on spinal BMD. A number of papers have been published reporting an increase in vertebral BMD after NaF (25-36 mg of elemental F/day[14-16]) or MFP treatment (20-38 mg F/day[17-22]).

Pharmacokinetic studies done in this laboratory with rats[6] and human volunteers[7] showed that the bioavaila-bility of F of MFP was twice that of NaF. The difference is the consequence of the metabolic characteristics of this drug[4, 6]. It is not related to absorption as shown in the rat[4] and confirmed in this work by the urinary F excretion. The latter variable rose in proportion to the daily dose of F.

The trial reported in this paper was designed to check the prediction of pharmacokinetic studies. We report that the effect on spinal BMD obtained with 30 mg F/day (as NaF) was not significantly different to that obtained with one half the dose of MFP.

The F species in serum. In untreated subjects with low spontaneous F intake, serum F is composed of two fractions: ionic fluoride, and F associated to high molecular weight molecules (non ionic, not diffusible F)[8]. The former species has mitogenic activity on osteoblasts[23] and is assumed to be responsible for the increase in bone mass produced by chronic administration of fluoride. The non ionic F fraction, associated to high molecular weight molecules, is usually assumed to be deprived of biological activity. This fraction, however, has not yet been characterized in terms of its chemical nature, sources, biological significance or fate[8].

In MFP treated subjects, the increase in serum F derives mainly from F bound to proteins and peptides. A fraction of each MFP dose is absorbed without hydrolysis and binds to serum α2-macroglobulin and C3[4-6]. As shown in rat studies[9], these protein complexes are uptaken

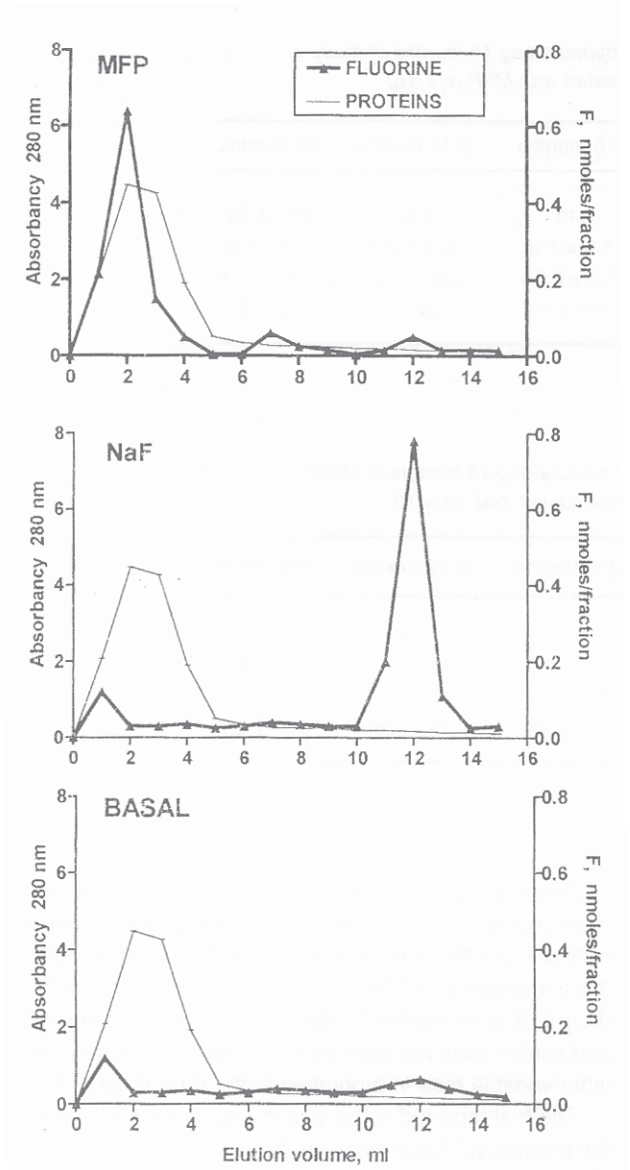

Fig. 1.– Patterns of F species in the sera of untreated patients (Basal) and patients treated with MFP or NaF. The points are the mean of six different sera. Error bars have been omitted to avoid clutter. See text for further details.

by liver and bone cells, degraded by intracellular proteases and recirculated as F-bound peptide/s. The serum diffusible F fraction is composed of approximately equal amounts of ionic fluoride and F bound to polypeptide/s of 2 200 ± 600 Da. The latter is confirmed in this report.

In agreement with reports showing that ionic F does not bind to serum proteins[8], the serum F of NaF treated subjects increased only as ionic fluoride.

It is often assumed that the bone mass increasing effect of fluoride therapy is related to changes in serum F[24, 25]. According to present data, this assumption is not sustained. The notion that increased serum fluoride is not required for bone mass increase had been imposed to us by experiments reported elsewhere[6]. In rats chronically treated with NaF or MFP dissolved in the water supply (which implies very low rates of intake and absorption), bone mass increased without significant changes in serum F. It is concluded that this bone increasing effect is not reflected or directly dependent upon serum F levels.

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References

1. Riggs BL, Melton LJ IIIrd., O'Fallon WM. Drug therapy for vertebral fractures in osteoporosis: evidence that decreases in bone turnover and increases in bone mass both determine antifracture efficacy. *Bone* 1991; 18: 3 (Suppl) 197S-201S.
2. Reginster JY, Zegels B, Meurmans L, et al. Monofluorophosphate decreases vertebral fracture rate in postmenopausal osteoporosis: a randomized placebo-controlled, double blind study. *Osteoporosis Int* 1996; 6 (Suppl 1): 239.
3. Meunier PJ, Sebert JL, Reginster JY, et al. Fluoride salts are no better at preventing new vertebral fractures than calcium-vitamin D in postmenopausal osteoporosis: the FAVOStudy. *Osteoporosis Int* 1998; 8: 4-12.
4. Rigalli A, Cabrerizo M, Beinlich AD, Puche RC. Gastric and intestinal absorption of monofluorphosphate and F in the rat. *Arzneim Forsch/Drug Res* 1994; 44: 651-5.
5. Rigalli A, Estebal L, Pera L, Puche RC. Binding of monofluorophosphate to α2-macroglublin and C3. *Calcif Tissue Int* 1997; 60: 86-9.
6. Rigalli A, Ballin JC, Beinlich A, Alloatti R, Puche RC. Phar-macokinetic differences between sodium fluoride and sodium monofluorophosphate and comparative bone mass increasing activity of both compounds in the rat. *Arzneim Forsch/Drug Res* 1994; 44: 762-6.
7. Rigalli A, Morosano M, Puche RC. Bioavailability of F administered as sodium fluoride or sodium monofluorophosphate to human volunteers. *Arzneim Forsch/Drug Res* 1996; 46: 531-3.
8. Whitford G.M. The metabolism and toxicity of fluoride. Basel Karger 1989, p 21.
9. Esteban L, Rigalli A, Puche RC. Metabolism of the complex sodium monofluorophosphate-α2-macroglobulin in the rat. *Medicine (Buenos Aires)* 1999; 59: 151-6
10. Puche RC, Morosano M, Masoni M, et al. The natural history of kyphosis in postmenopausal women. *Bone* 1996; 17: 239-46.
11. Taves D. Separation of F by rapid diffusion using hexametildisiloxane. *Talanta* 1968; 15: 969-74.
12. The United States Pharmacopeia. The National Formulary, United States Pharmacopeial Convention Inc., Rockville, USA 1990.
13. Hallsworth AS, Weatherell JA, Deutsch D. Determination of subnanogram amounts of fluoride with electrode. *Anal Chem* 1976; 48: 1660-4.
14. Snedecor WG, Cochran GW. Statistical Methods. Ames, IA: Iowa State University Press, 1966.
15. Mamelle N, Dusan R, Martin JL, et al. Risk-benefit of sodium fluoride treatment in primary vertebral osteoporosis. *Lancet* 1998; 13: 361-5.
16. Riggs BL, O'Fallon WM, Lane A., et al. Clinical trial of

fluo-ride therapy in postmenopausal osteoporotic women: extended observations and additional analysis. *J Bone Min Res* 1994; 9: 265-75.
17. Delmas PD, Dupuis J, Duboeuf F, et al. Treatment of vertebral osteoporosis with disodium monofluorophosphate: comparison with sodium F. *J Bone Min Res* 1990; 5 (Suppl 1): S143-S147.
18. Ringe JD. Monofluorophosphate prevents fractures in postmenopausal osteoporosis. *In:* Genazzani AR, Petraglia, F (eds), Current Research in Gynecology and Obstetrics. Carnforth: Parthenon Publ, 1991; 533-8.
19. Bröll H, Peichl P, Wilfert H. Effect of sodium monofluorophosphate and calcium on bone density and fracture rate in post osteoporosis. *Lancet* 1990; 8: 1446-7.
20. Affinito P, Di Carlo C, Primizia M, et al. A new F preparation for the prevention of postmenopausal osteoporosis: calcium monofluorophosphate. *Gynecol Endocrinol* 1993; 7: 201-9.
21. Rizzoli R, Chevalley T, Slosman DO, et al. Sodium monofluorophosphate increases vertebral bone mineral density in patients with corticosteroid-induced osteoporosis. *Osteoporosis Int* 1995; 5: 39-46.
22. Sebert JL, Richard PR, Mennecier I, Bisst JP, Loeb G. Mo-nofluorophosphate increases lumbar bone density in osteopenic patients: a double-masked randomized study. *Osteoporosis Int* 1995; 5: 108-14.
23. Lau KH, Farley JR, Freeman TK, Baylink DJ. A proposed mechanism of the mitogenic action of fluoride on bone cells: inhibition of the activity of an osteoblastic acid phosphatase. *Metabolism* 1989; 38: 858-68.
24. Taves DR. New approach to the treatment of bone disease with fluoride. *Fed Proc* 1970; 29: 1185-7.
25. Baylink DJ. Serum fluoride Levels. *In*: MJ Favus (ed) Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism. 2nd ed., New York: Raven Press 1993; 262-3.

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... pero admita que aunque Cristo no fuera más que una gran leyenda, el hecho de que esta leyenda haya podido ser imaginada y querida por estos bípedos sin plumas que sólo saben que nada saben, sería tan milagroso (milagrosamente misterioso) como el hecho de que el hijo de un Dios real fuera verdaderamente encarnado. Este misterio natural y terreno no cesaría de turbar y hacer mejor el corazón de quien no cree. Por ello considero que en sus puntos fundamentales, una ética natural –respetada en la profunda religiosidad que la anima– puede salir al encuentro de los principios de una ética fundada sobre la fe en la trascendencia, la cual no deja de reconocer que los principios naturales han sido esculpidos en nuestro corazón sobre la base de un programa de salvación. Si quedan, como lógicamente quedarán, ciertos márgenes irreconciliables, no serán diferentes de los que aparecen en el encuentro entre religiones distintas. Y en los conflictos de la fe deben prevalecer la Caridad y la Prudencia.

Umberto Eco

? En qué creen los que no creen? Un diálogo sobre la ética en el fin del milenio. Umberto Eco, Carlo María Martini (Obispo de Milán), Buenos Aires: Planeta, 1998, p 97