

FLUORIDE INHIBITION OF OXYGEN CONSUMPTION AND INCREASED OXIDATIVE STRESS IN RATS

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SUMMARY: The aim of this work was to evaluate the effect of fluoride (F) on oxygen consumption (VO_2) in rats and how it might affect the respiratory chain and the levels of reactive oxygen species (ROS). Eighteen Sprague-Dawley rats were divided into three groups: Control, NaF20, and NaF40, which received 0, 20, and 40 $\mu\text{mol F}/100\text{ g bw/day}$ for 30 days, respectively. *In vivo*, VO_2 decreased 90 min after treatment with F, whereas TBARS, CAT, and GPx were higher. *In vitro*, F decreased VO_2 in liver and in mitochondria. These results support the hypothesis that the bone inflammatory foci observed in treatments with NaF could involve an inhibition of the respiratory chain that would cause increase in reactive oxygen species (ROS).

Keywords: Liver; Fluoride and oxygen in rats; Mitochondria; Oxidative stress; Rat oxygen consumption; Reactive oxygen species.

INTRODUCTION

Recently, we found an unforeseen result in the form of bone inflammatory foci from treatment of rats with NaF, which could help account for the lack of the expected effect of fluoride (F).¹ This finding agrees with the fact that F causes an increase in levels of reactive oxygen species (ROS) and decreases antioxidant enzymes in osteoblast cultures.^{2,3} It is also known that the onset of ROS production and inflammatory response occur in mitochondria, where the key parameter of mitochondrial respiratory chain activity is the rate of oxygen consumption (VO_2). The overall objective of this work, therefore, was to determine the effect of F on VO_2 and oxidative stress in rats *in vivo*, and, *in vitro*, on their liver and mitochondria with F concentrations found in plasma after an oral dose of F.

MATERIALS AND METHODS

The Bioethics Committee of the School of Medicine, Rosario National University, Argentina, approved the design of the experiments, which were conducted in accordance with the *Guide to the care and use of experimental animals*.⁴

Eighteen 21-day-old female Sprague-Dawley rats from the School of Medicine, National University of Rosario, were divided into three groups of six and were given the following individual oral gavage treatments: (1) Control: distilled water; (2) NaF20: 20 $\mu\text{mol NaF}/100\text{ g bw/day}$; (3) NaF40: 40 $\mu\text{mol NaF}/100\text{ g bw/day}$.

After 30 days, 24-hr urine samples were collected, blood was obtained by heart puncture,⁵ euthanasia was performed by carbon dioxide inhalation,⁶ and liver tissues were collected. Fluoremia, fluoruria,⁷ TBARS,⁸ glutathione peroxidase,⁹ and catalase¹⁰ were measured by standard procedures as cited.

The following different kinds of experiments were carried out to investigate the effect of F on VO_2 and oxidative stress:

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(a) The effect of F on VO_2 was measured *in vivo*, both before and 90 min after the first F dose in the three groups. Simultaneously, fluoremia was measured. Plasma fluoride levels after the first dose of NaF were $120.0 \pm 17.22 \mu\text{mol/L}$. Taking these values into consideration, F concentrations between 0 and $100 \mu\text{mol/L}$ were used to assess the effect of F on *in vitro* VO_2 .

(b) The effect of the addition of 0, 10, 50, and $100 \mu\text{mol NaF/L}$ on VO_2 was tested *in vitro* in liver from control rats.

(c) The effect of $100 \mu\text{mol NaF/L}$ on VO_2 in mitochondrial states 3 and 4 was measured in liver-isolated mitochondria from the control rats.

(d) The effect of $100 \mu\text{mol NaF/L}$ on the activity of respiratory chain complexes was tested.

(e) The chronic effect of F on the oxidative stress was evaluated by the activities of GPx and CAT and the levels of TBARS in plasma and in isolated mitochondria.

VO_2 was measured with a Clark-type electrode (Gilson, Middleton, USA). *In vivo* VO_2 measurement was carried out for 10 min to obtain basal VO_2 . Then each rat received the F dose and VO_2 was measured again after 90 min. *In vitro* VO_2 was measured in liver¹¹ after the addition of NaF at 10, 50, and $100 \mu\text{mol/L}$. Isolated mitochondria were obtained and resting mitochondrial VO_2 (state 4) and active VO_2 (state 3) were measured¹² before and after the addition of $100 \mu\text{mol NaF/L}$. Respiratory control was calculated as the relationship between state 3 respiration and state 4 respiration. Total protein concentration was measured with a commercial kit (ProtiU/LCR, Wiener Lab, Rosario, Argentina).

Activity of mitochondrial complexes was measured in submitochondrial membranes with and without the addition of NaF up to a final concentration of $100 \mu\text{mol NaF/L}$. Determination of the activities of NADH-cytochrome c reductase (complex I–III) and succinate-cytochrome c reductase (complex II–III) was based on the reduction of cytochrome c^{3+} to cytochrome c^{2+} . Determination of the activity of cytochrome oxidase (complex IV) was based on the oxidation of cytochrome c^{2+} to cytochrome c^{3+} .¹³

Statistical analysis: Differences among more than two groups were analyzed with One-way Analysis of Variance (ANOVA) and comparison of each variable overtime was performed with the Repeated Measures ANOVA test. In both cases, differences among means were analyzed with Bonferroni test. Unpaired or paired Student's t test were used to compare two independent or dependent samples, respectively. Differences were considered significant if $p < 0.05$.

RESULTS

In vivo oxygen consumption: The NaF20 group had a slight decrease in VO_2 that became significant in the NaF40 group (Table 1).

In addition, a significant negative linear correlation between *in vivo* VO_2 and fluoremia was observed. Pearson correlation test, $p = 0.0187$, $r = 0.427$.

In vitro oxygen consumption: The addition of F significantly decreased liver VO_2 (Figure 1).

Table 1. *In vivo* VO₂ (μmol O₂/min 100 g bw) of experimental groups at 0 (basal) and 90 minutes after a NaF orogastric dose. Values are mean ± SEM, n=6.

	Control	NaF20	NaF40
Basal	205.9 ± 42.23	181.1 ± 17.08	175.8 ± 15.67
90 min	206.6 ± 42.94	174.8 ± 15.91	156.0 ± 16.62*

*Significant differences with respect to basal VO₂; paired Student's t test, p=0.0004.

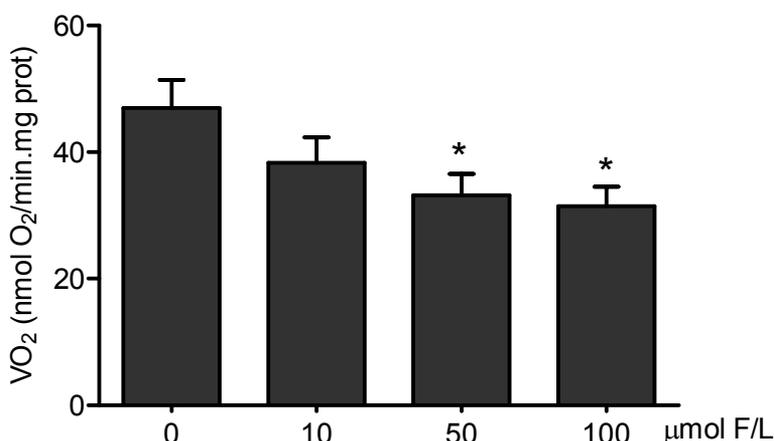


Figure 1. *In vitro* liver VO₂ after addition of different concentrations of F. *Significant differences compared to 0 μmol/L of F. Repeated Measures ANOVA, Bonferroni's Multiple Comparison post-test, n = 12, p = 0.0078.

The addition of 100 μmol NaF/L caused a significant decrease in mitochondrial respiration, both in state 4 and state 3 (Figure 2).

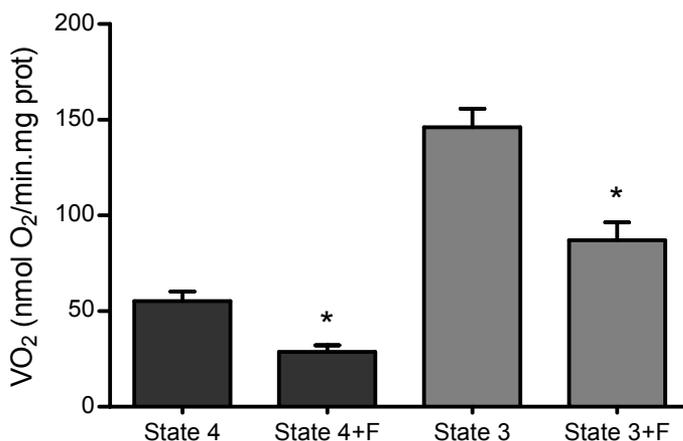


Figure 2. Fluoride effects on VO₂ of liver mitochondria in states 4 and 3 before and after adding 100 μmol NaF/L. *Significant differences of state 4+F and state 3+F compared to state 4 and state 3, respectively. Paired Student's t test, n = 12, p<0.0001.

The Respiratory Control confirmed a good coupling of the isolated mitochondria (RC = 2.58 ± 0.20).

Furthermore, there was a significant decrease of 9.76% in the activity of complex I-III after the addition of 100 $\mu\text{mol F/L}$. This effect was not observed in the activities of complex II-III and cytochrome c oxidase (Table 2).

Table 2. Activities of complex I-III, complex II-III ($\mu\text{mol/min mg prot}$) and complex IV ($\text{min}^{-1}/\text{mg prot}$) without and with addition of 100 $\mu\text{mol NaF/L}$. Values are mean \pm SEM, n=6.

	$\mu\text{mol NaF/L}$	
	0	100
Complex I-III	1906.0 \pm 125.0	1720.0 \pm 71.3*
Complex II-III	225.6 \pm 12.9	261.6 \pm 58.4
Complex IV	77.3 \pm 16.5	72.2 \pm 12.0

*Significant differences compared to 0 $\mu\text{mol/L}$ of F; paired Student's t test, p=0.0433.

Biochemical determinations: Mitochondrial CAT, GPx, and TBARS increased significantly in the NaF-treated groups by the end of the treatment. Moreover, the NaF-treated rats showed a significant increase in plasma TBARS and GPx (Table 3).

Table 3. Plasma and mitochondrial (Mit) TBARS, GPx, and CAT of experimental groups expressed as percentage of control group. Values are mean \pm SEM, n=6.

	TBARS		GPx		CAT	
	Plasma	Mit	Plasma	Mit	Plasma	Mit
Control	100 \pm 8	99 \pm 6	100 \pm 6	100 \pm 11	undetectable	100 \pm 12
NaF20	126 \pm 15	95 \pm 2	114 \pm 4*	98 \pm 2	undetectable	125 \pm 6*
NaF40	143 \pm 13*	106 \pm 2*	127 \pm 13*	117 \pm 4**	undetectable	135 \pm 5**

*Significant differences from 100%; unpaired Student's t test, *p<0.05, **p<0.005.

A significant increase in 24-hr F urinary excretion ($\mu\text{mol F/24 hr}$) in NaF-treated groups confirmed that NaF treatments were properly applied: Control 1.86 ± 0.531 ; NaF20 $5.90 \pm 0.955^*$; NaF40 $6.94 \pm 1.286^*$. The significant differences are based on comparison to the control group, ANOVA, and Bonferroni's Multiple Comparison post-test (p=0.0053).

DISCUSSION

Bone inflammation has been observed in rats treated with NaF.¹ Inflammation could be the consequence of an increase in oxidative stress due to an increase in ROS or a decrease in antioxidant mechanisms. This paper gives evidence for the

effect of F on tissue VO_2 with the concentrations found in plasma and tissues *in vivo* where F concentrations are below $100 \mu\text{mol/L}$.¹⁴ Such concentrations can be found after the intake of water with high levels of F.

Administration of an oral F dose caused a decrease in VO_2 , agreeing with the decrease in VO_2 in liver and isolated mitochondria and, with the inhibition of the activity of respiratory chain complex I-III after the addition of F. Finally, mitochondrial CAT and GPx activities, together with TBARS levels, increased significantly in the NaF-treated groups. Although change in the plasma activity of CAT was undetectable, plasma TBARS levels and plasma GPx activity were higher in those animals treated with F. These results indicate an increase in both tissue damage and oxidative stress in treated groups. Moreover, after each dose, F momentarily inhibited the respiratory chain, thereby increasing the release of superoxide anions, which would increase both ROS and oxidative stress and may be the cause of the inflammatory foci observed in the bone.

In agreement with our findings, the production of ROS induced by F has been shown to be reversed by the addition of respiratory chain inhibitors.¹⁵ As complex I and III are the main sites of superoxide anion synthesis, F could enhance its production by inhibiting the mitochondrial activity at complex I level.

Finally, the stimulating role of F on the formation of ROS, which is independent of the respiratory chain, should not be overlooked. In their formation, ROS also consume oxygen, but the available evidence indicates the release of ROS accounts for only 0.1–0.2% of the oxygen consumed by mitochondrial respiration.¹⁷ In our study, we have demonstrated F inhibition of VO_2 in systems of different complexity (whole animal, animal tissues, mitochondria, and submitochondrial membranes), but the inhibition of VO_2 in isolated mitochondria and in complex I in submitochondrial membranes clearly shows that F affects the respiratory chain.

ACKNOWLEDGEMENTS

We thank Eugenia Da Ros and Nicolas Rigalli for technical assistance. This research was presented in part at the XXXth Conference of the International Society for Fluoride Research, Advances in Fluoride Research, September 5–8, 2012, Szczecin, Poland.¹⁶ We are grateful for funding by Consejo Nacional de Investigaciones Científicas y Técnicas Grant 046.

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