G. NAPPI[1], S. DE LUCA[1], P. MAGRASSI[1], S. BENEDETTI[2], M. COCCHI[3], R. MATTINA[1]

Effects of oral administration of the "LETE" mineral water on Reactive Oxygen Metabolites (ROM) and on Biological Antioxidant Potential (BAP)

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TITOLO
Effetti del consumo di acqua minerale "LETE" sui metaboliti reattivi dell'ossigeno (ROM) ed il potenziale biologico antiossidante (BAP)

KEY WORDS
Natural mineral waters, oxidative stress, reactive oxygen metabolites, antioxidant barrier

PAROLE CHIAVE
Acque minerali naturali, stress ossidativo, metaboliti reattivi dell'ossigeno, barriera antiossidante

[1] Centro Studi e Ricerche sulle Acque Minerali, Università degli Studi di Milano
[2] Dipartimento di Scienze Biomolecolari, Sezione di Biochimica Clinica, Università degli Studi di Urbino "Carlo Bo"
[3] Facoltà di Medicina Veterinaria, Alma Mater Studiorum, Università di Bologna

Indirizzo per la corrispondenza:
Prof. Giuseppe Nappi
Centro Studi e Ricerche sulle Acque Minerali, Università degli Studi di Milano
Via Cicognara 7, 20129 Milano
Tel. 02.503.18440
Fax 02.503.18448
E-mail: giuseppe.nappi@unimi.it

Summary
Previous investigations led to recognize the anti-radical actions belonging to some natural spring waters. This study was performed in order to investigate the potential antioxidant activity of a natural mineral water effervescent natural, slightly acid, calcic, containing bicarbonate and called "Lete" [Pratella (CE), Italy]. Subjects (n = 61) were submitted for 15 days to hydric supplementation with 2 litres of LETE water with a standardized protocol. Before and after treatment (day 1 and day 15) plasma levels of reactive oxygen metabolites (ROM) and of antioxidant barrier (BAP) were determined. Both parameters demonstrated a statistically significant ($p<0.05$) improvement after treatment. In particular, marked improvements were observed in 24 of 47 subjects who showed significant alterations of ROM values before treatment, and in 15 of them ROM values returned to within normal range. In addition, 20 out of 26 with BAP deficit before treatment improved after treatment: in 15 of them BAP values returned to within normal limits. This study demonstrates that natural mineral waters, when administered in appropriate quantities and modalities, show anti-oxidant actions in the human body. These results are to be considered indicative, but further studies are needed to define mechanisms of action.

Riassunto
Come osservato in letteratura, alcune acque di sorgente naturale possono presentare un'azione anti-radicalica. Questo studio è stato condotto al fine di indagare la potenziale attività antiossidante di un'acqua minerale naturale effervescente, leggermente acida, calcica, contenente bicarbonato, chiamata "Lete" [Pratella (CE), Italia]. I soggetti coinvolti nella ricerca (n = 61) sono stati sottoposti per 15 giorni a supplementazione con 2 litri di acqua "Lete" secondo un protocollo standardizzato. Prima e dopo il trattamento (giorno 1 e giorno 15) sono stati determinati i livelli plasmatici dei metaboliti reattivi dell'ossigeno (ROM) e della barriera antiossidante (BAP). Per entrambi i parametri è stato evidenziato un miglioramento statisticamente significativo ($p <0,05$) dopo il trattamento. In particolare, i miglioramenti sono stati osservati in 24 dei 47 soggetti che presentavano alterazioni significative dei valori di ROM prima del

trattamento, in 15 di essi i livelli di ROM sono rientrati entro i valori normali. Inoltre, in 20 su 26 soggetti con deficit di BAP prima del trattamento si è evidenziato un miglioramento dopo il trattamento; in 15 di essi i valori di BAP sono rientrati entro i limiti normali. Questo studio dimostra che le acque minerali naturali, se somministrate in quantità e con modalità adeguate, possono avere azione antiossidante all'interno dell'organismo. Questi risultati sono da ritenersi indicativi, ma ulteriori studi sono necessari per definire i meccanismi di azione.

Introduction

The present European Regulation, confirmed in Italy by the DL N° 105 on January 25, 1992 and its further modifications, establishes: "*Natural mineral waters are defined as those who originate from an underground aquifer or layer, who originate from one or more underground natural or perforated springs or wells, who have peculiar hygienic characteristics, and possibly healthy properties*". These statements introduce, therefore, the concept that hydropinic waters may not only have an aspecific hydrating action, but also can possess specific actions on biological mechanisms favoring wellbeing.

Another fundamental point is that natural mineral water must not be submitted to processes which might modify its chemical characteristics. As a consequence, those intervention such as chlorination, purification etc. which are usually allowed and often requested as mandatory for drinking water utilized for urban population distribution for its domestic and alimentary utilization, are forbidden in natural mineral drinking waters. Frequently, purified waters for domestic use have similar chemical composition and origin to those of natural mineral waters, but physical/chemical processing to which they are submitted modify, according to the European regulations, the original characteristics and, therefore, their potential properties which could induce health improvement.

Over the last ten years natural mineral waters and thermal waters are submitted to experimental and clinical studies to assess their possible antioxidant properties. They include investigations on the Saturnia sulfuric waters (1, 2), on thermal mud and thermal bath therapy with sulfuric (3) and non-sulfuric (4) characteristics, on balneotherapy with bicarbonate (5) and sulfuric (6) waters. In addition, studies were performed on hydropinic commercial waters (7), which is utilized through either a drinking methodology and/or as a thermal therapeutic method as mud/balneotherapy (8).

With different motivations other countries with traditions in thermal medicine were also involved in clinical research: Hungary with chloride-alkaline waters (9), Russia and Austria with sulfuric waters (10, 11), Japan in experimental models with thermal waters (12), Germany in assessing balneotherapy with thermal waters containing iodine and selenium (13). Most investigations followed observational study designs and were able to only demonstrate variations of some of the indicators of oxidative stress, but not the biological and biochemical factors which were responsible for the results, with the exception of some hypothesis on the reducing power of SH-groups in sulfuric waters (14).

The purpose of this investigation

was to evaluate the potential antioxidant effect of the "Lete" natural mineral water, utilizing as indicators the total oxidative effect of serum with the d-reactive oxygen metabolites (d-ROMs) test and the efficiency of the plasmatic antioxidant barrier with the biological antioxidant potential (BAP) test.

Materials and methods

Subjects

A sample size of 61 male adults was investigated. These subjects were selected from a population of 95 volunteers on the base of inclusion/exclusion criteria, and all presented with baseline ROM and BAP levels higher than normal for these tests.

The selection was performed through a complete medical examination. All selected subjects were asked to continue following their normal lifestyle and alimentary habits. After giving their informed consent, all subjects were submitted to a drinking regimen of 2 liters of "Lete" water per day, distributed during the 24 hours on the base of a specific protocol (Tab. 1).

The oxidative conditions of the subjects were monitored by dosing the reactive oxygen metabolites (ROM) and plasma antioxidant barrier (BAP), before and after 15 days of treatment.

Table 1 - Clinical trial protocol

	Day 1	Day 2	Day 3 to day 15
Morning	600 ml	1000 ml	1400 ml
Afternoon	400 ml	500 ml	600 ml

Table 2 - Physical-chemical analysis of the "Lete" mineral water

Parameters	Expression of results	Results
Spring temperature	°C	14,5
Hydrogen ions activity	pH	6,2
Electric conductibility	μS cm^{-1} a 20°C	1280
Fixed residue at 180°C	mg/l	840
Oxidizability	mg/l O_2	0,30
Free Carbon dioxide (at spring)	mg/l CO_2	1920
Silica	mg/l SiO_2	18
Bicarbonate	mg/l HCO_3	980
Chlorine	mg/l Cl	9,50
Sulphates	mg/l SO_4	5,60
Sodium	mg/l Na	4,90
Potassium	mg/l K	1,70
Calcium	mg/l Ca	314
Magnesium	mg/l Mg	14,50
Dissolved Iron	mg/l Fe	0,01
Ammonium	mg/l NH_4	<0,01
Total Phosphorus	mg/l PO_4	<0,01
Hydrogen sulfide	mg/l H_2S	<0,03
Strontium	mg/l Sr	0,21
Lithium	mg/l Li	0,001
Aluminium	mg/l Al	0,002
Bromine	mg/l Br	<0,10
Iodine	mg/l I	<0,02

The "Lete" water spring is located in the Municipality of Pratella (Caserta, Italy), and according to the Italian law: D.L. N° 105, 1992, is classified as "Natural mineral water, naturally effervescent, slightly acid, calcic, containing bicarbonate" (Tab. 2).

Inclusion criteria

The patients who were enrolled did not have significant relevant medical diseases: the objective of the study was, in fact, not to verify the efficacy of the treatment on a specific disease, but rather to as-

sess its potential action as a reducer of oxidative stress, which could have been sustained hypothetically by lifestyle and/or alimentary factors inducing a decrement of antioxidant cellular activity.

Exclusion criteria

- Presence of factors which could increase the levels of free radicals: acute and chronic diseases, drug treatments, tabagism, etc.
- Risk of events that during the treatment observational period could presumably modify the levels of free radicals: patients subject to recurrent diseases, patients who could suggest, at the screening visit, that they might incur changes of alimentary/lifestyle habits or conditions which might require drug administration, etc.
- Lack of reliability in complying with the specifications of administration rules of the treatment protocol (dosage, time, modality of administration in relation to meals and other activities).

Drop-out criteria

- Patients who did not complete at least 90% of the therapy.
- Patients who did not follow the standardized modalities of treatment.
- Patients with discontinuous adherence to treatment modalities.
- Overweight with BMI >25.
- Patients who, during the treatment period, were subject to events which might have induced an increase in oxygen radicals: inflammatory diseases, traumas, drug treatments, climatic factors (environmental heat), substantial increase of normal physical activities, etc.
- Patients who during the time of hydropinic therapy, incurred in events, not related to thermal therapy, which could have caused a decrease of oxygen radicals: oral intake of antioxidant agents, etc.

At the end of recruiting, the sample size (n = 61) was composed of 61 males (100%) with a mean age of 37.9 ± 9.7, median = 37, with a minimal age of 21 and a maximal age of 78 (Tab. 3).

According to the World Health Organization, BMI classification (consulted 5/05/2010) (15), none of the subjects were found to be overweight, the BMI average 21.8 ± 2.4, median of 22 with minimum 16.9 and maximum 24.9 (Tab. 3).

Forty-seven of them presented with baseline ROM values superior to normal, and 26 BAP values inferior to normal (Tab. 4). Only 12 of them presented with baseline abnormal values of both ROM and BAP. This can be explained considering that ROM is indicating oxidative intracellular stress, while BAP expresses the plasmatic defense reaction: therefore the two parameters represent different biological compartments.

Table 3 - Descriptive statistics of the population

Total N.	61
Age	years
Means ± SD	37.9 ± 9.7
Median	37
Minimum	21
Maximum	78
BMI	
Means ± SD	21.8 ± 2.4
Median	22
Minimum	16.9
Maximum	24.9

The control group in this study should have been represented by the same group of patients who, after a wash-out period, should have been submitted to a 15 days of administration of tap water regimen. Unfortunately the patients belonged to different living locations where tap water was distributed by different municipal waterworks, each with different physical-chemical characteristics. As a consequence there was no uniformity in composition of what should have been utilized as a placebo treatment.

Considering the possibility to obtain a valid statistical analysis utilizing only a pre/post treatment trial design, the study was therefore conducted as previously described.

Table 4 - ROMs and BAP: pre-treatment and post-treatment values (n.v.= normal value)

Number	ROMs (U. Carr.)		BAP (μmoles/L of reduced iron)		Number	ROMs (U. Carr.)		BAP (μmoles/L of reduced iron)	
	Pre	Post	Pre	Post		Pre	Post	Pre	Post
1	*n.v.*	-	2084.8	2241.9	32	375	330	*n.v.*	-
2	325	312	2181.2	> 3000	33	*n.v.*	-	2027.1	2601.7
3	309	315	*n.v.*	-	34	321	316	*n.v.*	-
4	308	270	*n.v.*	-	35	278	269	*n.v.*	-
5	*n.v.*	-	1537.1	2555.8	36	317	300	1997.3	2168.1
6	337	323	*n.v.*	-	37	319	325	*n.v.*	-
7	333	269	*n.v.*	-	38	282	258	*n.v.*	-
8	290	250	2179.2	2268.7	39	296	304	*n.v.*	-
9	383	382	*n.v.*	-	40	301	326	*n.v.*	-
10	366	331	1860.5	2222.3	41	284	270	*n.v.*	-
11	*n.v.*	-	2052.8	2495.4	42	318	322	*n.v.*	-
12	*n.v.*	-	2047.6	2190.3	43	316	302	*n.v.*	-
13	346	336	*n.v.*	-	44	*n.v.*	-	2196.1	2229.1
14	347	331	*n.v.*	-	45	297	293	2164.8	2159.6
15	280	256	*n.v.*	-	46	322	307	*n.v.*	-
16	303	291	1938.7	2176.1	47	*n.v.*	-	2063.7	2291.9
17	280	250	1992.6	2223.9	48	289	273	1902.8	1928.4
18	297	250	*n.v.*	-	49	*n.v.*	-	1670.7	2123.7
19	*n.v.*	-	1869.1	2164.6	50	275	267	*n.v.*	-
20	332	267	*n.v.*	-	51	*n.v.*	-	1981	2060.5
21	297	277	*n.v.*	-	52	320	318	*n.v.*	-
22	*n.v.*	-	2109.8	2175	53	279	288	*n.v.*	-
23	304	287	*n.v.*	-	54	*n.v.*	-	2169.9	2244.1
24	308	298	2072.4	> 3000	55	354	297	*n.v.*	-
25	319	317	*n.v.*	-	56	293	278	*n.v.*	-
26	*n.v.*	-	1661.3	> 3000	57	300	304	1997.3	> 3000
27	304	303	2142.2	2239.3	58	275	275	*n.v.*	-
28	289	250	1614.7	2475.7	59	297	281	*n.v.*	-
29	318	261	*n.v.*	-	60	353	354	*n.v.*	-
30	*n.v.*	-	2032.2	2176.9	61	322	305	*n.v.*	-
31	306	262	*n.v.*	-					

F.R.E.E. photometer

In this investigation the quantitative analysis of ROM and BAP was performed with a special photometer (Model F.R.E.E., Diacron International, Grosseto, Italy). The F.R.E.E. photometer allows measurements in absorbance, concentration (end-point), kinetics, fixed time and differential end-point.

Evaluation of the pro-oxidant status: the d-ROMs test

The ROMs contained in the biological samples are mainly constituted by hydroperoxides (ROOH). The evaluation method followed reference standards (16-18). In the presence of iron (liberated from plasmatic proteins utilizing an acid buffering solution: the R2 kit reactant), the ROMs liberate, because of the Fenton reaction, alkoxyl (R-O*) and peroxyl (R-OO*) radicals. These radicals, when in contact with a substituted aromatic amine (A-NH2), contained in a chromo-

genic mixture (the R1 reactant of the kit), generate its oxidation and transformation into a rose-colored derivative.
The ROM plasma concentration is then obtained by photometric analysis of the sample and is directly proportional to the color intensity, as established by the Lambert-Beer law. This is a kinetic-type reaction. The photometric values are determined utilizing a wavelength of 546 nm (540-550 nm). The test is performed on heparinized plasma at a temperature of 37°C, in order to properly quantify ROM concentration.
The results are expressed in Carr Units (U CARR): 1 U CARR corresponds to 0.08 mg of hydrogen peroxide/dL. Following the criteria suggested by the analytic apparatus producer, the sample size was stratified as follows: <275 U Carr: normal values; 275-320: borderline values; 321-340: mild oxidative stress; >341: marked oxidative stress.

BAP (Biological Antioxidant Potential) test

The BAP Test (19,20) is based on the ability of ferric ions (Fe^{3+}), in presence of a specific chromogen, to discolor when ferric Fe^{3+} ions are transformed into divalent ferrous Fe^{2+} ions. This reaction is made possible by adding the solution with an adequate reducing system, represented by plasma.
In the BAP test the plasma sample is dissolved in a colored solution, obtained by adding ferric chloride ($FeCl_3$) – source of the ferric ions and R2 kit reactant – to a specific chromogen: thiocyanate (R1 kit reactant). After an incubation of 5', the solution discolors, and the discoloration degree, within the selected time interval, is directly proportional to the plasma reducing power of the ferric ions initially present and responsible for the new chromatic complexes. The intensity of discoloration is evaluated by photometry, allowing to evaluate the amount of reduced ferric ions and therefore the reducing antioxidant capacity of the tested plasma.
This antioxidant power is to be interpreted in relation to the substrate being investigated: the ferric ions. Considering that these ions are present in our organism, the BAP test delivers a reliable measurement of the antioxidant power of the fraction of the plasmatic barrier which is directly involved with oxidation, because of redox potentials, in defense from the attack of free radicals in "physiological" conditions.
This test is performed at 37°C. The photometric readings utilize a wavelength of 505 nm (500-510 nm). Final results express the plasmatic iron-reducing antioxidant physiological power directly in μmoles/liter of Vitamin C (considered as the antioxidant of reference). The normal value is >2200 μmoles/L of reduced iron. Following the criteria suggested by the analytic apparatus producer, the sample size was stratified as follows: >2200 μmoles/L: normal values; 2200-2000: borderline values; 2000-1800: mild deficiency of the antioxidant defense status; <1800: moderate-severe deficiency.

Statistical analysis

The evaluation before and after treatment of the same subject followed by stratification (which led to a scale of nominal measurements with polytomic variables), required a statistical analysis based on the Bowker symmetry test (21, 22).

Results

All patients selected were clinically in a healthy state. Therefore none of them presented extreme levels in excess of ROMs (max: 383 U Carr.) or deficit of BAP (min: 1537.1 μmol/l). For this reason we eliminated from analysis the extreme values.
Of the 47 subjects who had altered values of ROM at baseline only 3 slightly worsened after treatment, 20 remained stable, 24 consistently improved, while 15 returned to normal values (Fig. 1). Favorable results were confirmed with BAP evaluation (Fig. 2). Of

the 26 patients with defense deficit at baseline only 6 remained stable, while 20 improved and 15 returned to normal values.

Figure 1 - ROM: pre-treatment vs. post-treatment (P<0,05)

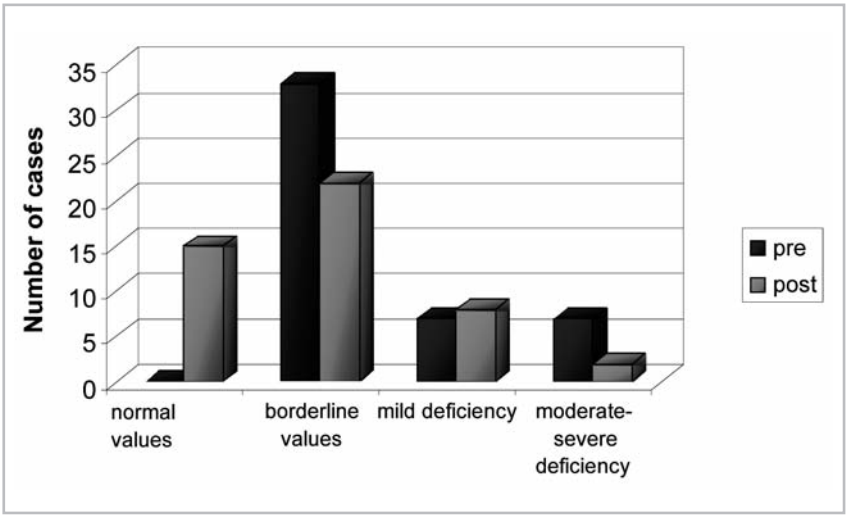
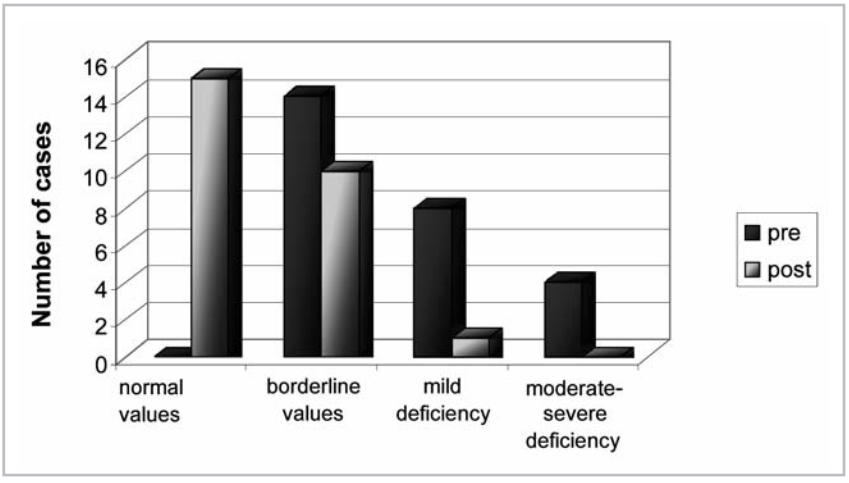


Figure 2 - BAP: pre-treatment vs. post-treatment (P<0,05)



Discussion

This investigation clearly demonstrates the positive antioxidant role of hydric supplementation with the "Lete" mineral water. Still, it is not possible to ascertain at the present time whether its action is specific or aspecific. There are not as yet specific bibliographic references which could orient our conclusions towards one of these two possibilities. Similar clinical studies were conducted with different mineral waters and reported an evident disparity of results (1, 2, 7, 13).

These considerations can indicate a different role of specific physical-chemical characteristics for specific waters determining their biological effects. Further investigations concerning the biological response to a "neutral" water will allow to perform interesting comparisons.

And therefore it is important to establish if any of the mineral waters have relevant antioxidant actions and it will be very important to establish a way of quantifying this in ORAC units as for other food and dietary substances.

The daily use of a natural alimentary product such as mineral water, for its anti-radical actions, could be an interesting supplement to the Mediterranean diet, at this point still held as the most likely reason for the longevity of the Italian population, which has the longest lifespan in Europe, and this is the basis for the recent data from ISTAT.

Nutrition, including hydratory input, must in addition be considered as the most important anti-oxidant remedy, in this case even more important than dietary supplements, whose possible collateral effects must not be overlooked; they are related not only to the active ingredients but also the excipients, which can trigger adverse reactions such as the sensitization, allergic reactions, intolerance, and pharmacological interactions (23, 24).

In addition, the consumption of "natural mineral water" based on recent findings, can without any doubt be regarded as a healthy choice, considering that some water purification processes to render the water suitable for human con-

sumption alter its physical and physicochemical characteristics, also introducing elements which can be detrimental to health (25-27).

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