

Natural mineral-rich water ingestion by ovariectomized fructose-fed Sprague-Dawley rats: effects on sirtuin 1 and glucocorticoid signaling pathways

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Abstract

Objective: Prevention or induction of metabolic disorders and obesity depend on estrogen signaling and/or exogenous factors, such as mineral content in diet. The protective effects of a Portuguese natural mineral-rich water against the induction of metabolic syndrome in fructose-fed male Sprague-Dawley rats have been reported. The present study was designed to assess the impact of this mineral-rich water on fructose-fed estrogen-deficient female Sprague-Dawley rats.

Methods: Ovariectomized rats had access to tap (TWO) or mineral-rich (MWO) waters, with and without 10% fructose (10-wk treatment). A sham-operated (tap water supplied) group was included and each of the five groups included six rats. Plasma biochemical and metabolic parameters were evaluated by routine clinical measurements. Western blotting was used to assess hepatic protein expression of sirtuins (Sirt) 1 and 3, phosphorylated AMP-activated protein kinase- α (p-AMPK α), peroxisome proliferator-activated receptor gamma coactivator-1- α (PGC1 α), glucocorticoid receptor, and 11 β -hydroxysteroid dehydrogenase type 1 (11 β HSD1).

Results: Ovariectomy increased plasma total cholesterol (46%/P < 0.05), but had no significant effects on hepatic protein expression. Fructose intake by ovariectomized rats increased PGC1 α and 11 β HSD1 (fructose in tap water [TWFO] vs TWO: 65%/P < 0.05 and 38%/P = 0.05, respectively) as well as glucocorticoid receptor (TWFO and fructose in natural mineral-rich water [MWFO] vs TWO and MWO: 107%/P = 0.05 and 182%/P < 0.05, respectively). Mineral-rich water ingestion exerted an increasing shape on Sirt1 (MWO vs TWO: 76%/P < 0.05; MWFO vs TWFO: 76%/P = 0.06), PGC1 α (MWO vs TWO: 77%/P < 0.01), p-AMPK α (MWO vs TWO: 152%/P = 0.01; MWFO vs TWFO: 107%/P = 0.01), and 11 β HSD1 (MWO vs TWO: 91%/P = 0.05; MWFO vs TWFO: 47%/P = 0.05).

Conclusions: Mineral-rich water ingestion may have a prime role on the activation of Sirt1 signaling and the modulation of glucocorticoid signaling in the postmenopause.

Key Words: Fructose – Glucocorticoid signaling – Liver – Natural mineral-rich water – Ovariectomy – PGC1 α – Phosphorylated AMPK α – Sirtuins 1 and 3 – Sprague-Dawley rats.

The development, prevention, and management of metabolic disorders (which may include insulin resistance, metabolic syndrome, and type 2 diabetes mellitus) and obesity can be associated with specific dietary

patterns.¹⁻⁸ Estrogen signaling is known to protect from metabolic disorders and obesity, whereas estrogen deficiency may accentuate the risk for their development.⁹⁻¹³ If dietary habits capable of inducing metabolic disorders and obesity

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occur in the absence of estrogen signaling, the metabolic dysfunction and/or obesity already present is expected to be aggravated.⁹

Fructose consumption induces obesity and metabolic changes similar to those observed in postmenopause, as demonstrated in humans and in rodent experimental models.^{2,3,9-14} Deficiency in potassium, calcium, and magnesium is frequent in metabolic dysfunction and obesity-inducing diets.^{1,6,8} This apparently strengthens the deleterious effects of fructose consumption² considering that mineral ingestion protects against metabolic disorders associated with obesity.^{1,6-8,14,15} Minerals from natural mineral-rich waters are highly bioavailable^{1,16} and could be considered an important supplementary dietary source.^{1,17-19} We have previously shown the beneficial impact of the ingestion of a Portuguese natural mineral-rich water (rich in magnesium, calcium, potassium, and bicarbonate), which proved to prevent/ameliorate features of metabolic dysfunction, prevent/improve impaired signaling pathways, and induce stress responses in fructose-fed male CD Sprague-Dawley rats.^{14,15,20-23}

In this context, sirtuins (Sirt) and glucocorticoids play relevant roles, although in opposite directions. Sirt1 and Sirt3 regulate critical cellular processes (glucose homeostasis, insulin action, adiposity, lipolysis, mitochondrial biogenesis and function, lipid oxidation, cholesterol metabolism, and antioxidant protection) to maintain or improve metabolic homeostasis,²⁴⁻²⁹ whereas the glucocorticoid signaling pathway has been linked to metabolic dysfunction and obesity (through insulin resistance, for instance).^{20,30-35}

Sirt1 is the most widely studied member of the Sirt family of proteins and much of the recent research has been directed toward the identification of Sirt1 activator molecules. Key targets of Sirt1 and Sirt3, in the beneficial coordinated regulation of metabolic homeostasis, include peroxisome proliferator-activated receptor gamma coactivator-1- α (PGC1 α) and AMP-activated protein kinase (AMPK). Sirt1 and Sirt3 are, in turn, positively regulated by PGC1 α and, in addition, activated AMPK (phosphorylated AMPK [p-AMPK]) also positively regulates Sirt1 and PGC1 α . Increased activity of these four important metabolic sensors has been associated with numerous metabolic benefits.^{24,25,27,29,36-40}

Another possible mechanism for the protective metabolic effects of Sirt1 could be the negative regulation of the glucocorticoid signaling pathway, by preventing the functioning of the glucocorticoid receptor (GR).^{32,33,41} An increased activity of GR may correlate with deleterious metabolic effects, which may be exacerbated by increased activity of the enzyme 11 β -hydroxysteroid dehydrogenase type 1 (11 β HSD1) that converts tissue cortisone into cortisol in humans (11-dehydrocorticosterone to corticosterone in rodents). Genetic, pharmacological, and/or nutritional inhibition of GR and 11 β HSD1 has proven to be beneficial against metabolic dysfunction and obesity.^{20,30,32,34,35}

The aim of this study was to assess, for the first time, (1) Sirt1, Sirt3, PGC1 α , p-AMPK α , GR, and 11 β HSD1 protein expression on an animal model of metabolic dysfunction

associated with obesity induced simultaneously by ovariectomy and high-fructose diet, and (2) the outcome of the Portuguese natural mineral-rich water mentioned above in these important metabolic sensors. There are some published data on the independent effects of high-fructose ingestion and ovariectomy in those proteins. The study of the aforementioned mechanisms was focused on the liver given its central role in metabolic regulation and fructose metabolism.^{42,43}

METHODS

Reagents

The chemical reagents used in the study were of analytical grade, as previously described.²⁰ Modifications in the reagents and suppliers have been described here. The chemical characteristics of the natural mineral-rich and tap waters are given in Supplementary Table 1, Supplemental Digital Content 1, <http://links.lww.com/MENO/A198>. The Portuguese natural mineral-rich water (Pedras Salgadas) contains a higher amount of calcium, magnesium, and potassium than the tap water and is rich in sodium bicarbonate. Pedras Salgadas is classified as a hypersaline sodium-rich naturally sparkling mineral water, in conformity with the European Community Council guidelines for natural mineral waters (2009/54/EEC), and was kindly provided by Unicer Bebidas, S.A. (Leça do Balio, Matosinhos, Portugal).

In vivo animal experimentation and treatments

The study was carried out in 30 female CD Sprague-Dawley rats (13 wk old), from Charles River Laboratories (Chatillon/Chalaronne, France). Twenty-four animals were bilaterally ovariectomized, whereas the others were sham-operated. Surgeries were performed by Charles River. Ovariectomized rodents are traditionally used as a model for postmenopausal women as they mimic estrogen insufficiency.⁴⁴ Upon arrival, rats were housed in an enriched environment (two per cage) and maintained on a daily photoperiod of a 12-hour lighting schedule (20°C-22°C) with free access to standard laboratory pellet food (Rodent Maintenance Diet from Harlan Interfauna Iberica S.A., Barcelona, Spain) and water. Acclimatization took place for 10 days before starting the experimental protocol. The handling and care of the animals were conducted in conformity with the European Community Council guidelines for the use of experimental animals (86/609/EEC) and Act 129/92.

Animals were divided into five groups (six animals each) with free access to four different drinking solutions: (1) sham-operated rats, with tap water (STW), (2) ovariectomized rats, with tap water (TWO), (3) ovariectomized rats, with natural mineral-rich water (MWO), (4) ovariectomized rats, with 10% fructose in tap water (TWFO), and (5) ovariectomized rats, with 10% fructose in natural mineral-rich water (MWFO). Splitting up of ovariectomized females into the TWO, MWO, TWFO, and MWFO groups assured a similar initial mean body weight distribution among these groups. The dietary manipulation lasted 10 weeks. All experimental groups were fed ad libitum with the standard laboratory chow

diet mentioned above. During the acclimatization period, the animals included in the MWO and MWFO groups had access to the Portuguese natural mineral-rich water to allow adjustment to water flavor and sparkles,¹⁴ whereas the other three groups had access to tap water. Body weight and food and fluid ingestion values were recorded throughout the study.

Sample collection

Overnight-fasted rats were used to collect blood and the liver. Sodium pentobarbital (80 mg/kg of body weight) was used as deep anesthesia, before collecting blood, from the left ventricle of rats, into heparinized syringes. After transcardiac perfusion with ice-cold isotonic sodium chloride solution, the liver was rapidly removed and washed in cold saline solution. Both plasma aliquots and liver fragments were stored at -80°C , until further use.

Assessment of plasma biochemical and metabolic markers

Plasma concentrations of glucose, triacylglycerols, total cholesterol, high-density lipoprotein cholesterol, total proteins, urea, creatinine, and uric acid were evaluated. All these quantifications were made at the Clinical Pathology Department of São João Hospital Centre, Porto, Portugal, using standardized methods for human sample routine hospital measurements.

Assessment of hepatic proteins expression by semiquantitative western blotting analysis

Protein extraction from the hepatic tissue was carried out by mechanical homogenization (Teflon-glass homogenizer) of the liver fragments (300–450 mg/each) in equal volume of protein extraction buffer (50 mM Tris-base, 150 mM NaCl, pH 7.4, 1% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) sodium dodecyl sulphate, 1 mM EDTA, tablets of protease inhibitor cocktail, and phosphatase inhibitors [10 mM sodium orthovanadate and 100 mM sodium fluoride]), with subsequent agitation, for 30 minutes at 4°C . The samples were then centrifuged at 13,000 g, for 20 minutes at 4°C , and the infranant protein solution collected and kept at -80°C , until further analysis.

Proteins were dissolved (1:1) in $2\times$ loading buffer (Bio-Rad Laboratories, Hercules, CA), supplemented with 100 mM β -mercaptoethanol (Sigma-Aldrich, Barcelona, Spain), and denatured for 5 minutes at 95°C . After denaturation, 50 μg of hepatic proteins (quantified as described in reference 20) were loaded per well and separated using 10% or 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis. The proteins were then transferred to PVDF membranes (Immobilon-FL, Merck-Millipore, Darmstadt, Germany). The membranes were incubated in blocking solution composed of Tris-base-buffered saline with 0.1% (v/v) Tween-20 (TBST) containing 5% (w/v) bovine serum albumin or nonfat dry powdered milk Sveltesse in TBST. The incubation in the blocking solution was carried out at room temperature, for 1 hour. The membranes were subsequently incubated with the primary antibody with gentle agitation overnight, at 4°C

(Supplementary Table 2, Supplemental Digital Content 1, <http://links.lww.com/MENO/A198>). Furthermore, the membranes were washed with TBST and incubated with the secondary antibody conjugated to horseradish peroxidase (Supplementary Table 2, Supplemental Digital Content 1, <http://links.lww.com/MENO/A198>). The protein bands were detected with enhanced chemiluminescence reagent (with ChemiDoc XRS + System [Bio-Rad Laboratories]); the intensity of the bands was quantified using ImageLab software. The protein expression was normalized by using β -actin as the loading control.

The hepatic protein expression of Sirt1, PGC1 α , p-AMPK α ,⁴⁵ Sirt3, GR, and 11 β HSD1 was assessed by western blotting. The concentrations of antibodies used for the detection of these proteins are listed in Supplementary Table 2, Supplemental Digital Content 1, <http://links.lww.com/MENO/A198>.

Statistical analysis

The association between the outcomes (body weight and food ingestion) and the interaction of dietary intervention with time (evaluated in days), *longitudinal statistical analysis*, was measured with interaction fixed regression coefficients terms (β), which were estimated by mixed effects model with random effect in the intercept.⁴⁶

As predictors the mixed effects model included the dietary intervention, linear time and square of time as well as the interaction between dietary intervention and both terms of time. The linear coefficients (β_L) represent the increase/decrease of the outcomes compared with the increase/decrease of the reference group. The squared coefficients (β_s) represent the increase/decrease in the gain per day of the outcomes divided by two compared with the increase/decrease of the reference group. At the end of the dietary intervention, ANOVA (indicating the global P [Gp] values) followed by Student's t test (with P -value correction for the number of comparisons) or Kruskal-Wallis (also indicating the Gp values) followed by Mann-Whitney U test (also with P -value correction) (IBM SPSS Statistics software, Armonk, NY) were used for the evaluation of significance of differences among groups for [(body weight at the end of the final week) minus (body weight at the beginning of the initial week)] as well as for plasma biochemical and metabolic markers and Sirt and glucocorticoid signaling pathway parameters. Student's t test (Microsoft Excel 2010 [Redmond, WA]) was used to assess differences in fructose ingestion between TWFO and MWFO groups within each week (at several time points). $P \leq 0.05$ was considered statistically significant (a tendency was considered whenever $0.05 < P \leq 0.1$).

RESULTS

Body weight

Over time, a significant increase of body weight in STW rats was observed ($P < 0.01$, data not shown). Moreover, throughout the study, the increase of body weight of TWO ($P < 0.01$), TWFO ($P < 0.01$), and MWO ($P = 0.02$) groups

was significantly higher than that of the STW group (Fig. 1A and Table 1). Over time, TWO, TWFO, and MWO rats showed a significantly lower daily body weight gain ($P < 0.01$, $P < 0.01$, and $P = 0.02$, respectively) than STW rats (Fig. 1B and Table 2), as evidenced by the slope of the corresponding lines, indicating an earlier stabilization of their body weight (Fig. 1B).

MWFO and STW rats behaved similarly with regard to body weight evolution and rhythm of daily body weight gain (Fig. 1A and Table 1 and Fig. 1B and Table 2, respectively). Over time, MWFO rats showed a tendency ($P = 0.06$) to a lower body weight increase than MWO rats (Fig. 1A and Table 1). Those displayed a significantly ($P = 0.05$) lower body weight increase that also varied significantly slowly ($P = 0.03$) along time versus TWFO rats (Fig. 1A and Table 1 and Fig. 1B and Table 2, respectively).

There were no significant differences comparing the final with initial body weights [(body weight at the end of the final week) minus (body weight at the beginning of the initial week)] (Fig. 2).

Food and fructose ingestion

Over time, TWFO, MWFO, and MWO rats significantly reduced their food ingestion when compared with STW rats ($P < 0.01$, $P < 0.01$, and $P = 0.02$, respectively) (Fig. 1C and Table 1). In addition, over time, the decrease of food intake was significantly higher in TWFO versus TWO ($P < 0.01$) and MWFO versus MWO ($P = 0.04$) groups. A similar behavior was observed in MWO versus TWO rats ($P = 0.05$; Fig. 1C and Table 1).

Over time, daily food ingestion gain was significantly higher in ovariectomized fructose-fed rats versus STW

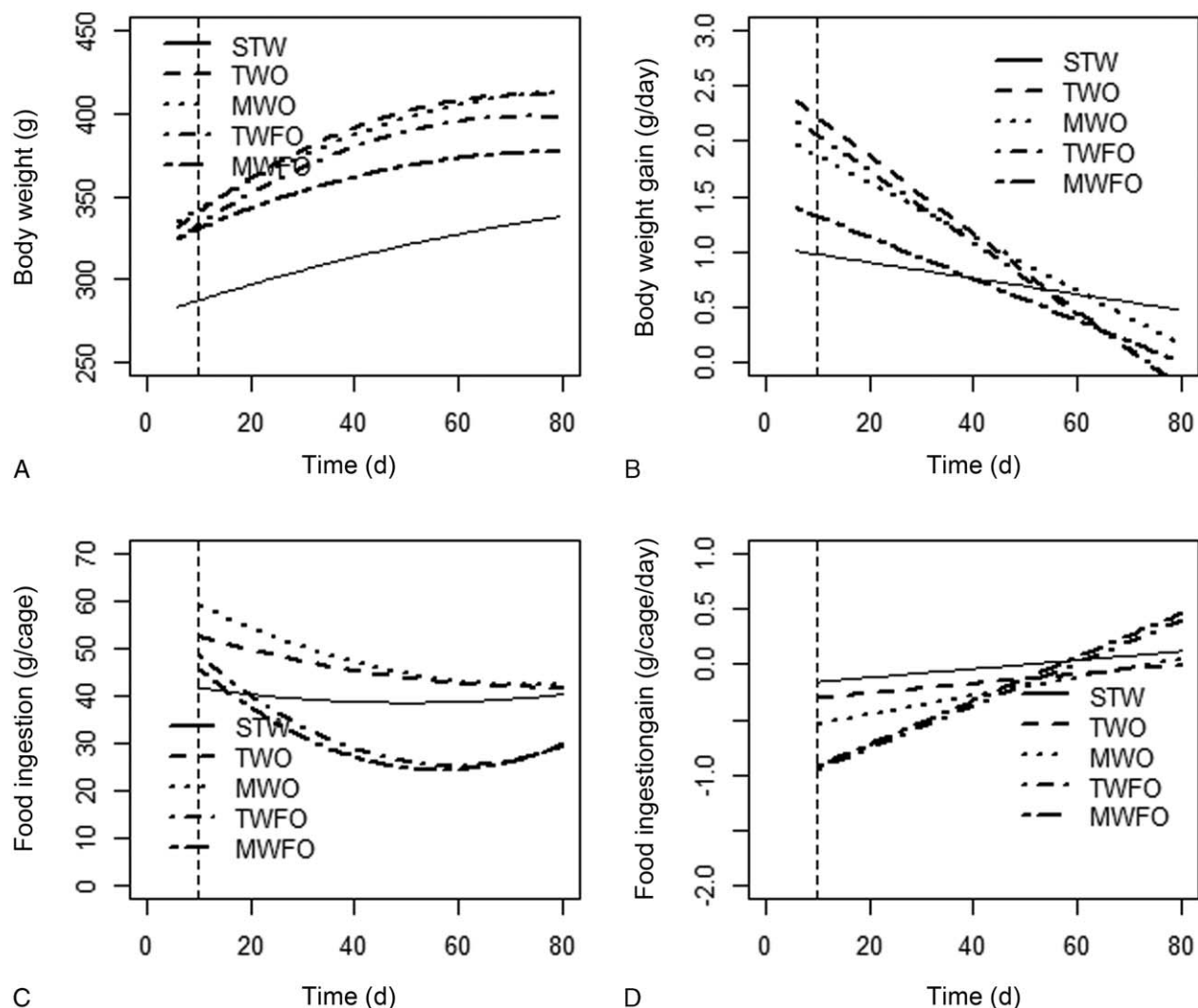


FIG. 1. Estimation of body weight and food ingestion. Body weight evolution (g; $n = 6$) (A), body weight gain (g/d; $n = 6$) (B), food ingestion evolution (g/cage; $n = 6$) (C), and food ingestion gain (g/cage/d; $n = 6$) (D). MWFO, ovariectomized Sprague-Dawley rats with access to 10% fructose in natural mineral-rich water; MWO, ovariectomized Sprague-Dawley rats with access to natural mineral-rich water; STW, sham-operated Sprague-Dawley rats with access to tap water; TWFO, ovariectomized Sprague-Dawley rats with access to 10% fructose in tap water; TWO, ovariectomized Sprague-Dawley rats with access to tap water. Results presented in the graphs (A) to (D) were estimated by mixed-effects model with random effect in the intercept.⁴⁶ Statistical significance relating these results is shown in Tables 1 and 2.

TABLE 1. Interaction terms (β_L), for linear time, estimated by mixed-effects model with random effect in the intercept, for longitudinal statistical analysis of body weight and food ingestion evolutions

Interaction terms (β_L) estimated by mixed-effects model with random effect in the intercept					
Body weight, g	β_L	<i>P</i>	Food ingestion, g/cage	β_L	<i>P</i>
STW	Reference		STW	Reference	
TWO	1.23923	<0.01	TWO	-0.14863	0.34
MWO	0.89138	0.02	MWO	-0.37684	0.02
TWFO	1.07752	<0.01	TWFO	-0.79213	<0.01
MWFO	0.34501	0.36	MWFO	-0.76181	<0.01
TWO	Reference		TWO	Reference	
MWO	-0.3478	0.40	MWO	-0.22821	0.05
TWFO	Reference		TWFO	Reference	
MWFO	-0.7349	0.05	MWFO	0.03032	0.88
TWO	Reference		TWO	Reference	
TWFO	-0.1617	0.73	TWFO	-0.64351	<0.01
MWO	Reference		MWO	Reference	
MWFO	-0.5464	0.06	MWFO	-0.38497	0.04

MWFO, ovariectomized Sprague-Dawley rats with access to 10% fructose in natural mineral-rich water; MWO, ovariectomized Sprague-Dawley rats with access to natural mineral-rich water; STW, sham-operated Sprague-Dawley rats with access to tap water; TWFO, ovariectomized Sprague-Dawley rats with access to 10% fructose in tap water; TWO, ovariectomized Sprague-Dawley rats with access to tap water.

animals ($P < 0.01$ for both TWFO and MWFO groups). As can be seen by the slope of daily food ingestion gain lines, over time, the increasing shape of daily food ingestion gain was significantly higher in TWFO versus TWO ($P < 0.01$) and MWFO versus MWO ($P = 0.03$) groups (Fig. 1D and Table 2).

Globally, MWFO and TWFO rats ingested a similar quantity of fructose. The difference in mean fructose ingestion values between TWFO and MWFO groups was statistically significant ($P < 0.05$) at two time points only: at day 8 (week 1, higher in MWFO) and at day 50 (week 7, higher in TWFO; Fig. 3).

Plasma biochemical and metabolic markers

Total cholesterol levels were higher in TWO versus STW rats (46%, $P < 0.05$), demonstrating the influence of estrogen signaling loss in this parameter.⁴⁷ Total protein levels presented an overall statistical significance of $P < 0.05$. No significant differences were observed for glucose,

triacylglycerols, high-density lipoprotein cholesterol, proteins, urea, creatinine, and uric acid levels either overall and/or when considering the effect of ovariectomy, fructose ingestion, or natural mineral-rich water ingestion, with and without fructose consumption (Fig. 4).

Sirt1, PGC1 α , p-AMPK α , and Sirt3 hepatic protein expression

In our study, significant overall alterations were found (ANOVA or Kruskal-Wallis) in the expression levels of Sirt1, PGC1 α , p-AMPK α ($G_p < 0.01$), and Sirt3 ($G_p < 0.05$).

Neither ovariectomy (TWO vs STW) nor fructose ingestion by ovariectomized rats (TWFO and MWFO vs TWO and MWO, respectively) had any relevant effect on Sirt1. Natural mineral-rich water intake showed an increasing shape for Sirt1 in ovariectomized rats (as we have previously reported for fructose-fed male rats^{20,22,23}), with and without fructose ingestion (76%, $P < 0.05$ for MWO vs TWO; 76%, $P = 0.06$ for MWFO vs TWFO) (Fig. 5A).

TABLE 2. Interaction terms (β_s), for square of time, estimated by mixed-effects model with random effect in the intercept, for longitudinal statistical analysis of body weight and food ingestion gains

Interaction terms (β_s) estimated by mixed-effects model with random effect in the intercept					
Body weight gain, g/d	β_s	<i>P</i>	Food ingestion gain, g/cage/d	β_s	<i>P</i>
STW	Reference		STW	Reference	
TWO	-0.01392	<0.01	TWO	0.00021	0.92
MWO	-0.00863	0.02	MWO	0.00222	0.26
TWFO	-0.01256	<0.01	TWFO	0.00766	<0.01
MWFO	-0.00584	0.11	MWFO	0.00792	<0.01
TWO	Reference		TWO	Reference	
MWO	0.0053	0.19	MWO	0.00201	0.16
TWFO	Reference		TWFO	Reference	
MWFO	0.0068	0.03	MWFO	0.00026	0.92
TWO	Reference		TWO	Reference	
TWFO	0.0014	0.72	TWFO	0.00745	<0.01
MWO	Reference		MWO	Reference	
MWFO	0.0028	0.40	MWFO	0.00570	0.03

MWFO, ovariectomized Sprague-Dawley rats with access to 10% fructose in natural mineral-rich water; MWO, ovariectomized Sprague-Dawley rats with access to natural mineral-rich water; STW, sham-operated Sprague-Dawley rats with access to tap water; TWFO, ovariectomized Sprague-Dawley rats with access to 10% fructose in tap water; TWO, ovariectomized Sprague-Dawley rats with access to tap water.

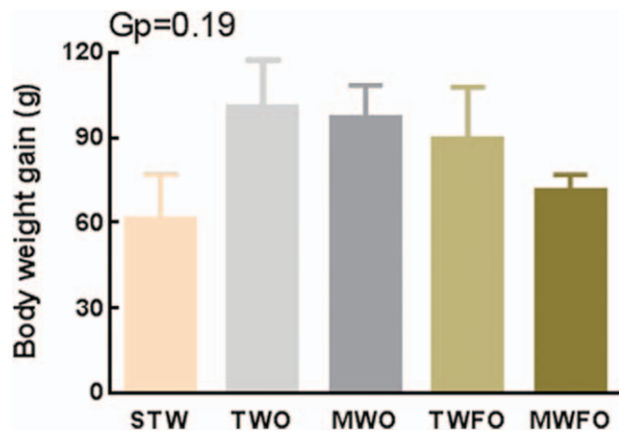


FIG. 2. Body weight gain (g; $n=6$). Gp, global P ; MWFO, ovariectomized Sprague-Dawley rats with access to 10% fructose in natural mineral-rich water; MWO, ovariectomized Sprague-Dawley rats with access to natural mineral-rich water; STW, sham-operated Sprague-Dawley rats with access to tap water; TWFO, ovariectomized Sprague-Dawley rats with access to 10% fructose in tap water; TWO, ovariectomized Sprague-Dawley rats with access to tap water. Results [(body weight at the end of the final week) minus (body weight at the beginning of the initial week)] are presented as mean \pm SEM. Percentage body weight gain at the end of the experiment for each group calculated as [(mean body weight gain minus mean body weight gain STW)/mean body weight STW \times 100] was 11.7, 10.6, 8.4, and 3.0 for TWO, MWO, TWFO, and MWFO rats, respectively.

Ovariectomy had no significant effect on PGC1 α , but fructose ingestion by ovariectomized rats significantly increased PGC1 α in the tap water arm of the study (TWFO vs TWO: 65%, $P < 0.05$). A significant increase of 77% was found for PGC1 α when comparing MWO with TWO ($P < 0.01$) (Fig. 5B).

Importantly, p-AMPK α protein expression was very similar to the one observed for Sirt1. Nevertheless, the effects of natural mineral-rich water ingestion on p-AMPK α were stronger than on Sirt1 (152%, $P = 0.01$ for MWO vs TWO; 107%, $P = 0.01$ for MWFO vs TWFO) (Fig. 5C).

No statistically significant differences among groups were observed for Sirt3 (Fig. 5D).

GR and 11 β HSD1 hepatic protein expression

Significant overall alterations were found (ANOVA or Kruskal-Wallis) in the expressions levels of GR (Gp < 0.01) and 11 β HSD1 (Gp < 0.05).

Ovariectomy showed no significant effect on GR, but fructose ingestion significantly increased GR, independently of the water ingested: 107% for TWFO versus TWO ($P = 0.05$) and 182% for MWFO versus MWO ($P < 0.05$). As we have previously reported for fructose-fed male rats,²⁰ although not statistically significant, a downward modulation seemed evident in GR with natural mineral-rich water ingestion by ovariectomized rats, fructose-fed or not (17% for MWFO vs TWFO and 39% for MWO vs TWO) (Table 3).

Similarly, ovariectomy showed no significant effect on 11 β HSD1. Fructose ingestion by ovariectomized rats significantly increased 11 β HSD1 only in the tap water arm of the study (38% for TWFO vs TWO, $P = 0.05$). MWO and MWFO

presented similar 11 β HSD1 values. These were significantly higher ($P = 0.05$) than in TWO (91%) and TWFO (47%) groups, respectively (Table 3).

DISCUSSION

Positive effects of natural mineral-rich waters ingestion on metabolic syndrome features (included or not in its definition) and metabolic syndrome complications have been published.¹ In fact, our group showed the beneficial effects of the ingestion of a Portuguese natural mineral-rich water on fructose-fed male CD Sprague-Dawley rats, which represents an animal model of environmentally (diet)-induced metabolic syndrome.^{14,15,20-23} The ingestion of the Portuguese natural mineral-rich water (1) reduced and/or prevented most of the changes induced by fructose intake, such as increased heart rate and triglycerides, insulin and leptin plasma levels, decreased hepatic PGC1 α and Sirt1 protein expression, and altered hepatic redox state, (2) strengthened compensatory mechanisms observed in response to stress induced by fructose ingestion (such as the increased shape of hepatic phosphorylated extracellular signal-regulated kinase to total extracellular signal-regulated kinase ratio), and (3) induced protective pathways per se (eg. increased Sirt1 protein expression in the liver and corpus cavernosum).^{14,20-22} In healthy Wistar Han rats, the same natural mineral-rich water caused an increase in hepatic catechol-O-methyltransferase activity.⁴⁸

Ovariectomy increased plasma total cholesterol. Fructose intake, on the contrary, increased hepatic 11 β HSD1, PGC1 α (in the tap water arm of the study), and GR (in both arms of the study). In the present work, the independent impact of ovariectomy and fructose ingestion did not always accord with available data on plasma biochemical and/or metabolic markers and signaling pathway players. Apart from obvious differences in species, rat strains, sex, tissues, and body weight evolution between published studies and our animal protocol, the age and the nutritional state of rats, along with time-sensitive regulatory mechanisms of the parameters assessed, may also have contributed to the differences observed.^{14,20,44,45,47,49-54} In

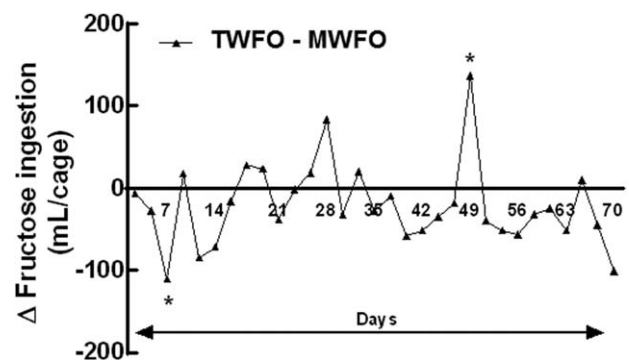


FIG. 3. Difference in fructose ingestion mean values (mL drinking solution/cage) between TWFO and MWFO groups. In each cage there were two rats. $n = 6$ for TWFO and MWFO. * $P < 0.05$. MWFO, ovariectomized Sprague-Dawley rats with access to 10% fructose in natural mineral-rich water; TWFO, ovariectomized Sprague-Dawley rats with access to 10% fructose in tap water.

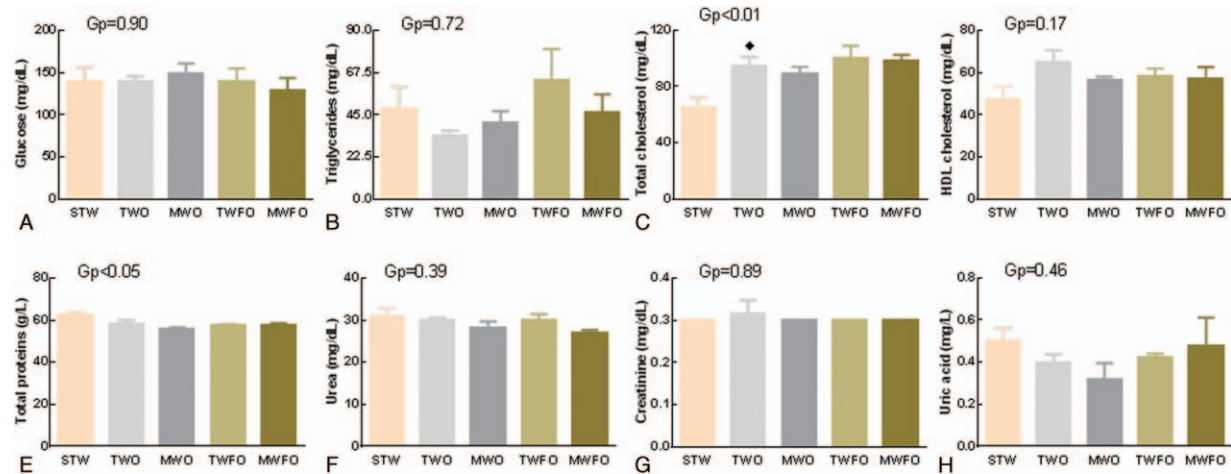


FIG. 4. Evaluation of plasma biochemical and metabolic markers. (A) Glucose ($n = 5$ for TWO and MWFO; $n = 6$ for STW, MWO, and TWFO); (B) triglycerides ($n = 6$ for all groups, but for TWO [$n = 5$]); (C) total cholesterol ($n = 5$ for all groups); (D) HDL cholesterol ($n = 6$ for all groups, but for TWFO [$n = 5$]); (E) total proteins ($n = 6$ for all groups, but for TWFO [$n = 4$]); (F) urea ($n = 4$ for TWO and MWFO; $n = 5$ for TWFO; $n = 6$ for STW and MWO); (G) creatinine ($n = 4$ for STW; $n = 5$ for MWO, TWFO, and MWFO; $n = 6$ for TWO); (H) uric acid ($n = 5$ for STW, TWFO, and MWFO; $n = 6$ for TWO and MWO). Gp, global P ; HDL cholesterol, high-density lipoprotein cholesterol; MWFO, ovariectomized Sprague-Dawley rats with access to natural mineral-rich water; STW, sham-operated Sprague-Dawley rats with access to tap water; TWFO, ovariectomized Sprague-Dawley rats with access to 10% fructose in tap water; TWO, ovariectomized Sprague-Dawley rats with access to tap water. Results are presented as mean \pm SEM. $\blacklozenge P < 0.05$ for TWO versus STW.

addition, the metabolic adjustment that might have occurred in this specific animal protocol, as a consequence of decreased food ingestion compensating for fructose ingestion, should be taken into consideration.

Natural mineral-rich water ingestion did not interfere in the food/fructose compensatory mechanism, once similar food and fructose ingestion patterns were observed. The results obtained here are in line with our previous findings and confirm the activation of Sirt1 signaling and the modulation of glucocorticoid signaling by the ingestion of a Portuguese natural mineral-rich water. Sirt1, p-AMPK α , and 11 β HSD1 hepatic protein expression presented an akin increasing shape when considering natural mineral-rich water ingestion (MWO vs TWO and MWFO vs TWFO; MWO and MWFO groups showing similar values for each one of the proteins). An upward effect in PGC1 α was also seen after natural mineral-rich water ingestion, without fructose consumption. Considering the cross talk among Sirt1, p-AMPK, and PGC1 α ^{24,27,29,36,37} (Fig. 6), one would expect that the effect of natural mineral-rich water ingestion would be more embracing in the fructose-free arm of this animal protocol.

Transgenic male mice (in a C57BL/6 and 129/Sv mixed genetic background) overexpressing Sirt1 are leaner and more metabolically active than littermate controls and display reduction in blood cholesterol, adipokines, insulin, and fasted glucose levels, without changes in blood triglycerides.³⁹ The strong increasing shape observed here for Sirt1 and p-AMPK α , irrespective of fructose ingestion, along with our previous Sirt1 results, would seem to suggest that natural mineral-rich water ingestion may lead to such a model. Nevertheless, further research is warranted to fully characterize the specificity of natural mineral-rich water activity on body tissues and its consequences.

Although the benefits of Sirt1 activators have been demonstrated in many recent studies, careful consideration is required while selecting such activators. For example, the Sirt1 activators SRT1720 and SA3 (Sirt1 activator 3) were shown to have an adverse impact by increasing fructose-induced Sirt1-dependent gluconeogenesis in primary rat hepatocytes and rat H411EC3 hepatoma cells.⁵⁵ So far, no adverse effects have been observed with the ingestion of the Portuguese natural mineral-rich water.^{14,20-22,48,56}

Assuming that natural mineral-rich water ingestion would increase Sirt1 and p-AMPK α in other tissues than the liver, it could have a protective effect against several physiological and psychological health complications that emerge after menopause.

An alteration in substrate utilization (decreased whole body fat oxidation) in the postmenopausal period, both at rest and during exercise, could, in part, explain the changed metabolic profile leading to an increased incidence of metabolic diseases after menopause.⁵⁷ In this regard, natural mineral-rich water ingestion would be beneficial as it could increase Sirt1 and p-AMPK α and, consequently, fatty acid oxidation.

Sirt1 signaling leads to induction of osteogenic gene transcription as well as to repression of genes promoting adipogenic differentiation.⁵⁸ Considering osteoporosis the result of impaired osteogenesis, natural mineral-rich water ingestion may be considered a promising strategy for preventing/treating postmenopausal bone loss.

A deficiency in the Sirt1/p-AMPK axis in postmenopausal metabolic syndrome may explain the occurrence of insulin resistance and cardiovascular dysfunction, through reduction of NO bioavailability, insulin-induced vasodilatation, and cardiomyocytes protection from apoptosis as well as impairment of homeostasis of cardiomyocytes in stress conditions.⁵⁹ Interestingly, ingestion of a

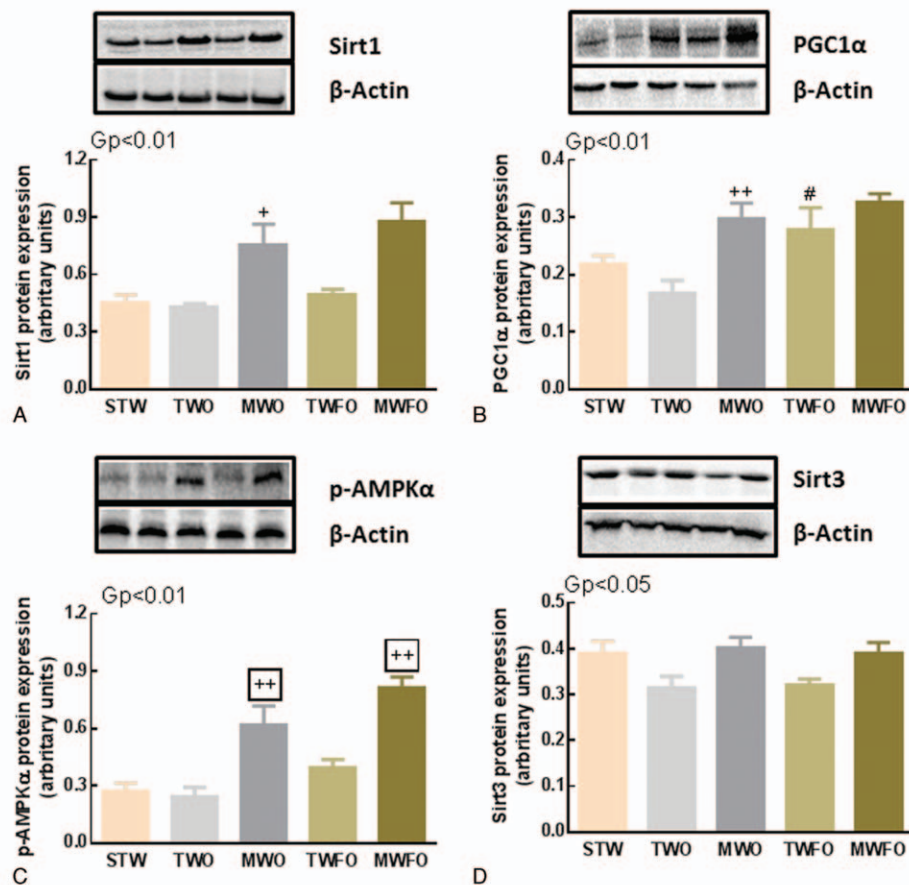


FIG. 5. Representative bands of semi quantitative western blotting analysis. (A) Sirt1 (n = 4 for TWFO; n = 5 for STW and TWO; n = 6 for MWO and MWFO); (B) PGC1α (n = 3 for TWFO; n = 5 for MWO; n = 6 for STW, TWO, and MWFO); (C) p-AMPKα (n = 3 for all groups); (D) Sirt3 (n = 5 for TWO, MWO, and TWFO; n = 6 for STW and MWFO). Gp, global P; MWFO, ovariectomized Sprague-Dawley rats with access to 10% fructose in natural mineral-rich water; MWO, ovariectomized Sprague-Dawley rats with access to natural mineral-rich water; p-AMPKα, phosphorylated AMP-activated protein kinase-α; PGC1α, peroxisome proliferator-activated receptor gamma coactivator-1-α; Sirt1, Sirtuin 1; Sirt3, Sirtuin 3; STW, sham-operated Sprague-Dawley rats with access to tap water; TWFO, ovariectomized Sprague-Dawley rats with access to 10% fructose in tap water; TWO, ovariectomized Sprague-Dawley rats with access to tap water. The graph indicates the mean ± SEM after normalization with β-actin, for each experimental group. ⁺P < 0.05 for MWO versus TWO; ⁺⁺P < 0.01 for MWO versus TWO; [#]P < 0.05 for TWFO versus TWO; ^{boxed ++}P = 0.01 for MWO versus TWO and MWFO versus TWFO.

bicarbonated natural mineral water, rich in sodium, chloride, and potassium, and with high bicarbonate to sodium ratio, by healthy postmenopausal women, decreased circulating endothelial dysfunction markers and glucose levels (reviewed in reference 1).

The weakening of Sirt1 signaling also impairs cognitive abilities, including immediate memory, classical conditioning, and spatial learning. Natural mineral-rich water ingestion could have a positive effect against cognitive decline and brain Sirt1 deficiency described in estrogen deficiency.^{60,61}

TABLE 3. Semiquantitative western blotting analysis of GR and 11βHSD1

	STW	TWO	MWO	TWFO	MWFO	P
GR	0.168 ± 0.025 (n = 6)	0.092 ± 0.010 (n = 5)	0.056 ± 0.005 (n = 6)	0.190 ± 0.023 ^a (n = 5)	0.158 ± 0.019 ^b (n = 5)	Gp < 0.01; P = 0.05 ^a (TWFO vs TWO); P < 0.05 ^b (MWFO vs MWO)
11βHSD1	0.225 ± 0.053 (n = 6)	0.092 ± 0.010 (n = 5)	0.175 ± 0.011 ^c (n = 5)	0.127 ± 0.005 ^a (n = 5)	0.186 ± 0.011 ^c (n = 5)	Gp < 0.05; P = 0.05 ^c (MWO vs TWO and MWFO vs TWFO); P = 0.05 ^a (TWFO vs TWO)

Values represent mean ± SEM (arbitrary units) after normalization with β-actin, for each experimental group.

Superscript letters (^{a,b,c}) correspond to statistically significant differences found between groups, as specified in the table.

11βHSD1, 11β-hydroxysteroid dehydrogenase type 1; Gp, global P; GR, glucocorticoid receptor; MWFO, ovariectomized Sprague-Dawley rats with access to 10% fructose in natural mineral-rich water; MWO, ovariectomized Sprague-Dawley rats with access to natural mineral-rich water; STW, sham-operated Sprague-Dawley rats with access to tap water; TWFO, ovariectomized Sprague-Dawley rats with access to 10% fructose in tap water; TWO, ovariectomized Sprague-Dawley rats with access to tap water.

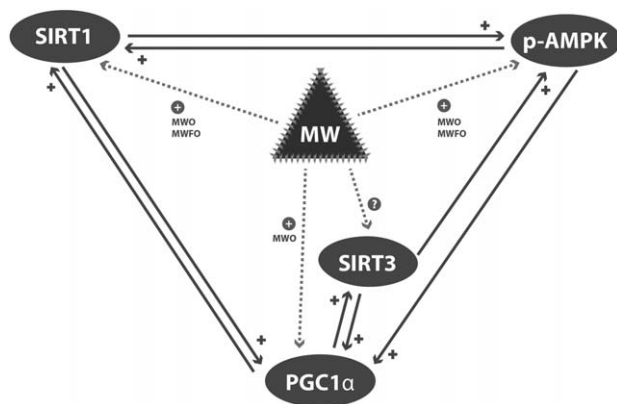


FIG. 6. Schematic representation of the putative positive impact of natural mineral-rich water ingestion in the relationship among sirtuin 1 signaling pathway players, in an animal model of fructose-fed ovariectomized female Sprague-Dawley rats. Some details on the relationship between Sirt1, Sirt3, PGC1 α , and p-AMPK. PGC1 α activity is increased by both Sirt1-dependent deacetylation and p-AMPK-mediated phosphorylation (p-AMPK phosphorylates PGC1 α , priming it for deacetylation and activation by Sirt1). p-AMPK also increases PGC1 α expression (only in the presence of PGC1 α protein that directly interacts with Sirt1, among other factors). Sirt1 and Sirt3 induce PGC1 α gene expression (the latter through activation of cAMP response element-binding protein [CREB] phosphorylation). PGC1 α induces Sirt3 gene expression (through binding to an estrogen-related receptor binding element [ERRE] within Sirt3 promoter region). PGC1 α can itself stimulate Sirt1 expression after interaction with Sirt1 gene promoter. Sirt1 increases Sirt3 expression via PGC1 α deacetylation. p-AMPK and PGC1 α relationship play a role in Sirt3 regulation. Sirt1 and Sirt3 increase AMPK phosphorylation (through activation of AMPK-activating protein kinase, the liver kinase B1), activating it. p-AMPK activates Sirt1 (through the increase of NAD⁺ availability).^{24,27,29,36,37} +, positive modulation; MW, Portuguese natural mineral-rich water; MWFO, ovariectomized Sprague-Dawley rats with access to 10% fructose in natural mineral-rich water; MWO, ovariectomized Sprague-Dawley rats with access to natural mineral-rich water; p-AMPK, phosphorylated AMP-activated protein kinase; PGC1 α , peroxisome proliferator-activated receptor gamma coactivator-1- α ; Sirt1, sirtuin 1; Sirt3, sirtuin 3.

There is a relevant interplay among 11 β HSD1, GR, PGC1 α , Sirt1, and p-AMPK in the glucocorticoid signaling. Although 11 β HSD1 activates cortisone conversion to cortisol in humans (11-dehydrocorticosterone to corticosterone in rodents), PGC1 α is a necessary mediator for GR genomic effects. Sirt1 and p-AMPK, in turn, impair GR association with p300, a transcriptional cofactor that positively regulates GR action.^{20,30-35,62} Taken together, the results obtained for 11 β HSD1, GR, PGC1 α , Sirt1, and p-AMPK led us to hypothesize that glucocorticoid signaling could be less active in ovariectomized rat groups with access to natural mineral-rich water. This could be beneficial in postmenopause, as high hepatic cortisol levels contribute to metabolic dysfunction.^{30,34,63} Comparing MWFO versus TWFO and MWO versus TWO, the putative higher availability of active glucocorticoids would be counteracted by the Sirt1 and p-AMPK α increase.

The extension of differences among groups did not reach statistical significance for all parameters assessed here. With regard to hepatic GR protein expression, the variations observed with natural mineral-rich water ingestion in male²⁰ and ovariectomized female Sprague-Dawley rats were consistent. In our opinion, this strengthens their biological

relevance and substantiates their brief inclusion in the discussion.

The evaluation of plasma insulin levels, plasma and hepatic corticosterone levels, hepatic Sirt1 activity and the phosphorylation level of hepatic p-AMPK α substrates, hepatic GR cellular location, and hepatic insulin signaling crucial players would enable us to fully characterize the effect of natural mineral-rich water ingestion in metabolic regulation, in the absence of estrogen signaling, with and without fructose feeding, through its impact upon the liver.

CONCLUSIONS

Confirming our previous results in the liver of fructose-fed male CD Sprague-Dawley rats,²⁰ natural mineral-rich water ingestion could have a prime role on the activation of Sirt1 signaling and the modulation of glucocorticoid signaling.

Figure 6 depicts a summary of the influence of natural mineral-rich water ingestion, in the absence of estrogen signaling, with and without fructose feeding, on the Sirt1, PGC1 α , and p-AMPK cross talk.

Potential clinical value

Although more studies are needed to unravel the specificity of natural mineral-rich water activity on body tissues as well as its consequences, the intake of this water may prove to be a complement, or even an alternative, to hormone therapy with the potential to mitigate its adverse side effects.

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