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Assessment of potential toxicological aspects of dietary exposure to silicon-rich spirulina in rats

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ABSTRACT

Silicon has beneficial effects especially on bones and skin and is important in cardiovascular pathophysiology. Furthermore, in spontaneously hypertensive rats, it reduces hypertension and increases antihypertensive and antiatherogenic gene expressions in the aorta. Thus, incorporating silicon into spirulina could be a way to produce a bioavailable food supplement.

The potential toxic effects of silicon-rich spirulina (SES) through haematological and biochemical parameters and inflammatory and oxidative status were evaluated in rats' blood and liver tissue. The study consisted in a 90-day experiment on female and male rats supplemented with three doses (28.5, 57 and 285 mg/kg BW/day) of SES. No mortality, abnormal clinical signs, behavioural changes or macroscopic findings were observed whatever the groups. Haematological parameters were not modified in SES treated-groups. No marked change was recorded in biochemical parameters The liver endogenous antioxidant enzymes (SOD, GPx, catalase) activities were not modified whatever the gender and the dose, just as markers of oxidative stress ($O_2^{\cdot-}$, TBARS, thiols) and inflammation such as IL-6 and TNF-alpha.

Our findings indicate that dietary supplementation of silicon-rich spirulina on rats has no harmful side nor toxic effects and could be beneficial especially in the case of suspicion or installation of pathologies due to oxidative stress.

1. Introduction

Silicon is a very important element in the living world (Exley, 1998). It is soluble essentially in the form of orthosilicic acid $Si(OH)_4$ in the surface waters. But beyond 2 mM and at neutral pH, this acid polymerizes into various forms of silica (colloidal solid) (Refitt et al., 2003) little or no soluble. At biological pH, silicon chemistry in solution mainly relates orthosilicic acid (Knight and Kinrade, 2001) which can form stable complexes with molecules containing hydroxyl groups (Ingri, 1978). Silicon has been identified associated with various biomolecules including proteins and carbon hydrates (Bond and McAuliffe, 2003). Silicon is indispensable for cell growth and trophic tissues in humans as in animals, and as such it is an essential element. Its digestive absorption is a function of its chemical presentation. Naturally, silicon is essentially in insoluble form, so poorly available (mineral silicon). On the other hand, even in a soluble form, silicon is slightly stable because it tends to polymerize rapidly at high concentration, which is the case of orthosilicic acid $Si(OH)_4$ which is the more soluble form of silicon and which has the better bioavailability (Calomme and Vanden Berghe, 1997; Jugdaohsingh et al., 2002), although it remains in a mineral form.

The silicon absorption in humans is largely performed in the form of orthosilicic acid $Si(OH)_4$ found in the drinking water or formed after food hydrolysis through the digestive tract (Refitt et al., 1999). The bulk of the silicon being in the form of colloidal silica is poorly absorbed by the body. In humans, daily intake is about 20–50 mg and the body needs 5–6 mg/day (EFSA, 2004).

Biological importance of silicon has to be considered in the context of its body distribution. The highest concentrations are found in bone and connective tissues such as aorta, trachea, tendons and skin, where silicon appears as an actor in turnover processes through collagen and elastin synthesis (Seaborn and Nielsen, 1993). Silicon supplementation has been reported to have beneficial effects on these tissues and especially bones (Jugdaohsingh, 2007) and skin (Calomme and Vanden Berghe, 1997). Silicon deficiency, in contrast, has been associated to detrimental effects on bone mineralization and growth or skin elasticity and healing (Carlisle,

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1976). The importance of silicon has also been demonstrated in cardiovascular pathophysiology and especially in the prevention of atherosclerosis (Loeper et al., 1979). An inverse relationship between the ingestion of silicon and the development of atherosclerosis has been reported (Loeper et al., 1988), and in spontaneously hypertensive rats, silicon supplementation reduces hypertension and increases antihypertensive and antiatherogenic gene expressions in the aorta (Maehira et al., 2011). Thus, a supplementation may be justified in preventing these effects, especially in the case of suspicion or installation of pathologies.

A fundamental step is to transform silicon in an organic form that is much more assimilated by the body than mineral forms. This is what we have achieved by incorporating it into spirulina.

The cyanobacteria Spirulina platensis is commercially available for human consumption. Spirulina are a good source of vitamins and minerals and are used as nutraceutical food supplements with no toxic side effects (Chamorro et al., 1988). They are particularly suitable for the production of specific bioactive compounds that they are able to accumulate in an organically bio-transformed form. Thus, new kind of food supplements has been developed and could serve as a rich source of trace elements (Mazo et al., 2007). Silicon is one of those trace elements essential in human nutrition.

Incorporating silicon into spirulina is a way to produce a bioavailable food supplement. Thus, given the beneficial effects of silicon and the antioxidant, hypolipidaemic and anti-inflammatory properties of spirulina (Deng and Chow, 2010) a food supplement was prepared. It has been shown that spirulina had no toxic effects *in vivo* and *in vitro* and did not release toxins (Heussner et al., 2012; Yang et al., 2011).

Whereas Buesen et al. (2014) reported that silicon dioxide nanomaterials neither cause local nor systemic effects upon subacute oral administration in rats and Horie et al. (2014) demonstrated that silicon dioxide nanoparticles do not exert potent cytotoxic effects on cells in culture, others have shown that (i) silicon dioxide increased enzymatic activities in the plasma, indicating an endothelial lung damage (Deb et al., 2012), (ii) silica nanoparticles exposure triggered a pro-inflammatory status in human bronchial epithelial BEAS-2B cells (Skuland et al., 2014), (iii) silicon in the form of silicon carbide induced no cytotoxicity but triggered pro-oxidative and pro-inflammatory responses of variable intensity on RAW 264.7 macrophages (Boudard et al., 2014).

Thus, we aimed to investigate here for the first time the safety and potential toxicity of repeated doses administration of silicon-rich spirulina (for 90 days) on rats. The maximum dose administered corresponds to 5 times the daily human recommended dose by the European Food Safety Authority.

2. Materials and methods

2.1. Materials

Spirulina (produced at PhycoBiotech, Lunel, France) grown in 25 m^3 pools under greenhouse with natural light, and temperature is controlled at 33 °C, in a Zarouk's medium in the presence of sodium metasilicate (Na_2O_3Si). This medium contained $NaHCO_3$, 16.8 g/L; K_2HPO_4, 0.5 g/L; $NaNO_3$, 2.5 g/L; K_2SO_4, 1.0 g/L; NaCl, 1.0 g/L; $MgSO_4.7H_20$, 0.2 g/L; $CaCl_3$, 0.04 g/L; $FeSO_4.7H_20$, 0.01 g/L; EDTA, 0.08 g/L; H_3BO_3, 2.86 mg/L; $MnCl_2.4H_2O$, 220 mg/L; $CuSO_4.5H_2O$, 79 mg/L; MoO_3, 15 mg/L; and Na_2MoO_4, 21 mg/L and was supplied with light aeration (30 L/min) and the addition of 0.03% CO_2. The pool is continuously stirred with pumps. At the end of the growth, the biomass was recovered and filtered trough a 40 mm membrane, thoroughly washed with distilled water, frozen and lyophilized. Resultant spirulina contained 1% silicon, as indicated by the manufacturer.

2.2. Animals and experimental design

Thirty male and thirty female Sprague-Dawley rats (Janvier-Labs, Le Genest-St-Isle, France) weighing ~100 g were housed at 23 ± 1 °C and were randomly divided in five groups of six animals for each sex, then subjected to a 12 h light/dark cycle with free access to both food and water. They were handled in compliance with European Union rules and according to the guidelines of the NIH (National Research Council, 1985) and the Committee for Animal Care at the University of Montpellier (France).

They were fed *ad libitum* on a standard diet supplied by SAFE (Scientific Animal Food and Engineering, Augy, France) consisting of 236 g/kg casein, 3.5 g/kg L-methionine, 300 g/kg corn starch, 30 g/kg maltodextrin 10, 290.5 g/kg sucrose, 50 g/kg cellulose, 45 g/kg vegetable oil (10.3% energy of the diet), 35 g/kg mineral mix and 10 mg/kg vitamin mix. Vitamin and mineral mixes were formulated according to AIN-93 guidelines (Reeves et al., 1993). Rats and uneaten food were weighed every day. For each sex, rats of each group received daily by gavage either tap water (Control, CTR) or an aqueous suspension of crude spirulina (SP) at a dose of 285 mg/kg BW, or silicon-rich spirulina (SES) at a dose of 28.5 mg/kg BW (SES1 group) or SES at a dose of 57 mg/kg BW (SES2 group), or SES at a dose of 285 mg/kg BW (SES3) for 90 days. All animals were observed twice daily for general appearance, behaviour, signs of morbidity and mortality (once before treatment and once after). The SES2 dose (57 mg/kg BW/day) corresponds to the EFSA recommendations adapted to the rat (EFSA, 2004). The SES1 dose (28.5 mg/kg BW/d) is a two-fold lower dose, while the SES3 dose is fivefold times higher than SES2.

2.3. Sampling

At the end of the experimental period, rats were deprived of food overnight and blood samples were collected under anaesthesia by cardiac puncture. The liver was perfused with 0.15 M NaCl to remove residual blood, rapidly excised, weighed, sectioned for analyses, and stored at –80 °C. Heart, spleen and kidneys were rapidly excised, blotted dry and weighed.

2.4. Haematological and biochemical plasma parameters

All samples were collected early in the working day to reduce biological variation.

Test tubes containing EDTA, as an anticoagulant, were used for haematology. Haematological parameters (red blood cells (RBCs), haemoglobin, haematocrit, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), white blood cells (WBCs), neutrophils, eosinophils, basophils, lymphocytes, monocytes, platelets) were determined with an ABX Pentra DX 120 whole blood automated analyser (Horiba ABX, Montpellier, France).

Heparinized tubes were used for blood biochemistry. All parameters (urea, creatinine, cholesterol (CH), triglyceride (TG), chloride (Cl), calcium (Ca), phosphorus (P), bilirubin, aspartate transaminase (ASAT), alanine transaminase (ALAT) and gamma-glutamyl transferase (GGT)) were determined in plasma samples, after centrifugation (2500 rpm, 15 min), with a Cobas® 6000 automated analyser (Roche, Meylan, France).

2.5. Oxidative status

Liver superoxide anion ($O_2^{\cdot -}$) production by NADPH oxidase were evaluated by the intensity of lucigenin-enhanced chemiluminescence (10 µM lucigenin), measured with a luminometer (Perkin Elmer Wallac, Victor, Turku, Finland) on tissue homogenates, as previously described (Romain et al., 2012). Results were expressed as relative light units (RLU/mg protein).

The index of lipid peroxidation was determined from the liver homogenate by measuring thiobarbituric acid reactive species (TBARS) according to Sunderman et al. (1985). After thiobarbituric acid had been reacted with malondialdehyde, the reaction product was measured spectrometrically at 532 nm. Optical imperfections were corrected according to Allen (1950) at 508 and 556 nm. Results were expressed as nanomoles of TBARS per gram of tissue.

Total thiols (SH) were determined according to Faure and Lafond (1995). The method is based on the reaction of 5,5′-dithiobis (2-nitrobenzoic) (DTNB) with the samples producing a yellow product, the thionitrobenzoic acid (TNB), measured spectrometrically at 412 nm. Results were expressed as nanomoles of SH per milligram of protein.

Protein content was determined by using a commercial protein assay (Sigma, Saint Quentin Fallavier, France) according to the method of Smith et al. (1985) and using bovine serum albumin as standard.

Liver tissue was homogenized in 50 mM phosphate buffer (pH 7.0) and spun at 3500 *g* for 15 min at 4 °C. The superoxide dismutase activity (SOD) was assayed in the supernatant using the method of Marklund and Marklund (1974) and Marklund (1976), based on a competition between the oxidation reaction of pyrogallol by $O_2^{\cdot -}$ and the dismutation of $O_2^{\cdot -}$ by SOD. The reaction product was measured spectrometrically at 320 nm. Results were expressed as unity activity per milligram of protein. The glutathione peroxidase activity (GPx) was evaluated using the method of Flohé and Günzler (1984), and activity was expressed as milli-units per milligram of protein. The activity of catalase (CAT) was measured according to Beers and Sizer (1952). The enzyme catalyses the decomposition of hydrogen peroxide into water molecule. Activity was expressed as units per milligram of protein.

2.6. Inflammatory factors evaluation

For pro-inflammatory cytokines determination, liver tissue was homogenized in 10 mM Tris buffer (pH 7.4) containing 2M NaCl, 1 mM EDTA, 0.01% Tween 80, 1 mM phenylmethylsulfonyl fluoride, and centrifuged at 9000 *g* for 30 min at 4 °C. The

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