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Investigation on the role of *Spirulina platensis* in ameliorating behavioural changes, thyroid dysfunction and oxidative stress in offspring of pregnant rats exposed to fluoride

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ABSTRACT

The study investigated the role of *Spirulina platensis* in reversing sodium fluoride-induced thyroid, neurodevelopment and oxidative alterations in offspring of pregnant rats. The total antioxidant activity, phycocyanins, and β carotene content were quantified in *Spirulina*. Thirty female pregnant rats were allocated to six groups and treatment initiated orally from embryonic day (ED) 6 to postnatal day (PND) 15. Treatment groups included control, *Spirulina* alone, sodium fluoride (20 mg/kg) alone, and sodium fluoride along with *Spirulina* (250 and 500 mg/kg). Serum fluoride levels were determined on ED 20 and PND 11. Offspring were subjected to behavioural testing, estimation of thyroid levels, oxidative measurements in brain mitochondrial fraction and histological evaluation of the cerebellum. Fluoride-induced alterations in thyroid hormones, behaviour and increased oxidative stress. *Spirulina* augmented the displacement of fluoride, facilitated antioxidant formation, improved behaviour and protected Purkinje cells. Supplementing *Spirulina* during pregnancy could reduce the risk of fluoride toxicity in offspring.

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1. Introduction

Fluorine, a strongly electronegative member of the halogen group is one of the most reactive elements, forming fluoride ions in solution (Hem, 1989). Groundwater contains variable concentration of fluoride depending upon the nature of the rocks and the occurrence of fluoride-bearing minerals. Dissolution of fluorite, apatite and topaz from local bedrock raises the level of fluoride in groundwater. Endemic fluorosis continues to be a challenge and a widely studied national health problem in India. Nalgonda, a district in Andhra Pradesh in southern India is seriously affected with endemic fluorosis, with levels of fluoride ranging from 0.1 to 8.8 mg/l in ground water (Brindha, Rajesh, Murugan, & Elango, 2011). This has led to the innovation of a local technology called Nalgonda technique, which is being utilised by developing countries to defluoridate water (Ayoob, Gupta, & Bhat, 2008); however, this technique is suitable only for small communities.

Thyroid gland is particularly sensitive to the deleterious effects of fluoride (Bouaziz, Soussia, Guermazi, & Zeghal, 2005; Shashi, 1988; Susheela, Bhatnagar, Vig, & Mondal, 2005). Follicular epithelial cells of the thyroid gland undergo structural and functional changes on exposure to fluorides (Wang et al., 2005) characterised by a decline in the colloidal content, vacuolation and damage to the endoplasmic reticulum (Assmaa, Manal, & Iman, 2012). All this can disrupt the synthesis of thyroid hormones (Bouaziz et al., 2004). Balanced thyroid hormone status is essential during pregnancy as it facilitates differentiation and maturation of the brain. Fluoride can induce neuroendocrinal changes, triggering developmental disability in offspring.

Natural antioxidants, such as β carotene, tocopherol, chlorophyll, and flavonoids, have gained tremendous attention, owing to their ability to support the physiological system against oxidative stress. *Spirulina platensis* (*Spirulina*), a single celled spiral-shaped blue-green alga (Family Oscillatoriaceae) referred to as a "super food" is nutritionally rich in chlorophyll, β carotene, phycocyanin, and minerals (Annapurna, Deosthale, & Bamji, 1991). *Spirulina* strengthens the immune system, and is considered as the poor man's HIV/AIDS anti-retroviral therapy (Teas, Herbert, Fitton, & Zimba, 2004). Chronic treatment with blueberry, spinach and *Spirulina* prevented ischaemia/reperfusion-induced apoptosis (Wang et al., 2005) and treatment of aged rats with *Spirulina* produced considerable improvement in motor abilities (Bickford et al., 2000).

Spirulina has been evaluated for various activities including neuroprotection in rodents (Bickford, Shukitt-Hale, & Joseph, 1999; Strömberg, Gemma, Vila, & Paula, 2005). It is extensively used the world over as a natural food supplement without being subjected to further extraction. We, therefore, presumed that the

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plethora of nutrients and antioxidants in *Spirulina* might facilitate the displacement of fluoride from tissues and minimise toxicity. To the best of our knowledge, no study has been undertaken so far to examine the protection exerted by *Spirulina* against fluoride-induced thyroid toxicity and its impact on neurodevelopment. Keeping this in perspective, we have investigated the role of *Spirulina* in ameliorating fluoride-induced thyroid toxicity, behavioural alterations, oxidative changes and histopathological changes in the cerebellum.

2. Methods

2.1. Drugs and chemicals

Sodium fluoride was procured from Universal Laboratories Pvt. Ltd, Mumbai; *Spirulina*, a dark blue green alga, as a spray dried powder was purchased from Parry Nutraceuticals, Chennai, India having a composition of proteins (65.38%), phycocyanin (15.37%), mineral (7.95%), total carotenoids (4.3 mg/g), and β-carotene (1.67 mg/g); 2–2′-diphenyl-1-picrylhydrazyl (DPPH), glutathione and corticosterone were purchased from Sigma–Aldrich (St Louis, MO). ELISA kits for the estimation of rat-specific TSH were obtained from GenXbio Health Sciences (P) Ltd., New Delhi, India. All other chemicals used were of analytical grade.

2.2. In vitro evaluation

2.2.1. Quantitative determination of phycobiliproteins

Spirulina powder (1 g) was suspended in 100 ml of sodium–phosphate buffer (0.1 M, pH 7.0, containing 1 mM sodium azide), and sonicated for 60 s. It was then subjected to repeated freezing at −20 °C and thawing at room temperature in the dark to facilitate the extraction of phycobiliproteins. The mixture was subsequently centrifuged at 10,000*g* for 30 min at 4 °C and phycobiliprotein containing clear supernatant was collected. The absorbance of phycobiliprotein-containing supernatant was measured on a UV–vis spectrophotometer at 620, 652, and 562 nm in triplicate and averaged to determine the concentrations of C-phycocyanin (CPC), allophycocyanin (APC), and phycobiliprotein (PE), respectively, using the following equations (Bennett & Bogorad, 1973):

$$\text{CPC (mg/ml)} = [A_{620} - 0.474\ (A_{652})]/5.34$$

$$\text{APC (mg/ml)} = [A_{652} - 0.208\ (A_{620})]/5.09$$

$$\text{PE (mg/ml)} = [A_{562} - 2.41\ (\text{PC}) - 0.849\ (\text{APC})]/9.62$$

2.2.2. Determination of total carotenoids

Methanolic solutions of *Spirulina* (5 mg/ml) were prepared by sonicating powdered *Spirulina* with 70% aqueous methanol for 15 min. The extract was filtered and the methanolic solutions of *Spirulina* (5 mg/ml) were analysed in triplicate for the presence of total carotenoids by recording the absorbance at 470 nm, using a UV/vis spectrophotometer. The concentration of chlorophyll a, and chlorophyll b was also determined in the same extract at 653 and 666 nm respectively. The content of total carotenoids, chlorophyll a, and chlorophyll b was calculated based on the formula proposed by Lichtenthaler and Wellburn (1985). The concentration of chlorophyll a and b was utilised to calculate the total carotenoid content.

$$\text{Chlorophyll a} = 15.65\ A_{666} - 7.340\ A_{653}$$

$$\text{Chlorophyll b} = 27.05\ A_{653} - 11.21\ A_{666}$$

$$\text{Total carotenoids (mg/l)} = 1000\ A_{470} - 2.860\ Ca - 129.2\ Cb/245$$

where C*a* indicates Chlorophyll a and C*b* corresponds to chlorophyll b.

2.2.3. Determination of β-carotene/linoleic acid assay

β-Carotene/linoleic acid were determined by the method of Dapkevicius, Venskutonis, Van Beek, and Linssen (1998). *Spirulina* extract (5 mg/ml) was prepared by shaking the powder with 0.4% (w/v) Tween 40 solution followed by centrifugation at 600*g* for 10 min. Butylated hydroxyl toluene (5 mg/ml) was also shaken with 0.4% (w/v) Tween 40 solution. β-Carotene (0.5 mg) was dissolved in 1 ml of chloroform, 25 μl of linoleic acid and 0.8 ml Tween 20 were added to the above mixture. The chloroform extract was evaporated under vacuum; and 100 ml of distilled water was added to the residue. Aliquots (2500 μl) of the β-carotene/linoleic acid emulsion was transferred to tubes to which *Spirulina* extract or BHT (500 μl) was added. The test tubes were incubated for 2 h at 50 °C together with the control sample. The absorbance was measured at the beginning (t = 0 min) and after the experiment (t = 120 min) at 470 nm. Standard used was butylated hydroxytoluene which was also subjected to the same procedure. All determinations were carried out in triplicate and averaged. The antioxidant activity (AA) was calculated as percentage inhibition of oxidation using the following equation:

$$\%\text{AA} = [1 - (A - A_{120\ \text{min}})/(A_{\text{control}} - A_{\text{control}\ 120\ \text{min}})] \times 100$$

2.2.4. Determination of 2,2′-diphenyl-1-picrylhydazyl radical (DPPH) free radical scavenging activity

Spirulina extract (5 mg/ml) was prepared by sonicating *Spirulina* powder with methanol for 15 min followed by centrifugation at 600*g* for 10 min. To 1 ml of the supernatant, 5 ml of 0.004% methanolic solution of DPPH were added and the absorbance was measured at 517 nm after 30 min. Control (without any additive) and standard (BHT) were also subjected to the same procedure for comparison (Burits & Bucar, 2000a, 2000b). The ability to scavenge DPPH radicals was calculated using the following equation:

$$\text{DPPH scavenging effect (\%)} = 100 \times (A_c - A_t)/A_c$$

where A_c is the absorbance of control, and A_t is the absorbance of test sample.

2.3. Animals, dose selection and experimental design

Healthy adult Wistar rats (180–200 g) were obtained from the National Institute of Nutrition, Hyderabad. They were kept in polypropylene cages, housed in a room at 22 ± 2 °C on alternative 12 h light–dark cycle, fed with standard chow diet (National Institute of Nutrition), and provided with water *ad libitum*. After 1 week of acclimation, one male and two females were placed together in the cage for mating. The females were separated and moved to separate cages after the appearance of the vaginal plug. All the experiments were carried out in accordance with the guidelines set forth by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India and have been approved by the Institutional Animal Ethical Committee (IAEC) bearing number NCOP/IAEC/approved/23/2011 dated 20.09.2011.

Sodium fluoride was administered in a dose of 20 mg/kg based on earlier reports (Paul, Ekambaram, & Jayakumar, 1998). The doses of *S. platensis* selected were 250 and 500 mg/kg based on previous findings (Simsek, Karadeniz, Kalkan, Keles, & Unal, 2009).

Pregnant animals were randomised into six groups with each group consisting of six animals. They were housed separately from gestational day 17 and the litters were culled to eight

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