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Neuroimmunomodulatory role of *Spinacea oleracea* on blastogenic activity in penicillin induced experimental epileptic rat model

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Abstract

Background

Epilepsy - a central nervous system disorder is associated with the production of Reactive oxidative species (ROS) leading to immunosuppression by HPA axis. High levels of glucocorticoids inactivates macrophages, damage DNA resulting in blast cells formation. *Spinacea oleracea* (SO), commonly known as Spinach; is a rich source of antioxidants, thus, can easily modify the ROS level contributing to neuroimmunomodulation and preventing the immunosuppressive damage of Epilepsy.

Methods

Twenty four Holtzman strain male rats were used for this study. The animals were divided into four groups, viz, the control, SO treated control, Penicillin (PCN) induced experimental epileptic group and the SO pretreated PCN induced experimental epileptic group. Experimental epileptogenesis was induced with PCN. After the completion of SO pretreatment, behavioral analysis was done. Peripheral blood mononuclear cells were separated (PMC) for blastogenic activity. Brain tissue samples were used to study the alteration in the levels of antioxidants and neurotransmitters.

Results

Pretreatment with SO leaf extract showed a significant decrease in the blast cell formation unlike the PCN induced experimental epileptic group. The seizure score, ictal phase and LPO level were significantly decreased with an increase in interictal phase, SOD, CAT, GSH level in the SO pretreated epileptic group.

Conclusion

SO, through its high antioxidant content decreases blast cell formation thereby, immunomodulating the oxidative damage during epilepsy.

Key words

Blast cells, epilepsy, neuroimmunomodulation, Penicillin, *Spinacea oleracea*

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Background

Epilepsy is known to be a distinctive brain disorder with seizure activity, affecting people worldwide. It is found to have an effect on all age groups and social classes. The prevalence in developing countries has been estimated to be 4 - 10 per 1000 [1].

Oxidative damage has an important role in the progression of epilepsy - related to brain inflammation with pro-inflammatory cytokine gene expression and DNA damage [2, 3]. Inflammatory stress increases the plasma cytokine levels and thereby, ROS as well as IL -Ib increases and activate the HPA axis [4]. Induced PCN experimental epileptic rat develops immunosuppression due to ROS as epilepsy is related to ROS production [5]. Cytokines production from white blood corpuscles (WBC) increases. Thereby, the Hypothalamus-Pituitary-Adrenal axis (HPA axis) is activated [3]. This boosts the secretion of cortisol - the immunosuppressant [4]. The inactivation of macrophages is associated with the generation of ROS. DNA damage is related to oxidative cellular damage and probably the reason behind blastogenesis as apoptosis marks the end point in blastogenesis [6]. Blastogenesis is the morphological alteration of small lymphocytes into larger cells resembling blast cells on exposure to immunosuppressive agents like glucocorticoids (GCs). Research showed apoptosis is induced by GCs [7]. A series of studies explains that people suffering with epilepsy has a weak immune defense system due to the low endogenous antioxidant content [8].

Studies on human population suggest that Vitamin A, C and E enhance the brain functions and provide a protection against oxidative damages [9, 10]. SO with its high content of antioxidants - the flavonoids and the phenolic compounds exhibit to be a potent free radical scavenger. The water extract of spinach leaves; natural antioxidant mixture (NAO) is known to have antiproliferative and anti-inflammatory properties [3].

In view of the neuroprotective study with SO leaves earlier; this study was conducted to explore the neuroimmunomodulatory role of SO, as no work was carried out on this aspect.

Material and Methods

Study Period

This study was done at Sachindra Nath Pradhan Center for Neurosciences, University of Calcutta, West Bengal, India, for a period of 1 year (2009 Aug. - 2010 Aug.). This study included animal maintenance, their habituation, treatment, behavioral analysis followed by biochemical and immunological studies.

Study design and collection of data

24 male Holtzman Strain adult albino rats (200-250 gms b.w) were used for our study. In accordance with 'Institutional Ethical Committee' rules and regulations standard laboratory conditions were preserved. The animals were individually maintained in steel cages at room temperature (28°C) and at a photoperiod cycle of 12h: 12h (light-dark). Ad libitum drinking water and normal diet of food pellets (Hindustan Unilever Ltd., India), milk, bread and gram powder was provided. Body weights of rats were measured on a routine basis.

The motor behavior, food, drinking habits, micturition and fecal output of the animals were noted along with their habituation to the laboratory condition for 7 days, for further studies.

The behavioral procedure was observed for a two hours period (12:00-14:00 hr)

Collection and preparation of water extract from SO leaves

Fresh, young, healthy leaves of SO were collected from Agricultural Horticulture Society of India, Belvedre, Kolkata and was authenticated by the Botanical Survey of India, Howrah (Voucher specimen no - CHN/I-I/(239)/2008/Tech.II/278). These were stored at S. N. Pradhan Centre for Neurosciences, Calcutta University, for further study purpose. The leaves were handpicked, cleaned, washed, shade dried and grinded (electrical grinder) to obtain a free flowing powder, which was spread over tray with shifting of materials daily to avoid growth of fungus. This powder was subjected to extraction with water (1:3) at room temperature for 24 hours, then filtered through Whatman No. 1 filter paper and vacuum dried at 400-500°C to obtain a brown colored sticky mass. This was refrigerated for further use. Before the pretreatment the extract was dissolved in double distilled water for final use. The final yield was 17% [3].

Treatment

Orogastric cannula was used to orally administer the SO leaf extract at the dose of 400mg/kg body weight for 14 consecutive days between 12:00 and 1:00 hrs. Prior to the experiment the dose was standardized in our lab [11]. The behavior parameters such as seizure score, ictal phase and interictal phase were studied during this period between 12:00 and 1:00 hrs.

Grouping of Animal

The animals were divided into four groups, viz; (1) Control rats (2) only SO treated control (3) PCN induced epileptic rat model (4) SO treated PCN induced epileptic rat model. For fourteen consecutive days the experimental groups were given aqueous leaf extract of SO orally [11].

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Preparation of experimental Epileptic rat model by PCN induced models resembles human generalized epilepsy, thus, this model helps us to understand and study about the basic mechanism of this disease.

Microinjection of penicillin (benzyl penicillin sodium, Alembic Ltd., India) was prepared freshly with isotonic saline. Hamilton Syringe was inserted vertically through a burr hole with the help of a stereotaxic set to inject PCN very slowly in 0.05 ml volume (100 IU) for 5 minutes at the specific loci into the somatosensory cortex (3.2 mm posterior to bregma, 2.25 mm lateral to the midline and depth 1 mm) perpendicular to the surface. Prevention was taken; not to agitate any deeper brain areas and to prevent any reflux of the solution. After withdrawing the syringe, the hole was closed with gel foam and aseptic bone wax. The muscle and the skin were sutured with braided silk finally [12]. Proper antibiotic preventive care was taken for three days by the intramuscular injection of penicillin.

For postoperative care, all sort of sterility was maintained after the surgery, emphasis was given to the feeding until the animals recovered from operative stress. Penicillin at a dose of 10,000 IU was given postoperatively to all animals daily for consecutive three days by intramuscular route. The animals were microinjected for consecutive 3 days [12].

Sham operated control animals were prepared by injecting the vehicle, i.e., isotonic saline of the equal volume at the same site.

Behavioral analysis

The development of seizure activity was observed in the PCN induced experimental epileptic rat model for every 15 minutes for 2 hours according to a modified version of Patel *et al* [13].

No behavioral change - '0'; Facial movements, ear twitching and tail raising - '1'; Myoclonic jerks of the whole body - '2'; Clonic convulsion with rearing - '3'; Forelimb clonus - '4'; Clonic convulsion with falling down and loss of body control - '5'.

Measurement of serum Cortisol

Sample preparation: blood was collected from the tail vein on the 15th day after the behavioral study. Serum and peripheral blood mononuclear cells (PMC) were separated.

Serum cortisol: Serum cortisol was estimated by using Assay Design's Correlate-EIA™ Cortisol Enzyme Immunoassay Kit; Catalog No. 900-071.The reagents were brought to room temperature for at least 30 minutes prior to opening it.

Blastogenesis

The PMC were separated and cultured in presence and absence of:

Conventional mitogens (eg. Phytohemagglutinin PHA, Concavalin A). The supernatant was collected at the end of 72-96 hours and stored at 20°C. The cell viability was measured by MTT assay [3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide] [14]. In another approach, the cells were stimulated with the mitogen and the influence of the plant products on mitogenesis was assessed.

Tissue preparation

Immediately after the behavioral study and collection of blood, rats were sacrificed by cervical dislocation. The brain tissues of Cerebral cortex (CC), Cerebellum (CB), Caudate nucleus (CN), Pons and Medulla (PM) and Midbrain (MB) were dissected out, weighed and homogenized with 5 ml of ice-cold 0.1 M phosphate buffer (pH-7.4) buffer for biochemical estimation.

Measurement of Catalase (CAT)

The homogenates was centrifuged at 3000 rpm for 10 min. The precipitate was stirred with 15 ml of ice-cold 0.1 M phosphate buffer. Then it was allowed to stand in cold condition with occasional shaking (repeated for three times). The rate of decomposition of H_2O_2 was measured spectrophotometrically at 350 nm, by adding, 1 ml of sample with 9 ml of H_2O_2. The activity of Catalase was expressed as % inhibition unit [15].

Measurement of Superoxide dismutase (SOD)

The homogenized samples were centrifuged at 3000 rpm for 10 min. 0.8 ml of TDB was added to 0.1 ml of sample and mixed. With the addition of 4 µl of NADH the reaction began. Then 25 µl of EDTA-$MnCl_2$ mixture was added. The spectrophotometric readings were recorded at 340 nm, followed by a second spectrophotometric reading at 340 nm after adding 0.1 ml of Mercaptoethanol [16].

Measurement of Reduced glutathione (GSH)

To equal quantity of homogenate 10% trichloroacetic acid (TCA) was added and centrifuged - to separate the proteins. 2 ml of phosphate buffer (pH 8.4), 0.5 ml of 5, 5-dithiobis (2-nitrobenzoic acid) and 0.4 ml of double distilled water were added to 0.01 ml of the supernatant. The mixture was vortexed and the absorbance was read at 412 nm within 15 min. The concentration of reduced glutathione was expressed as µg/g of tissue [17].

Measurement of Lipid peroxidation (LPO)

Homogenized brain tissue samples were centrifuged at 3000 rpm for 10 min. 1 ml of TDB was added to 0.5 ml of sample and incubated at 37°C for 1 hour. To this 0.5 ml of TCA was added, vortexed and the absorbance was read at 350 nm. A second reading was taken at an absorbance 350 nm by adding 1 ml sample with 500 µl Mercaptoethanol [16].

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Inclusion criteria
Holtzman strain adult male albino rats weighing between 200-250gms were used for our study.

Exclusion criteria
Female rats were not used for our study as their hormonal level varies during estrous cycle. Moreover, since; the dose of SO was standardized on rats weighing between 200-250gms, rats weighing >250gms and <200gms were excluded from our study.

Ethical committee approval
Animal ethical committee approval was obtained (from animal ethical committee of S. N. Pradhan Center for Neurosciences) before our experiment. This research was conducted according to the declaration of Helsinki (Latest version).

Sample size calculation
We have used two means - Hypothesis testing for two means (equal variances) sample size calculation formulae. After a pilot study on 12 rats from four groups, for getting statistical significance in all groups we kept standard deviation in PCN group = 0.035, Standard deviation in SO+PCN group = 0.045, Mean difference = 2.8, Effect size = 70, Alpha Error(%) = 1, Power(%)= 99, sided = 2, required sample size per group was 5.

Outcome variable
The outcome variables are glucocorticoids, blast cells, CAT, SOD, LPO and GSH.

Explanatory variables
The explanatory variables of our study are SO, PCN, Control, SO+PCN

Data management and statistical analysis
The data were expressed as Mean±SEM. To decide on the level of significance between the means, two-tailed Student 't' test was performed. Difference below the probability level 0.01 was considered statistically significant.

Results

Table – 1 Changes in duration of seizure score, ictal phase and interictal phase of SO pretreated PCN induced experimental epileptic rat model

Group	Seizure score	Ictal phase (in sec.)	Interictal phase (in sec.)	Percentile protection (%)
PCN	94.00±0.92	79.00±0.21	128.00±0.38	0
SO+PCN	40.00±0.41	29.00±0.23	362.00±0.42	69. 79

SO pretreatment of the experimental epileptic group showed a significant decline in the behavioral analysis as compared to the epileptic group. The seizure score and ictal phase were significantly decreased and the interictal phase was significantly increased in SO pretreated control group compared to that of control group (Table-1).

Table – 2 Effect of SO on serum cortisol level

Models	Serum cortisol (ng/ml)	P value
Control	2.37 ± 0.19	0.0001†
SO	2.30 ± 0.13	
PCN	7.5 ± 0.19	0.0001†
SO + PCN	5.1 ± 0.04	

†p < 0.01 statistically significant

The serum cortisol level was increased in PCN induced experimental epileptic group as compared to the SO pretreated experimental epileptic group. SO leaf extract lead to a significant decline in serum cortisol level in SO pretreated control group as compared to the control group (Table-2).

Table – 3 Number of blast cell formation in SO pretreated PCN induced experimental epileptic rat model

Group	Number of blast cells (%)	P value
Control	8.5 ± 6.7	0.0001†
SO	3.7 ± 3.93	
PCN	48.50 ± 8.4	0.0001†
SO + PCN	31.25 ± 7.04	

†p < 0.01 statistically significant

The number of blast cell production increased (p<0.001) in the experimental epileptic group as compared to the SO pretreated epileptic group. The number of blast cell formation decreased (p<0.001) in SO pretreated control group as compared to the control group (Table-3).

A pretreatment with SO leaf extract showed significant alteration in the endogenous antioxidant levels. SOD, CAT and GSH levels in different areas of brain were increased in the SO pretreated PCN induced experimental group (Table-4).

Lipid peroxidation level was higher in the PCN induced group compared to that of SO pretreated PCN induced group. The levels of SOD, CAT and GSH were significantly increased (p<0.001) in only SO pretreated control group as compared to the control group (table-4). Likewise the LPO activity was significantly decreased (P<0.001) in SO pretreated control groups rather than control groups (Table-5).

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Table – 4 Effect of SO on SOD, CAT and GSH level in PCN induced seizure

Group	CC	P value	CB	P value	CN	P value	MB	P value	PM	P value
SOD (% inhibition unit)										
Control	13.30±0.41	0.0001†	11.28±0.32	0.0001†	11.31±0.28	0.0001†	11.12±0.14	0.0001†	12.47±0.19	0.0001†
SO	10.31±0.22		9.82±0.18	0.0001†	9.96±0.11		9.79±0.21		10.27±0.26	
PCN	22.68±0.3	0.0001†	18.91±0.55	0.0001†	20.11±0.42	0.0001†	19.58±0.36	0.0001†	24.85±0.32	0.0001†
SO + PCN	18.34±0.17		13.89±0.64	0.0001†	13.91±0.36		15.11±0.41		15.86±0.32	
CAT (% inhibition unit)										
Control	13.98±0.09	0.0001†	12.30±0.14	0.0001†	12.54±0.19	0.0001†	13.19±0.22	0.0001†	12.16±0.17	0.0001†
SO	12.27± 0.25		10.81±0.15	0.0001†	10.66±0.16		11.79±0.18		10.55±0.18	
PCN	21.35±0.12	0.0001†	21.10±0.29	0.0001†	18.99±0.29	0.0001†	21.72±0.30	0.0001†	20.12±0.68	0.0001†
SO + PCN	14.12± 0.15		12.83± 0.56	0.0001†	12.45± 0.20		14.30± 0.34		12.97±0.19	
Reduced glutathione (µg/g of tissue)										
Control	30.54±0.76	0.0001†	29.62±0.81	0.0001†	29.61±0.44	0.0001†	23.9±0.26	0.0001†	26.65±0.43	0.0001†
SO	31.21±0.77		31.2±0.61		28.08±0.58		26.71±0.41		27.68±0.81	
PCN	20.11±0.4	0.0001†	18.18±0.42	0.0001†	14.86±0.12	0.0001†	15.25±0.44	0.0001†	17.12±0.45	0.0001†
SO + PCN	25.11±0.54		25.11±0.61		23.97±0.17		25.45±0.72		25.12±0.63	

†p < 0.01 statistically significant

Table – 5 Effect of SO on LPO level on PCN induced seizure

Group	LPO (nmol of TBARS / gm mol of tissue)									
	CC	P value	CB	P value	CN	P value	MB	P value	PM	P value
Control	4.03±0.10	0.0001†	3.99±0.46	0.0001†	3.46±0.07	0.0001†	3.89±0.07	0.0001†	3.72±0.12	0.0001†
SO	2.93±0.11		2.90±0.07		3.19±0.34		2.89±0.18		3.35±0.28	
PCN	9.11±0.23	0.0001†	6.98±0.24	0.0001†	7.56±0.36	0.0001†	7.67±0.32	0.0001†	6.98±0.23	0.0001†
SO + PCN	5.19±0.16		3.83±0.25		4.19±0.32		4.53±0.35		4.45±0.23	

†p < 0.01 statistically significant

Discussion

Our study explains the neuroimmunomodulatory effect of SO in PCN induced experimental epileptic rat model. The behavioral study showed that at a dose of 400mg/kg body weight SO leaf extract significantly decreased the seizure activity, ictal phase and increased the interictal phase. Besides the cortisol level, rate of blast cell formation also decreased with SO pretreatment.

Epilepsy and neuroimmunomodulation

From our study it is manifested, that, epilepsy is associated with significant high levels of cortisol causing immunosuppression in the epileptic rat model. Probably this is related to the brain inflammation associated with epilepsy [2]. Inflammatory stress is related to high plasma cytokine levels and activation of the HPA axis. Immune system is also modulated by the hypothalamus - through a direct innervations and an endocrine mechanism. Thymus and spleen receive a direct impulse from the autonomic nervous system [18]. The role of hypothalamus regulating pituitary - adrenal activity is an example of neuroimmunomodulation [4]. Activation of HPA axis increases secretion of corticotrophin releasing hormone (CRH). This triggers the anterior pituitary to secrete ACTH.

Excess ACTH causes excess secretion of glucocorticoids which modify macrophage function (inactivates) and control the host's immune response to pathogens [4]. Our study explained this with high levels of cortisol in PCN induced experimental epileptic model as compared to the SO pretreated experimental group. In lymphoid cells culture studies, apoptosis was evident by immunosuppressive agent like GCs [7]. On exposure to GCs, blast cells production increases. A significant rise in the blast cell formation and in the cortisol level in epileptic group as compared to the SO pretreated group. SO being a rich source of antioxidants modulates the immune functions of the body. This is evident from the results, a significant decrease in the glucocorticoids levels and blast cell formation in SO treated control and SO pretreated experimental epileptic group as compared to control and PCN induced experimental epileptic group respectively.

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Antioxidants and neuroimmunomodulation

A rise in LPO levels with low SOD, CAT and GSH values ensuing blast cell formation in experimental epileptic group denoting the oxidative cellular damage [19]. The endogenous antioxidants viz; SOD, CAT, GSH is known to scavenge the free radicals [3]. CAT helps in the removal H_2O_2 and thus provides a protection against oxidative cellular damage [20]. Similarly SOD contributes to this protective action by destroying the superoxide radical [3]. With the help of the enzyme GPX reduced form of glutathione is converted to its oxidized form to prevent the generation hydroxyl radicals. So, ROS depletes the endogenous antioxidant levels leading to the increase in cortisol level and formation of blast cells observed in this research. SO pretreatment reduced the blast cell formation by enhancing the immune functions of the body through modulations in the endogenous antioxidant levels. Spinach is rich in the antioxidants like beta-carotene and lutein. Natural antioxidant mixture (NAO) obtained from SO leaf extract has antioxidative antiproliferative and anti-inflammatory properties [21-23].

Conclusion

Based on this present study SO can be categorized as a nootropic agent. The neuroimmunomodulatory role of SO enhanced the endogenous antioxidant levels with a decrease in cortisol level, leading to immunomodulation. Decrease in oxidative cellular damage improves the immune functions. Thus, this study provides a probable solution, an herbal remedy for the management of epilepsy.

Limitations & future scope of the study

Clinical trials are needed to be carried on humans. Moreover the active component of SO needs to be isolated and advance studies on the endogenous antioxidants level should be done for the evaluation of its therapeutic significance.

Abbreviations

Adrenocorticotropic hormone (ACTH), Catalase (CAT), Cerebellum (CB), Cerebral cortex (CC), Caudate Nucleus (CN), Central Nervous System (CNS), Corticotrophin releasing hormone (CRH), Deoxyribonucleic acid (DNA), Ethylenediaminetetraacetic acid (EDTA), Gamma Aminobutyric acid (GABA), Glucocorticoids (GCs), Reduced Glutathione (GSH), Glutathione peroxidase (GPX), Hydrogen peroxide (H_2O_2), Hypothalamic Pituitary axis (HPA axis), Interleukin Ib (IL-Ib), Lipid peroxidation (LPO), Midbrain (MB), Manganese dichloride (MnCl2) 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT assay), Nicotinamide adenine dinucleotide (NADH), Natural antioxidant mixture (NAO), Nitric Oxide (NO), Penicillin (PCN), Peripheral mononuclear cells (PMC), (PHA), Pons medulla (PM), Reactive Oxidative species (ROS), *Spinacea oleracea* (SO), Superoxide Dismutase (SOD), Trichloroacetic acid (TCA), Triethanolamine - diethanolamine (100 mM each)- HCl buffer, pH 7.4 (TDB), White blood cells (WBC).

Competing interests

None declared. This study was not supported by any funding agency.

Authors' contribution

Mondal M performed the experiments, collected the data and drafted the manuscript. Guha D, Maity S, Sinha S directed this research work and helped with the critical analysis. Sathian B helped with the statistical analysis of the data. Final manuscript was accepted for publication by all authors.

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