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Immunomodulatory Effects of *Nigella sativa* (Black Cumin) on Cyclosporin Induced Toxicity in Spleen of Rat

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NY and ABA conceived the idea of study. NY, ABA, MI and HA contributed to the implementation of research. SF, HA and SM analyzed the results. NY and ABA and SM finalized the manuscript. All authors discussed the results and commented on the manuscript.

Key words
***Nigella sativa*, Immunomodulation, Toxicity, Cyclosporin, Immunohistochemical staining**

ABSTRACT

Nigella sativa is a popular herb, known for its immunomodulatory effects. Studies have proved its ability to enhance lymphocyte population and reduce toxicity of drugs through its anti-oxidative property. Cyclosporin is a commonly used immunosuppressive agent which acts by inhibiting T cells activation. It induces toxicity in many organs including spleen. This study was planned to observe the immunotoxic effects of cyclosporin on rat spleen and its immunomodulation by *N. sativa*, through morphometric and micrometric analysis on routine hematoxylin and eosin (H and E) staining and CD3 immunohistochemical staining. Sprague Dawley rats were randomly divided into three groups. Group A served as control, group B was administered oral cyclosporin and group C was administered oral cyclosporin with *N. sativa*. At the end of the study period, spleen was harvested for H and E and CD3 immunohistochemical (IHC) staining. Histomorphometric examination of H and E-stained sections showed thickened capsule of spleen, reduced white pulp with decrease in peri-arteriolar lymphoid sheath (PALS), congested red pulp and central artery showing vacuolization; while immune-stained sections of spleen showed significant (P=0.003) reduction in diameter of PALS and significant (P<0.001) reduction in number of T lymphocytes per reticule in cyclosporin treated group B. *N. sativa* improved the above parameters. In conclusion, *N. sativa* was able to modulate the immunotoxic effects of cyclosporin in rat model as seen on spleen histological sections.

INTRODUCTION

Black cumin or *Nigella sativa* seeds are black and triangular with pungent smell and bitter taste. They belong to the Ranunculaceae family. *N. sativa* is popularly known as Kalonji in the South Asian region and Habbat-ul-Sauda or Habbat-ul-Baraka, meaning Seeds of Blessing in Arab countries (Benhaddou-Andaloussi *et al*., 2011). The seeds of *N. sativa* are highly nutritive, containing 20-85% proteins, in form of amino acids like arginine, tyrosine, and others; 31.94% carbohydrates; minerals like copper, phosphorus, zinc, iron and others; and 7-94% fiber. Seeds also contain 26-34% fixed oils and 0.4%–2.5% essential or volatile oils. These oils have been isolated to study their individual properties. Thymoquinone is one such important oil which has been extracted. In Tibbe-Nabvi, it is believed to have cure for all illnesses except death. In Ayurvedic medicine, it has been used for centuries as a carminative, diuretic, liver tonic, antidiarrhoeal, and a natural spice (Yimer *et al*., 2019).

Kalonji's or *N. sativa*'s most documented effects includes antioxidation and immunomodulation. Its other common pharmacological effects have been listed in literature as: anti-inflammatory, anti-bacterial, anti-histaminic, anti-helminthic, anti-fungal, anti-diabetic and anti-hypertensive. It also shows various neuroprotective effects on conditions like depression and anxiety, epilepsy, Alzheimer's disease and Parkinsonism (Yimer *et al*., 2019).

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Various studies have proven that *N. sativa* has an immunostimulant effect on lymphoid organs, including spleen and lymph nodes (Mahmoud *et al*., 2021). It is also known to increase the WBC production and enhance cell mediated immune responses in animal models (Mahmoud *et al*., 2021; Mokhtari-Zaer *et al*., 2020). It can increase T cell and natural killer cells production and can ameliorate the age-induced T cell and hemoglobin decline (Tutuncu, 2020). In lymph nodes, *N. sativa* has also shown to induce lymphoid hyperplasia in parafollicular and medullary regions with increase in overall number of lymphocytes (Mahmoud *et al*., 2021). It has furthermore shown to correct splenic lymphocytic depletion and marginal zone enlargements, thereby reducing splenomegaly (Ebaid *et al*., 2011).

The spleen's parenchyma is divided into two well-defined morphological red and white pulp compartments. The red pulp consists of blood-filled sinusoids while the white pulp consists of lymphocytes. These lymphocytes are arranged either around the central artery as PALS (peri-arteriolar lymphoid sheath) with predominantly T-cells or in the form of lymphatic nodules with predominantly B-cells. The marginal zone is situated at the interface of white and red pulp. The main function of red pulp is to remove damaged/worn out erythrocytes and the white pulp is the site of differentiation of helper T cells into its different phenotypes (Kim and Liu, 2020; Borch *et al*., 2019). The helper T cells, in turn, activate cellular immunity and the germinal centers of B lymphocyte follicles to produce antibodies (Borch *et al*., 2019). Cyclosporin is a common immunosuppressant which acts by inhibition of T lymphocyte signaling and eventually preventing it from activation and proliferation in target tissues. It is used for the prevention of allograft rejections. It is also prescribed in autoimmune diseases like rheumatoid arthritis and psoriasis. Cyclosporin is a calcineurin inhibitor which in turn leads to inhibition of gene transcription of interleukins, interferon and other lymphokines. It also causes calcineurin inhibition in non-lymphatic tissue and may prove toxic to these organs. It exerts its toxic effects by increasing the reactive oxygen species (ROS) and decreasing antioxidant enzymes like super oxide dismutase (SOD) and catalases (Amber and Tabassum, 2020; Omar *et al*., 2013).

Studies have shown cyclosporin to have detrimental effects on the immune system. After the ingestion of cyclosporin, the lymphoid organs demonstrated various pyknotic lymphocytes, the lymph nodes were found to be hypocellular and disorganized (Legrand *et al*., 2013) and the thymic medulla was considerably reduced (Sawanobori *et al*., 2021). As spleen has the largest secondary lymphoid aggregates in the body, the effect of cyclosporin toxicity is clearly observable in it. Cyclosporin reduces splenic white pulp, by acting on the lymphocytes present in PALS and marginal zone (Omar *et al*., 2013; Alberti *et al*., 2021). Arteriolar hyalinosis, a hallmark feature of cyclosporin toxicity, is also observed in splenic section (Al-Houri *et al*., 2019).

The present study was designed to observe the immunotoxic effect of cyclosporin and its amelioration by *N. sativa* on the spleen of rats.

MATERIALS AND METHODS

Animals and treatment

Sprague Dawley rats (n=45, 10-12 weeks old, 165-205g) were used for this study. They were housed in plastic cages and were given standard rat diet *ad libitum*. The temperature was controlled at 30° C with 8/16 hours day/night cycle. The animals were randomly and equally divided into three groups. Group A (control group) received no intervention, Group B (treatment group) received oral cyclosporin by gastric gavage at a dose of 15mg/kg/day for 21 days and Group C (prevention group) received oral *N. sativa* seeds at a dose of 450 mg/kg/day in addition to oral cyclosporin by gastric gavage in the same dose as Group B for 21 days.

Assessment of splenic damage

For assessing spleen damage, the spleen was fixed in 10% formaldehyde solution, processed for 4–5 µm thick sections after paraffin embedding. Sections were stained with hematoxylin and eosine (H and E) and morphology of splenic tissue was observed under light microscope.

For immunohistochemical staining 4-5 µm thick sections were subjected to the Pan CD3 antibody treatment which stains only T lymphocytes giving them brown colour (Al-Houri *et al*., 2019). Hematoxylin contrast was used which stains the surrounding tissue blue. T lymphocytes predominate in PALS region while B lymphocytes predominate in lymphatic nodules. For morphometric measurements the diameter of PALS and number of T lymphocytes were noted at 100 X and 1000 X magnifications, respectively; and means for both the diameter of PALS and T lymphocyte count per reticule in different groups were calculated.

Statistical analysis

All data was analyzed using SPSS version 22.0. Difference in groups were analyzed by one-way analysis of variance (ANOVA) followed by post hoc test Tuckey. The significance difference between groups was accepted at $P < 0.05$ at 95% confidence interval.

RESULTS

Histopathological structure of spleen

Morphological/histopathological examination of H

and E-stained sections of spleen from control group showed a covering stromal connective tissue capsule, made up of dense fibrous tissue and a fine cellular stroma, made up of a network of reticular connective tissue supporting the splenic tissue. The parenchyma was easily distinguishable as red pulp containing sinusoids filled with blood cells, and white pulp containing basophilic lymphocytes, arranged as PALS around central artery and lymphatic nodules (Fig. 1A). In between the interface of white and red pulp, the marginal zone is situated. At a higher magnification, the central artery of PALS showed uninterrupted endothelium with underlying smooth muscle layers.

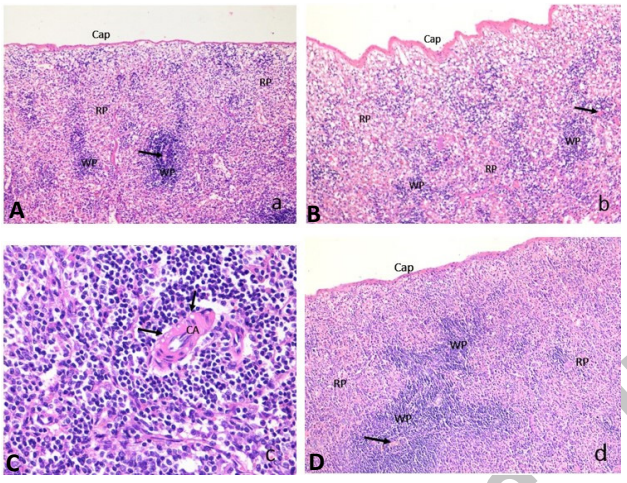

Fig. 1. Histological structure of H and E-stained sections of rat spleen. **A**: Control group A spleen showing parenchyma, divided into white pulp (WP) and red pulp (RP) and arrow showing central artery, covered by connective tissue capsule (Cap) 40X. **B**: Cyclosporin treated group B spleen showing congested red pulp (RP), reduced white pulp (WP) with peri-arterial lymphoid sheath (PALS) surrounding central artery (arrow) and increased marginal zone (MZ) 40X. **C**: Cyclosporin treated group B spleen showing vacuolization (arrows) in the wall of central artery (CA) 400X. **D**: *N. sativa* protected group C spleen showing slightly thick capsule (Cap) and parenchyma showing increased white pulp (WP) surrounding central artery (arrow) 40X.

Cyclosporin treatment group B animals showed thickened capsule of spleen. Within the parenchyma, there was reduction in size and cellularity of PALS, congested sinusoids of red pulp, and increased thickness of marginal zone (Fig. 1B). At higher magnification, the central artery showed hyalinosis and vacuolization within the intimal endothelial layer (Fig. 1C).

In *N. sativa* protected group D animals showed less thickened capsule, parenchyma reaching its normal proportion with increased size and cellularity of PALS and lymphatic nodules in white pulp, less congested red pulp sinusoids; and thickened marginal zone (Fig. 1D). The central artery showed no deposits of hyalinosis and vacuolization in endothelium.

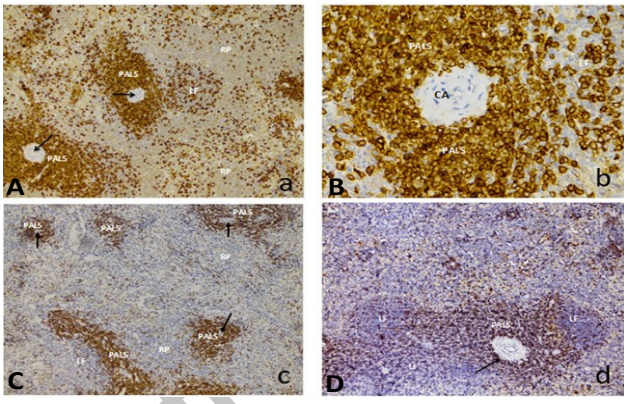

Fig. 2. Histological structure of rat spleen. **A**: Control group A showing normal distribution of brown colour CD3 positive T lymphocytes in peri-arteriolar lymphoid sheath PALS, surrounding the central artery (black arrow), circular lymphoid follicle (LF) 100X. **B**: Control group A showing PALS containing brown T lymphocytes surrounding central artery (CA) 400X. **C**: Treated group B showing reduced density of PALS surrounding central artery (black arrow), red pulp (RP) and lymphoid follicle (LF) are also appreciated 100X. **D**: Protected group C showing normal density and size of PALS surrounding central artery (black arrow), abundant lymphoid follicles (LF) which are also increased in size 100X.

Morphometric examination in immuno-stained tissue

The immuno-stained sections from spleen of control group A showed brown CD3 positive T lymphocytes with a blue background (Fig. 2A). The white pulp can be demarcated as a circular area of PALS showing highest density of brown pigment, indicating the presence of T-lymphocytes around the central artery (Fig. 2B); and less highlighted circular lymphoid follicles (Fig. 2A). Red pulp can also be appreciated around the white pulp (Fig. 2A). In cyclosporin treated group B sections, the area of PALS and density of brown pigment is reduced (Fig. 2C). The size of PALS is increased in *N. sativa* protected group as compared to group B; moreover, there is an appreciable increase in number and size of lymphoid follicles (Fig. 2D).

The mean diameter of PALS in spleen of control group A was found to be 290.00±48.72, which significantly (P=0.00) decreased 213.00±30.20 in treated group B. While it increased 232.00±24.40 significantly (P=0.03) in spleen of protected group C rats when compared to group

A and insignificantly (P=0.47) when compared to group B (Table I).

Table I. Mean diameter of PALS and T lymphocyte count of different groups.

Group	Diameter of PALS (µm)	T count per reticule (µm2)
A (n=20)	290.00 ± 48.72	10.37±0.76
B (n=20)	213.00 ± 30.20*	7.53±1.04*
C (n=20)	232.00 ± 24.40*	11.37±0.64*/**

Values are written as Mean ± SD (Standard Deviation). *Significant in comparison to group A; **Significant in comparison to group B.
For details of groups, see Figs 1 and 2. PALS, peri-arteriolar lymphoid sheath; T, T lymphocyte.

The mean T lymphocyte count/reticule in spleen of control group A was observed to be 10.37±0.76 (Table I), which was decreased 7.53±1.04 significantly (P=0.00) in treated group B; while the counts increased 11.37±0.64 significantly in protected group C in comparison to group A (P=0.03) as well group B (P=0.00) (Table I).

DISCUSSION

This study established that splenic injuries brought about by cyclosporin were impeded by the simultaneous use of *N. sativa*.

Morphology of splenic tissue was distorted in cyclosporin treated group B, which included thickening of capsule, decrease in white pulp (specially PALS), congestion of red pulp, and arterial hyalinosis and vacuolization as shown in other studies (Alberti *et al*., 2021).

Arterial vacuolization is the most common toxic feature of cyclosporin treatment observed in transplanted kidneys (Hamasaki *et al*., 2017). This feature has also been demonstrated in other immune suppression conditions, like diabetes (Al-Harbi *et al*., 2019) and nephrotoxicity (Oyouni *et al*., 2018); but is reversible (Hamasaki *et al*., 2017). The pathogenesis of vacuolization has been linked to degeneration of basement membrane and smooth muscle cells, leading to protein leakage from the circulating blood (Mencke *et al*., 2019); which still stands true with vasoconstriction being its precursor (Lusco *et al*., 2017). Amador *et al*. (2016) clarified the molecular mechanism of vasoactive control of smooth muscle and endothelial cells. Cyclosporin induction experiments clearly linked mineralocorticoid receptors' involvement in vasoactive modulations leading to vacuolization *in vitro* and *vivo* mouse model. Mencke *et al*. (2019), talked about hyalinosis in renal vasculature as being an ageing process as well as drug induced; hasten by the deficiency of klotho, an anti-aging protein expressed in renal tubular cells. The above-mentioned distorted changes were improved by the antioxidant effects of *N. sativa* protected group C, which were same as observed by Mahmoud *et al*. (2021), Ebaid *et al*. (2011), Essawy *et al*. (2010) and Ahmad *et al*. (2019), in spleen and other organs. In accordance with the study of Mahmoud *et al*. (2021), *N. sativa* protected group showed an increase in PALS and other areas of white pulp.

Continuous proliferation of T cells makes them more exposed to xenobiotic compounds, which translates as degenerative alterations in the lymphocytes. These alterations were noted in cyclosporin treated group but not in *N. sativa* protected group (Figs. 1 and 2). Cyclosporin has intracellular binding receptors in T cells. After binding, it initiates a cascade of reactions, which causes cell death by dedifferentiation (Omar *et al*., 2013). Low density of lymphocytes with congested red pulp and pyknosis were also seen by Mazen *et al*. (2017), in silver nanoparticles toxicity. The active ingredient of *N. sativa*, thymoquinone was able to improve the nephrotoxic changes of cyclosporin due to its antioxidant and anti-inflammatory effects (Alrashedi *et al*., 2018); same as demonstrated by Alkis *et al*. (2021), with renal changes secondary to radiation.

Polyclonal CD3 antibody was used in this study for immuno-staining, which is a lineage specific, multimeric protein complex and a pan T-cell marker (expressed in all T cells), presenting on the surface of mature T lymphocytes and in cytoplasm of immature T lymphocytes (Rehg *et al*., 2012). Since, CD4 and CD8 markers are specific for helper and cytotoxic T cells respectively, they were not included in this study (Natalini *et al*., 2021). The expression of CD3 positive lymphocytes, concentrated in PALS around central artery, was reduced with cyclosporin treatment, were well-matched with the results of Omar *et al*. (2013) and Alberti *et al*. (2021). This effect is due to the inhibition of calcineurin dependent interleukin-2 production by cyclosporin, as debated by Fellman *et al*. (2019). In *N. sativa* protected group, the number of CD3 positive T cells and diameter of PALS increased but the area of PALS around central artery appeared less dense (Fig. 2) because CD3 positive T cells were dispersed. Moreover, the size and number of lymphoid follicles were reactively increased, which had been recognized as *N. sativa's* anti-oxidative property by Mahmoud *et al*. (2021); and immunomodulatory properties which augments T cells and natural killer cells mediated immune responses (Khazdair *et al*., 2021). This has also been proven by hematological studies, which showed *N. sativa* increasing TLC (Younus *et al*., 2020). Enhanced splenocyte proliferation was seen by *N. sativa*, *in vivo* in a dose-responsive fashion, as seen by Liang *et al*. (2021). In the protected group, new lymphoid follicles and immature

immunoblastic cells were seen, as B cell proliferation is independent of helper T cells and could be due to response to antigenic challenges.

CONCLUSION

It can be concluded from the results of this study that the herb, *N. sativa* was able to mitigate the toxic effects of cyclosporin in spleen of adult Sprague Dawley rats. This study could be a gateway for *in vivo* human research and substitution in transplant and immuno-compromised patients.

DECLARATIONS

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IRB approval

Institutional Review Board at Baqai Medical University, Karachi approved the study (Ref: BMU-EC/2016-03).

Ethical statement

The study was conducted after approval from the Ethics Committee at Baqai Medical University, Karachi.

Statement of conflict of interest

The authors have declared no conflict of interest.

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