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Original Article

The Toxic Effects of Cyclosporine on Splenic Tissue of Rat and it's Amelioration with Nigella Sativa

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ABSTRACT

Background: Cyclosporine is an immunosuppressive drug. Its mechanism of action is inhibition of T cells activation. Cyclosporine has toxic effects on many organs including spleen. Nigella sativa is an immunomodulatory agent. It increases lymphocyte population and also has promising anti-oxidative effects.
Objective: Observe the toxicity of cyclosporine and evaluate the ameliorative effects of Nigella Sativa in spleen
Material and Methods: This study was conducted in Baqai Medical University from November 2016 to December 2016. Sprague Dawley rats were randomly divided into three groups. Group A was control, group B was given oral cyclosporine only and group C was given cyclosporine with Nigella sativa. At the end of study period animals were sacrificed and spleen was harvested.
Results: Body weight of cyclosporine group B was decreased significantly (P<0.001) in comparison to control A and increased in Nigella sativa and cyclosporine group C. Absolute and relative spleen weights were also decreased in cyclosporine group B and increased in Nigella sativa and cyclosporine group C. Morphometric examination of splenic tissue showed thickened capsule, congested red pulp, reduced white pulp and hyalinosis and vacuolization in central artery in cyclosporine group B while these features were reduced in Nigella sativa and cyclosporine group C.
Conclusion: Cyclosporine caused toxicity in spleen while Nigella sativa was helpful in ameliorated these effects.
Keywords: Cyclosporine, Nigella Sativa, Spleen, morphometric Study, arterial vacuolization, Arterial hyalinosis.

Introduction

Cyclosporine is a cyclic polypeptide consisting of 11 amino acids.[1] It is a metabolic product of fungus species *Tolypocladium inflatum*.[2] It is an immunosuppressant drug used in solid organ transplant rejections and autoimmune diseases like rheumatoid arthritis and psoriasis. [1, 2, 3] It functions by inhibiting T lymphocyte signaling thus preventing its activation and proliferation in target cells. [1, 4]

Cyclosporine enters into the cell, binds to the cyclophilin, a cytosolic receptor protein and forms cyclosporine cyclophilin complex; [1] which then binds to calcineurin, a three component protein phosphatase. [4]

This binding inhibits the translocation of nuclear factor for activated T cells (NFAT) from cytosol to nucleus thus inhibiting gene transcription of interleukins, interferon and other lymphokines. [1, 5] Cyclosporine also inhibits calcineurin in non-lymphatic tissues which is one of the causes for the toxic effects of cyclosporine seen in other tissues. [5] Other cause of toxicity is increase in reactive oxygen species and decrease in activities of antioxidant enzymes like Super oxide Dismutase (SOD) and Catalases. [3]

Spleen is the largest lymphoid organ in body. Its parenchyma is divided into two distinct morphological and functional compartments; the red and white pulps. Red pulp removes damaged and worn out erythrocytes and circulating foreign materials. [6] White pulp is linked to immune system where differentiation of Helper T lymphocytes into phenotypes occur; [7] which is responsible for activation of cellular immunity as well as germinal center of B lymphocytes to produce antibodies. [7, 8]

Cyclosporine causes reduction in total percentage of splenic white pulp, reduction in periarteriolar lymphoid sheaths (PALS) and marginal zones of white pulp and loss of lymphocytes within PALS and marginal zones. [3] The hallmark feature of cyclosporine

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toxicity, the arterial hyalinosis is also seen in spleen.[10, 11]

Nigella sativa (Kalonji) is a dicotyledonous seed of the Ranunculaceae family. [12] The seeds are triangular in shape and black in colour with pungent smell. In Arabic countries its name is "Habbat-ul-Baraka", meaning "Seed of Blessing". [12] It is believed to have curative properties for all diseases except death. [13] Its most documented pharmacological property is anti-oxidation, [14] which is attributed for its immunological effects. [12] It has shown to increase the weight of lymphoid organs, [15] increases the white blood cell production; enhance the cell mediated immune response and antibody titers. [16] In spleen, it has shown to increase lymphoid follicles and to enlarge marginal zone. [17, 18]

The current study was planned; to investigate the adverse effects of cyclosporine on the body weight, on spleen weight, morphological changes in spleen at microscopy level and to evaluate the amelioration of Nigella sativa by its immunomodulatory and antioxidant effects at microscopy.

Material & Methods

This study was conducted in Department of Anatomy, Baqai Medical University, Karachi, after approval from the Ethics Committee of Baqai Medical University. Time period of study was 2 months from November 2016 to December 2016.

ANIMALS;

Thirty Sprague Dawley rats of age 10-12 weeks and weight 165-205 grams were used in this study. The animals were housed in Baqai medical university animal house under controlled environment of temperature 30° C with 8 hours day and 16 hours night cycle. They were kept in plastic cages and given standard rat diet ad libitum.

CHEMICALS;

Cyclosporine (Sandimmune Neoral® oral solution, USP) was purchased. It is present as clear, viscous, yellow liquid supplied in 50 ml bottles containing 100mg/ml cyclosporine. It is distributed by Novartis Pharmaceuticals Pakistan. Nigella sativa whole seeds were purchased from local market and identified by pharmacists. They were cleaned and then used in this study.[9]

EXPERIMENTAL PROTOCOL;

The rats were randomly divided into three groups, each group having ten rats each.

Group A (control group) was given no intervention. They were kept on same diet ad libitum as was given to other groups at different times.

Group B was treated orally with cyclosporine at a dose of 15mg/kg/day for 21 days [3] through gastric gavages. [8]

Group C was treated orally with cyclosporine at a dose of 15mg/kg/day for 21 days; concomitantly this group was given Nigella sativa seeds orally at a dose of 450 mg/kg/day one week prior to treatment and continued for 21 days.

COLLECTION OF SAMPLES;

Weight of the animals was recorded at the beginning and at the end of experimental period. Then the animals were anesthetized with ether and sacrificed by exsanguination. Spleen was dissected out. Its absolute weight was recorded and relative weight or splenic index was calculated with the help of the formula; [19]

$$\textit{Relative spleen weight} = \frac{\textit{Weight of spleen (gm)}}{\textit{Final body weight (gm)}} \times 100$$

HEMATOXYLIN & EOSIN STAINED STUDY;

Spleen was fixed in 10% neutral formalin. It was processed and paraffin blocks were made. Four to five micrometer thick sections were made and then stained with H & E for morphometric study of splenic architecture under light microscope.

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's test) accepted at $P < 0.05$.

Results

BODY AND SPLENIC WEIGHTS;

Body weight of the animals in group A controls was increased significantly ($P<0.001$) over the period of time.

Final body weight of Cyclosporine treatment group was decreased significantly ($p=0.001$) (Tabe le 1). Final body weight of protected Nigella sativa group C increased but was not significant ($P=0.082$). There was significant difference in body weight change of cyclosporine treated group B ($P<0.001$) and nigella sativa protected group C ($P=0.001$) when compared to weight change of controls. Absolute and Relative

Table-1: Body weights and spleen weights of different groups

Experimental groups	Initial body weight (gm)	Final body weight (gm)	Absolute Spleen weight (gm)	Relative Spleen weight (gm)
Group A No Treatment	184.50±4.61	195.30±3.97**	0.715 ± 0.043	0.366 ± 0.022
Group B Cyclosporine	183.80±4.14	172.60±3.87*	0.593±0.048	0.346 ± 0.031
Group C Cyclosporine+ Nigella Sativa	185.63±3.65	186.25±3.58	0.665 ± 0.047	0.356 ± 0.025

Values are written as Mean ± SEM (Standard Error of Mean).
* (p=0.001) statistically significant **(P<0.001) statistically significant

weight of spleen of cyclosporine treatment group B and nigella sativa protected group C decreased as compared to controls but was not significant (Table 1).

MICROSCOPIC EXAMINATION:

Normal architecture of control rat spleen as examined is shown in Figure-1.

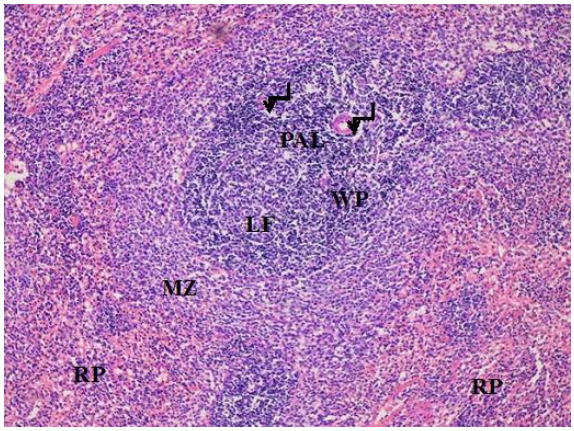

Figure-1: Control spleen showing normal architecture. Parenchyma is divided into White pulp (WP) and Red Pulp (RP). White pulp comprises of Peri-arteriolar lymphoid sheath (PALS) surrounding central artery (arrow), lymphoid follicle (LF) and Marginal Zone (MZ). Marginal zone separates white pulp from red pulp. H & E x 100

The spleen was covered with dense irregular connective tissue capsule with one to two layers of fibroblasts. There were prominent trabeculae containing blood vessels in the parenchyma. Parenchyma was well demarcated as red and white pulp. White pulp consisted of basophilic lymphocytes, which were arranged as **periarterial lymphatic sheaths** (PALS) around central artery and lymphatic nodules at different locations. The red pulp showed anastomosing cords of reticular tissue with sinusoids filled with blood cells. Marginal zone is situated between red and white pulp. Central artery appeared normal with layers of smooth muscles and intact endothelium.

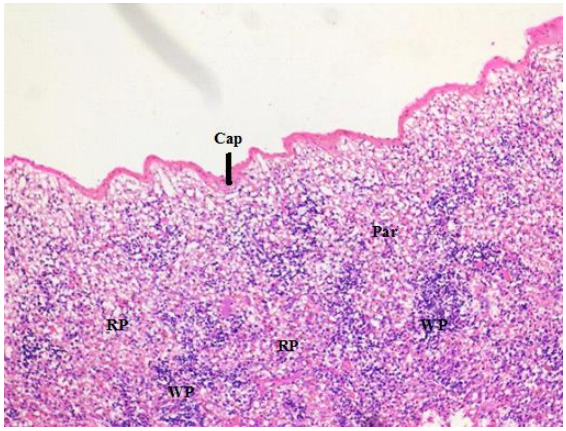

Figure-2: Cyclosporine treated spleen showing thick capsule (Cap). Parenchyma (Par) is disturbed. White pulp (WP) is reduced in size and red pulp (RP) is congested. (H & E x 400)

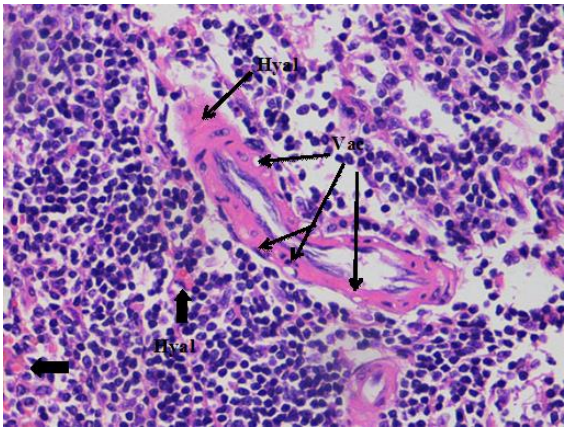

Figure-3: Cyclosporine treated spleen showing central artery nodular hyalinosis (Hyal). Arterial vacuolization (Vac) is also clearly visible. Hemosiderin laden macrophages are also seen (bold arrows). (H & E x 400)

The morphology of cyclosporine treated spleens as we examined are shown in figures 2 & 3. There was

thickened capsule with three or more layers of fibroblasts. White pulp area was reduced in comparison to red pulp. The area of PALS and its cellularity was also reduce. The red pulp showed short anastomosing cords of reticular tissue with congested sinusoids. Marginal zone was increased in thickness. The central artery showed hyalinosis (Figure 3) which appeared as concentric/nodular hyaline deposition in intimal layer. Central artery also showed vacuoles in endothelial layer (Figure 3). Hemosiderin laden histiocytes and macrophages were also seen (Figure 3).

In Nigella sativa protected group the spleen capsule was less thickened than treated group with two to three layers of fibroblasts (Figure 4). White pulp area was increased in comparison to red pulp. The area of PALS and its cellularity was increased. The areas of lymphoid follicle and marginal zone are increased. Red pulp showed short anastomosing cords of reticular tissue with congested sinusoids (Figure 4). The Central artery showed very few or no arterial hyalinosis deposits and vacuoles (Figure 4).

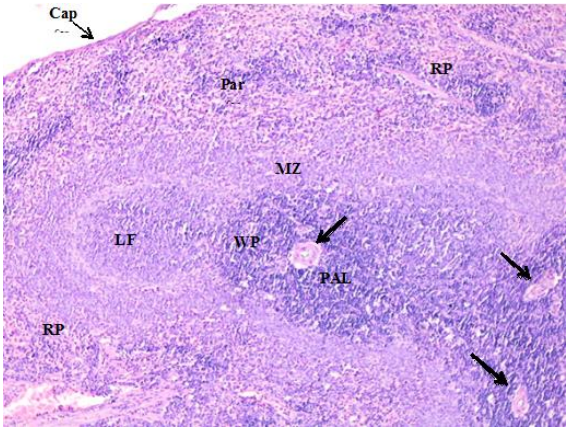

Figure-4: Nigella sativa protected spleen showing capsule (cap) and parenchyma (Par). White pulp (WP) area is increased. Lymphoid follicle (LF) and marginal zone (MZ) is increased more than PALS. PALS is surrounding the central artery (bold arrow) which doesn't show hyalinosis or vacuolization. (H & E x 100)

Discussion

The observations and results of the present study demonstrated that cyclosporine induced spleen toxicity and Nigella sativa reduced those toxic effects.

Body weight of animals was reduced after taking cyclosporine in this study. This effect is same as observed by Baldwin et al [20] where cyclosporine attenuated animals from taking food thus causing loss in body weight. Experimental group of Jiang et al [21] also had lower weights than controls whose mechanism was explained to be cyclosporine induced down regulation of gluconeogenesis. Animals from Nigella sativa protected group C showed increase in body weight in comparison to treated group B as Nigella sativa intake is believed to increase body weight directly as it did in boilers. [15] Secondly Nigella sativa had an effect on decreasing oral bioavailability of cyclosporine by increasing clearance at absorption site.[22]

Organ weight can be the most sensitive indicator of organ toxicity even when no morphological changes are present. [23, 24] Spleen weight alone is a relative insensitive tool as it can be affected by many factors like strain of animal, age, sex, stress, housing and environmental conditions. [23] Thus organ weight should be normalized with body weight; the organ to body weight index or relative organ weight; then organ weight will correlate well with the toxicity. [23, 24] In this study the absolute and relative spleen weights were decreased in cyclosporine treated groups pointing towards organ toxicity. Nigella sativa protected the effects of cyclosporine in group C which was seen by comparative less decrease in absolute and relative weights than group B. This protection was also seen by Kaleem et al where Nigella sativa increased the body weight and weight of liver. [25]

Toxic injury to spleen could be seen as reactive lesions in architecture that is white or red pulp or stroma including capsule and trabeculae of spleen. [26] In this study, general architecture was disturbed. The capsule was thickened in cyclosporine treated group. This feature was also observed by Hossam Ebaid et al where chloramphenicol treatment thickened capsule and disturbed the trabecular pattern within the parenchyma. [18] In this same study, Nigella sativa showed protection from toxic effects: almost normal capsule and minimal toxic effects on parenchyma. Same findings were seen in the present study in Nigella sativa protected group.

Vacuolization can be seen as a toxic effect of many basic drugs (e.g. procaine, nicotine, etc). [27] Cyclosporine is also known to cause vacuolization in kidneys when given in transplant patients, which was a feature of drug toxicity not rejection in such patients. [28] Vacuoles were seen in cyclosporine treated group as compared to control group in arterial wall and were less in Nigella sativa protected group. Same effects were observed by Nahed A Omar] in spleen after treatment by aflatoxins.[29] Vacuolization occurs when

weak bases enter the acidic organelles of cells like lysosomes and endosome, become positively charged at low pH and get trapped in these organelles. The water is then forced in due to charged osmosis pressure.[27] Nigella sativa treated group showed less vacuolization as compared to treated group. The protection against carbon tetrachloride induced vacuolar degeneration by Nigella sativa was also seen in liver by Essawy et al which was attributed to its antioxidant effect. [30]

Arterial hyalinosis is a hallmark feature of cyclosporine toxicity, which starts as a nodular deposit and can progress concentrically to whole thickness of arteriolar wall. [10,11] In kidneys, the arteriolar hyalinosis is a sign of chronic drug toxicity specially calcineurin inhibitor (cyclosporine) toxicity. [31] Arterial hyalinosis was seen in this study in cyclosporine treated group in central and trabecular arteries in form of nodular deposits, same as seen by Krawczynski.[10] Arterial hyalinosis refers to a degenerative lesion which causes thickening of intimal layer of arterial walls by homogenous pink hyaline deposits in routine staining. This deposit is without inflammation or necrosis of the vessel wall. [10, 32] Different theories are given to explain the exact nature of these deposits which are; the plasma proteins leaking from circulating blood, [32] the thickening due to degenerating basement membrane materials or degenerating smooth muscle cells. [10] The deposits are composed of fibrin and γ globulin as proposed by Gupta et al and Krawczynski. [10, 32] Krawczynski had seen that these deposits were made up of immune deposits, IgG, IgM, globulin and fibrin, the soluble plasma proteins. [10] In present study almost no deposits were seen in Nigella sativa protected group. Same protective effect was observed by Uz et al [33] in chronic nephrotoxic effects of cyclosporine. The mechanism of protection postulated by Uz et al was the antioxidant effects of Nigella sativa. Same antioxidant effect of Nigella sativa ameliorating diabetes induced hyalinosis in kidneys had also seen by Mahood et al. [34]

Conclusion

It was concluded from above study that the cyclosporine caused toxicity in rats which was seen by decreased body and spleen weights and morphometric changes in splenic architecture. Nigella sativa was helpful in ameliorating these toxic effects in rats. This animal study can be taken as first step towards human research where side effects of cyclosporine might be reduced by adjuvant therapy with Nigella sativa.

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C. Analysis/Interpretation/Discussion
D. Manuscript Writing
E. Critical Review
F. Facilitated for Reagents/Material/Analysis