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Anti-inflammatory Effects of Diazepam on Different Models of Inflammation: Roles of Peripheral Benzodiazepine Receptors and Genes for Corticosterone, Nitric Oxide and Cytokines Biosynthesis

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Abstract

Background: It was reported that Peripheral Benzodiazepine Receptors (PBRs) agonists as diazepam possesses immunomodulatory and anti-inflammatory activities that suppress inflammation.

Aim of the study: This study aims to investigate the anti-inflammatory effects of diazepam on the different inflammatory responses in the rats with exploring the possible underlying mechanisms.

Methods and animals: 88 Albino rats used in acute and chronic inflammatory study of carrageenan–induced inflammatory paw edema of the rats (CIPE), and Freund's adjuvant induced polyarthritis in rats respectively. Tested rat groups in both models received diazepam (I.P). Bilateral adrenalectomy was done in some rat groups of acute and chronic models as reference groups to diazepam treated groups with intact adrenals glands, and indomethacin was used as a reference anti-inflammatory agent in acute and chronic models of inflammation. The hind paw thickness and plasma Nitric Oxide (NOx) were assessed in each sub-groups of acute inflammatory response. The chronic inflammatory response was assessed by measuring the paw thickness, serum C-reactive protein, serum albumin and serum corticosterone of these arthritic rats.

Results: Diazepam significantly reduced the increase in the paw thickness as well as NOx levels compared to other groups of acute study. Administration of diazepam for arthritic rats significantly decreased paw thickness, reduced increase in C-reactive protein, increased serum albumin, and increased serum corticosterone in comparison to administration of indomethacin to the arthritic rats. Diazepam had no any inhibitory anti-inflammatory actions on paw edema thickness, and on others measured serum biochemical when administrated to adrenalectomized rats in both acute and chronic inflammatory studies.

Conclusion: Diazepam through enhancement of activity of genes responsible for synthesis of corticosterone via its stimulatory action on Peripheral Benzodiazepine Receptors (PBRs) in adrenal glands and modulating activity of immune cells could be of high pharmacological interest as a potential anti-inflammatory agent.

Keywords: Diazepam; Peripheral Benzodiazepine Receptors (PBR); Inflammation; Corticosterone; Nitric oxide; Cytokines

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Introduction

Inflammation is a pathological series of protective responses generated by the host in response to infection or any other insults [1]. Inflammatory events are initiated, and coordinated by the actions of various chemical mediators. There are several body cell types, such as mast cells, platelets, and leukocytes are responsible for the release of inflammatory mediators and chemicals; that play an important role in the development of inflammation. The elevated levels of cytokines and chemokines can promote and amplify the inflammatory response [2]. These cytokines and chemokines are generally proteins, in addition to the low-molecular weight lipids derived from arachidonic acid, are playing an important proinflammatory role. In addition to this, gases like nitric oxide and carbon monoxide are recognized also as inflammatory mediators [3]. Experimental and clinical evidences suggested that benzodiazepines could influence immune cell properties, such as phagocyte activity, chemotaxis and the production of superoxide, and cytokines; either by reducing the stress and anxiety or through stimulation of Peripheral Benzodiazepine Receptors (PBRS) [4]. These PBRS have been identified mainly into the mitochondria of non-neuronal peripheral cells as adrenocortical cells [5] and also, in the cellular components of the immune system, such as monocytes, neutrophils, macrophages [6].

Ligands of PBRs have been found to regulate activity of genes responsible for biosynthesis of cholesterol derivatives as corticosterone modulate monocyte functions such as chemotaxis [7,8]. Also, these ligands have been reported to suppress the production of IL-1, IL-2, IL-6, TNF-α, IFN-γ, and to increase glucocorticoid production [9]. Therefore, this study aims to investigate the possible anti-inflammatory effects of the diazepam on acute and chronic inflammatory responses in the rats with exploring the possible underlying mechanisms involved in these anti-inflammatory actions.

Material and Methods

Chemicals and drugs: Were used in the study

Complete Freunds adjuvant, Sodium Carrageenan and Kits for measurement of Serum corticosterone, serum albumin and serum C-reactive protein (Sigma Chemical Co., USA). Diazepam (2 mg ampoules, Memphis Pharmaceutical Co., Cairo, A.R.E.). Indomethacin (5 mg ampoules, Merk, Sharp and Dohme, USA). Hydrocortisone 100 mg vial (Aventis pharmaceutical, Co., Cario, A.R.E.) was used.

Acute experimental models of inflammation: Carrageen induced acute paw edema in rats: Forty rats of both sexes, weighing about 200-250 g each were used in this experiment; the hind paw edema was induced by sub-plantar injection of 0.1 ml of 1% sterile carrageenan suspension in normal saline into one of the hind paws of the rat while the other contralateral hind paw of the rat was injected with 0.1 ml of sterile normal saline solution according to Winter et al. [10].

Rats were divided into 5 equal sub-groups (in each sub-group; the rats were received normal saline, diazepam or Indomethacin intraperitoneally (I.P) 1 h before carrageenan administration: Control Saline-treated rats: Rats were given 1 ml normal saline 0.9% (I.P) and served as a control group. Diazepam-treated rats: Rats were given diazepam in a single dose of 10 mg/kg (I.P) [11]. Adrenalectomized sham rats: Rats were subjected to bilateral surgical adrenalectomy and were given 1 ml normal saline 0.9% (I.P) [12]. Adrenalectomized+diazepam treated rats: Rats were subjected to bilateral surgical adrenalectomy, and then one week later these rats were given diazepam in a single dose of 10 mg/kg (I.P) [12]. Indomethacin–treated rats: Rats were given indomethacin in a single dose of 10 mg/kg (I.P) and served as reference anti-inflammatory group [10].

Chronic experimental model of inflammation (Group of adjuvant arthritis): 48 rats of both sexes, weighing about 200-250 g each were used. 8 rats were kept as non-arthritic saline treated group and the remaining 40 rats were used to induce adjuvant arthritis by single intradermal injection of 0.1 ml complete Freund's adjuvant into the base of each rat's tail [13]. The arthritic rats were subdivided into 5 equal subgroups according to the following schedule: Arthritic Saline-treated group: Rats were given 1 ml normal saline 0.9% (I.P) 3 times/week for 2 weeks and served as control arthritic group. Arthritic Diazepam- treated group: Rats were given diazepam in a dose of 10 mg/kg (I.P) 3 times/week for 2 weeks [12]. Arthritic adrenalectomized (ADX) Sham group: Rats were subjected to bilateral surgical adrenalectomy then one week later, they were given 1 ml normal saline 0.9% (I.P) 3 times/week for 2 weeks. Arthritic Adrenalectomized (ADX)+diazepam treated group: Rats were subjected to bilateral surgical adrenalectomy and the one week later, they were given diazepam as in subgroup II-b. Arthritic Indomethacin–treated group: Rats were given indomethacin in a dose of 10 mg/kg (I.P) 3 times/week for 2 weeks and served as a reference group [10,14]. At the end of the experiment, rats were subjected to the following procedures:

1) The anti-inflammatory effect was assessed by paw edema test using the water displacement method (Plethysmometer-Harvard Apparatus) described by Winder et al. [15] **(Figure 1)**.

2) Blood samples: Rats were sacrificed and blood was collected for measurement of the following parameters:

A) Acute phase proteins as C-reactive protein and serum albumin.

B) Serum corticosterone.

C) Determination of Nitric Oxide (NO): Nitric oxide was determined in the plasma by measuring NO metabolites (NOx) based on the method of Moshage et al. [16].

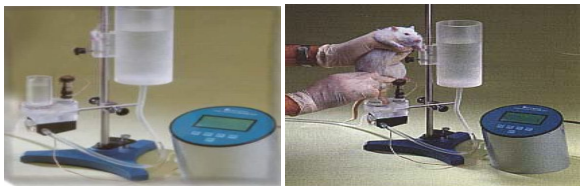


Figure 1 Plethysmometer-Harvard apparatus.

Statistical Analysis

Results are expressed as mean ± S.E.M. Differences between two groups are composed by Student's t-test. P<0.05 is considered statistically significant.

Results

Effects of Diazepam on acute inflammatory study

A single sub-plantar injection of carrageenan induced an increase in the paw thickness over 24 h. The rat group pretreated with Diazepam had a significantly reduced (p<0.05) an increase in the paw thickness after 24 h with 34.6% reduction of the paw edema as well as NOx level. While rats group pretreated with indomethacin produced 36.88% reduction the paw edema and NOx level. The sub-plantar injection of carrageenan into the right hind paws of the adrenalectomized rats, produced a significant (p<0.05) increase in the right hind thickness over the first 24 h later to carrageenan injection. Diazepam had no any inhibitory effects on the paw thickness as well as NOx level in adrenalectomized+diazepam treated rats (p>0.05) **(Figure 2, Histogram 1 and Table 1)**.

Effect of Diazepam on chronic adjuvant arthritic inflammatory study

40 rats injected with Freund's adjuvant developed systemic polyarthrits as manifested by redness and swelling of joints assessed by significant (p<0.05) increase in paw thickness, in addition to the significant (p<0.05) increase in serum C-reactive protein, and significant decrease in both serum albumin (p<0.05) and serum corticosterone (p< 0.05) when compared to the non-arthritic control group.

Administration of diazepam for 2 weeks after development of arthritis in subgroup II-b, produced significant (p<0.05) decrease in paw thickness, significant (p<0.05) decrease in C-reactive protein, significant (p<0.05) increase in serum albumin, and significant increase in serum corticosterone (p<0.05) as compared to arthritic saline-treated rat group. In Arthritic adrenalectomized rats, there were insignificant changes in the above mentioned parameters when compared to arthritic saline treated group. Also, the administration of diazepam to the adrenalectomized arthritic rats had no effects on the above mentioned parameters. Administration of indomethacin to the arthritic rats produced a significant decrease in paw thickness (p<0.05) but it did not affect the changes in the other parameters **(Figure 3, Histogram 2 and Table 2)**.

Discussion

The results of the study revealed that, diazepam (prototype benzodiazepine agonist) has a significant anti-inflammatory effect on the different experimental models of inflammation whether, acute or chronic. In the acute inflammatory study, diazepam produced significant reduction of Carrageenan-induced Paw Edema (CIPE) and serum NOx levels in rats. While in the chronic inflammatory study, diazepam produced significant reduction the paw thickness, significant reduction in the serum

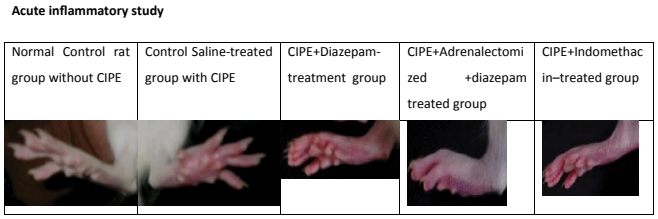


Figure 2 Carrageenan induced acute inflammatory paw edema in different rats groups and effects of diazepam pretreatment on CIPE in comparison to other rat groups of acute inflammation study (CIPE: Carrageenan–Induced Paw Edema).

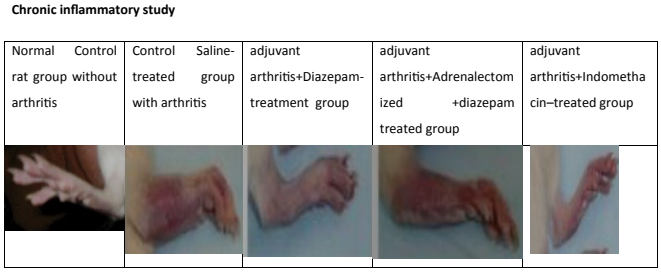
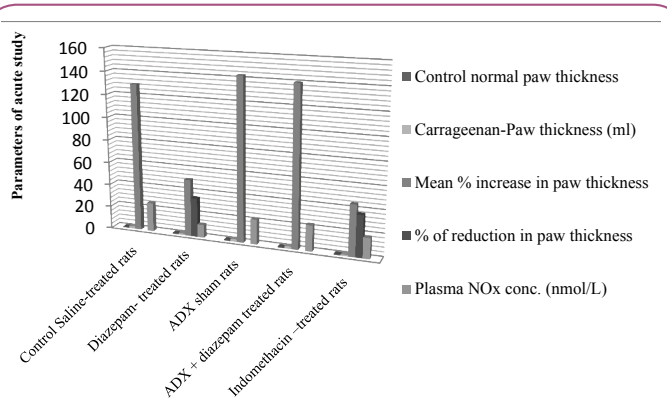
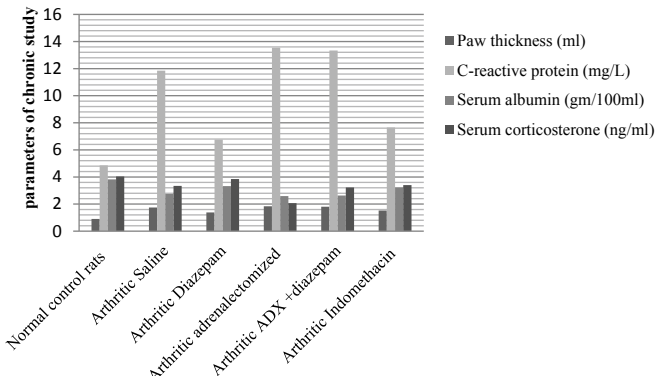


Figure 3 Freund's adjuvant induced polyarthrits in different groups and effects of diazepam treatment on adjuvant arthritis in comparison to other rat groups of chronic inflammatory study.



Histogram 1 Effects of diazepam on paw thickness, mean % increase in paw thickness, plasma NOx concentration of rats with acute paw edema compared to other rat groups of acute inflammatory study.

C-reactive protein, significant increase in serum albumin and significant increase in the serum corticosterone level. The results of the acute inflammatory study of diazepam on CIPE and on serum NOx levels in rats; were in agreement with all of the previous reports and results of Lazzarini et al. [11,12,17], who reported that the high doses of diazepam (10 mg/kg) injected (I.P) produced anti-inflammatory effects as was demonstrated by the significant reduction in the CIPE in rats and it was found that this high dose of the diazepam was in the same range of benzodiazepine doses reported to modify immune reaction [9]. Also, present results are similar to the results of Torres et al.; Farges et al. [18,19], who reported that the peripheral



Histogram 2 Effects of diazepam on paw thickness, C-reactive protein, serum albumin, and serum corticosterone of adjuvant arthritic rats compared to others rat groups of chronic arthritic study.

benzodiazepine receptor ligands 4-chlorodiazepam and PK 11195 produced a significant reduction in the mouse paw edema induced by carrageenan. The results of chronic inflammatory study shown that adjuvant arthritis in rats induce a significant decrease in serum corticosterone and serum albumin but a significant increase in both serums. C-reactive protein and the average paw thickness. A similar finding had been reported in adjuvant arthritic rats [20] and also, in the patients with rheumatoid arthritis [21]. From the results of chronic study, it was found that diazepam produced significant reduction in the paw thickness, significant reduction the serum C-reactive protein, significant increase in serum albumin and significant increase in the serum corticosterone level. While, indomethacin administration reduced significantly only the paw thickness and the joint swelling without any effect on the remaining parameters. This consistent with the previous report of Stecher et al. [22], who noticed that agents like NSAIDs may provide only symptomatic relief of arthritis without altering the progression of the disease and do not affect the disturbed parameters of the arthritis. The serum corticosterone level increased after administration of diazepam in arthritic diazepam treated rats, indicating that the observed anti-inflammatory effects of diazepam could be due to induction and activation of endogenous underlying genes responsible for corticosterone production by the adrenal glands. Such explanation is in agreement with results of Lazzarini et al. [11,12], who reported that a, single dose (10 mg/kg I.P) of diazepam treatment to carrageenan-injected rats produced a 2.4-fold increase in the serum corticosterone level. Also, it is consistent with results of Righi et al. [23], who reported that diazepam treatment (3 mg/kg I.P) daily for 30 days produced a significant increase in the serum corticosterone levels in the treated hamsters in relation to those measured in the animals of control saline treated group. Also, from the results of chronic inflammatory study, revealed that diazepam significantly increased serum albumin level and decreasing significantly serum C-reactive protein, which is an acute phase protein commonly used to assess the disease activity in the inflammatory rheumatic diseases, and this effect of diazepam could be explained by the fact that it can suppress secretion of pro-inflammatory cytokines from mouse macrophages such as TNF-α, IL-1, and IL-6 which is the main stimulator of acute phase protein synthesis during acute and chronic inflammation [9,21,24]. Bilateral adrenalectomized were done in some rat groups of acute and chronic inflammatory studies of the present work to show that the anti-inflammatory effects of diazepam were due to stimulation of genes responsible for endogenous corticosterone secretion from the adrenal glands, where the adrenalectomy significantly precluded and prevented the anti-inflammatory effects attributed to diazepam on CIPE in rats, also on the adjuvant arthritic rats as was demonstrated by the inability of diazepam to affect the different arthritic parameters of the adrenalectomized arthritic rats. This consistent with the results of Lazzarini et al. [12] who reported that the adrenalectomy significantly abrogated the anti-inflammatory effects of the diazepam on both; CIPE and the carrageenan-induced pleurisy model in rats and this was confirmed previously by Yang et al. [25] who reported that adrenalectomy leads to exacerbation of adjuvant arthritis in rats. The possible underlying anti-inflammatory mechanisms of diazepam on acute and chronic inflammation could be occurred at least at six levels. The first level, the anti-inflammatory effects of diazepam may be due to the stimulation of genes responsible for steroid synthesis, which is one of the best characterized functions of PBRs by the PBR agonist like diazepam [26,27] where diazepam stimulates steroid synthesis mainly in adrenal gland cells and also, in the placenta, testis and ovaries; by enhancing the translocation of cholesterol from the outer to inner mitochondrial membranes [28,29]. Our results in accordance with previous findings of an experimental study reported that pretreatment with diazepam (5 mg/kg i.p) exhibited anti-inflammatory effects in cerulein-induced acute pancreatitis, as demonstrated with a significant reduction in the inflammatory response of acute pancreatitis; by ameliorating pancreatic edema, serum levels of amylase and lipase, myeloperoxidase activity, pancreatic TNF-α, and pathological alterations compared to control group not treated with diazepam. They assumed that some of beneficial anti-inflammatory effects of diazepam on pancreatitis could be mediated possibly through PBRs, where diazepam decreased interleukin release from macrophages and suppressed neutrophil activities [30]. The second level could be explaining the significant reduction of the inflammation in adjuvant arthritis with diazepam treatment, where it was assuming that PBRs expression was down-regulated in the chondrocytes when rheumatoid arthritis developed and the expression of these receptors in the chondrocytes was restored and increased following the treatment with a PBR ligand as diazepam or 4'-chloro- diazepam and this may account for the preventative and therapeutic actions of these PBR ligands on the rheumatoid arthritis, where the progressive erosion of cartilage and bone destruction may occur through a PBR-mediated modulation of apoptosis in chondrocytes, where PBRs are characterized by the modulation and control of the apoptosis process and the PBR ligands exhibit a potent anti-apoptotic activities, thus limiting erosion of cartilage and bone destruction in arthritis [31] and this hypothesis is consistent with the results of Bribes et al. [32], who reported that the PBR ligands as 4-chloro-diazepam could be effective in the management of the rheumatoid arthritis in the mice. At the third level, the PBRs anti-inflammatory effects could be explained also by the modification of genes responsible for cytokines production and secretions.

Table 1 Effect of diazepam on rat paw edema & NOx measurements.

Parameters / Rat groups	Paw thickness (ml) (Mean ± S.E)		Mean % increase in paw thickness ± S.E	% of reduction in paw thickness from Control Saline-treated rats	Plasma NOx conc. (nmol/L)
	Carrageenan injected tested paw	Saline –treated paw (control paw)			
Control Saline-treated rats	2.25 ± 0.08	0.98 ± 0.03	129.60 ± 9.87	-	25.51 ± 2.11
Diazepam- treated rats	1.47 ± 0.05	0.98 ± 0.03	50.34 ± 3.40*	34.66	11.53 ± 1.01
Adrenalectomized sham rats	2.38 ± 0.12	0.98 ± 0.03	142.85 ± 9.07	-	22.1 ± 1.91
Adrenalectomized +diazepam treated rats	2.35 ± 0.08	0.98 ± 0.03	139.79 ± 9.89#	-	23.0 ± 2.11
Indomethacin –treated rats	1.42 ± 0.07	0.98 ± 0.03	44.89 ± 4.57*	36.88	18.31 ± 1.50

Number of the rats in each group = 8 rats & Parameters values expressed in Mean ± S.E
*P value: Comparing the mean % increase in the rat paw thickness of all tested rat groups with that of control saline-treated rat group.
#P value: Comparing the mean % increase in the rat paw thickness of Adrenalectomized+diazepam treated rats with that of diazepam- treated rats.

Table 2 Effect of diazepam on the paw thickness, serum C- reactive protein, serum albumin and serum corticosterone in adjuvant arthritic rats (Mean ± S.E).

Parameters / Rat groups	Control Non-arthritic rats	Arthritic rats				
		Arthritic Saline-treated	Arthritic Diazepam-treated	Arthritic adrenalectomized Sham group	Arthritic Adrenalectomized +diazepam treated	Arthritic Indomethacin – treated
Paw thickness (ml)	0.90 ± 0.09	1.75 ± 0.11*	1.38 ± 0.06#	1.84 ± 0.09	1.80 ± 0.07¥	1.52 ± 0.04#
C-reactive protein (mg/L)	4.86 ± 0.13	11.85 ± 1.14*	6.76 ± 0.43#	13.54 ± 1.30	13.34 ± 1.10¥	7.65 ± 0.85
Serum albumin (g/100 `ml)	3.82 ± 0.11	2.75 ± 0.13*	3.33 ± 0.03#	2.59 ± 0.20	2.63 ± 0.04¥	3.23 ± 0.05
Serum corticosterone (ng/ml)	4.04 ± 0.03	3.34 ± 0.04*	3.85 ± 0.05#	2.07± 0.17#	2.10± 0.20#¥	3.40 ± 0.19

Number of the rats in each group = 8 rats & Parameters values expressed in Mean ± S.E

* *P* value: Comparing the result of Arthritic Saline-treated with that of non-arthritic group.

P value: Comparing the result of Arthritic Diazepam- treated, Arthritic adrenalectomized Sham group, Arthritic Adrenalectomized +diazepam treated and Arthritic Indomethacin –treated with that of Arthritic Saline-treated.

¥ *P* value: Comparing the result of Arthritic Adrenalectomized +diazepam treated with that Arthritic Diazepam- treated.

Indeed, cartilage destruction is a major characteristic of rheumatoid arthritis and is linked to aberrant cytokines and growth factor expression in affected tissues [33]. It is well known that, IL-1, IL-6, TNF-α and IFN-γ can affect chondrocytes function [34]. IL-6 has been shown to boost progression from an initial inflammation to a chronic state [35]. Diazepam treatment was found to reduce and to suppress macrophage secretions of IL-1, IL-6 and TNF-α [9]. Also, the PBR ligands as 4'-chloro-diazepam reduces dramatically both IL-6 and IL-13 expression in the pleural exudation of mice injected with carrageenan [18]. Our results were in agreement with results of recent study reported that diazepam and resveratrol, are possessing obvious potential anti-inflammatory effects comparable to that of sulfasalazine on dextran sulphate sodium model of colitis as demonstrated with a significant decrease in serum levels of IL-6, TNF-α, PGE2, increase in serum corticosterone, decrease in colonic rat tissue leucocytes count, MOP, MDA and with a significant marked increase in antioxidant enzymes CAT and SOD in colonic tissues of DSS-induced colitis rat group treated concomitantly with diazepam and resveratrol [36]. The fourth level, a previous study was suggested an important role of NO on the effects of diazepam on CIPE, where these anti-inflammatory effects of diazepam reflect its direct action on the PBRs present in the endothelial cells of the microvascular ambient and/or on immune and inflammatory cells that migrated to the inflammatory site at the rat paw thus, leading to a decrease in NO generation by NO synthase, which leading to a decrease in CIPE [17]. This is consistent with the previous report of Handy and Moore [37], who reported that compounds have the ability to inhibit NO biosynthesis can reduce both phases of carrageenan inflammatory response and thus, may provide a viable targets for the development of novel anti-inflammatory compounds. While in the fifth level [38], was reported that there are functional and biochemical evidence for diazepam as a cyclic nucleotide phosphodiesterase type 4 inhibitor. It was found that inhibition of PDE-4 isoenzyme can elicit some anti-inflammatory effects [39]. The effect of diazepam on phosphodiesterases especially PDE-4 could be also part of the mechanisms involved in some experimental and clinical effects of benzodiazepines which are not well understood. Interestingly, diazepam has some effects such as airway smooth muscle relaxation [40] and hyperglycemia which are shared by PDE-4 inhibitors [41] and also, it is known that diazepam inhibits antigen-induced eosinophil infiltration into guinea-pig conjunctiva [42] as well as, other immune cell functions such as phagocytosis

of human polymorphonuclear leukocytes and monocytes or human natural killer cell activity [43]. Lastly at the six levels, it was reported that Calcium Channel Blockers (CCBs) reduce the CIPE in rats and was attributing this anti-inflammatory effect due to stimulation of the HPA axis by CCBs [44]. This is consistent with the report of Schaufele et al. [45] who reported that diazepam is acting through a common mechanism with calcium antagonists, where some of peripheral and central effects of diazepam are thought to be induced by inhibition of adenosine uptake or by inhibiting calcium channels.

Conclusion

The results of present study with underlying anti-inflammatory mechanisms of diazepam suggesting that the peripheral benzodiazepine receptor agonists like diazepam could be of high pharmacological interest as a potential anti-inflammatory agent and also a useful tool to increase our understanding of the inflammatory mechanisms and to provide fertile ground for development of new, safe and more effective anti-inflammatory agents.

Conflict of Interest

The author declares no conflict of interest.

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