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TIME COURSE OF SYSTEMIC AND BRAIN CYTOKINE EXPRESSION BY ENDOTOXIN AND ROLE OF DEXAMETHASONE

Shuvranshu Praharaj [*1], V. K. Kalaichelvan [2], V. Murugan [3] and Ishtiyaque Ahmad [1]

Jubilant Biosys Ltd, Bangalore [1], Annamalai University [2], College of Pharmaceutical Sciences, Dayananda Sagar University [3], Bangalore - 560022, Karnataka, India.

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Correspondence to Author:
Mr. Shuvranshu Praharaj

Sr. Research Scientist,
#96, Industrial Suburb, 2nd Stage,
Industrial Area, Yeshwanthpur,
Bangalore - 560022, Karnataka, India.

E-mail: shuvranshup@gmail.com

ABSTRACT: Literature evidence suggests that neuroinflammation plays a key role that activates and propagates the neurodegenerative process associated with many neurological diseases. Different stimuli, such as lipopolysaccharide (LPS) are the endotoxin that activates the immune system to create neuroinflammation associated with neurodegeneration. The peak expression of different cytokines (TNF-α, IL-1β & IL-6) post LPS injection is time-dependent. Those inflammatory cytokines in plasma and the brain were measured by enzyme-linked immune sorbent assay (ELISA). To inhibit the cytokine expression, the mice were orally given a single dose (1 mg/kg) of Dexamethasone (Dexa), 0.5 h prior to LPS injection. Above cytokines were observed at their peak expression time points and the plasma & brain concentrations of Dexa were analysed using LC-MS/MS. Brain/plasma ratio (B/P) was calculated. Our results indicate that Dexa (1 mg/kg, po) has better anti-inflammatory effect in plasma with good exposure and minimal efficacy in brain may be due to low B/P ratio.

INTRODUCTION: Association between inflammation & neurodegeneration was well established in both clinical & pre-clinical stages. Studies in depressed patients reported an elevated level of peripheral inflammatory markers (plasma IL-6, TNF-α and IL-6) [16]. Cytokines are the factors that regulate homeostatic responses among different tissues. In the wall of gram-negative bacteria, lipopolysaccharide (LPS) was found to be a major component [6]. It activates pro-inflammatory cytokine cascades through plasma membrane proteins, *e.g.* the toll like receptor (TLR4) and CD14, which leads to the production of TNF-α and other pro inflammatory cytokines [1, 4]. In addition, they affect the central nervous system function. LPS injection induces TNF-α production in plasma and brain within one h [2]. The major finding from these studies is that TNF-α from serum or plasma quickly activates the pro-inflammatory cascade in the brain [17]. Central cytokines are activated by intra cerebro ventricular (ICV) administration of LPS, which can develop depression-like behaviour in rodents [5]. A high dose of LPS is found to be toxic & sometimes lethal to the mice [12].

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LPS-induced animal model is more frequently used amid non-genetically modified neuroinflammation models [14]. After stimulation with the LPS, C57BL/6 mice, a prototypical Th1 type strain was capable of producing higher levels of TNF-α and IL-12 than BALB/c mice, a prototypical Th2 type

strain [13]. In the present study, we used C57BL/6 mice with single systemic injection of LPS (endotoxin), which strongly activates the immune system & produced major cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor (TNF) [3]. These cytokines, which affect neural, neuroendocrine, and behavioral functions, are synthesized both in the periphery and the brain [8]. We measured the major pro-inflammatory cytokines TNF-α, IL-1β & IL-6 since they are mainly involved in the process of pathological pain & associated with neuro-degeneration. These pro-inflammatory cytokines are responsible for sickness and depressive disorders in physically ill patients [3]. The release of TNF-α & IL-1β follows that of IL-6 [4].

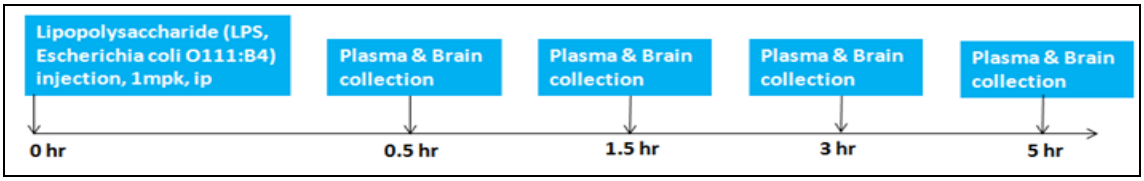
Cortisol and other glucocorticoids bind to the glucocorticoid receptor, which is also known as NR3C1 (nuclear receptor subfamily 3, group C, member 1. Dexa, a broadly prescribed corticosteroid, has long been a choice for the treatment of inflammation. It's anti-inflammatory, and immunosuppressive effects are approximately 30 times more potent than cortisol [9]. The downstream pharmacological effect of Dexa and it's plasma & brain concentration were characterized in this paper.

MATERIALS AND METHODS:

Animals: Male C57BL/6 mice (20-25 gm) were obtained from a single source, Vivo biotech and housed in a group of 3 in stainless steel cages. Food and water were available *ad libitum* & were kept in a temperature-controlled and humidity-controlled room maintained with a 12 h light-dark cycle. The animal protocol was approved by Institutional Animal Ethics Committee, Jubilant Biosys Ltd, Bengaluru, with registration number IAEC/JDC/2018/150.

Reagents: Lipopolysaccharide (LPS, *Escherichia coli* O111:B4, L2630, Lot# 028M4022V, Sigma), Dexamethasone (D1756, Lot # BCBR0315V, Sigma), RIPA buffer (9806, Cell Signaling Technology), Protease Inhibitor Cocktail (P8340, Sigma) were used for this study. TNF-α, IL-1β, and IL-6 ELISA kits were purchased from R & D Systems Inc. All other reagents came from Sigma Chemical Co.

Experiment 1:

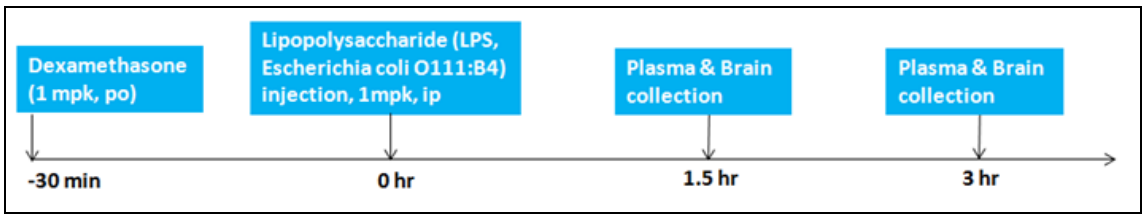


Animal Treatments Male C57Bl/6 mice were divided into eight groups (n=6), with the following treatments to each group. In the normal control group, the mice were intraperitoneally injected with 0.9% saline & the disease control group mice received a single dose of LPS @ 1 mg/kg.

Groups	Dose	Route	Time of necropsy	n
Normal control	0.9% saline	i.p.	0.5 h	6
Disease control	LPS @ 1 mg/kg	i.p.	0.5 h	6
Normal control	0.9% saline	i.p.	1.5 h	6
Disease control	LPS @ 1 mg/kg	i.p.	1.5 h	6
Normal control	0.9% saline	i.p.	3 h	6
Disease control	LPS @ 1 mg/kg	i.p.	3 h	6
Normal control	0.9% saline	i.p.	5 h	6
Disease control	LPS @ 1 mg/kg	i.p.	5 h	6

At the time of necropsy (0.5 h, 1.5 h, 3 h and 5 h following saline/LPS injection), mice were slightly anesthetized; blood collected using Na-EDTA as anticoagulant. Plasma was obtained by centrifugation (5000 × g, 4 °C, 5 min) and kept at -80 °C till analysis. Then animals were under CO_2 asphyxia; the whole brain was removed & immediately snap-frozen in liquid nitrogen & stored at -80 °C till analysis.

Experiment 2:



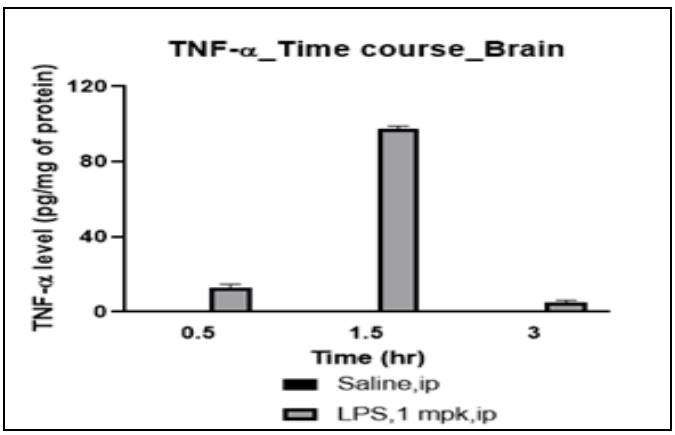
Groups	Dose	Route	Time of necropsy	n
Normal control	0.9% saline	i.p.	1.5 h	6
Disease control	LPS @ 1 mg/kg	i.p.	1.5 h	6
Dexamethasone 1 mpk,po	LPS @ 1 mg/kg	i.p.	1.5 h	6
Normal control	0.9% saline	i.p.	3 h	6
Disease control	LPS @ 1 mg/kg	i.p.	3 h	6
Dexamethasone 1 mpk,po	LPS @ 1 mg/kg	i.p.	3 h	6

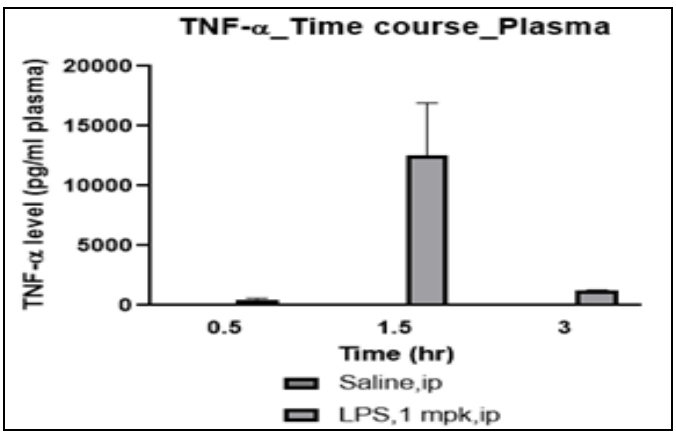
The groups were (1) normal control group; (2) LPS group; (3) dexamethasone group, where mice were pretreated with a dose of dexamethasone (1 mg/kg, po) for 0.5 h and then injected i.p. with LPS (1mg/kg). Dexamethasone was triturated with 0.1% Tween-80 & the dose-volume (10 ml/kg) was made up with 0.5% methylcellulose to the appropriate concentration.

Brain Homogenate Preparation: Frozen brains were brought to room temperature. Cut into 1-2 mm pieces. Added 1:5 ml of RIPA buffer containing protease inhibitors and lyse tissues with a genogrinder at a cold temperature for 2 min cycle for 2-3 times. Centrifuge at 12000*g for 10 min & collect the supernatant. Quantify total protein concentration using a total protein assay. The RIPA Buffer was prepared by adding 9 ml of MilliQ water + 1 ml of RIPA Buffer + 100 µl of Protease inhibitor cocktail [2]. The levels of TNF-α, IL-1β and IL-6 in plasma and brain were measured with TNF-α, IL-1β, and IL-6 commercial enzyme-linked immune sorbent assay (ELISA) kits from R&D Systems (Minneapolis, MN), as described in the kit manual. Absorbance was read in the Spark multi-mode microplate reader, Te can.

Sample Preparation for Dexamethasone LC/MS Assay: To an aliquot of 50 µl plasma sample, 10 µl of the IS working stock solutions were added and vortex mixed for 10 sec. Thereafter, 300 µl of acetonitrile was added and vortex mixed for 2 min, followed by centrifugation for 5 min at 14000 rpm in a refrigerated centrifuge (Eppendorf 5424R) maintained at 5 °C. Post centrifugation, a clear supernatant organic layer (200 µl was separated) and 5 µl were injected onto the column for LC-MS/MS analysis [11].

Statistical Analysis: Data are presented as mean ± SEM of 6 animals. Statistical evaluation was done using one-way analysis of variance (ANOVA) with Graph pad Prizm (version 9.0.0 Graph Pad Software, San Diego, CA, USA). A Dunnett's test was used for post hoc analysis with significance determined at a value of $P < 0.05$.





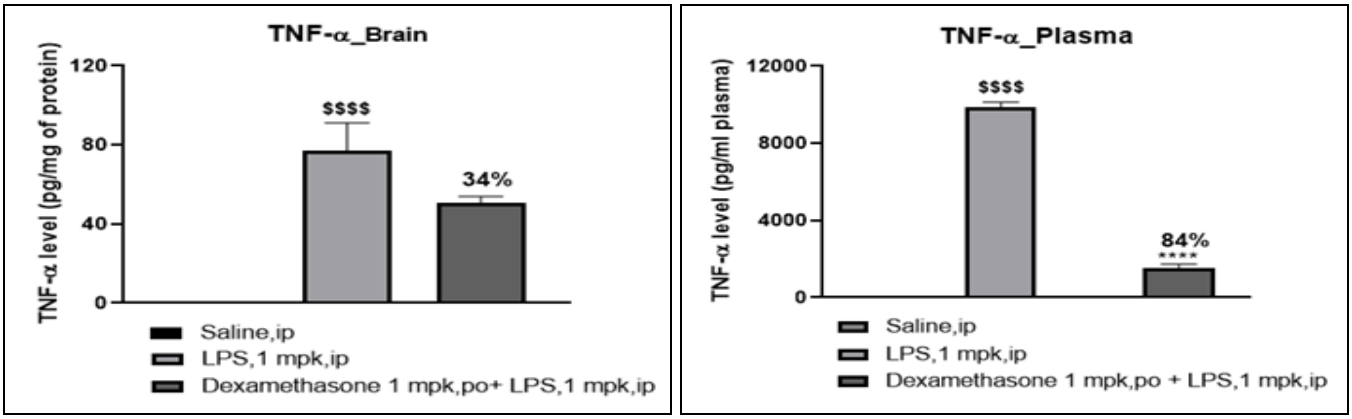


FIG. 1: (TOP PANEL) TIME COURSE OF TNF-A RELEASE POST LPS INJECTION. (BOTTOM PANEL) EFFECTS OF DEXA ON TNF-α RELEASE IN BRAIN & PLASMA

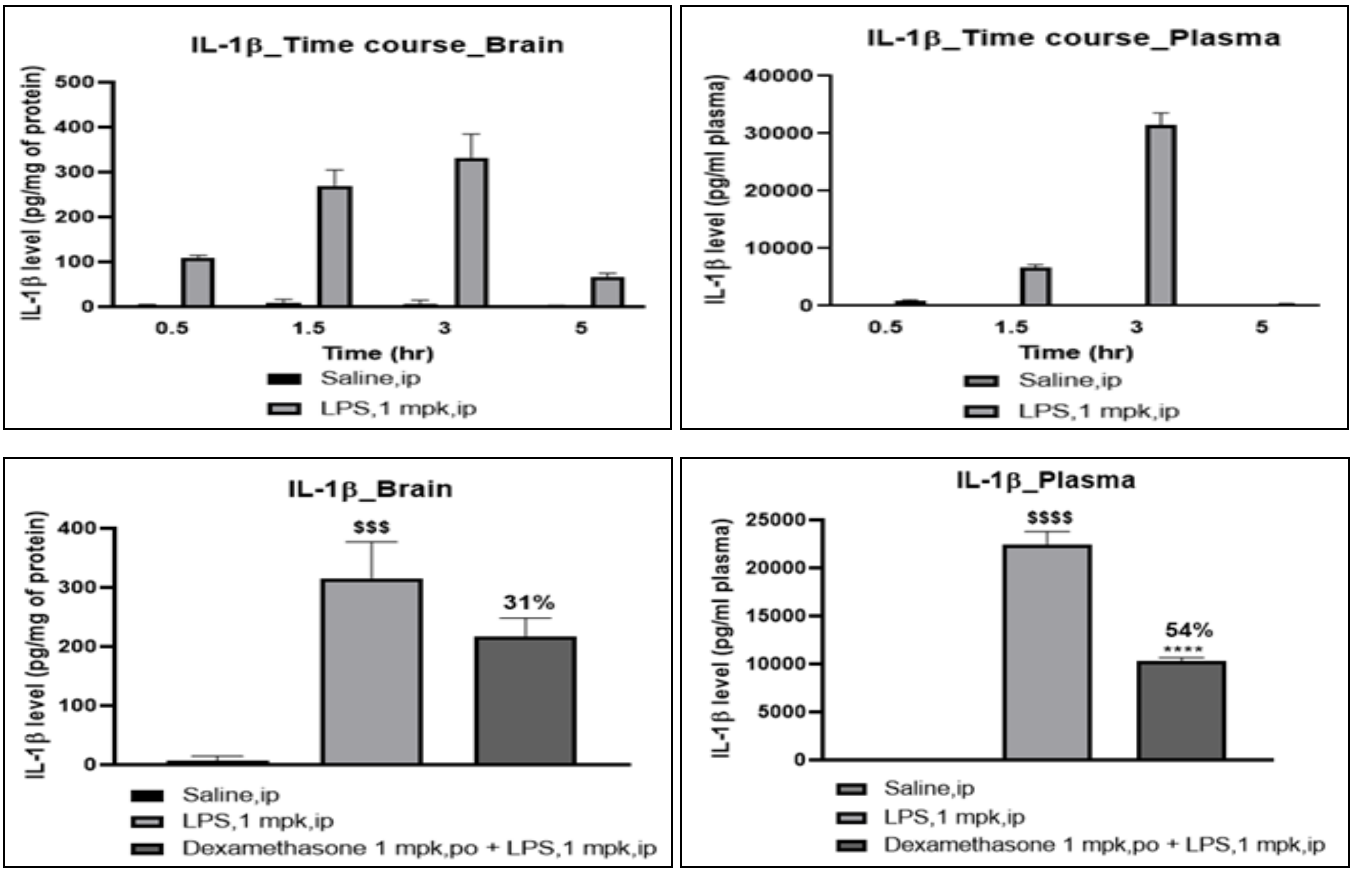
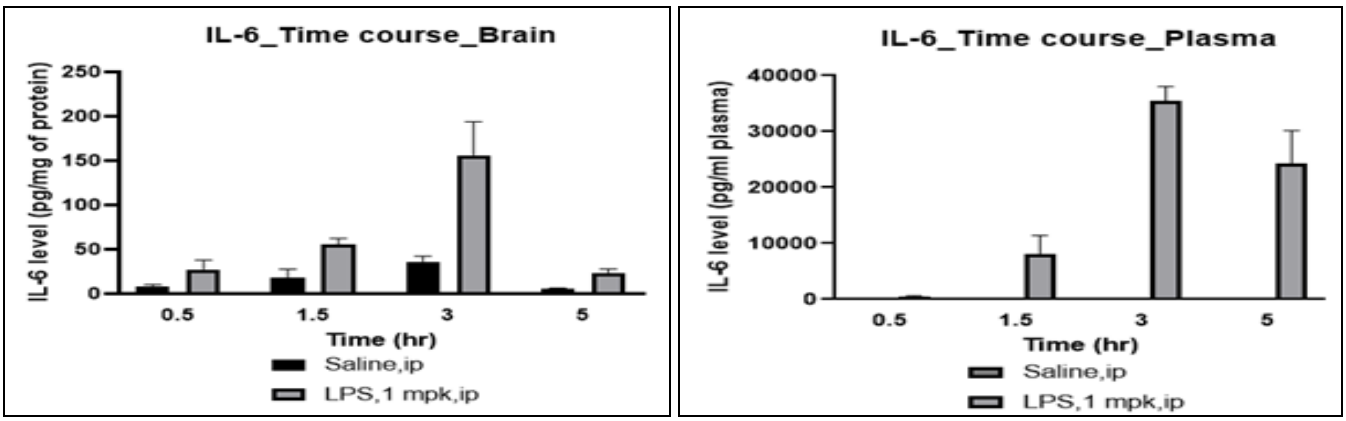


FIG. 2: (TOP PANEL) TIME COURSE OF IL-1B RELEASE POST LPS INJECTION. (BOTTOM PANEL) EFFECTS OF DEXA ON IL-1β RELEASE IN BRAIN & PLASMA



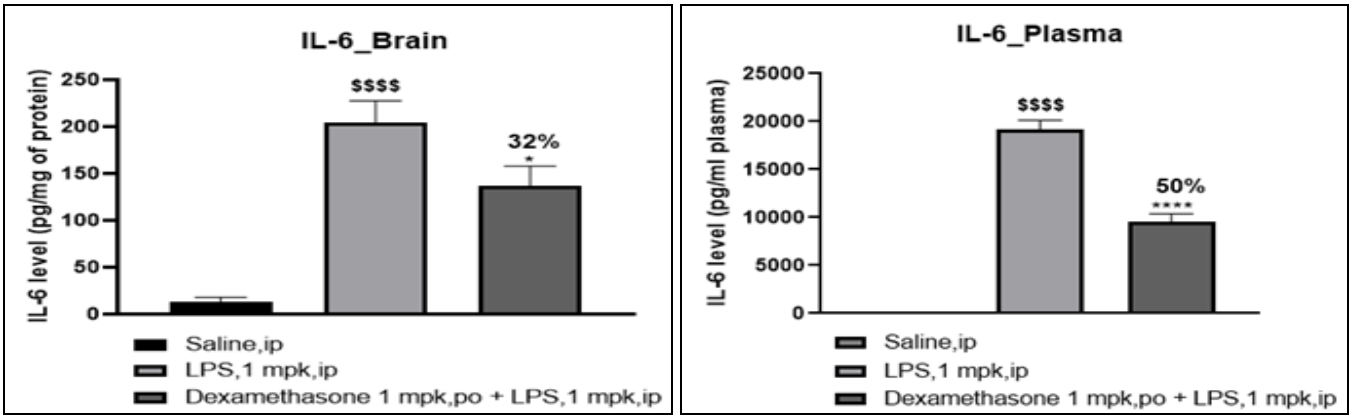


FIG. 3: (TOP PANEL) TIME COURSE OF IL-6 RELEASE POST LPS INJECTION. (BOTTOM PANEL) EFFECTS OF DEXA ON IL-6 RELEASE IN BRAIN & PLASMA

TABLE 1: CYTOKINES LEVEL IN BRAIN (PG/MG PROTEIN) AND IN PLASMA (PG/ML) AT 0.5 H, 1.5H & 3H POST LPS INJECTION

Cytokines	Time points	Brain (pg/mg of protein) Avg ± SEM	Plasma (pg/ml) Avg ± SEM
TNF-α	0.5 h	12.9 ± 1.8	497.2 ± 98.5
	1.5 h	97.4 ± 1.6	12512.4 ± 4437.2
	3 h	5.1 ± 1.2	1244.7 ± 48.5
IL-1β	0.5 h	109.7 ± 4.8	859.7 ± 181.2
	1.5 h	269.7 ± 34.5	6619.5 ± 572.3
	3 h	332.4 ± 52.5	31405.5 ± 2185.8
	5 h	67.4 ± 6.9	384 ± 40.2
IL-6	0.5 h	27.3 ± 10.4	572.7 ± 99.1
	1.5 h	55.6 ± 6.5	8008 ± 3425.3
	3 h	156.2 ± 37.6	35537.5 ± 2509.9
	5 h	23.6 ± 4.4	24326 ± 5845.5

TABLE 2: EFFECT OF DEXA ON CYTOKINE RELEASE IN BRAIN & PLASMA

Parameters	Group	Brain (pg/mg of protein) Avg ± SEM	Plasma (pg/ml) Avg ± SEM
TNF-α at 1.5 h	LPS	77.2 ± 14	9844.5 ± 288.7
	Dexamethasone	50.7 ± 3.2	1529.8 ± 190.7
IL-1β at 3 h	LPS	315.8 ± 61.4	22405.5 ± 1390.2
	Dexamethasone	217.4 ± 30.8	10282.4 ± 395.2
IL-6 at 3 h	LPS	203.9 ± 23.8	19220.5 ± 945.4
	Dexamethasone	136.9 ± 21	9520.3 ± 874.7

TABLE 3: EXPOSURE ANALYSIS FOR THE LEVELS OF DEXA MEASURED IN BRAIN AND PLASMA

Time points	Brain (ng/gm) Avg ± SEM	Plasma (ng/ml) Avg ± SEM	B/P ratio
1 h	1.9 ± 0.4	157.1 ± 12.8	0.012 ± 0.003
2 h	0.6 ± 0.3	118.4 ± 12.3	0.006 ± 0.003
3.5 h	BLQ	36.9 ± 6.2	0

RESULTS:

Plasma and Brain Pro-inflammatory Cytokines: To investigate plasma and brain cytokines, endotoxin (LPS, 1 mg/kg, i.p.) was administered and cytokine levels were determined. LPS induced enhanced expression of TNF-α protein levels in plasma and brain peaked at about 1.5 h **Fig. 1**. TNF-α level was increased to 97.4 pg/mg & 12512.4 pg/ml in brain & plasma respectively at 1.5 h and was declined to 5.1 pg/mg & 1244.7 pg/ml in brain & plasma respectively at 3 h. To evaluate the effect of Dexamethasone, mice were dosed orally at a dose of 1 mg/kg, 0.5 h prior to LPS stimulation. It significantly inhibits ($p<0.0001$) plasma TNF-α expression & improves brain TNF-α expression. LPS induced enhanced expression of IL-1β and IL-6 protein levels in plasma and brain observed peak at 3 h **Fig. 2** & **3.** The IL-1β level was time-dependently increased to 332.4 pg/mg & 31405.5 pg/ml in brain & plasma respectively at 3 h and declined to 67.4 pg/mg & 384 pg/ml brain & plasma respectively at 5 h. Dexamethasone, was dosed orally 0.5 h prior to LPS injection at a dose of 1 mg/kg, significantly

inhibit ($p<0.0001$) plasma IL-1β expression & improves brain IL-1β expression. IL-6 level was slowly increased to 156.2 pg/mg & 35537.5 pg/ml in brain & plasma respectively at 3 h and was declined to 23.6 pg/mg & 24326 pg/ml in brain & plasma respectively at 5 h. Dexamethasone was dosed orally 0.5 h prior to LPS injection at a dose of 1 mg/kg, significantly inhibit plasma IL-6 expression ($p<0.0001$) & brain IL-6 expression ($p<0.05$). Pharmacokinetics analysis for the levels of Dexa measured in plasma and brain from experiment 2 **Table 3.** Mice were sacrificed at different time points (1, 2 & 3.5 h after oral administration of Dexa (1 mg/kg). LoQ = Limit of quantitation of the LCMS/MS method: brain, 0.2 ng/g; plasma, 10.2 ng/mL.

Dexa level measured in plasma at 1 h, 2 h & 3.5 h was 157.1 ng/ml, 118.4 ng/ml & 36.9 ng/ml respectively & in brain at 1hr, 2 h & 3.5 h was 1.9 ng/ml, 0.6 ng/ml & BLQ. respectively. The brain/plasma ratio at 1, 2 & 3.5 h were 0.012, 0.006 and 0 respectively.

DISCUSSION: Notably, the various protocols on the experimental design, serotypes, dose and route of administration, vehicle & dose of LPS, frequency of LPS injections induce varied pathological responses and source, species gender age of animal may show different results [15]. However, LPS is always an important inflammatory agent to investigate the pathophysiological mechanisms of neurodegenerative diseases [7]. Male mice reported higher production of TNF-α, IL-1β, and IL-6 after LPS challenge [10]. In line with cytokine inhibition results in plasma, Dexa was more efficacious in reducing TNF-α (84%), IL-1β (54%) & IL-6 (50%) production in plasma, which was well correlated with good plasma exposure of Dexa. In line with cytokine inhibition results in the brain, Dexa showed minimal efficacy in reducing TNF-α (34%), IL-1β (31%) & IL-6 (32%) production in the whole brain, which was well correlated with very low exposure of Dexa with very low B/P ratio 0.012, 0.006 & 0 at 1 h, 2 h & 3.5 h respectively.

Corticosteroids (CS) have long been focused on their potent immunosuppressive and anti-inflammatory actions. Upon chronic use, CS have their dose-related adverse effects [9]. Hence, further study is necessary to elucidate a drug with a good B/P ratio & less adverse effect may be a choice to observe significant neuroinflammation inhibition.

CONCLUSION: The present study demonstrated that dexa was correlating well between the anti-inflammatory effect and it's exposure in plasma. Our finding is that, due to low brain penetration, dexa showed minimal efficacy in brain cytokine inhibition, suggesting that B/P ratio may be a potential factor to show better neuro inflammation inhibition.

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CONFLICTS OF INTEREST: The other authors declare no conflict of interest.

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