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BACTERIAL LIPOPOLYSACCHARIDE INDUCES A 'DOUBLE HIT' WITH DIFFERENTIAL EFFECTS CENTRALLY AND PERIPHERALLY IN A DIETHYLNITROSAMINE-TREATED WISTAR RAT MODEL OF HEPATIC CANCER

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ABSTRACT: Bio behavioural aspects, immunity, and disease course are interlinked in a majority of conditions, particularly cancer. Peripheral stressors or their products regulate immunity by various ways. When there is added insult in an already compromised immune state such as cancer, as is done here using lipopolysaccharide (LPS), the system is said to be 'double-hit. Behavioural and brain tissue-associated biochemical changes on LPS challenge have been assayed in a Diethylnitrosamine (DEN)-treated Wistar rat model of hepatic cancer. Peripheral and central effects of LPS challenge have been screened at levels of neuro behaviours, liver enzymes and brain enzymes. While LPS challenge reduced exploratory and grooming behaviours across groups after 2 h, the effect disappeared on testing after 4 h, 6 h post LPS injection, rats demonstrated increased open arm time, reduced closed arm time and increased entries into both arms of the elevated plus-maze. DEN treatment induced similar results. LPS challenge in DEN-treated rats however, led to reduced open arm time, increased closed-arm time, and reduced entries into both arms. LPS challenge reduced locomotion in DEN-treated rats, an effect not observed in controls. DEN treatment induced a reduction in brain AChE that was similar also on LPS challenge. There was a concomitant increase in nNOS in the DEN-treated group, with nNOS not affected by LPS challenge. Central and peripheral effects on antioxidants and lipid peroxidation as a result of DEN treatment and LPS challenge were observed. Results indicate that neuro behaviours, the activity of brain and liver tissue enzymes vary in cancer-induced, altered immune states.

INTRODUCTION: The interface that facilitates adaptation and homeostasis while connecting intrinsically interlinked components of cancer, immune system, brain, and behaviour is well. Established Peripheral and central immune processes affect brain activity and the brain can influence the body's immune processes [1]. In contrast, the immune system works to restore homeostasis internally.

The brain mirrors the peripheral infection by activating important metabolic and behavioural pathways leading to homeostasis, mediated by cytokines with specific, direct neural effects [2]. Stressors that upset internal homeostasis could be physical or pathological and orchestrate a set of

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mechanisms leading to cellular stress manifested in oxygen radical production and membrane lipid peroxidation, offset by antioxidant activity. Neuronal stress pathways particularly are activated in response to peripheral and local inflammation-inducing oxidative stress and neuronal dysfunction [3]. Activation of the brain's local immune cells, particularly through microglia and local production of immune-modulatory molecules, induces a different kind of neuronal activity, which is not involved in maintaining homeostasis [4].

Several collative evidences point to a correlation between cancer, the immune system and stress. A down-regulation in immunity occurs when the system is exposed to different kinds of stressors. A cancer patient shows much higher levels of stress than healthy individuals, with cancer being associated with immune down-regulation and heightened distress [4, 5]. This is because, in cancer, existing pathways from the brain acting via the sympathetic adrenergic neurons to the immune system and terminating in lymphoid organs are hyper-activated in cases of environmental/social stressors and/or undergo modulation of existing neuro-endocrine-immune pathways through the release of hormones [6]. Disrupted hypothalamus-pituitary-adrenal (HPA) axis signaling resulting in altered release of glucocorticoid hormones, or disrupted glucocorticoid receptor function, might sustain inflammation.

Stress can increase the peripheral production of pro-inflammatory cytokines, with elevated serum levels being linked to risks for several conditions, including some cancers. Additionally, considerable evidence points to cancer progression and abnormal cellular immune responses, such as decreased cytokines and reduced cell-mediated effector mechanisms [4]. Severe cases of cancer and neurodegenerative disorders are accompanied by clinical depression, characterized by depressed mood and cognitive impairments indicating similar underlying mechanisms [7]. Thus, bio behavioural aspects, immunity, and disease course are interlinked [1]. Furthermore, peripheral stressors or their products have a variety of ways to regulate immunity in vulnerable/diseased conditions as in cancer, where due to lowered immunity, susceptibility to infectious illnesses increases. Therefore, when there is added insult [8] as is proposed to be studied here using lipo-polysaccharide (LPS) in vulnerable/diseased conditions, as in cancer, the system is said to be 'double-hit'. To model this, a Diethylnitrosomine (DEN)-induced model of hepatic cancer or hepatic cell carcinoma (HCC), which is one of the most accepted and widely used experimental cancer models, was used. LPS, a major component of outer membrane of gram-negative bacteria, and a potent mitogen stimulate immune mechanisms *via* activated macrophages and B lymphocytes, thereby bringing about a humoral immune response [9, 10]. Centrally, LPS induces a coordinated set of adaptive behavioural changes collectively known as sickness behaviour that are similar in humans and other mammals [9, 11].

During a systemic infection, the immune cells release cytokines that induce neuronal cells to respond to and help the body coordinate an adaptive behavioural response [1, 2, 12, 13]. The pro-inflammatory cytokines act on the hypothalamus and other brain areas inducing neuroinflammation, accompanied by profound behavioural deficits like prolonged sleepiness, depression, reduced levels of mobility, anxiety, food and water intake [2, 14]. These are proposed to be assayed here in rats on exposure to different familiar and novelty-based paradigms at different time intervals post-LPS injection. LPS-induced neuroinflammation [9, 14, 16] can lead to oxidative stress by the accumulation of free radicals and reactive oxygen species (ROS). ROS react with cellular bio molecules inducing a chain reaction leading to membrane lipid per oxidation-associated membrane porosity which can be assayed using malondialdehyde (MDA) levels [7, 17].

One potent antioxidant, free radical scavenging molecule, is the primary ubiquitous non-enzymatic Glutathione (GSH) which donates its electron to ROS, thus lowering its adverse effect. Peripheral inflammation, as elicited by LPS, can also influence ubiquitous brain enzymes[3] acetyl-cholinesterase (AChE), which causes rapid hydrolysis of ACh into inactive metabolites acetate and choline at the synaptic cleft region allowing the molecules to be recycled and rebuilt for the next nerve impulse, and neuronal nitric oxide synthase (nNOS) which synthesizes nitric oxide (NO), a prominent gaseous neuronal signaling molecule in neurons [18].

LPS-induced antioxidant activity centrally and peripherally and nNOS and AChE activity were assayed spectrophotometrically. The interplay between adaptation and homeostasis on LPS challenge in rodents can be quantified using a series of measures. Here we have used a set of rodent-specific neurobehavioural paradigms [7, 16, 19] at two-hour intervals each after the LPS injection to assess effect on behaviour. First, grooming activity (behavioral adaptation for removing litter particles, pathogenic microbes, and parasites from animal fur and skin) and rearing (where the rat stands on the rear limbs and explores its surround either by lifting the fore limbs or placing them on the wall for support), which is indicative of exploratory behaviour were measured in a familiar environment (home cage).

Rodent-specific neurobehaviours manifest differently in a novel environment, so to assay this, a novel activity box was used to measure overt locomotory behaviour and exploration (frequency of rearing) four hours post LPS injection. Exploratory behaviour here is defined as a measure of the tendency to explore or investigate a novel environment.

Finally, at six hours post LPS injection, anxiogenic neurobehaviours are assayed in the novel elevated plus maze (EPM), consisting of two open arms and two closed arms at right angles. As the maze is elevated, it is a tool for measuring the rat's inherent tendency to explore new spaces *vis*-à-*viz* its fear of open, illuminated spaces and is, therefore, an index for anxiety levels in rodents [8], [20, 21]. Exploration of mainly open and closed arms is assessed as anxiety-like behaviour [22].

The compromised immune system is expected to respond differently to subsequent immune challenges. Therefore, this study proposed to screen for neurobehavioural differences and plasma cytokine levels (Il-4) on the immune challenge in an already compromised condition such as cancer, which was histopathologically verified by immunohistochemistry for proliferating cells, when compared to controls. Further, ready indicators, such as neuronal and peripheral antioxidants and ubiquitous neuronal enzymes, with altered levels impacting physiology, were assayed in a rat model of DEN-induced HCC.

MATERIALS AND METHODS:

Animal Housing and Handling: Young adult male Wistar rats (100-160 g) were housed in polypropylene cages in groups of three, under standard laboratory conditions with food and water ad libitum (ambient temperature of 24 ± 2 ℃; 12-h: 12 h light/dark cycle). All animals were handled (picking up the animal) and gentled (gentle stroking to familiarize the animal with the experimenter) on three consecutive days before the experiments began. All experiments were conducted in the light cycle 9:00-17:00 h and in accordance with the ethical regulations for animal care, use and experimentation laid by CPCSEA and cleared by the Institutional Animal Ethics Committee.

DEN Treatment: Rats were randomly divided into DEN-treated (n=19) and Control (n=17) groups. Diethylnitrosomine (DEN, Sigma) was dissolved in physiological saline and administered intra-peritoneally at a dosage of 200 mg⁄ml/kg body weight. The control group received only saline. The animals were left undisturbed for ten weeks.

LPS Administration: One set of DEN-treated (DEN; n=14) and Control (CONTROL; n=12) rats were randomly, but equally divided into two groups each. One group (LPS: DEN: n=7; CONTROL: n=6) was intraperitoneally injected with LPS (Escherichia coli 0111: B4; Sigma) dissolved in physiological saline at a dose of 1 mg⁄ml/kg body weight. The other group was injected with the Vehicle (VEH: DEN: n=7; CONTROL: n=6). Both groups were left undisturbed until behavioural testing (see below at Neuro-behavioural Testing).

Ki 67 Immunohistochemistry: Another set of DEN-treated (n=5) and Control (n=5) rats were put under deep anesthesia with an intraperitoneal injection of Thiopentone and sacrificed for harvesting of liver tissue for immunohistochemistry of Ki-67 and plasma samples which were frozen and maintained at -80 ℃ in a deep freezer till quantification of IL-4 levels. Frozen liver samples were sectioned on a cryostat (Leica CM 1510S) and mounted on gelatinized slides. Sections were washed in 0.01 M Phosphate buffered saline (PBS; pH 7.4) for 3 × 10 min and were fixed in 10% methanol for 10 min. Sections were washed for 30 min using PBS and incubated in 3% hydrogen

peroxide for 15 min. After washing, they were placed for 5 min in skimmed milk powder dissolved in PBS for blocking of non-specific binding sites. Sections were then incubated in primary antibody *i.e.* Ki 67 (H-300, Santacruz Biotechnology) overignht. They were then washed in PBS for 30 min and incubated with biotinylated secondary antibody (anti-Goat IgG, made in Rabbit; Vectastain PK-6101, Vector Laboratories) for 30 min and incubated in Vectastain A-B complex before being immersed in Diamino-benzidine hydrochloride (DAB, Sigma) with 3% hydrogen peroxide in Tris-PBS for 15 min and washed with deionized water. Sections were dehydrated in alcohol series, cleared in Xylene, and coverslipped. Sections were observed, and photomicrographs taken using LAS software and camera connected to a Leica DM2500 microscope. For quantitative analysis, the central vein of each liver section was located at the center and immuno-positive nuclei were counted in an area of 160 × 120 um. The average of ten measures of each liver tissue sample was taken for statistical analysis.

IL-4 ELISA: The plasma was assayed for IL-4 using an ELISA kit (RayBio® Rat IL-4, ELR-IL-4). This assay employs an anti-IL-4 antibody coated on a 96-well plate. Standards (range: 0.66 – 160 pg) and samples were pipetted out into the wells and IL-4 present in a sample was bound to the wells by the immobilized antibody. The wells were washed, and biotinylated anti-rat IL-4 antibody was added. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin was pipetted into the wells. The wells were again washed, TMB substrate solution was added to the wells and blue color developed. The Stop Solution changed the color to yellow, which was read at 450 nm using Thermo Scientific multiskan GO. The minimum detectable dose of rat IL-4 was determined to be 1.5 pg/ml.

Neuro-behavioural Testing: At intervals of 2 hours each post LPS injection, the animals were exposed to different behavioural testing paradigms such as cage, activity box (AB), and elevated plus maze (EPM). Video recording was done using Panasonic WV CP500 CCD camera which was mounted in the ceiling and fed to a Piccolo frame grabber card and analyzed using Ethovision XT version 8.5® (Noldus, Netherlands).

All recordings were done for 5 min under 8-8.5 lx. Video recordings were analysed either automatically using Ethovision or manually as described in detail elsewhere [20, 21, 23, 24].

Two hours post-injection, the animal was placed in the centre of the cage and its behavior was observed for 5 min. Behavioural measures recorded were: time spent in grooming (min) and a number of rearings (where the rat stands on the rear limbs and explores its surround either by lifting the forelimbs or placing them on the wall for support), which are indicative of exploratory behaviour. Four hours post-injection, the animal was placed in the center of the box of activity box with 44 °C 44 × 3 0.5 cms dimensions, which is placed 50 cms above the ground, and its behavior was observed for 5 min. This is commonly used to measure general locomotor activity and exploratory behaviour in rodents. Behaviours recorded here were the same as that of the cage test.

Six hours post LPS injection, animals were placed in the centre of the Elevated Plus Maze, and behaviour was measured for 5 min. The EPM consists of two open arms (50 × 10 cms) and two closed arms (50 × 10 × 40 cms) at right angles to each other and an open square (10 × 10 cms) in the centre. The maze is elevated 50 cms above the floor and is a tool for measuring anxiety levels in rodents. The number of entries onto open and closed arms, the time spent in open and closed arms, distance moved, number of rears, latency to enter an arm were scored.

Brain and Liver Tissue Harvesting: 6 h after the LPS injection, rats were sacrificed as described above. Brain and liver tissues were harvested and frozen at -180 °C in liquid Nitrogen and kept in a deep freezer maintained at -80 °C until the assays were conducted.

Enzyme Assays: Brain AChE activity was assayed as per Ellman *et al.* [25] by measuring the rate of hydrolysis of substrate Acetyl thio choline iodide (ATCI). The reaction mixture contained tissue homogenate (10% in 50 mM tris buffer, pH 7.4) in 0.1 M PO4 buffer (pH 8.0), 1 mM DTNB, and 1 mM ATCI at room temperature. The addition of substrate started the reaction, and absorbance was measured after 10 min at 415 nm.

Enzyme activity was calculated by using extinction coefficient (E415 = 14.3 * 104 m/cm) for the yellow anion 5-thio-2-nitrobenzoate. AChE activity was expressed as mmols of ATCI hydrolyzed/min/mg of protein. For brain NADPH Diaphorase (nNOS) activity, brain tissue was homogenized in 50 mM Tris HCl Buffer (pH 7.4) containing 2 mM EDTA and centrifuged at high speed for 15 min at 4°C and assayed as per Dawson *et al.* [26] Briefly, the reaction mixture which contained the supernatant, EDTA (5 mM), NBT (0.5 mM), NADPH (0.5 mM) in 50 mM Tris buffer (pH 7.4) was incubated for 15 min at 37 °C and the colour developed was read at 585 nm (Molar Extinction Coefficient of NBT at 585 nm is 16,000 m/cm).

Oxidative Stress-Related Measures: Glutathione (GSH) was assayed in both liver and brain with modifications after Ellman [27] Briefly, 0.2 g of tissue was homogenized in 1:1 ratio of 10% TCA and 10 mM EDTA, centrifuged at 4 ºC at high speed for 15 min. To 200 µl of the supernatant, 3 ml of 0.2 M Tris HCl and 50 µl of 10 mM DTNB dissolved in 1% Sodium citrate were added and incubated for 10 min at room temperature. Absorbance was read at 412 nm, and Glutathione content was expressed as µg GSH/mg protein. Lipid Peroxidation (LPO) was measured as per Ohkawa *et al.* [28] by estimating the formation of Thio-barbituric acid reactive substances (TBARS). Briefly, brain and liver tissue were homogenized in 0.25M sucrose and centrifuged at high speed for 5 min. To 1 ml of supernatant, 1.5 ml of 20% TCA and 2.5 ml of 0.67% TBA were added, boiled for 30 min and cooled, after which 2 ml of butanol was added, then centrifuged again as above. The top layer formed was read at absorption maxima of 535 nm. Total protein estimation of the liver and brain samples used for enzyme assays and oxidative stress-related measures was carried out as per Lowry *et al.* [29] using bovine serum albumin as standard. The spectrophotometer used for all the above enzyme assays was UV-Vis spectrophotometer (Spectro 105, Systronics).

Statistical Analysis: Data are represented as mean ± SE. For specific effects of LPS administration in DEN-treated and control rats, two-way ANOVA followed by Bonferroni post-hoc test was performed. Two-group data were subjected to an unpaired t-test, while before-after analysis was subjected to a paired t-test. A value of $p < 0.05$ was considered as statistically significant.

RESULTS: As DEN is toxic, the number of rats in the DEN-treated group was two more than the Controls, though no mortality was observed over the ten weeks. DEN treatment did not induce any adverse effect on body weight. While Control rats demonstrated a bodyweight increase from 133.4 ± 7.13 g to 196.2 ± 14.43 g ($t16 = 5.253$; $p = 0.0004$; paired t-test), DEN treated rats also demonstrated an increase in body weight from 144.9 ± 5.07 to 233.9 ± 7.90 ($t18 = 12.94$; $p < 0.0001$; paired t-test). After ten weeks there was an increase in body weight in DEN-treated rats when compared to controls ($t34 = 2.477$; $p = 0.0204$; unpaired t-test).

Liver Ki-67 Immunoreactivity: Ki-67-positive hepatocytes were observed distributed evenly in transverse sections of liver tissue **Fig. 1A and B.** Ki-67 immunoreactive cells could be differentiated from immuno-negative cells. The central vein of each liver section was located at the center of the visual field and darkly stained nuclei of hepatocytes were counted. DEN-treated liver tissue demonstrated 84.80 ± 4.36 in relation to Controls 57.50 ± 3.10, the difference being significant ($t8 = 5.103$; $p < 0.0001$).

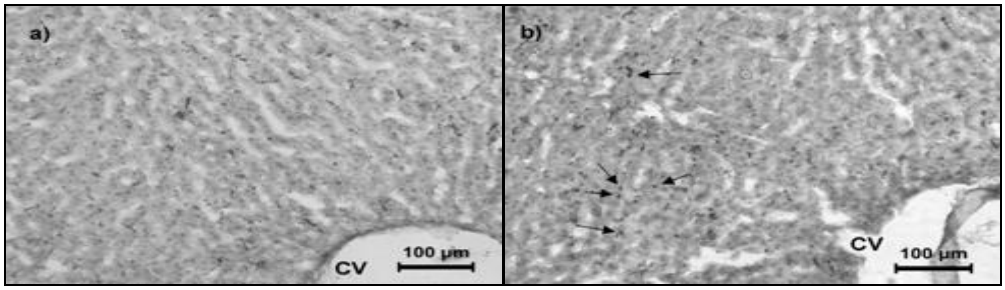

FIG. 1: PHOTOMICROGRAPHS OF KI-67 IMMUNOREACTIVITY IN LIVER TISSUE OF DEN-TREATED RATS (B) AS COMPARED TO CONTROLS (A). Arrows point to representative immunopositive cells that were quantified. CV: central vein.

Plasma IL-4: DEN treatment led to a significant increase in plasma IL-4 levels (2.07 ± 0.17 pg/ml) when compared to saline-treated Control group (1.00 ± 0.17 pg/ml), the difference being significant (t8 = 4.298; p = 0.0006).

LPS – Neurobehaviours: Two hours post LPS challenge when tested in the home cage, rearing behaviour was significantly impacted by LPS (F(1,22) = 44.21; p < 0.0001) with a significant reduction **Fig. 2A.** in both the DEN-treated rats (p < 0.001) as well as controls (p < 0001). Rearing behaviour was not affected by DEN treatment (F(1,22)= 1.20; p = 0.2837; LPS x DEN interaction: F(1,22) = 0.01; p = 0.9234). Grooming behaviour **Fig. 2B.** was also significantly affected by LPS (F(1,24) = 29.27; p < 0.0001), with a significant reduction in both the DEN-treated (p < 0.001) as well as Control groups (p < 0.05). Effects (F(1,22) = 0.13; p = 0.7231) of DEN treatment per se on grooming behaviour were again not observed (LPS × DEN interaction: F(1,22) = 0.70; p = 0.4104). Four hours post LPS injection, when exposed to a novel open field **Fig. 2C.** A significant effect of LPS challenge on rearing behaviour (F(1,22) = 6.51; p = 0.0175) was observed with DEN-treated LPS-challenged rats demonstrating a significant difference in rearing (p < 0.01) as compared to LPS-challenged Controls.

An effect of DEN treatment per se (F (1,22) = 7.46; p = 0.0117) on rearing frequency was also observed, with a significant increase (p < 0.01), when compared to un-treated controls (LPS × DEN interaction: F(1,22) = 5.69; p = 0.0253).

Grooming behaviour **Fig. 2D.** demonstrated some effect of LPS (F (1,24) = 7.01; p = 0.014), though effects F(1,22) = 1.59; p = 0.2201) of DEN treatment were not observed (LPS × DEN interaction: F(1,22) = 0.36; p = 0.5567) and no specific group effects were returned.

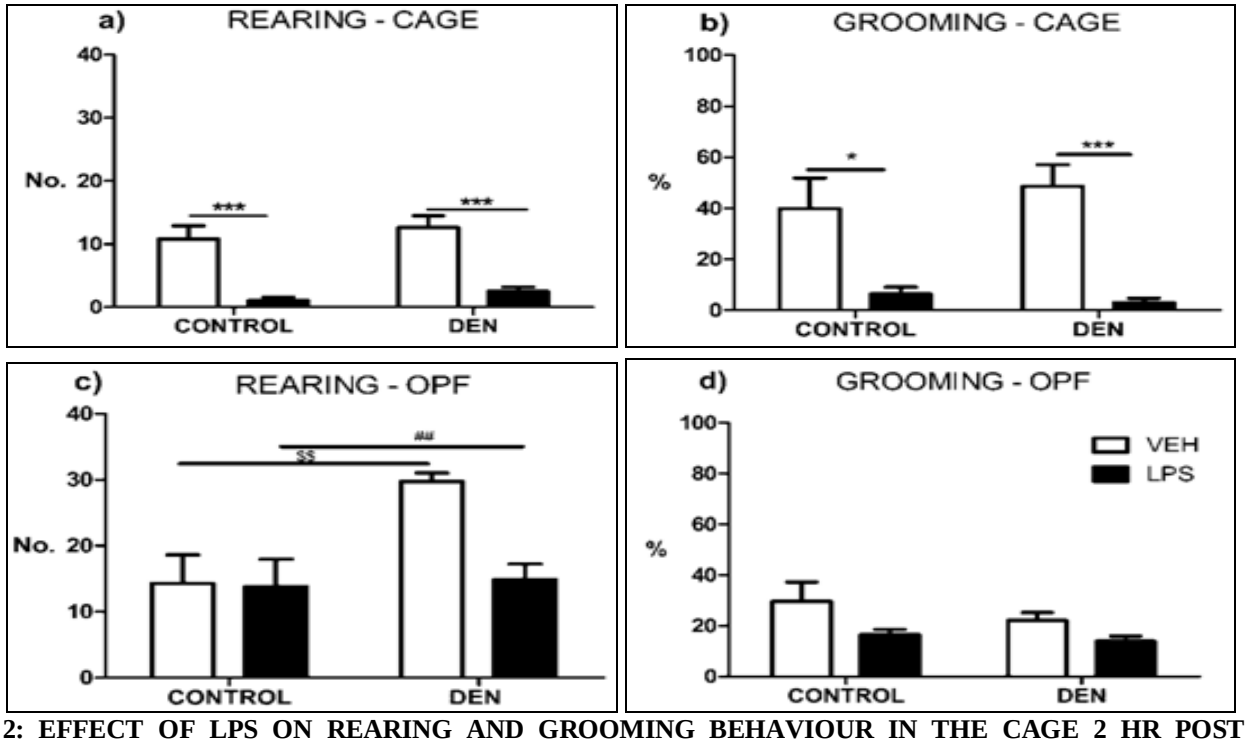

FIG. 2: EFFECT OF LPS ON REARING AND GROOMING BEHAVIOUR IN THE CAGE 2 HR POST LPS CHALLENGE (A+B) AND IN A NOVEL OPEN FIELD 4 HR POST LPS CHALLENGE (C+D). Animals were tested for 5 min each. Data are represented as Mean ± SEM. a) Rearing: 10.83 ± 2.07; 1.0 ± 0.63; 12.63 ±1.90; 2.50 ±0.71. b) Grooming: 38.89±12.01; 6.50 ± 2.64; 48.63± 8.52; 3.0± 1.76. c) Rearing 14.33 ± 4.33; 13.83 ± 4.16; 29.75 ± 1.31; 14.88 ± 2.39 d) Grooming 29.67 ± 7.63; 16.56 ± 2.06; 22.18 ± 3.13; 13.88 ± 2.20 *indicates significant differences between Vehicle vs. LPS in both Controls and DEN-treated rats; #Controls vs. DEN-treated; $Controls vs. DEN-treated in LPS-challenged rats p < 0.05, p < 0.01, p < 0.001.

Anxiety-Related Neurobehaviours: A significant effect of LPS (F (1,22) = 12.61; p = 0.0023) was observed on open arm time or duration **Fig. 3A**, with LPS challenged rats demonstrating significantly increased open arm duration in Controls (p < 0.001), while in the DEN-treated

group, LPS induced a significant reduction ($p < 0.05$) in open arm time. A significant effect of DEN treatment was also observed ($F(1,22) = 8.40$; $p = 0.0096$) with increased open arm time ($p < 0.05$) in DEN-treated rats when compared to controls. An LPS challenge in DEN-treated rats, reduced open arm time significantly ($p < 0.001$) when compared to Control LPS group (LPS × DEN interaction: $F(1,22) = 49.72$; $p < 0.0001$). For closed arm time (fig. 3b), no effect of the LPS challenge ($F(1,22) = 0.42$; $p = 0.5234$) or of DEN treatment ($F(1,22) = 0.43$; $p = 0.5180$) were observed, due to significant LPS x DEN interaction ($F(1,22) = 11.18$; $p = 0.0029$). However, post tests comparing groups indicated that DEN-treated rats demonstrated significantly reduced closed arm time ($p < 0.05$) in relation to non-treated controls, and on LPS challenge, DEN-treated rats demonstrated significantly increased ($p<0.05$) closed arm time, when compared to non-challenged DEN-treated rats **Fig. 2B.** Time spent in the centre (fig. 3c) was not affected by LPS challenge ($F(1,22) = 1.52$; $p = 0.2315$) or by DEN treatment ($F(1,22) = 0.01$; $p = 0.9112$), though on LPS-challenge, DEN-treated rats demonstrated significantly reduced centre time ($p < 0.05$) when compared to non-challenged DEN-treated rats.

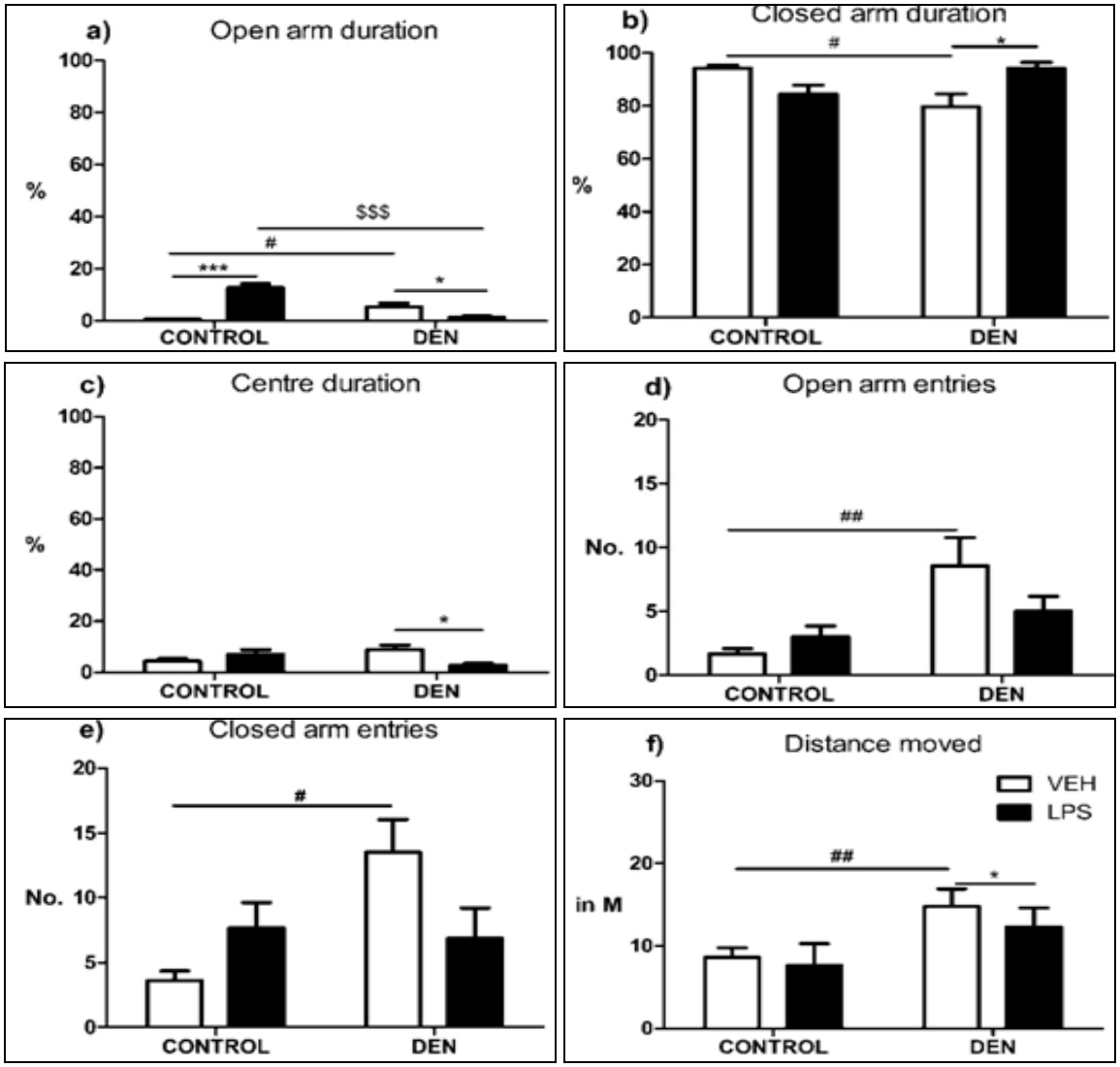

FIG 3: IN THE ANXIOGENIC EPM, MEASURES OF CONTROL AND DEN TREATED WITH VEHICLE AND LPS. Animals were tested for 5 min. Data are represented as Mean ± SEM. a) Open arm time 0.61 ± 0.17; 12.75 ± 1.46; 5.37 ± 1.48; 1.36 ± 0.44 b) Closed arm time 94.23 ± 1.12; 84.39 ± 3.45; 79.62 ± 4.87; 94.20 ± 2.27) c) Centre time 4.31 ± 0.95; 6.94 ± 1.79; 8.89 ± 1.76; 2.70 ± 0.80 in %. d) Open arm entries 1.67 ± 0.42; 3.00 ± 0.86; 8.57 ± 2.20; 5.00 ± 1.19 e) Closed arm entries 3.60 ± 0.75; 7.67 ± 1.96; 13.50 ± 2.52; 6.86 ± 2.35. f) Distance moved in meters: 7.53 ± 1.18; 9.06 ± 1.96; 16.85 ± 2.04; 10.67 ± 1.91; Significant differences: $p < 0.05$, $p < 0.01$; $p < 0.001$. *: Vehicle vs. LPS in both Controls and DEN-treated rats; #: Controls vs. DEN-treated; $ Controls vs. DEN-treated in LPS-challenged rats

There was also a significant LPS × DEN interaction: F(1,22) = 9.35; p=0.0060). Open arm entries were not impacted by LPS challenge (F(1,22) = 0.61; p = 0.4425; fig. 3d), though there was an effect of DEN treatment (F(1,22) = 9.68; p = 0.0051) with DEN-treated rats demonstrating significantly increased open arm entries ($p < 0.01$) in comparison with controls (LPS × DEN interaction: F(1,22) = 2.94; p = 0.1006). No effect of the LPS challenge (F(1,22) = 0.32; p = 0.5764) was observed on the frequency of closed arm entries **Fig. 3E**, though there was an effect of DEN treatment (F(1,22) = 4.01; p = 0.05) with DEN-treated rats demonstrating significantly increased closed arm entries ($p < 0.05$) in comparison with vehicle-treated controls (LPS x DEN interaction: F(1,22) = 5.56; p=0.0277).

Ambulation or distance moved **Fig. 3F.** was not affected by the LPS challenge (F(1,22) = 1.53; p = 0.2289) per se, though LPS challenge in DEN-treated rats induced a significant reduction ($p < 0.05$) in ambulation when compared to unchallenged DEN-treated rats, there being a significant LPS × DEN interaction (F(1,22) = 4.20; p = 0.05). An effect of DEN treatment (F(1,22) = 8.42; p = 0.0080) was further evident with DEN-treated rats demonstrating significantly increased ambulation ($p < 0.01$) in comparison with vehicle-treated controls. For all other measures see **Table 1.** Rearing behaviour **Table 1.** was significantly affected by LPS challenge (F(1, 22) = 17.18; p = 0.004) with DEN-treated LPS-challenged rats demonstrating significantly reduced exploratory behaviour ($p < 0.001$) when compared to non-challenged DEN-treated rats. An effect of DEN treatment (F(1, 22) = 7.75; p = 0.0103) was also observed with DEN-treated rats exhibiting increased ($p < 0.05$) rearing behaviour when compared to Controls (LPS x DEN interaction: F(1,22) = 0.17; p = 0.6845).

Latency **Table 1.** to enter the open arm was reduced in LPS-challenged groups in both Controls as well as DEN-treated groups, but they were not significant F(1,22) = 0.135; p = 0.7150). Latency to enter the closed arm was significantly affected (F(1, 22) = 7.13; p = 0.0134) with LPS inducing a significant reduction ($p < 0.001$) in DEN-treated rats, an effect not observed in the LPS-challenged Controls. A significant main effect of DEN treatment was also observed (F(1, 22) = 12.23; p = 0.0019) with increased ($p < 0.001$) latency to enter the closed arm in the DEN-treated group as compared to Controls (LPS x DEN interaction: F(1,22) = 12.23; p = 0.0019). Latency to re-enter centre was also significantly increased with LPS challenge in DEN-treated rats ($p < 0.001$).

TABLE 1: EXPLORATORY (REARING BEHAVIOUR) AND ANXIETY-RELATED PROFILES (LATENCIES) IN EPM FROM LPS-CHALLENGE AND DEN-TREATMENT

Variables	CONTROL		DEN	
	Vehicle	LPS	Vehicle	LPS
Rearing (no.) Latency (s)	10.83 ± 2.07	1.00 ± 0.63	19.25 ± 3.72	7.25 ± 2.02
Open arm	20.68 ± 10.38	13.57 ± 5.43	21.27 ± 8.91	11.11 ± 7.13
Closed arm	4.33 ± 4.17	5.03 ± 3.78	110.70 ± 23.15##	17.46 ± 7.52***
Centre	3.48 ± 1.86	8.92 ± 3.38	6.66 ± 2.55	25.23 ± 10.36***

Animals were tested for 5 min. n=7 in each group. Data are represented as Mean ± SEM. Significant differences: $p < 0.05$, $p < 0.01$; $p < 0.001$. *Vehicle vs. LPS in both Controls and DEN-treated rats; #Controls vs. DEN-treated rats.

Central Enzymes: AChE as measured in mmoles/min/mg of protein is reduced in DEN-treated rats. AChE activity (0.18 ± 0.02 mmoles/min/mg of protein) was significantly (F(1,22) = 9.29; p = 0.0059) reduced in the DEN-treated group ($p < 0.05$) when compared

To Controls (0.28 ± 0.04 mmoles/min/mg of protein). Six hours post LPS challenge, brain AChE levels were reduced demonstrating a trend (F(1,22) = 3.52; p = 0.0738; LPS x DEN interaction: F(1,22) = 0.61; p = 0.4423) in Vehicle group. AChE activity was not affected as a result of LPS challenge in the DEN-treated group. For Mean ± SEM data and graph see **Fig. 4A.** nNOS in mmoles/min/mg of protein was increased in DEN-treated rats with LPS not having an effect in either group.

A significant main effect of DEN-treatment (F(1,22) = 20.17; p = 0.0002) was demonstrated as DEN-treated rats depicted a significant ($p < 0.01$) increase in nNOS activity (6.25 ± 0.74 mmoles/min/mg of protein) when compared to the

vehicle-treated group (2.58 ± 0.13 mmoles/min/mg of protein). LPS challenge did not affect nNOS activity when assayed 6 h after the injection. No main effect was observed for LPS challenge ($F(1,22) = 0.40$, $p = 0.5537$; LPS x DEN interaction: $F(1,22) = 1.41$; $p = 0.2468$).

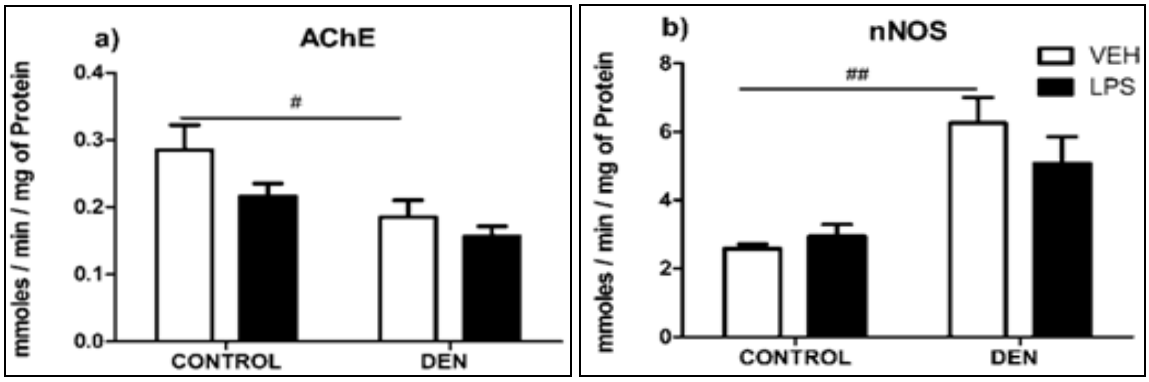

FIG 4: BRAIN ENZYME PROFILES ACHE AND NNOS. Data are represented as Mean ± SEM. a) Brain AChE levels: 0.28 ± 0.04; 0.21 ± 0.02; 0.18 ± 0.02; 0.16 ± 0.01 mmoles/min/mg protein and b) Brain NOS levels: 2.58 ± 0.13; 2.94 ± 0.35; 6.25±0.75; 5.08 ± 0.77 mmoles/min/mg protein. Significant differences: #indicates significant differences between Controls and DEN treatment: $p < 0.05$, $p < 0.01$.

Brain MDA levels (6.25 ± 0.74 × 10-12 moles / min / mg of protein) were significantly ($p < 0.01$) increased on LPS challenge **Fig. 5A.** in relation to controls (5.40 ± 0.62 × 10-12 moles of MDA / min / mg of protein; $F(1,22) = 3.81$; $p = 0.0627$). DEN treatment also significantly impacted brain MDA levels ($F(1,22) = 25.56$, $p < 0.0001$) with LPS-challenged, DEN-treated rats demonstrated significantly ($p < 0.001$) reduced MDA levels (1.86 ± 0.11 × 10-12 moles of MDA / min / mg of protein) than LPS-challenged Controls (vs. 5.40 ± 0.62 × 10-12 moles of MDA / min / mg of protein) (LPS × DEN interaction: $F(1,22) = 11.39$; $p = 0.0025$). Brain GSH activity was not

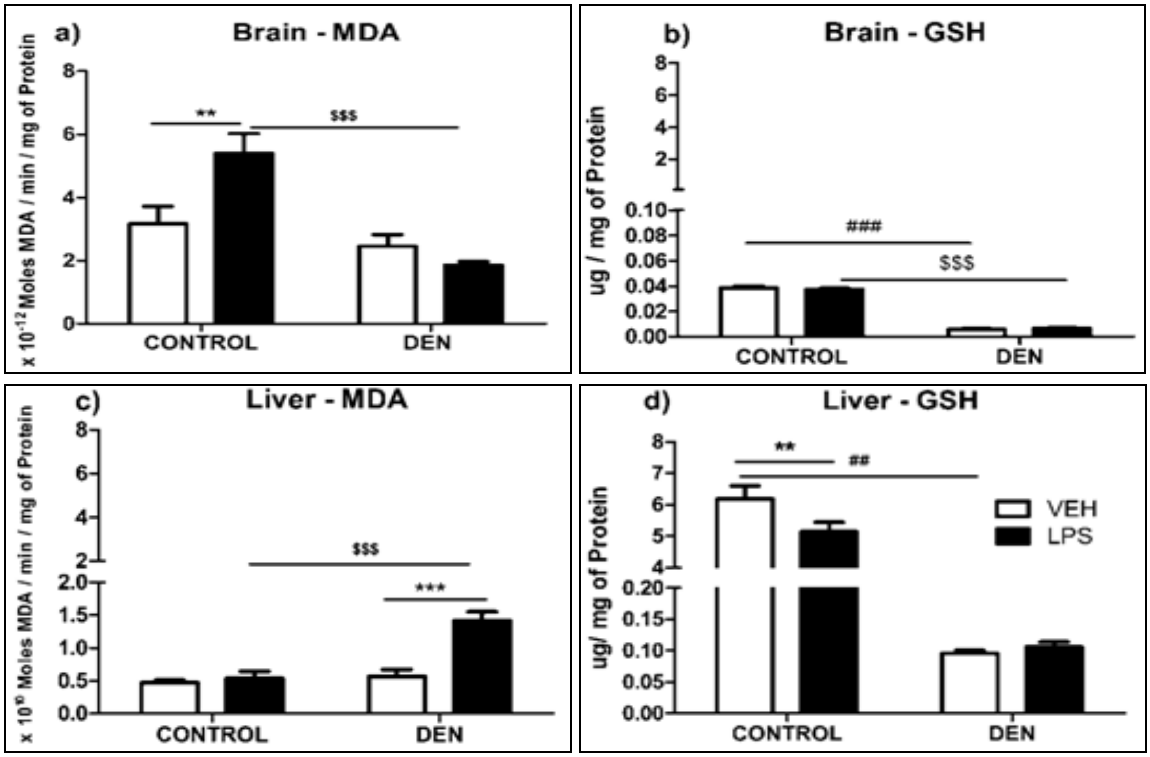

FIG 5: BRAIN (a+b) AND LIVER (c+d) ENZYME PROFILES OF LIPID PEROXIDATION AND GLUTATHIONE (GSH). MDA IS AN INDICATOR OF THE EXTENT OF LIPID PEROXIDATION AT THE MEMBRANE LEVEL. Data are represented as Mean ± SEM. Significant differences: $p < 0.01$; $p < 0.001$. *Vehicle vs. LPS in both Controls and DEN-treated rats; #Controls vs. DEN-treated; $Controls vs. DEN-treated in LPS-challenged rats.

Affected by LPS challenge **Fig. 5B.** F (1,22) = 0.20; p = 0.6574) but reduced in DEN-treated rats, with DEN-treatment (F(1,22) = 1762.31, p < 0.0001) inducing a significant reduction in GSH activity in both groups - unchallenged Controls (p<0.001) and LPS-challenged (p < 0.001); LPS x DEN interaction (F(1,22) = 1.42; p = 0.2470).

Peripheral Enzymes: Six hours post LPS challenge, LPS induced (F(1,22) = 15.74; p = 0.0007) a significant (p < 0.001) increase in liver MDA levels (1.41 ± 0.14 x 1010 moles / min / mg of protein) in DEN-treated rats when compared to DEN-treated vehicle group (0.56 ± 0.10 x 1010 moles of MDA / min / mg of protein; fig. 5c). DEN treatment also affected MDA levels (F(1,22) = 17.56, p = 0.0004) with the same LPS-challenged DEN-treated rats demonstrating significantly (p < 0.001) increased liver MDA levels than non-DEN LPS-challenged rats (0.54 ± 0.11×1010 moles of MDA / min / mg of protein; LPS x DEN interaction: F(1,22) = 11.33; p = 0.0028). Six hours post LPS challenge, liver GSH activity (fig. 5d) was affected (F(1,24) = 5.56; p = 0.0269) with LPS-challenged rats (5.14 ± 0.30µg/mg protein) demonstrating significantly reduced (p < 0.01) liver GSH activity when compared to the Vehicle group (6.18 ± 0.41 µg/mg protein). DEN treatment also affected liver GSH activity (F(1,24) = 647.34, p < 0.0001) with DEN-treatment inducing a significant reduction (p < 0.01) in GSH activity (0.10 ± 0.00) when compared to Controls (6.18 ± 0.41 µg/mg protein); LPS x DEN interaction: F(1,24) = 5.79; p = 0.0242).

DISCUSSION: DEN is an important enviro-nmental carcinogen, that primarily induces tumors of the liver with potent carcinogenic activity via a simple metabolic pathway [30]. In DEN-induced experimental liver carcinogenesis, early preneoplastic foci are said to appear and exhibit moderately elevated rate of proliferation [31]. These complex phenotypic foci, which are the precursors of the benign neoplastic nodules appear to be hyperproliferative [32], a condition that is evident here, ten weeks after the DEN injection, in the increased frequency of Ki-67 label led, immuno-positive cells. Besides demonstrating carcinogenicity as a potent alkylating agent, it is also said to induce powerful toxicity [33], though no mortality was observed in this study. Beha-viourally, the DEN-treated group demonstrated increased open arm time and open arm entries, indicative of reduced anxiety. This induced a concomitant reduction in closed arm time, though closed arm entries were high, which could have been due to the increased ambulation in the DEN-treated group. The DEN-treated rats also demonstrated increased exploratory behaviour.

Dysregulation of the immune system with increased production of pro-inflammatory cytokines linked to cancer [34, 35] and here plasma IL-4 levels were significantly increased in DEN-treated group. IL-4, produced by TH2 cells, along with IL-17α, acting *via* STAT6 signaling pathways, is responsible for increased hydrogen peroxide, up-regulation of dual oxidases such as NOS that contribute to ROS, and increased inflammation in cancer cells [34]. Cytokine administration induces unfavorable symptoms with features common to depression. In fact, immune-mediated cancer symptoms are classified into early-onset neurovegetative and somatic symptoms of depression, which involve flu-like symptoms, fatigue, anorexia, pain and sleep disorders, and late-onset psychological symptoms of depression include mild cognitive alterations and anxiety. Cytokines produced during early or late phases of inflammation activate adaptive CNS responses14, including acute stress responses mediated by the HPA, so in cancer, which is purported to be a state of immune compromise, it is a double hit model.

LPS induced immune-system alterations on the affective dimensions of rats expressing sickness behaviour as has been shown earlier [36] and in recent times, correlated with brain region-specific cytokine expression [14]. The reduced exploratory and grooming behaviours or restricted activity observed here two hours post LPS injection indicate as others have shown [7, 37] that the animals show little or no interest in their environment, display hunched postures, and occupy a corner of the cage. Lethargy, one of the symptoms of sickness behaviour results from increased expression of pro-inflammatory cytokines, probably released by stromal cells of leptomeninges and choroid plexus stromal and epithelial cells, which induce responses in a dose- and time-dependent manner [14]. Four hours post LPS injection, differences between controls and

DEN treatment emerge, with the increased rearing of DEN-treated controls being significantly reduced on LPS challenge. Grooming behaviour through reduced is not different.

Complex behavioral changes on LPS challenge, such as reduced general activity and exploration, observed at two hours here and at different time points of testing in other studies, also include increased anxiety, cognitive dysfunction, reduced social motivation and social withdrawal [13]. The two hours LPS effect may be underlined by a significant increase in amygdaloid activity and increase in extracellular noradrenaline levels which correlated with increase in anxiety-like behaviour and decreased locomotor activity and exploration in the open field [13].

Six hours post injection, LPS challenge per se led to significantly increased open arm time, reduced closed arm time and increased entries into both arms, indicative of hyperactivity and an anxiolytic state. This could be again related to the early cytokines such as IL-6 and TNFs, which increase in brain, 4 h post-LPS injection, observed with a higher dose of 3 mg/kg b.w., and indicative not of passive diffusion, but of signal transduction mechanisms [14]. In DEN-treated rats, LPS challenge had the opposite effect, leading to increased anxiety as recorded by reduced open arm time and entries and an increase in closed arm time. The same group also demonstrated reduced locomotor activity, an effect not existent in controls as LPS did not change locomotor activity, though at this time point rearing continued to be reduced in both controls and DEN-treated groups.

While changes in open arm time indicate changes in anxiety [38], closed arm entry frequency is also a measure of locomotor activity [45]. DEN increased distance traveled as well as closed arm entries, while LPS challenge in DEN-treated rats reduced these measures indicating that social motivation to explore is reduced as an effect of LPS. Increased entries into both arms and open arm time, generating anxiety-like behaviours, reflected a dose-dependent conflict inside the animal's emotional drive, with a peak being reached at 200 µg/kg b.w. of LPS [12]. With a dosage of 1 mg/kg b.w. we show reduced anxiety-like profiles indicating that different time windows of testing and different doses induce differential but not incongruous results. For instance, a 3 mg/kg b.w. dose induced wide-spread brain response to the endotoxemia, with underlying cytokine responses inducing delirium and cognitive impairments [14].

Though the brain is an immunologically privileged site, there is some level of immune surveillance of the CNS [18, 39,] and under conditions where the blood-brain barrier (BBB) function is compromised [40,] peripheral leukocytes enter, mirroring the peripheral inflammatory response. However, in acute reactions to cerebral injury or direct central nervous system (CNS) infections or in the absence of BBB breakdown 9, there is a subtler response of the brain's local immune system that includes activation of resident microglia and astrocytes inducing neuroinflammation [14].

LPS has been shown to activate neuroimmune pathways and processes without inducing or being dependent on BBB disruption, as it may be a neuromodulator that is not only pathological, but induces differential responses in tissues, including brain [14, 16, 39]. When LPS gains entry into the CNS/brain through a leaky vascularisation [41], it is said to induce a cascade of intensely pro-inflammatory events leading to tissue damage *etc* [7]. mediated among others by the NF-kB and MAPK signalling pathways, leading to strong cytokine response [18, 42].

LPS challenge also reduces glucose metabolism in dorsal prefrontal cortex but increases it in cerebellum and basal ganglia indicative of the energetics underlying neurovegetative symptoms [7, 43]. The medial preoptic area, supraoptic nucleus, central amygdala and paraventricular nucleus of the hypothalamus are activated in the first two-four hours after LPS injection that correlated with behaviours characteristic of sickness behaviour, including fever, lethargy *etc*. Three and half hours after the injection another brain area activated is the bed nucleus of the Stria terminalis.

No activity, however, was observed in motor, cingulate and piriform cortices, noradrenergic brain stem locus coeruleus and Nucleus accumbens [43] which may underlie delirium and cognitive impairments [14]. Systemic LPS injections in a model of HCC as used here, caused retardation of motor

activity, reduced interest in exploration at six hours indicative of a decrease in social activities, loss of interest or pleasure (anhedonia) and impaired cognitive function [12]. These together constitute symptoms of a depressive-like profile, as symptoms of sickness behaviour and major depression have been shown to overlap considerably and are associated with cancer [44], as subjects show a transient, substantial increase in levels of anxiety and depressed mood43 that are mediated by brain cytokines IL-6, IL-8, IL-1β and TNF-α [7, 36].

Thus, LPS induces inflammatory cascades on sickness behaviour, as peripheral LPS administration increases plasma and central levels of multiple pro-inflammatory cytokines in a time-dependent manner [14, 36]. LPS is therefore, a stressor [16, 45] that activates common physiological responses (brain cytokines expression, HPA axis activation) as a stressor would, making it useful to study the double-hit hypothesis as in immune challenge in cancer. Activated macrophages produce cytokines [18, 42, 46] that control local and systemic immune responses to pathogens and

Therefore, cytokines and other pro-inflammatory molecules, including enzymes and free radicals, are responsible for most cellular and molecular pathophysiology associated with bacterial LPS-induced infection [7, 47]. IL-4, which was up-regulated here in the DEN-treated rats, is a pleiotropic cytokine produced by myeloid and lymphoid immune cells that directs the response towards humoral immunity peripherally, that is TH2 cells and has been shown to have also a modulatory, anti-inflammatory and healing promoting role in restoring brain homeostasis [18].

Here, in the brains of DEN-treated rats, a reduction in brain AChE and a concomitant increase in nNOS was observed with LPS having not to effect on AChE and nNOS activity when sampled six hours post LPS injection at the dose tested, which could be explained by anti-inflammatory activity [18, 48] of upregulated IL-4 in DEN-treated group, or that the time window of iNOS activity [34] was not considered in this study. Brain MDA levels were not affected by DEN treatment per se, indicating no differences in lipid peroxidation in this model of HCC. However, on LPS-challenge, it was increased, as has been shown by others, with such a challenge inducing astrocyte-released cytokines to express microglial NOS and prime microglia to produce to generate more radicals upon activation in all brain areas except the brain stem. Increased MDA activity on LPS challenge as observed here and by others [7, 49] was not evident in the DEN-treated group, which cannot be explained in the current study. In the liver, on the other hand, MDA was not affected by LPS challenge in controls at the dose of 1 mg/kg b.w. However, in the DEN-induced HCC liver, there is a significant increase in MDA levels indicating up regulated hepatic tissue damage and causative oxidative stress [50].

Thus, LPS has a more profound effect in HCC liver tissue, rather than normal liver tissue as observed by elevated levels of lipid per oxidation in DEN-treated, LPS-challenged rats which was significantly higher than that in control LPS-challenged rats.

Here, brain GSH which is a substrate for cytosolic Glutathione peroxidase reduction of hydroperoxides [51] was significantly reduced in the DEN-treated group irrespective of LPS challenge. Others have shown both; an LPS-induced 25% decrease [7] or an increase in hippocampal GSH, which would be expected to reverse LPS-induced ensuing lipid peroxidation and oxidative stress. HCC liver GSH activity was also reduced in DEN-treated rats, indicating compromised non-enzymatic antioxidant mechanisms, though not to the extent of brain GSH. The LPS-induced reduction in HCC liver GSH levels is indicative of reduced scavenging of ROS. In fact, DEN treatment has been shown to elevate tissue-damaging enzymes [52].

Whereas enzymatic antioxidants such as superoxide dismutase, catalase and peroxidases and non-enzymatic antioxidant GSH were reduced also on LPS challenge [9], both indicative of oxidative stress-induced damage [53]. The differential response of brain and liver to lipid peroxidation in both DEN-treatment and LPS-challenged groups and the overall low levels of GSH in the DEN-treated groups could indicate attempts made by the organism to tide over the challenge of inflammation and cope with its effects via different stoichiometries, both at the level of the brain and in

the periphery (liver). Subsequent and sustained immune activity prolongs the generation of ROS and NOS and would eventually lead to increased lipid peroxidation, membrane damage-inducing energy deficit, and brain tissue injury. The differences in LPS effects in controls and DEN-treated rats, whether in neurobehaviours and antioxidant measures at the level of brain and periphery indicate that in cancer or other immune compromised states, existing pathways from the brain to the periphery and vice-versa are hyper-activated and/or undergo modulation and therefore warrant differential approaches in diagnostics and therapeutics.

CONCLUSION: Nil

ACKNOWLEDGEMENT: Nil

CONFLICTS OF INTEREST: Nil

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