Prednisone Induces Anxiety and Glial Cerebral Changes in Rats

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ABSTRACT. ***Objective.*** To assess whether prednisone (PDN) produces anxiety and/or cerebral glial changes in rats.

Methods. Male Wistar rats were studied and 3 groups were formed (8 rats per group). The moderate-dose group received 5 mg/kg/day PDN released from a subcutaneous implant. In the high-dose group, implants containing PDN equivalent to 60 mg/kg/day were applied. In the control group implants contained no PDN. Anxiety was assessed using an open field and elevated plus-maze devices. The number of cells and cytoplasmic transformation of astrocytes and microglia cells were assessed by immunohistochemical analyses.

Results. Anxiety was documented in both groups of PDN treated rats compared with controls. The magnitude of transformation of the microglia assessed by the number of intersections was significantly higher in the PDN groups than in controls in the prefrontal cortex (moderate-dose, 24.1; high-dose, 23.6; controls 18.7; $p < 0.01$) and striatum (moderate-dose 25.6; high-dose 26.3; controls 18.9; $p < 0.01$), but not in hippocampus. The number of stained microglia cells was significantly higher in the PDN treated groups in the prefrontal cortex than in controls (moderate-dose, 29.1; high-dose, 28.4; control, 17.7 cells per field; $p < 0.01$). Stained microglia cells were significantly more numerous striatum and hippocampus in the high-dose group compared to controls.

Conclusion. Subacute exposure to PDN induced anxiety and reactivity of microglia. The relevance of these features for patients using PDN remains to be elucidated. (J Rheumatol 2001;28:2529–34)

Key Indexing Terms:
PREDNISONE COGNITIVE IMPAIRMENT ANXIETY GLUCOCORTICOIDS

Increasing evidence suggests that endogenous hypersecretion of glucocorticoids is a key neurobiological determinant of the presentation and course of several mood disorders and cognitive dysfunction. For instance, higher cortisol levels have been associated with greater impairment of cognition function in aging and Alzheimer's disease[1-4]. Patients with Cushing's syndrome exhibit cognitive dysfunction that correlates with hypercortisolemia and reduced hippocampal volume[5]. Endogenous and synthetic glucocorticoids interact with the human immunodeficiency virus coat protein gp120 to exacerbate the neurotoxic effects of this protein in patients with AIDS related dementia complex[6].

Prednisone (PDN) is a synthetic glucocorticoid widely used for many medical conditions including chronic rheumatic and nonrheumatic diseases. Studies suggest that PDN may directly affect the brain, participating in the development of cognitive dysfunction, cerebral atrophy, depression, and anxiety, besides the well known effect of steroid psychosis. For instance, acute administration of high dose PDN or dexamethasone to healthy volunteers produced impairment in some aspects of cognitive functioning[7,8]. Patients with asthma or Crohn's disease had mild subclinical psychiatric disturbance and selective neuropsychological deficits associated with steroid treatment[9]. Some mood disturbances were also found in PDN treated asthmatic children[10]. Although findings are controversial, PDN has also been implicated in producing cognitive dysfunction and/or cerebral atrophy in a subgroup of patients with systemic lupus erythematosus (SLE) with no history of central nervous system involvement[11].

There is scanty information on the effects on mood and brain structural changes associated with PDN use. We chose a pharmacological approach using rats to assess the effects of PDN on anxiety and brain histomorphology. Anxiety was assessed by 2 different and commonly used behavioral tests, and the brain regions considered for histomorphology were

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those with higher glucocorticoid receptors, such as CA1 hippocampus, prefrontal cortex, and striatum[12]. In these regions immunocytochemical analyses of astrocyte and microglial populations were performed. These cells are commonly studied to assess different kinds of nervous tissue response to injury.

MATERIALS AND METHODS

Experimental animals. Experiments were performed on adult male Wistar rats weighing 180–240 g. Animals were housed at 22 ± 1ºC under 12 h light/dark cycle and were allowed free access to food and tap water. Three groups of 8 rats per group were assembled to assess anxiety and number and transformation of glial cells. All assessments were performed by the same person. This protocol was approved by the ethics committee for animal care and management of the Centro de Investigacion Biomedica de Occidente.

Drugs. A subcutaneous (sc) implant of agar containing PDN was used as follows: prednisone (Sigma, P-6254) was dissolved in 1.25% agar solution at 45ºC. Under ether anesthesia 2 ml of this mixture was injected subcutaneously into the back of rats with a syringe using a 22G needle. Preliminary experiments showed that these implants produce a longlasting delivery of PDN from day 3 to day 8 after sc implantation. The implants of moderate-dose PDN contained 10.5 mg PDN, to attain a daily expected liberation rate equivalent to 5 mg/kg day, while the high-dose PDN group received an implant containing 630 mg PDN for an expected liberation rate equivalent to 60 mg/kg/day. These doses were similar to those used in other reports[13]. Control rats received agar implants with no PDN.

Anxiety tests. Anxiety status was assessed 5 days after PDN implantation over 3 consecutive days, using an open-field and elevated-plus maze devices. The open-field locomotor activity was performed on a 60 × 60 cm board divided into 36 equal size quadrants, and 4 walls 15 cm high were used to enclose the field. A video camera was secured above the chamber to record all experiments without disturbance. Rats were placed at the center of the field and were left free to ambulate for 5 min on each day of 3 consecutive days. Trials always started at 10:00 AM. The number of crossed squares was recorded each session and total distance covered was calculated. It revealed the motivational status and anxiety level of rats; longer walked distance means lower anxiety levels, and vice versa[14].

The elevated-plus maze is a device consisting of 2 open arms and 2 closed arms, forming a cross. This device was placed at 50 cm above the floor suspended on a support invisible to rats. As reported[15], each rat was placed at the center of the cross and allowed to explore freely for 5 min; rats were exposed to this device on 3 consecutive days. Sessions always started at 11:00 AM. The total time that rats spent on the open arms, number of entries to closed arms, total arm entries, and number of line crossings were registered. Longer time on open arms was interpreted as indicating less anxiety.

Tissue processing. After anxiety assessment, all animals were sacrificed by transcardiac perfusion. Briefly, animals were anesthetized with intraperitoneal injection of pentobarbital (50 mg/kg) and thoracotomy was performed to expose left cardiac ventricle. A catheter was inserted into it and 150 ml distilled 0.9% NaCl and then 250 ml fixative (4% paraformaldehyde) in 0.1 M phosphate buffered saline (PBS), pH 7.2, was injected. The first solution was prewarmed at 37ºC. After perfusion, brains were removed and immersed in the same fixative at 4ºC for 24 h. Four brains per group were transferred to a cryoprotective solution (30% saccharose, 0.5% Arabic gum in distilled water) until brains sank. Coronal sections were cut at a thickness of 30–40 mm using a cryostatome and kept in PBS at 4ºC. To study prefrontal cortex, striatum corpus, and CA1 hippocampus, tissue sections were cut from –0.7 mm to –3.80 mm from bregma, according to the Paxinos-Watson atlas[16]. All sections from control and PDN treated animals were processed simultaneously.

Immunocytochemical identification of astrocytes. Washes and incubations were performed on free-floating tissue sections under moderate shaking. Tissue sections were first incubated in 1% hydrogen peroxide and 10% methanol for 20 min to block endogenous peroxidase staining. Then slices were washed twice for 20 min with 0.1% bovine serum albumin and 0.1 % Triton X-100 in 0.1 M PBS. This buffer was also used for the following washes and incubations. Sections were then incubated overnight at 4ºC with an anti-glial fibrillary acidic protein (GFAP) polyclonal antiserum (Dako Z-0334) at dilution of 1:800. The sections were then washed twice in buffer, and incubated 2 h at 22ºC with the secondary antibody (anti-rabbit IgG, Dako Z-0196, diluted 1:250), and washed again 3 times with PBS. Peroxidase-antiperoxidase system incubation was performed for 2 h in darkness (Dako Z-113, diluted 1:200). Peroxidase activity was revealed with 0.01% hydrogen peroxide, using 3,3' diaminobenzidine as chromogen.

Immunostained sections were analyzed using an imaging analyzer (Leica Q500IW). The number of GFAP-immunoreactive cells was recorded counting the total number of cells coming into focus within a counting frame delimiting 0.78 mm^2 in the section. In addition, quantitative evaluation for cytoplasmic transformation of astrocytes was performed on the upper focal plane of each section by using a stereological grid, according to the Weibel point-counting method[17] and modified as follows: test grid consisted of 5 concentric circles with a separation between them of 90 mm. The nuclei of astrocytes were placed at the center of the circle and the number of points at which immunoreactive profiles crossed the test grid lines was recorded, at 1000× magnification. All immunopositive profiles seen in the same plane of focus were considered for quantification at each cerebral area studied.

Histochemical staining of microglia. Histochemical techniques were performed on free-floating sections under gentle shaking. To inhibit endogenous peroxidase activity the sections were first incubated 15 min with 10% methanol/1% H_2O_2; tissues were rinsed in 0.1 M PBS (2 × 15 min) and incubated 10 min in a cationic solution (0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$, 0.1 mM $MnCl_2$) dissolved in 0.1 M PBS, pH 7.4. After rinsing in 0.1 M PBS (2 × 15 min), the tissues were incubated overnight at 4ºC in peroxidase labeled (Sigma L-5391) isolectin B4 (Griffonia simplicifolia) dissolved in 0.1 M PBS, pH 7.3, at 1:100 dilution. After 3 washes in buffer, peroxidase activity was visualized with 0.07% 3,3'-diaminobenzidine (Sigma) plus 0.01% hydrogen peroxide in 0.1 M phosphate buffer (pH 7.3) for 10–15 min. All tissues were processed at the same time.

Microglia cell population was assessed in the same way as for astrocytes. Cytoplasmic transformation was analyzed using a lucid camera attached to a microscope, with a stereological grid composed of twenty-five 30.4 mm squares.

Statistical analysis. Results are expressed as means ± SEM. Differences between 3 continuous variables of the anxiety tests were determined by Kruskal-Wallis test. Differences between continuous variables in the morphometrical analysis were determined by one-way ANOVA and Bonferroni test. Significant p values were set at ≤ 0.05.

RESULTS

No rat showed local infections after implants and no casualties were seen during the study. Routine necropsy performed in all the 32 studied animals showed incipient acute bacterial bronchitis in 2. Both these rats were in the high-dose PDN group.

Results of the anxiety tests are shown in Table 1. Both groups of PDN exposed rats had significantly less exploratory activity on the open-field compared with controls. No differences between the 2 PDN groups were found.

In the elevated plus-maze trail, PDN exposed groups

Table 1. Effects of prednisone on anxiety in rats.

	Control, n = 8	Prednisone Moderate n = 8	Prednisone High–dose, n = 8
Walked distance on open–field, cm	539 ± 60.5	383 ± 32†	414 ± 29†
Time spent on open arms of elevated plus-maze, s	69 ± 8	6.8 ± 4†‡	47 ± 6†‡
No. of closed entries	8.6 ± 0.4	8 ± 0.5	8 ± 0.6
Total arm entries	13 ± 3.3	12 ± 4.2	11 ± 0.76
No. of line crossings	2.7 ± 0.4	2.7 ± 0.3	2.7 ± 0.44

Values are mean ± SEM. † Differences between PDN treated groups vs control, $p < 0.05$, Kruskal–Wallis test. ‡ Indicates individual differences between both PDN groups, $p < 0.05$, Kruskal–Wallis test.

spent significantly less time on the open arms compared with controls. However, the moderate-dose PDN group showed significantly less time spent on the open arms compared to the high-dose PDN group. Locomotor activity variables such as total arm entries, number of closed-arm entries, and number of line crossings were not significantly different among groups (Table 1).

Microglial response is illustrated in Figures 1, 2, 3. The histochemical reactivity of these cells was increased after PDN treatment compared to controls. Morphometric analysis confirmed the qualitative observations. Table 2 shows the number of stained microglial cells and their cytoplasmic intersections by cerebral regions. The high-dose PDN group had a significantly higher number of stained microglial cells in the 3 studied regions compared to controls. The moderate-dose PDN group had a significantly higher number of cells only at the prefrontal cortex compared with controls. Microglial cytoplasmic transformation as assessed by the number of intersections showed significant differences at the prefrontal cortex and striatum regions in both PDN treated groups compared with controls (Table 2).

Table 3 shows the number of astrocytes and their cytoplasmic intersections by cerebral regions. There were no significant differences among groups in any examined region.

DISCUSSION

Increasing evidence suggests that endogenous hypersecretion and exogenous administration of glucocorticoids participate in the development of several mood disorders and cognitive dysfunction in aging and diseases such as Alzheimer's, Cushing's, asthma, Crohn's, and perhaps SLE[1-5,7-11,18]. Moreover, there are methodological and ethical difficulties in assessing the effect of PDN on mood and brain structures in humans. Thus we chose a pharmacolog-

Table 2. Effects of prednisone on microglial population.

	Control	Prednisone Moderate–dose	Prednisone High–dose
Number of cells/field			
Prefrontal cortex	17.7 ± 0.57	29.2 ± 1.41†	28.4 ± 1.77†
Hippocampus (CA1)	23.2 ±1.01	25.1 ± 2.04	30.3 ± 1.90†
Striatum	14.3 ± 1.05	18.3 ± 1.73	22.6 ± 1.20†
Number of intersections			
Prefrontal cortex	18.7 ± 0.65	24.1 ± 0.59†	23.6 ± 0.63†
Hippocampus (CA1)	27.2 ± 0.65	25.8 ± 0.49	25.3 ± 0.61
Striatum	18.9 ± 0.55	25.6 ± 0.53†	26.3 ± 0.59†

Values are mean ± SEM of 120 microscopic fields (number of cells) and 600 cells (cytoplasmic transformation) per cerebral area for each group. † $p < 0.05$, ANOVA–Bonferroni.

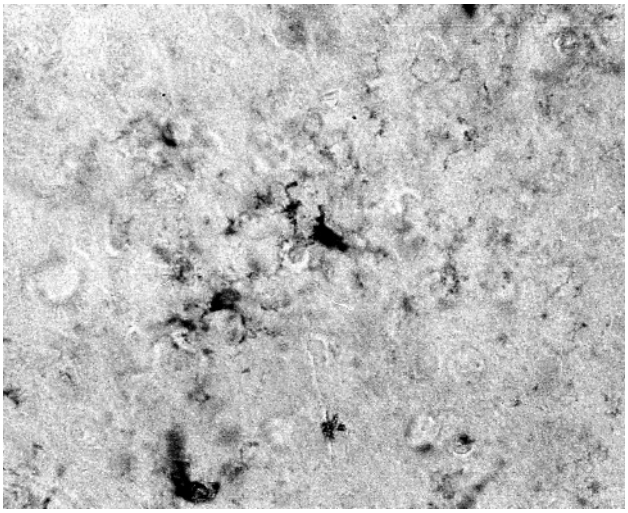

Figure 1. Microglia cells of the control group (no PDN) — prefrontal cortex stained by isolectin-B4 histochemical technique. Original magnification ×400.

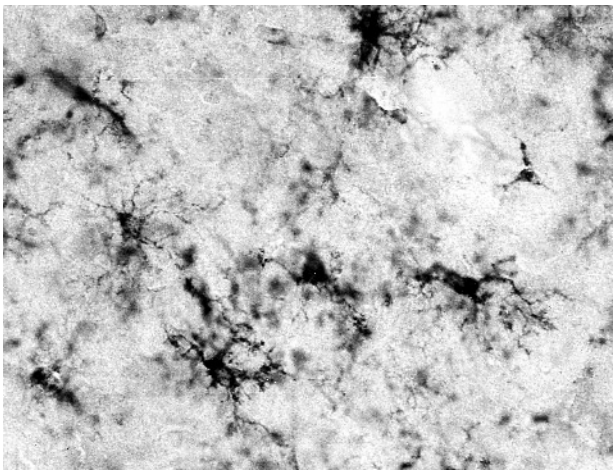

Figure 2. Isolectin stained microglia cells of the moderate-dose PDN group — prefrontal cortex. Note increased number of cells and length of cytoplasmic branches. Original magnification ×400.

Table 3. Effects of prednisone on astrocyte population in rat brains.

	Control	Prednisone	
		Moderate-dose	High-dose
Number of cells/field			
Prefrontal cortex*	69.0 ± 2.17	66.9 ± 2.71	73.1 ± 2.54
Hippocampus (CA1)*	95.0 ± 2.52	94.7 ± 3.45	94.7 ± 1.86
Striatum*	56.0 ± 2.99	62.8 ± 2.36	56.1 ± 3.66
Number of intersections			
Prefrontal cortex*	63.7 ± 0.67	62.2 ± 0.55	64.2 ± 0.66
Hippocampus (CA1)*	78.9 ± 0.89	78.9 ± 1.00	81.5 ± 0.86
Striatum*	51.7 ± 0.83	50.5 ± 0.71	52.1 ± 0.53

Values are mean ± SEM of 120 microscopic fields (number of cells) and 600 cells (cytoplasmic transformation) per cerebal area for each group.
*p >0.09, ANOVA-Bonferroni

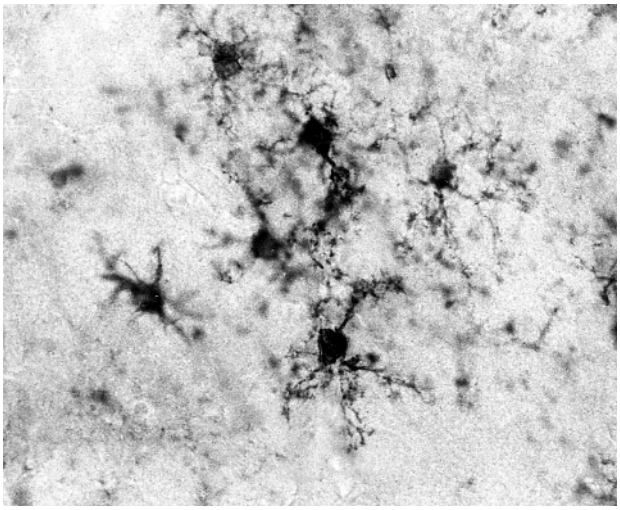

Figure 3. Isolectin stained microglia cells of the high-dose PDN group — prefrontal cortex. Number of cells and length of cytoplasmic branches are increased. Original magnification ×400.

ical approach using rats to specifically assess whether PDN induces anxiety and glial cerebral changes.

No complications were seen from the agar implants, which offered both good tolerance and low infection risks, as described[19]. Further, both PDN doses used in this study are usually utilized in the rodent model; the higher doses of PDN per unit body weight used in the rat are commensurate with its higher metabolic rate. Thus according to interspecies pharmacological scaling the doses are roughly equivalent to 0.625 and 8 mg/kg for humans[20].

Open-field and elevated plus-maze measures are reliable and commonly used to assess anxiety in rodents[14,21-25]. Each test assesses different components of the anxiety behavior — the elevated plus-maze evaluates mainly exploration behavior[22,25], while the open-field assesses escape behavior[23,24]. Our findings on the elevated plus-maze showed that administration of both moderate and high doses of PDN produced significant anxiety compared with controls. It seems that anxiety levels were dose related; thus moderate doses produced more anxiety than high doses. This paradoxical effect on anxiety level might be related to a dose-dependent dynamic regulation on mineralocorticoid receptors, which is a common feature in the presence of psychological stressors[26]. A similar dose-dependent paradoxical effect has also been reported using this behavioral test with GABAergic drugs[27], but further studies are required to characterize its relationship. The anxiety phenomenon observed in our study is not explained by depression or locomotor changes associated with PDN, as the locomotor activity of the rats was normal, as assessed by the number of entries into the open and closed arms of the elevated plus-maze. The open-field test also showed significant anxiety levels in the PDN treated groups compared with controls. Yet no differences were found between the high-dose and moderate-dose PDN groups. PDN may induce anxiety-like behavior by means of the same mechanisms reported for other glucocorticoids. These may induce anxiety through an inhibition of hippocampal cholinergic modulation and/or intra-amygdaloid circuitry that regulates the hypothalamic-pituitary-adrenal axis (HPA)[28,29].

Histological analyses revealed changes of the number and cytoplasmic transformation of stained microglia cells at the prefrontal cortex, striatum, and CA1 hippocampus. These are cerebral regions with higher density of glucocorticoid receptors[12] related to anxiety and locomotion control. Several hypotheses may explain the microglial changes found in this study. The number and cytoplasmic transformation of stained microglial cells were mainly observed at the prefrontal cortex, likely due to intense glutamatergic regulation at this area[12]. These morphometric changes have also been reported using cortisone[30] and dexamethasone[31]. Our findings of increased isolectin-labeled microglia cells are coincident with those reported with prenatal dexamethasone treatment[31]. The overexpression of galactosyl glycoproteins on microglia can explain the increased number of stained cells induced by glucocorticoids[31]. If that were the case, the increased microglia population might be explained by enhancement of histochemical reactivity. The increment of the microglia population may also be explained by activation of these cells. This assumption is supported by the fact that the concentration of isolectin B4 used in our study (1:100) stains mainly activated microglia[32]. Thus an increase of the number and transformation of stained microglia cells could be associated with an indirect induction of neuro-excitotoxicity by glutamate. Such neuronal overstimulation would induce alterations in cellular homeostasis and the consequent increment of the number and cytoplasmic transformation of microglia[33,34]. Nevertheless, other authors have reported a downregulation of microglia induced by glucocorticoids[35-37], but these results are not

unexpected because it is well known that corticosteroids can mediate opposite effects on microglia functions, acting as inhibitors through glucocorticoid receptors and as stimulators through mineralocorticoid receptors[38]. Other mechanisms including cellular proliferation and monocytic migration from bloodstream seem unlikely[39].

Astrocyte analysis showed no differences in PDN treated rats compared with controls. This suggests that cerebral damage was not produced by PDN, at least at doses and time used in this study. However, whether PDN induced microglial activation produces irreversible cytotoxic damage was not determined, and further studies using PDN for longer periods are required. Yet there is evidence that other glucocorticoids produce dendritic atrophy[40,41] and hippocampal neuron loss in rats[42,43].

In summary, subacute exposure to PDN produced selected mood disturbances and brain structural changes in rats, including anxiety and microglial reactivity. The relevance of these features to patients treated with PDN remains to be elucidated.

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