Effects of progesterone vs. dexamethasone on brain oedema and inflammatory responses following experimental brain resection

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Abstract

**Background:** Dexamethasone (DEXA) is commonly used to reduce brain swelling during neurosurgical procedures. DEXA, however, has many side-effects that can increase the risks of post-operative complications. In contrast, progesterone (PRO) has fewer side-effects and has been found to be neuroprotective on traumatic brain injury (TBI). Whether PRO may be used as an alternative to DEXA during routine procedures has not been fully explored.

**Object:** To compare the effects of DEXA and PRO on surgical brain injury (SBI).

**Methods:** Seventy-five adult male Sprague Dawley rats were randomized into five groups: (1) SBI + drug vehicle (peanut oil, 1 ml kg\(^{-1}\)); (2) SBI + DEXA (1 mg kg\(^{-1}\)); (3) SBI + low-dose PRO (10 mg kg\(^{-1}\)); (4) SBI + high-dose PRO (20 mg kg\(^{-1}\)); and (5) sham SBI + drug vehicle. Magnetic resonance imaging study and assessments of brain water content (BWC), blood–brain barrier (BBB) permeability, cellular inflammatory responses and matrix metalloproteinase 9 (MMP-9) expression were conducted.

**Results:** This model consistently resulted in increased BWC and BBB disruption. PRO reduced astrocyte and microglia responses and attenuated brain oedema with preservation of BBB. A significant down-regulation of MMP-9 expression occurred in the PRO 20 group.

**Conclusions:** PRO is as effective as DEXA in reducing brain oedema and inflammation following SBI; 10 mg kg\(^{-1}\) of PRO was demonstrated to be more effective in relieving acute cellular inflammatory responses.

Introduction

Glucocorticoids are effective in maintaining blood–brain barrier (BBB) integrity and reducing peritumoural oedema [1,2]. In daily neurosurgical practice, dexamethasone (DEXA) is the form of glucocorticoids most commonly used during elective brain resection, with the rationale that it may facilitate surgery and patient recovery by reducing brain swelling and inflammatory responses [3,4]. DEXA, however, has many side-effects such as glucose intolerance, immunosuppression, osteoporosis, myopathy, gastric erosion and neuropsychiatric problems, all of which may cause post-operative morbidities or even mortalities [3]. Furthermore, it has now been established that the use of glucocorticoids is detrimental and contraindicated in patients with traumatic brain injury (TBI) [5]. Elective brain resection essentially constitutes a form of penetrating surgical brain injury (SBI).

Although the pathophysiology may differ, the unfavourable pharmacodynamics of DEXA is an argument for seeking and using an alternative adjunct during routine brain operations. The ideal agent should have well-defined neuroprotective properties and few side-effects, as well as being inexpensive and readily available.

Progesterone (PRO) is a potential candidate. Its neuroprotective effects have already been demonstrated in many pre-clinical studies for a variety of neurologic conditions, including TBI [6], stroke [7,8], spinal cord injury [9] and chronic pain [10]. Clinically, two randomized controlled studies have independently demonstrated the beneficial effects of PRO in TBI. PRO resulted in better neurologic recovery, was well tolerated and had minimal side-effects [11,12]. This contrasts with DEXA and provides support for studying PRO as an alternative peri-operative adjunct. A previous report has shown that PRO could reduce brain oedema following experimental cortical ablation, but no comparison with DEXA was made [13]. The present study...
used an animal model mimicking clinical brain resection to compare DEXA with two dosages of PRO. It was hypothesized that PRO would at least be as effective as DEXA in reducing oedema and inflammation around the resection cavity. The aim was to provide pre-clinical evidence to support the clinical testing of PRO as a superior alternative to DEXA, given the former’s more favourable pharmacodynamics.

Materials and methods

Study design

Seventy-five adult male Sprague Dawley rats (250–300 grams) were randomized into five treatment groups: Group 1, SBI + drug vehicle (peanut oil, 1 ml kg\(^{-1}\), \(n = 15\)); Group 2, SBI + DEXA (1 mg kg\(^{-1}\), \(n = 15\)); Group 3, SBI + low-dose PRO (10 mg kg\(^{-1}\), \(n = 15\)); Group 4, SBI + high-dose PRO (20 mg kg, \(n = 15\)); and Group 5, sham surgery + drug vehicle (peanut oil, 1 ml kg\(^{-1}\), \(n = 15\)). Treatments were administrated intraperitoneally (i.p.) at five time points: 12 hours before surgery and then at 1, 24, 48 and 72 hours after surgery. The brain samples for western blot (WB) and immunohistochemistry (IHC) were taken from the boundary of lesion site on the same 25 rats with five rats from each group (Figure 1(A), WB part (green arrow) and IHC part (yellow arrow)). Both PRO and DEXA were obtained from Sigma-Aldrich (St. Louis, MO). The study was approved by the Committee for the Use of Live Animals in Teaching and Research of the corresponding authors’ institution.

Surgical procedures

The animal model has been described previously [14]. Briefly, following the induction of general anaesthesia with 10% chloral hydrate (50 mg/100 g, i.p.), the rats were placed in a prone position on a stereotaxic frame under a surgical operating microscope. A craniotomy was made over the right frontal skull such that the left lower corner of the craniotomy was at the bregma. The dura was opened to expose the right frontal lobe. Using a micro-knife, two brain incisions were made leading away from the bregma along the sagittal and coronal planes to sever an area of brain 2 mm lateral to the sagittal and 1 mm anterior to the coronal planes. The depth of the incisions extended to the base of the skull, which effectively resulted in a standardized partial frontal lobectomy (Figure 1(A)). Intraoperative surgical packing with gelatin sponge (Gelfoam\(^{\circledR}\), Pfizer, New York, NY) was used to secure hemostasis. The dura was closed with 10/0 nylon sutures (Ethicon, Johnson & Johnson, Edinburgh, UK). The skull window was covered with paraffin film and the skin sutured with 4/0 silk (Ethicon). As the control group, animals in Group 5 received sham surgery that included only the craniotomy without any dural opening or brain resection.

Figure 1. (A) Intra-operative pictures showing the craniotomy window (left), the resection site (middle) and a harvested brain specimen with frontal lobe resection (right, WB part (green arrow) and IHC part (yellow arrow)). (B) MRI studies taken on post-operative day 3, showing the areas of oedema with T2-hyperintensity (red arrow). (C, left) BWC in different brain regions in the control and sham groups. (C, right) BWC around the operative site in different treatment groups. \(\# p < 0.05\) vs. sham. \(* p < 0.05\) vs. control.
Magnetic resonance imaging (MRI)

Seventy-two hours after surgery but before sacrifice for the rest tests, three rats from each group were randomly selected for MRI. The animals were anaesthetized with 1.5% isoflurane and placed in a plastic stereotactic device. All MRI data were acquired on a 7-Tesla scanner with a maximum gradient of 360 mT m⁻¹ (70/16 PharmaScan, BrukerBiospin GmbH, Germany). Multi-slice, proton-density weighted scout images were acquired using two-dimensional rapid acquisition with refocused echoes (RARE) sequence along the coronal, axial and sagittal planes to accurately locate the oedema region and to position the following MRI slices. T²-weighted images were acquired with 17 coronal slices, each of 0.5 mm thickness and without inter-slice gap. They were positioned to cover the rat brain around the bregma (−3.46 mm to 5.04 mm) and were scanned with the following parameters: RARE, TR/TE = 4800/120 ms, FOV = 35 × 35 mm², data matrix = 256 × 256, NEX = 6.

Brain water content (BWC)

Animals (n = 5 for each group) were sacrificed after an overdose of general anaesthesia 3 days after surgery. The brains were removed and divided into six portions: frontal ipsilateral (to the resection), frontal contralateral, parietal ipsilateral, parietal contralateral, brainstem and cerebellum. These parts were weighed immediately (wet weight) and again after drying in an oven at 120 °C were weighed immediately (wet weight) and again after drying in an oven at 120 °C for 24 hours (dry weight). The percentage of water content was calculated as [(wet weight – dry weight)/wet weight] × 100%.

Blood–brain-barrier permeability

Two per cent Evans-blue (EB) (4 ml kg⁻¹, Sigma-Aldrich) in 0.9% saline was injected through the tail vein 3 days after surgery (n = 5 for each group). Animals were kept anaesthetized for 1 hour after the injection and then perfused transcardially with saline to clear the cerebral circulation of EB. The brain was then removed and photographed. To quantify EB extravasation, the operated hemisphere was homogenized in 3 ml of N,N-dimethylformamide (Sigma-Aldrich), then incubated at 56 °C for 48 hours and centrifuged. The absorption of the supernatant was measured at 620 nm using a spectrophotometer (Thermo Scientific, Waltham, MA).

Western blot (WB)

Three days after surgery, brain tissue samples (n = 5 for each group) were taken and homogenized on ice and extracted using the RIPA Buffer (Cell Signaling Technologies, Inc., Danvers, MA) containing a concentration of Protease Inhibitor Cocktail, according to the manufacturer’s instructions (Roche Diagnostics, Mannheim, Germany). Protein lysates (50 µg per sample) were separated by 12% SDS-PAGE and transferred onto a PVDF membrane (Bio-Rad Laboratories, Hercules, CA). After milk-block for 1 hour at room temperature, membranes were incubated overnight at 4 °C with the primary antibodies to matrix metalloproteinase (MMP)-9 at 1:700 dilution and β-actin as loading control at 1:1000 dilution (both from Cell Signaling Technologies).

Membranes probed with β-actin were then probed with horseradish peroxidase (HRP)-conjugated secondary antibody against rabbit at 1:10 000 dilution (Santa Cruz Biotechnology) for 1 hour at room temperature, while those with MMP-9 were probed with Biotin goat-anti-rabbit secondary antibody at 1:5000 dilution (Invitrogen, CA), followed by Streptavidin HRP Conjugate tertiary antibody (Zymed, Invitrogen) at 1:15 000 dilution for 1 hour at room temperature. Immobilon Western HRP Substrate (Merck Millipore, Billerica, MA) was used to detect immunoreactivity signals.

Immunohistochemistry (IHC)

The right frontal lobes from the remaining animals were harvested 72 hours after surgery (n = 5 for each group). The sham-operated rats were used as negative controls. Immunohistochemical staining for GFAP (for astrocytes) and IBA1 (for microglia) was performed on consecutive 6 µm thick paraffin-embedded tissue sections. Briefly, tissue sections were deparaffinized with xylene and antigens retrieved in 10 mM sodium citrate (pH = 6.0). After peroxidase blocking, the specimens were blocked with 5% normal goat serum (Dako, Glostrup, Denmark) for 1 hour at room temperature, followed by incubation with mouse monoclonal anti-GFAP antibody (1:300 dilution; Cell Signaling Technology, Inc.) and rabbit polyclonal anti-IBA1 antibody (1:500 dilution; Wako, Inc.) at 4 °C overnight. After washing with phosphate-buffered saline, sections were incubated with ready-to-use DAKO En Vision + Kit (Dako). Signals were detected using DAB (Dako) and subsequently counterstained with haematoxylin (Vector Laboratories, Burlingame, CA).

In the GFAP/IBA1-immunostained sections, positive reactivities around the lesion sites were quantified on four randomly selected sections per brain. On each section, four randomly selected images were captured along the edge of lesion boundary zone. These were input to the image analysis system, Meta Morph Offline and the total area of positive reactivities was automatically measured. The percentage of positive reactivities was calculated using the following formula: Ap/Af = 100, where Ap is the total area of positive reactivities and Af is area of the whole field.

Statistical analysis

SPSS (version 18.0) software was used to conduct the statistical analysis. The correlation of the investigated parameters was analysed by the use of Student t-test or one-way ANOVA with a post-hoc Bonferroni test. Results were established as significant if p < 0.05.

Results

Magnetic resonance imaging

Representative T²-weighted images are shown in Figure 1(B). These were taken at 2.54 mm anterior to the bregma (slice thickness = 0.5 mm) on day 3 post-surgery. Control group rats showed significant T²-hyperintensity along the upper border of the resection site, indicating brain oedema. The oedematous cortex swelled and protruded through the bone window.
In contrast, DEXA- and PRO-treated animals displayed less significant hyper-intense areas and less swelling.

**Brain water content (BWC)**

Comparing between the sham and control groups, significantly greater BWC was found in the latter around the operative site in the residual right frontal lobe (Figure 1(C), left, \( p < 0.001 \), Student \( t \)-test). There was no difference between these two groups in other regions of the brain, indicating that the experimental model could consistently induce localized brain oedema. All surgical groups (1–4) also showed higher BWC than the sham group over the right frontal lobe (\( p < 0.05 \), Bonferroni test). When compared with the control group, both DEXA and PRO treatments reduced BWC to significantly lower levels (Figure 1(C), right, \( p < 0.05 \), Bonferroni test). No significant difference was found between the three hormone-treated groups.

**Blood–brain barrier disruption**

EB extravasation in the control group was markedly increased when compared with the sham group (Figure 2(A), \( p = 0.000 \), Bonferroni test), indicating that the SBI model could induce a significant and localized BBB breakdown. All three hormone-treated groups showed significantly reduced EB extravasation surrounding the operative site when compared with the control group (\( p < 0.05 \), Bonferroni test). However, there was no difference among these three groups.

**Cellular inflammatory responses**

GFAP (for astrocyte) and IBA1 (for microglia) expressions were studied as markers of acute cellular inflammatory responses. The expressions of both markers were significantly increased in the control group (\( p < 0.05 \), Bonferroni test) when compared with sham treatment (Figure 2(B)). With hormone treatments, both markers showed significantly lower levels of expression than the control group (Figure 2(B)). Furthermore, low-dose PRO resulted in significantly less cellular inflammatory responses than DEXA and high-dose PRO (Figure 2(B), \( p < 0.05 \), Bonferroni test).

**MMP-9 expression**

This study also investigated the potential mechanism underlying the effect of PRO by studying the expression of MMP-9. In comparison with the sham group, this SBI model induced a significantly higher level of expression of MMP-9 in the control group (Figure 3). Except the PRO 10 group, both

![Figure 2](image-url). (A) The upper panel shows representative pictures of EB dye extravasation in different groups. The lower panel shows the quantitative analysis of EB extravasation. \#\( p < 0.05 \) vs. sham. *\( p < 0.05 \) vs. control. (B) Representative pictures of immunohistochemical staining of GFAP and IBA1. The bar charts show the amount of positively stained area around the operative sites in each group. \#\( p < 0.05 \) vs. sham. *\( p < 0.05 \) vs. control. **\( p < 0.05 \) vs. DEXA and PRO 20.
DEXA and PRO 20 groups caused substantial down-regulation of MMP-9 (p < 0.05, Bonferroni test) when compared with the control group.

Discussion

Surgical brain injury

Neurosurgical procedures, whether performed in an emergency or elective setting, may result in additional trauma due to cortical incision, parenchymal resection, brain retraction, haemorrhages and thermal injuries from electrocautery. These may cause serious post-operative complications such as brain swelling, ischaemia, raised intracranial pressure, functional deficits or even deaths. Various medications and methods are being used to minimize these including osmotic agents, glucocorticoids, ‘neuroprotective’ anaesthetic drugs and induced hypothermia [15]. Given the large number of neurosurgical procedures performed on a daily basis and the potential negative impact of SBI on patient outcome, further research effort in finding an effective perioperative adjunct is of major clinical importance [16]. The present study took into considerations recent pre-clinical and clinical findings on PRO and investigated its use in modulating SBI.

Progesterone as a neurosteroids

PRO is commonly known as a gonadal hormone. It is, however, also synthesized by oligodendrocytes and neurons within the central nervous system (CNS), where it serves many important functions [17]. During pregnancy, PRO protects the foetal brain from oxidative stress and immune-inflammatory reactions around the time of neuronal development. It is currently thought that healing after CNS injury and degeneration recapitulates many of these pre-natal events [18] and that exogenous PRO may improve the survival, differentiation and functioning of post-natal neurons and glial cell [19]. There is now a growing body of evidence that PRO may be a safe and effective treatment for TBI and other neurologic disorders in humans [8,20–22].

This study compared PRO with DEXA because the latter has been in use for decades to reduce brain swelling and inflammatory responses around the time of surgery [3]. DEXA has many unwanted side-effects [5] and there is now definitive evidence that it should not be used in TBI patients. This somewhat poses a conceptual problem with its use during routine brain surgery, except for its ability to reduce peritumoural oedema. In contrast, PRO has fewer side-effects and is well tolerated and effective in various forms of CNS insults [23]. Moreover, PRO can enter the CNS readily with a wide therapeutic window. Given its safety, low cost and accessibility, it is potentially a superior alternative to DEXA in the management of SBI.

Progesterone in SBI

Most reported studies on PRO and TBI used animal models that simulate blunt head injuries [24]. PRO was found to reduce oedema formation after frontal cortical contusion [25] and impact-acceleration diffuse TBI [26]. An inverse correlation between serum PRO level and the degree of oedema has been described [27]. To date, only a handful of reports focused on cortical resection injuries such as ours. Atella et al. [28] found a lesser degree of oedema in pseudopregnant rats (with higher PRO level) than in normal cycling ones after prefrontal cortex lesion. However, the authors suggested that the difference was due to different levels of vasopressin. Asbury et al. [13] also reported decreased neuronal death and better functional outcomes after bilateral medial pre-frontal cortex ablation in animals given PRO. Other investigators, however, did not find any benefit from PRO after sensorimotor cortex ablations in rats [29]. Previous work showed that systemically administered PRO could ameliorate brain injury induced by electrocautery [30]. The present study was conducted to further investigate the effects of PRO following brain resection. Brain swelling after brain injury may be vasogenic and/or cytotoxic in nature. The former commonly occurs within a few hours post-injury [31,32], followed by the latter that may persist for weeks [31]. Brain oedema usually peaks on 3–7 days after surgery and then decreases gradually. In this study, considering the available number of the animals in total for one time point has reached 75, it was decided to mainly focus on investigating the early stage of brain oedema instead of the late stage [33,34]. Although the observed differences in BWC between groups in this study may appear to be small (ranging from 82.4 ± 1.5 to 85.1 ± 1.2%), it must be emphasized that even a small increase in BWC can lead to a significant increase in tissue volume and intracranial pressure [35].

The mechanisms underlying the observed effects of PRO are not fully understood. The occurrence of brain oedema is closely related to the integrity of the BBB [33]. In general, the BBB becomes maximally permeable from 4–6 hours after injury, before regaining integrity throughout a 7-days period [36,37]. PRO is thought to maintain BBB integrity during the early phase by modulating the expression of many important mediators such as matrix metalloproteinases (MMPs) [38,39], p-glycoprotein efflux pump [40] and aquaporin-4 [25]. MMPs, in particular, are capable of degrading extracellular matrix proteins such as tight junction proteins and the neurovascular basal lamina of the BBB. Indeed, dysregulated MMPs expressions are commonly observed with BBB breakdown after TBI, neurodegeneration and stroke [41–44].
Amongst different MMPs, MMP-9 is the most up-regulated one after TBI [45] and is involved in tissue remodelling following many pathologic conditions [33]. MMP-9 gene knock-down, on the other hand, may protect against TBI and ischaemia due to reduced BBB breakdown, brain oedema and inflammatory responses [46,47]. The present study found that MMP-9 down-regulation was also likely to play a role in reducing post-operative oedema.

The activation and migration of inflammatory cells and mediators can initiate a cascade of damaging inflammatory reactions. Cellular responses such as reactive astrogliosis and microglial activation contribute significantly towards secondary injuries seen in TBI [48]. It is now thought that PRO and its active metabolites may attenuate post-traumatic inflammation by inhibiting cytokine release [49,50] as well as immune cell activation and migration [23]. It was found that PRO could reduce astrocyte and microglia development in response to SBI, although between-group differences did not correlate fully with those of BWC levels, suggesting that other mechanisms may be at work.

Timing and dosage

The dosing regimen of PRO requires careful considerations. Roof et al. [51] showed that earlier treatments resulted in greater effects, although treatment given as late as 24 hours post-injury may still be beneficial. For obvious reasons, only post-injury treatment is feasible for accidental TBI. However, as an adjunct for elective operations, both pre- and post-operative treatments are possible, if not desirable. In a previous study on electrocautery-induced injury, just a single pre-operative dose of PRO was already effective in reducing astrocytic hypertrophy and macrophage infiltration [30]. The present study used both pre- and post-operative treatments to mimic the common regimen of DEXA in order to enhance clinical relevance and found it to be beneficial. Regarding the optimal dosage, Goss et al. [52] reported that the efficacy of PRO followed a U-shaped curve, with the most effective dosages being between 8–16 mg kg\(^{-1}\); lower (4 mg kg\(^{-1}\)) and higher (32 mg kg\(^{-1}\)) dosages were less effective. It was found that, in terms of reducing acute cellular inflammatory responses, PRO was more effective at 10 mg kg\(^{-1}\) than at a higher dosage. Conversely, the result showed that there was no statistical difference between low dose and high dose PRO on reducing brain oedema and protecting the integrity of the BBB. However, within the two groups, the p-values of these two parameters (p = 0.075 for BWC analysis and p = 0.072 for EB extravasation, ANOVA) were very close to a significantly statistical level (p < 0.05). Whether or not low dose PRO has a better effect on reducing oedema and BBB leakage than high dose needs to be investigated with more animal samples.

Limitations and future studies

This model only studied brain resection without testing other common causes of SBI such as brain retraction. The establishment of the model strictly followed Yamaguchi et al.’s [14] method and, since they had already proved its stabilization and reproducibility, the variation between groups was not quantified. Without using a tumour implant, the additional impact of pre-existing peritumoural oedema was unknown. Blood PRO level, known to correlate with brain oedema, was not measured [27]. Furthermore, only pathologic outcomes at one early post-operative time-point were studied. How PRO may affect later events such as apoptosis, cavitation, glial scarring and functional recovery require further investigations. Since MMP-9 expression has been proved to have a positive relationship with the severity of brain oedema or BBB breakdown, the guess is that it may also change after PRO 10 treatment in a different time compared with control. Future studies should focus on these as well as different treatment regimens and the effects of combinatorial therapy. For instance, PRO may be used together with DEXA to assess the former’s glucocorticoids-sparing effect or with vitamin D, which is known to act synergistically with PRO [53]. Whether PRO would exert similar effects in animals (and patients) of different genders, age groups and endogenous PRO levels also need to be carefully evaluated in order to inform future clinical trials and applications.

Conclusions

Brain resection can cause significant post-operative morbidities in daily neurosurgical practice. This pilot study demonstrates that, like DEXA, PRO can reduce brain oedema, BBB leakage and inflammatory responses surrounding a resection cavity. Down-regulation of MMP-9 expression may play a role in reducing oedema after brain resection. A lower dose (10 mg kg\(^{-1}\)) of PRO was demonstrated to be more effective than a higher dose (20 mg kg\(^{-1}\)) in relieving acute cellular inflammatory responses. Given the safety, availability and more favourable pharmacodynamics of PRO, its use for the protection against brain resection and other forms of SBI deserves further investigation.

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Declaration of interest

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