LABORATORY STUDY

Retinal maturation is delayed by repeated, but not single, maternal injections of betamethasone in sheep

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Abstract

Purpose **The safety and efficacy of prescribing a single maternal course of corticosteroid during pregnancy has been documented in human trials. However, the current trend is to prescribe repeated courses of corticosteroid. We investigated an aspect of the safety of this practice in an animal model.**

Methods **Date-mated ewes received saline, single or four corticosteroid injections between days 104 and 124 of gestation (term = 150). Lambs were delivered on day 125 or 145 by caesarian section after spinal anaesthesia. Eye diameters were measured and semi-thin toluidine-blue-stained transverse sections of retinae were analysed using an Optimus Image Analysis program.**

Results **At 125 days, retinal measures in the ventral periphery and area centralis were significantly thinner than control (p = 0.0001). At 145 days, total eye size was significantly reduced compared with control (p = 0.03), and retinal measures in the ventral periphery (p = 0.0001), but not the area centralis (p = 0.19), remained significantly different from control.**

Conclusion **Repeated maternal administration of corticosteroid may affect retinal maturation in the fetus.**

Key words Corticosteroid, Development, Pregnancy, Preterm, Retina, Sheep

Systemic corticosteroids are administered to pregnant women for a wide variety of medical conditions including severe asthma, rheumatoid arthritis and systemic lupus erythematosus.[1,2] Furthermore, it is common in obstetric practice to prescribe a single course of corticosteroid to women at high risk of delivering a very preterm infant. The regimen has been proven to be a safe and effective way of reducing the incidence of respiratory distress syndrome, intraventricular haemorrhage, necrotising enterocolitis and preterm infant mortality.[3] Whilst the safety of a single course of corticosteroid has been documented in three large randomised trials that followed children to 12 years of age,[3] the trend in practice is to prescribe repeated maternal courses of corticosteroid in high-risk pregnancy where the risk of preterm birth persists or recurs.[4] No randomised data of outcomes in human pregnancy exist to support the safety of repeated administration of corticosteroid.

Here we have investigated the effect of repeated corticosteroid therapy on the development of the central nervous system by examining retinal maturation. The retina is of particular clinical interest for several reasons. The mammalian retina is produced in two major phases of cell generation. The 'homochrony' hypothesis predicts that in humans the second phase of retinal cell generation occurs from 15 to 24 weeks of gestation.[5] A survey of clinical practice suggests that corticosteroids are prescribed under 24 weeks gestation,[4] thus coinciding with the latter part of the second phase of retinal cell generation. During this phase, the retinae also undergo marked lateral expansion, particularly in the periphery, through the differential addition of second-phase cells to peripheral rather than central retina.[6] The retina is therefore potentially directly vulnerable to corticosteroid therapy, which has known antimitotic effects on DNA, RNA and protein synthesis in the nervous system.[7,8] Finally, although there is general agreement that low-birthweight preterm infants are at risk for impaired motor development, recent evidence suggests they are also specifically vulnerable to impaired visual development.[9–11]

We predicted that the weekly administration of corticosteroids could affect retinal maturation. Calculations derived utilising 'homochrony' hypothesis assumptions[5,12,13] revealed that the second phase of ovine retinal cell generation would be taking place from day 71 to 115 of gestation. To examine the effects of single and repeated exposures to corticosteroid in a clinically relevant animal model, we examined retinal maturation in sheep during the latter part of this gestational period. Retinal maturation was quantified using eye growth as

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a measure of lateral expansion of the retinae, and retinal thickness as a measure of the maturation of cells added in the second phase of neurogenesis.

Methods

Institutional animal ethics committee approval was obtained and animal maintenance and care conformed to the principles outlined by the NH&MRC (Australia). Findings on birthweight have been reported previously.[14] Date-mate ewes were randomised into one of three treatment groups: control (n = 12), single (n =12) and repeated corticosteroid (n = 12) treatments. Ewes received an injection of betamethasone (Celestone Chrondose 0.5 mg/kg, i.m.) on day 104 of gestation in the single corticosteroid group, and on days 104, 111, 118 and 124 in the repeated corticosteroid group. Injections of sterile normal saline of equivalent volume were given on days 111, 118 and 124 in the single corticosteroid group, and on all four days in the control group. All animals had previously received an intramuscular injection of medroxyprogesterone acetate (Depo-Provera, Upjohn, 150 mg) on day 100 to minimise the risk of preterm birth.

On day 125 or 145, ewes were premedicated with ketamine (100 mg/ml, 12 ml i.m.; Parke Davis, Ireland) and lambs delivered by caesarean section after spinal anaesthesia with lignocaine (Xylocaine 1%, 4 ml intraspinal, Astra, Australia). Newborns were killed (ketamine 5 ml i.v.; Parke Davis, Ireland: Lethobarb 2 ml i.v.) and then transcardially perfused with heparinised saline followed by modified Karnovsky's fixative in cacodylate buffer (0.1 M, 300 mosmol, pH 7.4). Skulls were opened from a ventral approach and dissection of the eyes completed with the aid of an operating microscope. The maximum diameter of each eye was recorded in two axes (nasal–temporal, ventral–dorsal) using Vernier calipers, with measurements rounded to the nearest millimetre.

The cornea, iris and lens were removed with iridectomy scissors and the vitreous removed with cotton buds. In all ungulates, a streak of high cell density extends along a straight horizontal line, dorsal to the optic disc.[15] The visual streak was clearly defined by a yellow band that widened at the area centralis, an area of high retinal ganglion cell density immediately temporal to the optic disc. Two millimetre square segments of retina from the area centralis and ventral periphery were removed, representing an area of high and low ganglion cell density respectively.

Tissue was postfixed for 2 h in osmium tetroxide, dehydrated in graded ethanols and propylene oxide, and embedded in Epon-Araldite. Tissue blocks were labelled with a code to mask the operator who would perform the subsequent measurements. Transverse semi-thin sections cut with a microtome and stained with toluidine blue were used to estimate the total retinal width, and that of its respective cellular and plexiform layers, which were clearly defined in the light microscope (Figs. 1, 2). Data were collected using frame-grabbed images of the semi-thin sections and analysed with an Optimus image analysis system, calibrated with a standard graticule. Measurements from each retina were repeated on three separate sections to obtain a mean for each animal.

A mixed model analysis of variance was applied to generate p values. Multivariate ANOVAs were applied to control for any effect of maternal weight, birthweight,

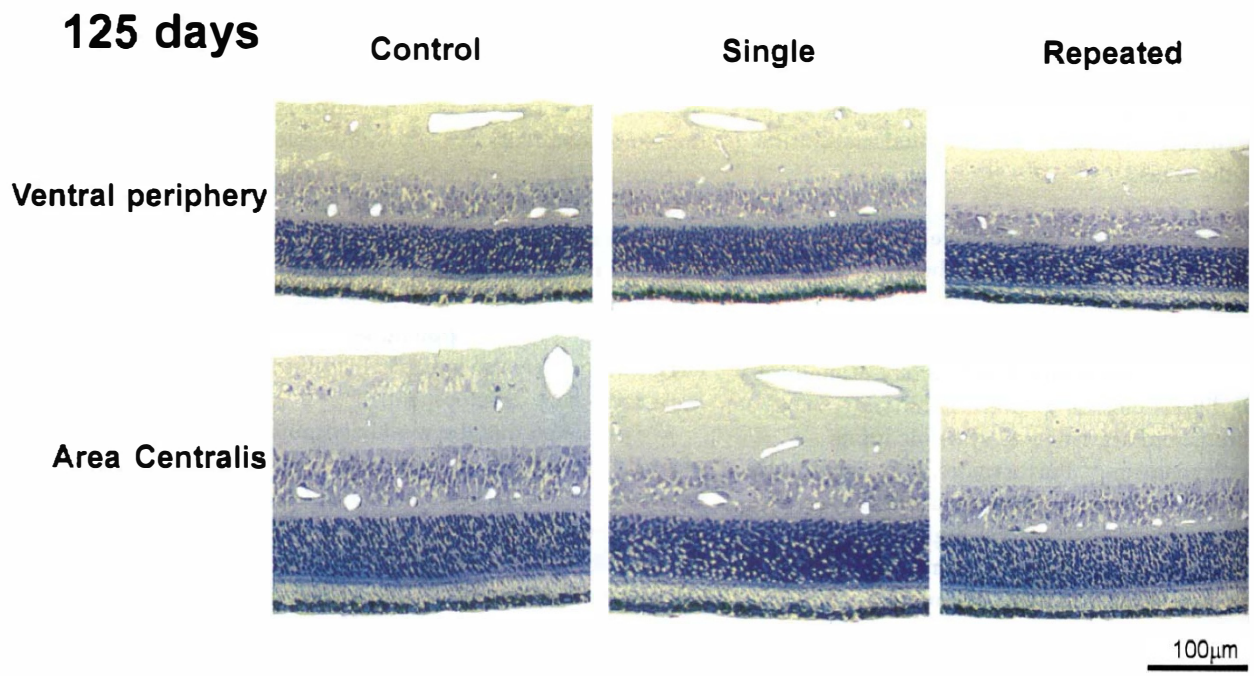

Fig. 1. *The effect on retinal thickness of single and repeated corticosteroid therapy compared with control in the ventral periphery and area centralis following delivery at 125 days. Retinae from animals exposed to repeated, but not single, corticosteroid treatments were significantly thinner than control at both the ventral periphery and area centralis* ($p < 0.0001$).

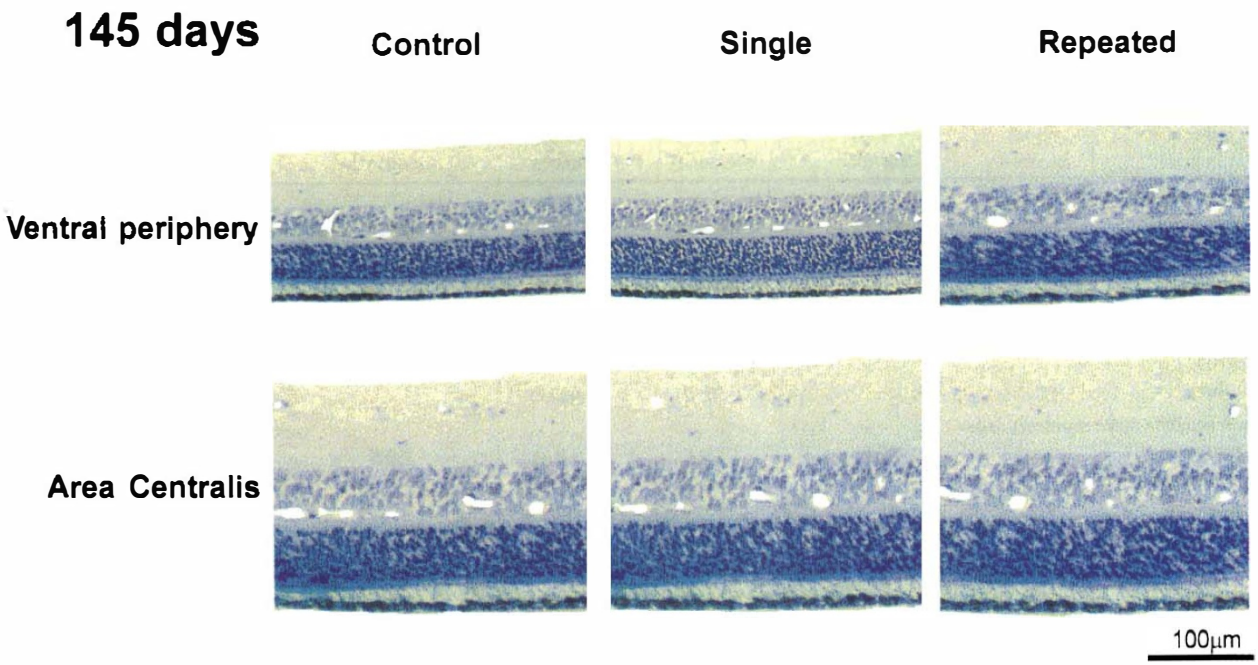

Fig. 2. *The effect on retinal thickness of single and repeated corticosteroid therapy compared with control in the ventral periphery and area centralis following delivery at 145 days. Retinae from animals exposed to repeated, but not single, corticosteroid treatments were significantly thicker than control in the ventral periphery* (p < *0.0001).*

gender or laterality upon outcome measures. A p value of 0.03 was considered significant because of the multiple estimations. Results are presented as adjusted mean and 95% confidence interval of the difference between the means.

Results

In control animals, eye diameter increased significantly between 125 and 145 days (39% increase, $p = 0.02$; Fig. 3). There was a concurrent thinning of the retinae which was more marked at the ventral periphery (15% reduction, $p = 0.0001$) than at the area centralis (9% reduction, $p = 0.0001$). Individual retinal layers showed variation in their thinning. In the ventral periphery, the most marked thinning was found in the ganglion cell layer (34% reduction, $p = 0.0001$), but in the area centralis the photoreceptor outer segments layer was most affected (46% reduction, $p = 0.0001$; Tables 1, 2).

Eye diameter was similar in the control and single corticosteroid groups at both 125 and 145 days ($p > 0.81$; Fig. 3). Similarly, no significant differences were detected in the values of retinal thickness between the control and single corticosteroid treatment at either gestational age ($p > 0.09$; Tables 1, 2, Figs. 1, 2).

In contrast, repeated corticosteroid therapy significantly altered patterns of eye growth. At 125 days, eye diameters were not significantly smaller compared with controls ($p = 0.45$), but at 145 days values were significantly reduced ($p = 0.03$; Fig. 3). Repeated corticosteroid treatment thus resulted in a relative failure of the eye to increase in diameter between days 125 and 145 of gestation, even though no further corticosteroid was administered during this period.

Despite eye size being similar to controls at 125 days following repeated corticosteroid therapy, the retinae were significantly thinner in both the ventral periphery (11% thinner, $p = 0.0001$) and area centralis (18% thinner, $p = 0.0001$). Specifically, repeated corticosteroid therapy resulted in a thinning of all retinal layers with the exception of the inner nuclear layer at the ventral periphery, and the nerve fibre layer at the area centralis (Table 1, Fig. 1).

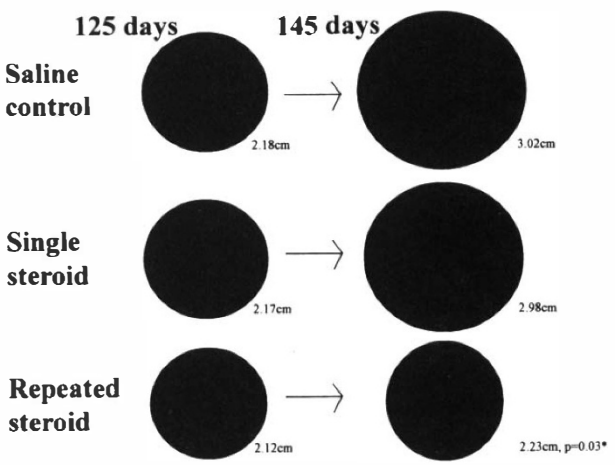

Fig. 3. *Mean eye diameter in the three treatment groups at 125 and 145 days gestational age. Eyes from animals exposed to repeated corticosteroid treatments failed to increase significantly in diameter between 125 and 145 days, despite no further drug being administered in this period. Thus, at 145 days, eye size was significantly reduced following repeated corticosteroid treatment compared with saline control* (p = *0.03).*

Table 1. *The effect of single and repeated corticosteroid treatments compared with saline control at 125 days on total retinal width and that of its respective cellular and plexiform layers*

	Ventral periphery (μm)			Area centralis (μm)		
	Saline control	Single steroid	Repeated steroid	Saline control	Single steroid	Repeated steroid
Retinal width						
Mean	173.1	171.4	**154.0**	231.9	225.5	**190.6**
95% CI		−3.5 to 6.9	**15.2 to 22.9**		−3.3 to 16.0	**34.4 to 48.1**
p value		0.51	**0.0001**		0.19	**0.0001**
Pigment layer						
Mean	10.8	10.5	**8.9**	10.7	10.6	**8.5**
95% CI		−0.8 to 1.4	**1.1 to 2.8**		−0.5 to 0.6	**1.7 to 2.6**
p value		0.58	**0.0001**		0.86	**0.0001**
Photoreceptor outer segments layer						
Mean	12.8	12.8	**9.8**	30.8	30.5	**20.9**
95% CI		−1.5 to 1.5	**1.9 to 4.1**		−2.8 to 3.5	**7.7 to 12.2**
p value		0.99	**0.0001**		0.84	**0.0001**
Outer nuclear layer						
Mean	39.8	39.0	**37.4**	51.9	51.0	**57.2**
95% CI		−1.8 to 3.3	**0.5 to 4.3**		−4.6 to 6.4	−9.3 to 1.5
p value		0.56	**0.02**		0.75	**0.008**
Outer plexiform layer						
Mean	10.0	10.2	**6.8**	11.2	11.7	**8.3**
95% CI		−2.1 to 1.8	**1.8 to 4.6**		−2.0 to 1.1	**1.8 to 4.0**
p value		0.88	**0.0001**		0.57	**0.0001**
Inner nuclear layer						
Mean	33.1	30.0	33.5	39.6	38.4	**32.4**
95% CI		−0.5 to 6.8	3.0 to 2.3		−1.4 to 3.9	**5.4 to 9.1**
p value		0.09	0.79		0.35	**0.0001**
Inner plexiform layer						
Mean	32.6	31.6	**27.0**	45.3	44.3	**31.0**
95% CI		−2.2 to 4.2	**3.3 to 8.0**		−3.1 to 5.0	**11.4 to 17.2**
p value		0.52	**0.0001**		0.64	**0.0001**
Ganglion cell layer						
Mean	25.6	26.6	**17.8**	24.0	24.1	**17.0**
95% CI		−5.2 to 3.1	**4.7 to 10.8**		−4.3 to 4.2	**4.0 to 10.0**
p value		0.62	**0.0001**		0.98	**0.0001**
Nerve fibre layer						
Mean	8.9	8.9	**11.3**	18.9	18.0	15.8
95% CI		−1.9 to 1.9	**−3.8 to** −1.0		−5.2 to 6.8	−1.1 to 7.4
p value		0.97	**0.001**		0.78	0.15

Between 125 and 145 days, a period during which control eyes increased in size and retinae significantly thinned, the pattern of development of the eyes exposed to repeated corticosteroid was markedly different. Not only did the eyes fail to increase in overall size, but retinal width significantly thickened (ventral periphery: 10% increase, $p = 0.0002$; area centralis: 12% increase, $p = 0.0001$). At the ventral periphery, the thickening occurred principally in the photoreceptor outer segments (56% increase, $p = 0.0001$), whereas at the area centralis it occurred primarily in the inner plexiform layer (47% increase, $p = 0.0001$). Thus, at 145 days gestation, the photoreceptor outer segments, outer and inner nuclear, and inner plexiform layers of the ventral periphery, and the photoreceptor outer segments, inner plexiform, ganglion cell and nerve fibre layers of the area centralis all remained significantly different compared with controls (Table 2, Fig. 2).

Discussion

The findings of the present study support our knowledge of normal mammalian retinal development[5,6,12,13,15–18] and suggest that repeated prenatal exposure to maternally administered corticosteroids may delay this development. The absence of significant differences between retinae of control and single corticosteroid exposed newborns also reinforces the safety findings of a single course of corticosteroid therapy derived from prospective clinical trials in humans.[3]

We predicted that in sheep, between days 71 and 115 days of gestation, the retinae would thicken as second phase retinal cells were generated. By 125 days, we anticipated the second phase of retinal cell generation would be complete; in accord with this assumption, mitotic figures were not seen in the retinae at E125. However, overall retinal growth would still be in progress in terms of the generation of extracellular matrix, cellular processes, synapse formation and

Table 2. *The effect of single and repeated corticosteroid treatments compared with saline control at 145 days on total retinal width and that of its respective cellular and plexiform layers*

	Ventral periphery (μm)			Area centralis (μm)		
	Saline control	Single steroid	Repeated steroid	Saline control	Single steroid	Repeated steroid
Retinal width						
Mean	146.8	144.9	**169.3**	210.1	212.3	214.4
95% CI		−8.2 to 12.1	29.6 to 15.3		−11.6 to 7.1	−1.1 to 1.5
p value		0.70	**0.0001**		0.63	0.19
Pigment layer						
Mean	8.5	9.0	9.0	9.2	8.9	8.3
95% CI		−1.5 to 0.90	−1.1 to 0.2		−1.1 to 1.5	−0.1 to 1.8
p value		0.24	0.14		0.72	0.08
Photoreceptor outer segments layer						
Mean	18.2	16.8	**15.3**	16.6	16.8	**21.9**
95% CI		−1.1 to 3.9	**1.1 to 4.6**		−3.9 to 3.5	**−8.0 to −2.8**
p value		0.27	**0.002**		0.90	**0.0002**
Outer nuclear layer						
Mean	31.3	30.9	**39.8**	49.0	48.7	50.5
95% CI		−2.9 to 3.9	**−10.9 to −6.0**		−4.5 to 5.2	−4.9 to 2.0
p value		0.79	**0.0001**		0.89	0.39
Outer plexiform layer						
Mean	7.3	7.9	7.7	8.7	8.9	**8.7**
95% CI		−1.5 to 0.4	−1.0 to 0.3		−2.1 to 1.7	−1.4 to 1.3
p value		0.24	0.30		0.82	0.97
Inner nuclear layer						
Mean	27.7	28.6	**36.2**	34.2	34.8	36.8
95% CI		−3.2 to 1.5	**−10.1 to −6.8**		−5.3 to 4.1	−6.0 to 0.7
p value		0.46	**0.0001**		0.78	0.11
Inner plexiform layer						
Mean	27.7	28.1	**35.2**	41.5	42.9	**45.5**
95% CI		−3.4 to 2.7	**−9.7 to −5.3**		−4.4 to 1.5	**−6.1 to −2.0**
p value		0.81	**0.0001**		0.34	**0.0004**
Ganglion cell layer						
Mean	16.8	15.3	16.1	24.8	25.4	**21.9**
95% CI		−2.3 to 5.3	−1.9 to 3.4		−3.0 to 1.9	**1.2 to 4.7**
p value		0.43	0.58		0.65	**0.001**
Nerve fibre layer						
Mean	9.2	9.6	10.5	25.0	26.1	**21.5**
95% CI		−3.4 to 2.6	**−3.5 to** −0.7		−4.6 to 2.5	**−1.0 to 6.1**
p value		0.80	0.19		0.56	**0.07**

dendritic elaboration. The growth would largely be in the plexiform, rather than the cellular, layers of the retinae and allows for continuing lateral expansion of the retinae, particularly in the periphery. We observed such a pattern, with the eye size of control animals significantly increasing between day 125 and 145, with concomitant thinning of some retinal cellular layers, predominantly in the periphery where lateral expansion is most marked.

Repeated corticosteroid therapy disrupted these patterns of retinal development, a disruption that persisted at term, 20 days after the cessation of treatment. At 125 days, immediately after the completion of the second phase of retinal cell generation, retinae in the repeated corticosteroid treatment group were significantly thinner than controls. The finding suggests that repeated corticosteroid treatment may have delayed retinal maturation by extending the duration of the second phase of retinal cell generation. Consequent delays would therefore take place in events such as the development of extracellular matrix and cell processes. Between day 125 and 145 of gestation, a phase of lateral retinal expansion, when control eyes grew in size and retinae thinned, the diameters of the eyes in the repeated corticosteroid treatment group failed to increase significantly and achieved an increase in retinal width only equivalent to that achieved in controls 20 days earlier. Thus, the delays in retinal development appear to correlate with the 20 day time period during which repeated corticosteroid therapy was administered, suggesting a temporary arrest of retinal development occurred at this stage.

Corticosteroids are known to affect DNA, RNA and protein synthesis in the central nervous system.[7,8] The outcome of their action upon the fetus seems to be dependent upon the cellular events that occur during the period of administration.[19–22] For example, biochemical analysis has demonstrated that myelination in the ovine central nervous system is in progress between 104 and 125 days.[23] The effect of corticosteroid administration

during this period is to delay myelinogenesis, although subsequent recovery takes place in some systems. We found delays in myelination in the ovine optic nerve after exposure to repeated corticosteroid treatment following delivery at 125 days,[19] but full recovery had occurred by day 145.[20] Neurological recovery is also possible. Newborn rats treated with cortisol on the first 4 postnatal days have reduced cell numbers with a 20% and 30% deficit in the cerebrum and cerebellum respectively, but compensation of the cell deficit occurs by postnatal day 20.[8]

However, permanent deficits have been reported in the frontal lobe of mice exposed to corticosteroid therapy during the critical phase of cell generation in the frontal lobe.[21] Furthermore, a dose-dependent loss of pyramidal neurones in the hippocampal CA1, 2, 3 and 4 regions and granular neurones in the dentate gyrus has been reported in the monkey following four repeated prenatal doses of corticosteroid.[22]

These findings on retinal maturation are of direct clinical relevance. Caecal period predictions for human pregnancy indicate that the second major phase of retinal cell generation occurs between 104 (15 weeks) and 169 post-conception days (24 weeks). The gestational period from 104 to 124 days in the sheep equates to approximately 22 to 26 weeks gestation in the human, with the second phase of retinal cell generation concluding under normal conditions around 24 weeks.[5,12,13,17,18] There is evidence that some clinicians are prescribing repeated maternal courses of corticosteroid in high-risk pregnancies under 24 weeks gestation where the fetus is at risk of early preterm delivery.[4] Furthermore, repeated courses of systemic corticosteroids are administered to pregnant women with severe asthma, rheumatoid arthritis and systemic lupus erythematosus.[1,2] Some of these steroid hormones, particularly dexamethasone and betamethasone, are capable of crossing the placental barrier and could influence the developing fetus.

Low-birthweight preterm infants are known to be at risk of impaired visual development, particularly if compromised during sensitive periods of development.[9] Infants born preterm, even in the absence of other neurological signs[11] and with normal imaging,[10] are at risk of abnormal visual function and perceptual-motor difficulties including abnormalities of linear acuity and stereopsis.[11] It will be important to investigate visual function in all children who were exposed to repeated prenatal courses of corticosteroid.

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