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Efficacy and Safety of Antenatal Steroids

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52   **[Abstract; Word Count = 253]**

53   **Abstract**

54   Antenatal steroids (ANS) are among the most important and widely utilised interventions to improve

55   outcomes for preterm infants. A significant body of evidence demonstrates improved outcomes in

56   preterm infants (24-34 weeks) delivered between 1 and 7 days after the administration of a single

57   course of antenatal steroids (ANS). Moreover, ANS have the advantage of being widely available, low

58   cost, and easily administered via maternal intramuscular injection.

59

60   The use of ANS to mature the fetal lung is, however, not without contention. Their use in pregnancy

61   is not FDA approved and treatment doses and regimens remain largely un-optimized. Their mode of

62   use varies considerably between countries, and there are lingering concerns regarding the safety of

63   exposing the fetus to high doses of exogenous steroids. A significant proportion of women deliver

64   outside of the 1-7 day therapeutic window after ANS treatment, and this delay may be associated

65   with an increased risk of adverse outcomes for both mother and baby.

66

67   Today, animal-based studies are one means by which key questions of dosing and safety relating to

68   ANS may be resolved, allowing for further refinement/s of this important therapy. Complementary

69   approaches using non-human primates, sheep and rodents have provided invaluable advances to

70   our understanding of how exogenous steroid exposure impacts fetal development. Focussing on

71   these three major model groups, this review highlights the role of three key animal models (sheep,

72   non-human primates, rodents) in the development of antenatal steroid therapy, and provides an up-

73   to-date synthesis of current efforts to refine this therapy in an era of personalised medicine.

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77   [Key Words]

78   **Key Words**

79   Preterm birth

80   Antenatal steroids

81   Animal model

82   Rodent

83   Sheep

84   Primate

85   Fetal maturation

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[Main Text; Word Count = 10,473]

Introduction

Preterm birth, defined by the WHO as delivery before 37 weeks' completed gestation, is a leading cause of neonatal death. Globally, there are in excess of 15 million preterm infants born each year, and of these, around one million will die (13). Rates of prematurity co-localise with social and economic disadvantage, such that the greatest numbers of preterm births occur in parts of Sub-Saharan Africa, South and South East Asia, and the United States of America (13). Prematurity has a substantial socio-economic cost in elevated heathcare costs, and lost economic opportunity for preterm infants and their families (72). The incremental cost per preterm infant surviving to 18 years of age varies substantially with gestational age at delivery; an extremely preterm infant born in 2006 costs, for example, approximately 95,000 UK pounds more than a term-born survivor (72).

Preterm birth is a multifactorial syndrome, making timely diagnosis and effective intervention especially difficult (35, 95). To date, although there have been some successes in identifying and treating women at elevated risk (81), marked reductions in the rate of prematurity remain elusive. As such the development of therapeutics to improve outcomes for preterm infants is of particular importance.

The past few decades have seen significant improvements in outcomes for all but the most extremely preterm infants (103, 104), due to the introduction of sophisticated neonatal ventilation, the use of exogenous surfactant, and the administration of antenatal steroid (ANS) therapy, the focus of the present review.

It is important to note that, when used appropriately, ANS are among the most important and beneficial treatments used in perinatal medicine today. The two glucocorticoids in most common clinical use are dexamethasone and betamethasone. They have similar pharmacokinetic properties

128 and affinities for glucocorticoid receptor binding (52). Dexamethasone is commonly administered as

129 dexamethasone phosphate, 4 x 6mg intramuscular injections spaced by 12 hours. Betamethasone is

130 commonly administered as a combined preparation of betamethasone phosphate and

131 betamethasone acetate, 2 x 12mg intramuscular injections spaced by 24 hours (52). There remains

132 some debate as to which drug offers the best maturational response and optimal safety profile (52).

133 As discussed below, some of the key outstanding questions in relation to ANS therapy include

134 defining the impact of single and multiple ANS doses on acute and long-term fetal development and

135 optimising the choice and dose of drug administered.

136

137 The use of ANS to mature the fetal lung is, however, not without contention. Their use in pregnancy

138 is not FDA approved and treatment doses and regimens remain largely un-optimized. Their mode of

139 use varies considerably between countries, and there are lingering concerns regarding the safety of

140 exposing the fetus to high doses of exogenous steroids. A significant proportion of women deliver

141 outside of the putative 1-7 day therapeutic window after ANS treatment, and this delay may be

142 associated with an increased risk of adverse outcomes for both mother and baby (74). The lack of

143 empirical dose optimisation performed to date makes it difficult to determine whether or not the

144 full therapeutic potential (maximum benefit with minimum side-effects) of this important agent has

145 yet been realised.

146

147 Beginning with a summary of data from clinical studies, this paper will review the contributions of

148 three major classes of animal models, rodents, sheep and non-human primates, to our

149 understanding of how ANS works, and how contemporary questions relating to safety and dose

150 optimisation might be answered.

Clinical Studies

Uncertainties regarding antenatal steroids for fetal lung maturity – evidence from clinical trials

As with any medical treatment, when considering administrating antenatal steroids the benefits of treatment need to be weighed against the potential harms. A further consideration must be the likelihood of preterm delivery - steroids have only been shown clinically to improve neonatal health if delivery occurs within 24 hours to 7 days of administration. The timing of steroids is of key importance when trying to balance the risks and harms (107).

Uncertainties in the Benefits of Antenatal Steroids

In 2017 a Cochrane Review updated the meta-analysis of trials of a single course of antenatal corticosteroids for accelerating fetal lung maturation in women at risk of preterm birth, including 30 trials (29 of which contributed data to outcomes) of 7,774 women and 8,158 babies (93). By meta-analysis, there are many benefits of appropriate antenatal corticosteroid administration, including around one quarter fewer neonatal deaths, one-third fewer cases of respiratory distress syndrome; and around a 50% reduction the number of cases of cerebroventricular haemorrhage (93). There is no doubt that, when given to the right women and babies at the right time, steroids save lives. However, when examining the population, intervention, comparator and outcomes (PICO) of the included trials, uncertainties in this evidence base are evident.

Population

The populations included in trials were often highly selective, and women with complications such as chorioamnionitis (frequently associated with preterm birth) were usually excluded, and important exception being the initial report by Liggins and Howie (66). Interestingly, a subsequent analysis of steroid-treated women with rupture of membranes in the original Auckland Trial failed to demonstrate a reduction in respiratory distress; however, benefit was shown when these data were incorporated into a larger data set comprising 15 controlled trials (37).

177   The majority of women included were at extremely high risk of imminent preterm birth, as

178   illustrated by the observation that in at least half the included studies more than 90% of participants

179   delivered preterm. This does not reflect current clinical practice, and a significant percentage of

180   women treated with ANS do not deliver within the proposed 1-7d optimal treatment window. In a

181   study from the USA, for example, more than half of women given steroids to promote fetal lung

182   maturity remained pregnant 7 days later (69).

183

184   In the single course Cochrane Review (93), the majority of trial participants delivered beyond 28

185   weeks gestation, and there is less evidence about benefits of antenatal steroids at extreme preterm

186   gestations. Half of the 26 trials were carried out in North America, whilst no trials that were

187   performed in low-income countries were included in the meta-analysis. The benefits of antenatal

188   steroids in low and middle-income settings are therefore unclear.

189

190   ***Intervention***

191   Twenty of the 29 trials that contributed data to the Cochrane Review of a single course of steroids

192   (93) used Betamethasone; usually given as Celestone, a mix of 6 mg betamethasone phosphate and

193   6 mg betamethasone acetate with two doses 24 hours apart (total dose 24mg). Celestone, however,

194   is not widely used outside USA, Europe (excluding the UK), Australia and New Zealand. Elsewhere,

195   betamethasone is frequently available only as betamethasone phosphate, and it is 24mg of this that

196   is administered to pregnant women. The distinction is crucial, as betamethasone phosphate and

197   acetate have very different pharmacokinetics. Betamethasone acetate is insoluble, and provides a

198   low and sustained release of betamethasone into the maternal and fetal circulations (53) . In

199   contrast, betamethasone phosphate is soluble and rapidly gives a peak in maternal and fetal plasma,

200   then quickly declines. The benefits (and potential harms) of a 24mg dose of betamethasone

201   phosphate may thus be different to those of Celestone.

202 Only seven of the trials in the Cochrane Review (1) examined dexamethasone (as phosphate), which

203 less expensive and more widely available than Celestone. The dexamethasone therapy

204 recommended by the World Health Organization (WHO) is given as 4, 12 hourly doses of 6 mg.

205 However, other doses and treatment intervals are widespread.

206

207 *Comparator*

208 The comparators in trials included in the single course Cochrane review were essentially standard

209 neonatal care (93). However, 20 of the 29 studies contributing outcome data to the meta-analysis

210 report on babies born more than 20 years ago. Since that time there have been significant changes

211 in neonatal care, not least the introduction of surfactant for the management of Respiratory Distress

212 Syndrome. In high income countries Respiratory Distress Syndrome is no longer the main driver of

213 neonatal mortality in premature neonates (51). The benefits of steroids on the background of

214 modern neonatal care are less certain.

215

216 *Outcome*

217 The age of the trials in the Cochrane Review (93) also impacts interpretation in other ways. In the

218 past 20 years there have been advances in clinical trial design, regulation and definition of core

219 outcomes. In older trials the quality of the trials and the risk of bias are not well documented.

220 Outcomes were variably defined by authors, and many clinically important outcomes were reported

221 only as secondary outcomes, if at all. It is also difficult to be sure that neonatal morbidity and

222 mortality were ascertained in the control group as carefully for the treatment group. Several trials

223 had post-randomization exclusion criteria censoring women not delivered by a certain gestation,

224 thus outcomes in these babies were not reported (93).

225

226 In summary, although the Cochrane Review (93) provides compelling evidence regarding the

227 benefits of ANS, it is important to assess these data in light of the fact that the majority of trials were

228 carried out more than two decades ago in high-income countries, and often in selected groups of

229 women. Importantly, there is very limited outcome data on babies who did not deliver preterm. The

230 degree to which these summary findings can be generalised therefore needs to be carefully

231 considered.

232

233 ***Uncertainties regarding potential harms of steroids***

234 More recently, studies have suggested that antenatal steroids may also cause harm, particularly in

235 babies that do not actually deliver preterm after maternal steroid administration. Balancing these

236 concerns are data from both mid-childhood and adult follow-up studies suggesting that, relative to

237 placebo, prenatal exposure to betamethasone does not alter long-term cognitive function, body size

238 or cardiovascular function, but may result in insulin resistance by 30 years of age (23, 24).

239

240 ***Potential Harms: Evidence from Observational Studies***

241 Indications of potential harms have come from observational studies showing that antenatal steroids

242 are associated with decreased birth length, weight and head circumference in babies (26) and

243 increased cortisol reactivity to acute psychosocial stress at 6- to 11-yr-old (3) in babies born at term

244 when compared to matched cohort controls. Such observational studies are vulnerable to

245 confounding due to intrinsic differences between the two comparison groups. However, evidence

246 from randomized control trials and their associated follow up studies have also shown the potential

247 for harms.

248

249 ***Potential Harms: Evidence from trials of multiple courses of steroids***

250

251 Questions relating to the safety and efficacy of using repeat or 'rescue' courses of ANS are of

252 particular importance given: **i)** the significant number of women receiving ANS who either deliver

253 outside of the proposed 1-7d ANS efficacy window (74); **ii)** the significant number of women

254 receiving ANS who deliver at or close to term (92); **iii)** the significant number of studies reporting

255 adverse fetal programming effects in association with high endogenous or exogenous steroid

256 exposures (53); and **iv)** a number of studies (included those discussed below) that conclude modest

257 treatment benefit in association with potential harm (75).

258

259 The Trial of Multiple Course of Antenatal Steroids (MACS) (78) included 1, 858 women between 25

260 and 32 weeks gestation who remained at high risk of preterm birth after antenatal corticosteroids

261 given 14-21 days previously. Women were randomised to Celestone every two weeks until 33 weeks

262 or delivery (whichever was sooner). The primary outcome was a composite outcome of death or

263 serious neonatal morbidity. There were no improvements in preterm birth outcomes. However,

264 babies in the steroid arm were lighter (2216 g vs 2330g; p=0·0026), shorter (44·5 cm vs 45·4 cm;

265 p<0·001) and had reduced head circumference (31·1 cm vs 31·7 cm; p<0·001) compared to those in

266 the control arm. In a pre-specified analysis in the 5 year follow up study, children in the steroid

267 group who had delivered at term were significantly more likely to have death or

268 neurodevelopmental disability (Odds Ratio [OR] 1.69 [95% CI 1.04-2.77]) and nearly four times as

269 likely to have neurosensory disability, than counterparts in the control group (OR 3.70 [95% CI 1.57-

270 8.75]) (6).

271

272 These results are different from those in another, smaller, trial – the Australian Collaborative Trial of

273 Repeat Doses of Steroids (ACTORDS), where steroids were given weekly to women at high risk (20).

274 ACTORDS did identify a significant reduction in respiratory distress syndrome in neonates born after

275 antenatal steroids (Relative Risk [RR] 0.82 [95% CI 0.71-0.95]). Babies in the steroid treatment group

276 were lighter at birth but not discharge, and follow up studies have been reassuring, although small

277 differences in blood pressure are evident by late childhood (19, 29).

278

279 The different results of the MACs (78) and ACTORDS (20) trials might relate to differences in the

280 proportion of women with 'appropriate' steroid administration.   In ACTORDS 82% of women

281 delivered preterm and, by trial design, all women were delivered within 7 days of study drug

282 administration. Strikingly, in the MACS trial 32% of participants actually delivered at term and three

283 quarters of all participants delivered more than seven days after the last dose of study drug. It is

284 perhaps not surprising then, that in MACS, there were no benefits seen from steroids. Furthermore,

285 the high- proportion of women receiving multiple doses of steroids inappropriately (i.e. without

286 delivering preterm or within 7 days) may have allowed harms to be detected (6, 78).

287 A Cochrane meta-analysis of ten trials (4733 women and 5700 babies) of multiple doses of

288 corticosteroids, performed by the ACTORDS authors reported a statistically significant reduction in

289 respiratory distress syndrome in neonates born after antenatal steroids (RR 0.83[95% CI 0.75-0.91]),

290 but analysis for this outcome did not include data from the MACS trial (21). Results of a pending

291 individual patient data level meta-analysis may help more clearly determine risks and benefits.

292

293 *Potential Harms: Evidence from trials in low resource settings*

294 Althabe *et al* performed a cluster-randomized trial of a strategy to implement antenatal steroid

295 administration versus standard care, in low and middle-income countries – the ACT trial (4). This was

296 performed in 6 countries, and included 99,742 mothers and 100, 705 babies. The aims of the study

297 were to determine the feasibility of scaling up antenatal corticosteroids at all levels of care in low

298 and middle income countries and determine the effectiveness and safety of antenatal steroids in

299 these settings. The intervention was the introduction of tools aimed to help identify those at risk of

300 preterm birth (because of signs of preterm labour, preterm prelabour rupture of membranes,

301 bleeding or raised blood pressure,) and dexamethasone 24 mg in 4 divided doses given to those at

302 risk, along with recommendations to refer to hospital for delivery. The intervention increased

303 coverage of antenatal steroids from 10% to 45%. However, it is striking that only 16% of women

304 treated with antenatal steroids gave birth to a preterm baby (defined as birthweight less-than-5th-

305   percentile, as ultrasound dating was generally not available). In preterm babies there were no

306   benefits of antenatal steroids. In the whole population there was an increase in neonatal mortality

307   (RR 1.12, 95% CI 1.02–1.22, p=0.013) and stillbirth (RR 1.11, 95% CI 1.02–1.22, p=0.018), as well as

308   an increase in suspected maternal infection (OR 1.45, 95% CI 1.33–1.58, p<0.0001). The increase in

309   mortality was evident only in babies >25th weight centile, indicating the increase in mortality was

310   predominantly driven by babies born at term (4).

311

312   ***Evidence from other trials***

313   Another trial suggests potential neurodevelopmental harms of steroids, if steroids are administered

314   at term. Stutchfield *et al* performed an open label trial to determine if Betamethasone 24mg in two

315   divided doses, improved outcomes in 998 women having elective caesarean section at 37 weeks or

316   beyond, compared to standard care (105). Contrary to current recommendations whereby elective

317   deliveries should only be undertaken at 39 weeks or beyond, in this study 55% of participants had

318   elective caesarean delivery before 39 weeks gestation. There were no overall differences in neonatal

319   unit admission between the two groups, although there was a reduction in admission to neonatal

320   unit with respiratory distress (mainly driven by a reduction in transient tachypnea of the newborn –

321   generally a benign and self-limiting condition). A subset of these babies were followed up, and at

322   school age children exposed to steroids before elective delivery at term were twice as likely to be in

323   the bottom quartile of academic ability than children who did not receive steroids (8.5% [14/190] vs

324   17.7% [33/217]; p0.03) (106).

325

326

327   ***Recommendations and the timing of steroids***

328   In 2015 the World Health Organization made recommendations on interventions to improve

329   preterm birth outcomes (18). These included the recommendation that antenatal steroid therapy

330   should be given to women at risk of preterm birth from 24 weeks to 34 weeks of gestation, but only

331   when gestational age can be accurately assessed; there is no clinical evidence of maternal infection;

332   adequate childbirth care is available; the preterm newborn can receive adequate care if needed; and

333   preterm birth is considered imminent. The guidelines also suggest that a single rescue dose of

334   antenatal corticosteroids should be considered in women who remain at imminent risk of preterm

335   birth seven days or more after the initial dose (18).

336

337   Imminent delivery is an important ANS effect modifier; an updated meta-analysis identified a

338   reduction in neonatal mortality only if delivery occurred within 24 - 48 hours of antenatal steroid

339   therapy (Neonatal mortality RR was 0.6 [95% CI 0.39-0.94] if steroids were given within 24 hours of

340   delivery; neonatal mortality RR was 0.59 (0.41-0.86) if steroids were given within 48 hours of

341   delivery) (18). Respiratory Distress Syndrome was decreased for up to 7 days after steroid

342   administration, but there was no reduction in neonatal mortality after 48 hours. This suggests that

343   the obstetric guidelines for appropriate steroids being given 48 hours to 7 days before delivery may

344   be outdated because RDS is now much less likely to cause death. Instead, clinicians should be aiming

345   to give steroids only if imminent preterm delivery is very likely, but unfortunately estimating the

346   time of delivery is not precise (2,3).

347

348   A clinical trial evaluating the potential benefits of steroids in late preterm delivery that was

349   published subsequent to the WHO guidance illustrates the importance of appropriate timing of

350   steroids (36). Gyamfi-Bannerman *et al* randomised 2,831 women with singleton pregnancy at 34+0

351   to 36+5 weeks gestation at high risk for delivery to Celestone or placebo in tertiary centres in USA

352   (36). The primary outcome was a neonatal composite outcome of treatment in the first 72 hours of

353   life, and there were short-term benefits of steroids in these late preterm infants, with a significant

354   reduction in respiratory morbidity (14.4% in control group vs 11.6% in steroid group; p=0.023).

355   Women were only included in the trial if they had a high probability of delivery, defined as either

356   planned medically indicated delivery, or preterm labor with intact membranes and at least 3 cm

357   dilation or 75% cervical effacement, or spontaneous rupture of the membranes. An exclusion

358   criterion was cervical dilation of 8cm or more, suggesting that many of these women were actually

359   in labour at the time of treatment. Indeed 40% delivered within 24 hours (before the second dose of

360   Celestone could be given) and more than 85% delivered within 7 days. The benefits within 24 hours

361   of administration support the findings in the WHO recommendations (18). It is important that these

362   results should not be extrapolated to women at lower risk of imminent delivery. Indeed, there were

363   harms, even in this group with near 'optimal' steroid administration, with a significant increase in

364   hypoglycemia in the steroid treated group (15% in control group vs 24% in steroid group; p<0.001).

365

366   As noted above, there is clear evidence to show that, when administered to the right women and

367   babies at the right time, steroids save lives, reduce disease, and convey relative little risk. However it

368   is also clear that the appropriate selection of women at risk of preterm labour, which remains

369   something of a difficult challenge in many cases, plays a key role in determining treatment

370   outcomes. Contemporary trends in the use of antenatal steroids include the administration of

371   multiple courses, an increase in the range of gestations (notably late gestation) at which these

372   agents are used, and substantial regional differences in the agents, doses and dosing schedules

373   employed. Given the clear importance of treating 'the right women at the right time', we suggest

374   that adapting the use of antenatal steroids away from the original single-course evidence base (for

375   which the data showing benefit is strongest) should be done with substantial caution. In addition,

376   further research attention should be devoted to understanding the optimal use of ANS in

377   complicated pregnancies, such as those involving growth restricted fetuses, diabetes, multiple

378   gestations, and where there is a risk of hypoxia-ischaemia.

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385 **Animal Models**

386 The demonstrated efficacy of ANS therapy relative to placebo is also arguably the largest

387 impediment to the further refinement of this important therapy, most notably from the perspective

388 of exploring ANS dosing. Well-designed human studies remain the gold standard for assessing ANS

389 treatment efficacy and safety, both from an acute and long-term outcome perspective. However

390 with overall benefit now well established, ethical considerations rightly limit an investigator's ability

391 to conduct, for example, the controlled studies necessary to optimise (especially via reduced dosing)

392 the ANS dose-response relationship. Moreover, the availability of well controlled human tissues is

393 extremely limited, making molecular and structural analyses of ANS effects difficult in human

394 populations. Well-designed animal studies thus offer the best means by which ANS therapy can be

395 further refined, by providing the data necessary to justify subsequent human studies of lowered

396 dosing or altered dosing schedules.

397

398 One may quite reasonably argue that the observed delay in the adoption of ANS therapy after the

399 first clinical trials may be partly attributable to data from animal studies demonstrating that

400 antenatal exposure to exogenous glucocorticoids caused adverse changes in fetal development. From

401 another perspective, however, one may also argue that such data should clearly have been

402 interepreted with reference to the animal model from which it was derived, the nature of ANS

403 exposure used, and the clinical need at the time. Moreover, it is entirely reasonable to suggest that

404 had a comprehensive battery of dose-response, toxicity and outcome studies been performed in

405 appropriate animal models in the mid-1970's, the uptake of ANS therapy may have proceeded much

406 more rapidly, and in a far more uniform manner. Indeed, such studies would have been entirely in

407 keeping with additional work proposed by Liggins and Howie in their conclusion of their landmark

408 clinical study report (66).

409

17

410 Studies that allow a better understanding of the cellular and molecular processes that underpin the

411 milleiu of fetal effects caused by ANS have, and will, remain an important focus of animal-based

412 research. However, from a translational perspective, animal studies that address outstanding

413 questions in relation to ANS dose optimisation, with specific reference to optimal dose, dosing

414 regimen (rather than solely characterising positive or negative effects *per se*) using clinically relevant

415 doses of steroids seem at this point in time to be of particular importance.

416

417 The following sections explore the use of rodent, sheep and non-human primate models of

418 pregnancy in advancing our understanding of the effect of steroid exposure on the developing fetus.

419 It is important to note that the studies included for discussion below are intended to provide the

420 reader with an introduction to the use of each animal model in attempts to better understand the

421 mode of ANS action in pregnancy. Given the size of the field, it is not possible to include all studies of

422 importance in a particular model system, or all models that have been used to address a particular

423 function of ANS therapy (e.g. fetal growth programming). In the clinical study section above, both

424 strengths and weaknesses of particular clinical trials were highlighted to assist in the interpretation

425 of those data.   For animal-based studies, the ARRIVE reporting guidelines provide a useful

426 standardisation framework for interpreting the results of *in vivo* studies, and may ultimately assist in

427 the application of meta-analyses similar to those commonly performed for the findings of clinical

428 studies (57). One such attempt at performing a systematic review of animal studies evaluating

429 antenatal corticosteroids to prevent neonatal respiratory distress syndrome was performed by Perel

430 and colleagues, which reported respiratory distress syndrome was reduced in the three studies

431 selected for inclusion (86). Although somewhat limited in scope with regards ANS studies in animals,

432 this report does highlight significant variation in experimental objectives between studies, and

433 adherence to experimental guidelines (such as those described in the ARRIVE reporting guidelines),

434 and consequently in the potential for the introduction of reporting bias. On this basis, it seems

435 reasonable to conclude that greater transparency in animal study randomisation, blinding and

436 reporting procedures (e.g. establishing *a priori* a justification schema for omitting subjects) would be

437 beneficial to both critical review of publications and the potential translation of research findings. To

438 this end, our treatment of individual studies will include, as appropriate, reference to the doses of

439 steroids administered, treatment intervals, and the breeds of animals used.

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456 **Rodent Models**

457 *Model Considerations*

458 Given their size, ease of housing, rapid sexual maturation, brief gestation and large litter sizes,

459 rodent models continue to be of great use for studies of steroid effects on the fetus and newborn.

460 Accordingly, a number of studies have focussed on ANS-driven alterations in the fetal growth, dose

461   timing, placental effects, and comparative analyses of betamethasone and dexamethasone efficacy

462   and safety. Studies in rats, in particular, have provided insights into the effects of ANS exposure on

463   the developing fetal brain. Mouse models have contributed to our understanding of the molecular

464   events causing fetal lung development, and the impact exerted by exogenous glucocorticoid

465   exposure on this intricately controlled developmental process. The subject of several excellent

466   reviews, total and selective gene knockout mouse models have also been central to allowing

467   researchers to perform molecular and spatial dissection of key developmental signalling pathways,

468   including the responses to glucocorticoid-driven activation of the glucocorticoid receptor (25).

469

470   Before moving to discuss a selection of the rodent-based studies that underpin our understanding of

471   how exogenous ANS exposure impacts the fetus, it is useful to touch briefly on some elements of

472   rodent reproduction that are pertinent to the translation of these data to human fetal development.

473   Mice (*Mus musculus*) reach sexual maturity at approximately 6-7 weeks and have large litters,

474   generally 4-15 pups, with gestation being a comparatively brief 19-21 days (term is 37 weeks'

475   completed gestation in humans). Rats (*Rattus norvegicus*) have a similar reproductive cycle to mice;

476   puberty occurs at approximately 60 postnatal days and rats have between 6 and 12 pups with

477   gestation lasting between 21 and 23 days (114).

478

479   Due to the brief gestations, modelling either short-term or chronic fetal ANS exposure is difficult in

480   mice and rats. At 80% gestation, the human fetus gains around 1.6% of total weight every day; in

481   contrast, fetal mice have a 97% day-on-day increase in weight and rats a 65% increase at the same

482   gestations. Given this difference, the rodent fetus will likely be more susceptible to the growth-

483   restrictive effects of ANS therapy than the slower developing human fetus. Moreover, it is important

484   to note that given their comparatively brief pregnancies, the relative duration of materno-fetal ANS

485   exposure in rodent-based studies will be significantly greater than in sheep, non-human primates, or

486   humans.

487

488 The murine placenta has a haemotrichorial structure, with three trophoblast layers separating the

489 maternal and fetal circulations, in contrast to the haemomonochorial human placenta (28). There

490 are also significant differences between human and murine placenta with regards villous

491 architecture and endocrine function, potentially translating into differences in trans-placental

492 steroid trafficking (71). There are also marked differences in maturation between rats, mice and

493 humans that should be taken into account. For example, development of the limbic system in the rat

494 brain occurs much later than in humans, with a significant proportion of neuroendocrine system

495 development occurring in the postnatal period (5). The sensitivity of the rodent GR to glucocorticoid

496 stimulation is also much higher than in primates (5).

497

498 Alveolarization in humans commences at approximately 34 weeks' gestation and is reasonably

499 advanced at term; in mice and rats, however, alveolarization predominantly occurs in the post-natal

500 period. In mice, the alveoli are around 80 μm mean linear intercept (MLI), making them significantly

501 smaller than in the rat (100 μm MLI) or human (210 μm MLI) (42).   Given the significant

502 developmental differences between species, these factors need to be taken into account when

503 assessing the implications of animal data on human therapeutic outcomes.

504

505 Lastly, data from rodent models of ANS should be interpreted with reference to the strain of animal

506 used, as different laboratory rodents appear to possess pronounced differences in steroid

507 responsiveness. For example, Ersek and colleagues reported that, when treated with prednisolone,

508 adult CD-1 mice had significant reductions in femur strength and elasticity; in contrast C57BL/6J mice

509 exhibited no changes in femur strength or elasticity after receiving the same treatment, suggesting

510 that C57BL/6J osteoclasts were less sensitive to glucocorticoid treatment and were tolerant of

511 higher steroid doses (32). In contrast, C57BL/6J mice were more sensitive to inhibition of

512 hippocampal cell proliferation than MRL/MpJ mice when chronically treated with corticosterone

513 exposure (38). For brevity, strains of mice used in the studies introduced below are contained in

514 Table 1.

515

516 *Rodent Responses to Antenatal Steroid Treatments*

517 Several groups used mouse models to study the impact of exogenous glucocorticoid exposure on the

518 developing fetus. Stewart and co-workers used betamethasone or placebo (saline) to investigate the

519 effect of two (0.2 mg once on gestational day 14), four (0.1 mg once daily on gestational days 13 -16

520 or twice daily on days 14 and 15) or eight (0.1 mg twice daily on gestational days 13 to 16) antenatal

521 doses of glucocorticoids (102). Pregnancy outcomes, growth, locomotor responses and reproductive

522 function were assessed from the early perinatal period to post-natal day 120. Functional

523 development tasks were also assessed.

524

525 In keeping with earlier studies in multiple species, the highest dose of ANS treatment was associated

526 with transient intrauterine growth restriction that resolved by post-natal day 7, and characterised by

527 lower birth weight, smaller length and narrower head width. The authors reported a negative linear

528 relationship between the number of betamethasone doses and the number of live pups per litter,

529 with male pups disproportionately affected at all but the highest dose. No persistent differences in

530 geotaxis task performance, locomotor tests, or reproductive capability were identified between

531 treatment groups and placebo control (102). Keeping in mind the particularly high doses used in this

532 study, these findings are in keeping with follow-up data from the MACS study, which associated

533 repeated ANS exposure with reduced birth weight, birth length and head circumference (78). These

534 data are also in keeping with the clinical observation that ANS-associated reductions in preterm

535 human birthweights are offset by accelerated neonatal growth (11).

536

537 Rayburn and colleagues investigated the potential developmental effects of a single dose of

538 antenatal betamethasone or dexamethasone at 14 days' gestation (91). Dams received a single,

539   intraperitoneal treatment of either betamethasone acetate (0.1mg), dexamethasone sodium

540   phosphate (0.1mg) or placebo (0.9% NaCl). Litters were standardised to eight pups (equal sex

541   distribution) on post-natal day 4, and a battery of neuro-behavioural tests were performed at

542   multiple ages between perinatal to young adult (maximum 135 days) life. Dexamethasone exposure

543   induced a transient developmental delay, although steroid exposure did not alter key motor,

544   learning, or sensory test results. Although there was no difference in pregnancy characteristics, the

545   authors concluded that (p.842) "subtle differences in offspring performances of neurobehavioral

546   development tasks favoured antenatal betamethasone rather than dexamethasone". No

547   pharmacokinetic data were presented in this study; however, given the steroid preparations used, it

548   is likely that fetal Cmax in the dexamethasone phosphate group was significantly higher, and

549   maximum maternal and fetal drug levels were reached much more rapidly compared with animals

550   treated with betamethasone acetate. As noted by the authors, the subtle differences in anxiety and

551   memory identified in the study may have derived from pharmacokinetic differences, rather than

552   functional differences between the two corticosteroids (91). A subsequent study by Christensen *et*

553   *al* with the offspring of repeat course betamethasone acetate and phosphate-treated mice showed

554   no difference in long-term cognitive outcomes compared to placebo (saline)-treated mice (17).

555

556   Ozdemir *et al* used a pregnant mice (n=30/group) at 14 days of gestation in a placebo-controlled

557   study to investigate the *in utero* impact of single (one dose on day 14) and repeated (two doses on

558   days 14 and 15) exposure to either 0.1mg betamethasone (as Celestone Chronodose), 0.1 mg

559   dexamethasone, or 0.25mL NaCl delivered by subcutaneous injection (85).   A breathing score

560   analysis conducted at day 16.5 showed that a single dose of betamethasone was associated with

561   improved breathing compared with a single dose of dexamethasone; a similar finding was evident

562   when comparing repeat treatment groups. In their repeated dosing experiment, Ozdemir and

563   colleagues did not detect a sex-difference in pup survival with multiple doses, but did report a lower

564   live litter size in the repeated dexamethasone group. Repeated steroid treatments were associated

565   with the greatest reduction in lung weight, with the effect most pronounced in dexamethasone-

566   treated animals. Lung, liver and body weight variables for male pups were significantly reduced in

567   the repeated steroid groups (both betamethasone and dexamethasone) relative to saline control,

568   with the most pronounced differences with dexamethasone-exposed animals. Again, although no

569   pharmacokinetic data were presented, it is worth noting that dexamethasone-group animals were

570   likely exposed to higher feto-maternal plasma steroid levels of a shorter duration than those

571   achieved with betamethasone (combined acetate and phosphate preparation), which is in keeping

572   with the reported lower breathing scores and greater growth resctriction in the dexamethasone

573   groups (85).

574

575   Given the importance of the placenta, the impact of exogenous steroid exposure on placental

576   development and function, the impact of changes on fetal growth is of particular interest, especially

577   in ANS-treated pregnancies that continue to term. Of note is work by Petropoulos and co-workers,

578   who demonstrated that repeated (daily between gestational days 9.5 and 15.5) intraperitoneal

579   injections of 1 mg/kg dexamethasone significantly reduced placental weight at gestational day 15.5

580   (but not at day 18.5) and fetal body weight at both day 15.5 and 18.5 (87). Interestingly, although an

581   identical dosing regimen of 0.1 mg/kg was associated a 22 % reduction in pup weight on gestational

582   day 15.5, there was no difference relative to control at day 18.5, nor was there any difference in

583   placental weight at day 15.5. (87).Baisden and colleagues administered either saline or 0.5mg/kg

584   dexamethasone via intraperitoneal injection on gestational days 15, 16 and 17 (n=9/group).

585   Compared to saline-exposed placental tissue harvested at gestational day 20, dexamethasone

586   exposure was associated with reduced placental weight, pronounced trophoblast apoptosis (likely

587   mediated by an observed increased in caspase 1 and 3 expression) and reduced fetal weight (8).

588

589   In addition to sex-linked observations by Stewart and Odzemir (85, 102), a number of murine

590   studies, including those of Cuffe (22) and O'Connell (83, 84) investigated sex-specific changes in

591   placental structure (i.e. branching morphology) and function following exogenous antenatal

592   glucocorticoid exposure. Cuffe and colleagues showed that at embryonic day 14.5, dexamethasone

593   infusions (1µg/kg/h) were associated with reduced pup weight in male and female fetuses, but

594   reduced placental weight in female fetuses only, with decreased mitogen-activated protein kinase 1

595   expression implicated as a contributing factor. In animals examined at day 17.5, there were no

596   difffernces in fetal or placental weights between groups, and differences in mitogen-activated

597   protein kinase expression had resolved (22).

598

599   Functional studies in the spiny mouse (which has an extended gestation of approximately 40 days)

600   revealed that dexamethasone exposure influenced placental glycogen storage in a sex-dependant

601   manner, with reduced histologically-detectable glycogen storage in females only, despite

602   perturbation of glycogen regulatory networks in placentas of both sexes (84). One set of studies

603   investigating glucocorticoid-driven changes in placental function focussed on the impact of

604   dexamethasone on placental system-A mediated amino acid transfer (7). Reduced system-A function

605   is associated with fetal growth restriction(43). Mice were given subcutaneous injections of either

606   0.1mg dexamethasone or saline. In these experiments, antenatal dexamethasone exposure did not

607   alter system-A function acutely (i.e. 24 h or 72 h post-treatment), but did result in a significant

608   functional reduction immediately before term, at 18.5 days. The authors did not identify a reduction

609   in fetal growth in association with a 40-50% reduction in system-A function, although placental

610   weight was reduced relative to control in female fetuses at day 18.5. The observed lack of changes in

611   fetal weight may be due, as the authors note, to the changes in amino acid transport occurring too

612   close to delivery to have a meaningful effect. More recent work by Vaughn *et al.* (110) suggested

613   that dexamethasone-induced changes in placental amino acid transport activity may be transmitted

614   between generations. Interestingly, the authors observed that these changes in F_2 placetal samples

615   were only seen when the F_0 treatment occurred close to term.   Work by Moisiadis and *et al.* in

616   guinea pigs showed that ANS caused transgenerational changes in programming (prefrontal cortex

617   and hypothalamic paraventricular nucleus gene expression) to F_3 (77).

618

619   The impact of excessive glucocorticoid exposure on the developing fetal brain is of particular interest

620   from a therapeutic safety perspective and has been the subject of a large body of research over

621   several decades (for in-depth review the reader is referred to analyses by Maleeb and

622   Stonestreet(70)). A number of rodent-based studies have contributed to our understanding of the

623   impact of ANS exposure on the developing fetal brain; a high level of fetal dexamethasone exposure

624   from repeated antenatal dosing (0.1 mg/kg daily from embryonic day 11-17) was associated with

625   regional alterations in the integrity of the blood brain barrier (increased competency in the cortex,

626   decreased in the paraventricular nucleus), and reductions in blood vessel length (13% reduction

627   relative to control) and density in the hypothalamic paraventricular nucleus (34). Similarly, Neuhaus

628   *et al.* showed that repeated administration of dexamethasone (0.1 mg/kg on embryonic days 15, 16,

629   and 17) was associated with alterations in the expression of key blood brain barrier and angiogenesis

630   markers in pups at post-natal day 4 (79).

631

632   Work by Maturana showed thathigh levels of dexamethasone exposure (0.5 mg/kg daily for the final

633   8 days of gestation) resulted in the upregulated expression of inflammasome components and

634   cytokines including interleukin 1-β and tumour necrosis factor-α in hippocampal oligodendrocytes -

635   a pro-inflammatory state the authors propose may have an involvement with demyelinating

636   diseases (73). Similarly, alterations in central nervous system myelination (30) and changes in

637   peripheral nerve development (90) have been identified in sheep models of repeated fetal ANS

638   exposure. A single dose of dexamethasone phosphate (0.4 mg/kg) given at gestational day 15.5

639   resulted in transient changes in fetal hippocampal volume, apoptosis and proliferation, with the

640   observed reduction in proliferating cells persisting into adulthood. Mean body weight changes

641   identified at post-natal day 10 and 20 also resolved by adulthood (82). Slotkin *et al.* (101)

642   demonstrated rapid (within 1-4 hours) changes in the expression of fetal brain nuclear transcription

643   factors after a single low-dose (0.05 mg/kg) treatment in rats. Using rats treated with either 0.2

644   mg/kg or 0.8 mg/kg dexamethasone phosphate on gestational days 17, 18 and 19, Carlos and

645   colleagues (16) demonstrated a region specific impact on brain development characterised by

646   alterations in cell acquisition rather than regional weight. Of note was the observation that areas of

647   the brain undergoing active mitosis appeared to be the most vulnerable to lasting developmental

648   abnormalities, and occurred in a dose-dependent manner (16).

649

650   In summary, there is good experimental evidence that antenatal steroid exposure exerts an impact

651   on many areas of fetal growth in rodents. One of the recurrent, and perhaps most important of the

652   themes that emerges from an assessment of rodent ANS studies is the existence of a dose-

653   dependent association between the scope and magnitude of adverse outcomes - most notably in

654   alterations to fetal growth profile. Although data suggest that conservative or low-level doses of ANS

655   exposure are to be associated with shorter acting, less pronounced deviations in growth, it is also

656   worth noting that even very low-levels of steroid exposure elicit changes in key organs such as the

657   rodent brain. This observation is pertinent given a trend towards administering antenatal steroids to

658   previable pregnancies, for late preterm birth (36), and recent data showing that in infants born at 34

659   weeks' gestation the number need to treat with ANS to prevent one death before discharge is 798

660   pregnancies (107).

661   In assessing how to translate data from rodent studies to the use of ANS in humans, it is important

662   to keep in mind a number of the interspecies differences in fetal development discussed above -

663   especially with regards the ontogeny of organ development and the duration of steroid exposure

664   relative to total gestation length. Moreover, readers should note that several of the studies

665   discussed above administered supra-clinical doses of steroids (e.g. Rayburn and colleagues (91) used

666   a standard dose of 0.1 mg dexamethasone sodium phosphate; at a reported 2 mg/kg estimated

667   dose, this weight-normalised exposure is in excess of a 24 mg standard course of Celestone

Chronodose delivered, for example, over 48 hours to a 60kg woman). Moreover, these studies further reinforce the need for ANS dosing optimisation, to determine the minimum level and minimum duration of materno-fetal antenatal steroid exposure required to functionally mature the preterm lung.

Lead Investigator	Model	Strain	Agent	Reference
Stewart	Mouse	CD-1	Betamethasone	102
Rayburn	Mouse	CD-1	Betamethasone or Dexamethasone	91
Christensen	Mouse	CD-1	Betamethasone	17
Ozdemir	Mouse	Swiss Albino	Betamethasone or Dexamethasone	85
Petropolous	Mouse	FVB	Dexamethasone	87
Baisden	Mouse	C57/BL6	Dexamethasone	8
Cuffe	Mouse	C57/BL6	Dexamethasone	22
O'Connell	Mouse	Spiny	Dexamethasone	83
Audette	Mouse	C57/BL6	Dexamethasone	7
Vaughn	Mouse	C57/BL6/J	Dexamethasone	110
Frahm	Mouse	FVB/N	Dexamethasone	34
Neuhaus	Mouse	C57BL6/JRccHsd	Dexamethasone	79
Maturana	Mouse	C57/BL6	Dexamethasone	73
Noorlander	Mouse	C57/BL6-JIco	Dexamethasone	82
Slotkin	Rat	Sprague-Dawley	Dexamethasone	101
Carlos	Rat	Sprague-Dawley	Dexamethasone	16
Moisiadis	Guniea Pig	N/A	Betamethasone	77

Table 1. *Summary of rodent strains and antenatal steroids used*

Sheep Models

Contributions of Sheep Models to ANS

As reviewed by Longo (68), sheep models have been critical for the field of fetal physiology notably developed by Barcroft and Dawes. Subsequently Meschia and Barron described fetal catheterization in 1965 to study the undisturbed fetus for the first time (76). Adams and colleagues at UCLA measured surfactant in the fetal lung fluid of sheep in 1963 (2). Brumley and colleagues reported surfactant characterizations of fetal sheep lungs in 1967 (14). These early reports set the stage for

682 Liggins seminal observation in 1969 that prolonged infusions of dexamethasone in catheterized fetal

683 sheep caused premature delivery and the preterm lambs unexpectedly breathed and had aerated

684 lungs (65). That observation was quickly operationalized to a randomized clinical trial demonstrating

685 that maternal treatments with a mixture of betamethasone acetate and betamethasone phosphate

686 decreased respiratory distress syndrome and death in preterm infants (66). That trial stimulated

687 research using sheep models that to this day are being used to better understand the effects of

688 maturational mediators on the fetus. The sheep model was ideal for asking translational research

689 questions because of the gestational length, the accessibility of a single fetus for catheterization, and

690 a fetal size similar to the human to facilitate delivery studies using standard clinical techniques.

691 Further, observations in sheep models to test lung maturation have generally translated well to the

692 human.

693

694 ***Timing of ANS responses***

695 A clinically important question was how long a period was required for effects of ANS on the fetal

696 lung to be sufficient to decrease RDS. A difficulty imposed by the sheep model was that preterm

697 delivery caused by ANS could prematurely terminate the experiment. Platzer and colleagues in San

698 Francisco in 1975 infused fetal sheep with dexamethasone and measured no changes in fetal lung

699 fluid flow but an increase in surfactant content 1.5 to 4 days after initiation of the steroid infusion

700 (88). Thus, a ANS effect on surfactant could be relatively rapid. A subsequent report by Kitterman

701 and colleagues demonstrated that the normal increase in cortisol in the fetal sheep correlated with

702 biochemical and structural indicators of lung maturation, indicating that endogenous cortisol was a

703 hormone regulating normal lung maturation (58).

704

705 While ANS treatments for women at risk of preterm labor are given as IM injections of fluorinated

706 steroids that cross the placenta, much of the research with fetal sheep has used infusions or

707 ultrasound directed fetal IM injections. Single dose fetal IM treatments with 0.5 or 2 mg/kg

708   Celestone 24 hr. or 48 hr. prior to preterm delivery improved lung function as assessed by gas

709   exchange and compliance assessed with mechanical ventilation (48). There were no effects for a

710   lower dose of 0.1 mg/kg or with a single IM dose of 12.5 mg/kg cortisol. A subsequent experiment

711   evaluated treatment to delivery intervals of 8, 15, and 24 hrs. (40). The fetal dose of Celestone

712   increased blood pressure and decreased albumin recovery in the fetal lungs but had no effect on

713   lung function at 8hr. After the 15-hr. treatment to delivery interval, the newborn also had high blood

714   pressure and decreased albumin recovery in lung tissue and alveolar wash plus improved lung

715   function comparable to 24 hr. or 48 hr. ANS exposure. There was no increase in surfactant amount

716   at 8 or 24 hrs. The fetal sheep lung had a large improvement in function without an increase in

717   surfactant, an effect explained in part by rapid decreases in mesenchyme that increased airspace

718   volumes (40).

719

720   ***Other Maturational Agents and ANS***

721   Multiple other hormones and drugs such as T_3, T_4, TRH, epidermal growth factor and beta – agonists

722   were tested in rodent and *in vitro* systems for their potential as maturational agents, often with

723   positive results. These mediators were evaluated in fetal sheep models by Liggins, *et al.* (67) using

724   infusion experiments where cortisone infusions alone had minimal effects. T3, epinephrine,

725   epidermal growth factor, or prolactin, and combinations of cortisol with epinephrine, T_3 or cortisone

726   plus T_3 and prolactin increased lung gas volumes as measured by pressure volumes curves and

727   increased surfactant in alveolar washes. T3 and T4 were also evaluated in combination with fetal IM

728   Celestone (89). The ANS effect was augmented by T_4 but not T_3 after 48 hr. exposures. The thyroid

729   hormones and other agents do not cross the placenta, but TRH does and will increase thyroid

730   hormones in the fetus. Schellenberg and colleagues reported that 84 hr. cortisol infusions had no

731   effect on alveolar surfactant or lung gas volumes (96). However, the combination of TRH and cortisol

732   greatly increased both indicators of lung maturation. Using a similar 60 hr. cortisol infusion model,

733   Ikegami and colleagues (41) reported no effects of TRH alone and comparable increases in postnatal

734 lung function assessed by ventilation for the cortisol and cortisol + TRH groups. Thus, the effects of

735 TRH on cortisol exposed fetal sheep lung were inconsistent in the reports. When tested with a large

736 clinical trial, maternal TRH plus ANS were not superior to ANS alone (10). The enticing possibility that

737 fetal exposure to multiple agents could improve lung function in preterm infants has not worked in

738 practice. Research with sheep models helped frame this research agenda.

739

740 ***Chorioamnionitis and ANS***

741 Chorioamnionitis and its surrogate preterm rupture of membranes is associated with perhaps 50% of

742 preterm deliveries at <30 weeks gestational age(95). Conversely, in high resource environments, 80

743 to 90% of preterm deliveries are exposed to ANS (113). Thus, it is clinically important to evaluate

744 these two exposures, which has been done primarily in sheep models, with additional studies in rat

745 models of chorioamnionitis. Lee and colleagues, for example, compared in rats the effect of

746 intraamniotic LPS (1.0 μg/sac) on bronchoalveolar lavage neutrophil counts with and without co-

747 administration of two maternal intramuscular betamethasone injections (170 μg/kg) at gestational

748 day 20 (64). The authors reported a significant reduction of lavage neutrophil counts in dual LPS- and

749 betamethasone-treated animals 2 days post treatment. Interestingly, only animals exposed

750 antenatally to both LPS and betamethasone had significant disruption in alveolarisation at days 5

751 and 14 post-treatment. Fetal sheep exposed by intraamniotic injection to LPS, interleukin 1, or live

752 *Ureaplasma* spp. develop chorioamnionitis and lung inflammation that resolves with striking lung

753 maturation (80). The inflammation-exposed fetal lamb lung has and additive maturation response to

754 ANS given either after or simultaneously with the inflammatory mediator (63). Although clearly not a

755 viable therapeutic option, it is interesting to note that the maturational effects of LPS or IL-1b are

756 more consistent and larger than ANS. The dose of LPS to achieve a maximal lung maturational effect

757 is >1 mg given by intraamniotic injection, and remarkably, intraamniotic doses to up 100 mg cause

758 lung maturation (60). LPS is a sterile pro-inflammatory agent that does not replicate a number of

759 features inherent to infection-driven chorioamnionitis (i.e. dynamic growth of microorganisms,

760 tissue invasion, multiple agonist stimulation) . ANS exposure causes growth restriction in fetal sheep

761 while LPS does not. The mechanism underlying these different responses remains unclear, but may

762 related to steroid-induced changes in placental blood flow (44). Interestingly. a simultaneous fetal

763 exposure to ANS and intraamniotic LPS blocks the ANS mediated growth restriction, a result that

764 remains enigmatic (80).

765

766 ***ANS and Postnatal Surfactant***

767 Another clinically relevant interaction is fetal exposure to ANS followed by surfactant treatment

768 after delivery. In an observational study, infants exposed to ANS and then treated with surfactant

769 after delivery had better lung function, fewer complications and lower mortality than either

770 treatment alone (45). This association has not been formally tested in a clinical RCT because

771 standard of care is to give ANS and then selectively treat with surfactant for respiratory failure. The

772 fetal sheep model has proved useful for exploring this interaction. Following delivery of fetal sheep

773 exposed to ANS, surfactant treatments work better than for animals not exposed to ANS (108). The

774 beneficial interactions result from multiple effects: the ANS exposed lungs are less leaky, the lungs

775 will hold more gas, the ventilated lung inactivates the surfactant less rapidly, there is an intrinsic

776 improvement in surfactant function, and the dose-response curve for surfactant is improved. Thus,

777 based on sheep models, ANS "primes" the preterm lung to have favourable responses to surfactant

778 treatment.

779

780 ***Drugs and doses for ANS***

781 As noted in the clinical review above, there are substantial gaps in knowledge as to which steroid

782 should be used at what dose, because ANS were developed by investigators without input from

783 Pharma or regulatory agencies. Liggins selected the mixture of betamethasone phosphate and

784 betamethasone acetate (Celestone) to achieve a prolonged fetal exposure from the slowly

785 deacylated betamethasone acetate (66). The other recommended therapy by the World Health

786   Organization (WHO) is a 4-dose treatment at 12 hr. intervals with 6 mg dexamethasone phosphate.

787   These therapies are not equivalent, and both drugs and other drugs are used worldwide without

788   standardization. The body of sheep research to support treatment strategies is confounded by

789   combinations of maternal and fetal treatments and treatments given by infusion or by IM injection

790   to the mother or fetus. An example of the different outcomes by route of injection is our finding that

791   fetal IM Celestone causes less lung maturation than a maternal IM treatment. Maternal treatment

792   results in much lower fetal plasma levels of betamethasone (46). Surprisingly, the maternal dosing

793   caused fetal growth restriction while the high dose exposure from a fetal treatment did not. The

794   results suggest that there are presently unknown pharmacokinetics or mechanisms of action of ANS

795   that explain the effects of route of treatment.

796

797   The dosing schedules for ANS should expose the fetus to the lowest effective doses to minimize

798   adverse off-target effects. Nathanielsz and his colleagues have argued that the clinical dose of

799   betamethasone is higher than necessary based on cardiovascular effects (elevated blood pressure)

800   on the fetus (100). They reported the pharmacokinetics of maternal IM betamethasone in maternal

801   and fetal compartments. The betamethasone phosphate preparation causes high peak fetal plasma

802   betamethasone levels, although equivalent changes in fetal blood pressure were identified between

803   different, clinically relevant doses suggesting a supramaximal cardiovascular response at the highest

804   doses (100). We reported similar findings and noted single dose of betamethasone phosphate was

805   not effective for lung maturation (47). However, a single dose of 0.25 mg/kg betamethasone acetate

806   was comparable to two doses of Celestone delivering a total of 0.5 mg/kg. Dexamethasone

807   phosphate or betamethasone phosphate given as 2 doses at 24 hr intervals were not as effective for

808   lung maturation as was 2 doses of Celestone. Shorter treatment intervals for the 2 doses of

809   betamethasone phosphate also were not as effective (98). We now think that the phosphate esters

810   of betamethasone or dexamethasone cause unnecessarily high fetal plasma drug levels for too short

811   an interval to effectively induce lung maturation(54, 97, 99). In ongoing studies, a single dose of

812   0.125 mg/kg betamethasone – acetate may still be unnecessarily high for adequate lung maturation.

813   Fetal plasma levels are about 10% of maternal levels in the sheep while primate and human fetuses

814   have plasma levels that are about 30% of maternal levels (9, 54, 97, 99). Thus, much lower dosing

815   may be possible in the human. As more and more women are being exposed to ANS for indications

816   such as preterm gestation >34 weeks and for elective cesarean section at term, a priority should be

817   the identification of an effective minimal dose. An approach is to use constant infusions of

818   betamethasone in sheep to test different target dosing levels and infusions times to establish the

819   optimal drug exposures (55).

820

821   ***Repeated doses of ANS***

822   Research in sheep models demonstrate that the repeated dosing of the fetus improve respiratory

823   outcomes, but with adverse effects on the fetal growth. Kutzler et al treated ewes with 3 low doses

824   of dexamethasone and noted decreased birth weight relative to controls at 119d gestation (62).

825   Following spontaneous birth at term, brain weight was decreased. Ewes treated with 1 to 4 weekly

826   doses of 0.5 mg/kg Celestone were delivered of singletons fetuses at 125d gestation (39). The

827   newborns weighted less for each additional dose of ANS, but lung function and lung gas volumes

828   increased for each additional dose of ANS. However, the lungs of preterm animals exposed to

829   multiple doses of ANS and controls were not different at term (111). The fetal lung does not seem to

830   have permanent changes in structure or function after ANS in sheep models.

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851 **Nonhuman Primate Models**

852 *Initial studies in Nonhuman Primates*

853

854 A major reason that ANS were not widely used clinically between 1972 (the Liggins trial) and the

855 1994 NIH Consensus Conference that supported wide use of ANS were concerns about adverse

856 effects on the fetus (1, 66). Concerns about growth effects and adverse effects on the fetal lung and

857   brain reflected the early reports of fetal exposures to steroids in nonhuman primates. For

858   perspective on dosing of ANS in nonhuman primates, the standard total clinical doses of the

859   betamethasone acetate plus phosphate or dexamethasone phosphate are 24 mg or about 0.34

860   mg/Kg for the 70 Kg pregnant woman. Rhesus macaque monkeys were treated with two 1.5 mg

861   doses of betamethasone acetate plus phosphate (0.4 mg/kg total dose), and accelerated

862   differentiation of lung, liver, kidney, and adrenals 48 hrs. later was noted (31). Liver glycogen

863   increased 4-fold and there were some anatomic indications of brain injury. This limited study

864   identified pleotropic effects of ANS with concerns for injury with clinically relevant steroid dosing.

865   Johnson and colleagues treated Rhesus with 3 doses of betamethasone acetate plus phosphate at a

866   total dose of about 1 mg/kg and reported decreased fetal weight 72 hrs. after the first dose (50).

867   Lung gas volumes increased greatly but there was not much change in deflation stability with

868   pressure-volume curves and no changes in lung phospholipid amounts. They interpreted these

869   results to indicate steroid effects on distal lung tissue without increased surfactant. A subsequent

870   report by the same group measured an increased collagen to elastin ratio and decreased alveoli per

871   tissue volume (12). These reports demonstrated that ANS inhibited septation in the fetal lung at

872   short intervals after treatment but with increased gas volumes. These changes in lung anatomy

873   translate to more normal lung function in subsequent studies in monkeys and sheep (56, 112). The

874   Rhesus study by Kessler and colleagues used 3-2 mg doses of dexamethasone phosphate to deliver a

875   total dose of about 1 mg/kg beginning 72 hrs. before preterm delivery and ventilation of the

876   preterm animals (56). The steroid treatment decreased the incidence and severity of RDS and

877   increased lung gas volumes and surfactant lipids - a clear demonstration of lung functional

878   improvements in nonhuman primates with ANS.

879

880   In 1984, Bunton and Plopper reported effects of ANS on fetal Rhesus at very early gestational ages

881   and close to term (15). They treated dams with triamcinolone actinide with a high dose of 10 mg or

882   a low dose of 1 mg, which may translate to about 0.3 m/kg (high) or 0.03 mg/kg (low)

883　dexamethasone equivalent, although the placental transfer of triamcinolone is not known. Separate

884　groups of preterm monkeys were treated at 63-65 days' gestational age and 110-112 days'

885　gestational age and delivered at 90, 120 or 150 days' gestation. Independent of gestational age at

886　treatment, the ANS accelerated maturation of the interstitium and epithelium of the fetal lung. The

887　early gestation treatment accelerated alveolarization, primarily with the high dose treatment. This

888　report is important because it demonstrates large effects on the fetal lung as early as 0.4 of

889　gestation, although the steroid dose was high. Johnson *et al.* (49) in 1981 also used a high total

890　exposure of about 3 mg/kg (2 mg betamethasone acetate plus phosphate for 13 days from 120 to

891　133 days' gestational age). Groups delivered at 134 days' gestational age or at term had decreased

892　body and organ weights. Lung gas volumes normalized to body weight were increased by ANS at

893　133 days, but were decreased at term. This latter result suggests adverse effects at term after ANS

894　exposure of the preterm fetal lung. These two reports are unique in that they describe large

895　maturation and developmental changes in response to fetal steroid exposures that appear to

896　depend on gestation of exposure and dose. The animals received high total doses with the 13-day

897　treatment, but the results indicate caution about dosing and duration of treatment in the primate

898　(15, 49).

899

900　The major focus of concerns about ANS relate to possible adverse effects on neurodevelopment.

901　The report by Uno and colleagues was influential because they described clear hippocampal injury

902　with fetal exposure to dexamethasone (109). They tested 0.5, 5, and 10 mg/kg maternal weight as a

903　single dose of dexamethasone in Rhesus at 133d or the same total doses divided into 4-12 hrs.

904　interval injections. The animals were evaluated at 72 hrs. (135d gestation) or at term. Multiple

905　indicators of injury were evident at 135 days, were dose dependent, and were more pronounced

906　with the multiple dose treatment. Injury remained at term even for the lowest dose. These early

907　reports of both benefits for the lung and potential for injury were generally performed with ANS

908   dosing higher than is used clinically. But, the results with nonhuman primates were similar to effects

909   that were observed with other animal models (109).

910

911   ***Risks related to developmental programming***

912

913   Developmental programming can be defined as a response to a specific challenge to the mammalian

914   organism during a critical developmental time window that alters the trajectory of development

915   with persistent effects on offspring phenotype. Given the powerful effects of glucocorticoids as

916   orchestrators of developmental preparation for an independent postnatal life and newer knowledge

917   of the role of glucocorticoids in developmental programming, it is important to use nonhuman

918   primates to test for clinically relevant risks from ANS. Nathanielsz and colleagues hypothesized that

919   antenatal betamethasone administration to pregnant baboons in doses equivalent to those

920   administered clinically cause adverse cardiometabolic, behavioral and endocrine outcomes by

921   developmental programming. Pregnant baboons were given betamethasone phosphate, as two

922   intramuscular injections of 0.18 mg/kg spaced by 24h at weekly intervals at 0.60, 0.64, and 0.68

923   gestation, equivalent to 24, 26, and 28 weeks of human pregnancy (59). This dose approximated the

924   human clinical dose, weight-adjusted for a 70kg woman. Using a chronically catheterised fetal

925   baboon preparation, fetal blood pressure was increased more than maternal blood pressure, but

926   there were no effects over the 48 hours after the first ANS injection on pH, electrolytes or blood

927   gases. The episodes of hypertension could potentially affect the setting of the baroceptor regulatory

928   reflexes throughout life. These findings are well-aligned with earlier work undertaken using the

929   sheep model of pregnancy. A numer of studies, including those by Derks (27) *et al*., and Fletcher (33)

930   and colleagues, showed that 48h fetal infusions of either betamethasone or dexamethasone

931   significantly increased fetal blood pressure, and altered basal fetal cardiovascular functions. Of

932   particular importance is the observation that a low-dose infusion of dexamethasone (2.06 ± 0.13

933 ug/kg/hr) modified the fetal baroreceptor set point, with a right-shift (accommodation of increased

934 blood pressure) that persisted 48 hours after cessation of treatment.

935

936 Very long term effects of fetal ANS exposure evaluated hepatic and pericardial lipid deposition in 10-

937 year-old baboons (human equivalent 40 years) exposed to the three weekly ANS courses during fetal

938 life (61). The ANS exposed primates delivered spontaneously at term, as do many ANS exposed

939 human neonates. At 10-years of age (human equivalent 40) pericardial fat and hepatic lipid content

940 were quantified with magnetic resonance imaging and spectroscopy in males. The prenatal ANS

941 exposure increased pericardial fat deposition and inceased hepatic fatty acids. It is of considerable

942 importance that these lipid abnormalities following ANS exposure occurred without birth weight

943 differences.   Thus there is abnormal fat deposition and adult body composition in mid-life in

944 primates after antenatal sGC therapy. The results illustrate how long-term nonhuman primate

945 studies could identify risks of ANS in humans.

946

947 ***Prenatal ANS alters cognitive function and behavior***

948

949 Zürcher and co-workers tested in baboons the Cambridge Neuropsychological Test Automated

950 Battery commonly used in human studies for assessment of cognition and behavior (115). Male and

951 female baboons of both ANS- and vehicle-treated mothers were assessed at 2.6-3.2 years (i.e. just

952 prior to puberty) for motivation associative learning, rule change plasticity, and for attention

953 allocation.   Motivation was reduced in both sexes by exposure to ANS in fetal life. In

954 intra/extradimensional testing, exposed females made more errors in the simple discrimination

955 reversal, compound discrimination and compound reversal testing compared to exposed males (94).

956 These findings indicate that ANS change central nervous system developmental programming in a

957 sex dependent manner. These results demonstrate that long-term cognitive effects of repeated fetal

958 ANS exposures with clinically relevant dosing occur in the primate.

959

960

961   *Testing new dosing strategies*

962

963   As pointed out throughout this review, ANS have not been rigorously evaluated for choice of drug,

964   dose or treatment interval. The presently used clinical treatments appear to be similarly effective,

965   but the randomized clinical trial is a blunt instrument for comparative trials. Further, as ANS have

966   important outcome benefits and are standard of care, a placebo treated group is generally unethical.

967   Large animal and nonhuman primate models will be essential prior to any testing in this very high-

968   risk population of pregnant women at risk of preterm delivery. We are evaluating treatment

969   strategies that use lower doses that may be safer in low resource environments for example. We

970   demonstrated in fetal sheep that a single dose of 0.125 mg/kg of betamethasone acetate can

971   mature the fetal lung comparably to 2 doses of 0.25 mg/kg betamethasone acetate plus phosphate

972   (97, 99). In initial testing in the Rhesus, the same low dose is effective as a lung maturational agent.

973   There is a way forward to develop better and presumably safer ANS treatments prior to clinical

974   trials.

975

976   **Conclusions**

977   Despite significant efforts over the past 50 years, preterm birth continues to be a leading cause of

978   perinatal death and disease. Of the limited antenatal interventions available to improve outcomes

979   for preterm infants, the administration of steroids in anticipation of preterm birth remains perhaps

980   the most effective and widely used.

981

982   Today, the gestational age range over which antenatal steroids are administered continues to widen,

983   most notably into late preterm deliveries, meaning that a greater percentage of women and fetuses

984   are likely to receive antenatal steroid therapy. Despite an appreciation for their potency, and wide-

985   range of actions, the most commonly employed dosing regimens for antenatal steroid delivery

986   remain un-optimised. Together, these observations highlight the importance of studies to: i) better

987   improve our understanding of the mode of antenatal steroid action; and ii) refine the dosing

988   regimen for antenatal steroids such that the safest, and most effective treatment possible may be

989   determined on the basis of empirical data.

990

991   **Perspectives and Significance**

992   Animal experiments have been critical to our understanding of antenatal steroid function. These

993   same animal models will be essential for future efforts to further refine this important therapy. Due

994   to a wide range of factors no one model system serves as an ideal, 'stand-alone' system to further

995   develop antenatal steroid therapy. Rather, the rigorous use of each of the model systems (rodent,

996   sheep, non-human primate) described above provide an important platform to allow investigators to

997   identify the molecular, pharmacological, physiological and long-term developmental responses of

998   the mother, the fetus, and the developing infant, to antenatal steroid exposure.

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1004   [Acknowledgements]

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