High Dilution of Dexamethasone in gestation and fetal development of mice

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ABSTRACT

Background: Recently, the use of homeopathy in veterinary medicine has grown significantly, mainly for farm animal practice, because of its usefulness in organic production and low cost. There is a wide range of veterinary products available in the market often used in females. However, the effect of these products in the litter and derived products for human consumption is completely unknown. **Aims:** this study sought to develop an experimental model to study the putative effects of high diluted substances in newborns after chronic exposure of females. **Methods:** based on previous studies, the chosen test substance was dexamethasone 15cH; adult female Balb/c mice were divided into 4 groups: a) treated with PBS (control); b) treated with dexamethasone 15 cH; c) treated with dexamethasone 15cH + dexamethasone 4 mg/kg and d) treated with dexamethasone 4 mg/kg. All medicines were administered subcutaneously, 3 times a week, in females from the first day of pregnancy up to the 20th day after parturition (end of lactation period). Development of the offspring was evaluated daily for 15 days after birth. Parameters evaluated were: female and offspring viability, number of newborns, time for eye opening, pinna opening, fur growth and postural reflex. **Results:** the group treated with dexamethasone 15cH showed 39% increase in mortality rate 39 days after the beginning of treatment and 35% increase in fetal mortality at the end of gestation ($p=0.0049$). Females treated with dexamethasone 4mg/kg + dexamethasone 15cH showed 100% of fetal mortality. After parturition newborn survival in animals exposed to dexamethasone 4 mg/kg was higher than the control ($p=0.0002$). All other parameters of neonatal development were unchanged among groups. **Conclusions:** these data point to adverse effect when using high diluted dexamethasone during gestation detectable by this experimental model in Balb/c mice.

Keywords: high dilution, homeopathy, neonatal, dexamethasone, toxicity.

Introduction

Recently, the use of homeopathy in veterinary medicine has grown significantly in some countries, mainly in farm animal practice, because of its usefulness in organic production and low cost [1-7]. There is a large range of veterinary products available in the current markets, which are often used in pregnant cows. However, the effect of these products in the litter and derived products for human consumption is poorly known.

Among the few studies available in the literature, Sommer *et al.* [8] tested different potencies of Sabina up to 30x to reduce placenta retention in cattle, in a study performed in 70 cows from 8 farms. The result was significant but increase in the incidence of mastitis and laminitis was also observed in treated groups. Recently, several studies about the use of homeopathic complexes as zootechnical tools in swine and chicken farms have been described [4-6].

On the other hand, it is classically known that corticoid hormones are critical agents of delivery outbreak and fetal development [9]. In previous studies, several effects of high diluted of dexamethasone have been

described in different experimental models, including cell migration, proliferation and differentiation, inflammation and tumor development [10, 11].

In Bonamin *et al.* [10] and co-workers described the property of dexamethasone 7cH and 15cH (mathematically equivalent to 10^{-17} M and 10^{-33} M) to modulate the acute inflammation induced by carrageenan and leukocyte migration to Ehrlich tumor site. In both cases, the simultaneous administration of high diluted dexamethasone blocked the action of the same dexamethasone in pharmacological doses (Bonamin *et al.* [10]). In Martinho *et al.* [11] and collaborators described the effects of high diluted dexamethasone (7cH) to increase the pre-neoplastic lesions development in rat liver induced by carcinogenic substances (Martinho *et al.* [11]). These studies are in line with the recent trend to establish appropriate models to study the peculiar features of high dilutions biological effects, using single cell cultures, plants or animals [12-18].

The aim of this work was to propose an easy experimental model to study the putative effects of high diluted substances in newborns after mother chronic exposition.

Material and methods

a. Animals

Sixty days old female Balb/c mice were used in this experiment. They were maintained in conventional cages in a controlled environment, with artificial light cycle (12 hours light; 12 hours dark) and temperature fixed at $22\pm 3^{\circ}\text{C}$. Commercial food and potable water were offered *ad libitum*.

b. Groups and treatment

Group I - Control (PBS)

Group II - Dexamethasone 15 cH

Group III - Dexamethasone 15cH mixed with Dexamethasone (4 mg/kg) in the same shot

Group IV - Dexamethasone (4mg/Kg)

Treatments were done three times a week, subcutaneously, in a volume equal to 0.1ml/10g of weight, during all gestational (21 days) and lactation (20 days) period. Each group was composed by 15 female and 15 male mice. Male mice were used only for procreation.

c. Drugs

PBS – Phosphate buffer saline (SIGMA) prepared in distilled water. It was used as negative control.

Dexamethasone 15 cH – The centesimal dilutions of Decadron ® were prepared in a serial manner in distilled water up to 14cH. The last dilution (15cH) was made in PBS, observing the same proportions. The final solution reached the concentration mathematically equal to 10^{-33} moles/liter. All dilutions were succussed according to the classical Hahnemannean method.

Dexamethasone 4 mg/kg – The commercial form of dexamethasone (Decadron ®) was diluted in PBS without succussion in order to reach the dose of 4.0 mg/kg.

Mixed Dexamethasone 15cH and 4 mg/kg - The commercial form of dexamethasone (Decadron ®) was diluted in the homeopathic form dexamethasone 15cH in order to reach the dose of 4.0 mg/kg.

The potency 15cH was chosen because, although beyond the Avogadro's number, is low enough to interfere in acute-physical parameters, according to its traditional homeopathic clinical use. Also, previous data related to the effects of dexamethasone 15cH in inflammatory process were already related by us previously (Bonamin *et al.* [10]). The fact to be beyond Avogadro's number excludes completely the possibility of any molecular transmission between mother and fetuses via placenta, which could result in false positive results.

d. Control of treatment period

Day zero of pregnancy: Was determined as the first day in which females were put together with males. This procedure lasted up to one week for all tested females.

Final of treatment: weaning, twenty days after parturition.

e. Post-natal evolution

After parturition, the number of days corresponding to the pregnancy period and the number of viable newborns were registered.

From the day of birth, the following parameters would be also registered:

1. day of fur appearance
2. day of pinna opening
3. day of testicle dehiscence
4. day of complete development of postural reflex

The postural reflex is evaluated by the capacity of newborn to recover immediately the quadruped position after be lay in dorsal decubitus.

After weaning, females were sacrificed and the number of implantations was registered.

f. Statistical analysis

For evaluation of all newborn parameters, including mother mortality, the Fisher test was used, being $p \leq 0.05$. This test was done using the Graph Pad InStat for Windows software. The Fisher test was chosen because it is the most adequate test to compare proportions between groups within little samples.

Results

a. Mortality

A clear trend of dexamethasone 15cH to increase mother mortality in relation to control (39%) was observed between days zero and 30, although without statistical significance (Figure 1). After death, these animals were necropsied and general fetal death was observed.

Figure 1 – Proportion of live (red column) and dead (blue column) female in the first 30 days of the experiment. Y axis = number of pregnant females; GI = control; GII = dexamethasone 15cH; GIII = dexamethasone (4mg/kg) + dexamethasone 15cH; GIV = dexamethasone 4mg/kg. Fisher's test.

b. Pregnancy and cannibalism

There was no statistical significance between groups regarding to pregnancy duration. The group treated with both, dexamethasone 15cH and dexamethasone 4mg/kg at the same time, presented 100% of newborn mortality. In the other groups there was no mortality, but cannibalism was detected (Table 1).

Table 1. Mother parameters during perinatal period.

	control	Dexamethasone 15cH	Dexamethasone 15cH + 4mg/kg	Dexamethasone 4mg/kg
Pregnancy duration (days)*	22	24	#	23
Cannibalism	yes	yes	#	yes

100% of precocious fetal mortality; Fisher's test.

c. Newborn evolution parameters

Since cannibalism was observed, the later parameters, such as the day of testicle dehiscence and the day of complete development of postural reflex could not be registered. In relation to fur appearance and pinna opening, no statistically significance was observed (Table 2).

Table 2. Newborn development. The data represent the median of days needed to observation of each parameter.

	Control	Dexamethasone 15cH	Dexamethasone 4mg/kg
Fur appearance (days)	2	2	2
Pinna opening (days)	2	2	2

Fisher's test.

The third group was not represented because all newborns were dead.

d. Number of newborn

The number of newborns at the end of pregnancy in dexamethasone 15cH treated group was superior to the other groups, although without statistical significance. However, the ratio number of newborns/number of fetuses at the final stage of pregnancy was 35% smaller than the control group ($p=0.0047$). No implantations were seen in mice treated with dexamethasone 15cH and dexamethasone 4 mg/kg at the same time. The group treated with dexamethasone 4mg/kg alone presented the ratio equal to 1 ($p=0.0002$) (Table 3).

Table 3. Number of newborns / number of fetuses' ratio among groups.

	control	Dexamethasone 15cH	Dexamethasone 15cH + 4mg/kg	Dexamethasone 4mg/kg
Total of fetuses	13	33	0	7
Total of newborns	5	1	0	7
Newborns/fetuses ratio	0.38	0.03*	0	1#

* Fisher test, $p=0.0049$; # Fisher test, $p=0.0002$ in relation to control

Discussion

The deleterious effect of corticoid during gestation is well known. This toxic effect is related to the role of this hormone in the early parturition induction. Physiologically, during delivery, the release of corticosterone by mice fetal adrenals induces inversion of progesterone/oestrogenous ratio in mother serum. These changes in endocrine panel result in prostaglandin secretion by endometrial cells and oxytocin release by posterior pituitary, followed by the expulsion of fetuses [19]. In the veterinary practice, the use of corticoid to synchronize parturitions in farm animals was already mentioned, although placenta retention can occur in some cases [20].

In this study, several interesting findings about high dilutions of dexamethasone were observed. Female treated with dexamethasone 15cH during pregnancy suffered higher percentage of mortality than the control, with associated fetuses' death. The dexamethasone 15cH seems to potentiate the toxic effect of dexamethasone 4mg/kg regarding fetal absorption. The exposition of mothers to dexamethasone 15cH during gestation resulted in significant decrease of newborn viability; the association of dexamethasone 4mg/kg with high diluted dexamethasone potentiated this toxic effect, since no implantations were observed in this group. These results suggest some kind of "antagonism" of high diluted dexamethasone regarding the endogenous cycle of corticosterone in pregnant mice, although more experiments have to be done to confirm the data and explain their mechanisms. to explain their mechanism. Additional studies using other mouse strain or species (such as rats) would be useful to identify the general impact of high diluted dexamethasone in pregnancy, since Balb/c mice is too much sensitive to stress during gestation. Thus, the exacerbated results observed here could be related to it. This hypothesis is sustained by previous results obtained by our group demonstrating the interference of high diluted dexamethasone in other physiopathological situations [10, 11].

Several data about the effects of high dilutions of endogenous substances on fetal development have been described in the last 15 years. Youbicier-Simo *et al.* [21] observed that *in ovo* treatment of bursectomized

chicken embryos with high diluted Bursin is able to recover B lymphocyte function – that was lost by bursectomy – and also all endocrine functions related to it, such as melatonin, ACTH and corticoid serum levels. One of the most studied models about the effect of high dilution of hormones in differentiation process is that created by PC Endler and co-workers. In multi-centric studies, they demonstrated that high dilutions of thyroxin modulate the metamorphosis rate of *Rana temporaria* [22, 23]. These results were also reproduced in different environmental conditions by Guedes *et al.* [24].

The use of homeopathic products in farm animals has been increased in the last years [1-7, 25], but few studies about the influence of these products in pregnancy and offspring are reported. In 2006, Rajkumar *et al.* [26] demonstrated that the treatment of cows with a complex composed by *Calcarea phosphorica* 30c, *Sepia* 30c and *Phosphorus* 30c in equal proportions increased oestradiol plasma concentration and was effective in inducing oestrus in anoestrus animals but no information is provided about fetal development and pregnancy. In human clinical practice, the lack of information regarding this approach is also noticed. In 2006, a case report about induction of labor using *Caulophyllum thalictroides* and *Cimicifuga racemosa* 30c in a 28 years old woman was published. The effects in fetal development were neither described (Kistin *et al.*, [27]).

Recently, a large review about animal experimental models to study high dilutions was published (Bonamin; Endler, [14]), but no pregnancy model was found in the literature. Herein, the proposed model was sensible enough to identify putative effects of high dilutions of endogenous substances in pregnancy and fetal development, indicating it could be a good tool for the characterization of this particular aspect of homeopathy biological activity. The implications of the use of homeopathic medicines according to the strict similia principle in pregnant females still remain unsolved.

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Efeitos de altas diluições de dexametasona na gestação e no desenvolvimento fetal de camundongos

RESUMO

Justificativa: a utilização da homeopatia na medicina veterinária tem crescido significativamente devido a questões relacionadas à produção orgânica e ao baixo custo. Atualmente há uma grande oferta de medicamentos homeopáticos para animais de criação, sobretudo em fase de gestação e lactação, mas pouco se sabe sobre as consequências desta exposição para a cria ou nos produtos derivados para consumo humano. **Objetivos:** este trabalho visa o desenvolvimento de um modelo experimental para estudar os efeitos da exposição crônica das fêmeas a substâncias altamente diluídas nos recém-nascidos. **Métodos:** com base em estudos anteriores, a substância escolhida foi dexametasona 15cH; camundongas adultas Balb/c foram divididas em 4 grupos: a) tratado com PBS (controle), b) tratado com dexametasona 15 cH; c) tratado com dexametasona 15cH + dexametasona 4 mg / kg; e d) tratado com dexametasona 4 mg/kg. Todos os medicamentos foram administrados por via subcutânea, 3 vezes por semana, nas fêmeas, a partir do primeiro dia de gravidez até o dia 20 após o parto (final do período de lactação). O desenvolvimento da prole foi avaliado diariamente durante 15 dias após o nascimento. Os parâmetros avaliados foram: viabilidade das fêmeas e crias, número de recém-nascidos, tempo para a abertura dos olhos, descolamento de orelha, crescimento do pelame e reflexo postural. **Resultados:** faz fêmeas tratadas com dexametasona 15cH apresentaram 39% de aumento no índice de mortalidade após 39 dias do início do tratamento e um aumento de 35% na mortalidade fetal no final da gestação ($p = 0,0049$). Fêmeas tratadas com dexametasona 4mg/kg + dexametasona 15cH apresentaram 100% de mortalidade fetal. Após o parto, a sobrevivência dos animais recém-nascidos expostos a dexametasona 4 mg/kg foi melhor do que o controle ($p = 0,0002$). Todos os outros parâmetros de desenvolvimento neonatal não foram alterados, entre os grupos. **Conclusões:** os dados apontam para a existência de efeitos adversos da dexametasona altamente diluída durante a gestação, detectáveis por este modelo experimental em camundongos Balb/c.

Palavras-chave: altas diluições, gestação, neonatos, dexametasona, toxicidade, ratos.

Efectos de las altas diluciones de dexametasona durante la gestación y el desarrollo fetal de ratones

RESUMO

Justificación: el uso de la homeopatía en la medicina veterinaria ha crecido considerablemente debido a cuestiones relacionadas con la producción agroecológica y el bajo costo. Actualmente hay una amplia gama de medicamentos homeopáticos para animales de granja, especialmente en el proceso de gestación y la lactancia, pero poco se sabe sobre las consecuencias de esta exposición en las crías o en los productos para consumo humano. **Objetivos:** este trabajo tiene como objetivo desarrollar un modelo experimental para estudiar los efectos de la exposición crónica de las hembras a sustancias altamente diluidas en los recién nacidos. **Métodos:** sobre la base de estudios anteriores, la sustancia elegida fue dexametasona 15cH; ; ratones adultos hembra Balb/c fueron divididos en 4 grupos: a) tratado con PBS (control); b) tratado con dexametasona 15 cH; c) tratado con dexametasona 15cH + dexametasona 4 mg/kg; y d) tratado con dexametasona 4 mg/kg. Todos los fármacos fueron administrados por vía subcutánea 3 veces por semana en las hembras desde los primeros días del embarazo hasta 20 días después del parto (final de la lactancia). El desarrollo de las crías fue evaluado durante 15 días después del nacimiento. Los parámetros evaluados fueron: viabilidad de hembras y fetos, número de recién nacidos, tiempo para abrir los ojos, separación de las orejas, crecimiento del pelo y reflejos posturales. **Resultados:** las hembras tratadas con dexametasona 15cH presentaron un incremento del 39% en la tasa de mortalidad después de 39 días de iniciado el tratamiento y un aumento del 35% en la mortalidad fetal tardía en el embarazo ($p = 0,0049$). Hembras tratadas con dexametasona 4 mg/kg + dexametasona 15cH presentaron 100% de mortalidad fetal. Después del parto, la supervivencia de los animales recién nacidos expuestos a dexametasona 4 mg/kg fue mejor que el control ($p = 0,0002$). Todos los demás parámetros de desarrollo neonatal no cambiaron entre los grupos. **Conclusiones:** estos datos indican la existencia de efectos adversos de la dexametasona altamente diluida durante la gestación, detectables mediante este modelo experimental en ratones Balb/c.

Palabras clave: altas diluciones, gestación, neonatos, dexametasona, toxicidad, ratones.

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