Animal Biology, Vol. 56, No. 1, pp. 63-68 (2006)

Also available online - www.brill.nl

Evolutionary conserved structures as indicators of medical risks: increased incidence of cervical ribs after ovarian hyperstimulation in mice

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Abstract—The presence of a rib on the seventh cervical vertebra (a cervical rib) represents one of the most common intraspecific variations of the number of cervical vertebrae in mammals. Cervical ribs are highly associated with stillbirths, congenital abnormalities and embryonal tumours. These associations indicate strong stabilising selection against such a change to the highly conserved number of cervical vertebrae in humans. We propose, therefore, that the presence of variation for this highly conserved trait can be used as an indicator of medical risks. We have tested for prolonged effects of controlled ovarian hyperstimulation treatments (OHS) in mice by analysing the frequency of cervical ribs in the offspring of females that had received OHS treatment. We found that OHS treatment in mice had several significant effects on the offspring after adjusting for multiple pregnancy: these included an increase in cervical rib incidence, gestational period and nest size, and a decrease in birth weight and ossification, indicating growth retardation.

The high incidence of cervical ribs in the OHS group compared to the control group (39.5% vs. 4.7%) indicates that the OHS treatment affects embryogenesis during a period that is highly sensitive to disturbance, the early organogenesis stage (phylotypic stage). This implies that in mice OHS treatment of the mother has a prolonged effect and continues during early pregnancy.

Keywords: cervical ribs; evolutionary conservation; homeotic transformation, IVF; organogenesis; ovarian hyperstimulation; Barker hypothesis.

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INTRODUCTION

The number of cervical vertebrae is remarkably constant in mammalian species and is nearly always seven. Nonetheless, variations frequently occur within species and there is, therefore, extremely low interspecific variation, as well as high intraspecific variation (Galis, 1999). We have earlier hypothesised that conservation of the number of cervical vertebrae is due to strong selection against changes of this number due to association with negative pleiotropic effects (Galis, 1999; Galis and Metz, 2003). The presence of cervical ribs (a transformation of the seventh cervical vertebra into a thoracic one, hence a decrease in the number of cervical vertebrae) is one of the most common anomalies in human stillbirths and occurs in up to 50% of stillborn foetuses (Noback and Robertson, 1951; Meyer, 1978; Galis et al., subm.). This implies strong selection against variation in the number of cervical vertebrae. In addition, cervical ribs in humans are associated with an increased incidence of abnormalities and cancer (Gladstone and Wakeley, 1931-1932; Narod et al., 1997; Schumacher et al., 1992; Galis and Metz, 2003). In mice, cervical ribs can be induced at embryonic days 7-8 (Abdulrazzaq et al., 1997), at the beginning of the sensitive early organogenesis stage (Galis and Metz, 2001). Cervical ribs, therefore, appear to be a good indicator of disturbances of early organogenesis that presumably lead to medical risks.

Controlled ovarian hyperstimulation (OHS) in humans is frequently used in ovarian stimulation and ovulation induction to enhance the chance of becoming spontaneously pregnant, but also as part of assisted reproductive technologies. Assisted reproductive technologies are frequently associated with an increased frequency of spontaneous abortions, congenital anomalies, low birth weight, preterm birth and perinatal mortality, albeit with a low prevalence (Helmerhorst et al., 2004; Jackson et al., 2004; Bonduelle et al., 2005; Hansen et al., 2005). Similar abnormalities have been found for OHS treatment alone (Olivennes et al., 1993; Brinton et al., 2004). A low birth weight, often reflecting a disturbance early in life, is associated with cardiovascular diseases later in life (Holt, 2002; Phillips, 2002), an association first hypothesised by Barker (Barker, 1992). In addition, early embryogenesis is, in general, the most sensitive period for disturbances (Galis and Metz, 2001). Ovarian hyperstimulation and other assisted reproductive techniques, therefore, may disturb early embryogenesis, including the very sensitive phase of early organogenesis. To investigate possible effects of OHS treatments on early embryogenesis, the frequency of cervical ribs in the offspring of female mice with and without OHS treatment was measured, and used as an indication of disturbance of early embryogenesis.

MATERIALS AND METHODS

Virgin adult female CD1 mice (8-10 weeks, Charles River, Germany) were randomly assigned to the different experimental groups and mated with randomly assigned CD1 males. Each male mated only once. Females were used irrespective of the day of the cycle and 142 were intraperitoneally injected with Metrodin (purified urinary hFSH; 5 units in 0.1 ml saline; Serono, Coinsins, Switzerland) at 12:00 and 48 h later with Pregnyl (urinary hCG; 5 units in 0.1 ml saline; Organon, Oss, The Netherlands). Control females (n = 115) received saline injections. Females were examined every morning for vaginal plugs indicating fertilisation (day of detection was considered as embryonic day (E.D. 0)). After birth the mother and newborn mice were weighed. For the control group 115 females were exposed to males, and for the OHS group 142, resulting in 38 (33.04%) and 53 (37.32%) conceptions, respectively. For the experiments, nests of five females were used for each group. Directly after birth, newborn mice were euthanised (peritoneal Nembutal injection), fixed in 4% formaldehyde and stained in a 0.2% silver nitrate solution for 2 weeks. X-ray photographs were taken (15 A, 20 kV, 20 s) and analysed for the number of cervical and thoracic vertebrae. X-ray photographs with insufficient staining were excluded from analysis. All animal experiments were in accordance with governmental guidelines for care and use of laboratory animals and approved by the Animal Care Committee of the University of Leiden.

RESULTS

The OHS group had a more than eight-fold increased incidence of cervical ribs compared to the control group (39.5% of OHS-treated mice showed cervical ribs vs. 4.7% in the control group, $\chi^2 = 19.14$, df = 2, $P < 0.01$, table 1). Part of the increased incidence of cervical ribs was due to the larger litter size in the OHS group, as there was a significant positive correlation between the incidence of cervical vertebrae and the weighted average of litter size between nests from the OHS group ($R^2 = 0.12$, df = 1, $F = 48.52$, $P < 0.01$). The OHS treatment was responsible for at least part of the increased incidence of cervical ribs, because there was also a significant increase in the smaller nests of the OHS group (within the size range of the control group) compared to those of the control group (Log Linear Model, $G^2 = 9.32$, df = 1, $P < 0.01$, litters <20 individuals).

The duration of pregnancy was longer (10.32%) in the OHS group than in the control group (ANOVA, df = 1, $F = 177.99$, $P < 0.001$, table 1) but, despite the longer pregnancy length, there were no significant differences in weight of the offspring (Pearson = 0.032, $P > 0.05$) and growth was thus slower in the treatment groups. Females from the OHS group had a significantly, although only moderately, larger average litter size (32.46%) compared with the control group (control litter size = 15.25, OHS litter size = 20.20, ANOVA, df = 1, $F = 22.88$, $P < 0.01$). In the OHS group the average weight of an individual at birth was negatively correlated with the litter size, but not in the control group (OHS group: $R^2 = 0.74$, df = 1, $F = 134.28$, $P < 0.01$, control group: $R^2 = 0.024$, df = 1, $F = 1.45$, $P > 0.1$). The absence of a litter size effect in the control group may have been due to the smaller size of the nests, i.e., there may be a threshold before litter size negatively affects weight.

Table 1.
Effects of OHS treatment on mice.

	OHS (N)	Control (N)	df	*F*	*P*
Incidence of cervical ribs in the total number of offspring per group (OHS and Control)	39.53% (n = 62)	4.65% (n = 61)	2	$\chi^2 = 19.14$	<0.01
Average duration of pregnancy, days (mean value ± standard error, SE)	20.96 ± 1.46 (n = 5)	19.0 ± 0 (n = 5)	1	177.99	<0.001
Average litter size (mean value ± SE)	20.20 ± 7.10 (n = 5)	15.25 ± 3.23 (n = 5)	1	22.88	<0.01
Average weight of siblings, grams (mean value ± SE)	1.39 ± 0.31 (n = 111)	1.43 ± 0.15 (n = 79)	1	6.97	<0.01
Average weight of mothers, grams (mean value ± SE)	38.78 ± 0.18 (n = 6)	35.57 ± 0.43 (n = 5)			
% new-born mice not analysed (rejection X-ray photographs, delayed ossification)	44.14% (n = 111)	22.78% (n = 79)	2	$\chi^2 = 21.77$	<0.01

The OHS treatment had an effect on individual weight. When comparing the weighted average individual nestling weight, corrected for the influence of litter number on litter weight, the weight at birth of individuals was significantly lower (2.80%) in the OHS group than in the controls (General Linear Model, df = 1, $F = 117.098$, $P < 0.01$).

Significantly more individuals from the OHS group could not be analysed compared to the control group (93.42%) due to insufficient staining of the X-ray photographs, indicating a delay in ossification at birth in the OHS treatment group (44.1%, $N = 111$ vs. 22.8 %, $N = 79$, $\chi^2 = 21.77$, df = 2, $P < 0.01$, table 1). The delay in ossification appears to be due to a direct effect of the treatment itself, because in the OHS group there was no significant difference in rejection rate of photographs between larger litters (larger than the average litter size for the control group, >15) and smaller ones (ANOVA, df = 1, $F = 2.96$, $P > 0.05$). Furthermore, in the smaller litters of the OHS group the rejection rate was also higher than in the control group (49.1% vs. 22.8%, respectively). However, this difference was not significant, presumably due to the low number of small litters in the OHS group (ANOVA, df = 1, $F = 3.26$, $P > 0.1$).

DISCUSSION

OHS treatment with urinary gonadotrophins in mice resulted in a significant increase in the frequency of cervical ribs in the offspring. In addition, we observed a prolonged gestational period, an increased litter size and a low birth weight, in agreement with earlier results on the effect of OHS treatment in mice (Ertzeid and

Storeng, 2001; Van der Auwera and D'Hooghe, 2001; Sibug et al., 2002, 2004). The prolonged gestational period and low birth weight in the treatment group indicate growth retardation. Ossification was also delayed by the OHS treatment, as apparent from the diminished response to silver nitrate.

In mice, cervical ribs are induced at E.D. 7 and 8, during the early organogenesis stage (Abdulrazzaq et al., 1997). The high frequency of cervical ribs, therefore, indicates that the OHS treatment affects early embryogenesis, a period that is highly sensitive to teratogenesis (Galis and Metz, 2001). This implies that, at least in our experiments with mice, the OHS treatment of the mother has a prolonged effect and continues during the early vulnerable stages of pregnancy. Although the response of mice to the OHS treatment may be different from those of humans undergoing OHS treatment, it is possible that similar processes take place in humans. The high incidence of cervical ribs in our experiments and the many associations of cervical ribs with serious abnormalities in humans suggest that these data may have implications for the use of OHS treatments in humans.

We propose that more general variations of highly conserved traits such as the number of cervical vertebrae and the number of digits (see Galis et al., 2002) may be useful as indicators of medical risks.

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