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# ETHANOL EXPOSURE DURING GESTATION ALTERS *Igf2* EXPRESSION IN DEVELOPING MOUSE EMBRYOS

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**Summary:** Ethanol is a classic teratogen capable of inducing a wide range of developmental abnormalities. They include fetal growth retardation, craniofacial anomalies and neurological disorders. The etiology of this multiple defect syndrome, known as fetal alcohol syndrome (FAS), was studied in animal models that reproduce many of the attributes of the human disease. In this study, we examined how the expression of the imprinted gene *Igf2* was affected when ethanol was introduced during the preimplantation period. Alcohol was applied to pregnant mice from C57BL/6 strain on 3.5 and 4.5 day *post coitus* (dpc). P0, P1, P2 and P3 promoters of the *Igf2* gene were examined on 11.5 dpc after treatment with two different ethanol concentrations in order to mimic typical consumptions observed in the human society. We found that ethanol altered gene expression in a dose-dependent manner in the developing embryos. These results suggest that ethanol does affect the expression of growth-related imprinted genes and help us to understand the observed phenotypes and the role of epigenetics in FAS.

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**Key words:** Ethanol; fetal alcohol syndrome, imprinted genes, mouse embryo.

**Abbreviations:** Cq – quantification cycle; CNS – central nervous system; DMRs – differentially methylated regions; dpc – days *post coitus*; E – efficiency; FAS – fetal alcohol syndrome; ICR – imprinting control region; REST – relative expression software tool; RT-PCR – real time polymerase chain reaction.

## INTRODUCTION

Gestational exposure to alcohol leads to a range of effects collectively known as FAS. It is associated with broad domains: prenatal and/or postnatal growth retardation, central nervous system (CNS) abnormalities and distinctive facial features (Sampson et al., 1997). Timing of exposure, duration, amount, as well as genetic susceptibility determines the specific effects of ethanol (Michelle et al., 2013).

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During mouse embryogenesis there are some important developmental periods, most susceptible to teratogens like alcohol - preimplantation and gastrulation. The preimplantation period begins with fertilization and ends with blastocyst hatching and implantation in the uterus. During this period methylation patterns of the genome are reprogrammed. Epigenetic reprogramming is critical for the developing embryo in this early stage and affects epigenetic modifications and genome function (Reik et al., 2001). Experimental evidence suggests that ethanol can inhibit DNA methyltransferase and alter normal patterns of DNA methylation. Epigenetic modifications and correct levels of methylation are especially important for the proper regulation and expression of imprinted genes.

One of the best characterized imprinted gene cluster in mammalian genome is *Igf2/H19*, located on chromosome 7 in mouse and position 11p15.5 in humans. Depending on promoter usage, the *Igf2* gene expresses four different transcripts in mice. P0 transcript is the only one expressed in the labyrinthine layer of the mouse placenta, whereas transcripts P1 to P3 are found throughout the developing embryo and placenta (Moore et al., 1997). The product of the *Igf2* gene is a protein called insulin-like growth factor 2. This protein plays an essential role in the growth and development before birth and promotes cell growth and proliferation in primordial germ layers.

In a study performed in the laboratory of Chris Downing (Downing et al., 2011) it was shown that C57BL/6J (B6) mice were susceptible to growth retardation and a number of morphological malformations following *in utero* ethanol exposure. The FAS-like phenotypes observed in mouse models have been connected with a change of gene expression (Michelle et al., 2013).

Surprisingly, little attention has been given to the effect of *in utero* alcohol treatment on the expression of imprinted genes in early pregnancy. The effect of ethanol introduced on 3.5 and 4.5 dpc on the embryo growth as well as on the *Igf2* expression level was investigated.

## MATERIALS AND METHODS

### Alcohol treatments

Alcohol was applied to pregnant mice from C57BL/6 strain. 25% ethanol at a dosage of 0.015ml/g (2.9 g/kg) was administered intraperitoneally on 3.5 and 4.5 dpc to pregnant females from group 1. On the same day, a group 2 of pregnant females were treated with an acute dose of 0.030 ml/g of 25% ethanol (5.8 g/kg). A non-treated group was used as the control. These doses were used according to the model of Padmanabhan and Hameed (1988). Fetuses and placenta were collected on 11.5 dpc. Student's *t*-test was used to determine whether data sets of non-treated and ethanol-treated groups were significantly different from each other.

### Analysis of *Igf2* mRNAs expression

We examined mRNA levels of several *Igf2* transcripts following *in utero* alcohol exposure, using Real Time- PCR. All embryonic tissues were pooled for RNA extraction. RNA was extracted by GenElute Mammalian Total RNA Miniprep kit (Sigma). To convert mRNA transcripts to cDNA, we used Enhanced Avian HS-RT-PCR kit (Sigma). The reaction was performed in two steps. Real

Time quantitative RT-PCR was performed on cDNAs using Maxima SYBR green qPCR master mix (2x) and PikoReal 96-real time PCR system, (Thermo Scientific). *Hsp90ab1* was used for normalization of qPCR data as an endogenous reference. The PCR cycling program for *Igf2* and *Hsp90ab1* consisted of 10 min at 95°C, followed by 40 cycles of 30 s at 95°C, 30 s at 58°C, and 30 s at 72°C. The melting curve program was performed at 60-95°C, with a heating rate of 0.2°C. The investigated transcripts showed high real-time PCR efficiency rates for: *Igf2* P2- 2.1; *Igf2* P3 -1.94; *Hsp90ab1*- 2.0 in the investigated range from 125 to 0.125 ng cDNA input (n=2) with good linearity. Primer sequences for *Igf2*- P0, P1, P2, P3 were according to Hagègea et al. (2006) and for *Hsp90ab1* according to Ficz et al. (2011).

The relative expression ratio was calculated based on E of the target gene and E of the endogenous reference. These were calculated by using the equation $E = 10^{(-1/slope)}$ (Rasmussen, 2001). Deviations of Cq values (ΔCq) for the target and reference genes were obtained by subtracting averaged Cq values of the sample from averaged Cq values of the calibrator: (CqCalibrator) – (CqSample). The relative fold change and statistical analysis were calculated by using the equation $((ETarget)^{\Delta Cq\ (Target)}) / ((EEnd.\ ref.)^{\Delta Cq\ (End.\ ref.)})$ (Pfaffl, 2001) and REST (Pfaffl et al., 2002).

## RESULTS

### Embryo characteristics

*Somite stage*

Mouse embryos were collected from control and ethanol-treated mother mice on 11.5dpc. The embryos exposed *in utero* to ethanol at the higher dose of 5.9 g/kg reached approximately 16-28 somite stage in contrast to the control and the embryos treated with 2.9 g/kg ethanol which developed to the 25–40 somite stage. The control embryos and those treated with 2.9 g/kg ethanol had a similar appearance, while some of the embryos from the group treated with 5.8 g/kg ethanol revealed distinct craniofacial abnormalities and internal hemorrhages during the whole period.

*Weight Data*

The embryonic weights on 11.5 day of gestation were as follows: for the non-treated control group the average body weight was 52.8 ± 2.71 mg whereas for the ethanol-treated groups the average body weight was: on 3.5 dpc and administration of 2.9 g/kg – 48 ± 3.54 mg ($p<0.5$); on 3.5 dpc and administration of 5.8 g/kg – 34.14 ± 1.78 mg ($p<0.001$); on 4.5 dpc and administration of 2.9 g/kg – 49.8 ± 0.98 mg ($p<0.5$); on 4.5 dpc and administration of 5.8 g/kg – 35.38 ± 2.11 mg ($p<0.001$). The effects of 5.8 g/kg alcohol on embryo weights are indicated in Figs.1 and 4.

*Embryos resorption*

Embryo resorptions were identified on 11.5 dpc for all test groups. In total, the resorption of non-treated embryos was 10.8%. The rate of resorption of ethanol-exposed embryos was significantly increased. Embryos from females treated with 2.9 g/kg on 3.5 dpc and 4.5 dpc showed a 6-fold increased resorption rate. At the higher dose of 5.8 g/kg and the same time interval resorptions increased approximately 7-fold (data not shown).

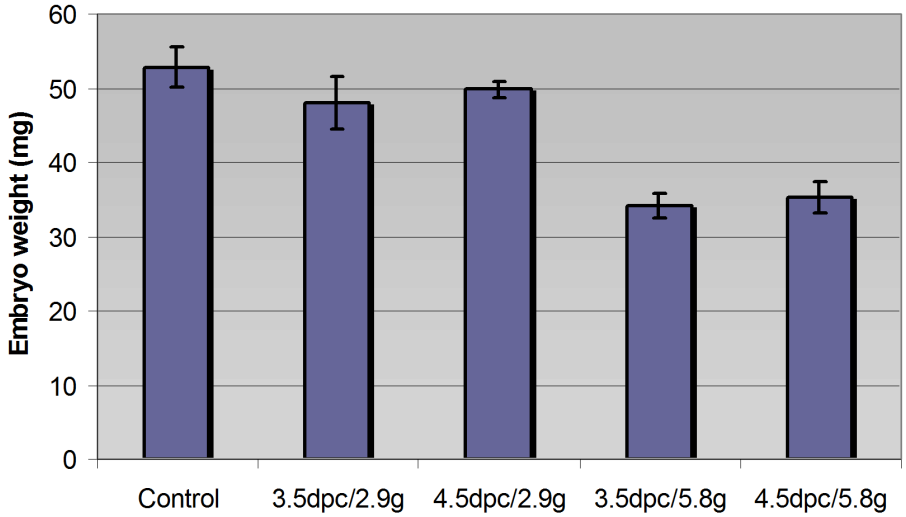


**Figure 1.** Mean weight of the offspring of gestational ethanol-treated groups on 3.5 dpc and 4.5 dpc treated with 2.9 g/kg and 5.8 g/kg ethanol and harvested on 11.5 dpc. Data are presented as means ± SE.

**Expression of *Igf2* mRNAs**

*Analysis of Igf2 relative gene expression with fluorescent quantitative RT-PCR*

The Pfaffl method (Pfaffl M., 2001) was used for analysis of all four promoters of *Igf2* (P0, P1, P2 and P3) on 11.5 dpc. *Hsp90ab1* was chosen as a reference gene or endogenous standard because of its constant expression, independent from alcohol treatment. *Igf2*-P0 is a specific placental promoter. In the embryo we found a low expression level, which remained unchanged after exposure to ethanol. Similarly, there was no difference in the expression of P1 promoter.

*Expression of Igf2-P2 mRNAs*

Real-time quantitative PCR analysis revealed that *Igf2*-P2 mRNA expression in embryos from females exposed to 2.9 g/kg ethanol on 3.5 dpc in comparison with that in the control non-treated embryos was up-regulated 2.27-fold ($p$<0.07) and did not change in embryos from females exposed to 2,9 g/kg ethanol on 4.5 dpc. In the embryos from females treated with the higher dose of ethanol (5,8 g/kg) on 3.5 dpc, the expression of *Igf2*-P2 mRNA was up-regulated 4.38-fold ($p$< 0.05), and with the same dose on 4.5 dpc the expression was changed by a factor of 3.37 ($p$<0.7) compared to non-treated smaples. The effects of alcohol on *Igf2-P2* expression are shown in Fig 2.

*Expression of Igf2-P3 mRNAs*

In embryos from females exposed to 2.9 g/kg ethanol on 3.5 dpc the expression of *Igf2*-P3 was up-regulated 1.6-fold ($p$<0.01) whereas after treatment with the same dose on 4.5 dpc it was up-regulated 2.5-fold ($p$<0.05). The higher dose of 5.8 g/kg ethanol on 3.5 dpc caused up-regulation of P3 expression by a factor of 3.1 ($p$<0.01). The most significant change of the expression level was found in embryos from females treated on 4.5 dpc with 5.8 g/kg ethanol (6.8-fold , $p$<0.01) (Fig. 3) .

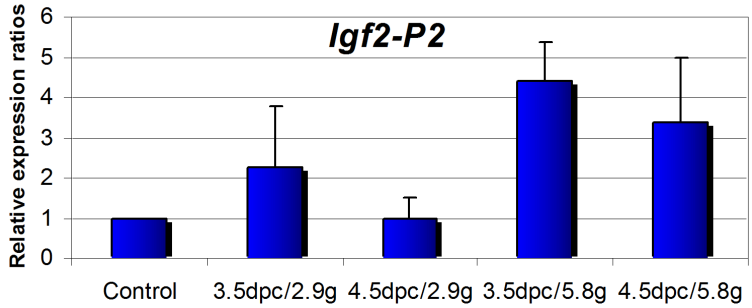


**Figure 2.** Analysis of relative *Igf2*-P2 gene expression with fluorescent quantitative RT-PCR. The Pfaffl method was used to analyze *Igf2*-P2 expression on 11.5 dpc after treatment with: 2.9 g/kg ethanol on 3.5 dpc and 4.5 dpc; 5.8 g/kg ethanol on 3.5 dpc and 4.5 dpc. *Hsp90ab1* was used as a reference gene. Data are presented as means ± SE.

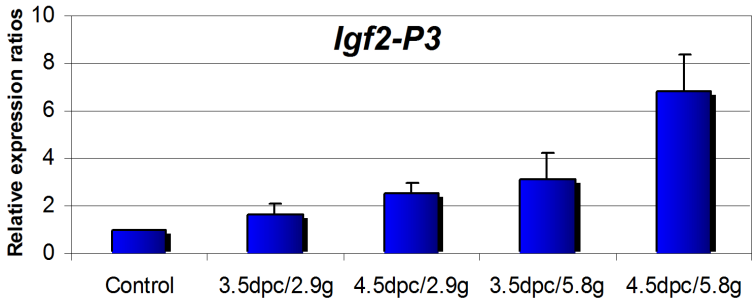


**Figure 3.** Analysis of relative *Igf2*-P3 gene expression with fluorescent quantitative RT-PCR. The Pfaffl method was used to analyze *Igf2*-P3 expression on 11.5 dpc after treatment with: 2.9 g/kg ethanol on 3.5 dpc and 4.5 dpc; 5.8 g/kg ethanol on 3.5 dpc and 4.5 dpc. *Hsp90ab1* was used as a reference gene. Data are presented as means ± SE.

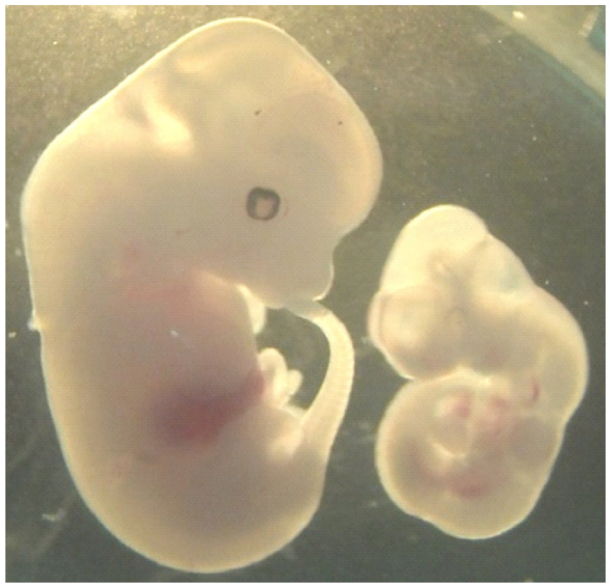

**Figure 4.** Normal embryo (on the left) and embryo after treatment with the higher dose of ethanol (5.8 g/kg) on 3.5 dpc, (on the right). Both embryos are at the same stage - 11.5 dpc**.**

## DISCUSSION

### Growth retardation and resorption process

Our results indicated that administration of ethanol during the preimplantation period of pregnancy induced growth retardation in close agreement with previous studies dealing with *in vivo* effect of ethanol during the same period (Mitchell et al., 1994). Padmanabhan and Hameed (1988) reported strict mouse embryos growth retardation in 80% -100% of embryos assessed in midgestation on 15 dpc after administration of 5.8 g/kg ethanol in the period 1.5–6.5 dpc.

In our study, we found significant dose-dependent changes in the embryo weight. Resorption rates increased dramatically in the group injected with 2.9 g/kg ethanol as well as with the higher dose of 5.8 g/kg ethanol compared to control. In contrast to the study of Haycock and Ramsay (2009) showing significant weight differences, the resorption rates were not substantially different between the two treated groups.

Based on our data growth retardation can not be strongly associated with the resorption process. The low alcohol dose (2.9 g/kg) affected embryo growth to a smaller extent in comparison to the higher dose (5.8 g/kg) and did not cause pathological forms, but increased resorption rates to a level similar to that of 5.8 g/kg. The higher ethanol dose was a potentially teratogenic dose, influencing negatively normal growth and development of embryos, leading in some embryos to craniofacial abnormalities and internal hemorrhages. Our results are in agreement with previous studies in which alcohol was administered to mice during blastocyst implantation and resulted in resorptions, reduced fetal weight and malformations (Padmanabhan et al., 1988).

### Analyses of gene expression

Imprinting is sensitive to the preimplantation environment and as suggested by Haycock and Ramsay (2009) ethanol-induced imprinting defects might be a mechanism of ethanol teratogenesis. In a previous study (Downing et al., 2011) it was found that 5.8 g/kg ethanol exposure *in utero* on 9.5 days of gestation altered the *Igf2* expression and metylation levels. Our results showed no change in the expresson of P0 and P1 promoters of *Igf2*. Statistically significant up-regulation was found in the expression of P2 after treatment with 2.9 g/kg ethanol on 4.5 dpc ($p<0.05$) and at the higher dose (5.8 g/kg ethanol) on 3.5 dpc ($p<0.05$). Normally P3 was the most expressed promoter among all 4 analysed promoters and its expression increased significantly after 2 days of treatment with both ethanol doses.

The function of *Igf2/H19* locus is determined by a strict methylation level of three DMRs associated with *Igf2* and a single DMR associated with *H19* ICR. The human studies have shown that alcohol consumption decreases methylation level of *Igf2/H19* DMRs (Meyers, 2012). Our hypothesis was that *in utero* ethanol exposure would result to modulation of DNA methylation in paternal alelle with a subsequent decrease in *Igf2* mRNA levels. Surprisingly, the level of *Igf2* expression increased in developing embryos, while in the placenta it was found to decrease (unpublished results). Alcohol can affect the placenta and the fetus by different mechanisms, or the mechanism could be

the same, but the expression level in the embryo increases in order to compensate the reduced function of the placenta. The data presented in this study are preliminary and further studies are need in order to examine the levels of methylation and their interaction with gene expression and the imprinted status of the gene after ethanol exposure.

## ACKNOWLEDGEMENTS

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