

GAPDH SHOWS ALTERED GENE EXPRESSION IN ALCOHOL MODELS

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Abstract. Background and objectives. Though glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) is one of the most commonly used housekeeping genes for comparison of gene expression data, studies have revealed that *Gapdh* expression is not constant at different developmental stages, and is modulated by many factors including ethanol. In view of this, in the present study, we investigated the effect of ethanol on *Gapdh* stability and expression levels in different model systems. **Methods.** Evaluation of *Gapdh* stability was determined by comparison with three commonly used housekeeping genes in alcohol-exposed and control mouse blastocysts, embryos, and placentas (*ActB*, *Hsp90ab1*, and *Atp5b*), and in alcohol-dependent and healthy humans (*ACTB*, *ATP5B*, and *HSPCB*), and was ranked by the software program RefFinder. To detect the *Gapdh* expression patterns as a target gene, qRT-PCR analysis was applied. **Results.** The order of expression stabilities obtained by the RefFinder ranked *Gapdh* as the most unstable reference gene in studied groups. The study of the expression dynamics of *Gapdh* showed significant upregulation in ethanol-exposed mouse blastocysts and embryos ($FC = 1.56$, $p = 0.05$ and $FC = 6.0$, $p = 0.01$) and downregulation in placentas ($FC = 0.60$, $p = 0.01$). **Conclusions.** Our results revealed that the expression of *Gapdh* can vary in different model systems under ethanol exposure and imply the need for caution when using it as an internal control. This is the first report for statistically significant change in *Gapdh* expression after ethanol exposure during preimplantation mouse development.

Key words: *Gapdh*, *ACTB*, *ATP5B*, *HSPCB*, alcohol, qRT-PCR

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INTRODUCTION

Alcohol dependence (AD) is a chronic and relapsing disorder, which includes a combination of physiological, behavioural and cognitive phenomena. Data provided by the World Health Organisation (WHO) shows that about 2.3 billion people aged 15 years and above had abused with drinking

alcohol in the previous 12 months. Total alcohol per capita consumption rose from 5.5 liters of pure alcohol in 2005 to 6.4 liters in 2016. The condition has been identified as a component of about 200 diseases, injuries and other health issues. Alcohol usage during pregnancy may cause a number of teratogenic abnormalities including growth restraint, craniofacial malformations and neurologic impairment [1].

Alteration in gene expression patterns is one mechanism by which psychoactive substances may induce physiological and behavioral dysfunction. Alcohol intake is shown to cause changes in gene expression patterns, yielding in cellular and molecular alterations. Specific ethanol-binding sites on a number of proteins were shown to act directly or indirectly to produce a biological effect [2]. Of the techniques developed to measure target gene expression, the quantitative reverse transcription PCR (qRT-PCR) is most widely applied [3]. The accuracy of the method, however, strongly depends on the internal normalization of gene expression data by reference genes [4, 5]. An endogenous reference gene is a housekeeping or maintenance gene, which encodes factors engaged in cellular processes that are essential for the survival of the cell, and are therefore expressed constitutively [6]. However, studies have shown that different biotic and abiotic factors are able to cause variations in the expression of housekeeping genes, which are otherwise stable [7-9].

Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) is one of the most commonly used housekeeping gene for comparison of gene expression data. It is a key regulatory enzyme, which catalyzes the oxidative phosphorylation of glyceraldehyde-3-phosphate during glycolysis and is involved in a variety of cellular processes like DNA repair, membrane fusion, and cell death. Though *Gapdh* has been traditionally applied as an endogenous control in qRT-PCR to normalize gene expression data, problems concerning its usage have been pointed out. First, many studies have provided a powerful demonstration that the transcription levels of *Gapdh* are not constant at different developmental stages, in the different model systems, organs, and tissue samples [10-12]. Second, *Gapdh* expression is sensitive to environmental and experimental conditions. Data have revealed that *Gapdh* mRNA levels are modulated from many factors such as heat shock, oxidative stress, iron-chelating agent, growth factors, insulin [13], as well as ethanol [14].

Based on these, in the present study, it was hypothesized that *Gapdh* gene expression is sensitive to change under ethanol exposure. To address this, we investigated the effect of ethanol on *Gapdh* stability in different model systems. Prior to examination of the expression levels of *Gapdh* as a target gene, we evaluated its stability as a reference gene, comparing it to three commonly used housekeeping genes – *ActB*, *Hsp90ab1*, and *Atp5b*. Stability analysis was evaluated and ranked by RefFinder. For evaluation of *Gapdh* sensitivity in different experimental conditions, we chose four different ethanol-exposed model systems: *in vitro* exposed mouse blastocysts; trans-

planted embryos, and their placentas obtained after transplantation of the exposed blastocysts; *in vivo* exposed mouse embryos and their placentas; and alcohol-dependent humans.

MATERIALS AND METHODS

Mouse blastocysts, embryos, and placentas

The C57BL/6 strain was purchased from the Velaz, s.r.o. (Prague, Czech Republic). Blastocysts were obtained at 3.5 dpc as described previously [15]. Briefly, blastocysts were obtained by flushing the oviduct and the uterus with Dulbecco's buffer (Sigma) and cultured at 37°C for 4 hr in sealed tubes containing 500 µl P1 medium optimized by adding 400 mg/100 ml albumin, and EDTA to concentration of 100 µM. A filtered gas mixture of 5% O₂, 5% CO₂, and 90% N₂ was used to achieve optimal pH (7.2-7.4). In total, 176 blastocysts were obtained from 22 pregnant mice. The blastocysts were separated into two groups: 50% were used for control and 50% were cultivated with the addition of 0.2% ethyl alcohol (v/v) for 4 hr at the above conditions. The dose of 0.2% ethanol was chosen according to our previous results [15], determining it as the most accelerating blastocyst growth and development. The dose is suitable for obtaining more blastocyst, matured for transplantation as well as for the investigation of ethanol's effects.

Transplanted embryos and their placentas were obtained from *in vitro* cultivated blastocysts in 0.2% ethanol for 4 hr, as described in the previous section, and implanted in pseudo-pregnant females. Six biological replicates were used: six embryos and six placentas, and the same set for controls, harvested at 11.5 dpc.

Embryos and their placentas were obtained according to the experimental model of Padmanabhan and Hameed [16]. The model investigated, the *in vivo* effect of an acute dose of 5,8 g/kg ethanol and its half – 2,9 g/kg ethanol on mouse embryogenesis. Briefly, doses of 0.015 ml/g (2,9 g/kg) and 0.030 ml/g (5,8 g/kg) of 25% ethanol for the first group and the second group, respectively, were administered intraperitoneally at 3.5 dpc to pregnant females. A non-treated group was used as a control. Embryos and placentas were removed from the uterus at 11.5 dpc. In total, there were 10 samples in each group: 2 embryonal and 2 placental samples from 5 mothers were pooled. All animal procedures were performed according to the Principles of Laboratory Animal Care and approved by the Ethical Committee for Animal Experiments at the National Veterinary Service of Bulgaria (3/11.04.2007).

Alcohol-dependent human participants and healthy controls

Additionally, the study recruited 13 patients with Alcohol Dependence (AD) (mean age $\pm$ SD = 41 $\pm$ 9 years, 8 males and 5 females), who were included in detoxification and psychosocial rehabilitation program at the Sofia State Psychiatric Hospital for Treatment of Drug and Alcohol Addictions, and 8 clinically healthy volunteers (mean age $\pm$ SD = 44 $\pm$ 9 years, 2 males and 6 females). Diagnosis was done according to the ICD-10 diagnostic system and confirmed by review of the existing medical records. All participants donated a 5-6 ml blood sample, which was transferred to the laboratory and processed within 2 hr to avoid RNA degradation. Written informed consent was obtained from each participant prior to the procedure. The study was carried out in accordance with the Declaration of Helsinki and approved by the Local Research Ethical Committee (IPPG-BAS 1/20.03.2019).

Total RNA isolation and cDNA synthesis

RNA was extracted from mouse blastocysts, embryos and placentas, and from human blood using Quick-RNA Micro Prep kit (Zymo Research, USA), GenElute Mammalian Total RNA Miniprep Kit (Sigma, USA), and QiAamp RNA Blood mini kit (Qiagen, Germany), respectively as described by the manufacturer. cDNA was synthesized using Sensiscript Reverse Transcription (Qiagen, Germany) for mouse blastocysts, Enhanced Avian HS RT-PCR kit (Sigma, USA) for embryos and placentas, and FIREScript RT cDNA Synthesis Mix (Solis Biodyne, Estonia) for human blood.

qRT-PCR analysis

qRT-PCR was performed on mouse cDNAs using Maxima SYBR Green qPCR master mix (Thermo Scientific, USA). PCR cycling and melting curve programs for *ActB*, *Atp5b*, *Hsp90ab1*, and *Gapdh* and primer sequences for *Hsp90ab1* and *Atp5b* are described elsewhere [17]. Primer sequences for *Gapdh* and *ActB* are according to Kharroubi et al. [18] and Bohn et al. [19], respectively. qRT-PCR was performed on human blood cDNA using 5x HOT FIREPol EvaGreen qPCR Mix Plus (Solis Biodyne, Estonia) as described by the manufacturer. The PCR cycling program included 15 min at 95°C, followed by 40 cycles of 15 sec at 95°C, 30 sec at 60°C, 45 sec at 72°C, and final elongation for 5 min at 72°C. Primer sequences for *Gapdh*, *ACTB*, and for *HSPCB* and *ATP5B* are according to Kala et al. [20], Gandhirajan et al. [21], and Cavalcanti et al. [22], respectively. All assays were conducted in a PikoReal 96-real time PCR system (Thermo Scientific, USA).

Data analysis

Statistical evaluation of gene expression was performed using Relative Expression Software Tool (REST) [23]. The expression stability of the analyzed reference genes was evaluated and ranked by RefFinder (<https://www.heartcure.com.au/reffinder/>) [24].

RESULTS

Evaluation of *Gapdh* stability

Evaluation of *Gapdh* stability was determined by comparison with three other housekeeping gene transcripts in alcohol-exposed and control mouse blastocysts, embryos, and placentas (*ActB*, *Hsp90ab1*, and *Atp5b*), and in alcohol-dependent and healthy humans (*ACTB*, *ATP5B*, and *HSPCB*) (Fig. 1). The order of expression stabilities obtained by the RefFinder ranked *Gapdh* as the most unstable gene. In eight of the twelve studied groups, *Gapdh* was at the bottom of the ranking. In the group of embryos exposed to 5,8 g/kg ethanol, the average expression stability values crossed the cut-off value of 1.0 (data not shown), indicating that it cannot be a reliable endogenous standard for normalization in the studied model systems.

Expression dynamics of *Gapdh*

We further investigated the expression dynamics of *Gapdh* as a target gene in all alcohol-exposed mouse groups and in the group of humans with AD (Fig. 2). For normalization of *Gapdh* relative expression, we used the first two ranked reference genes for each group. We found upregulation of *Gapdh* to 1.56 in mouse blastocyst exposed to 0.2% ethanol, and a significant increase in *Gapdh* expression to 6.0 in mouse embryos exposed to 5,8 g/kg ethanol compared to control. The higher dosage of 5,8 g/kg of ethanol reduced *Gapdh* expression in mouse placentas down to 0.60. *Gapdh* mRNA expression did not show a significant change in the other studied groups.

DISCUSSION

Though glyceraldehyde-3-phosphate dehydrogenase is one of the most commonly used housekeeping gene for comparison of gene expression data, studies have revealed that *Gapdh* expression is not constant at different developmental stages, and is modulated by many factors including ethanol. In this study, we aimed to investigate *Gapdh* expression patterns as a reference and a target gene upon ethanol exposure in *in vivo* and *in vitro* mouse model systems and in humans with alcohol-dependence.

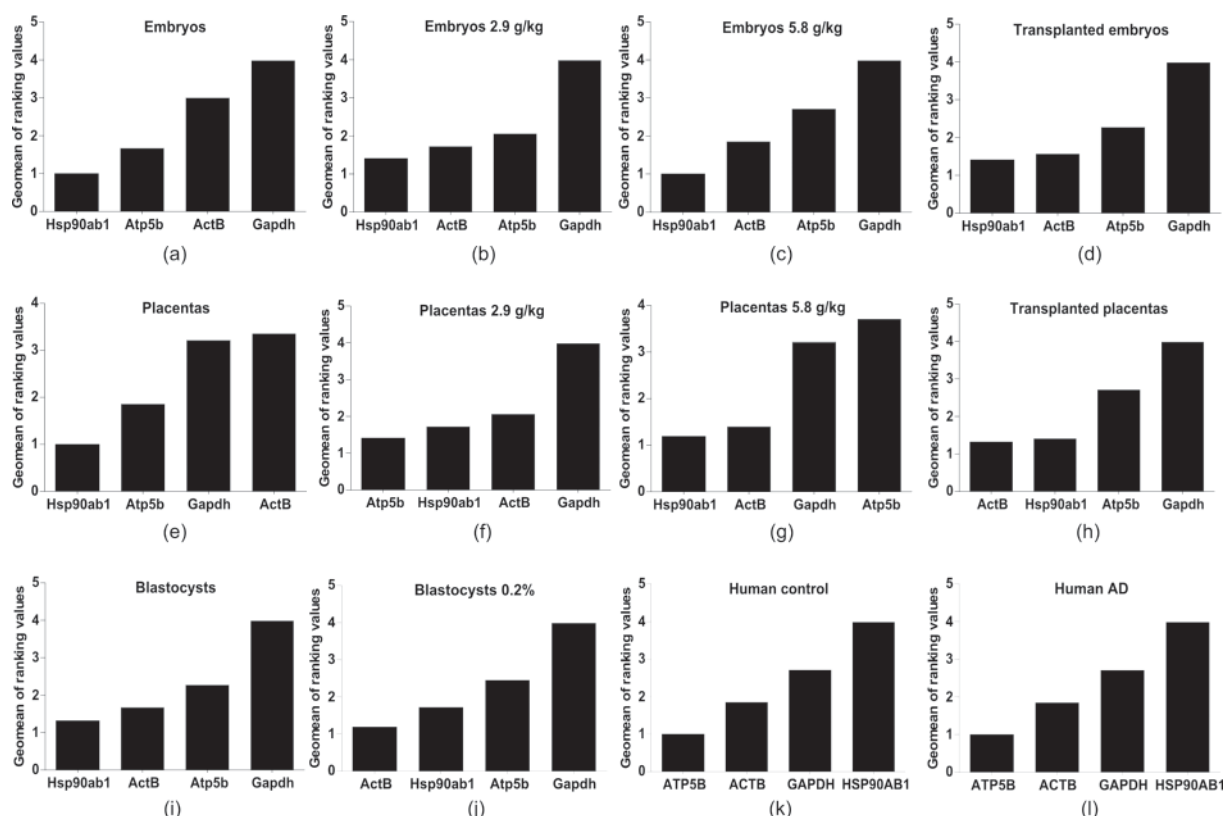


Fig. 1. *Gapdh* ranking by RefFinder. The lower ranking value indicated the higher stability of gene expression. (a) control mouse embryos; (b) embryos exposed to 2,9 g/kg ethanol; (c) embryos exposed to 5,8 g/kg ethanol; (d) transplanted embryos; (e) control mouse placentas; (f) placentas exposed to 2,9 g/kg ethanol; (g) placentas exposed to 5,8 g/kg ethanol; (h) placentas of transplanted embryos; (i) control mouse blastocysts; (j) blastocysts exposed to 0.2% ethanol; (k) human healthy control volunteers; (l) human participants with AD

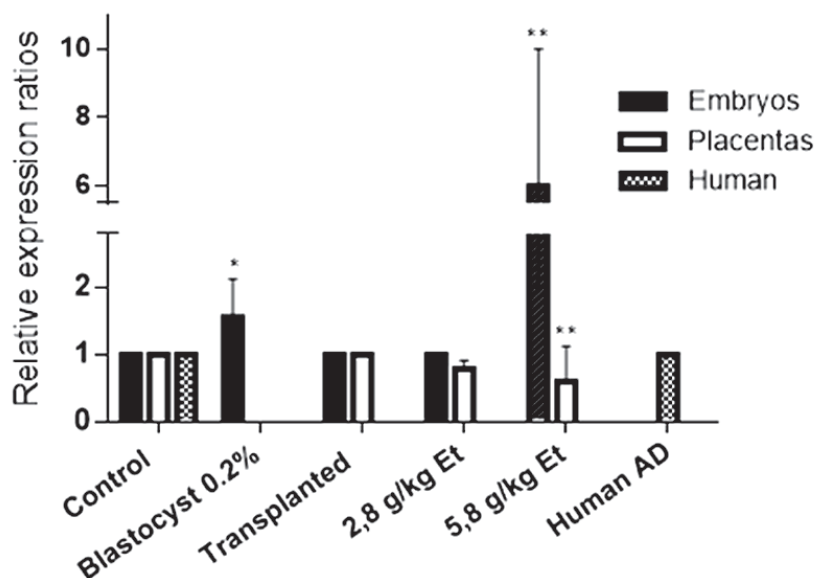


Fig. 2. Changes in *Gapdh* expression revealed by qRT-PCR in mouse embryos, blastocysts, and placentas exposed to ethanol and in human participants with AD

We evaluated *Gapdh* stability as a reference gene, comparing it to three commonly used housekeeping genes – *ActB*, *Hsp90ab1*, and *Atp5b*. The *ACTB* (β -actin) is widely used as control due to its relatively high level of expression in all cell types and its his-

torical use as a reference gene in nonquantitative assays such as northern blot analysis. The other two endogenous standards, Heat shock protein 90-beta (*Hsp90ab1*) and ATP synthase F1 subunit beta (*Atp5b*), were selected according to data by Ander-

sen et al. [25] classifying them among the top ranked candidates that will introduce the least systematic error when used.

Our results ranked *Gapdh* as the most unstable of the studied reference genes indicating that it cannot be a reliable endogenous standard for normalization in the studied model systems. The expression levels of *Gapdh* were shown to vary between individuals, during pregnancy, and in development [11,12]. In our experiments, we detected unstable *Gapdh* expression patterns in control, untreated blastocyst, embryos, and placentas which is in agreement with data obtained from Gu et al. [26], where *Gapdh* was considered one of the least stable genes at various mouse developmental stages. Moreover, ethanol exposure further increased its variability. The highest doses of ethanol applied *in vivo* can alter *Gapdh* expression to the point that makes it unsuitable for normalization control.

Based on these results and the available literature data revealing that *Gapdh* expression can vary strongly depending on the system being studied and the experimental conditions, we investigated the expression dynamics of the gene as a target one in all alcohol-exposed mouse groups and in the group of humans with AD. *Gapdh* mRNA levels were shown to be modulated under many circumstances, both *in vitro* and *in vivo*, including ethanol exposure. Ou et al. [14] investigated *Gapdh* protein levels in ethanol-treated *in vitro* models of glioblastoma and neuroblastoma, in the prefrontal cortex from human subjects with alcohol dependence and in rats fed with ethanol diet. The results showed a significant increase in *Gapdh* levels upon ethanol exposure. In another study *Gapdh* variability was examined in biopsy liver samples of patients with alcoholic liver disease [27]. The authors found that *Gapdh* was decreased with steatosis and increased with alcoholic hepatitis and fibrosis. In the present study, we found altered *Gapdh* expression both in *in vitro* and *in vivo* mouse models but not in the group of humans with AD. It is possible that these differences result from the different levels of proliferation and differentiation. The mouse models were exposed to ethanol during preimplantation – the period characterized by rapid proliferation. However, cellular proliferation is one of the most recognized modulators of *Gapdh* levels [13]. Thus, the high proliferation levels along with ethanol exposure can make *Gapdh* mRNA expression more sensitive in mouse models. Moreover, ethanol neutralization is limited in poorly differentiated preimplantation embryo cells due to their immature enzyme system, which suggests more strong toxic effect in this model. The effect of ethanol in different experimental mouse groups, however,

was different. *Gapdh* expression was up-regulated in the blastocysts and in embryos exposed to 5,8 g/kg ethanol, but not in the embryos exposed to low doses of ethanol and transplanted embryos. In blastocysts, the effect of 0,2% ethanol can be detected immediately after exposure, but after their transplantation, it is not retained through the middle of gestation (11.5 dpc). The effect on *Gapdh* mRNA levels was not detected also at 11.5 dpc after exposure of low dose of ethanol. These results indicate that only exposure to high doses of ethanol leads to long-term changes in gene expression. In placentas, the changed *Gapdh* expression can be detected again after exposure to high dose of ethanol; however, the effect was the opposite – down-regulation of *Gapdh* expression. The different response to ethanol exposure in embryos and placentas suggests that the mechanisms of alcohol action are tissue specific.

CONCLUSION

To summarize this, in our observation for *Gapdh* stability as a reference gene in eight of the twelve investigated groups, *Gapdh* was at the bottom of the ranking. The above results clearly indicate that the expression levels of *Gapdh* can vary in different model systems under ethanol exposure and imply the need for caution when using it as a control. The present study performed detailed analysis on the dynamics of *Gapdh* expression and showed significant upregulation in ethanol-exposed mouse blastocysts and embryos, and downregulation in placentas. Future investigation on alcohol-responsive *Gapdh* transcriptional changes in preimplantation mouse model systems are needed to assess the mechanisms underlying the observed changes in expression.

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