Effects of alcohol on H3K9 acetylation in mouse pre-implantation embryos

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ABSTRACT

It is well known that excessive long-term alcohol consumption is harmful, especially in pregnant women. In the present study, the Kunming white mouse was used as an animal model and indirect immunofluorescence was performed to analyze the toxic effects of alcohol on early pre-implantation embryos. H3K9 acetylation immunofluorescence could not be detected in MII oocytes. H3K9 acetylation levels in the treatment group were higher than in the control group during the morula stage, and contrary to results during the blastocyst stage. Other stages showed no obvious differences for in vivo embryos. For in vitro embryos, almost no difference was found between the two experimental groups across all stages, and both groups showed increasing H3K9 acetylation levels (except at the 2-cell stage). This study shows that H3K9 acetylation levels in early pre-implantation embryos are notably impacted by excessive alcohol ingestion by females. These data are the first step in understanding the epigenetic mechanism of alcohol toxicity in early pre-implantation mouse embryos.

Keywords: Alcohol intake; Pre-implantation embryos; H3K9 acetylation; Mice; Embryo development

INTRODUCTION

Long-term excessive drinking is severely harmful, especially in pregnant women. Fetal alcohol spectrum disorder (FASD), with syndromes including pre- and postnatal developmental retardation, craniofacial paramorphia, central nervous abnormalities, congenital heart diseases and mental and behavior disorders, can be induced by excessive drinking during progestation or gestational periods (Mattson et al, 2011). Studies into the toxicology of alcohol have mainly focused on the effects of excessive drinking on tissues, such as alcohol induced fatty liver (Li, 2003), cerebral injury (Zhao & Feng, 2012) and retinopathy (Jiang et al, 2007), or neonatal risks and syndromes following maternal alcohol exposure (Mattson et al, 2011). Few studies have been done on the toxicology of alcohol on early pre-implantation embryos in mice. Huang et al (2013a,b) found that alcoholism reduces germocyte activity and induces abnormal DNA methylation in early pre-implantation embryos. The aim of the present study is to understand the effects of alcoholism on H3K9 acetylation levels of pre-implantation embryos in mice from an epigenetic perspective.

The influence of epigenetic modification on biological phenotype has become a research hotspot. Most congenital diseases are the result of genetic and environmental factors and genetic mutation itself may not fully explain their nosogenesis. Epigenetic modifications include DNA methylation, genomic imprinting, histone modification and non-encoding RNA, etc. Histones are highly alkaline proteins found within chromosomes that package and order DNA into structural units. Histone H3 and H4 feature a N-terminal tail protruding from the globular nucleosome core, which can undergo several different types of post-translational modification that influence cellular processes, including reversible covalent modifications, such as histone acetylation, etc. Histone acetylation plays a vital role in the development of mammals and its abnormality results in chromatin reorganizing, and genetic transcriptional disorders in the cell cycle, cell differentiation and apoptosis (Vigushin & Coombes, 2002). Histone acetyltransferase (HATs) and histone deacetylases (HDACs) are a pair of enzymes that regulate genetic acetylation and deacetylation. By adjusting the

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electrostatic attraction and sterical hinderence between DNA and histone, thereafter influencing the tightness of chromosomes, HATs and HDACs can repress or initiate gene transcription (Haycock, 2009). Histone H3K9 acetylation is one of the signs of gene transcription initiation (Morinobu et al, 2004) and its abnormal acetylation can induce abnormal chromatin structure and abnormal gene expression. Fetal malformations or abnormal developments are usually the result of protein misexpression. For example, imbalanced histone acetylation can cause the misexpression of cardiogenesis-related genes and bring on obstacles during the differentiation of mesenchymal stem cells (MSCs) to myocardial cells (Lin, 1981). Fisher et al (1986) reported that alcohol elevates acetylation levels of histone H3K9 in myocardial progenitor cells and then induces misexpression of cardiogenesis-related genes.

Earlier studies from our group suggest that epigenetic modifications of pre-implantation embryos in mice are extremely sensitive to environmental change. Alcohol-induced genomic DNA methylation in germocytes and zygotes, H3K9 methylation and H3K27 acetylation could interfere with the orderly establishment of epigenetic patterns in pre-implantation embryos in mice (Huang et al, 2013a, 2013b). To better understand epigenetic patterns under the influence of alcohol we force-fed Kunming (KM) mice with alcohol to stimulate external inducing conditions of FASD, and then monitored the acetylation level of H3K9.

MATERIALS AND METHODS

Experimental animals

Female KM mice (4–5 weeks old, 30±2 g) were provided by the Experimental Animal Center of Anhui Medical University and were housed at 24±2 ℃ with lights on from 0800h–2200 h. Food and water were available *ad libitum*. Animals were allowed to adapt to the housing condition for one week. All animal procedures were carried out in accordance with Fuyang Teachers College Animal Care and Use Committee.

Experimental reagents and equipments

Pregnant mare serum hormone (PMSG) and human chorionic gonadotrophin (hCG) were products of the Second Hormone Factory of Ningbo, China. Saline water was from Shanghai Huayuan Anhui Jinhui Pharmaceutical, China. The antibody of H3K9 acetylation was from Epigentek Group Inc. (New York, USA). Secondary goat-anti-rabbit antibody, IgG-FITC, was from Bioss Biotechnology (Beijing, China). Petri dishes were from Corning and all other reagents were products of Sigma. Equipment included a stereomicroscope (SMZ1500, Nikon, Japan), CO_2 incubator (2323-2, Thermo, USA), electronic balance (AR224CN, Ohaus, USA) and laser scanning cofocal microscope (TCS-SP5, Leica, Germany).

Animal model establishment and animal grouping

Animal models were established according to previous publications (Dole & Gentry, 1984; Dole et al, 1985; Griffin et al, 2009; Hwa et al, 2011) indicating that the behavioral and physiological features in mice force-fed with 20% (4–5 g/kg) alcohol bear a resemblance to those in human alcoholics. Mice were randomly separated into *in vivo* and *in vitro* groups. In the *in vivo* group, animals were further divided into an alcohol group and control group. The alcohol treatment animals (*n*=20) were force-fed 20% (4-5 g/kg) alcohol for 30 days and then force-fed saline water (4-5 g/kg) for another 4 days, *ad libitum*; control animals (*n*=20) were force-fed saline water 34 days. In the *in vitro* group, animals were divided into an alcohol group and control group. The alcohol treatment animals (*n*=10) were force-fed 20% (4-5 g/kg) alcohol for 30 days *ad libitum*; the control animals (*n*=10) were force-fed saline water 30 days. Zygotes and embryos at different stages were cultured in Petri dishes for 4 days.

Collection of MII stage ovum and pre-implantation embryos from *in vivo* and *in vitro* groups

Animals were given 10 U PMSG (i.p.) at 1730h on the last day of force-feeding and given 10 U hCG (i.p.) 48 hours later.Five females were randomly selected to not mate with males and 14 hours after hCG injection, their ampulla were punctured to collect MII stage ovum. The other females were mated with adult males (1:1). At 0800h on the day after mating, vaginal suppositories were checked and if the vaginal suppository was identified then female fertilization was confirmed. *In vivo* flushing method (using syringe, HCZB fluid and observation under a stereomicroscope) was applied in the *in vivo* group to collect zygotes (at 14 hours after fertilization and zygotes can be obtained by puncturing ampulla directly), 2-cell-, 4-cell-, 8-cell-embryo, morula and blastula (at 30, 48, 60, 72 and 88 hours after fertilization) at different stages of embryonic development. For the *in vitro* group, zygotes collected 14 hours after fertilization were rinsed with CZB sugar-free culture fluid and then cultured with CZB fluid (sugar-free) in the incubator at 37 ℃, 5% CO_2. Forty-eight hours later, 4-cell-embryos were transferred into CZB fluid (with sugar) and cultured in the incubator at 37 ℃, 5% CO_2. Zygotes, 2-cell-, 4-cell-, 8-cell-embryo, morula and blastula collected *in vitro* were rinsed with PBS (0.1% PVA), fixed with 4% paraform and saved.

Indirect immunofluorescence detection of H3K9 acetylation in pre-implantation embryos at different developmental stages

Fixed zygotes and embryos at different stages were rinsed with PBS (0.1% PVA) and then incubated in PBS with 0.2% TritonX-100 at 37.5 ℃ for 1 hour; rinsed with PBS (0.1% BSA); sealed with PBS (0.1% BSA) for 1-2 hours at 37.5 ℃; incubated in H3K9 acetylation antibody (1:100, diluted with PBS (1% BSA)) over night at 37.5 ℃; rinsed for 5 min, 3 times with PBS (0.1% Tween 20 and 0.01% TritonX-100); incubated in secondary goat-anti-rabbit antibody, IgG-FITC (1:100, diluted with PBS (1% BSA), inspired with 488 nm waves), in the dark for 3 hours at 37.5 ℃; rinsed with PBS (0.1% PVA) 3 times and then stained in propidium iodide (PI, 10 μg/mL, inspired with 568 nm waves) in the dark for 10 min; and finally rinsed with PBS (0.1% PVA) and fixed on slides for observing and photographing under a laser scanning cofocal microscope.

Statistical analysis

Fluorescence densities were analyzed using LAS AF Lite and Image Pro-Plus. Every sample group was repeated three times and at least 20 zygotes or embryos at different stages were examined. Corresponding fluorescence data were obtained by running Image Pro-Plus. Data from alcohol treatment groups and control groups were compared with *t*-tests (Kim et al, 2002). GraphPad Prism 5 was used to produce histograms and fluorograms.

RESULTS

H3K9 acetylation of MII stage ovum

Indirect immunofluorescence detection of H3K9 acetylation in MII stage ovum showed that no immunofluorescence could be found in the alcohol treatment groups or controls. However, immunofluorescences were found by DNA PI staining (data not shown).

Immunofluorescence of H3K9 acetylation in pre-implantation embryos at different developmental stages in the *in vivo* groups

Indirect immunofluorescence detection of H3K9 acetylation in pre-implantation embryos at different developmental stages in the *in vivo* groups is shown in Figure 1.

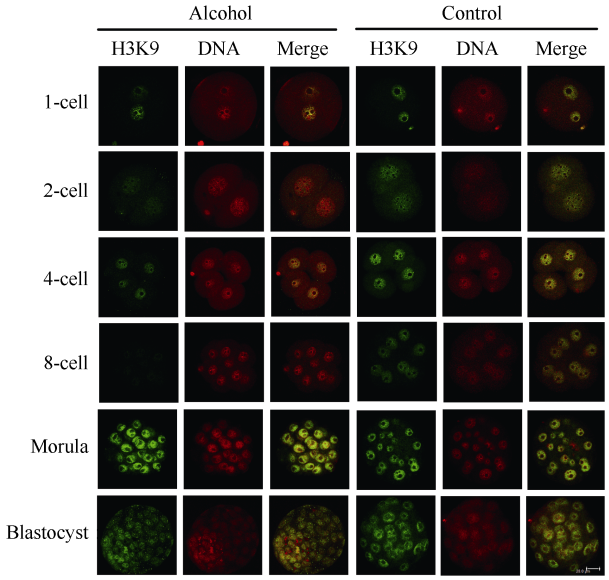

Figure 1 Different stages of mouse pre-implantation embryos and H3K9 acetylation immunofluorescence *in vivo*

1-cell, 2-cell, 4-cell, 8-cell, morula and blastocyst *in vivo* treatment and control groups collected separately. Embryos were immunostained with specific antibodies against resistance to H3K9 acetylation antibody (H3K9 in histone); in red (middle), DNA; Merged (right) . Scale bar=20 μm.

Semi-quantitative analysis of H3K9 acetylation in pre-implantation embryos at different developmental stages in the *in vivo* groups

The analysis of fluorescence data showed that at the morula stage, H3K9 acetylation levels in the *in vivo* alcohol treatment group were higher than in the control group, whereas at the blastocyst stage H3K9 acetylation levels in the control group were higher than in the alcohol treatment group. No differences were found for other stages. An abrupt reduction in H3K9 acetylation only occurred in the alcohol treatment group at the blastocyst stage. For other stages in both groups, H3K9 acetylation levels all initially decreased, then increased. High expression was found in the prokaryotic phase. Expression levels were rather low in the 2-cell stage and later increased.

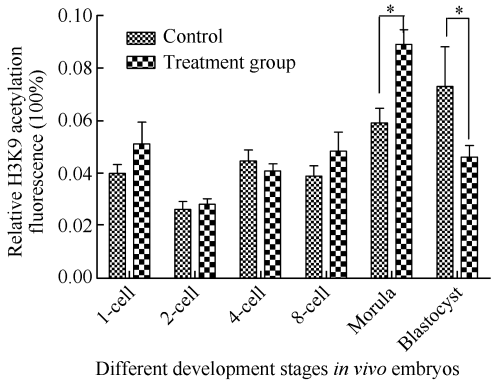

Figure 2 Semi-quantification of H3K9 acetylation levels of mouse pre-implantation embryos *in vivo*

H3K9 acetylation intensities of mouse pre-implantation embryos *in vivo* from control group and treatment group quantified with Image Pro-Plus 6.0. Different columns and bars represent mean±*SE*, *: $P<0.05$, control group *in vivo* compared with treatment group *in vivo*.

Immunofluorescence of H3K9 acetylation in pre-implantation embryos at different developmental stages in the *in vitro* groups

Indirect immunofluorescence detection of H3K9 acetylation in pre-implantation embryos at different developmental stages in the *in vitro* groups is shown in Figure 3.

Semi-quantitative analysis of H3K9 acetylation in pre-implantation embryos at different developmental stages in the *in vitro* groups

The analysis of fluorescence data showed that H3K9 acetylation levels of the *in vitro* alcoholic treatment and control groups were comparable (except at the 2-cell stage) and that H3K9 acetylation levels increased (Figure 4).

Patterns of H3K9 acetylation in the *in vivo* and *in vitro* alcohol treatment groups were different (Figure 2, 4). At the 2-cell stage *in vivo* a reduction in H3K9 acetylation was found, and then increased. *In vitro*, the H3K9 acetylation level increased, after which it decreased and increased again. For control groups, H3K9 acetylation levels *in vivo* at the 2-cell and blastocyst stages were lower than *in vitro*, while at the 4-cell-stage *in vivo* were higher than *in vitro*; no differences

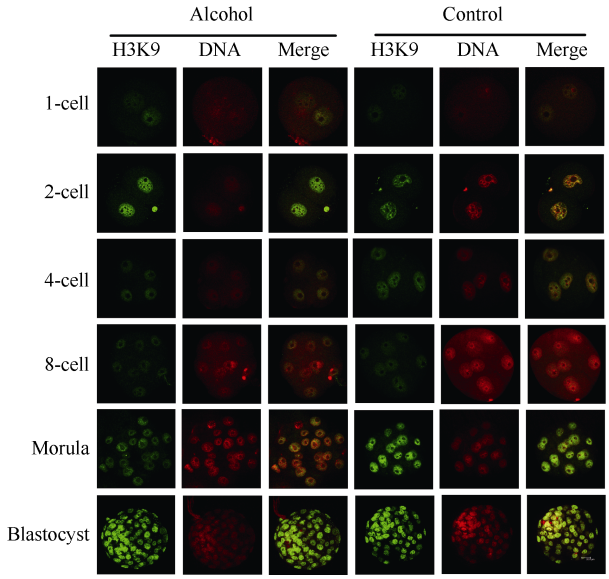

Figure 3 Different stages of mouse pre-implantation embryos and H3K9 acetylation immunofluorescence figure *in vitro*

1-cell, 2-cell, 4-cell, 8-cell, morula and blastocyst in the *in vitro* treatment and control groups were collected separately. Embryos were immunostained with specific antibodies against resistance to H3K9 acetylation antibody (H3K9 in histone); in red (middle), DNA ; Merged (right). Scale bar=20μm .

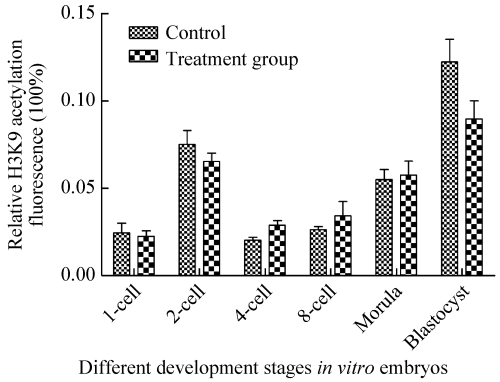

Figure 4 Semi-quantification of H3K9 acetylation levels of mouse pre-implantation embryos *in vitro*

Different columns and bars represent mean±*SE*, control group compared with treatment group *in vitro*.

were found for other stages. For alcohol treatment groups, H3K9 acetylation levels *in vivo* at the 1-cell and morula stages were higher than *in vitro*, while at the 2-cell and blastocyst stages *in vivo* were lower than *in vitro*. No differences were found for other stages.

We found that the blastocyst rates of *in vitro* alcohol treatment group and their controls were comparable. However, for the morula and blastocyst rates of *in vivo* groups, the alcohol treatment group was lower than the control group.

DISCUSSION

Histone acetylation influences chromatin remodeling and gene expression. As one of the signs of gene transcription initiation, H3K9 acetylation functions as an important form of histone acetylation and changes in its level can directly affect gene expression, induce protein misexpression and lead to phenotypic defects or maldevelopment. Here, no H3K9 acetylation indirect immunofluorescences in MII stage ovum were observed in either alcohol treatment groups or their controls and we assume this is because MII stage ovum were already in maturation and H3K9 deacetylated.

In vivo results showed that H3K9 acetylation levels in the 2-cell stage were low, and then increased. The mechanisms responsible is likely that during the 2-cell stage zygotic genes are activated, accumulated activation effects suppress gene transcription, and then under the regulation of zygotic genes many development-related genes start to transcript (Wang et al, 2013). Pan et al (2013) claim that increased H3K9 acetylation in mice embryos lays the ground for transcription initiation of development-related genes, which is in accordance with our data. From zygotes to the 8-cell stage in *vivo*, although H3K9 acetylation in the alcohol group was only slightly higher than the control group, a difference induced by alcohol occurred at the morula stage when many development-related genes would be activated. Under these circumstances, alcohol treated morula turned mature in advance and then cells began to deacetylate, and thereafter H3K9 acetylation levels were lower compared to the control group. Alcohol can harm female mice and affect H3K9 acetylation of pre-implantation embryos in multiple ways. Studies on hepatic cells show that alcohol related high histone acetylation may be correlated with the MAPK signal pathway (Yeh et al, 2008).

In vitro abnormal 4-cell stage H3K9 acetylation could be the result of activation delay of zygotic genes (Ma et al, 2001; Qiu et al, 2003). Zygotic gene activation (ZGA) is the initiation of embryo development from maternal regulation to embryonic regulation when the embryo will start to synthesize its own mRNAs and proteins and no long depends on maternally-sourced mRNAs and proteins (Kidder, 1993). The 2-cell stage is a vital period in mice embryonic development and is also the initiation phase of ZGA. *In vitro* culture may delay ZGA and prolong the procedure from maternal regulation to embryonic regulation.

Fluctuations in H3K9 acetylation levels observed in the *vivo* and *in vitro* control and alcohol groups from zygotes to blastocytes indicate that environmental differences can induce H3K9 acetylation imbalance in pre-implantation embryos. This imbalance may be correlated with the activation delay of zygotic genes and the postponing of 2-cell stage development; however, the mechanism for *in vitro* 2-cell stage development postponement remain unclear. Some studies have shown that at the 2-cell stage *in vitro*, the active oxygen content in embryos was much higher than that of other stages and the lesion caused by excessive active oxygen may be one of the reasons for 2-cell stage development postponement in mice

(Tang, 2006). Zhao et al (2005) reported that *in vitro* culture could suppress or decrease GCN5 and HDAC1 protein expressions in mice pre-implantation embryos and influence *in vitro* histone acetylation. Both *in vivo* and *in vitro* data suggest that long-term excessive alcohol consumption, even without alcohol intake during pregnancy, could still influence H3K9 acetylation in pre-implantation embryos because of remnant alcohol inside the body or metabolism products. Changes in H3K9 acetylation may also interfere with the correct establishment of epigenetic patterns in pre-implantation embryos. Alcohol may influence H3K9 acetylation in pre-implantation embryos and then induce gene misexpression by adjusting the activities of HATs; however, the underlying mechanisms at play here require further exploration.

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