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CHAPTER 7

Metabolic effects of carrier solvents and culture buffers in zebrafish embryos determined by ^{1}H NMR metabolomics.

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Abstract

Any bioactivity or toxicity test needs to dissolve the target compounds in an appropriate solvent. This is also the case in zebrafish embryo assays, an increasingly popular assay system in biomedical research. Water-soluble compounds can simply be dissolved in the aqueous medium that the zebrafish embryo grows in. This may be egg water (EW) or Hanks' balanced salt solution (HBSS). Non-polar compounds require in addition an organic solvent. It is important to know whether the solvent itself – including the type of buffer - has an effect on the organisms or assay. Lower concentrations of some organic solvents have been shown to produce a pronounced change in the metabolic profile of an organism. For these reasons, we evaluated the effect of two commonly-used organic carrier solvents in drug screening: Dimethyl sulfoxide (DMSO) and Ethanol; and two commonly used aqueous buffers. The effects of three concentrations (0.01, 0.1 and 1%) of these solvents were tested on the metabolic profile of 5 day old zebrafish embryos. The metabolic difference was determined by using the ^{1}H NMR based metabolomics. The zebrafish embryos were exposed at 24 hours post fertilization for 96 h, and then collected at 5 days post fertilization and analyzed by ^{1}H NMR. DMSO (0.1 and 1% but not 0.01%) exposure significantly decreased the level of adenosine triphosphate (ATP), betaine, alanine, histidine, lactate, acetate and creatine. By contrast, ethanol exposure did not alter the embryo metabolome at any concentration tested. We next examined the effect of HBSS and EW on the zebrafish embryo and found that the two media result in different embryo metabolomes. These results suggest that DMSO has relatively more pronounced effect on the embryo metabolome than ethanol, and used as a carrier solvent, DMSO concentration should be lower than 0.1%. These findings also show the significance of ^{1}H NMR based metabolomics which can detect even a slight change in embryo

metabolome caused by different media, and can successfully be used for the identification of markers of stress- or toxicity-induced metabolic shifts.

Introduction

Aquatic toxicity testing of potentially hazardous chemicals is an important issue in environmental hazard assessment . Water solubility is the major hurdle for the testing of hydrophobic compounds in aqueous test systems such as, for example, the zebrafish model. Hydrophobic compounds have to be homogeneously distributed in water (Herzel and Murty 1984). Therefore, carrier solvents are crucial for reliable studies of a wide range of compounds (Rufli et al. 1998) including lipophilic compounds, in aquatic bioassays (Chen et al. 2011). In fact, appropriate solvents for the delivery of xenobiotics is a major issue in *in vivo* toxicology studies (Nazir et al. 2003). Solvents may themselves affect the test organisms, leading to false positive or negative results (Rayburn et al. 1991). So, there is always need to analyze the effects of the solvents separately from that of the toxicant i.e. negative controls (Calleja and Persoone 1993).

For these and other reasons, it is necessary to first validate the use of carrier solvents before starting any toxicity study. This should result in a protocol that describes the type and maximum allowable concentrations (MACs) of solvents, which can be used in different studies without having effect on the experimental results. For this, United States environmental protection agency (US EPA) has set a maximum acceptable limit of 0.05% for solvents for acute toxicity tests and of 0.01% for chronic toxicity tests (Okumura et al. 2001).

Dimethyl sulfoxide (DMSO) and ethanol are commonly used solvents for hydrophobic compounds in toxicology studies. Both of these solvents have been used for developmental, reproductive and behavioral studies in zebrafish embryos, frog embryos, marine microalgae and *Drosophila melanogaster* (Chen

et al. 2011, Hallare A. et al. 2006, Nazir et al. 2003, Rayburn et al. 1991). These solvents have a great capability to solubilize a wide range of polar and nonpolar compounds. Therefore it is of importance to know the effects of these compounds on model organisms.

The zebrafish embryo assay has been used for the screening of a growing library of chemicals to understand their mechanisms and the indication of possible acute and long term adverse effects (Ali et al. 2011, Scholz et al. 2008). By comparison of the metabolome of the zebrafish embryo treated with certain test compounds, one may learn more about the kinds of metabolic 'signature'of certain drugs. For these studies, however, we need first of all to establish the metabolomic changes during normal development (in the absence of compound exposure) as well as the effect of the various carrier solvents (including the different aqueous buffers) used during compound exposure.

Previously, the zebrafish embryo model was used to study the behavioral effects of DMSO and ethanol(Chen et al. 2011). The effects of these solvents on the zebrafish embryo metabolome is not known. Here, we used nuclear magnetic resonance spectroscopy (NMR) based metabolomics to study the effects of DMSO and ethanol on the metabolic profile of zebrafish embryo.

Materials and methods

Ethics statement

All animal experimental procedures were conducted in accordance with national regulations, which are described in the *Wet op de dierproeven* (article 9) of Dutch Law administered by the Bureau of Animal Experiment Licensing. This national regulation serves as the implementation of "Guidelines on the protection of experimental animals" by the Council of Europe (1986), Directive 86/609/EEC, which allows zebrafish embryos to be used up to the moment of free-living (approximately 5-7 dpf).

Zebrafish

Male and female adult zebrafish (*Danio rerio)* of wild type were purchased from Selecta Aquarium Speciaalzaak (Leiden, The Netherlands) which obtain stock from Europet Bernina International BV (Gemert-Bakel, The Netherlands). The fish were kept at a maximum density of 100 individuals in glass recirculation aquaria (L 80 cm; H 50 cm, W 46 cm) on a 14 h light: 10 h dark cycle (lights on at 08:00). Water and air were temperature controlled (25 ± 0.5 ^{o}C and 23 ^{o}C, respectively). All the zebrafish handling was in accordance with national regulations. The fish were fed twice daily with 'Sprirulina' brand flake food (O.S.L. Marine Lab., Inc., and Burlingame, CA, USA) and twice a week with frozen food (Dutch Select Food, Aquadistri BV, Leiden, The Netherlands).

Defined embryo buffer

Ten percentage (v/v) of Hank's balanced salt solution was used (made from cell-culture tested, powdered Hank's salts, without sodium bicarbonate, Cat. No H6136-10X1L, Sigma-Aldrich, St Louis, MO, USA) at a concentration 0.98 g/L in Milli-Q water (resistivity = 18.2 MΩ·cm), with the addition of sodium bicarbonate at 0.035 g/L (Cell culture tested, Sigma), and adjusted to pH 7.46. A similar medium has been used previously (Irons et al. 2010, Macphail et al. 2009).

Embryo care

Eggs were obtained by random pair wise mating of zebrafish. Three adult males and four females were placed together in small breeding tanks (Ehret GmbH, Emmendingen, Germany) the evening before eggs were required. The breeding tanks (L 26 cm; H 12.5 cm, W 20 cm) had mesh egg traps to prevent the eggs from being eaten. The eggs were harvested the following morning and transferred into 92 mm plastic Petri dishes (50 eggs per dish) containing 40 ml

fresh embryo buffer. Eggs were washed four times to remove debris. Further unfertilized, unhealthy and dead embryos were screened under a dissecting microscope. At 3.5 hours post fertilization (hpf), embryos were again screened and any further dead and unhealthy embryos were removed. Throughout all procedures, the embryos and the solutions were kept at 28.5°C, in acclimatised room. All incubations of embryos were carried out in acclimatised room under a light cycle of 14 h light: 10 h dark.

Embryo Treatment and Collection

One hundred and twenty embryos per replicate were collected. After 24 hours the embryo were treated with 0.01%, 0.1% and 1% of both DMSO and ethanol. Embryos were collected on 5 days post fertilization (dpf) that is four days of exposure in falcon tube. The collected embryos were immediately frozen in liquid nitrogen and stored at -80° C till further analysis.

Sample preparation and Extraction

A fairly simple sample preparation method already was used for plant metabolomics (Abdel-Farid et al. 2009, Ali Kashif et al. 2010, Jahangir et al. 2008, Kim et al. 2010a, Kim et al. 2010b, Kim et al. 2010c). Embryos were freeze dried and transferred to a micro tubes (2 ml) to which 1ml of 50% CH_3OH-*d4* in D2O (KH_2PO_4 buffer, pH 6.0) containing 0.01% TMSP (w/w) was added. The mixture was vortexed for one min, sonicated for 20 min, and centrifuged at 13,000 rpm at room temperature for ten minutes. An aliquot (650 μL) was then transferred to a 5mm-NMR tube.

NMR measurements

NMR spectra were recorded at 25°C on a 500MHz Bruker DMX-500 spectrometer (Bruker, Karlsruhe, Germany) operating at a proton NMR frequency of 500.13 MHz. CH_3OH-d_4 was used as the internal lock. Each ^1H

NMR spectrum consisted of 128 scans requiring with the parameters as used by our group (Kim et al. 2010c)

Data Pre Processing and Analysis

Spectral intensities of 1H-NMR spectra were scaled to total intensity and reduced to integrated regions of equal width (0.04 ppm) corresponding to the region of δ 0.4–δ 10.0. The regions of δ 4.8–δ 4.9 and δ 3.30–δ 3.34 were excluded from the analysis because of the residual signal of the deuterated solvents. PCA was performed with the SIMCA-P software (v. 12.0, Umetrics, Umeå, Sweden) based on a Pareto scaling method. Statistical analyses were performed using GraphPad Prism (v. 5.03 for Windows, GraphPad Software, La Jolla CA, USA, www.graphpad.com). We used one-way analysis of variance and a Dunnett's Multiple comparison test with probability level of 5% as the minimal criterion of significance. All of the spectra were referenced, base line, phase-corrected and visually inspected by superimposing and stacking the spectra (Fig. 3) using MestReNova v.6.0.2 (Mestrelab research S.L., A Coruna, Spain).

Results

Effect of DMSO and Ethanol on embryos metabolome

In this study three concentrations (0.01, 0.1 and 1 %) of DMSO and Ethanol and two commonly used fish media were selected to analyze their metabolic effects on the 5 days old zebrafish embryos. The embryos were exposed to DMSO and Ethanol at 24 hpf and incubated for further 96 h in Hanks balanced salt solution (HBSS). The broad range identification ability of NMR makes it useful tool to identify macro metabolites. Mostly the metabolites identified are primary metabolites present in living organism like amino acids, energy related compounds, sugars and lipids. In the aliphatic region of NMR spectra amino acids like alanine, glycine, glutamine, glutamate, arginine, asparagines,

isoleucine, leucine and methionine; while organic acids like citric and lactic acid; bases like, creatine, choline and betaine; energy relating compounds, sugars like glucose and adenosine triphosphate, tyrosine and phenylalanine were identified. The identification of the metabolites was made by comparing the chemical shifts and splitting patterns with our in-house library of more than 700 common metabolites and comparison with available literature (Foxall et al. 1993, Govindaraju et al. 2000, Nicholson et al. 1995). The ^{1}H NMR spectra of the control zebrafish embryos (embryos raised only in HBSS) and those exposed to 1% of DMSO and Ethanol are shown in Fig. 1. It is evident that no

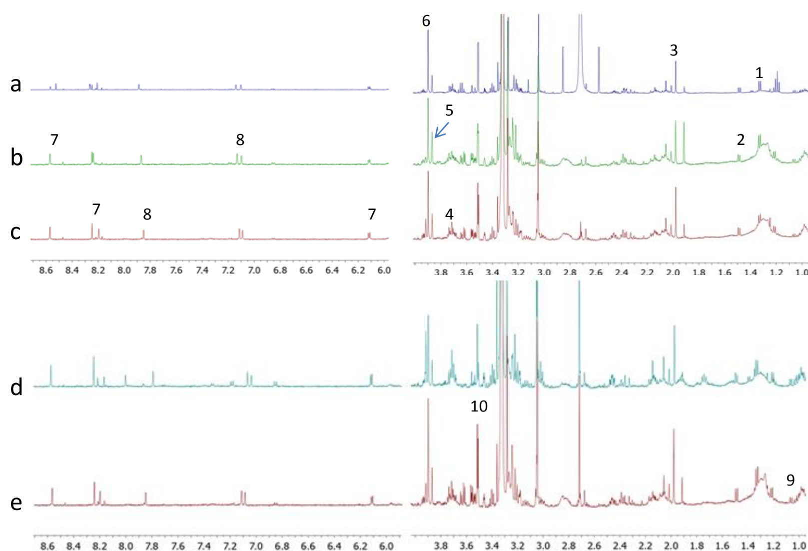

Figure 1. ^{1}H NMR spectra of 5 days old zebrafish embryo treated with (a) 1% of DMSO in HBSS, (b) 1% of ethanol in HBSS, (c) HBSS, (d) EW (e) HBSS,. 1, Lactate; 2, alanine; 3, acetate; 4, glutamate; 5, betaine; 6, creatine; 7, ATP; 8, Histidine; 9, valine; 10, glycine.

difference is present among the kind of metabolites, while significant differences exist among the level of metabolites. The signals of the discriminating metabolites are numbered (Fig 1). These signals were identified and are summarized in Table 1. Some of the major contributing metabolites

Table 1. Characteristic ^{1}H NMR chemical shifts and coupling contstants of indentified metabolites in 5 days old zebrafish embryo.

Metabolites	**Chemical shifts (δ) and Coupling constants (Hz)**
Acetate	δ 1.91 (s), δ 1.97 (s)
Alanine	δ 1.48 (d, J = 7.0 Hz)
ATP	δ 6.13 (d, J = 5.0 Hz), δ 8.26 (s), δ 8.56 (s)
Betaine	δ 3.29 (s), δ 3.87 (s)
Creatine	δ 3.06 (s), δ 3.91 (s)
Glucose	δ 4.60 (d, J = 7.94 Hz), δ 5.20 (d, 4.02 Hz)
Glutamate	δ 2.05 (m), δ 2.40 (m), δ 3.71 (m)
Glutamine	2.14,2.46,3.71
Glycine	δ 3.52 (s)
Histidine	δ 7.07 (s), δ 7.84 (s),
Lactate	δ 1.32(d, J= 7.0), δ 4.06 (m)
Valine	δ 1.01 (d, 7.01 Hz), δ 1.07 (d, J= 7.1 Hz)
3 methyl histidine	δ 7.05 (s)

were quantified and are shown in Fig. 2. The relative quantification was done by using the bucket data of ^{1}H NMR spectroscopy with the p value <0.01. It can be seen in the Fig. 2, compared to control group, the quantity of alanine, histidine, lactate, acetate, creatine and adenosine triphosphate (ATP) is greatly

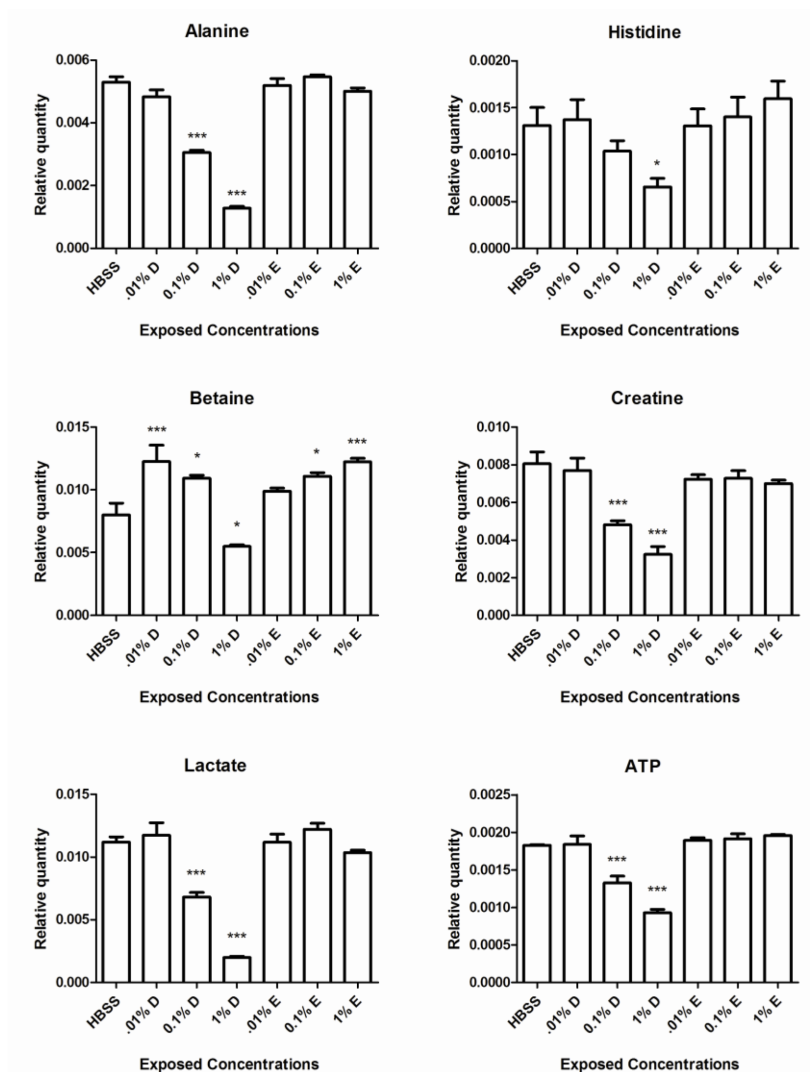

Figure 2. Relative quantification of the major differentiating metabolites based on mean peak area of the realted signals. “D” symbolizes the DMSO and “E” stands for Ethanol. * depict the differences between control (HBSS) and the different used concentrations of DMSO and Ethanol. Statistical icons: *= $p < 0.05$, **= $p < 0.01$ and ***= $p < 0.001$.

reduced after exposure to 0.1 and 1% of DMSO, However, 0.01% of DMSO does not show any effect on these metabolites but significantly increases the concentration of betaine. On the other hand, none of the ethanol concentrations affects any of the metabolite except betaine.

Multivariate data analyses (MvDA)

Principal component analysis (PCA) is a statistical technique used to analyze a large number of data by reducing the number of dimensions without losing important information. It is an unbiased and unsupervised method, which represents the data in a way that allows to observe similarities and differences in a score plot of PCA. The corresponding loading plot shows the chemical shifts of the discriminating metabolites responsible for the similarities and differences among the samples.

The PCA score plot shows the projections of Control (treated only with HBSS), DMSO and Ethanol treated groups (Fig. 3, A). A clear separation can be seen of the DMSO treated samples (0.1 and 1% DMSO) from control and Ethanol subjected groups. The major separation is characterized by PC1. The 0.1 and 1% DMSO samples were separated by PC2 and positioned in the positive and negative PC2 score, respectively. The 0.01% DMSO replicates were clustered more close to control having a positive PC1 score. The control and Ethanol groups were overlapping and placed on the positive side of PC1. The results show a clear metabolic change of the groups treated with higher concentrations of DMSO compared to control group. Whereas, there was hardly any distinction of metabolites observed between the control and the embryos exposed to Ethanol. The corresponding loading plot of the PCA was used to identify the ^{1}H chemical shifts of the subsequent metabolites responsible for the separation in score plot (not shown).

These results show that various solvents affect differently to zebrafish embryo metabolome. Dimethyl sulfoxide considerably changed the quantity of metabolites incubated in HBSS. To confirm these results, we also studied the effect of DMSO by growing the embryos in egg water containing 0.01, 0.1 and 1% of DMSO. The PCA score plot shows the separation of 0.1 and 1% of DMSO from 0.01% DMSO and control (embryo raised only in Egg water) group (Fig. 3B). The 95% of the separation was based on PC1 score. The 0.1

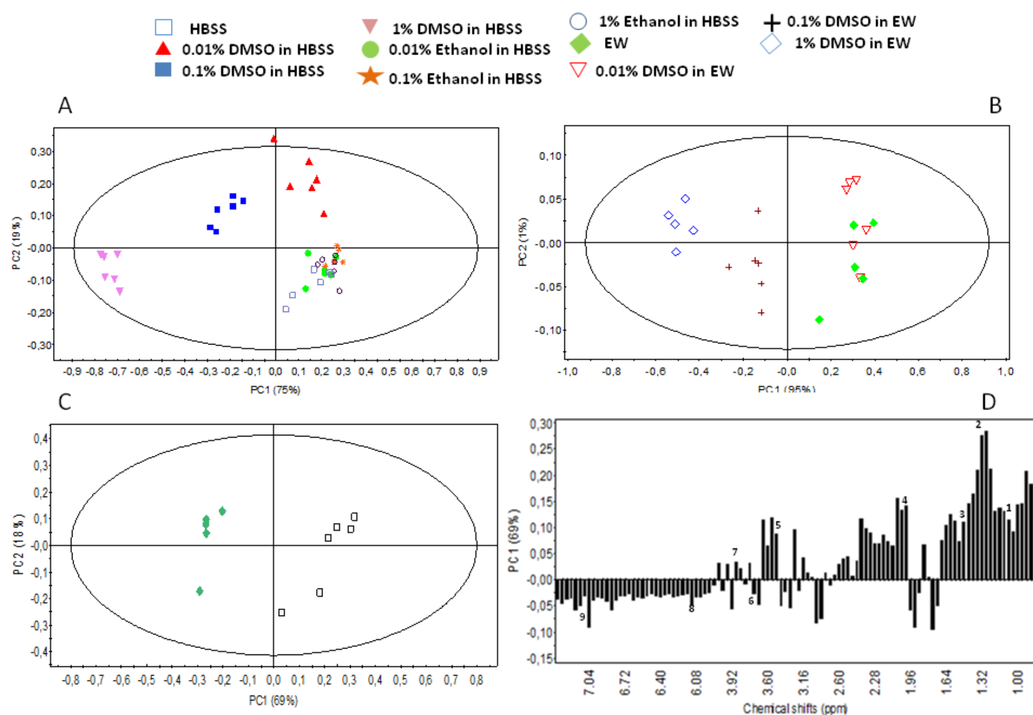

Figure 3. Score plot (PC1 vs PC2) of PCA based on whole range of ^{1}H NMR signals (δ 0.3- δ 10.0) of zebrafish embryo treated with A, .01, 0.1 and 1% of DMSO and Ethanol incubated in HBSS; B, .01, 0.1 and 1% of DMSO incubated in EW; C, HBSS and EW; D, Loading column plot of HBSS and EW. 1, valine; 2, lactate; 3, alanine; 4, acetate; 5 glycine; 6, glutamate; 7, ATP; 8, Histidine.

and 1% DMSO replicates were positioned on the negative, whereas 0.01% DMSO and control groups were overlapped on positive side of PC1. In the

corresponding loading column plot (not shown) all the signals were shifted to the positive side. The key metabolites responsible for the separation were quantified and are shown in Fig. 4. The 0.1 and 1% of DMSO showed a similar effect in EW and decreased the quantity of all those metabolites which were found suppressed in embryo populations exposed to 0.1 and 1% of DMSO and were raised in HBSS (Fig. 1a and Fig. 4). Whereas 0.01% of DMSO did not affect any of the metabolite compared to control group.

Effect of Aqueous media on embryos metabolome

We further analyzed the effect of commonly used fish media, Hanks balanced salt solution (HBSS) (Adams et al. 2005, Ali et al. 2011) and Egg water (EW) (Hentschel et al. 2005, Watanabe et al. 2010). An apparent separation can be observed by the PCA score plot for the PC1 score (Fig. 3C). The zebrafish embryo incubated in HBSS and Egg water is positioned on the positive and negative side of PC1, respectively. The differentiating metabolites were identified on the basis of chemical shifts obtained from the loading plot (Fig. 3D) and related signals are shown in the ^{1}H NMR spectra (Fig. 1d and 1e). The relative quantification (not shown) of these signals showed the reduction of valine, alanine, lactate, acetate, betaine and glycine levels in the samples raised in Egg water. Whereas glutamate, ATP and histidine were lower in the embryo incubated in HBSS. The comparison of HBSS and EW indicates that not only the choice of solvent but the selection of medium is also important for the reproducibility of the assay to study the effect of a drug on the zebrafish embryo metabolome.

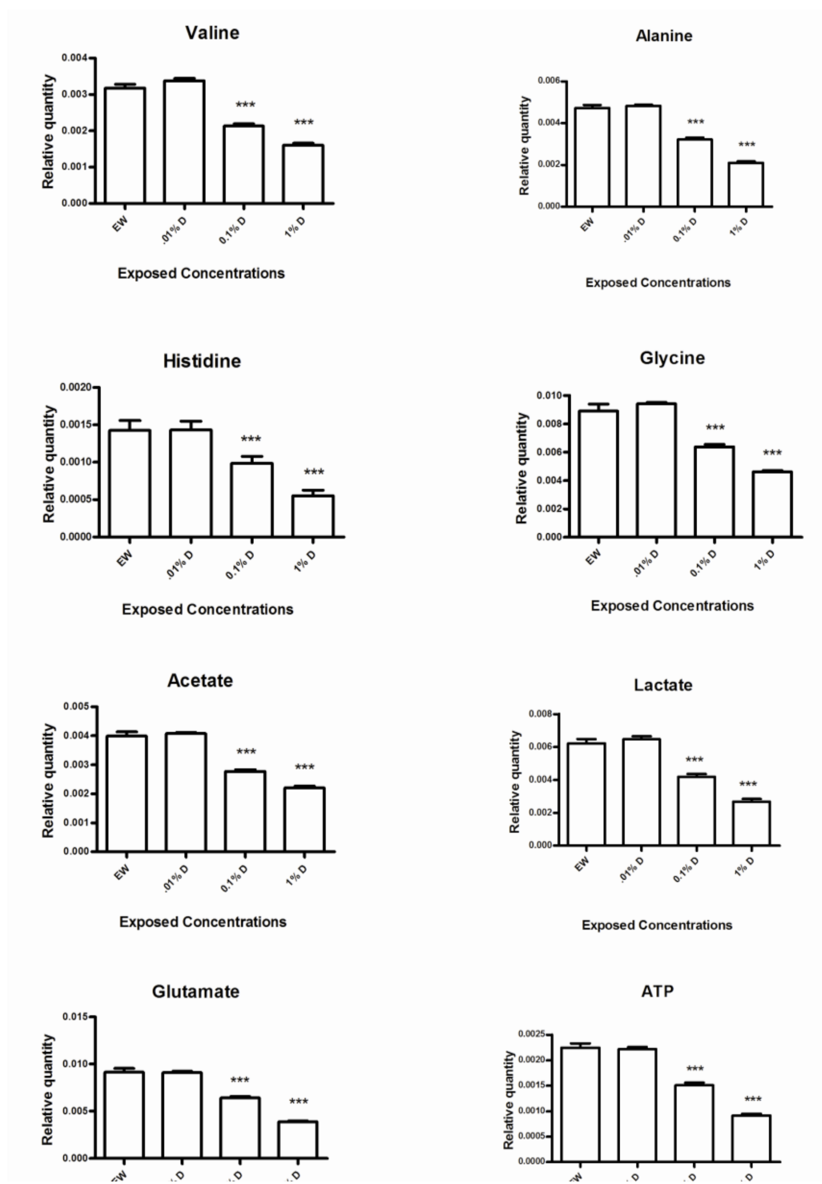

Figure 4. Relative quantification of the metabolites responsible for the separation in PCA score plot of DMSO treated samples (incubated in EW) and EW. The quantification based on mean peak area of the associated signals. * depict the differences between control (EW) and the different used concentrations of DMSO. Statistical icons: *= $p< 0.05$, **= $p< 0.01$ and ***= $p< 0.001$.

Discussion

In the present study, we examined the effects of 4 days of exposure to low concentrations of DMSO and ethanol on the metabolome of zebrafish embryo, as well as two aqueous buffers, EW and 10% HBSS. A significant difference was observed between the metabolic profiles of ^{1}H NMR analyses of embryo populations exposed to 0.1 and 1% DMSO and control (aqueous buffer only). DMSO was seen to have a large effect on the various metabolite levels and were well separated from ethanol and the aqueous buffer group in PCA by their PC1 and PC2 score. Particularly lower levels of amino and organic acids were observed in the treated groups. DMSO (0.1 and 1%) reduced the overall quantity of all the identified metabolites in a similar pattern. By contrast, ethanol did not alter the metabolite concentrations compared to control group. Finally, we noted that the choice of aqueous buffer (EW vs. HBSS) for raising zebrafish embryos had a significant effect on their metabolomic profiles.

Previously, NMR has been used to analyze the metabolic characterization of rat hepatocellular carcinoma formation, rat urine and serum, and human brain extracts for tumor biopsies (Maxwell et al. 1998, Wang et al. 2011, Wei et al. 2008). NMR based metabolomics and other targeted techniques (HPLC, LC-MS, GC-MS) have also been successfully applied for the metabolic fingerprinting of zebrafish embryo and liver of the adult fish (Ong et al. 2009, Papan and Chen 2009).

DMSO has previously been reported as a potential inducer of stress (Hallare et al. 2004). In a comparative study of embryotoxicity and proteotoxicity of carrier solvents to zebrafish embryos, DMSO at low concentrations (0.01, 0.05 and 0.1%) was shown to significantly increase the expression of the stress-related protein hsp 70 (heat shock protein). Ethanol and acetone showed such an activity at 1.5 and 0.1%, repectively (Hallare et al. 2006, Hallare et al. 2004).

As can be seen in Fig. 3 and 4 we find that DMSO exposure (0.1 and 1%) reduced the level of adenosine triphosphate (ATP). This is consistent with previous findings. Thus, it was reported that DMSO (0.1%) exposure, coupled with the inhibition of cytochrome P450 (CYPs) in zebrafish embryos, reduces the level of ATP in zebrafish ovarian follicles and fragments (David et al. 2012, Zampolla et al. 2009, Zampolla et al. 2011). CYP enzymes play an important role in the oxidative metabolism of endogenous and exogenous substances (Buhler and Wang-Buhler 1998). A further study found that in the kidneys of male wistar rats, DMSO reduced the level of ATP and slowed down the process of glycolysis by activating the FDPase (Fructose 1,6-diphaphatase) (Baxter and Lathe 1971).

Taken together, our results and previous findings could suggest that DMSO may cause a metabolic inhibition leading to greatly reduced levels of ATP, and in turn the suppression of primary metabolite levels in the zebrafish embryo. Further studies (including also, for example, proteomics and transcriptomics) are needed to confirm this hypothesis. What is perhaps surprising in our study is that ethanol exposure (0.01, 0.1 and 1.0%) had no significant effect on the zebrafish embryo metabolome, even though it is known to be a potent teratogen (Arenzana et al. 2006). These studies showed that even low concentrations of ethanol (0.05, 0.1 and 1%) can induce the hyper locomotor activity in zebrafish embryos (Echevarria et al. 2010). In a study of behavioral and teratological effects of ethanol on zebrafish larave, 0.01 and 0.1% of ethanol was found safe in not producing any developmental defect but still inducing hyper locomotion (Chen et al. 2011). By contrast, 1% ethanol not only altered the locomotor activity but also significantly increased the incidence of e.g pericardial edema, yolk sac edema, crooked body and cyclopia (Arenzana et al. 2006, Chen et al. 2011).

An interesting question is why, in our study, DMSO had such a pronounced metabolic effect, while previous studies showed it to have only mild phenotypic effects on embryos; while in contrast, ethanol had no effect on the metabolome in our study, even though it is known to have pronounced phenotypic effects on embryo development. The answer may lie in the fact that DMSO is known to produce pronounced effects on the liver enzymes and ATP production which might cause the large effect on embryo metabolism seen in our study. The use of DMSO should therefore be evaluated carefully, and the final concentration should be as low as possible; the concentration of 0.01% DMSO in either EW

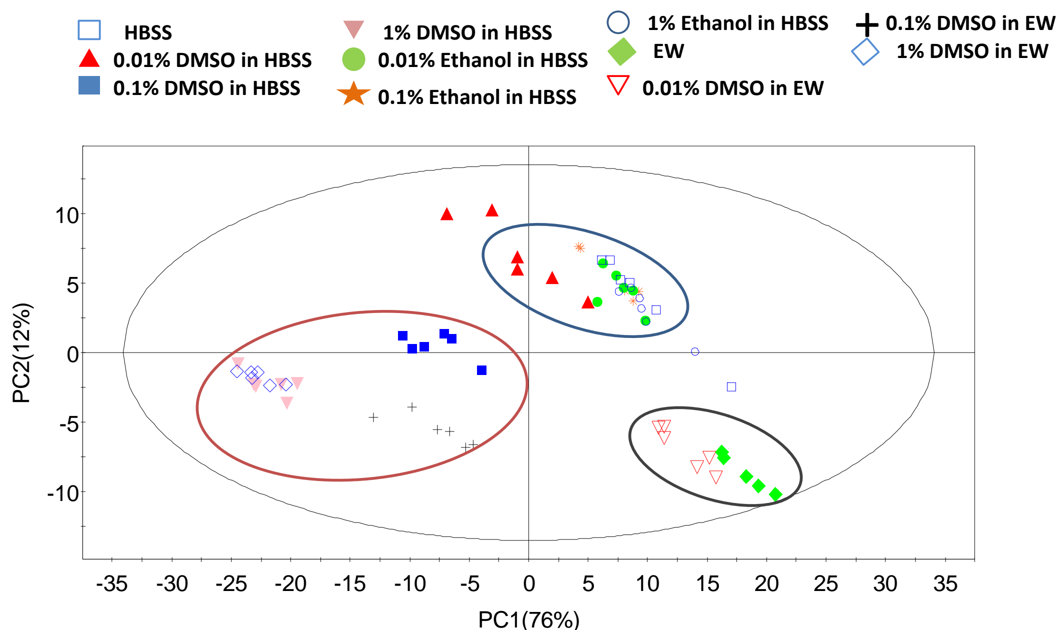

Figure 5: Score plot (PC1 vs PC2) of PCA based on whole range of ^{1}H NMR signals (δ 0.3- δ 10.0) of zebrafish embryo treated with Ethanol (0.01, 0.1 and 1%) raised in HBSS, DMSO (0.01, 0.1 and 1%) raised in HBSS and EW, raised only in HBSS and EW.

or HBSS had no significant effects on the zebrafish metabolome in this study.

Moreover, our findings suggest that the selection of the aqueous medium is also important because it may affect embryo metabolism. Given a fixed concentration of DMSO (0.01), the metabolic effect varies according to whether this concentration of DMSO was in HBSS or EW. It can be seen in the PCA analysis of all samples shown in Fig. 5 that, all three ethanol concentrations, 0.01% DMSO in HBSS and HBSS alone, were all clustered on the positive side of PC1. EW alone, and 0.01% of DMSO in EW replicates overlapped and had positive PC1 and PC2 scores. The points for DMSO (0.1%) in HBSS, and those for DMSO (0.1%) in EW, were separated by a small distance having negative PC1 score. Interestingly, the 1% DMSO samples (raised in either HBSS or EW) were found to cluster tightly together. Therefore, at this concentration of DMSO, the choice of aqueous medium no longer has an effect.

Conclusion

Our results demonstrate that NMR together with multivariate data analyses provides comprehensive information of amino and organic acids in the 5 dpf zebrafish embryo metabolome. NMR can successfully detect a even slight differences in metabolite levels induced by aqueous media or organic solvents, respectively. The study also shows that zebrafish embryos can be used for the preliminary screening of compounds by providing insight into the metabolic changes mediated by drugs or toxins. Finally, we recommend that for metabolomic studies of zebrafish embryos, the DMSO concentration should be lower than 0.1%.

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