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Original Articles

Zebrafish as a Model for Systems Medicine R&D: Rethinking the Metabolic Effects of Carrier Solvents and Culture Buffers Determined by ^{1}H NMR Metabolomics

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Abstract

Zebrafish is a frequently employed model organism in systems medicine and biomarker discovery. A crosscutting fundamental question, and one that has been overlooked in the field, is the ''system-wide'' (omics) effects induced in zebrafish by metabolic solvents and culture buffers. Indeed, any bioactivity or toxicity test requires that the target compounds are dissolved in an appropriate nonpolar solvent or aqueous media. It is important to know whether the solvent or the buffer itself has an effect on the zebrafish model organism. We evaluated the effects of two organic carrier solvents used in research with zebrafish, as well as in drug screening: dimethyl sulfoxide (DMSO) and ethanol, and two commonly used aqueous buffers (egg water and Hank's balanced salt solution). The effects of three concentrations (0.01, 0.1, and 1%) of DMSO and ethanol were tested in the 5-day-old zebrafish embryo using proton nuclear magnetic resonance (^{1}H NMR) based metabolomics. DMSO (1% and 0.1%, but not 0.01%) exposure significantly decreased the levels of adenosine triphosphate (ATP), betaine, alanine, histidine, lactate, acetate, and creatine ($p < 0.05$). By contrast, ethanol exposure did not alter the embryos' metabolome at any concentration tested. The two different aqueous media noted above impacted the zebrafish embryo metabolome as evidenced by changes in valine, alanine, lactate, acetate, betaine, glycine, glutamate, adenosine triphosphate, and histidine. These results show that DMSO has greater effects on the embryo metabolome than ethanol, and thus is used with caution as a carrier solvent in zebrafish biomarker research and oral medicine. Moreover, the DMSO concentration should not be higher than 0.01%. Careful attention is also warranted for the use of the buffers egg water and Hank's balanced salt solution in zebrafish. In conclusion, as zebrafish is widely used as a model organism in life sciences, metabolome changes induced by solvents and culture buffers warrant further attention for robust systems science, and precision biomarkers that will stand the test of time.

Introduction

Zebrafish is a frequently employed model organism in oral medicine, biomarker and systems medicine R&D (Cichoń et al., 2014). A crosscutting fundamental question, and one that has been overlooked in the field, is the ''system-wide'' (omics) effects induced in zebrafish by metabolic solvents and culture buffers. Indeed, any bioactivity or toxicity test requires that the target compounds are dissolved in an appropriate nonpolar solvent or aqueous media. It is important to know whether the solvent or the buffer itself has an effect on the zebrafish model organism. Moreover, aquatic toxicity testing of potentially hazardous chemicals is an important issue in environmental hazard assessment.

Water solubility is a major hurdle for the testing of hydrophobic compounds in aqueous test systems such as 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 setback for *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). Hence, there is a real need to analyze the effects of the solvents separately from that of the toxicant (i.e., negative controls) (Calleja and Persoone, 1993).

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For these reasons, it is necessary first to 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. The 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 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 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. NMR spectroscopy provides an unbiased and nontargeted approach for the comprehensive analyses of an organism's metabolome. It is widely used to examine the urine samples for disease diagnosis and drug toxicity assessment in different animal models (Bollard et al., 2005; Gavaghan et al., 2002).

NMR provides a platform to measure a wide range of the metabolites in an organism both qualitatively and quantitatively and provides a comprehensive metabolic picture of a living organism under certain conditions (Kim et al., 2010a). In comparison to other analytical platforms such as mass spectrometry coupled with liquid or gas chromatography, the attractive features of NMR are simple sample preparation; nondestructive sampling; short measurement time; and the ease of quantitation and high reproducibility (Kwon et al., 2011). Metabolomics and related systems scale technologies (Cuadrat et al. 2014; Montague et al. 2014) offer the ability to evaluate the effects of solvents and culture buffers at a systems scale as illustrated over the past decade in various facets of systems medicine, not to mention oral health (Cuevas-Córdoba et al., 2014; Perera et al., 2014; Sahu et al., 2014; Sitole et al., 2014; Zheng et al., 2014).

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 days post fertilization (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 temperature were controlled (25 ± 0.5°C and 23°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 and embryo care

The buffer (10% (v/v) of Hank's balanced salt solution) and embryo preparation was prepared according to Akhtar et al., (2013).

Embryo treatment and collection

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

Sample preparation and extraction

A fairly simple sample preparation method was followed that has already been used for plant metabolomics (Abdel-Farid et al., 2009; Ali et al., 2010; Jahangir et al., 2008; Kim et al., 2010a; 2010b). Embryos were freeze dried and transferred to a micro tube (2 mL) to which 1 mL 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 1 min, sonicated for 20 min, and centrifuged at 13,000 rpm at room temperature for 10 min. An aliquot (650 μL) was then transferred to a 5 mm-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 ^{1}H NMR spectrum consisted of 128 scans requiring with the parameters as used by our group (Kim et al., 2010c).

Data analysis

Spectral intensities of 1H-NMR spectra were scaled to total intensity and reduced to integrated regions of equal width

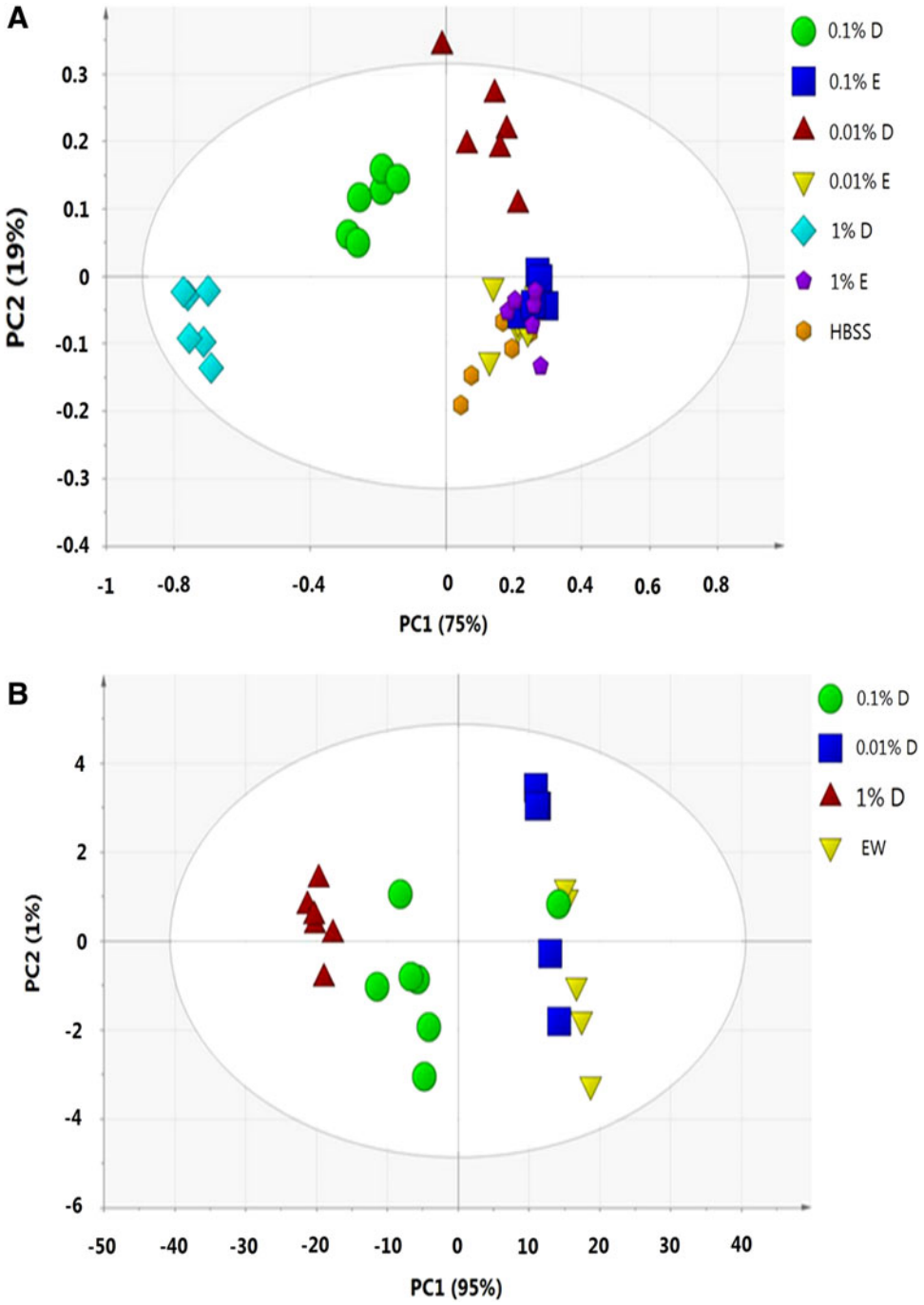

FIG. 1. Score plot (PC1 vs. PC2) of PCA based on whole range of ^{1}H NMR signals (δ 0.3–δ 10.0) of zebrafish embryo treated with **(A)** 0.01%, 0.1%, and 1% of DMSO and ethanol incubated in HBSS; **(B)** 0.01%, 0.1%, and 1% of DMSO incubated in EW.

(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. Principal component analysis (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). All of the spectra were referenced, base line corrected, 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

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-day-old

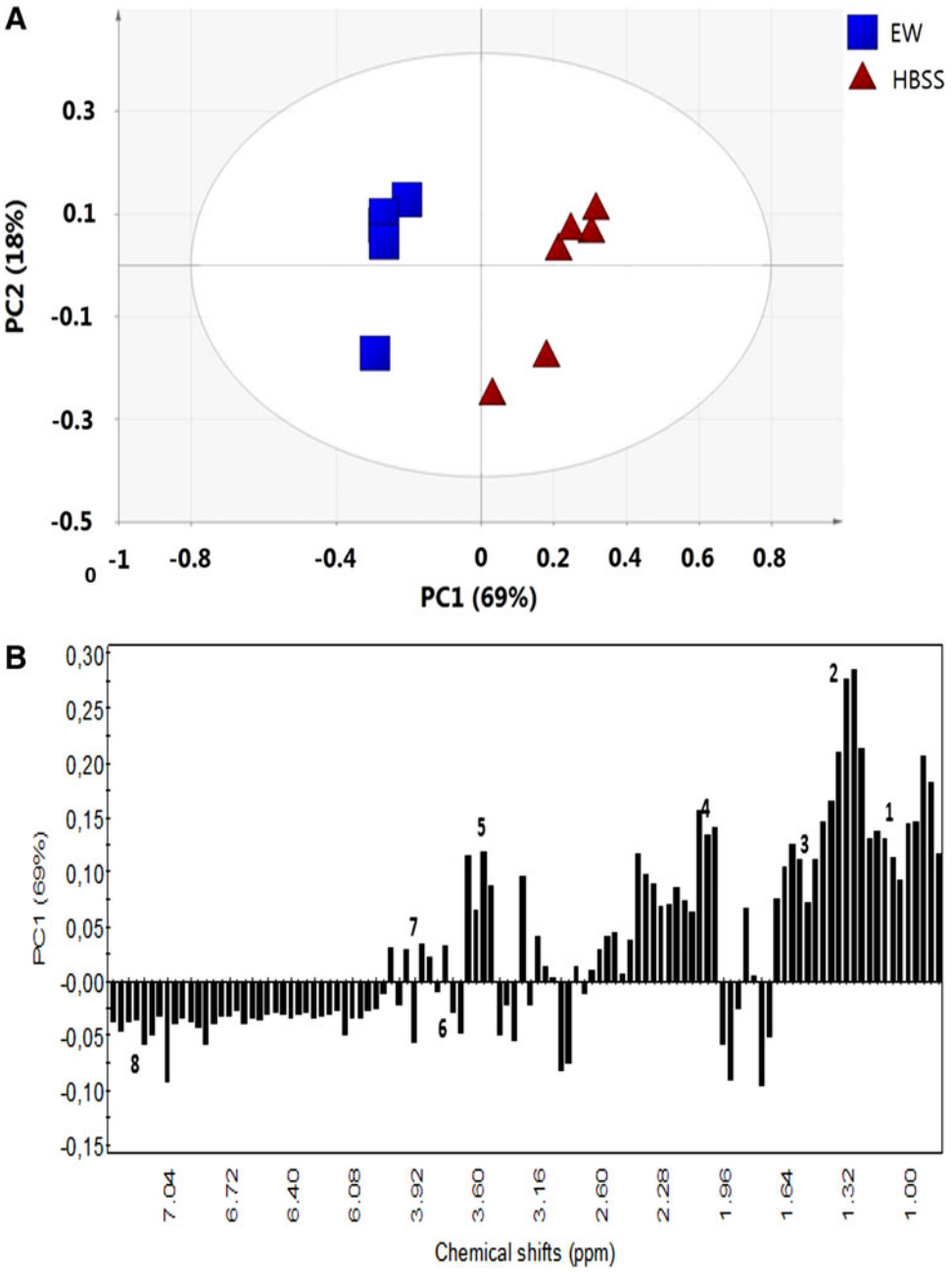

FIG. 2. **(A)** Score plot (PC1 vs. PC2) of PCA based on whole range of ^{1}H NMR signals (δ 0.3–δ 10.0) of zebrafish embryo raised in HBSS and EW; **(B)** Loading column plot of HBSS and EW: 1, valine; 2, lactate; 3, alanine; 4, acetate; 5 glycine; 6, glutamate; 7, ATP; 8, histidine.

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 made it possible to identify macro metabolites. Mostly the metabolites identified are primary metabolites present in living organism such as amino acids, energy-related compounds, sugars, and lipids. In the aliphatic region of NMR spectra, amino acids such as alanine, glycine, glutamine, glutamate, arginine, asparagines, isoleucine, leucine, and methionine; the organic acids citric and lactic acid; the bases creatine, choline, and betaine; energy-related compounds, sugars such as glucose and adenosine triphosphate (ATP), 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).

Effects of DMSO and ethanol on embryos' metabolome

We applied principal component analyses (PCA) to evaluate the variables representing the key metabolites regulated

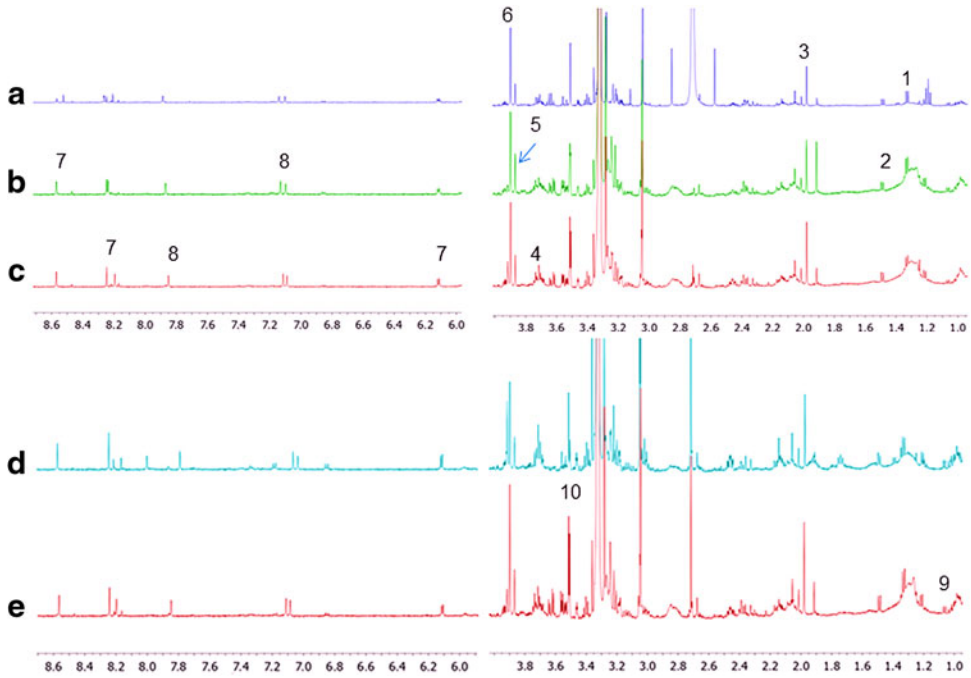

FIG. 3. [1]H NMR spectra of 5 days old zebrafish embryo treated with **(a)** 1% of DMSO in HBSS, **(b)** 1% of ethanol in HBSS, **(c)** HBSS, **(d)** EW, and (e) HBSS. 1, lactate; 2, alanine; 3, acetate; 4, glutamate; 5, betaine; 6, creatine; 7, ATP; 8, histidine; 9, valine; 10, glycine.

differently in the DMSO, ethanol, and nontreated zebrafish embryos. The PCA score plot shows the projections of control (treated only with HBSS), DMSO, and ethanol treated groups (Fig. 1A). A clear separation can be seen of the DMSO-treated samples (0.01% and 1% DMSO) from control and ethanol subjected groups.

The major separation is characterized by PC1 (75%). The 0.01% DMSO replicates were clustered closer to control having a positive PC1 score. The 0.01% and 1% DMSO samples were separated by PC2 and positioned in the positive and negative PC2 score, respectively. The control and ethanol groups were overlapping and placed on the positive side of PC1. The results show a clear metabolic change to 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 1H chemical shifts of the subsequent metabolites responsible for the separation in score plot (not shown).

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 Figure 3. It is evident that no difference is present among the kind of metabolites, while significant differences exist among the concentration of metabolites. The signals of the discriminating metabolites are numbered in Figure 3. These signals were identified and are summarized in Table 1.

Some of the major contributing metabolites were quantified and are shown in Figure 4. 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 Figure 4, compared to control group, the quantity of alanine, histidine, lactate, acetate, creatine, and adenosine triphosphate (ATP) is greatly

TABLE 1. CHARACTERISTICS OF [1]H NMR CHEMICAL SHIFTS AND COUPLING CONSTANTS OF IDENTIFIED METABOLITES IN 5-DAY-OLD ZEBRAFISH EMBRYO

ID No	*Metabolites*	*Chemical shifts (δ) and Coupling constants*
1	Acetate	δ 1.91 (s)
2	Alanine	δ 1.48 (d, J = 7.1 Hz)
3	ATP	δ 6.13 (d, J = 5.0 Hz), δ 8.26 (s), δ 8.56 (s)
4	Betaine	δ 3.29 (s)
5	Creatine	δ 3.06 (s), δ 3.91 (s)
6	Choline	δ 3.23 (s)
7	Glucose	δ 4.60 (d, J = 7.9 Hz), δ 5.20 (d, 4.0 Hz)
8	Glutamate	δ 2.05 (m), δ 2.40 (m), δ 3.71 (m)
9	Glutamine	δ 2.14 (m), δ 2.46 (m), δ 3.71 (m)
10	Glycine	δ 3.52 (s)
11	Histidine	δ 7.07 (d, J = 0.6), δ 7.84 (d, J = 1.1)
12	Lactate	δ 1.32 (d, J = 7.0), δ 4.06 (m)
13	Leucine	δ 0.99 (d, J = 7.0), δ 1.00 (d, J = 7.0), δ 1.68 (m), δ 3.68 (m)
14	Taurine	δ 3.21 (t, 6.47 Hz), δ,3.41 (t, J = 6.47 Hz)
15	Threonine	δ 1.33 (d, J = 6.86 Hz), δ 4.23 (m)
16	Tyrosine	δ 6.89 (d, J = 8.5), δ 7.20 (d, J = 8.5)
17	Valine	δ 1.01 (d, 7.01 Hz), δ 1.07 (d, J = 7.1 Hz)
18	3-Methyl histidine	δ 7.05 (S)

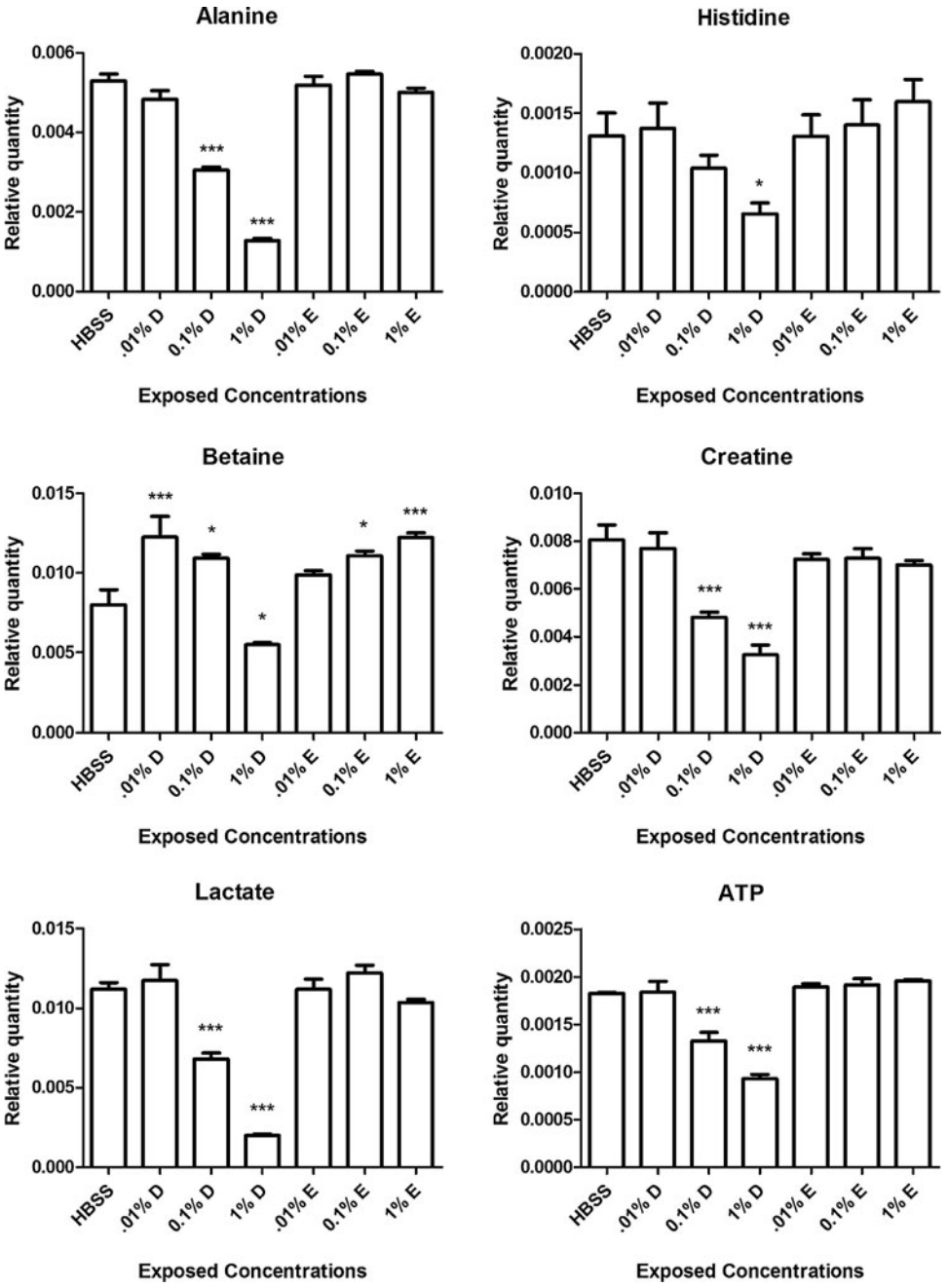

FIG. 4. Relative quantification of the major differentiating metabolites based on mean peak area of the related signals. ''D'' = DMSO and ''E'' = Ethanol. * depict the differences between control (HBSS) and the different concentrations of DMSO and Ethanol. Statistical differences of metabolites between groups were calculated by using one-way analysis of variance and a Dunnett's multiple comparison test. 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 affect any of the metabolite except betaine.

These results show that various solvents have different effects on the 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 (EW) 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. 1B). The 95% of the separation was based on PC1 score. The 0.1% and 1% DMSO replicates were positioned on the negative, whereas 0.01% DMSO and control groups were overlapped on positive side of PC1. The key metabolites responsible for the separation were quantified and are shown in Figure 5. The 0.1% and 1% of DMSO showed a similar effect in EW and decreased the quantity of all those metabolites that were found suppressed in embryo

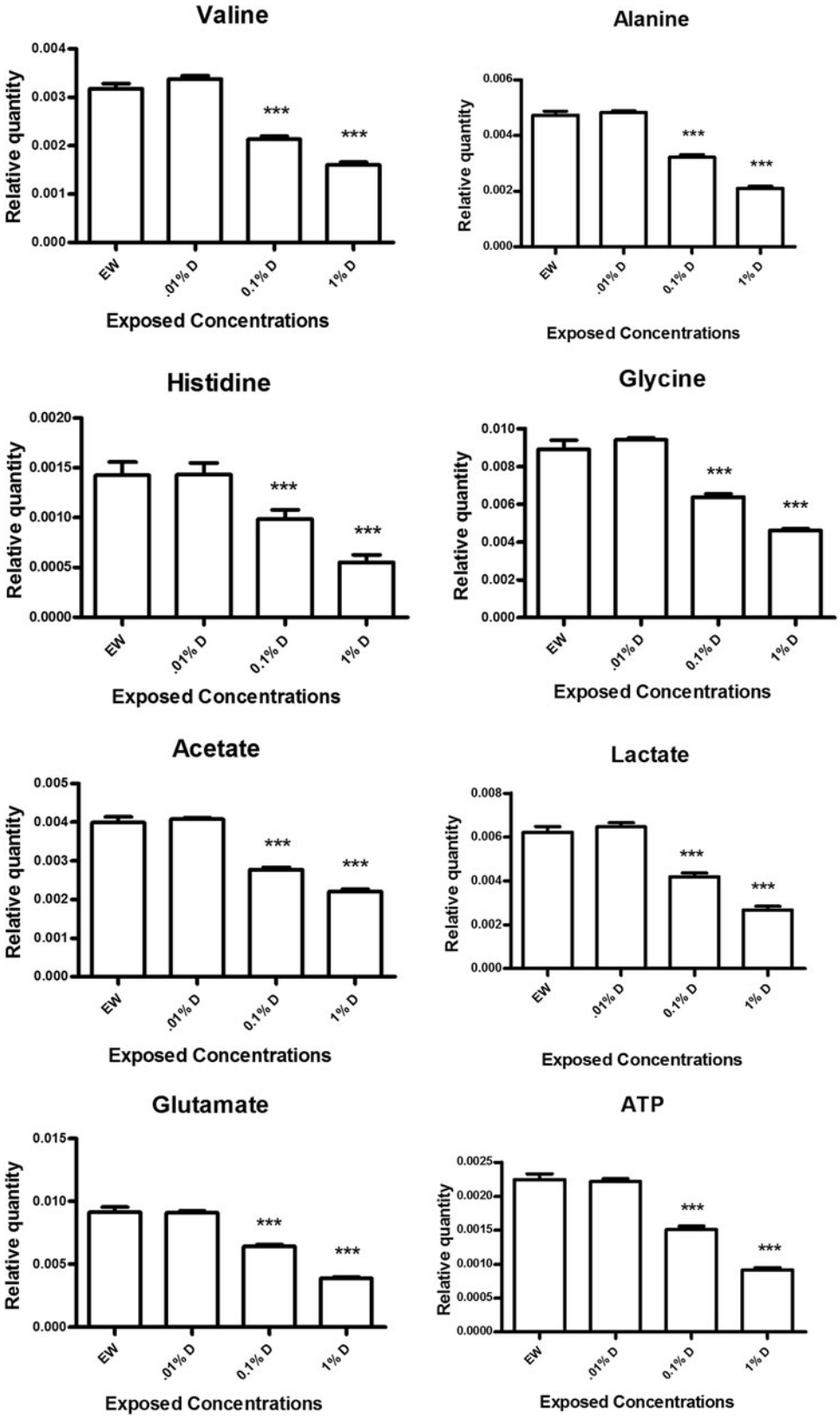

FIG. 5. Relative quantification of the metabolites responsible for the separation in PCA score plot of DMSO-treated samples (incubated in EW) and EW. The quantification based on mean peak area of the associated signals. * depict the differences between control (EW) and the different concentrations of DMSO. Statistical differences of metabolites between groups were calculated by using one-way analysis of variance and a Dunnett's multiple comparison test. Statistical icons: $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

populations exposed to 0.1% and 1% of DMSO and were raised in HBSS (Fig. 3a and Fig. 5), whereas 0.01% of DMSO did not affect any of the metabolite compared to the control group.

Effect of aqueous media on embryo metabolome

We further analyzed the effect of commonly used fish media, HBSS (Adams et al., 2005; Ali et al., 2011) and 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. 2A). The zebrafish embryo incubated in HBSS and EW 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. 2B) and related signals are shown in the ^{1}H NMR spectra (Fig. 3d and e).

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 EW, whereas glutamate, ATP, and histidine were lower in the embryos 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.

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 was 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%, respectively (Hallare et al., 2004; 2006).

As can be seen in Figures 1 and 5, 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; 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-diphophatase) (Baxter and Lathe, 1971).

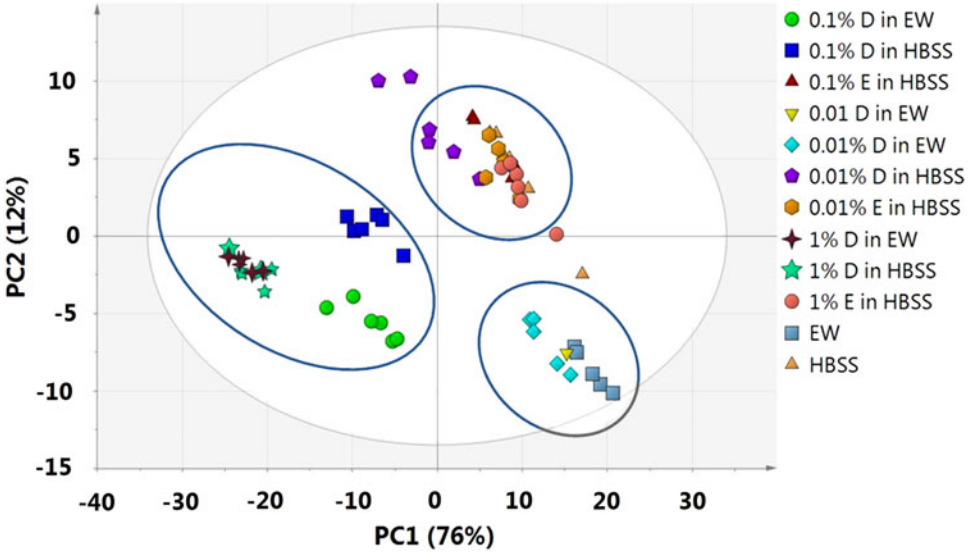

FIG. 6. Score plot (PC1 vs. PC2) of PCA based on whole range of ^{1}H NMR signals (δ 0.3–δ 10.0) of zebrafish embryo treated with Ethanol (0.01%, 0.1%, and 1%) raised in HBSS, DMSO (0.01%, 0.1%, and 1%) raised in HBSS and EW, raised only in HBSS and EW.

Taken together, our results and previous findings 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 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 pericardial edema, yolk sac edema, crooked body, and cyclopia (Arenzana et al., 2006; Chen et al., 2011).

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

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 Figure 6 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

This report shows comprehensive changes in amino and organic acids in the 5-day zebrafish embryo metabolome after exposure to solvents and buffers. NMR can successfully detect 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. We recommend that for metabolomics studies of zebrafish embryos, the DMSO concentration should not be higher than 0.01%. Careful attention is also warranted for the use of buffers EW and HBSS in zebrafish. Because zebrafish is widely used as a model organism in systems medicine and biomarker R&D, metabolome changes induced by solvents and culture buffers warrant further attention for robust systems science and precision biomarkers that stand the test of time.

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Author Disclosure Statement

The authors have no competing financial interests to declare.

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Abbreviations Used

Dpf = days post fertilization
EW = egg water
HBSS = Hanks balanced salt solution
Hpf = hours post fertilization
PCA = principal component analysis