The study of fibroblast proteome changes in the presence of Ethanol

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ABSTRACT

Ethanol known as ethyl alcohol is being widely used around the world. Many serious diseases are related with its consumption. Alcohol posses many divers effects on human body including risk of cirrhosis and/or hepatocellular carcinoma. Therefore, analysis of this component is prominent. Fibroblast cells were cultured in various dosages of ethanol. The effective dosage was then investigated by proteomic methods. Separated proteins of fibroblast cells by Two-Dimensional Gel (2DG) Electrophoresis method based on pI and MW were analyzed based on spots alteration by Same Spot Software. Furthered analysis was carried out with vigorous statistical analysis based on significant folding changes and one-way ANOVA. About 372 protein spots were identified and among them 65 of them were having significant expression profile, which is evaluated as $p < 0.05$. Therefore, ethanol can induce a great impact on protein profile of fibroblast. It is concluded that altering morphologic features and viability, as well as protein expression changes, confirm toxic properties of ethanol in human body.

Keywords: Ethanol; Human Fibroblast Cell line; Proteomics; 2-Dimensional gel electrophoresis; Progenesis SameSpots Software

INTRODUCTION

Ethanol has enormous consumption around the world [1] that trigger major health-related problems ranging from low-risk, at-risk, and problem drinking to alcohol addiction and chronic abuse [2]. Regular volume of alcohol consumption can increase risk of many chronic and acute diseases [3], including: tuberculosis, mouth, nasopharynx, other pharynx and oropharynx cancer, oesophageal cancer, colon and rectum cancer, alcoholic liver, liver cancer, female breast cancer, diabetes mellitus, alcohol use disorders, unipolar depressive disorders, epilepsy, hypertensive heart disease, ischaemic heart disease (IHD), ischaemic and haemorrhagic stroke, conduction disorders and other dysrhythmias, lower respiratory infections (pneumonia), cirrhosis of the liver, preterm birth complications, decrease fertility and fetal alcohol syndrome [3-9]. From the beginning of the history the alcoholic

beverages consumption reported for many diseases. In fact, there is a complex and multidimensional relationship between alcohol consumption and health outcomes. Generally, 4% of the worldwide diseases origin is associated with alcohol consumption, which is equal to the same rate of death and disability globally for tobacco and hypertension [10]. Even small part of brain can be damaged and cognitive dysfunction can be obtained in uncomplicated alcoholics who have no specific neurological or hepatic complications. While neuropsychological abnormalities such as alteration in CNS can be observed by advance imaging technology, high-throughput technologies such as DNA microarrays and proteomics can provide molecular changes view [11]. As states earlier, alcohol posses many adverse effects on human body including the risk of cirrhosis and/or hepatocellular carcinoma [12]. Studies show that protein interaction can be affected; in fact, chronic

alcohol intake cause liver dysfunction and consequently proteasome inactivation. This can happen by several alcohol metabolism mechanisms in body. As mentioned above, this includes protein-protein interaction and proteasome regularity system changes [13]. Proteomics is a promising method for assessing large sets of complex protein mixtures that enables a researcher to separate the whole proteins from a complex mixture of proteome of the real state of a cell, tissue or organism based on isoelectric point (pI), molecular mass (M_r), solubility, and relative abundance. In addition to this, alteration of protein expression, isoforms, and post-translational modifications can be obtained in this way [14, 15]. Since these mentioned adverse effects of this component, it is essential to evaluate the molecular basis of its function in human cells. As a result, here the proteome evaluation of fibroblast cells in the presents of ethanol is examined.

MATERIALS AND METHODS

The cell culture medium (RPMI), fetal bovine serum (FBS), penicillin and streptomycin were provided by Gibco BRL (Life Technologies, Paisley, Scotland). Cell lines were obtained from cell bank (Pastuer Institute, Tehran, Iran). 3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), Annexin V-FLOUS staining kit (Cat. No. 11 988 549 001) was provided from Roche Diagnostics GmbH (Germany). For MTT assay evaluations, cultures were maintained at 37°C in 5% CO2 and 95% air, and the medium changed two times per week. The cells were seeded, in the 96 well plates and incubated at 37 °C under 5% CO2 atmosphere for 48 h. Then, the various filtered concentrations of ethanol including: 0, 54,108,200,216,270,378, 432 mM concentrations applied. In addition, the concentration 0 as control pattern was induced into cells for 24h for MTT assay proposes. The inhibition of cellular proliferation was measured by the modified MTT 3-(4, 5 dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide]) assay, based on the ability of live cells to converting thiazolyl blue to dark blue formazan [25]. Approximately 2.5 - 103 cells were seeded into 96-well culture plates and treated with or without ethanol for 24 hrs.

After treatment, 20 ml MTT (5 g/l) was added into the wells and incubation continued at 37°C for 4 hrs, and 100 ml DMSO was pipette to solubilize the formazan product for 30 min at room temperature. The absorbency at 570 nm was measured using ELISA reader [16]. For proteome analysis, The harvested normal and treated fibroblast cells with 270 mM dosage of ethanol were washed three times using washing buffer (250 mM D-Sorbitol and 10 mMTris, pH 7.0), followed by lysis with the relevant buffer that is 8 M urea, 4% CHAPS (3-(3-cholamidopropyl) dimethylammonio- 1-propanesulfonate), 40 mM dithiothreitol (DTT), 2% pharmalyte (pH 3–10NL), 1 mMphenylmethylsulfonyl fluoride (PMSF) and 1 mM ethylene diamine tetra-acetic acid (EDTA) [16]. Sonication was performed for this aim for 3 minutes with 1 minute intervals. After that, centrifuge at 40 000 g for 30 min was performed. Bradford method was applied for protein quantifications, and then the supernatants were stored at -20C for furthered evaluations. The method of 2D electrophoresis was the next step for protein separation, in which Isoelectric focusing electrophoresis was carried out with 17 cm (pH 3–10NL) IPG strips at -20°C according to the manufacturer's instructions. The strips were rehydrated without voltage for 4 h and with 50 V for 8 h. The iso-electric focusing was programmed at a gradient mode, which was first focused for 3 hrs at the different voltages, 500, 1000 and 8000 V, respectively, then continued at 8000 V until a total of 50 KVh. After that, the focused strips were equilibrated in buffer with 6 M urea, 50 mM Tris–HCl, 30% glycerol, 2% SDS and trace bromophenol blue, and were subsequently treated by the reduction of DTT and alkylation of iodoacetamide. The treated strips were transferred onto 12% uniform SDS PAGE then performed in 2.5 W each gel for 30 min and 15 W each gel until the bromophenol blue dye line reached the bottom of the gel [17].

Then gels were visualized by silver staining. The gels were scanned by Bio Rad Image Scanner and finally SameSpot Progenesis was performed and spots were analyzed based on the degree of folding, and indicated by P ANOVA statistical analysis.

RESULTS

MTT assay can present significant properties related to viability quantification feature of the examined cells, providing data pertaining if the treated agents act up or downstream of mitochondrial dysfunction [18]. Here, after first treatment with different concentration of ethanol, 24hrs incubation was applied without ethanol in the culture; finally the samples were evaluated with MTT assay protocol. (See figue1) Invert microscopic studies have a noteworthy application in analyzing morphological changes of the live cells [19].

In this study, microscopic analysis of fibroblast before and after treatment by 270 and 378 mM dosages of alcohol as the most effective dosages was applied (See figure2).
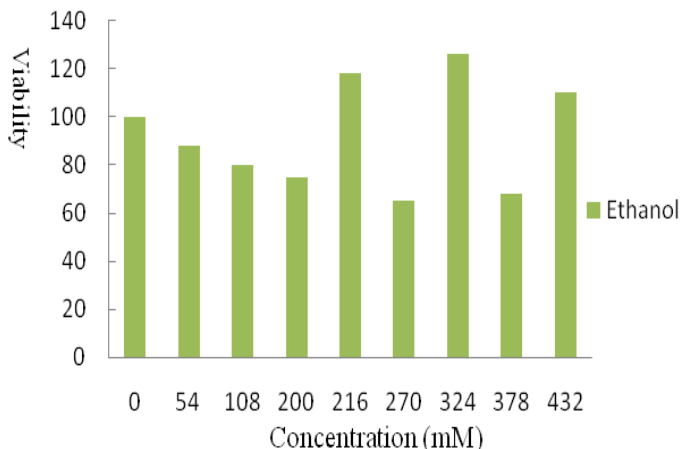

Figure1. Different applied dosages of ethanol on human fibroblast cells after 24 hrs. Viability indicated as percentage.
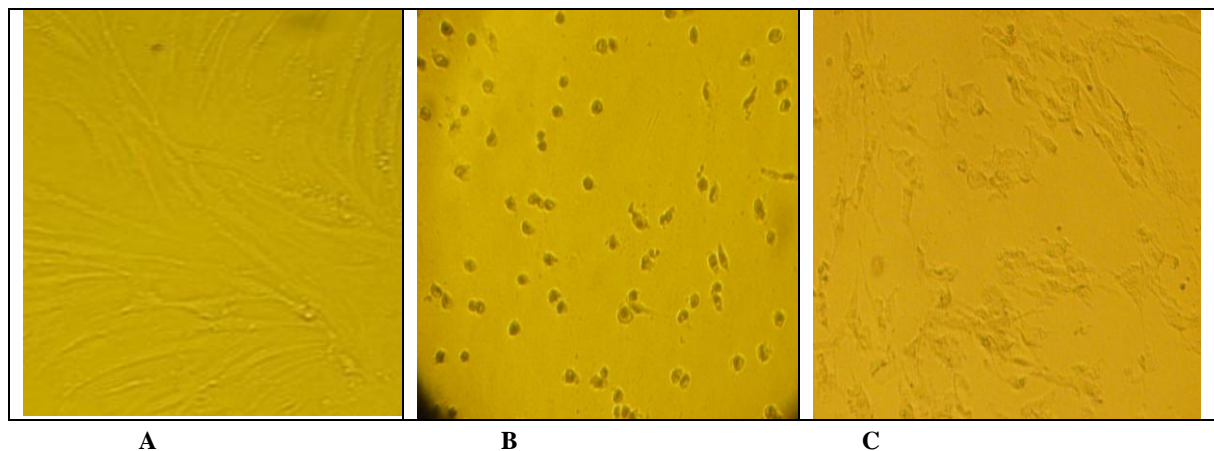

| A | B | C |

Figure 2. Different morphological study of ethanol effect on fibroblast cells: A: normal fibroblast cells without ethanol, B: treated cells with the 270 mM dosage of ethanol, C: treated cells with 378 mM of ethanol by the first exposition and then 24hrs incubation in the absence of ethanol in the culture captured by Invert microscope with 1000 magnification
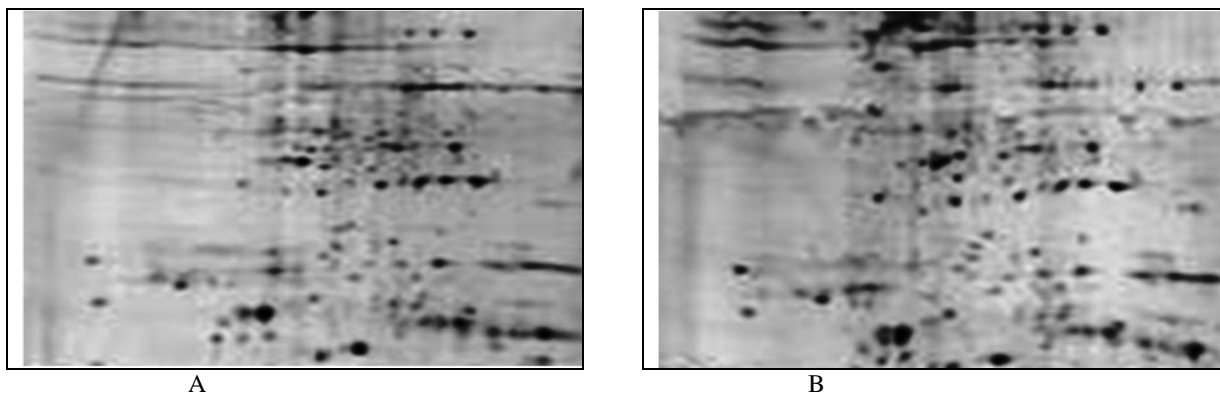

| A | B |

Figure3. Two-dimensional electrophoresis gels: A: normal sample of fibroblast B: Treated fibroblast sample with ethanol concentration of 270 mµ
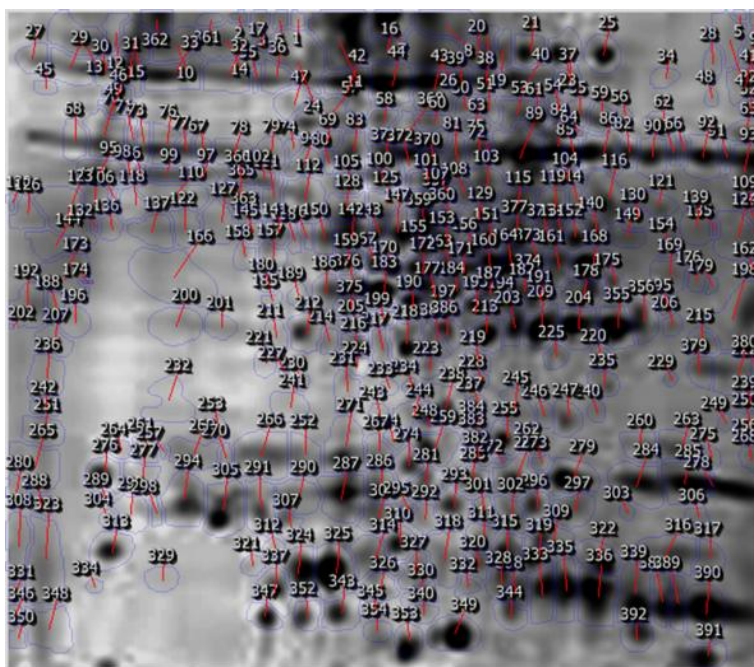
Figure4. Detected normalized protein spots on all obtained images of fibroblast normal and treated with alcohol are marked in an individual image as a reference gel (normal sample). Numbers signifies the 372 proteins of the samples.

Proteome study provides significant information pertaining to protein alterations. This aim can be facilities with proteomic studies [17]. Two electrophoresis gels related to fibroblast normal cells and treated with ethanol with dosage of 270 mM as the most effective concentration is applied for proteomic research as it is presented in figure3. Analyzing protein by gel-base method is useful in presenting a visual pattern of protein distribution [14]. These proteins are numbered in the treated sample as it is clear in figure4.

The level of protein expression in each gel is demonstrated by The Principal Component Analysis (PCA) for verifying the principle axes of expression variation [16]. It lets to separate the spots according to their expression level by transforming and plotting. In addition this is helpful in identifying gel outliers (See figure5).
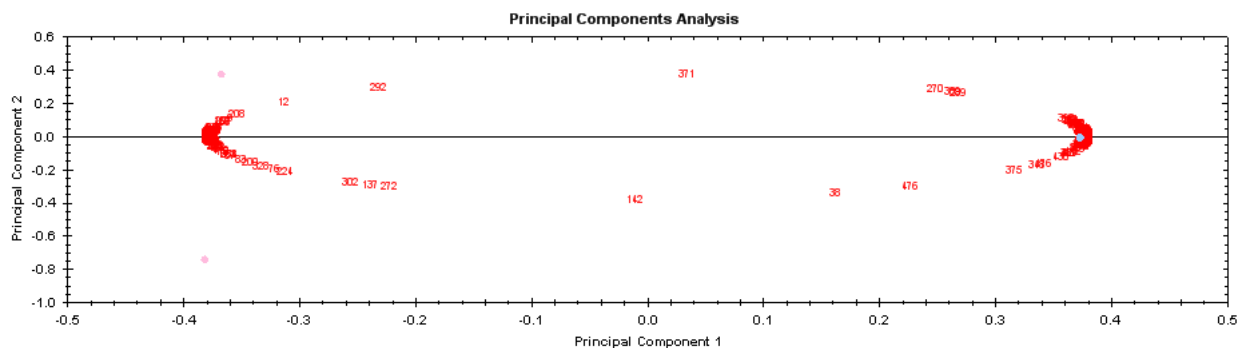

Figure5. Protein expression and gels position in biplot format of PCA. Gels from each group are indicated as the colored dots, in which spots around each gel in the positive side have high expression value, and the rest in the negative side shows low expression. The result is obtained by SameSpots software
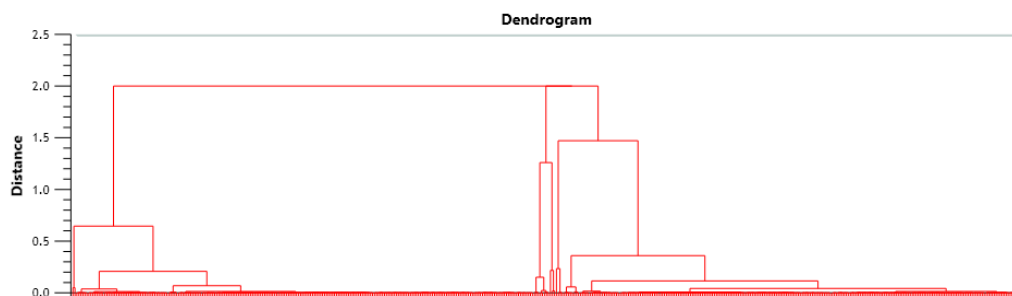
Figure 6. Protein spot clusters shown as visual representation based on similarity characters obtained by SamesSpots software
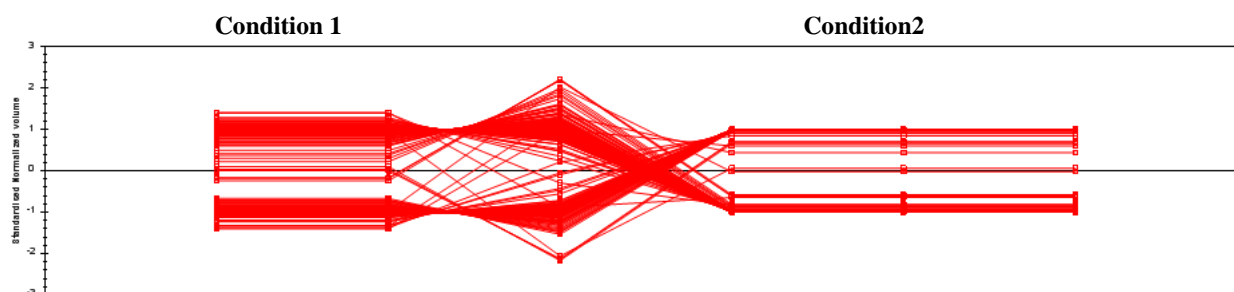

Figure7. Protein profiles that show correlation in expression pattern obtained by SameSpots software. Proteins with fold >2 alters significantly from condition 1 to 2.

Clustering is performed based on how much similar the protein spots are. Similar expression can be calculated by high correlation value (i.e. close to 1) whereas opposing expression can be achieved by high negative correlation value (i.e. close to -1). Leaf nodes are each spot located down the bottom of the dendrogram. By joining each spots or accessible spot clusters with a node, the spot clusters are created as hierarchical relationship [14]. (See figure6)

DISCUSSION

Proteome evaluations can emerge novel result due to integral role of proteins in many diseases [20]. Identifying the interactive pathways of proteins for diagnostic approaches is the final [17, 21] Ethanol showed to have some cytotoxic effects on human body based on previous studies [22]. By studying in-vitro analysis of ethanol effect on human fibroblast cells, here this aim is reachable. MTT assay was performed to estimate the effect of different concentrations of ethanol on fibroblast cells after 24hrs. In fact, cells were first treated with alcohol then incubated without ethanol. It is clear from figure1, that the different concentrations have different effect on cell

viability rate. The most decreased population rate of the cells is related to 270 and 378 mM of ethanol (P value<0.05). For this reason, the 270 mM dosage of ethanol has been applied for more evaluations so the data in figure2, confirms that the morphology of the cells changes vigorously after treatment with this dosage of ethanol. In a way that, the morphology of cells alters from spindle-shaped into rounded-shaped cells and the number of living cells decreases dramatically. Consequently, it is applied for more assessment in proteome level. Two dimensional gel electrophoresis is the one of the most applicable techniques in the field of proteomic study [23]. Proteome of normal cells of fibroblast cells compared with the treated sample with ethanol and amazingly, about 372 protein spots were identified and among them about 65 were having significant expression altration, in which 37 of them are up-regulated in ethanol-treated sample, and the rest are down-regulated. As it is indicated in figure 5, PCA analysis can bring out useful information related to clustering of proteins and also the evaluation of transformed proteins in each gel [17]. In PCA figure both gels and protein spots are captured in a same graph. The spread of

gels of each group indicates the biological variation. A cluster of spots are gathered around each gel, in which represents the most highly expressed ones in that particular group. Protein spots around the pink-colored dots (normal gels) signifies high expression in these samples while contain low expression level for the blue-colored ones (treated gels). This result can be observed for blue-colored gels in a converse manner. In addition, more analysis shows that proteins gathered together in biplot should have similar expression profiles and consequently should be clustered together in the dendrogram. For deciphering correlation analysis, dendrogram analysis is valuable [24, 25]. As figure 6 illustrates, proteins with similar expression levels (small clusters) have close distance while there is a farther distance between bigger clusters that shows general expression profile. A number of 37 proteins have close distances which are the up-regulated proteins, on the other hand, the total number of 28 proteins assigned for down-regulated ones that implies on close distance between them. As it is clear from protein expression profile in figure7, clusters of correlated proteins in expression level are notable between two samples. In fact, up-regulated

proteins change together and down-regulated ones alter in a same way; this shows the positive correlation between them and approving the earlier results and also suggests that these proteins within specific clusters can have similar biological characteristics including three main features: cell compartment, biological process, and molecular function which requires more evaluations. It seems that ethanol induces considerable changes in many biological processes in the treated cells.

CONCLUSION

It can be concluded that, ethanol has remarkable effect on molecular level of human cells. The proteome changes implies on this fact. Therefore, it is suggested that transformed proteins would be identified and the gene ontology of these significant changed proteins would be evaluated in further analysis.

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