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Profiling of Metabolites from Human Intervertebral Disc through Gas Chromatography - Mass Spectrometry

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Abstract

This work aims to identify the metabolites present in the human Intervertebral Disc (IVD). Metabolomic analysis of human IVD tissue has not been extensively done to date. Knowledge on the metabolites present in the IVD tissue in humans is very limited and many compounds are yet to be identified. In this study, we have carried out the metabolic profiling for human IVD through Gas Chromatography/Mass Spectrometry (GC/MS). This is the first initial study that has compared the metabolites of control and diseased IVD. We have identified 75 different chemical compounds in IVD, and also metabolites that are unique to the diseased IVD, suggesting that some of these metabolites might play a role in disc degenerative disease.

Keywords: DDD, Human, Intervertebral Disc, Metabolite Profiling

1. Introduction

Low Back Pain (LBP) is a global health problem in which more than 40% is caused by lumbar intervertebra[1] disc degeneration1 and one of the most important health care issues today. About 60 % and 80 % of the global population experiences LBP at least once in their lifetime[2]. The annual prevalence ranges from 15% to 45% with the point prevalence averaging 30% [3]. Only a small proportion (approximately 20%) of LBP cases can be attributed with reasonable certainty to a pathologic or anatomical entity. Thus, diagnosing the cause of LBP represents the biggest challenge for doctors in this field. Recent reports are showing enough evidences to the involvement of genetic factors and a number of genes like Vitamin D Receptor (VDR)[4], Collagen – factors[5], Interleukins[6], MMP3 (Matrix metalloproteinase-3)[7], aggrecan[8] and cartilage intermediate layer protein[2] are reported to be associated with DDD. A few candidate genes, which have a weak association with DDD (viz., (Vitamin D receptor) VDR, (collagen IX A2) COL9A2, (collagen IX A3) COL9A3, (Matrix metalloprotease-3) MMP3 and Aggrecan), have been identified using genetic polymorphisms[9]. However, functional studies of candidate genes will be an important step for testing whether a candidate gene is truly associated with Disc Degenerative Disease (DDD) or not.Hence systematic analysis of genes expressed in IVD through high throughput genomic tools viz., microarrays, proteomics and metabolomics are needed which will shed light on the major events associated with DDD and lead

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to the identification of metabolic pathways involved in this degeneration.

2. Materials and Methods

The IVD of control and patient were obtained from spine surgery unit and used after getting permission from Ethical Clearance Committee with informed consent. For method development and validation, a representative control and affected disc tissue was snap-frozen immediately following surgery and then stored at – 80°C. About 20mg of the stored tissue weighed accurately and ground with liquid nitrogen. Then 1mL of a mono-phasic mixture of chloroform/methanol/water in ratio of 20:50:20 (v/v/v) was added to each sample[10]. The samples were ultrasonicated in an ultra-sonicator bath at ambient temperature (24–28°C) for 100 min and then vortex-mixed for 2 min. The samples were subsequently centrifuged at 12000 rpm for 10 min and the supernatant was collected separately from each sample in different tubes. The collected supernatant was concentrated to complete dryness at 50°C for 30 min. A 100 µL of toluene (kept anhydrous with sodium sulfate) was added to each of the sample extracts, vortex-mixed for 5 min and again evaporated to complete dryness using vacuum evaporator in order to eliminate any trace amount of water which might interfere with the GC/MS analysis.

2.1 Derivatization Procedure

The dried samples were then derivatized by adding 100 µL of MSTFA with 1% TMCS to each sample. The samples were then vortex-mixed for 2 min and incubated at 70°C for 30 min. After incubation, samples were again vortex-mixed for 2 min and then transferred to glass vials for GC/MS analysis.

2.2 GC/MS Analysis

The analysis was performed on a Thermo GC - Trace Ultra Ver: 5.0, Thermo Ms DSQ II. A DB 5 - MS capillary standard non - polar column (30 x 0.25mm; 0.25µm flim thickness, Thermo Scientific). Helium was used as the carrier gas at 1.0 mL per min and the injector split ratio was set to 1:5. An injection volume of 1µl was used and the solvent cut off time was 5 min.

The injector and source temperatures were kept at 250°C and 200°C, respectively. The oven temperature was kept at 60°C for 3 min, increased at 7°C min^{-1} to 140°C where it was held for 4 min and further increased at 5°C min^{-1} to 310°C where it remained for 6 min. The mass spectrometer was operated in Electron Impact (EI) ionization mode at 70 eV. Data acquisition was performed in the full scan mode from m/z 50 to 650 with a scan time of 0.5 s.

3. Results

3.1 Metabolite Identification and Data Processing

GC/MS analysis from this study led to the identification of 75 different metabolites (Figure 1 and 2) belonging

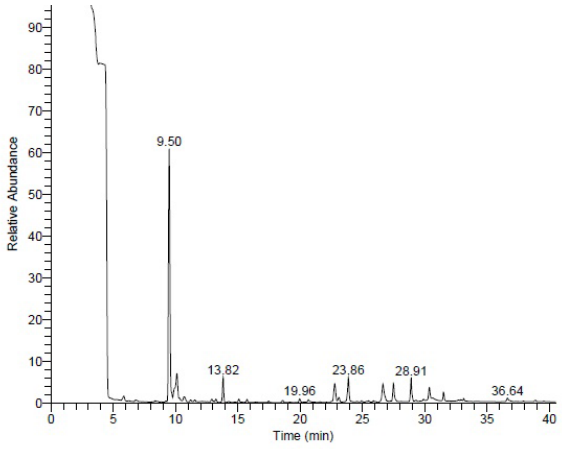

Figure 1. GC/MS chromatograms of control intervertebral disc.

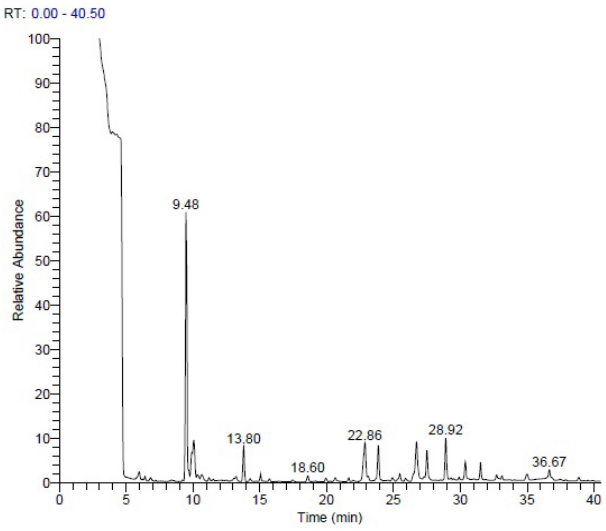

Figure 2. GC/MS chromatograms of degenerated intervertebral disc.

to diverse chemical classes such as amino acids, organic acids, fatty acids, phenolics, silanes, nitriles, ethers, amines, azides, hydrocarbons, alcohols, carbonyl compounds, heterocyclic compounds, spiro compounds and metal complex (Table 1 and 2). The identities of selected metabolites were recognized by the NIST mass spectral library.

4. Discussion

GC/MS-based metabolomics is a powerful tool to elucidate the impact of low back pain thereby providing novel insights into the pathomechanism underlying disc degenerative disease. This GC/MS based metabolomic approaches cover a large number of novel compounds.

Table 1. Identified metabolites from control intervertebral disc tissue through GC/MS against NIST library search

S. No	Metabolites	Retention time	Chemical groups identified
1	Cyclopropanecarboxylic acid, pent-2-en-4-ynyl ester	3.06	Organic acid and its derivatides
2	N1-(Formyl)-N2-(1-oxobut-2-en-1-yl)hydrazide	3.45	Azides
3	exo-5-hexyl-exo-4-oxa-tricyclo[5.2.1.0**2,6]dec-8-en-3-one	4.43	Carbonyl compounds
4	Methoxy-phenyl oxime	4.92	Carbonyl compounds
5	D-Lactic acid-DITMS	5.28	organic acid and its derivatives
6	Methyl 3-((aminocarbonyl)amino)-2-cyano-3-phenylpropenoate	5.87	Organic acid and its derivatides
7	2-[5-(2-hydroxyethyl)-2-thienyl]-4,4-dimethyloxazoline	6.28	Heterocyclic compounds
8	2-methyl Decane	6.77	Hydrocarbons
9	1,2-Dioxetane, 3,4,4-trimethyl-3-[[(trimethylsilyl)oxy] methyl]1,2-Dioxetane	7.38	Silanes
10	5,8-Diethoxy-7-methoxyquinoline	8.05	Heterocyclic compounds
11	Octanoic acid, trimethylsilyl ester	8.4	fatty acid (S)
12	1-Tert-buthoxy-6-trimethylsilyloxyhexane	8.79	Ethers
13	Bis(trimethylsilyl) 3-Ketovaleric acid	9.22	Amino acids
14	Tris(hydroxymethyl)aminomethane,O,O',O"-tris(trimethylsilyl) ether	9.5	Ethers
15	3-hydroxy Benzocycloheptene	10.12	Hydrocarbons
16	4-Methyl-6-cyanothieno[2,3-b]pyridine	10.31	Heterocyclic compounds
17	[(2-deuterio)-s-isobutyl]-2-propenyl-sulfoxide	10.73	Hydrocarbon
18	N-Acryloylmorpholine	11.21	Heterocyclic compounds
19	4,5-Dihydro-4,5-trans-di-n-propyl-2-ethoxyimidazole	11.54	Heterocyclic compounds
20	Docosane	12.16	Hydrocarbon
21	N-Nitrosomethylethylamine (à-D2)	12.57	Amines
22	[6-(4-tert-Butylphenyl)-1,3,5-hexatriynyl]trimethylsilane	12.9	Silanes
23	Hexadecane (CAS)	13.24	Hydrocarbon
24	3-phenyl-1,2-naphthoquinone	13.82	Hydrocarbon
25	Dodecanoic acid (CAS)	14.26	fatty acid (S)
26	Eicosane, 10-methyl- (CAS)	14.72	Hydrocarbon
27	Isopropyl Dodecanoate	15.09	Organic acid and its derivatives
28	2-Methoxy-8-Chloro-Dibenzofuran	15.27	Heterocyclic compounds
29	Eicosane, 2-methyl- (CAS)	15.74	Hydrocarbon

Continued ...

30	Oxirane, hexyl- (CAS)	15.98	Cyclic ether (Ethylene oxide)
31	Malonic acid, dodecyl 2-ethylbutyl ester	16.19	Organic acid and its derivatives
32	1-methoxymethyl-4-methylnaphthalene	16.41	Hydrocarbon
33	Nonadecanoic acid, 18-oxo-, methyl ester (CAS)	17.25	fatty acid (S)
34	17-Methyl-9-oxo-10-nor-14à-4,5-nitrilomorphinan	17.46	Heterocyclic compounds
35	Tetradecanoic acid (CAS)	18.59	fatty acid (S)(Myristic acid
36	2-(o-Hydroxymethylbenzyl)naphtho[2,3-b]thiophene	19.19	Heterocyclic compounds
37	Heneicosane	19.96	Hydrocarbon
38	(15à)-phylloclad-16-ene-15-carbaldehyde	20.65	Carbonyl compounds
39	Eicosane	21.55	Hydrocarbon
40	1-Hydroxy-17-(1-oxoethyl)-2-oxa-androst-4-en-3-one	22.14	Carbonyl compounds
41	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester (CAS)	22.79	Organic acid and its derivatives
42	2-Methoxycarbonyl-3-phenylsulfonylhydroquinone	23.1	Hydrocarbon
43	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	23.86	Carbonyl compounds
44	Bis[1,2-di(2-thienyl)-1,2-ethenedithiolene]nickel	24.57	Metal complex
45	Dibutyl phthalate	24.93	organic acid and its derivatives
46	4-(4-Fluorophenyl)-2-methyl-6-methylthiobenzonitrile	25.51	Hydrocarbon
47	Diethyl [2- (4'-methylphenyl) ethyl] phosphonate	25.89	Organic acid and its derivatives
48	2,2-Dichlorocyclobuta[a]cyclopent[3,4-a]azulenone	26.64	Carbonyl compounds
49	1,3-Dimethoxy-5,7-dihydrodibenz[c,e]oxepine	27.48	Heterocyclic compounds
50	Hentriacontane	28.12	Hydrocarbon
51	Octadecanoic acid, propyl ester (CAS)	28.31	Fatty acid (S) (Stearic acid
52	10,10-dimethylanthrone hydrazone	28.53	Carbonyl compounds
53	1,3-Dimethoxy-5,7-dihydrodibenz[c,e]oxepine	28.91	Heterocyclic compounds
54	9,10-Dihydrocyclobuta[a]triphenylene-11,12-dione	29	Carbonyl compounds
55	Pulchelstyrene D	29.91	Hydrocarbon
56	3,7-dimethoxy-1,9-dimethyldibenzofuran-4-carbaldehyde	30.36	Carbonyl compounds
57	3,18-Epoxyandrosta-5,7-dien-17-ol, 4,4-dimethyl-3-methoxy- (13á)	31.25	Epoxide (Hydrocarbons)
58	3,7-dimethoxy-1,9-dimethyldibenzofuran-4-carbaldehyde	31.5	Carbonyl compounds
59	2áBenzyl-8-oxo-4,6-dimethyl-3,5,7-trioxatetracyclo[7.2.1. 0(4,11).0 (6,10)]dodecane	31.94	Hydrocarbon
60	Pentacosane	32.87	Hydrocarbon
61	Di-(2-ethylhexyl)phthalate	33.14	Organic acid and its derivatives
62	Hexadecane, 2,6,10,14-tetramethyl- (CAS)	33.32	Hydrocarbon
63	Nonacosane (CAS)	33.59	Hydrocarbon
64	03027205002 Flavone	34.27	Carbonyl compounds
65	10-Benzyloxy-1,8-dihydroxy-9(10H)-anthracenone	35.21	Carbonyl compounds
66	2-Pentoxy-tetrahydropyran	35.59	Heterocyclic compounds
67	N-(5à-Cholestan-3á-yl)-acetamide	36.17	Organic acids and its derivatives

Continued ...

68	Octadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester (CAS)	36.64	Fatty acid
69	Tetratetracontane (CAS)	37.31	Hydrocarbon
70	1,4-Cyclohexadiene-1,2-dicarboxylic acid, 4,5-dimethyl-, dimethyl ester	37.92	Organic acids and its derivatives
71	Acetic acid, 4,5-dihydroxy-10,13-dimethyl-3-oxohexadecahydrocyclop enta[a]phenanthren-17-yl ester	38.21	Organic acids and its derivatives
72	Methyl 4-(4-methoxybenzoyl)-4-methyl-pent-2-enoate	38.5	Organic acids and its derivatives
73	13-Docosenamide, (Z)-	38.87	Organic acids and its derivatives
74	2-[3-(Aminomethyl)-5,7-Dimethyl-1-Adamantyl] Ethanamine	39.56	Amines
75	Dihydromonticamine	39.93	Amines

Table 2. Identified metabolites from degenerated intervertebral disc tissue through GC/MS against NIST library search

S. No	Metabolites	Retention time	Chemical groups identified
1	1-Benzyloxymethyl-1-Hydroxymethyl-2,5- Cyclohexadiene	3.1	Hydrocarbon
2	4-Chloro-4-(phenylsulfinyl)-3-heptanol isomer	3.51	Alcohols
3	(6á)-8a-(3',3'-Dimethylbut-1'-ynyl)-3,4,4a,5,6,8a-hexahydro-6-methoxy-3,3,6-trimethylnaphthalen-1(2H)-one	4.65	Carbonyl compound
4	4-[3-(Trimethylsilyl)-2-propinyl]oxy-2-butynoic acid	5.09	Organic acid and its derivatives
5	D-Lactic acid-DITMS	6	Organic acid and its derivatives
6	2-[5-(2-hydroxyethyl)-2-thienyl]-4,4-dimethyloxazoline	6.41	Heterocyclic compounds
7	Ethyl 3-(Trimethylsilyl)Propanoate #	6.84	Organic acid and its derivatives
8	3,7-Dioxa-2,8-disilanonane, 2,2,8,8-tetramethyl-5-[(trimethylsilyl)oxy]- (CAS)	7.22	Silanes
9	2-[4(or 5)-(2-Phenylimidazolyl)]propionitrile	7.59	Nitriles
10	3-Phenylnon-4-en-3-ol	8.06	Alcohols
11	Octanoic acid, trimethylsilyl ester	8.43	Organic acid and its derivatives
12	9,12,15-Octadecatrienoic acid,2-[(trimethylsilyl)oxy]-1-[[(trimethylsilyl)oxy]methyl]ethyl ester, (Z,Z,Z)- (CAS)	8.81	Organic acid and its derivatives
13	2-Aminoethanol, N-acetyl-, trimethylsilyl ether	9.04	Ethers
14	3-Oxovaleric acid TMS ether TMS ester	9.22	Amino acids
15	5-Benzoyl-1,2,3,4-tetrahydronaphthalene	9.48	Hydrocarbon
16	Dibenzo[c,e]thiin-2-thione	10.05	Carbonyl compound
17	4-Methyl-6-cyanothieno[2,3-b]pyridine	10.31	Heterocyclic compound
18	Eicosane (CAS)	10.65	Hydrocarbon
19	N-Acryloylmorpholine	11.21	Heterocyclic compound
20	Hexadecane, 2,6,11,15-tetramethyl- (CAS)	11.52	Hydrocarbon
21	1,2-Difluoro-3,4,5-trimethylbenzene	12.12	Hydrocarbon
22	N,N'-Dityrityl-1,5-diaminopentane	12.51	Hydrocarbon

Continued ...

23	Hexadecane (CAS)	13.22	Hydrocarbon
24	2-tert-Butyl-4-isopropyl-5-methylphenol	13.8	Phenolic
25	Dodecanoic acid (CAS)	14.3	Fatty acid (S)
26	2-(2-chinoxalinyl)-(all-às)cyclotetrathiophen	14.7	Heterocyclic compound
27	Dodecanoic acid, 1-methylethyl ester	15.07	Fatty acids
28	Spiro(1,3-dioxolane)-2,3'-[5'-androsten-16'-trimethylsilyloxy)-	15.26	Spiro compounds
29	Hexadecane, 2,6,10,14-tetramethyl- (CAS)	15.72	Hydrocarbon
30	11-Heneicosanone (CAS)	16.17	Carbonyl compound
31	1-methoxymethyl-4-methylnaphthalene	16.39	Hydrocarbon
32	Docosane (CAS)	16.57	Hydrocarbon
33	Octadecane (CAS)	17.46	Hydrocarbon
34	Tetradecanoic acid (CAS)	18.62	Fatty acid (S) (Myristic acid)
35	Tetradecanoic acid, trimethylsilyl ester (CAS)	19.17	Fatty acid (S) (Myristic acid)
36	1,1-Bis(p-tolyl)ethane	19.64	Hydrocarbon
37	Hexadecane, 2,6,10,14-tetramethyl- (CAS)	19.95	Hydrocarbon
38	Methyltetrahydrofurolacellicolic anhydride	20.26	Organic acid and its derivatives
39	1-Amino-2-cyano-3,4-dihydro-4-ethoxycarbonyl-3-phenylpyrido[1,2-a]benzimidazole	20.64	Heterocyclic compounds
40	Hexadecanoic acid, methyl ester (CAS)	21.64	Fatty acid (S) (Palmitic acid)
41	4-phenyl-6-(p-methylbenzoyl)bicyclo[3.3.0]octa-3,7-dien-2-one	22	Carbonyl compound
42	10-Cyano-3-amino-12-(4'-methoxyphenyl)-2-oxopyrano[4,3-d] pyrido[1,2-a]benzimidazole	22.88	Heterocyclic compounds
43	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	23.88	Carbonyl compound (Spiro)
44	Heptadecanoic acid (CAS)	24.6	Fatty acid (S) (Margaric acid)
45	Dibutyl phthalate	24.93	Organic acid and its derivatives
46	Octadecanoic acid, methyl ester (CAS)	25.47	Fatty acid (S) Stearic acid
47	1-[1-[[(tert-butyldimethylsilyl)oxy]methyl]-1-methyl-2-oxoethyl]perhydroazine	25.9	carbonyl compound derivatives
48	6,6-d2-.delta.2-5à-androsten-1-one	26.73	Carbonyl compound
49	10-Methylene-8-methoxy-3,4,4a,10-tetrahydro-2H,9H-anthracene-1,9-dione	27.13	Carbonyl compound
50	1-[1-[[(tert-butyldimethylsilyl)oxy]methyl]-1-methyl-2-oxoethyl]perhydroazine	27.52	carbonyl compound derivatives
51	Octadecane, 3-ethyl-5-(2-ethylbutyl)- (CAS)	28.12	Hydrocarbon
52	Ethanamine, 2,2'-oxybis[N,N-dimethyl- (CAS)	28.53	Amine
53	1,3-Dimethoxy-5,7-dihydrodibenz[c,e]oxepine	28.92	Heterocyclic compounds
54	Acetyl-Ginsenol	28.98	Alcohols
55	Butyl 9-octadecenoate or 9-18:1	29.49	Organic acid and its derivatives
56	3,7-dimethoxy-1,9-dimethyldibenzofuran-4-carbaldehyde	29.91	Carbonyl compound
57	3,7-dimethoxy-1,9-dimethyldibenzofuran-4-carbaldehyde	30.37	Carbonyl compound
58	Hept-enyl-2-acetate	31.05	Organic acid and its derivatives
59	(S)-(2l,6l,3")x)-(1'E)-2-[3'-(3"-Oxocyclohexyl)-1'-propenyl]-6-methyl-3-(1-methylethyl)-1,3,2-oxazaphosphorinane 2-Oxide	31.52	Heterocyclic compounds

Continued ...

60	2áBenzyl-8-oxo-4,6-dimethyl-3,5,7-trioxatetracyc lo[7.2.1.0(4,11).0(6,10)]dodecane	31.93	Hydrocarbon
61	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethylester	32.7	Organic acid and its derivatives
62	Di-(2-ethylhexyl)phthalate	33.13	Organic acid and its derivatives
63	Docosane, 11-decyl- (CAS)	33.34	Hydrocarbon
64	erythro-1,2-Epoxy-2-methyl-3-heptanol	33.68	Alcohols
65	7-(4'-Nitrophenyl)-5-imino-2-methyl-5H-thiazolo[3,2-a] pyridine-6-carbonitrile	34.05	Heterocyclic compounds
66	3-Formyl-1-oxyl-4-(pyren-1'-yl)-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrole	34.52	Heterocyclic compounds
67	1'-Benzyl-3-methoxynaphtho[16,17-b]estra-1,3,5(6)-triene	34.99	Hydrocarbon
68	Cholan-24-oic acid, 3,12-dihydroxy-, (3à,5á,12à)- (CAS)	35.66	Organic acids
69	Octadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethylester	36.67	Organic acid and its derivatives
70	3,5-dicyano-2,6-di(m-tolyl)-1-methylpyridine-4(1H)-thione	37.5	Heterocyclic compounds
71	2,6-Diisopropylanisole	37.91	Phenolic
72	13-Docosenamide, (Z)-	38.87	Organic acid and its derivatives
73	2,2,5,5-Tetramethyl-4-(p-methoxyphenyl)-3-oxazoline	38.97	Heterocyclic compounds
74	Benzoic acid, 2,4-dimethyl-, (3,5-dimethylphenyl)methylester	39.56	Organic acid and its derivatives
75	Hexadecanoic acid, hexadecyl ester (CAS)	39.93	Organic acid and its derivatives

Unlike to proteomics, metabolomics demands a large amount of manual evaluation like the validation and peak correction of identified metabolites. Mass spectrometric analysis could identify the metabolites present in the disc tissue sample through NIST library search.

Interestingly, the presence of lactic acid in our study indicates that lactic acid might play a role in disc degeneration. Intervertebral disc is the largest avascular tissue in the body, its metabolism is mainly anaerobic, and thus lactate is produced at a significant rate. The higher lactate concentration reduces the pH in the disc[11,12]. Experimental data from other studies have showed that acidic pH adversely affect the supply of nutrients to IVD, cellular activity of IVD[13,14], synthesis of proteoglycans (which play a major role in the load bearing capacity of the disc)[12], integrity of extracellular matrix of the IVD, even IVD viability[15], and thus acidification of IVD due to lactic acid may play a role in disc degeneration[13,14].

The presence of lactic acid in both control and degenerated disc indicates that quantification of lactic acid is necessary for correlating the quantity of lactic acid with the causation of IVD degeneration. Similarly further confirmatory studies are required to ascertain whether the compounds found in our study play real roles in disc degenerative disease. Our future studies will concentrate on the quantification of the metabolites from both control and affected tissue for obtaining valuable insights about the IVD degeneration.

5. Conclusion

Our GC/MS-based metabolic profiling study has profiled the metabolites present in both control and degenerated human IVD. Our study revealed that some of these metabolites present in the human IVD might play an important role in disc degenerative diseases.

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