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Elucidating of the metabolic impact of risperidone on brain microvascular endothelial cells using untargeted metabolomics-based LC-MS

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A R T I C L E I N F O

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A B S T R A C T

Risperidone is useful for the treatment of schizophrenia symptoms; however, it also has side effects, and an overdose can be harmful. The metabolic effects of risperidone at high therapeutic doses and its metabolites have not been elucidated. Endogenous cellular metabolites may be comprehensively analyzed using untargeted metabolomics-based liquid chromatography-mass spectrometry (LC-MS), which can reveal changes in cell regulation and metabolic pathways. By identifying the metabolites and pathway changes using a nontargeted metabolomics-based LC-MS approach, we aimed to shed light on the potential toxicological effects of high-dose risperidone on brain microvascular endothelial cells (MVECs) associated with the human blood brain barrier. A total of 42 metabolites were selected as significant putative metabolites of the toxicological response of high-dose risperidone in MVECs. Six highly correlated pathways were identified, including those involving diacylglycerol, fatty acid, ceramide, glycerophospholipid, amino acid, and tricarboxylic acid metabolism. We demonstrated that methods focused on metabolomics are useful for identifying metabolites that may be used to clarify the mechanism of drug-induced toxicity.

1. Introduction

Schizophrenia is a mental disorder that affects how a person thinks, feels, and behaves. It is a chronic condition that typically requires lifelong treatment, and it can be challenging to manage in urban areas because of the high levels of stress and exposure to triggers that can exacerbate symptoms [1,2]. Risperidone is commonly used to treat schizophrenia. It is an antipsychotic drug that works by changing the levels of certain chemicals in the brain that are associated with schizophrenia symptoms [3]. Although risperidone is effective at managing these symptoms, it also has side effects and overdoses can be dangerous. Symptoms of a risperidone overdose include drowsiness, dizziness, rapid heart rate, and low blood pressure [4–6]. In severe cases, risperidone overdose can result in coma or even death [7–9]. It is important for people taking risperidone to follow their doctor's instructions carefully and to be aware of its potential risks and side effects.

Although risperidone is generally considered safe and effective, it can cause side effects, including brain microvascular vascular endothelial cell (MVEC) dysfunction [10]. To transport vital nutrients and metabolites across the blood brain barrier (BBB), MVECs are equipped with a network of specialized transport systems including tight junctions, nutrient transporters, and polarized efflux transporters. MVECs are a protective barrier that surround the brain and restricts harmful substances from reaching the brain tissue [11–13]. When MVECs are functioning properly, they can prevent the entry of certain medications, toxins, and other substances into the brain; however, when they are

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damaged or disrupted, such substances can pass through more readily, potentially leading to adverse effects on brain function. Recently, a few studies have shown that high therapeutic doses of risperidone can disrupt the BBB, resulting in increased permeability and potential brain damage [10].

High therapeutic doses of risperidone often unintentionally have been linked to side effects that cause the MVECs of BBB to break down and become dysfunctional. According to X et al. [14], risperidone can significantly alter the permeability of iron within MVECs of the BBB. In addition, risperidone inhibits MVEC function in the BBB of bipolar disorder patients. Recently, Elmorsy et al. [10] demonstrated that oxidative stress, cell ultrastructure, morphology, and viability of MVECs are impaired at high therapeutic doses of risperidone. Risperidone can affect MVEC function at high doses; however, the underlying mechanism is unclear. Thus, it is important to identify the risperidone-mediated mechanisms responsible for MVEC breakdown and dysfunction as the metabolic effects of risperidone and its metabolites have not been reported.

Untargeted metabolomics-based LC-MS is a powerful tool for studying the chemical composition of biological samples. This approach enables the comprehensive identification and quantitation of metabolites present in a sample, thus providing a comprehensive view of the metabolic state of an organism [15–17]. There are several key benefits to using untargeted metabolomics-based LC-MS. First, it can detect a wide range of metabolites, including both known and unknown compounds. This is particularly importance for studying complex biological systems, in which the metabolic pathways and interactions are not fully understood [17]. Second, it enables the quantitative analysis of metabolites, which provides information on the relative abundance of each compound within a sample. This is useful for examining changes in metabolic pathways in response to various stimuli, such as environmental stress or drug treatments [18]. Third, this technique is a sensitive and specific analytical technique that provides high-resolution data on the chemical composition of samples. This enables one to identify subtle changes in metabolism that may not be detectable by other methods [19]. Overall, the use of untargeted metabolomics-based LC-MS is essential for understanding the complex metabolic networks present in biological systems and can provide valuable insights into the role of metabolism in health and disease. Therefore, we determined the metabolic changes caused by risperidone at high therapeutic doses in MVECs using untargeted metabolomics-based liquid chromatography-mass spectrometry (LC-MS).

2. Material and methods

2.1. Chemical and reagents

Complete Human Endothelial Cell Medium (CHECM) (containing essential and non-essential amino acids, vitamins, organic and inorganic compounds, hormones, growth factors, trace minerals) supplemented with endothelial cell growth supplement, antibiotics, and phosphate buffer saline solution (PBS) were purchased from Gibco (Bangkok, Thailand). All MS graded chemicals and water were obtained from Apex Chemical (Bangkok, Thailand).

2.2. MVECs

Primary human brain MVECs microvascular endothelial cells (MVECs), an important component of the BBB, were used in this study and provided by Faculty of Medicine & Health Sciences, University of Nottingham Medical School, Queen's Medical Centre, Nottingham, UK.

2.3. Cell viability

MVECs were seeded into 96-well plates at a density of 1×10^4 cells/well and allowed to attach for 24 h. The media was removed, and the cells were treated with vehicle (control) or 10 µl risperidone. After 4, 24, 48, and 72 h, MVECs viability was determined using the MTT assay (Gibthai, Bangkok, Thailand) according to the manufacturer's instructions.

2.4. MVEC experiments

A mature MVEC monolayer with a robust barrier phenotype was formed, grown, and maintained as previously described [20,21]. Briefly, MVECs were cultured in CHECM in a humidified incubator at 37°C containing 5 % CO_2 and 95 % air. Unattached MVECs and cell culture media were discarded after reaching 80 % confluence and the attached MVECs were washed with 5 ml of PBS. Cell culture medium (10 ml) was then added with or without risperidone and incubated for 48 h. After removing the media, the attached MVECs were washed with 5 ml of PBS and treated with 0.5 ml of cold methanol (4°C). After removing the cells from the flask with a cell scraper, the suspension was transferred to an Eppendorf tube and kept at −80°C for subsequent procedures. The concentrations of risperidone used in the experiments were similar to that measured in highly/over dosed individuals (10 µM) [10]. This concentration did not affect MVEC viability or morphology.

2.5. Control groups

To ensure the robustness and validity of our findings, we included multiple control groups in our study. These controls included:

- Vehicle Control: MVECs were treated with the vehicle (solvent used to dissolve risperidone) without the drug. This control helps to identify any effects that may be due to the solvent rather than risperidone itself.
- Untreated Control: MVECs were cultured under identical conditions without any treatment. This control provides a baseline for comparison against treated samples to assess the impact of risperidone.
- System Blank: Samples without cells, treated identically to other groups, were used to identify any background noise or contamination in the experimental setup.

By including these control groups, we could differentiate between the effects specifically attributable to risperidone and those arising from other variables, ensuring the reliability of our results.

2.6. Cellular metabolite extraction

The cell extraction method was previously described by Bligh and Dyer [22]. A 0.5 ml suspension of cell debris from each dish was transferred to an Eppendorf tube and vigorously vortexed for 5 min to extract cell metabolites. The suspension was added to 0.5 ml of 4°C chloroform and 0.5 ml of 4°C water, and then vigorously vortexed for 10 min at 4°C. The suspension was subsequently centrifuged at 15,000 g for 10 min at 4°C. Both the polar and non-polar fractions were collected separately, transferred to new Eppendorf tubes, and dried by speed vacuum for the non-polar fraction and freeze-dried for the polar fraction. The dried non-polar fraction was reconstituted in 50 µl of cold chloroform, and the dried polar fraction was reconstituted in 50 µl of cold methanol. To remove cell debris, the reconstituted samples were centrifuged at 15,000 g for 10 min at 4°C. The supernatant was transferred into an HPLC vial and chilled to −80°C until further analysis. Then, 10 µl of the treatment sample, the control, and the system blank were pooled to create a QC sample representing the polar and non-polar fractions.

2.7. Metabolomic analysis

Metabolic profiling was performed on an LC AccelaTM system (Thermo Scientific Ltd, Bangkok, Thailand) coupled with a high

-resolution mass spectrometer (Exactive, Thermo Scientific Ltd, Loughborough, UK). Non-polar chromatographic separations were performed using an Agilent SB C8 column (1.8 μm particle size, 2.1 × 100 mm, Crawford Scientific Ltd., Lanarkshire, UK) and polar chromatographic separations were performed using a C18 (2) column (2.5 μm particle size, 3 × 100 mm, Phenomenex Ltd, Cheshire, UK). The details of the LC and MS conditions are described in our previous study [23,24]. Briefly, Supple. Table 1 and 2 summarize the details of metabolomic analysis conditions. For both polar and non-polar profiling, six independent samples were analyzed for each group (treated vs. untreated) to account for any biological variability. The retention time consistency and mass accuracy were confirmed based on the pooled QC samples and used to evaluate the performance of the instrument [25].

2.8. Statistical analysis

The resulting LC-MS data were analyzed by principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA) using MetaboAnalyst 6.0 (https://www.metaboanalyst.ca/). For OPLS-DA modeling, the samples were grouped together. Score plots representing PCA and OPLS-DA results were used to visualize sample clustering and denote sample similarity. The variable importance in the projection (VIP) ranks >1.00 were used to screen for discriminatory metabolites between the risperidone-treated and untreated samples, and an ANOVA statistical analysis and a false discovery rate (FDR) with a significant level of 0.05 was used to validate the results. The target masses of the metabolites identified by the profiling method were searched over a constrained 5 ppm mass window using the HMDB, METLIN, and LIPIDMAP databases in accordance with the identity check based on raw data and peak features.

2.9. Biological metabolism analysis

To identify the pathways associated with the treatment conditions, pathway analysis was done by combining the findings from MetaboAnalyst 6.0. The results were reported graphically and in a table. The identified metabolic pathways and statistics were used to identify the putative effects of risperidone.

3. Results

3.1. The effect of high therapeutic risperidone on MVEC viability

The association between risperidone and MTT reduction for the HBMECs at 4 different time points (4, 24, 48, and 72 h) is shown in Fig. 1. Risperidone (10 μM) significantly decreased the ability of the cell to reduce MTT compared with the control ($p < 0.05$, ANOVA). A similar decrease was observed in the ability of the cells to exclude trypan blue.

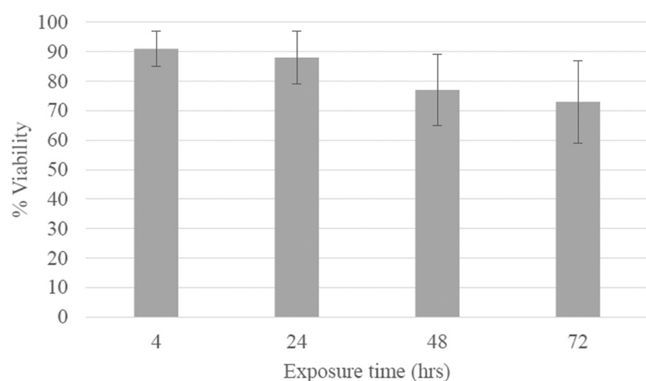

Fig. 1. Time course of MVEC viability following high-dose risperidone (10 μM) treatment as measured by the MTT assay ($p < 0.05$, ANOVA).

This indicates a loss of plasma-membrane integrity of the cells.

3.2. LC-MS analysis

A QC sample was created by pooling 10 μl of each sample to test the performance of the LC-MS instrument. The MS spectral data were aligned based on this sample. Importantly, the alignment process did not change how the samples were clustered and was applied to the aligned data to identify significant clusters in an unsupervised manner. Furthermore, the pooled samples (QC) were used for data normalization to reduce any metabolic changes caused by environmental factors. A total of 412 polar and 556 non-polar metabolites were identified, including amino acids, fatty acids, organic acids, and sugars, based on mass alignment using a mass spectral database and comparison with reliable standards and literature data (data not shown). The base MS peak chromatogram of the samples in ESI+ and ESI- are shown in Supple. Figs. 1 and 2, respectively.

3.3. Metabolomics analysis

Metabolomics was used to resolve the data collected from both ion modes. For statistical analysis, a broad multidimensional array containing RT, *m/z*, and peak height intensity between the control group and risperidone-treated group was analyzed using MetaboAnalyst 6.0. The PCA score plot of the metabolic route in the risperidone-treated sample was clearly discrete (Fig. 2A and B) for each polar and non-polar fraction, which was different from the orientation of the control group. The OPLS-DA score plots between the control and treated groups in both fraction modes (Fig. 2C and D) showed a clear alteration in the s-plot plot (Fig. 3A and B) and Volcano plot (Fig. 3A and B). The ions further from the origin contribute more to the change in the metabolic profile trajectory.

3.4. Differential MVEC metabolite recognition

The metabolites of the polar and non-polar fractions from the OPLS-DA with VIP values >1.00 were selected using an independent t-test. An FDR <0.05 and a fold-change >1.5 was used to cluster the differences between the various groups to identify significant metabolites. To confirm metabolite structure, retrieving information from online databases and searching reference materials was necessary after acquiring the MS spectrum and pertinent MS/MS fragments from the analysis. A total of 42 distinct metabolites were identified (Table 1) and 30 of 42 potentially significant metabolites were increased, whereas 10 were decreased following treatment. The top ten significant metabolites included Cer(d18:1/24:0), LysoPC (C24), (R)-3-hydroxybutyrylcarnitine, PS (C41), 2-methylbutyrylcarnitine, propionylcarnitine, isobutyryl-L-carnitine, (1 R,3 R)-1-aminocyclopentane-1,3-dicarboxylate, N6-methyladenosine, and LysoPE (C23), which exhibited significant differences compared with the control group. Fig. 4 shows the comparative signal strengths of these metabolites.

3.5. Biochemical metabolism analysis

The 42 potentially significant metabolites were used in a biochemical analysis based on the study conditions. The three major metabolic pathways (Fig. 5), which included fatty acids and derivatives, amino acids and derivatives and derivatives, and tricarboxylic acid (TCA) metabolism, were all involved in metabolite regulation by high-dose risperidone in the MVECs. These mechanisms are associated with MVEC function, including changes in energy production, membrane structure and function, and signaling pathways.

4. Discussion

High-dose risperidone can lead to serious effects on the BBB function,

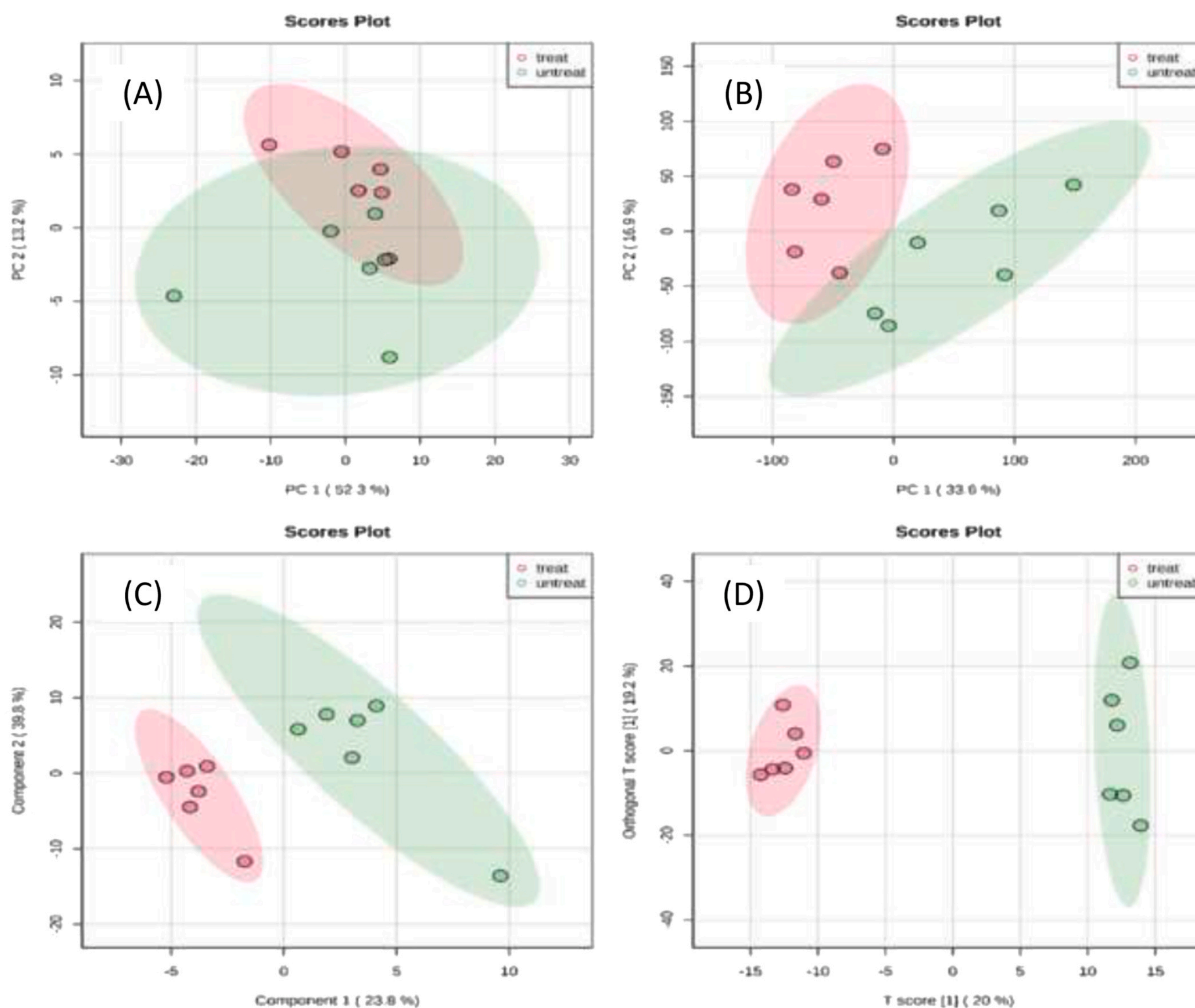

Fig. 2. PCA score plot of the cell metabolism trajectory in the high-dose risperidone-treated group in polar (A) and non-polar (B) fractions, and OPLS-DA score plot of the MVEC LC-MS spectra data between the control and high-dose risperidone-treated groups in the polar (C) and non-polar (D) fractions.

which is a protective barrier that prevents certain substances from entering the brain. When BBB function is impaired, it allows harmful substances to pass through and potentially cause damage to the brain. If the BBB function is significantly impaired, long-term neurological effects can result, such as memory problems and cognitive impairment. In severe cases, it can even lead to coma or death. There are few studies on the effects of a high-dose risperidone on MVECs of the BBB [10].

In the study, we examined the metabolic changes caused by risperidone at high doses in MVECs using untargeted metabolomics-based liquid chromatography-mass spectrometry (LC-MS). Our results indicate that alterations in the structure and function of MVECs may be impaired at high concentrations of risperidone through the metabolism of diacylglycerols and derivatives, fatty acids and derivatives, ceramides and derivatives, glycerophospholipids, amino acids and derivatives, and TCA.

4.1. Interpretation of findings

We identified significant changes in metabolites associated with diacylglycerol (DAG), fatty acid, ceramide, glycerophospholipid, and amino acid metabolism, as well as the TCA cycle. These metabolic disruptions likely contribute to the impairment of MVEC function and the integrity of the BBB. The observed alterations suggest that high-dose risperidone can compromise the BBB, increasing its permeability and potentially allowing harmful substances to enter the brain.

4.1.1. Diacylglycerol (DAG) metabolism

We identified five diacylglycerols (DAGs) significantly increased in the risperidone-treated group (Table 1). DAGs are crucial for the regulation of cell function and maintenance of cell membrane barrier properties [26]. In MVECs, DAGs are involved in the regulation of MVEC function and the maintenance of their barrier properties. MVECs are a specialized system of blood vessels that surrounds the brain and central nervous system and protects these sensitive tissues from potentially harmful substances. The integrity of MVECs is maintained by tight junctions between the endothelial cells that line the blood vessels, which prevent the passage of large molecules and cells from the bloodstream into the brain [27–29]. DAGs are produced in MVECs through the breakdown of phospholipids and function as second messengers in various signaling pathways. They play a role in the regulation of the barrier properties of the BBB by modulating the activity of the tight junction proteins [30–32]. Our results are supported by recent studies, in which it was shown that risperidone at the same concentration used in this study can alter the function of MVECs in the BBB, including changes

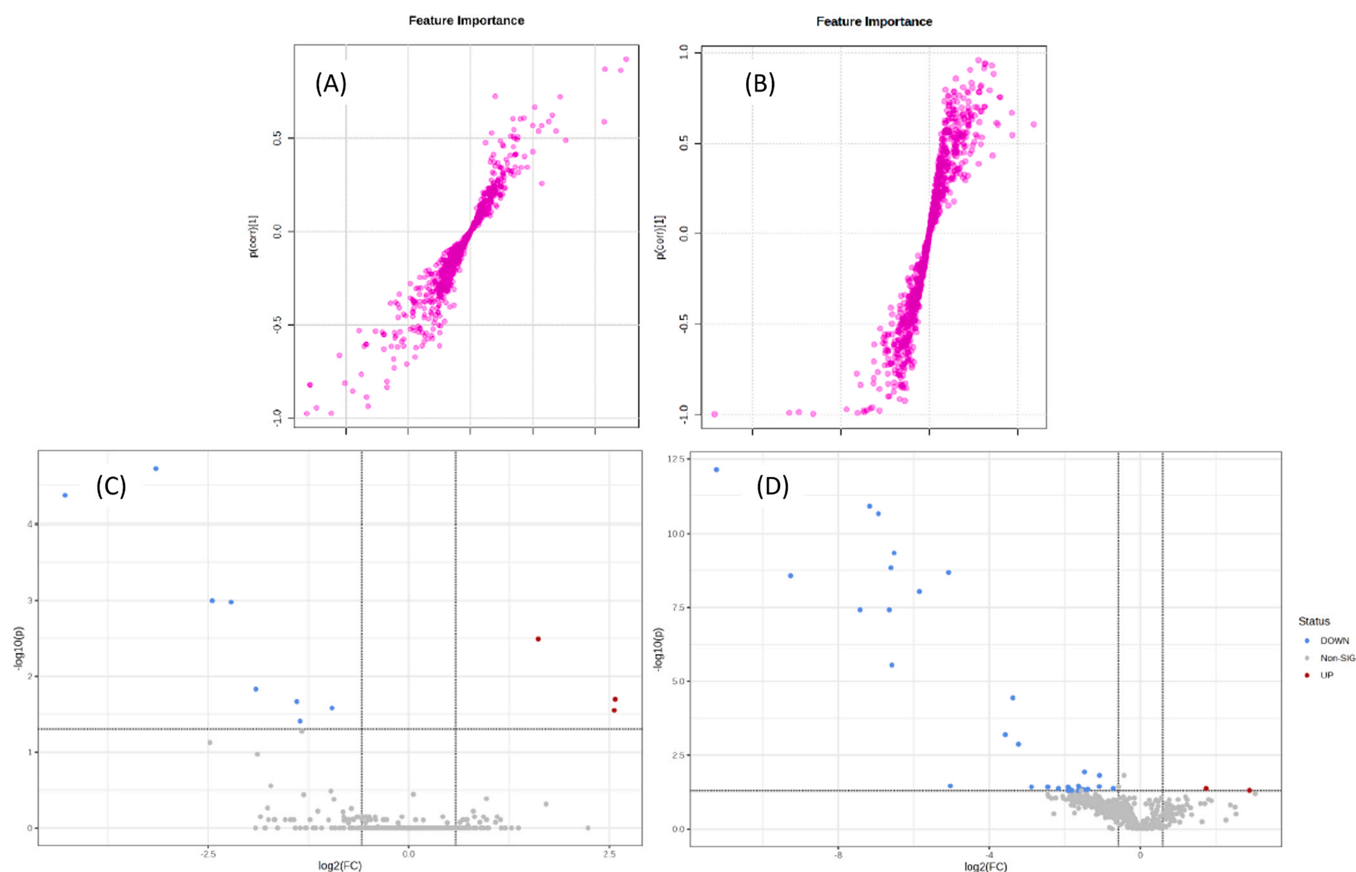

Fig. 3. S-plot of the OPLS-DA model of LC-MS spectra data between the control and high-dose risperidone-treated groups in the polar (A) and non-polar (B) fractions, and Volcano plot of the LC-MS spectra data between the control and high-dose risperidone-treated groups in the polar (C) and non-polar (D) fractions.

in the distribution of certain proteins, such as tight junction proteins [10]. The observed increase in DAG levels suggests that risperidone disrupts DAG metabolism, potentially impairing BBB integrity and increasing permeability to harmful substances.

4.1.2. Fatty acid metabolism

Fatty acids are essential for maintaining MVEC integrity and function. They help preserve the structural and functional integrity of MVECs, which regulate the transport of substances into and out of the brain [33,34]. One of the main functions of the MVECs is to regulate the transport of substances into and out of the brain, including nutrients, hormones, and drugs. Fatty acids are important to this process because they help to maintain the structural and functional integrity of the MVECs [35]. We found that L-acetylcarnitine, isobutyryl-L-carnitine, (R)-3-hydroxybutyrylcarnitine, 24-methyl-5Z,9Z-pentacosadienoic acid, 1-O-Palmitoyl-2-O-linoleoyl-D-glycerol, GlcCer{t18:1(8Z)/26:0 (2OH[R]}, and 3α-hydroxy-6-oxo-5β-cholan-24-oic acid were significantly increased in the risperidone-treated group (Table 1). One effect of risperidone on fatty acids in MVECs is to increase the synthesis of fatty acids. This increase in synthesis may result from the effect of the drug on the enzymes responsible for fatty acid synthesis, such as fatty acid synthase and acyl-CoA synthase [36]. Another effect of risperidone is that it alters the distribution of fatty acids within MVECs, including the mitochondria, which can affect the production of energy within cells [10]. These changes suggest that risperidone enhances fatty acid synthesis and alters their distribution within MVECs, leading to energy production disruptions and inflammation, consistent with other studies [10].

4.1.3. Ceramide metabolism

Two ceramides were increased in MVECs treated with high concentrations of risperidone (see Table 1). Ceramides play a vital role in the formation and maintenance of MVECs. They contribute to the impermeable barrier of the BBB and regulate nutrient transport into the brain [37]. These lipids form a continuous and impermeable barrier that prevents the passage of large molecules, such as drugs, toxins, and bacteria, into the brain. In addition, ceramides regulate the transport of nutrients and other essential molecules, such as glucose and oxygen, into the brain [38]. This is important for maintaining brain function and preventing neuronal damage. We observed increased levels of ceramides, such as Cer(d18:0/16:0) and Cer(d18:1/16:0), in risperidone-treated MVECs. This suggests that risperidone disrupts ceramide metabolism, which may compromise BBB function and reduce its protective capability.

4.1.4. Glycerophospholipid metabolism

Glycerophospholipids form the majority of the lipid bilayer in MVECs and are crucial for maintaining cell membrane integrity and function. They regulate the uptake and release of substances into and out of the BBB [40]. In addition, glycerophospholipids are important for the proper function of transporters and enzymes in the MVECs. These molecules regulate the uptake and release of various substances into and out of the BBB, ensuring that the brain receives the nutrients it requires, while also protecting it from harmful substances [39]. Changes in glycerophospholipids can alter the structure and function of MVECs. Our study found significant increases in glycerophospholipids such as LysoPE and LysoPC in the risperidone-treated group. These alterations indicate that high doses of risperidone impair the lipid bilayer of MVECs, potentially reducing cell function and barrier integrity.

Table 1
Differentiated metabolites identified in MVECs-treated with high therapeutic risperidone (10 μM) by LC-MS in the polar and non-polar fractions.

Fraction	*m/z*	rt (min.)	Tentative	Formula	M.W.	Adduct	Delta (ppm)	VIP	FC	FDR
Non-polar	121.0655	3.7	Lentialexin	C8H8O	120.0581	[M+H]+1	5	1.81	0.43	0.03
Non-polar	191.1178	3.76	5-Methoxytryptamine	C11H14N2O	190.1106	[M+H]+1	0	2.2	81.83	0.00
Non-polar	334.2353	3.91	N-tetradecanoyl-homoserine lactone	C18H33NO3	311.2461	[M+Na]+1	0	2.1	0.43	0.00
Non-polar	478.2927	5.08	LysoPE	C23H46NO7P	479.3002	[M-H]-1	2	2.06	1.6	0.00
Non-polar	713.4217	5.6	PI(12:0/13:0)	C34H65O13P	712.4145	[M+H]+1	3	1.87	1.65	0.02
Non-polar	482.3242	5.78	LysoPE(18:0/0:0)/LysoPE(0:0/18:0)	C23H48NO7P	481.3166	[M+H]+1	0	2.01	2.32	0.00
Non-polar	209.0808	7.08	5-(3′,4′-Dihydroxyphenyl)-gamma-valerolactone	C11H12O4	208.0736	[M+H]+1	0	1.95	1.79	0.00
Non-polar	149.0233	7.08	Phthalic anhydride	C8H4O3	148.016	[M+H]+1	0	1.87	2.16	0.02
Non-polar	167.0338	7.08	Phthalic acid	C8H6O4	166.0266	[M+H]+1	0	1.92	2.14	0.01
Non-polar	279.159	7.08	alpha-CEHC	C16H22O4	278.1518	[M+H]+1	0	1.85	1.91	0.02
Non-polar	391.2841	7.09	3α-Hydroxy-6-oxo-5β-cholan-24-oic Acid	C24H38O4	390.2768	[M+H]+1	0	1.85	2.11	0.02
Non-polar	496.3396	7.19	LysoPC(16:0/0:0)/LysoPC(0:0/16:0)	C24H50NO7P	495.3323	[M+H]+1	0	2.17	8.81	0.00
Non-polar	309.2792	7.22	9Z-Eicosenoic acid	C20H38O2	310.2865	[M-H]-1	2	1.76	0.63	0.05
Non-polar	480.3085	7.32	LysoPE	C23H48NO7P	481.3158	[M-H]-1	2	2.19	4.42	0.00
Non-polar	520.5088	8.8	Cer(d18:1/16:0)	C34H67NO3	537.5117	[M+H-H2O]+1	1	1.78	2.23	0.03
Non-polar	391.3573	9.01	24-methyl-5Z,9Z-pentacosadienoic acid	C26H48O2	392.3646	[M-H]-1	2	1.75	1.85	0.03
Non-polar	540.5352	9.03	Cer(d18:0/16:0)	C34H69NO3	539.5274	[M+H]+1	1	1.77	1.77	0.03
Non-polar	547.4031	9.15	cholesteryl beta-D-glucoside	C33H56O6	548.4103	[M-H]-1	5	1.83	0.38	0.03
Non-polar	610.5408	9.23	1-O-Palmitoyl-2-O-linoleoyl-D-glycerol	C37H68O5	592.507	[M+NH4]+1	0	1.91	1.62	0.02
Non-polar	636.5563	9.29	DG	C39H70O5	618.5226	[M+NH4]+1	0	2.04	1.76	0.00
Non-polar	662.5723	9.6	DG	C41H72O5	644.5383	[M+NH4]+1	0	1.89	1.82	0.01
Non-polar	617.5118	9.73	DG	C37H70O5	594.5226	[M+Na]+1	0	1.89	2.11	0.02
Non-polar	643.5275	9.84	DG	C39H72O5	620.5382	[M+Na]+1	0	1.84	2.17	0.02
Non-polar	664.5879	9.96	DG	C41H74O5	646.5539	[M+NH4]+1	0	1.85	1.66	0.01
Non-polar	872.7193	10.17	GlcCer(t18:1(8Z)/26:0(2OH[R]))	C50H97NO10	871.7121	[M+H]+1	1	1.86	2.18	0.03
Non-polar	648.629	11.91	Cer(d18:1/24:0)	C42H83NO3	649.6369	[M-H]-1	1	1.98	0.09	0.01
Non-polar	699.5954	12.36	DG	C45H78O5	698.5885	[M+H]+1	5	1.8	0.42	0.03
Non-polar	776.5441	14.3	PS	C41H80NO10P	777.5513	[M-H]-1	1	1.75	0.16	0.04
polar	172.0618	5.28	(1 R,3 R)-1-Aminocyclopentane-1,3-dicarboxylate	C7H11NO4	173.069	[M-H]-1	1	2.49	5.11	0.02
polar	220.118	5.6	Pantothenic acid	C9H17NO5	219.1107	[M+H]+1	0	2.48	1.75	0.03
polar	246.1699	7.21	2-Methylbutyrylcarnitine	C12H23NO4	245.1626	[M+H]+1	0	2.9	0.17	0.00
polar	232.1544	7.7	Isobutyryl-L-carnitine	C11H21NO4	231.1471	[M+H]+1	0	2.93	5.34	0.00
polar	218.1387	8.19	Propionylcarnitine	C10H19NO4	217.1314	[M+H]+1	0	2.87	0.17	0.00
polar	204.1231	8.72	L-Acetylcarnitine	C9H17NO4	203.1158	[M+H]+1	0	2.56	1.52	0.02
polar	248.1493	8.85	(R)-3-Hydroxybutyrylcarnitine	C11H21NO5	247.142	[M+H]+1	0	2.95	8.67	0.00
polar	162.1126	9.82	L-Carnitine	C7H15NO3	161.1053	[M+H]+1	1	2.9	0.3	0.00
polar	101.0244	10.54	2-Ketobutyric acid	C4H6O3	102.0317	[M-H]-1	0	2.64	2.46	0.01
polar	145.0142	10.55	Oxoglutaric acid	C5H6O5	146.0215	[M-H]-1	0	2.6	2.5	0.01
polar	282.1197	11.43	N6-Methyladenosine	C11H15N5O4	281.1124	[M+H]+1	0	2.92	4.66	0.00
polar	230.1864	13.06	N1,N8-Diacetylspermidine	C11H23N3O2	229.1791	[M+H]+1	0	2.43	1.91	0.02
polar	205.1548	13.5	3-Hydroxy-N6,N6,N6-trimethyl-L-lysine	C9H21N2O3	205.1552	[M+H]+1	2	2.42	0.54	0.04
polar	104.107	16.76	Choline	C5H14NO	104.1075	[M+H]+1	5	2.52	0.47	0.02
polar	255.0976	19.14	Nicotinamide riboside	C11H15N2O5	255.0981	[M+H]+1	2	2.84	3.85	0.00

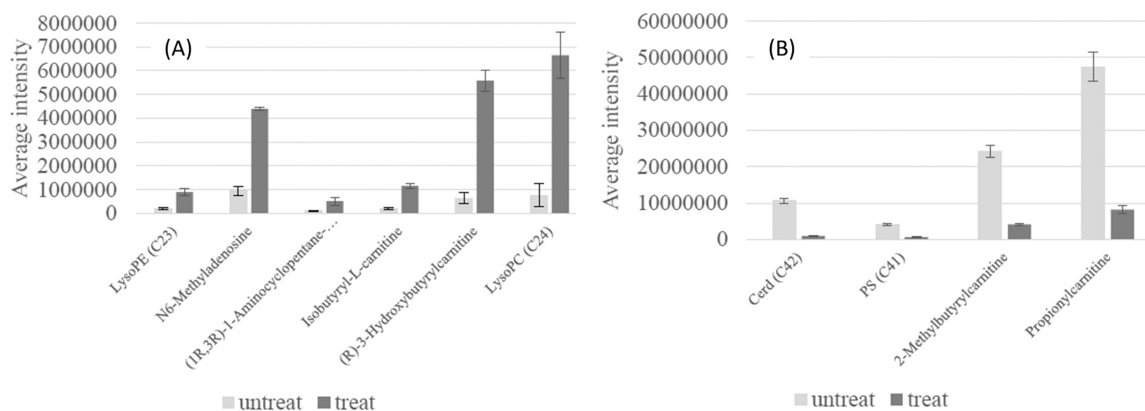

Fig. 4. Relative signal intensities of the increased (A) and decreased (B) metabolites identified in MVECs identified in the control and risperidone-treated groups along with the control group.

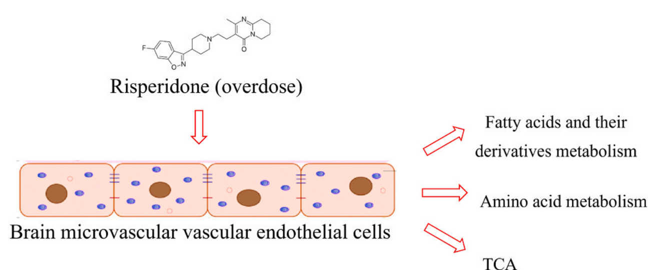

Fig. 5. Three major possible metabolite pathways were found to separate risperidone-treated groups from controls.

4.1.5. Amino acid metabolism

Amino acids and their derivatives are essential for the synthesis and maintenance of MVECs. They are involved in protein synthesis, neurotransmitter production, and nutrient transport across the BBB [41,42]. If the metabolism of amino acids and derivatives in MVECs is altered by drugs or toxins, the function of MVECs in the BBB will be impaired. We detected significant changes in amino acids such as (1 R, 3 R)-1-aminocyclopentane-1,3-dicarboxylate and N1, N8-diacetyl spermidine in risperidone-treated MVECs. These changes suggest that risperidone affects amino acid metabolism, impairing nutrient transport and cell function.

4.1.6. TCA

The TCA cycle, or citric acid cycle, is crucial for ATP production, which is the primary energy source for MVECs. The cycle also regulates the transport of nutrients into MVECs [43]. We found that pantothenic acid, phthalic acid, oxoglutaric acid, 2-ketobutyric acid, nicotinamide riboside were significantly altered in MVECs by high-dose risperidone treatment. These alterations could reduce ATP levels, impairing MVEC function and compromising BBB integrity.

In summary, our study reveals that high-dose risperidone significantly impacts the metabolism of MVECs, affecting diacylglycerols, fatty acids, ceramides, glycerophospholipids, amino acids, and the TCA cycle. These metabolic disruptions likely contribute to the impairment of MVEC function and the integrity of the BBB. Understanding these metabolic changes provides valuable insights into the mechanisms of risperidone-induced toxicity and highlights the importance of metabolic pathways in maintaining BBB function.

4.2. Limitations of the study

Despite the valuable insights gained from this study, several limitations must be acknowledged:

4.2.1. Sample size and replicates

One of the primary limitations is the relatively small sample size and the number of replicates used for the metabolomic analysis. Although we conducted six independent samples for each group (treated vs. untreated), this number may still be insufficient to capture the full extent of biological variability and to ensure the robustness of the findings. The limited sample size can compromise the statistical power of the study, reducing the ability to detect subtle but biologically significant changes in metabolite levels [44]. Furthermore, small sample sizes can increase the likelihood of type I and type II errors, potentially leading to false-positive or false-negative results [45].

4.2.2. Control groups and conditions

While we included vehicle controls, untreated controls, and system blanks to differentiate between the effects specifically attributable to risperidone and those arising from other variables, the control conditions might not fully account for all potential confounding factors. For example, variations in cell culture conditions or subtle differences in sample handling could introduce variability [46].

4.2.3. Metabolite identification and quantification

The untargeted metabolomics approach used in this study, while powerful, has limitations in metabolite identification and quantification. Some metabolites may have been misidentified or not detected due to the inherent limitations of the mass spectrometry technique and the databases used for metabolite identification. Additionally, the quantitative accuracy of the detected metabolites can be influenced by ion suppression, matrix effects, and other technical factors [47,48].

4.2.4. Biological relevance and mechanistic insights

Although we identified several metabolic pathways affected by risperidone treatment, the biological relevance, and mechanistic insights into how these metabolic changes translate to functional impairments in MVECs and BBB integrity require further investigation. In vitro findings may not fully replicate in vivo conditions, and additional studies using animal models or clinical samples would be necessary to validate and extend our observations.

4.2.5. Generalizability

The use of primary human brain MVECs provides a relevant model for studying BBB function, but results may not be directly applicable to other cell types or tissues. Additionally, the effects observed at high doses of risperidone may differ from those at therapeutic doses, limiting the generalizability of our findings to clinical scenarios.

4.3. Future directions

To address these limitations and build upon our findings, future studies should consider the following approaches:

4.3.1. Increasing sample size and replicates

Conducting studies with larger sample sizes and more biological replicates would improve the statistical power and reproducibility of the findings, providing more confidence in the identified metabolic changes and their implications.

4.3.2. Refining control conditions

Including additional control groups and conditions, such as time-matched controls or alternative cell types, could help further isolate the specific effects of risperidone and reduce potential confounding factors.

4.3.3. Advanced metabolomics techniques

Utilizing more advanced and complementary metabolomics techniques, such as targeted metabolomics or isotope-labeled metabolic flux analysis, could enhance metabolite identification and quantification accuracy, providing deeper insights into metabolic changes.

4.3.4. In vivo studies

Conducting in vivo studies using animal models or clinical samples would help validate the in vitro findings and provide a more comprehensive understanding of the metabolic impact of risperidone on BBB function and overall brain health.

4.3.5. Mechanistic studies

Investigating the underlying mechanisms by which risperidone affects metabolic pathways and BBB integrity through molecular and cellular approaches could provide deeper insights into the drug's toxicological effects and potential therapeutic targets for mitigating adverse outcomes.

5. Conclusion

Our study reveals that high-dose risperidone significantly impacts the metabolism of MVECs, affecting diacylglycerols, fatty acids, ceramides, glycerophospholipids, amino acids, and the TCA cycle. These metabolic disruptions likely contribute to the impairment of MVEC function and the integrity of the BBB. Understanding these metabolic changes provides valuable insights into the mechanisms of risperidone-induced toxicity and highlights the importance of metabolic pathways in maintaining BBB function. However, the limitations identified in this study underscore the need for further research to validate and extend these findings, ensuring their relevance and applicability to clinical settings.

CRediT authorship contribution statement

Napatarin Srikornvit: Formal analysis. **Patipol Hongthawonsiri:** Formal analysis. **Krittaboon Pornchokchai:** Formal analysis. **Siriphattarinya Wongpitoonmanachai:** Formal analysis. **Jiajun Mo:** Formal analysis. **Petchlada Pholkla:** Formal analysis. **Pracha Yambangyang:** Methodology. **Phichanan Duchda:** Resources. **Jenyuk Lohwacharin:** Resources. **Watcharaporn Devakul Na Ayutthaya:** Writing – review & editing, Writing – original draft. **Surachai Ngamratanapaiboon:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.toxrep.2024.101691.

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