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Research Article
Bee Pollen Effects on Methylmercury-Induced Intestinal Leakiness as Co-Morbidity of Autism: Experimental Approach

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Abstract

Background and Objective: Methylmercury (MeHg) is a common environmental toxin that has negative effects on the developing and adult neurological systems. The purpose of this study was to assess the therapeutic and protective efficacy of bee pollen grain in reducing increased intestinal permeability or leaky gut as a well-known co-morbidity in many neurodevelopmental disorders among which is Autism Spectrum Disorders (ASD). **Materials and Methods:** Five groups of ten male neonates each were delivered by: Control healthy moms (control group), (bee pollen group), (MeHg group), (therapeutic group) and (protective group) were taken. Zonulin, occludin, lipopolysaccharide- binding protein (LBP) and Intestinal Fatty Acid Binding Protein (I-FABP) together with some oxidative stress markers including GSH, GST, lipid peroxides and diamine oxidase were measured in the serum of control and treated rats of all studied groups. **Results:** While highly significant alterations of both gut leakiness and oxidative stress biomarkers were recorded in MeHg-intoxicated rats, bee pollen was effective to ameliorate the MeHg toxic effects. Overall, neonatal exposure to MeHg during the developing brain stages was highly effective in showing signs and symptoms of increased gut permeability and oxidative stress. **Conclusion:** Furthermore, bee pollen could be used safely to ameliorate gut leakiness and oxidative stress as critical mechanisms contribute to the etiology of ASD as a neurodevelopmental disorder.

Key words: Methylmercury, leaky gut, oxidative stress, autism spectrum disorders, bee pollen

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

A group of neurodevelopmental diseases known as Autism Spectrum Disorder (ASD) are typified by challenges with social interaction, social communication and repetitive activities. These clinical diagnostic standards are frequently associated with long-term issues and can be seen in children as early as one year old[1].

The ASD show significant and persistent inflammatory reactivity with concurrent neuronal damage. These results point to neuronal injury caused by external stimuli rather than some sort of developmental error. There have been numerous xenobiotics proposed as potential causes of this illness. One of the main environmental toxins whose exposure has been shown to be hazardous to people is mercury[2-4]. Mercury, organic and inorganic mercury are the three chemical types of mercury that are known to exist. Organic mercury molecule Methylmercury (MeHg) has harmful effects on both people and animals, including neurological and behavioral abnormalities[3,4]. Previous research has demonstrated that prenatal, postnatal and adult exposure to mercury compounds can result in the development of symptoms in mice that resemble autism through a variety of mechanisms, including the expression of pro-inflammatory cytokines and altered transcription factor signaling in the Central Nervous System (CNS)[5-7]. The MeHg was listed on the top ten environmental compounds suspected of causing autism and learning disabilities[8].

The gut microbiota and an intact intestinal barrier function may affect MeHg absorption, whereas intestinal barrier dysfunction has been associated to greater MeHg intestinal absorption, blood-brain barrier disruption and altered gut-brain axis signaling[9]. With no specific active excretion mechanism, MeHg is rapidly absorbed and translocated to numerous bodily tissues[10], with the CNS and peripheral nerve systems being the most commonly targeted organs[11]. Several investigations have found that in MeHg intoxications, the cerebellum and dorsal root ganglion are the most severely injured tissues[12]. The cerebral blood brain barrier (BBB) is expected to be more vulnerable to MeHg poisoning than the cerebellar BBB[13].

According to García-Domínguez *et al.*[14], a "leaky gut" may considerably raise the level of circulating lipopolysaccharides (LPS), produce peripheral inflammation and oxidative stress, as well as brain neuroinflammation with increased microglia priming and activation through the gut-brain axis. In rodent model of selected neurological disorders among which is ASD, circulating LPS levels have been connected to a decline in neurogenesis and a slight decline in cognitive function[15].

Zonulin is the only known physiological regulator of tight junctions and a biomarker for intestinal permeability. A transmembrane tight junction protein called occludin is necessary for the development and maintenance of such junctions. Recent research has demonstrated lower levels of intestinal tight junction (TJ) claudin in ASD patients[16] Serum zonulin, a marker of enhanced intestinal permeability, was significantly higher in autistic children than in healthy controls[17,18].

The LPS binding protein (LBP), mediates the inflammatory response to LPS. The LPS first binds to LBP, producing the LPS-LBP complex, which then binds to CD14 and the MD-4/MD-2 complex[19], ultimately activating signal transduction pathways and causing the generation of cytokines and other pro-inflammatory mediators[20]. Exposure to proinflammatory cytokines such as Interleukin (IL)-1, IL-6 and tumor necrosis factor has been shown to significantly increase LBP release by intestinal epithelial cells[21]. These cytokines are seen in higher amounts in sub-epithelial tissue during inflammatory bowel illness[22] and systemic inflammation[23]. Moreover, LBP was recorded as a GI biomarker associated with LPS-induced neurotoxicity[24].

Intestinal Fatty-Acid Binding Protein (I-FABP) is expressed in epithelial cells of the small intestine's mucosal layer. The I-FABP is released into the circulation and its plasma concentration rises when the intestinal mucosa is damaged. In this context, it could be used as a marker of increased intestinal permeability or leaky gut[25].

These findings initiate our interest in measuring serum zonulin, occludin, LBP and I-FABP as markers of gut leakiness in a prenatally MeHg-induced rat model of autism, as well as testing the protective and therapeutic benefits of bee pollen as a safe nutritional intervention.

MATERIALS AND METHODS

Study area: The study was carried out at Department of Biochemistry, College of Science, King Saud University, Riyadh, Saudi Arabia from January, 2023 to September, 2023.

Animals: In the current study, 40 healthy pregnant female Wister rats of about 8-10 weeks old and 180-200 g weight were randomly allocated into 5 groups, then after delivery, 10 male pups from each group were selected as shown in Fig. 1. Pups delivered by control moms who only got tap water or bee pollen treatment (200 mg/kg b.wt.) from postnatal day 0 for 4 weeks represented the control group or bee pollen group, respectively. Pups delivered by MeHg-orally treated moms who received, from gestational day 7 until postnatal

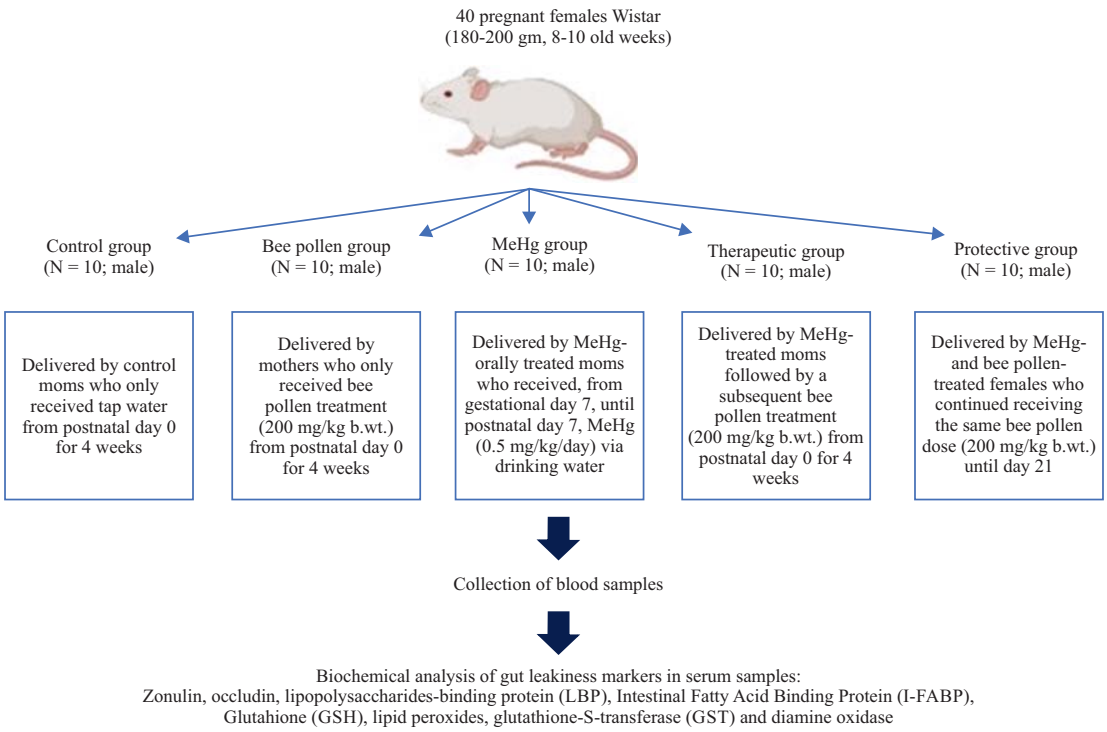

Fig. 1: Schematic diagram of the animal experiments

day 7, MeHg (0.5 mg/kg/day) via drinking water formed the MeHg-exposed group. Male pups delivered by MeHg and bee pollen-treated females who continued receiving the same bee pollen dose until day 21 formed as the protective group while the therapeutic group consisted of pups delivered by MeHg-treated moms followed by a subsequent bee pollen treatment (200 mg/kg b.wt.) from postnatal day 0 for 4 weeks.

After treatment, animals were scarified and blood samples were taken directly from the heart using a flat tube without anticoagulant. After centrifugation (10 min, 3000 rpm, 4°C), serum samples were collected and stored at -80°C until needed (Fig. 1).

Ethical consideration: The King Saud University ethics committee for animal research gave its preapproval (KSU-SE-17-10) to the experimental protocol.

Biochemical analysis: All serum samples were examined for glutathione-S-transferase (GST) activity, Glutathione (GSH) level and lipid oxidation in accordance with Mannervik[26], Adachi *et al.*[27] and Ruiz-Larrea *et al.*[28], respectively.

Using ELISA kits from MyBioSource and adhering to the manufacturer's instructions, serum levels of diamine oxidase (MBS700248), I-FABP (MBS265971), LBP (MBS2882282), occludin (MBS725124) and zonulin (MBS2606662) were measured in each group. Every measurement was done in triplicate and the average of the three results was determined.

Statistical analysis: One-way ANOVA followed by Tukey's multiple comparison test was performed to analyze obtained data. Only $p \leq 0.05$ were considered significant. Results illustrated as Mean ± Standard Error of the mean (SEM) were generated using GraphPad Prism (version 9.5.0). The Receiver Operating Characteristics (ROC) curve along with the area under the ROC curve (AUC) were also determined as a fundamental tool to assess the gut leakiness as co-morbidity rat model of ASD. In fact, ROC curves, which were created by plotting true (sensitivity) and false (specificity) positive rates corresponding to the biomarker values range on the y- and x-axis, respectively, were used to assess the predictive capacity of all investigated biomarkers. No discrimination (i.e., the capacity to diagnose individuals with and without the disease or condition based on the test) is generally indicated by AUC value of 0.5; values between 0.7 and 0.8 are regarded as acceptable; values between 0.8 and 0.9 are regarded as excellent and values greater than 0.9 are regarded as outstanding[29].

RESULTS

The current study looked at the impact of MeHg exposure on four important biomarkers linked to leaky gut as a co-morbidity in ASD. Briefly, all measured variables showed a highly significant rise in response to MeHg treatment in the rats (Fig. 2a-d). Additionally, rats given bee pollen protection or therapeutic treatment exhibited noticeably decreased levels of zonulin, occludin, PBP and I-FABP but remained distinct from controls (Fig. 2a-d).

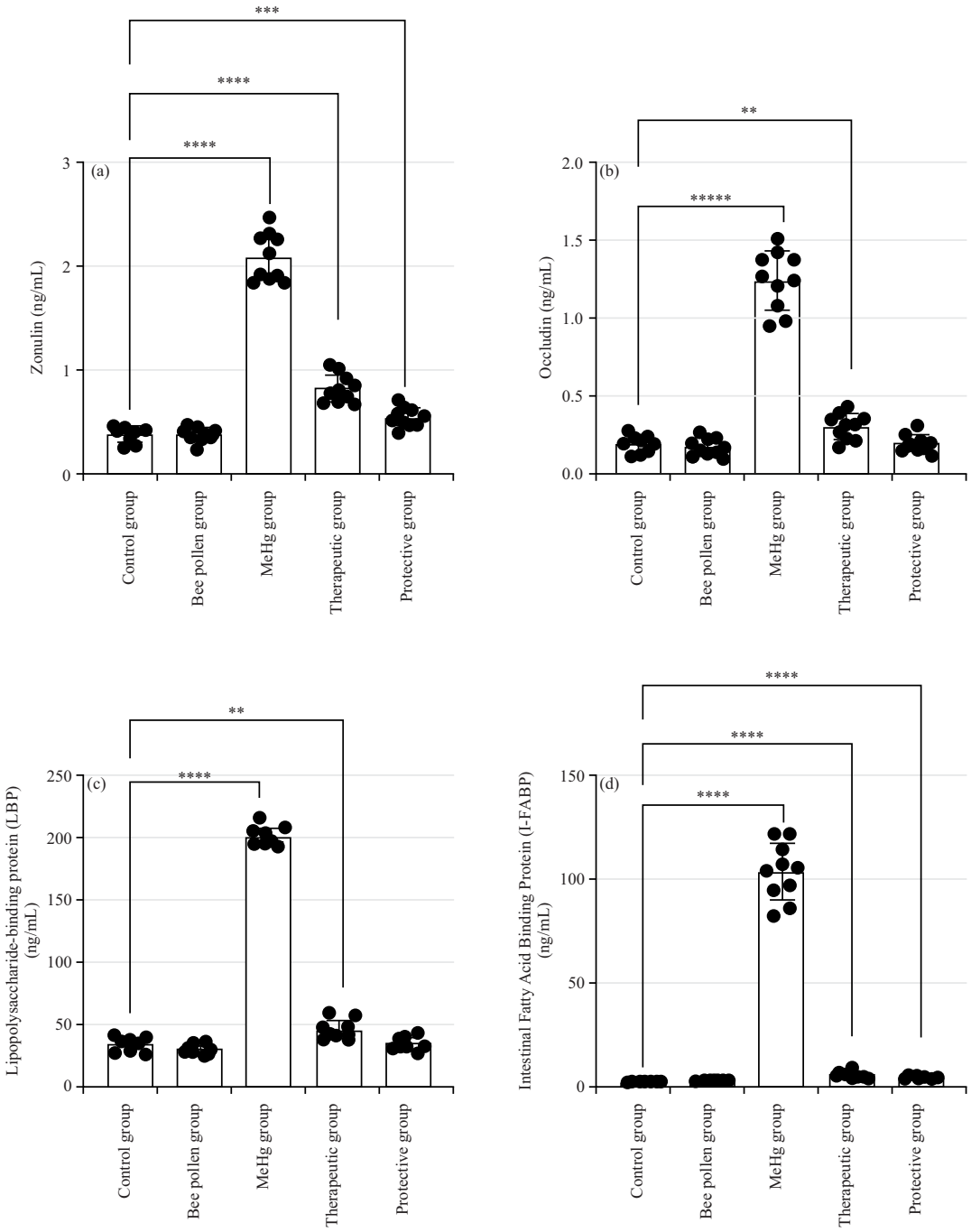

Fig. 2(a-d): Effect of bee pollen on (a) Serum zonulin, (b) Occludin, (c) LPB protein and (d) I-FABP in MeHg-induced gut leakiness as co-morbidity in rat model of ASD

Values are shown as Mean±SEM, n = 10 group, **p<0.01, ***p<0.001 and ****p<0.0001

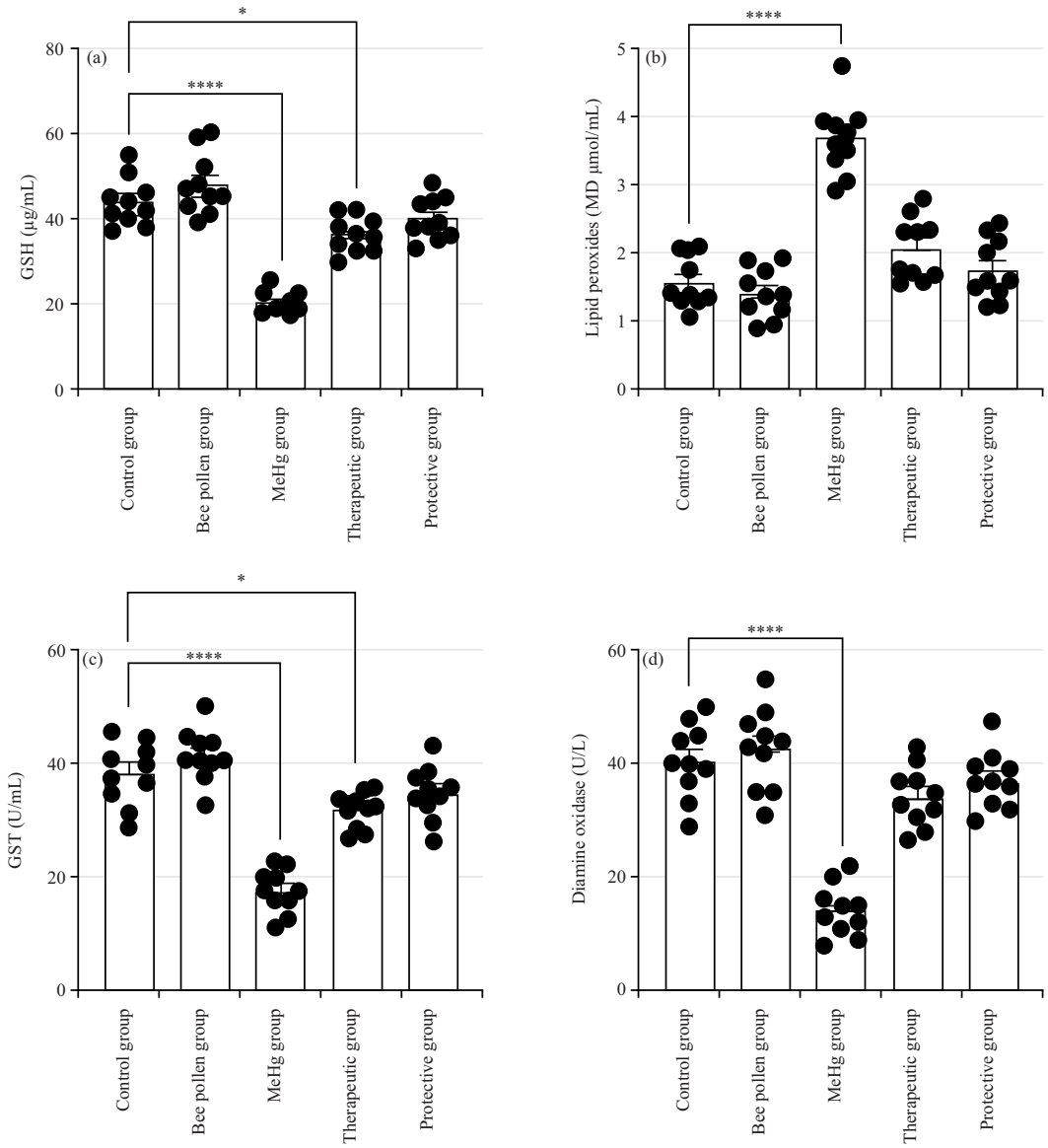

Fig. 3(a-d): Effect of bee pollen on serum (a) GSH, (b) Lipid peroxides, (c) GST and (d) Diamine oxidase in MeHg-induced gut leakiness as co-morbidity in rat model of ASD

Values are shown as Mean±SEM, n = 10 group, **p<0.01, ***p<0.001 and ****p<0.0001

Figure 3(a-d) showed a significant decrease in GSH, GST and diamine oxidase as non-enzymatic and enzymatic antioxidants, as well as an increase in lipid peroxides as a marker of oxidative stress in MeHg-treated animals. In the same image, the preventive and therapeutic benefits of bee pollen were depicted. While GSH, lipid peroxides and diamine-oxidase levels were not statistically different in the bee pollen-protected or medically treated groups compared to the control group, GST was considerably lower in the bee pollen-protected group.

The ROC analysis presented in Fig. 4(a-h) and Table 1 showed the AUCs of the measured parameters for all investigated animal groups. These values were calculated as a measure of the efficiency of the investigated variables as markers of intestinal leakiness or protective/therapeutic potency of the MeHg or bee pollen treatment, respectively, in animal modeling.

DISCUSSION

The ASD is linked to a higher prevalence of a diversity of medical conditions, including immunological dysfunction, disturbed sleep, anxiety, seizures and gastrointestinal (GI) problems along with a variety of developmental anomalies.

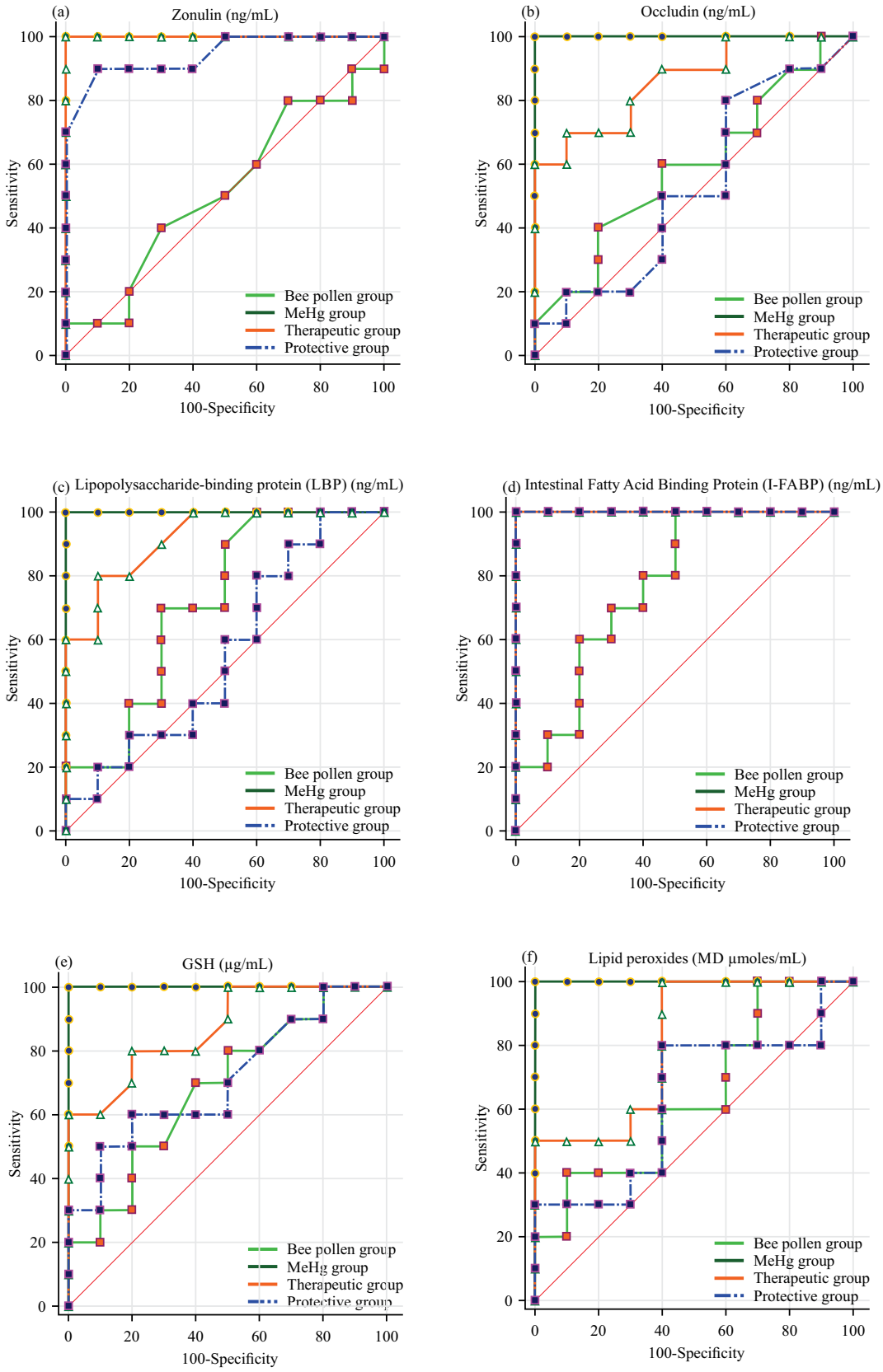

Fig. 4(a-h): Continue

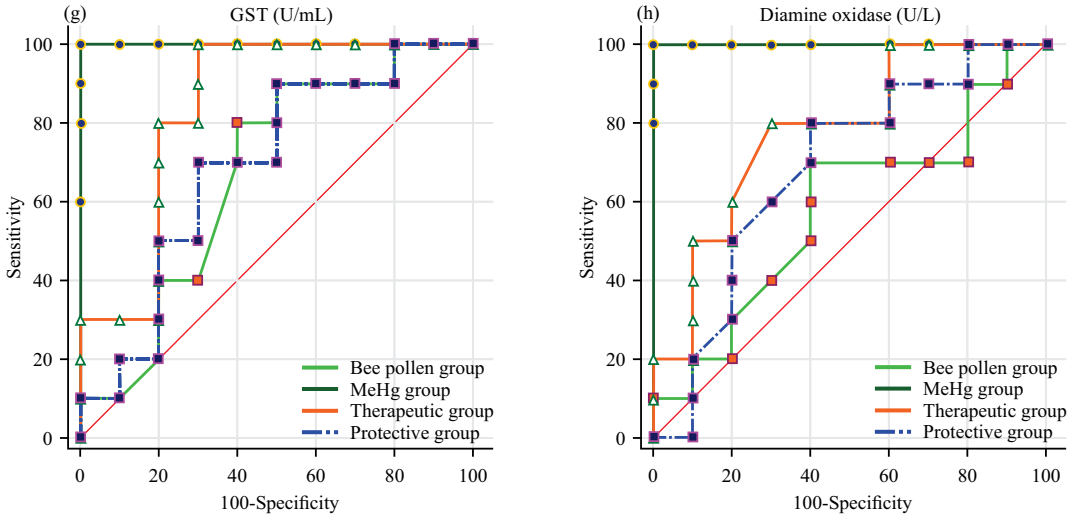

Fig. 4(a-h): Analysis of receiver operating characteristics of selective parameters measured in the serum of the four treated groups of rats, (a) Zonulin, (b) Occludin, (c) LPB protein, (d) I-FABP, (e) GSH, (f) Lipid peroxides, (g) GST and (h) Diamine oxidase

Table 1: ROC analysis of all measured parameters in all groups

Parameter	Groups	AUC
Zonulin (ng/mL)	Bee pollen group	0.515
	MeHg group	1.000
	Therapeutic group	1.000
	Protective group	0.945
Occludin (ng/mL)	Bee pollen group	0.590
	MeHg group	1.000
	Therapeutic group	0.865
	Protective group	0.530
Lipopolysaccharide-binding protein (LBP) (ng/mL)	Bee pollen group	0.715
	MeHg group	1.000
	Therapeutic group	0.920
	Protective group	0.560
Intestinal Fatty acid Binding Protein (I-FABP) (ng/mL)	Bee pollen group	0.760
	MeHg group	1.000
	Therapeutic group	1.000
	Protective group	1.000
GSH (µg/mL)	Bee pollen group	0.685
	MeHg group	1.000
	Therapeutic group	0.870
	Protective group	0.710
Lipid peroxides (MD µmoles/mL)	Bee pollen group	0.640
	MeHg group	1.000
	Therapeutic group	0.810
	Protective group	0.630
GST (U/mL)	Bee pollen group	0.670
	MeHg group	1.000
	Therapeutic group	0.840
	Protective group	0.690
Diamine oxidase (U/L)	Bee pollen group	0.580
	MeHg group	1.000
	Therapeutic group	0.780
	Protective group	0.685

Children with ASD are often reported to have GI problems, such as abdominal pain, constipation and diarrhea. Recent research indicates that GI comorbidity may have an effect on troublesome behaviors in ASD[30]. Although the gut-brain communication has long been thought to play an important role in ASD, studies of this interaction have just recently started and as a result, correlation between the pathophysiology of ASD and the gut microbiota have been found[31].

The high significant increase of zonulin, occludin, PBP and I-FABP as biomarkers of increased intestinal permeability recorded in the present investigation (Fig. 2a-d), can find support in the study of Rodríguez-Viso *et al.*[32] which recorded that Caco-2 intestinal cell monolayers show depressed redox state and impaired GSH metabolism along with an increase in intestinal permeability. They suggested that the p38 MAPK, JNK and NF-B inflammatory signaling pathways were responsible for all of these harmful consequences of MeHg exposure. Moreover, the obtained data can support the role of gut-brain axis in ASD. This could be proved by considering our earlier published work which recorded that MeHg treatment led to a rise in oxidative stress markers as well as a decline in glutathione levels in brains of treated animals[33]. Moreover, brain energy metabolism was compromised, as shown by the decrease of lactate dehydrogenase and creatine kinase activity[33]. Concentrations of Mg^{2+} and K^{+} sharply decreased which was easily related to imbalanced glutamate/GABA ratio as a well ascertained etiological mechanism greatly controlled by Mg^{2+} and K^{+}[33].

The data currently presented on increased intestinal permeability in MeHg-treated animals (Fig. 2a-d) and its detrimental effects on brain chemistry, including neuroinflammation, oxidative stress and mitochondrial dysfunction[33,34], may be used to prove the connection between leaky gut and altered brain chemistry in ASD. This could help to suggest that inflammation and oxidative stress induced by MeHg treatment, raise intestinal permeability[35-37], which in turn promotes the translocation of certain microbial antigens and toxins that eventually cross the gut lumen into the intestinal mucosa. This causes a vicious feed-forward cycle through a variety of different mechanisms, which worsens the pattern of brain and systemic inflammation as two autistic features in human and rodent models[38].

The significant increase in serum zonulin in the MeHg-induced rat model of ASD can be easily related to the contribution of the impaired gut-brain axis in the etiology of ASD (Fig. 2a). In dysbiotic autistic individuals or rodent models, the rise, in intestinal zonulin production, impaired gut permeability, increased blood-brain barrier (BBB) permeability and neuroinflammation are caused by the spread of gut-derived pathogenic microbial fragments, toxins and inflammatory factors, including zonulin, which eventually reach the brain across the blood[38,39].

Al Dera *et al.*[40] observed that rats fed on a diet high in gluten and casein can worsen intestinal permeability, increasing serum zonulin levels, as an indication of a leaky gut. Additionally, the effects of both diets are additive on dysbiosis, which is a factor in the development of GI comorbidity in ASD[40]. Dietary and microbiological therapies may be suggested to support a healthy microbiota profile in ASD patients[41,42]. Camara-Lemarroy *et al.*[41] reported that, through tight junction modulation, zonulin may regulate the breakdown of both the intestinal barrier and the BBB in gut dysbiosis. This could explain how the gut-brain axis influences neuroinflammation in many neurological disorders. Monitoring a leaky gut and identifying people at risk of developing many chronic illnesses among which is ASD may be made easier with the help of serum zonulin, in particular[43,44].

Nalbant *et al.*[45] showed that compared to the control group, children with ASD have altered epithelial barrier function and that examining the mechanism underlying the variation in occludin levels between ASD and controls is important to better understand the etiopathogenesis of ASD as well as its follow-up and treatment[45].

The recorded increased serum levels of zonulin and occludin in MeHg-treated rats can help to ascertain its deleterious effect on the integrity of intestinal cells and tight junction assembly (Fig. 2a-b). It is well known that tight junctions in the intestine are irreversibly disassembled by zonulin, often known as the "gate of the gut". Together, the occludin and zonulin proteins control how TJs are structurally organized and create a traditional barrier to the passage of solutes across the paracellular channel. They control the movement of immune cells, macromolecules and ions. Increased zonulin production results in reversible cell-to-cell tight junction unsealing, which raises intestinal barrier permeability and that, increased zonulin concentrations were accompanied by higher occludin values[46].

The role of gut leakiness reported in the current study (Fig. 2a-d) and the striking impairment in brain chemistry observed in our previous study[33,34], could be easily related to the high significant increase of LPB as a mediator of LPS inflammatory response and marker of increased intestinal permeability in MeHg-treated rats (Fig. 2c). Increased LPS localization (as LPB-LPS complex) in the colon of MeHg-treated rats exaggerates intestinal inflammation and decreases the frequency of regulatory T cells, increasing

epithelial TJ permeability via the release of IL-8 by intestinal epithelial cells[47]. This can be support through considering the report of Sochaczewska *et al.*[48], which proves that higher levels of excreted fecal occludin and zonulin were frequently associated with higher levels of fecal LPS[47]. Furthermore, research suggests that systemically increased LPS can increase BBB permeability by activating microglia throughout the brain[49].

The current study demonstrated that I-FABP levels in MeHg-treated rats are considerably greater than in healthy controls, indicating small intestine epithelial damage as a toxic response (Fig. 2d). This offers acceptance of the idea that I-FABP serum levels, as a measure of gut leakage, can be a useful biomarker for the early detection of ASD.

Figure 2(a-d) also showed that bee pollen has protective and curative effects on MeHg-induced gut leakiness. Occludin and LPB levels in the protected group did not differ significantly from controls, indicating the high potency of bee pollen. When compared to MeHg-treated rats, both showed much lower levels. Bee pollen was also beneficial as a therapeutic drug, lowering levels of zonulin, occludin, LPB and I-FABP, all of which are markers of increased gut permeability.

Numerous researches on the nutritional benefits of bee pollen confirm this result. Bee pollen extract was studied for its anti-inflammatory and antioxidant effects on the regulation of important cytokine gene expression[47], as well as its protective effects on intestinal barrier function. They also showed that pretreatment of Caco-2 cells with bee pollen extract dramatically reduced MAPK signalling pathway activation in response to dextran sulphate sodium (DSS)-induced cytotoxic damage to intestinal cells. In a more recent study, Li *et al.*[50] reported that pretreatment of DSS-intoxicated Caco-2 intestinal cells with bee pollen significantly regulated glycerophospholipid and sphingolipid metabolisms, potentially involved in building permeability barriers and alleviating intestinal oxidative stress.

Natural antioxidants have been used in a growing number of studies to reduce inflammation and oxidative stress in the intestinal system and they have shown encouraging results when used to treat gut leakiness in IBD patients[51,52]. As a result, pathogenesis-based treatments continue to be interesting for future drug research and development. In this work, we examined the antioxidant effect of bee pollen as a naturally occurring substance high in flavonoids and polyphenols. The GSH, GST and diamine oxidase levels were significantly higher in bee-pollen-treated groups, coincident with a significant decrease in lipid peroxides, suggesting a complete antioxidant effect. It's interesting to consider our findings in terms of the two key linkages between gut microbiota and the brain that have lately received attention in research: "Gut microbiota-oxidative stress-neurodegeneration" and "gut microbiota-antioxidant-neuroprotection". These connections may help explain why MeHg-intoxicated rats have a substantial increase in leaky gut markers and oxidative stress, which is mitigated in bee pollen-fed groups[53].

Table 1 and Fig. 3(a-h) demonstrate the data of ROC, AUCs as measures of the predictive value of the measured variables either of MeHg toxicity, or bee pollen protective and therapeutic potency. It can be easily noticed that in the MeHg-treated group, the four measured leaky gut biomarkers recorded AUCs of 1.0 (Fig. 3a-d). This means that zonulin, occludin, LBP and I-FABP are excellent predictive biomarkers of MeHg-induced leaky gut. The AUCs for the bee pollen-treated group vary from 0.515 to 0.716, meaning there is no discernible difference between them and the control group. This supports the idea that the consumption of bee pollen is safe. The ROC-AUCs provide good predictive values in measuring the effectiveness of bee pollen in treating leaky gut in the therapy group, which received MeHg followed by bee pollen (AUCs of the four variables are close to 1.0). Only zonulin and I-FABP recorded high AUCs (0.945 and 1.0, respectively) in the protected group, indicating their suitability to follow the potential protective effects of bee pollen, whereas occludin and LPB were useless as biomarkers of bee pollen anti-gut leakiness effect, with AUCs of (0.53 and 0.56), respectively. Table 1 and Fig. 3(e-h) also present the ROC-AUCs for the oxidative stress variables. The four variables demonstrate good predictive variables either for MeHg toxicity or bee pollen therapeutic potency and fare predictiveness values for bee pollen-protective effects.

The obtained ROC data can find support in the recent study of Tokuno *et al.*[54] in which they proved the usefulness of ROC analysis in studying selected biomarkers related to intestinal permeability and altered gut microbiota as a risk factor for multiple chronic diseases.

Most recently, Nowosad *et al.*[55] reported the effectiveness of bee pollen on the development of beneficially important gut microbiota, such as lactic acid-producing bacteria in African catfish[54]. Bee pollen has been suggested to have prebiotic effects, meaning it can provide nutrients to beneficial gut bacteria such as bifidobacteria and *Lactobacillus*, promoting their growth. A healthy gut microbiota is associated with a strong immune system. This suggestion can find great support in the recent work which showed a novel method to mitigate the neurotoxic effects of propionic acid, a short-chain fatty acid associated with the pathoetiology of autism, by combining bee pollen and probiotics as a therapeutic intervention technique. The use of a mixture of some healthy bacteria including *Bifidobacterium breve*, *Bifidobacterium infantis*, *Lactobacillus acidophilus*,

Lactobacillus bulgaricus, *Lacticaseibacillus casei*, *Lacticaseibacillus rhamnosus* and *Streptococcus thermophilus* in combination with bee pollen was more effective than each independently[56].

The data of the present study is in good agreement with the previous report that mercury appears to be a risk factor for ASD, with both direct and indirect effects.

Nevertheless, current study's limitations include the absence of gut microbiota assessment and the analysis of the molecular pathways linking the rise in the four markers in the MeHg-treated group with the development of behavioral autistic features in the rodent model, although the vast body of evidence indicating that mercury exposure causes and/or contributes to ASD.

CONCLUSION

The MeHg promotes intestinal permeability, leading to altered levels of leaky gut (zonulin, occludin, LBP and I-FABP) and oxidative stress (GSH, GST, lipid peroxides and diamine oxidase) indicators in the blood. Furthermore, bee pollen was effective in reducing dysbiosis, as a factor in the development of GI comorbidity in ASD. Thus, dietary interventions to promote a healthy microbiota composition and address leaky gut could be recommended.

SIGNIFICANCE STATEMENT

New research indicates that adhering to specific healthy diets may be a safe method to support gut health and restore a healthy gut microbiota, even though the precise cause-and-effect relationship between ASD and changed gut microbiota is yet unknown. This study examined bee pollen's potential as a prebiotic to treat gut leakiness brought on by MeHg, one of the top ten environmental pollutants thought to be linked to autism and learning disabilities.

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