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Effect of Sitagliptin on Sodium Valproate-Induced Autism in Mice Model

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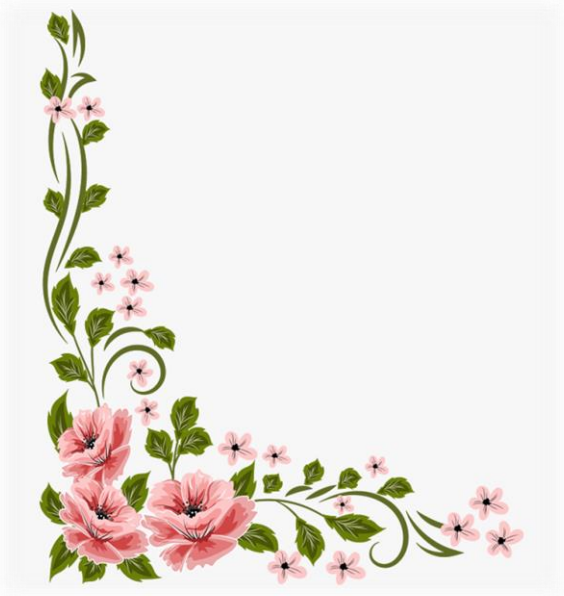

Ahmed Sudan Abbas

Summary

A neurodevelopmental illness known as autism spectrum disorder (ASD) is characterized by a decline in interpersonal relationships and communication that begins in early childhood.

This study was aimed to investigate the effect of sitagliptin on autism spectrum disorder (ASD) in mice after induction of ASD in this mice by sodium valproate. It was aimed to determine social communication of mice by social interaction test and locomotor activity and anxiety by open field test. Also, this study determined the antioxidant activity of sitagliptin by measuring levels of reduced glutathione (GSH), malondialdehyde (MDA) and determined the anti-inflammatory activity by measuring levels of interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α).

The study period lasted from 21/9/2022 to 5/2/2023. This study was conducted on 60 healthy adult albino mice (20 male and 40 female) weighting (26-39 g), divided into 20 mating groups (each group had 1 male and 2 female). After determination of day zero by seeing mice sperm under light microscope, female mice were then divided into two groups at 12^{th} day. One group was injected intraperitoneally (i.p.) with normal saline only and another group was injected with sodium valproate i.p. 600 mg/kg and waited until delivery. On the 40^{th} postpartum day, the newborn mice were separated from their mothers and randomly divided into eight groups, each group consisting of ten animals and being categorized as follows:

- The offspring in the control groups were given the following treatments orally: group 1 received normal saline, group 2 obtained sitagliptin 10 mg/kg, group 3 obtained sitagliptin 15 mg/kg, and group 4 got risperidone 1 mg/kg.
- VPA-induced offspring groups: group 5 obtained normal saline by oral administration, group 6 received sitagliptin 10 mg/kg via oral administration, group 7 got sitagliptin 15 mg/kg via oral administration, and group 8 obtained risperidone 1 mg/kg via oral administration.

The experiment was continued for 20 days, and then performs social interaction and open field tests. After performing behavioral tests, the mouse sacrificed by decapitation after 24 hours of the last dose of the treatment and took only two hemispheresof the mice's brain for estimation of oxidative stress (GSH and MDA) and anti-inflammatory activity (IL-6 and TNF-α) after performing tissue homogenization.

According to the findings of this research, sitagliptin, at both of the studied doses, increase time spent with stranger mouse in social interaction test. Also, it had anxiolytic activity by decreasing the symptoms of ASD (anxiety, fearing and hyperactivity) in open field test. Sitagliptin had potent antioxidant and free radical scavenging activities as compared with risperidone. The results of this study determined that 10 mg/kg sitagliptin had strong antioxidant effect on lipid peroxidation as compared with risperidone. While the anti-inflammatory effect of sitagliptin at both doses was less than risperidone; despite sitagliptin at both doses had potent anti-inflammatory activity in control offspring mice.

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List of abbreviations

Abbreviation	Description
5-HT1A	5-hydroxytryptamine 1A
Ab	Antibody
ADD	Attention-deficit disorder
ADHD	Attention Deficit Hyperactivity Disorder
AMPK	Adenosine monophosphate-activated protein kinase
ANOVA	One-way analysis of variance
ASD	Autism spectrum disorder
ATP	Adenosine triphosphate
AUC	Under the curve
BBB	Blood brain barrier
BDNF	Brain Derived Neurotrophic Factor
Ca	Calcium
CAT	Catalase
D2	Dopamine 2 receptor
DNA	Deoxyribonucleic acid
DPP-4	Dipeptidyl peptidase IV
DSM 5	Diagnostic and Statistical Manual of Mental Disorders, 5th Edition
DTNB	5,5- dithio-bis 2-nitrobenzoic acid
E-I	Excitatory-Inhibitory
eIF4E	Eukaryotic translation-initiating factor 4E
ELISA	Enzyme linked immunosorbent assay
Et al	And others
FDA	Food and drug administration

GABA	Gamma aminobutyric acid
GIP	Gastric inhibitory polypeptide
GLP 1	Glucagon like peptide-1
GLP-1R	Glucagon like peptide-1 receptor
GLT-1	Glutamate transporter-1
GPx	Glutathione peroxidase
GRx	Glutathione reductase
GSH	Reduced glutathione
HbA1c	Hemoglobin A1c
HCl	Hydrochloric acid
HDAC	Histone deacetylases
HRP	Horseradish peroxidase
i.p.	Intraperitoneal
IL-1	Interleukin-1
IL-6	Interleukin-6
IU	International unit
LHP	Lipid hydroperoxides
LSD	Least significant difference
MDA	Malondialdehyde
MIA	Maternal immune activation
Mmol/l	Millimoles per liter
NF-κB	Nuclear factor kappa B
NMDA	N-methyl-D-aspartate
Nrf2	Nuclear factor erythroid 2-related factor 2
OD	Optical density
OPC	Oligodendrocyte precursor cells
PAMP	Pathogen-associated molecular pattern molecules

PBS	Phosphate buffered saline
PGC1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PRR	Pattern-recognition receptors
PTEN	Phosphatase and tensin homolog
RIS	Rispridone
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SITA	Sitagliptin
SOD	Superoxide dismutase
SPSS	Statistical Package for Social Science
SSRI	Selective serotonin reuptake inhibitor
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid
TLR	Toll-like receptors
TNF-α	Tumor necrosis factor-α
UV	Ultraviolet
VOCs	Volatile organic compounds
VPA	Sodium Valproate

CHAPTER ONE

Introduction
and
Literature Review

1.1. Introduction

Autism spectrum disorders, abbreviated as ASD, are a group of complicated illnesses that are brought on by a confluence of epigenetic, environmental, and genetic factors during early prenatal as well as postnatal infancy (Mathieu Thabault *et al.*, 2022).

Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition, 2013 (DSM-5), states the following: the criteria for diagnosing ASD are solely behavioral. Hallmarks of ASD include deficiencies in interpersonal relationships and communication, in addition to compulsive behavior, attention deficits, and behavioral inflexibility. (Jill L. Silverman *et al.*, 2022).

Autism spectrum disease affects one in every 160 child around the world, as reported by the World Health Organization, and the prevalence rate is three boys for every one girl, which indicates that males are more susceptible to the condition. (Loomes R. *et al.*, 2017).

Prenatal variables, such as mother infections and pregnant toxin exposure, have been proven to increase the likelihood of having ASD. There was a correlation between being admitted to the hospital owing to a viral infection during childbirth and an elevated risk of ASD, according to the results of a meta-analysis that was presented in 2016 that included over than 40,000 instances of ASD (Jiang H.Y. *et al.*, 2016).

In response to this fact, one of the ideas that have been put forward is that a change in the immune system of the mother causes more proinflammatory cytokines to be made. There is not a universal agreement on how the cytokines would make their way to the brain of the fetus. They might get there by causing a breach in the brain–blood barrier (BBB) (Shigemoto-Mogami Y. *et al.*, 2018), or due to the impact of proinflammatory cytokines that are capable of piercing the blood-brain barrier (BBB) (Zawadzka A. *et al.*, 2021).

Some of the antiepileptic medications, including sodium valproate (VPA), are classified as teratogens. Prenatal exposure to volatile organic compounds (VOCs) has been linked to greater rates of a variety of major congenital malformations in humans, including neural tube, cardiovascular, craniofacial, and limbs deformities. This was already a known fact when the research was conducted (Bruna Lotufo Denucci *et al.*, 2021). In prenatal exposure to volatile organic compounds has also been linked to neurodevelopmental issues, such as impaired brain performance, learning difficulties, and attention-deficit hyperactivity disorder (T. Tomson *et al.*, 2019).

The type of treatment a child receives for autism spectrum disorder depends on their individual needs. Because ASD is a spectrum disorder (meaning some children have mild symptoms and others have severe symptoms) and each child who has it is unique, there are a variety of treatments. They can include different kinds of therapies to improve speech and behavior, and sometimes medications to help manage any medical conditions related to autism (Bruna Lotufo Denucci *et al.*, 2021). Risperidone and Aripiprazole are the only drugs approved by the Food and Drug Administration (FDA) for children with autism spectrum disorder, risperidone can be prescribed for children between 5 and 16 years old to help with irritability and aggression. Aripiprazole can be prescribed for children between 6 and 17 years old (S. Siafis *et al.*, 2020).

This investigation utilized sitagliptin, along with other members of the DPP-4 group, as a powerful neuroprotective drug because previous researchs (W. Hewedy, 2020; Suzan A. Khodir *et al.*, 2022; Esraa M. Elnahas *et al.*, 2022) indicated that it had potent antioxidant and anti-inflammatory effects on Alzheimer's disease, Parkinson's disease, and dementia. As a result of these findings, sitagliptin was useful in this investigation.

1.2. Aim of the study

In this study, the aims were:

1. To create sodium valproate-induced mice model of autism.

2. To evaluate the behavioral effect that sitagliptin has on the sodium valproate-induced mice model of autism.

3. To evaluate the effect of sitagliptin on malondialdehyde (MDA), reduced glutathione (GSH), interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α).

1.3. Literature Review

1.3.1.Autism spectrum disorder (ASD)

Autism spectrum disorder "ASD" is a difficult neurological disorder that can have a variety of causes. ASD manifests in early childhood (Lord *et al.,* 2020).

ASD is expressed by limitations in socialization, in addition to by habits, hobbies, or activities that are confined and repetitive. Those diagnosed with ASD may also demonstrate difficulties in the development of language, as well as self-injurious behavior, hyper-excitability, and motor abnormalities (Bhat *et al.,* 2014). In addition to these symptoms, people who have ASD frequently struggle to focus their attention are anxious, have trouble sleeping, and have an odd response to certain sensory inputs (Marotta *et al.,* 2020). Male are roughly three times more likely than female to experience it (Loomes *et al.,* 2017) and afflict white children more frequently than African American or Hispanic kids (Bhat *et al.,* 2014).

A number of diseases that fall into two major categories are included in ASD:

1. Genetic conditions that have characteristics with autism spectrum disease, such as fragile-X syndrome, mental retardation, in addition to tuberous sclerosis complex.
2. Idiopathic, meaning the causes are uncertain. Idiopathic variants of ASD were referred to it by a number of different labels throughout the years, including persistent developmental problems, as well as Asperger syndrome (Lord *et al.,* 2018).

The term 'autism spectrum disorder' (ASD) and other illnesses that share some features with autism were once classified under the umbrella term "pervasive developmental disorder". "Asperger syndrome" is a term used to describe high-functioning persons who have autism spectrum disorder; these are individuals who already have intellectual skills that are average or above average. Individuals who have Asperger syndrome might not have the typical issues in social communication

that people with ASD do. People who possess a genetic condition that is related to ASD may or mayn't exhibit the manifestations of ASD (Lord *et al.,* 2018).

1.3.1.1. Pathophysiology of Autism spectrum disorder (ASD)

Some experts believe that ASD is caused by a disruption in brain connections, which, in turn, affects the excitation/inhibition equity. The neuronal hypo-connectivity in the brain that is identified in people with ASD may illustrate the physical modification in brain size and hemispheric connections, according to some research. This balance between neurological inhibition and excitation is maintained by a number of biological as well as chemical mechanisms that change the geometry of synapses and the development of the brain. These processes can have an effect on the connections between the regions of the brain (Alma *et al.,* 2020).

By interfering with neurotransmission or altering axonal conductivity, astrocytes as well as oligodendrocytes may be responsible for the neurotransmitter abnormalities observed in autistic brains. In mice models of ASD, the inherited reduction of glutamate transporter-1 (GLT-1) in astrocytes increases excitatory neurotransmission, which is associated with a high rate of activity that is continuously repeated (Lee *et al.,* 2017). On the other hand, phosphatase and tensin homolog mislocation (PTEN) generates premature oligodendrocytes, which are associated with atypical myelin sheaths (Lee *et al.*, 2019).

According to a recent study, a genetic change in the microglia's eukaryotic translation-initiating factor 4E (eIF4E) alters the generation and functioning of synapses in lab mice, leading to behaviors that are identical to the symptoms that are associated with ASD (Xu *et al.*, 2020). As a result, behavioral dysfunctions may result from changes in glia-transmission, ion-channel regulation, synaptic plasticity, as well as oxidative stress caused by neurotoxicity and pro-inflammatory cytokines generated by microglia. It's interesting to note that, via altering the myelination process,

oxidative stress is also connected to the pathophysiological mechanisms of ASD (Kim *et al.*, 2020).

In this aspect, oxidant radicals can harm the group of oligodendrocytes that is unable to differentiate into mature oligodendrocytes that can create myelin and raise a large spread of oligodendrocyte precursor cells (OPCs), which as a result affects the entire myelination process. Since they possess large concentrations of sphingolipids and few concentrations of glutathione, a very effective antioxidant molecule, oligodendrocytes are a relatively vulnerable cell type to oxidative radicals. Hence, the oxidative stress found in the brain's ASD represents a plausible biological target for oligodendrocytes (Alma *et al.*, 2020).

1.3.1.2. The etiology of Autism spectrum disorder

A- A mixture of hereditary components and environmental variables is frequently used to define it. Genes associated with intellectual impairment and neurological disorders, genes involved in common pathways, genes associated with ASD, DNA mutations, environmental variables that impact expression of genes and the functions of proteins are among the hereditary factors that contribute to the causes of ASD (Devlin and Scherer, 2012).

Research has revealed a variety of factors that raise the chance of ASD. Autism-related symptoms were associated with perinatal and neonatal environmental risk variants, such as prenatal contact with chemicals (such as sodium valproate, misoprostol, alcohol drinking, heroin, and toxicants), infections, maternal and fetal inflammation, and diseases (such as diabetes) (Ornoy *et al.*, 2015).

The current study demonstrated a model of autism that is produced by VPA, because it impacts the white matter area, which has a significant impact in the neurodegenerative illness that is brought on by the physiology of glial cells, which is another cause of the ASD characteristics. The prenatal phase was when the

development of the brain first started, and the postnatal phase is when it continues. Similar to how astrocytes expand after birth, the amount of glial cells rises after birth. Following birth, within the sub-ventricular zone, oligodendrocytes and astrocytes are formed. They first arise during the latter few weeks of pregnancy. The separation of gray and white matter takes place during migration (Stouffer *et al.,* 2016).

B- Oxidative stress is the term given to the condition that occurs in the body when there is an abnormally high production of reactive oxygen species (ROS). It is an indication that there is a disconnect between the generation and buildup of ROS and the antioxidant defense mechanisms (Pawel Kowalczyk *et al.,* 2021). When created in excessive amounts, reactive oxygen species (ROS) turn into one of the biggest contributors of the injury caused to tissues and cells. ROS perform a biological role in intracellular signaling pathways when kept at a low level (Holmstrom and Finkel, 2014). The latter is the result of an action that is directly detrimental to biological structures such as proteins, fats, and nucleic acids (Pawel Kowalczyk *et al.,* 2021). Endogenous reactive oxygen species are produced as a by-product of oxygen metabolism, whereas exogenous oxidative stress can be caused by environmental factors such as ionizing or X-ray radiation, UV, chemical contaminants, smoking cigarettes, toxic metals, and certain drugs. Endogenous ROS are more prevalent than exogenous ROS. (Pizzino *et al.,* 2017). Chronic inflammatory processes within the organism exacerbate oxidative stress and accelerate the formation of reactive oxygen species (ROS) (Hussain *et al.*, 2016).

The mitochondria are the primary location within the cell that generates free radicals. In addition to their principal function, which is the production of ATP, mitochondria also have a part in the biosynthesis of lipids, nucleic acids, hemes, purines, and amino acids (Spinelli and Haigis, 2018; Kokkinopoulou and Moutsatsou, 2021). They are also responsible for maintaining the homeostasis of Ca^{2+} within the cell and regulating heat production, cell division, and the death of programmed cells (Spinelli and Haigis, 2018; Osellame *et al.*, 2012).

About one to two percent of the molecular oxygen that is pulled in by cells during the process of normal respiration is transformed to reactive oxygen species (ROS) during the strong oxidative metabolism that takes place in mitochondria. In point of fact, the vast majority of free radicals and, more specifically, superoxide anion are byproducts of mitochondrial respiration. They are produced when electrons move through complexes I, II, and III of the mitochondrial electron transport chain (Shadel and Horvath, 2015).

Oxidative stress, which is caused by exposure to hazardous substances during pregnancy, is widely recognized as a significant contributor to the development of neurodevelopmental problems (Nishimura *et al.,* 2021). In the case of autism, mitochondrial abnormalities, increased oxidative stress, and lower antioxidant capacity have all been documented in autistic persons; all of these factors, taken together, may be responsible for neuroinflammation and the pathology of autism (Balachandar *et al.,* 2020; Toscano *et al.,* 2021).

A recent study of blood samples taken from children diagnosed with autism spectrum disorders found anomalies in neural transduction as well as a reduction in total plasma peroxidase and overall antioxidant activity (Omotosho *et al.,* 2021).

According to the findings of another study (Zawadzka *et al.,* 2021), delayed brain development is the result of inflammatory processes that induce oxidative stress and mitochondrial dysfunction. These, in turn, worsen oxidative stress, which leads to more cellular damage. Studies in children with autism who used N-acetylcysteine or other antioxidants reported a reduction in some autistic behaviors, such as irritability and over-activity (Balachandar *et al.,* 2020). These findings lend credence to the theory that oxidative stress plays a role in the pathology of autism (Liu *et al.*, 2021).

- **Reduced glutathione**

Because of its function in the upkeep of the intracellular redox balance, the significance of glutathione in ASD is important. A glutathione redox imbalance has been implicated as a leading factor in ASD, and both ASD and many other neurodevelopmental disorders involve irregularities in the expression of glutathione-related enzymes in the blood or brain. Low levels of reduced glutathione (GSH), high concentration of oxidized glutathione (GSSG), and irregularities in the expression of glutathione-related enzymes have been linked to ASD. The metabolism of glutathione interferes with several pathways that are involved in the pathophysiology of autism spectrum disorder (ASD), either through its effect on the redox environment or through redox-independent mechanisms. It is possible that the modulation of glutamate excitotoxicity is influenced by glutathione-mediated regulation of glutamate receptors for example, the N-methyl-d-aspartate (NMDA) receptor, as well as the significance of glutamate as a substrate for glutathione production. On the other hand, the nature of the relationship between glutathione and glutamate in the process of disease progression in the brain can range from synergism to antagonism. Glutathione metabolism and autism spectrum disorder (ASD) may also have a substantial connection if mitochondrial malfunction and neuronal death are both involved. (Geir Bjorklund *et al.,* 2020).

- **Malondialdehyde**

Antioxidants are cytoprotective substances that stop free radicals from causing oxidative damage to cells (Khadijah *et al.,* 2021). As a result of unhealthy behaviors, reactive oxygen species (ROS) can reach increased levels that can evade or overwhelm the anti-oxidant protective mechanisms provided by the anti-oxidants found in cells and tissues. It brings about the depletion of anti-oxidants, which in turn brings about the accumulation of reactive oxygen species (ROS), which in turn brings about the condition known as oxidative stress (Katakwar *et al.,* 2016). Oxidative stress

causes a disturbance in cell metabolism, which manifests itself as an increase in the amount of intracellular free Ca^{2+} as well as destroys the membrane ion transporters. ROS is also responsible for the facilitation of punctual mutations, DNA base oxidations, and strand breakage, as well as the mutation of tumor suppressor genes and the activation of proto-oncogenes (Khadijah *et al.*, 2021).

ROS interactions with biological molecules result in damage to lipid biomembranes, the sulfhydryl linkages of proteins and carbohydrates, and other biological structures. The removal of hydrogen from fatty acids that are unsaturated is the first step in the process that leads to biomembrane lipid peroxidation damage. The formation of free radicals is an essential stage in the multi-stage process of carcinogenesis because it sets in motion the chain reaction that ultimately leads to the complete breakdown of the cell membrane (Khadijah *et al.*, 2021).

In addition, the breakdown of such peroxidized lipids is accompanied by a speedy disintegration and the formation of reactive carbon molecules. These chemicals include malondialdehyde (MDA) and lipid hydroperoxides (LHP). Such secondary compounds can be used as a marker of whether or not lipids have been peroxidized (Khadijah *et al.*, 2021). By triggering the signal transduction system, the products of lipid peroxidation have the ability to control cell proliferation as well as accelerate the progression of tumors. In addition to this, they contribute to the development of cancer by virtue of the significant cytotoxicity they exhibit (Metgud and Bajaj, 2014).

C- Cytokines: the innate immune system is the primary line of defense in the body, and it is triggered if a pathogen or another immunological insult is encountered. Neutrophils, monocytes, and their tissue-resident counterparts, macrophages, are examples of cellular components that make up the innate arm of the immune system. These cells produce pattern-recognition receptors (PRRs), such as Toll-like receptors (TLRs), that broadly recognize molecular patterns that are linked with pathogens associated molecular pattern molecules (PAMPs). During the earliest and early stages

of the immune response, this detection sets off a chain reaction of activation that ultimately results in inflammatory responses and the production of innate inflammatory cytokines. These cytokines include IL-1, IL-6, and a tumor necrosis factor (TNF) (Heather *et al.,* 2022).

Innate myeloid cells, such as monocytes and macrophages, are essential for the production of inflammatory cytokines, and their presence is a substantial help in this regard. Only a very few of those studies have isolated and triggered circulating monocytes to determine the changing performance of myeloid cells in ASD children, and the results of those studies have been mixed. Although many investigations have discovered increases in inflammatory cytokines in autism spectrum disorder (ASD), the results of only a small number of those studies have found increases in ASD (Heather *et al.*, 2022).

ASD inflammation may be brought on by disequilibrium between cytokines that promote inflammation as well as those that reduce it caused by a low quantity of anti-inflammatory cytokines. Infection during pregnancy may produce inflammatory markers that go over the placenta and cause inflammation of the fetus's neurons (figure 1.1) (Saghazadeh *et al.*, 2019).

D- Pre-eclampsia, fetal distress, threatened abortion, antepartum bleeding, caesarean section, age at delivery 36 weeks gestation, natural delivery, induced labor, or no labor, hypertension, or diabetes in the mother were the perinatal factors linked to a higher likelihood of having ASD. Infant weight loss, postpartum bleeding, male sex, and central nervous system abnormalities were the postnatal factors increase the risk for ASD (Wang *et al.,* 2017).

E- Selective serotonin reuptake inhibitor are using by the mother, especially in the first trimester, may increase the likelihood that her child would be diagnosed with ASD (Croen *et al.*, 2011; Andrade, 2017).

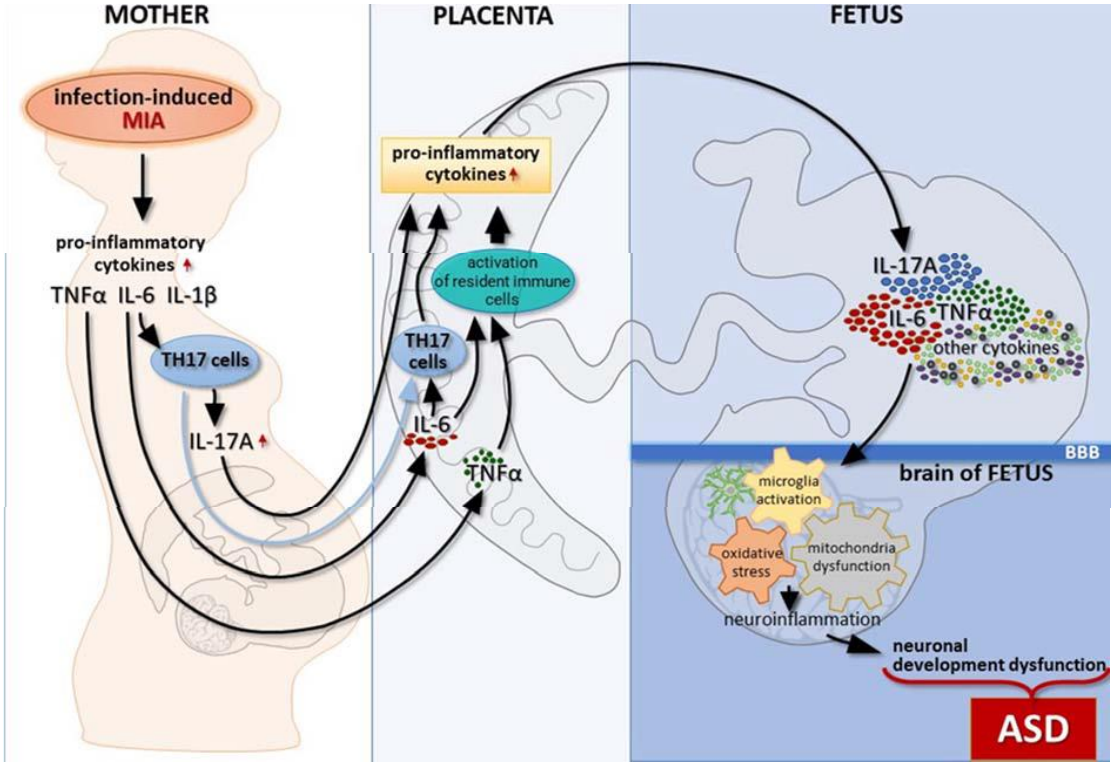

Figure 1.1. Immunological alterations in the placenta and the brain of the fetus as a result of systemic inflammation occurring in the mother during pregnancy, which leads to dysfunctional neuronal development in the offspring (Aleksandra Zawadzka *et al.,* 2022).

1.3.1.3. DSM-5 diagnostic criteria for autism spectrum disorder

The DSM-5 lists the signs and symptoms of autism spectrum disorder and states how many of these must be present to confirm a diagnosis of autism spectrum disorder. In DSM-5 autism was characterized by a triad of symptoms (figure 1.2), including the presence of persistent deficits in social communication, deficits in social interaction in various contexts, and restricted, repetitive patterns of behavior, interests, or activities (Ana *et al.*, 2021).

A. Difficulties in social communication: to be diagnosed with autism spectrum disorder, children must have difficulties and/or differences from what's typical in the area of social communication.

Signs in this area include:

- rarely using language to communicate with other people
- not speaking at all
- rarely responding when spoken to
- not sharing interests or achievements with parents
- rarely using or understanding gestures like pointing or waving
- using only limited facial expressions to communicate
- not showing an interest in friends or having difficulties making friends
- Rarely engaging in imaginative play (Rice *et al.,* 2022).

B. Restricted, repetitive and sensory behavior or interests

To be diagnosed with autism spectrum disorder, children must have difficulties and/or differences from what's typical in the area of restricted, repetitive and/or sensory behaviors or interests. Signs in this area include:

- lining up toys in a particular way over and over again
- frequently flicking switches or spinning objects
- speaking in a repetitive way
- having very narrow or intense interests
- needing things to always happen in the same way
- having trouble with changes to their schedule, or changing from one activity to another
- Showing signs of sensory sensitivities like becoming distressed by everyday sounds like hand dryers, not liking the feel of clothes labels, or licking or sniffing objects (Rice et al., 2022).

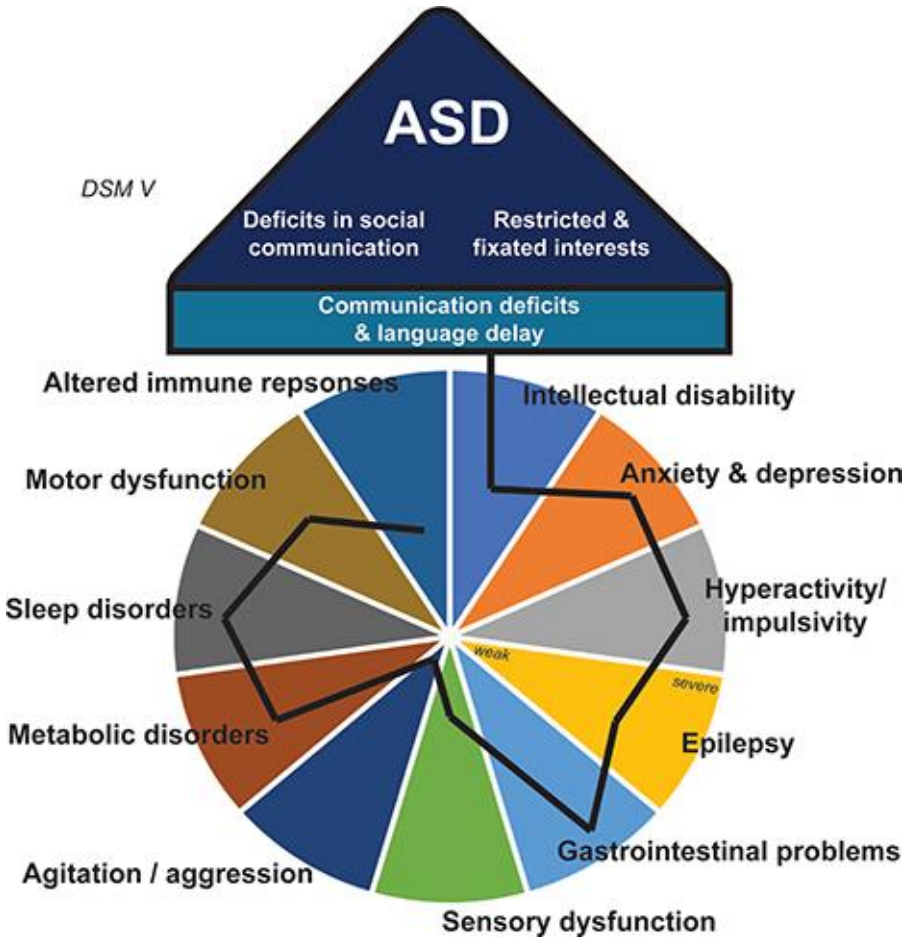

Figure 1.2. Show clinical traits of ASD. ASD is characterised by a wide range of observable behaviours and traits, the most prominent of which include difficulty in social communication, restricted and focused interests, speech issues, and language delays. According to DSM-5 the first two features are the ones that are used to diagnose individuals with ASD. Other indications and situations that may be present in those with ASD include cognitive impairment, anxiety, depressed mood, attention-deficit disorder (ADD), hyperactivity, seizures, gastrointestinal issues, sensory impairment, aggressive behavior, metabolic disturbances, sleep disturbances, motor dysfunction, and modified immunological responses. The severity of the signs and the accompanying conditions that are present in people with ASD varies, although not all of them are. Because of this, the behavioral traits found in individuals who have ASD can vary greatly from one individual to the next (Ann Katrin *et al.*, 2021)

1.3.1.4. Treatment of autism spectrum disorder

Individualized, intensive treatments that incorporate cognitive, behavioral components have proven to be the most effective treatments for ASD. When treatment is initiated during the earlier stages of infanthood, there is a greater chance that it will result in a favorable outcome. Because of this, it is extremely important to perform routine checks on newborns and toddlers to look for signs of ASD, as doing so permits the earlier diagnosis of affected individuals. As soon as symptoms or indicators of ASD emerge, the American Academy of Pediatrics advises referral for professional diagnostic and therapeutic approaches (Hyman *et al.*, 2020).

ASD can be treated using a variety of methods, including behavioral therapy, antipsychotics, antidepressants, mood stabilizers, and CNS stimulants. Nevertheless, no medication can reverse ASD. New pharmaceutical treatments must be created in order to address these symptoms and enhance the standard of living for those with ASD. The primary treatments and methods for treating ASD are also highlighted, with an emphasis on the pharmacological possibilities of peptides and bio-inspired chemicals discovered in animal venoms as a possible treatment for ASD in the near future (Bhat *et al.*, 2014).

A- Non pharmacologic therapy

- Individualized, focused special education
- Language, social, occupational, and physical treatments (such as exercise/physical therapy, auditory integration education, and therapy for sensory integration).
- Kids with ASD, including those with associated anxiety problems, benefit from social skill training (Antshel *et al.,* 2011). While social skills therapies for young people with ASD seem to be moderately helpful, the effects may not easily transfer to educational settings (Gates *et al.,* 2017).
- Cognitive behavioral therapy (Gates *et al*., 2017).

B- Pharmacologic therapy

Due to the variability of the illness and the paucity of a thorough understanding of its etiology, pharmacological therapies for ASD are scarce. Medical interventions are mostly used to manage ASD-related secondary symptoms. The only two pharmaceutical therapies recognized by the Food and Drug Administration (FDA) for the management of ASD symptoms are risperidone and aripiprazole. Both antipsychotic medications are used to treat aggression and irritability. In addition, various medications are frequently recommended to address ancillary symptoms despite the lack of valid trials to support their use. Antidepressants, antipsychotics, mood stabilisers, and stimulants are some of these treatments (Farmer *et al.,* 2013; Masi *et al.,* 2017).

It is challenging to conduct reliable research in order to develop and discover novel treatments for autism spectrum disorders. Small sample sizes, cultural differences in what is viewed as usual behavior changes, the complexity of the condition, placebos producing positive results, and the variability of the disorder are some of the issues (Masi *et al.,* 2017). However, finding effective therapies for the primary symptoms, such as communication and social behavior deficits, is a difficult task. In clinical trials using pharmaceuticals and dietary supplements, these efficient therapies revealed that

twenty percent of the participants in the placebo group exhibited a considerable enhancement in the fundamental symptoms they were suffering from, such as interpersonal contact, behavioral disturbances, and sensory irregularities (Siafis *et al.*, 2020).

- **Antipsychotic drugs:**

The FDA allowed the use of two antipsychotic medications after clinical research on ASD kids who displayed irritability issues as a secondary symptom. These medications improved the primary symptoms, but only for ASD children who also had this secondary symptom. The negative consequences of these medicines may outweigh the slight advantages they produce (Farmer *et al.*, 2013).

The first medication for treating ASD was risperidone (figure 1.3; A), which works by blocking serotonin and dopamine receptors in the brain. Research with ASD kids revealed significant enhancement in irritability, violence, and hyperactivity. Risperidone has a number of negative effects, despite being thought to be safe, such as increased hunger, weight gain, nasal congestion, weariness, dizziness, constipation, and diarrhoea (Lamberti *et al.*, 2016).

The second medication authorized for the therapy of irritability in ASD is aripiprazole (figure 1.3; B), an additional atypical antipsychotic. Aripiprazole works as a partial agonist on serotonin receptor type 1 (5-HT_{1A}) and dopamine (D_2) receptor antagonist. It also acts as a serotonin receptor type 2 (5-HT_{2A}) antagonists (Laurence *et al.*, 2020).The main symptoms of ASD, such as improper speech and social disengagement, are mildly improved, along with modest improvements in impatience and hyperactivity, thanks to this medication. Aripiprazole has been demonstrated to be safe, just like risperidone. Sedation, exhaustion, somnolence, increased hunger, vomiting, and diarrhea were a few of the negative effects that were noted (Mankoski *et al.*, 2013).

Chronic use of risperidone and aripiprazole has also been demonstrated to be beneficial for some primary and secondary symptoms of ASD in prenatal VPA-model animals, reducing prefrontal cortex dendritic spine density and improving deficiencies in sociability and recognition memory (Hara *et al.,* 2017); this finding supports clinical evidence that these medications are also helpful for treating ASD in humans (Varadinova, Bogdanov and Markova, 2019). Additionally, it implies that the prefrontal dopaminergic system in the prenatal VPA-model of ASD is a viable pharmacological target for novel medications (Hara *et al.,* 2017).

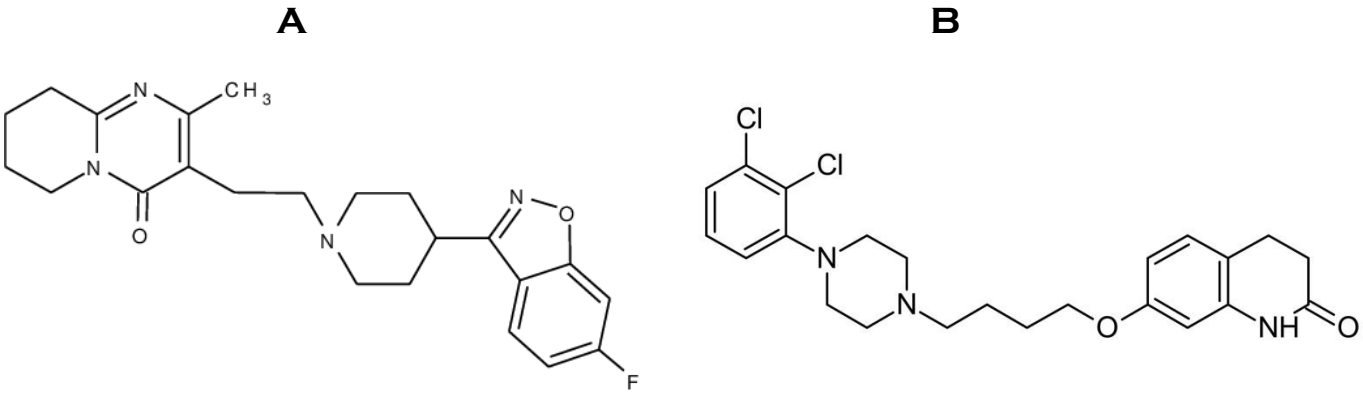

Figure 1.3. A- Chemical structure of risperidone B- chemical structure of aripiprazole (Guillermo *et al.,* 2021)

- **Attention Deficit Hyperactivity Disorder (ADHD) drugs:**

In spite of the fact that therapy with dopamine antagonists is successful, research has been conducted on additional medications. Methylphenidate and atomoxetine (figure 1.4), two medications typically prescribed for ADHD, were shown to enhance the behaviors associated with ASD. Several medications for ADHD are being considered as a possible therapy option for the characteristics that are associated with ASD. In a study, the use of methylphenidate and atomoxetine caused certain behavioral adjustments as well as dendritic spine architecture in a prenatal VPA model. These effects were caused by an increase in the release of dopamine in the prefrontal cortex. The long-term use of medication for attention deficit hyperactivity

disorder (ADHD) led to improvements in behavior and a slowing of the loss of spine density (Hara *et al.*, 2015).

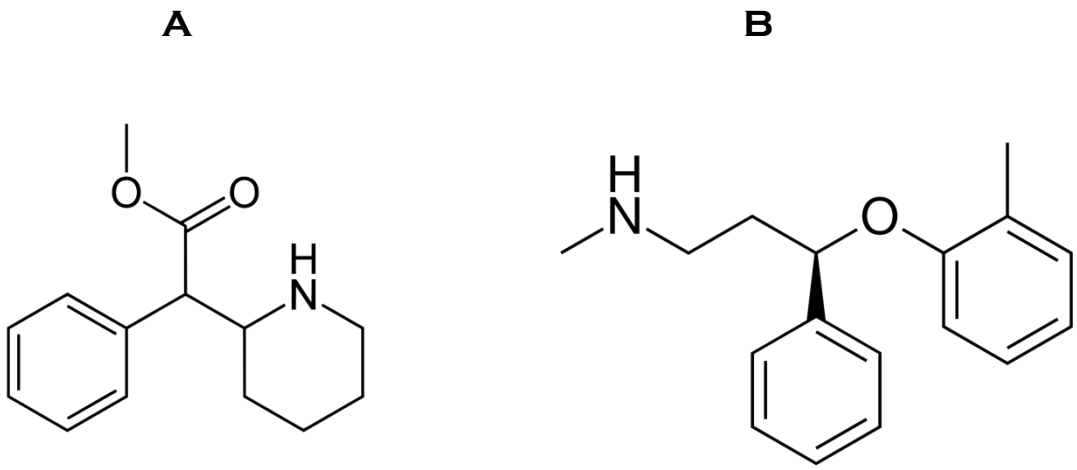

Figure 1.4. A- Chemical structure of methylphenidate B- chemical structure of atomoxetine (John Baele and John Block, 2011).

- **Drugs that are antagonists of the N-methyl-D-aspartate (NMDA) receptor:**

The use of agmatine is being investigated as a possible treatment for the primary symptoms of the disease. Excitatory-Inhibitory (E-I) imbalance is a disorder that is characterized by over-excitability in ASD individuals and produces a variety of signs, including cognitive and social difficulties, as well as seizures. E-I imbalance is the outcome of aberrant glutamatergic and gamma-aminobutyric acid (GABA) neurotransmission (Uzunova *et al.*, 2016). The E-I imbalance can be normalized with the help of a drug called agmatine (figure 1.5), which is an antagonist for the N-methyl-D-aspartate (NMDA) receptor. A specific treatment of agmatine and an agmatinase inhibitor, given before animal behavioral tests, restored the sociable, hyperactive, and repetitive behaviors, according to a study that was conducted with rats that were prenatally exposed to sodium valproate (VPA). In addition to this, it controlled the patient's seizures by regulating the overactivation of the prefrontal cortex and the hippocampus (Kim *et al.*, 2017).

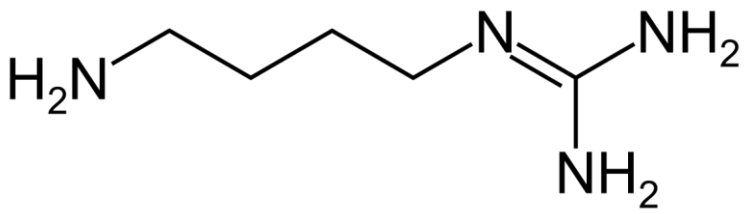

Figure 1.5. Chemical structure of agmatine (John Baele and John Block, 2011)

- **Selective Serotonin Reuptake Inhibitor (SSRI) drugs:**

In children diagnosed with ASD, hyperserotonemia is the most frequent serotonin-related disease. It is detected in around 25% of affected children (Muller *et al.*, 2016). In research using knockout mice as a model for hyperserotonemia, researchers saw an elevated level of anxiety as well as social abnormalities. These effects got better when tryptophan, which is a precursor of serotonin, was restricted in the diet (Tanaka *et al.*, 2018). Hence, manipulation of the levels of serotonin and transporter activity could be capable of reducing the severity of certain problems associated with autism spectrum disorder in humans (Bruna *et al.*, 2021).

Children who have been identified as having ASD and other diseases that are similar frequently receive prescriptions for drugs called selective serotonin reuptake inhibitors (SSRIs). Citalopram, escitalopram and fluoxetine have all been shown to have positive effects on ASD patients of all ages, including children and adolescents (Bruna *et al.*, 2021).

On the other hand, King and colleagues conducted a multicenter, randomized, controlled trial in which 149 children with ASD participated. They discovered that there was no variation between citalopram and placebo in terms of the number of children who were rated as having improved a great deal. Participants in the treatment group were given citalopram in liquid form once a day for a period of twelve weeks, with a maximum prescribed dose of 16.5 mg (maximum 20 mg). Virtually all of the people who took citalopram reported experiencing unwanted side effects, such as impulsiveness, hyperactivity and diarrhea (Bruna *et al.*, 2021).

- **Oxytocin**

Peptides are small polymers that are made up of amino acids that are joined together by peptide bonds. In comparison to proteins, peptides are much shorter and have a more flexible structural shape. These molecules have a higher activity per unit mass and are able to penetrate tissue more effectively than proteins, in addition to posing a lower risk of immunogenicity. These properties make them potentially useful in a variety of therapeutic applications. Peptides, on the other hand, have been shown to be more powerful, selective, and specific than some other small molecules, making them less poisonous and leading to fewer adverse medication interactions (Diao and Meibohm, 2013).

However, there are a few drawbacks associated with using peptides as therapeutic agents, including their susceptibility to proteolytic destruction, quick clearance from the blood circulation, poor metabolic stability and limited bioavailability (Diao and Meibohm, 2013). However, there are other alternative methods that are being taken in order to improve the compound's bioavailability. These include the substitution or replacement of amino acids, the modification of peptide amino or carboxy termini, the insertion of disulfide bridges, the conjugation of polymers and the use of nanoparticles (Bruna *et al.*, 2021).

The therapeutic technique makes use of neuropeptides like oxytocin, which is a molecule that has significance for the control of social behaviors and is being extensively researched as a potential treatment for autism spectrum disorder (ASD). Y.C. Dai and colleagues (2018) discovered that VPA rats had a lower concentration of this peptide in their bodies in comparison to the control group. They discovered that immediate oxytocin intranasal therapy at a concentration of 1 microgram per microliter improved the results of behavioral tests performed on VPA rats, partially correcting the reduced sociability that had been caused by the VPA (Dai *et al.*, 2018).

In addition, the injection of oxytocin subcutaneously and continuously to neonatal rats showed long-term effects, effectively addressing social deficits as well as repeated behaviors that were observed in adolescents (Dai *et al.*, 2018).

Another study discovered that administering oxytocin intra-nasally at a dose of 200 micrograms per kilogram can alleviate autism-like symptoms in VPA mice, leading to improvements in these animals' levels of anxiety, sadness and cognition (Wang *et al.*, 2018). In addition to that, it was shown that this neuropeptide reduced the amount of inflammation and oxidative stress that was present in the hippocampus and the amygdala. In addition to these findings, A. Tanaka *et al.*, (2018) conducted an investigation on the efficacy of oxytocin intranasal application in rats. They found that this route is extremely promising for the transport of this peptide to the brain in the treatment of ASD (Tanaka *et al.*, 2018).

In addition, the intranasal administration of oxytocin was studied in humans, and the brain alterations that were related with it were analyzed. Also, another study showed that treatment with multiple doses of oxytocin diminishes the action of the bilateral amygdala, which is related with improved behavioral gains. The participants in the study self-administered a daily intake of 24 IU for a total of four weeks. This finding is evidence that oxytocin has anxiolytic effects because it caused a downregulation of adverse effect and social discomfort after treatment, in addition to alleviating ASD symptoms including social responsiveness and compulsive behavior. Taking into account all of these findings, oxytocin has a significant amount of potential as a therapy approach for the primary social symptoms associated with ASD. This is due to the fact that it influences human cognition as well as social conduct (Bernaerts *et al.*, 2020).

1.3.2.Sitagliptin

1.3.2.1. Chemical structure

Sitagliptin Phosphate is the phosphate salt form of sitagliptin, an orally available drug. Sitagliptin is the first agent from gliptins that has been used for the treatment of diabetes mellitus (type II). International Union of Pure and Applied Chemistry (IUPAC) name for sitagliptin is (R)-4-oxo-4-[3-(trifluoromethyl)-5,6-dihydro[1,2,4]triazolo[4,3-a]pyrazin-7(8H)-yl]-1-(2,4,5-trifluorophenyl) butan-2-amine. Figure 1.6 show a chemical structure of sitagliptin (Samy *et al.,* 2021).

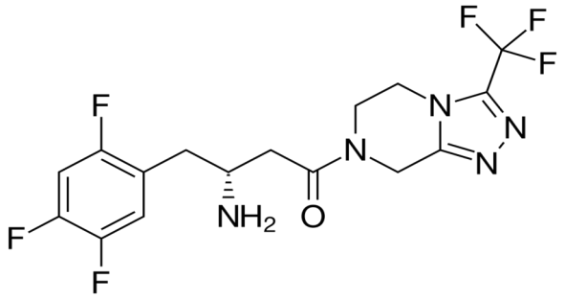

Figure 1.6. chemical structure of sitagliptin (Samy *et al.,* 2021).

1.3.2.2. Mechanism of action

The serine protease known as dipeptidyl peptidase IV (DPP-4) is disseminated across the body in a widespread manner. It is formed as such an ectoenzyme on vascular endothelial cells, on the membrane of T lymphocytes, and in a circulating state. It appears that DPP-4 is especially important for the deactivation of glucagon like peptide-1 (GLP-1) and gastric inhibitory polypeptide (GIP) because it separates the two N-terminal amino acids from peptides that include an alanine in the second position (Nauck M., 2016). The area under the curve (AUC) of GLP-1 and GIP that is triggered by the consumption of food is increased when DPP-4 inhibitors are used. Because of the very complete and long-lasting inhibition of DPP-4 that is provided by a number of different drugs, the proportion of active GLP-1 has increased from 10% to 20% of the total circulating GLP-1 immunoreactivity to nearly 100%. (Laurence *et al.,* 2020).

The DPP-4 enzyme is inhibited by sitagliptin, which acts as a competitive inhibitor. It is possible to administer it in doses that reduce the measured activity of DPP-4 for a period of 12 hours by more than 95%. Increased insulin secretion, decreased glucagon levels, and reductions in both postprandial and fasting hyperglycemia are related to this phenomenon, which raises plasma levels of active GIP and GLP-1 by a factor of more than two and is associated with these changes. It does not appear that inhibiting DPP-4 has any immediate effects on insulin sensitivity, stomach motility, or satiety, and prolonged treatment with a DPP-4 inhibitor has no effect on body weight (Laurence *et al.,* 2020).

1.3.2.3. Therapeutic uses

When administered as a monotherapy in patients with type 2 diabetes, the drug sitagliptin lowers A1c levels (also known as hemoglobin A1c or HbA1c) by an average of roughly 0.8%. When combined with the treatment that hyperglycemic patients receive insulin, metformin, thiazolidinediones, and sulfonylureas, these chemicals are also beneficial for chronic glucose management, resulting in a further reduction of A1c of around 0.5%. It appears that the actions of DPP-4 inhibitors in combination therapies have an additive effect (Laurence *et al.,* 2020; Hugo *et al.,* 2023).

1.3.2.4. Adverse effects

Sitagliptin is generally well tolerated, with nasopharyngitis and headache being the two side effects that occur most frequently. Pancreatitis has been linked to the use of DPP-4 inhibitors on occasion, despite the fact that this is not very common. (Karen *et al.,* 2019).

The Food and Drug Administration has warned consumers that this category of medications is only infrequently connected to severe joint pain. Lymphocytes are responsible for the expression of DPP-4, also known as the enzyme CD26. Because

more people are being treated with these drugs, it is essential to do studies on their impacts on immunological function (Laurence *et al.*, 2020).

1.3.3.Autism as illustrated by animal models

Validating animal models is one of the most difficult challenges that come with using them. Research that uses animal models is predicated on the idea showed there were neurobiological pathways that were shared between humans and animals that are related with activity that is complicated. Despite this, it is possible that biological substrates and metabolic pathways are distinct in animals and humans. It is possible for the same gene to produce two different phenotypes in the same species despite the fact that it is structurally comparable at the DNA levels, at the RNA organizational levels, or has equivalent functioning in the two different species (Bruna *et al.*, 2021).

Animal models are recommended for the investigation of ASD, and it is recommended that these models use a multimodal approach that takes into account the behavioral, neuropathological, physiological, and hereditary aspects of the condition. As more aspects of autism spectrum disorder (ASD) are taken into account in a model, the better that model will be at reflecting ASD in people. Hence, animal models are of tremendous utility because they help clarify complex mechanisms that are involved in neuropathological illnesses, even if these mechanisms are simplified by using the models. They are also helpful in understanding the link between biological and behavioural characteristics, as well as the ways in which both of these variables affect upon environmental elements (Bruna *et al.*, 2021).

The mice that carry a mutation in any one of the several ASD risk genes are used to create animal models the vast majority of the time (Satterstrom et al., 2020). Despite the fact that rats exhibit a significantly increased indication of social contact when compared to mice, which is the rodent model that is most commonly used, some researchers are beginning to emphasize the value of comparing mouse and rat rodent

models and the potential for cross-species convergence and divergence (Till *et al.*, 2022; Berg *et al.*, 2020).

The use of a targeted approach across species and the exploitation of the capabilities of each species could provide insight into the manner in which social data is analyzed by a variety of organisms. For instance, rodents are creatures that rely on their sense of smell, whereas non-human primates rely on their sense of sight (Jill *et al.*, 2022).

Rats employ auditory ultrasonic connection as pups and adults, whereas mice use it mostly during the neonatal period (for survival) and during mating. Rats also use it when they mature into adults (like instinctual). Rats communicate with one another by auditory and ultrasonic calls, which is a more complex habit. For instance, in their repertory find calls for making social contact, calls for playing, calls for tickling, calls for anticipating something, calls connected to fear and calls that warn of potential danger. Adult rats will let their young know about danger by making alarm cries. In the case of mice, this is not the case (Berg *et al.*, 2021).

1.3.4.Sodium valproate

1.3.4.1. Chemical structure

Sodium valproate is the sodium salt form of valproic acid with anti-epileptic activity. Sodium valproate is converted into its active form, valproate ion, in blood. IUPAC name for sodium valproate is 2-propylpentanoic acid. Figure 1.7 shows a chemical structure of sodium valproate (Samy *et al.*, 2021).

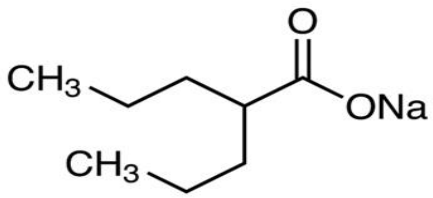

Figure 1.7. Chemical structure of sodium valproate (Thanigaimalai *et al.,* 2020)

1.3.4.2. Mechanism of action

At therapeutically relevant concentrations, sodium valproate inhibits sustained repetitive firing induced by depolarization of mouse's cortical or spinal cord neurons. The action appears to be mediated by a prolonged recovery of voltage-activated Na^+ channels from inactivation. In neurons isolated from the nodose ganglion, sodium valproate also produces small reductions of T-type Ca^{2+} currents at clinically relevant concentrations that are slightly higher than those that limit sustained repetitive firing. Together, these actions of limiting sustained repetitive firing and reducing T-type currents may contribute to the effectiveness of sodium valproate against focal and tonic-clonic seizures and absence seizures, respectively (Karen *et al.*, 2019; Laurence *et al.*, 2020).

In model systems, sodium valproate can increase brain content of GABA, stimulate GABA synthesis (by glutamate decarboxylase) and inhibit GABA degradation (by GABA transaminase and succinic semialdehyde dehydrogenase) (Laurence *et al.*, 2020).

1.3.4.3. Therapeutic uses

Sodium valproate is among the most adaptable and powerful anticonvulsant medications available today. It is frequently employed in the treatment of tonic-clonic, atonic and myoclonic seizures, as well as generalized onset tonic-clonic seizures. One example of the latter condition is Lennox-Gastaut syndrome (Katzung, 2018). When a

patient also experiences generalized tonic-clonic seizures, sodium valproate is frequently chosen over ethosuximide as the medication of choice to treat both types of seizures. Sodium valproate is also an effective treatment for widespread absence seizures. Sodium valproate is an effective treatment for focal seizures; nevertheless, it is possible that its efficacy is not on par with that of carbamazepine or phenytoin. Formulations that are administered intravenously are an option for treating status epilepticus (Clement *et al.*, 2023).

1.3.4.4. Adverse effects

Nausea, vomiting, as well as other gastrointestinal problems such as abdominal discomfort and heartburn are the most prevalent adverse effects of sodium valproate that are related to the dosage. The medication should be begun at a small amount, with subsequent increases to prevent unwanted side effects. Moreover, some people may experience reversible adverse effects including as weight gain, an increase in appetite and loss of hair (Katzung, 2018; Clement *et al.*, 2023).

Rarely, sodium valproate has been known to cause a kind of hepatotoxicity known as idiosyncratic toxicity, which can be severe and even fatal. Patients younger than 2 years old and patients taking numerous drugs are at a greater risk than other patients. (Katzung, 2018).

The usage of sodium valproate throughout the first period during pregnancy was already associated with a higher chance of developing neural tube abnormalities, including spina bifida, in the developing baby. Moreover, a higher prevalence of cardiovascular abnormalities, orofacial abnormalities, and digital anomalies has been observed. Last but not least, reports of cognitive damage in kids have emerged. These findings must be given careful thought to in order to select the appropriate medications for use in women who are or could become pregnant (Bruna *et al.*, 2021).

Even while it is well established that autism-like symptoms can be brought on by prenatal exposure to VPA, the exact processes by which this takes place are not yet completely understood. On the other hand, this effect might be linked to increased concentrations of GABA, neural tube abnormalities and decreased levels of folic acid (Nicolini, Fahnestock, 2018). In light of this, the use of VPA for the induction of autism in animal models demonstrates relevance for research into this disorder due to the fact that it contributes to a better understanding of the disorder's pathophysiological mechanisms and also provides a foundation for the development of potential treatments (Bruna *et al.,* 2021).

1.3.5. Behavioral tests

1.3.5.1. Social interaction test

The social interaction element of social performance is a measurement that is commonly used to evaluate the social disability criterion related to autism spectrum disorder (ASD) (Do Gyeong *et al.*, 2019).

Through direct contact with a stimulus mouse, this test is possible to determine the naturalistic social behavior phenotypes of mice that are being tested as subjects. During social interaction tests, mice inspect each other mostly by smelling their anogenital region, their head, or the remainder of their body, as nose-to-nose touch or oral-to-oral contact. (Do Gyeong *et al.,* 2019).

In most cases, the study will call for the manual monitoring of these behaviors, in addition to requiring a certain level of expertise and clarity in order to assess and quantify the many types of social interaction. Because it is free to walk around in the arena, the stimulus mouse has the ability to either initiate or prevent contacts with the stranger mice. This is a potential confounding variable for direct social interaction. There is also the possibility of aggressive behaviors being displayed by the subject mouse towards the stimulus mouse, or vice versa. This is another variable (Do Gyeong *et al.*, 2019).

1.3.5.2. Open field test

The Open Field test is one of the tools that is used the most frequently to measure anxiety and behaviors that are similar to locomotor-like activity in animal models. It is a quick and simple procedure that offers a variety of information regarding the subject animal's behavior, ranging from data regarding the animal's general ambulatory capacity to information regarding the animal's emotional state. In the context of the research of rodent models, the process makes it possible to investigate distinct types of mice or rats that have been developed in a laboratory or that have been captured in the wild. Also, the method lends itself very well to the research of various pharmacological substances for the purpose of determining whether or not they have anxiolytic or anxiogenic actions (Michael Seibenhener and Michael Wooten, 2015).

A walled enclosure in the open field serves as the component of an open field device. The wall's height must be sufficient to prevent the animal from eluding capture. The typical shape of a labyrinth is either circular or square, and it has a central region that is big enough, relative to the size of the animal being tested, to give them the impression that they are in an open space (Michael Seibenhener and Michael Wooten, 2015).

In the open field device, a variety of different factors can be scored, with the vast majority of the characteristics involving different kinds of motor activity. Although ambulation is the behavior that is most frequently investigated, other behaviors like latency or rearing may be evaluated (Michael Seibenhener and Michael Wooten, 2015).

CHAPTER TWO

Materials and Methods

2.1. Materials

2.1.1. Instruments and Equipment

Table 2.1 provides a listing of the devices and equipment, along with their respective vendors, that were used for the study:

NO.	Instruments / Equipment	Company	Country
1	Centrifuge	Hettich	Germany
2	ELISA reader	Biotek	USA
3	Incubator	Memmert	Germany
4	Light microscope	Huma Scope	Germany
5	Refrigerator	Concord	Lebanon
6	Sensitive balance	Camry	China
7	Tissue homogenizer	Thomas scientific	USA
8	UV/VIS spectrophotometer	Biotek	USA
9	Eppendorf tube	Sigma	England
10	Micropipettes	Dragon Lab	China
11	Microscope Slide	Canfort	China
12	Oral gavage 16 G	Instech laboratories	USA

2.1.2. Kits

Table 2.2 listings of kits that were utilized in the study, together with the companies that made them and the locations where they were manufactured:

NO.	Kit	Company	Country
1	IL-6	Elabscience	USA
2	TNF-α	Elabscience	USA

2.1.3. Chemical materials

Table 2.3 lists the chemical compounds that were used in this work, together with the companies that made them and the countries where they were produced:

NO.	Chemicals/ Drugs	Company	Country
1	Distilled water	Pioneer	Iraq
2	DTNB (5,5-dithio-bis-(2-nitrobenzoic acid)	Thermo-Fisher Scientific	USA
3	Hydrochloric acid	HCS Scientific and Chemicals	Singapore
4	Normal saline	Pioneer	Iraq
5	Risperidone 1 mg	Janssen	Belgium
6	Sitagliptin 100 mg	MSD	USA
7	Sodium hydroxide	FC-BIOS	Malaysia
8	Sodium phosphate buffer	MP Biomedicals	California
9	Sodium valproate	Sanofi	France
10	Thiobarbituric acid	Isolab	Germany
11	Trichloroacetic acid 99%	Sigma-Aldrich	USA

2.2. Methods

2.2.1. Experimental animals

This study was carried out on 60 healthy adult albino mice (40 female and 20 male) weighting (26-39 g) obtained from Ministry of Science and Technology (Baghdad, Iraq). They were housed in the College of Medicine-Babylon University Animal House in standard plastic cages under good conditions at room temperature 24±5 °C with a humidity levels of 65±5% and a light/dark cycle of twelve hours, as well as having unrestricted access to food pellets and water.

2.2.2. Mating and vaginal smear

The adult mice were split into twenty different mating groups (each group had 1 male and 2 females). Examine the female for the presence of a vaginal plug to confirm that mating has taken place. The clog is composed of coagulated secretions that are produced by the male's vesicular and coagulating glands. It is common for it to fill the vagina of the female, and it can last anywhere from 8 to 24 hours after breeding. Raise the female by the end of her tail, look inside her vaginal hole for a whitish mass, and then lower her back down. This will reveal the plug.

To obtain a vaginal swab for the purpose of determining when day zero of pregnancy occurred, with the use of a cotton-tipped swab, stretch the lips of the vaginal opening ever-so-slightly.. This will allow to collect the sample. After performing a vaginal smear, the vaginal specimen was placed on a new glass microscope slide by rubbing a swab across the surface of the slide. After allowing the slide to dry naturally, the material was examined under a microscope in order to identify sperm (Figure 2.1), which indicates that day zero has passed for the mouse that provided the sample.

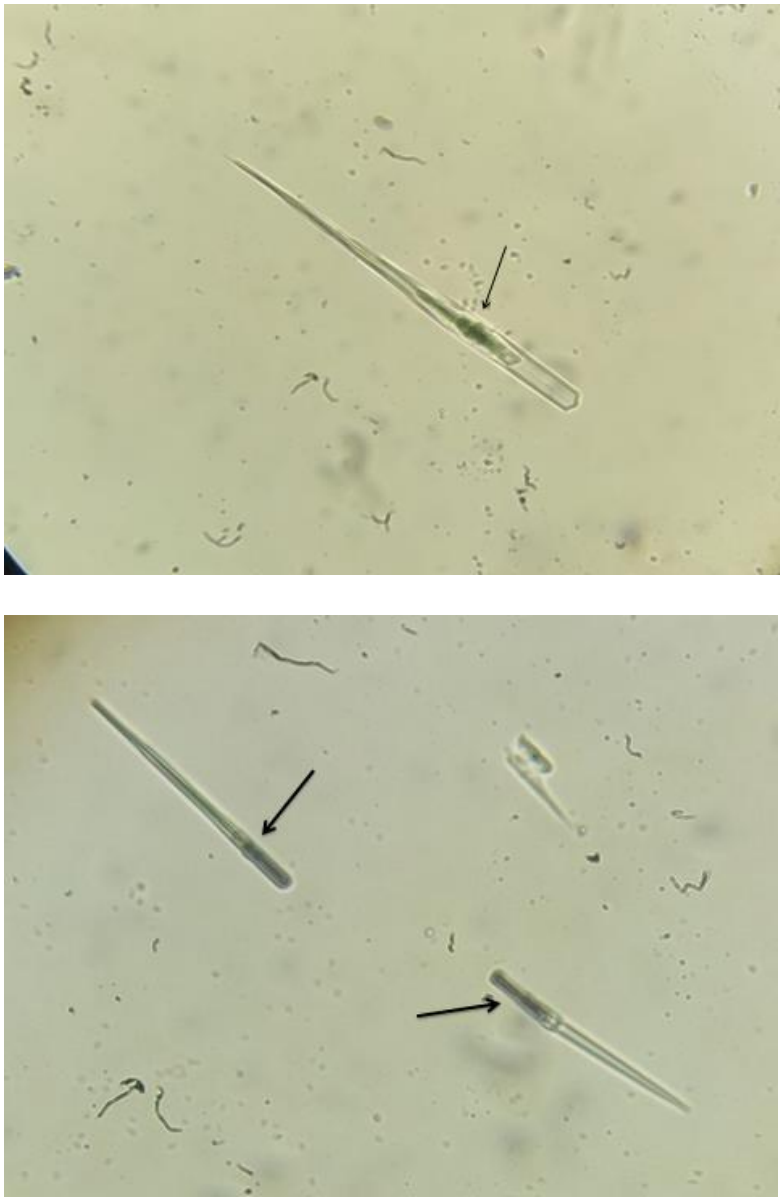

Figure 2.1. Show identification of mouse sperm by light microscope (x 100)

2.2.3. Preparation of animals and induction of autism

At the 12th day, the pregnant female animals were isolated into two groups. Control groups, which consisted of ten subgroups each one had two pregnant mice, were given only water for injection intraperitoneally (i.p.). Experimental groups, also consisted of ten subgroups each one had two pregnant mice, were given a single intraperitoneal injection of sodium valproate at a dose of 600 mg/kg to develop the experimental model of autism (Bruna *et al.*, 2021).

2.2.4. Preparation of drugs

Sodium valproate salt was used as a tablet dosage form from Sanofi Company. A tablet was crushed into powder using a mortar and pestle. The powder was subsequently mixed in water for injection at strength of 200 mg/2 ml, and a single injection of 600 mg/kg body weight was given intraperitoneally. (Bruna *et al.*, 2021).

Sitagliptin was used as a tablet dosage form from MSD Company. Oral administration of a dosage of 10 mg/kg (Suzan *et al.*, 2022) and 15 mg/kg body weight for twenty days was performed using a tablet that had been crushed into powder using a mortar and pestle. The resulting powder was then diluted in distilled water at a concentration of 100 mg/50 ml.

Risperidone was used as a tablet dosage form from Janssen Company. A tablet was crushed into powder using a mortar and pestle, and the resulting powder was dissolved in water at a concentration of 1 mg/10 ml. The powder was then delivered orally at a dosage of one mg/kg body weight for duration of twenty days (Esraa *et al.*, 2022).

2.2.5. Experimental design

The offspring mice were separated from the mothers on the 40th postnatal day and divided into 8 groups, each of them consisted of 10 animals:

Group 1 (control-saline group): received normal saline orally for 20 days.

Group 2 (control-sitagliptin group): received sitagliptin 10 mg/kg orally for 20 days (Suzan *et al.*, 2022).

Group 3 (control-sitagliptin group): received sitagliptin 15 mg/kg orally for 20 days.

Group 4 (control-risperidone group): received risperidone 1 mg/kg orally for 20 days (Esraa *et al.*, 2022).

Group 5 (sodium valproate [VPA]-saline group): received normal saline orally for 20 days.

Group 6 (VPA-sitagliptin group): received sitagliptin 10 mg/kg orally for 20 days (Suzan *et al.*, 2022).

Group 7 (VPA-sitagliptin group): received sitagliptin 15 mg/kg orally for 20 days.

Group 8 (VPA-risperidone group): received risperidone 1 mg/kg orally for 20 days (Esraa *et al.*, 2022).

Behavioral tests (social interaction and open field test) were performed after one day rest of the last dose of therapy as illustrated in figure (2.2). VPA: sodium valproate, SITA: sitagliptin, RIS: risperidone, IL-6: interleukin-6, TNF-α: tumor necrosis factor alpha, GSH: reduced glutathione, MDA: malondialdehyde.

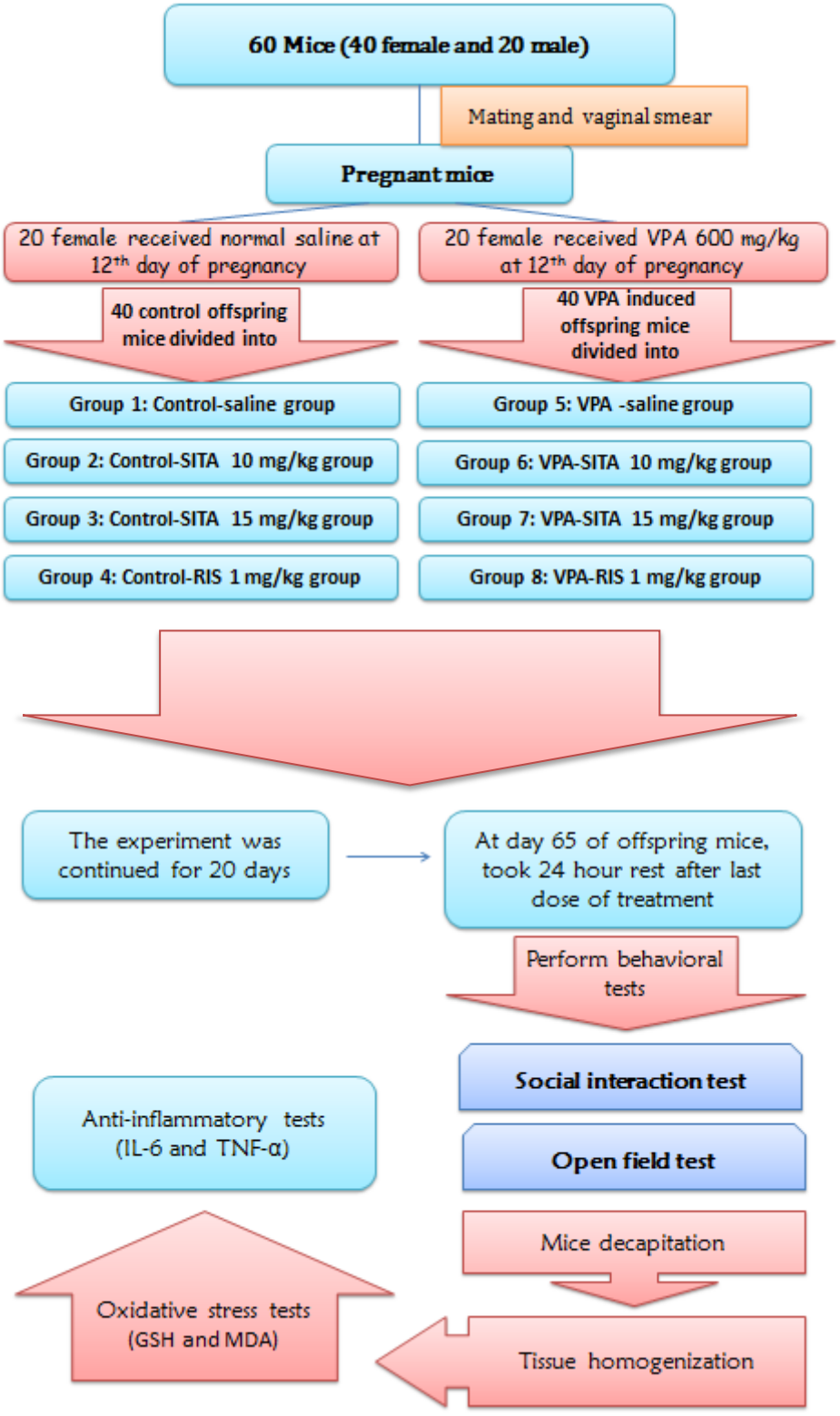

Figure 2.2. Displays a diagrammatic representation of the various groups of mice used in this research.

2.2.6. Behavioral tests

2.2.6.1. Social interaction test

The test was performed in three attached compartments in a rectangular shape (20 x 30 x 30 cm in each chamber). Access to each chamber was allowed by one of two holes measuring 10 x 7 cm. Each phase began in the central chamber, which served as the starting point.

The social interaction test lasts for 15 minutes and consists of three phases: habituation, familiarization and testing.

During phase I (habituation, 5 minutes), the experimental mouse was put in the midst of the compartment in the middle, and the doors to the other two compartments were locked. The mouse was then free to explore the environment.

During phase II (familiarization, 5 minutes), the experimental mouse was located in the middle compartment, the other two chambers were left open, and there was no other mouse introduced into the experiment. After that, the mouse used for testing was given the freedom to investigate all three compartments, and the researchers kept a tally of the number of times it moved from one area to another. These statistics provide a collection of information that can be used to shed light on the sociability of mice, as demonstrated in the figure below (Figure 2.3).

During phase III (testing, 5 minutes), the midsection of each side chamber was outfitted with a wire cage measuring 10 centimeters in diameter. The testing phase was initiated by placing the unfamiliar mouse in the wire cage located in the right compartment of the testing area, while the wire cage located in the left testing area was left empty. After being located in the central chamber, the experimental mouse was given free reign to investigate the other three compartments. The experiment was designed to induce social behavior through a comparison of the total length of time spent by a testing mouse and an untrained mouse (figure 2.4) versus the time period

the testing mouse spent alone in a wire cage chamber. The actions of everyone were captured on camera (Redmi Xiami, China).

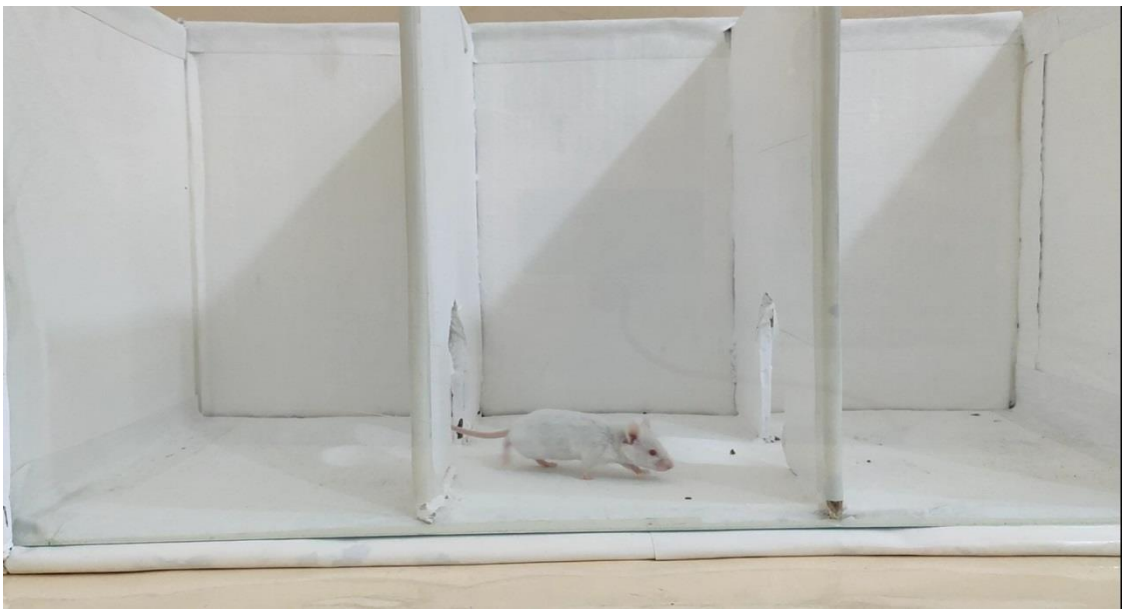

Figure 2.3. Show the movement of the mouse from chamber to chamber in social interaction test.

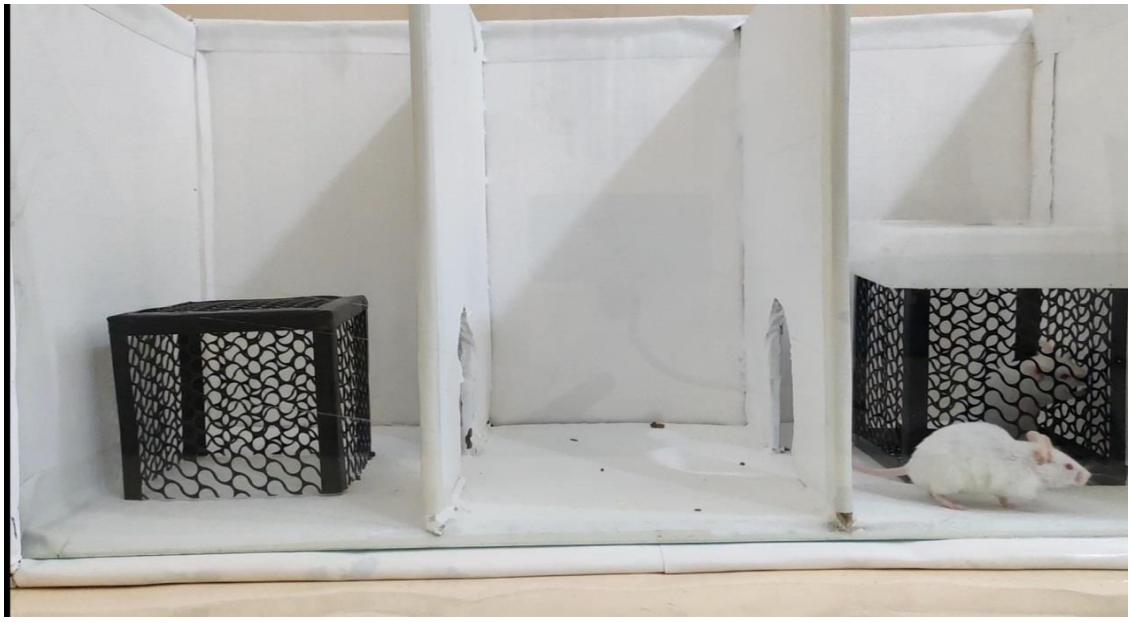

Figure 2.4. Show test mouse spend time with stranger mouse in social interaction test.

2.2.6.2. Open field test

The open field test method was made by utilizing colored wood as the material following dimensions (100 x 100 x 40 cm), and it consisted of a square ground separated into 100 cubes by thin white lines. The test was carried out in an open field (figure 2.5). After positioning each mouse within the apparatus's focal point for approximately 5 minutes, the resulting activity levels were tallied and analyzed. Following each trail, the device was disinfected with 70% ethanol in order to eliminate any olfactory traces that might have been left behind by the preceding mouse subject. When doing the test on the mouse, ensure that the ethanol has entirely evaporated.

Rearing and line crossings are two methods that can be utilized to determine a mouse's level of hyperactivity in an open field apparatus. The total number of squares that cross the line during the course of the test is referred to as line crossings (figure 2.6), and this number is utilized in order to evaluate the mice's level of locomotor activity. The term "rearing" refers to the total number of times that the mouse raises on its back legs with the intention of investigating the environment throughout the course of the experiment (figure 2.7). It is referred to as the latency time of the central area, and it is utilized as a measure of nervous behavior in mice. The overall amount of time that is spent on the central zone of the open field apparatus. The total amount of time spent on various grooming activities is referred to collectively as "grooming" (figure 2.8). The actions of everyone were captured on camera (Redmi Xiami, China).

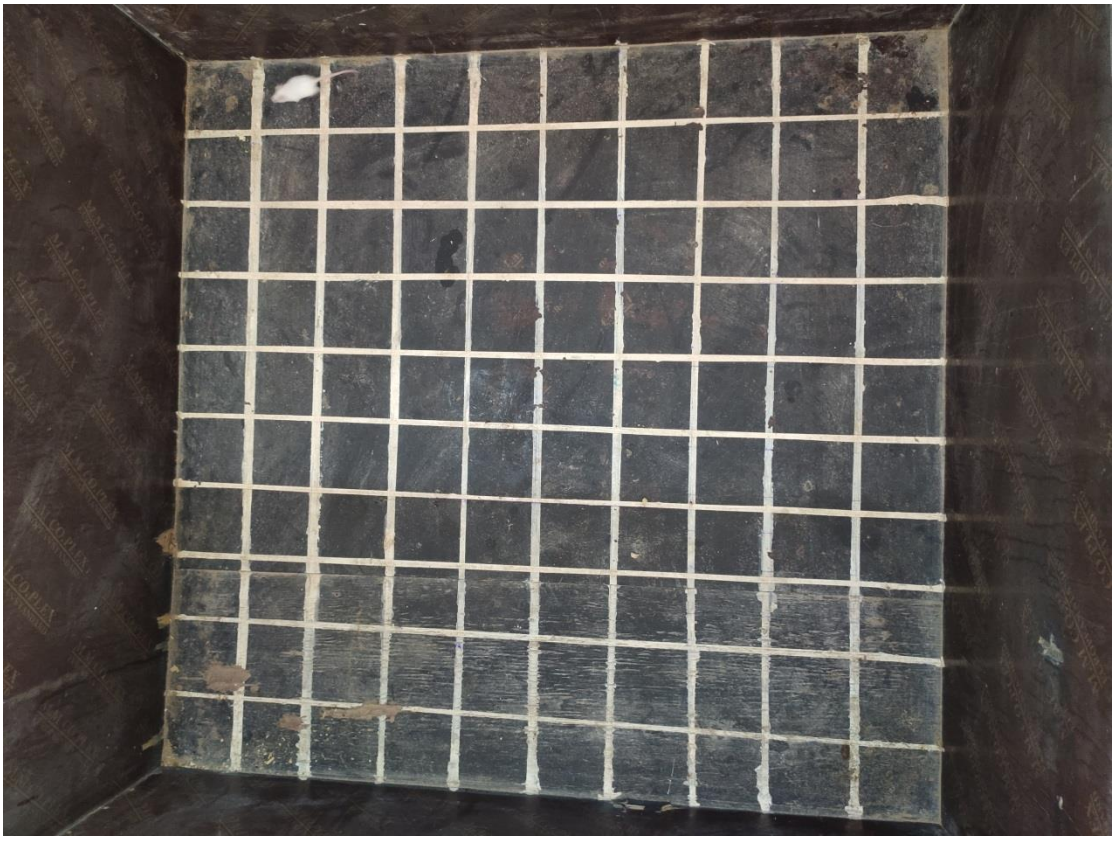

Figure 2.5. Open field test

Figure 2.6. line crossing in open field test

Figure 2.7. Rearing in open field test

Figure 2.8. Grooming in open field test.

2.2.7. Mice decapitation

Following the completion of a series of behavioral tests, the mouse was beheaded 24 hours after receiving its final dosage of therapy as a sort of sacrifice. Surgical shears were used to sever the head of the mouse. The skull was revealed by cutting the skin that was located on top of the head, and then the skin was flipped around the head in order to assist in catching the head. A horizontal incision was made on the surface of the skull with a scalpel. The skull was peeled back with forceps so that the researcher could see their brain (figure 2.9). The brain was extracted by carefully taking it out, beginning with the olfactory bulbs, and then eliminating any associated nerves that could have been holding the brain in position (figure 2.10, A). Only two hemispheres of the brain were removed during the procedure since the olfactory bulbs and cerebellum had to be removed first. In order to bring the temperature of the brain down as quickly as possible, the two hemispheres were rinsed with 10% (w/v) PBS phosphate buffered saline (30 mmol/l, ph. 7.0), and then they were placed in a flat tube that contained saline that was placed on ice. After placing the tube in the refrigerator, it was chilled to -20 degrees Celsius (Figure 2.10, B).

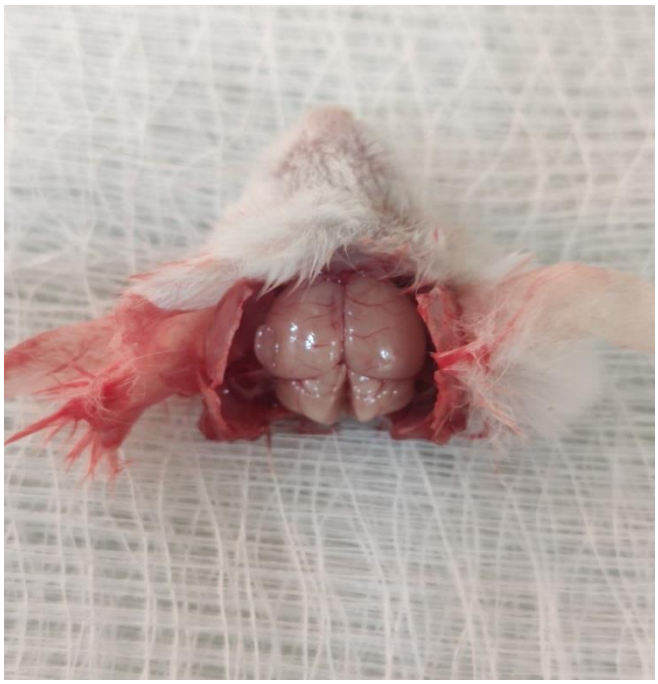

Figure 2.9. Show brain of the mouse after made a cut across the skull.

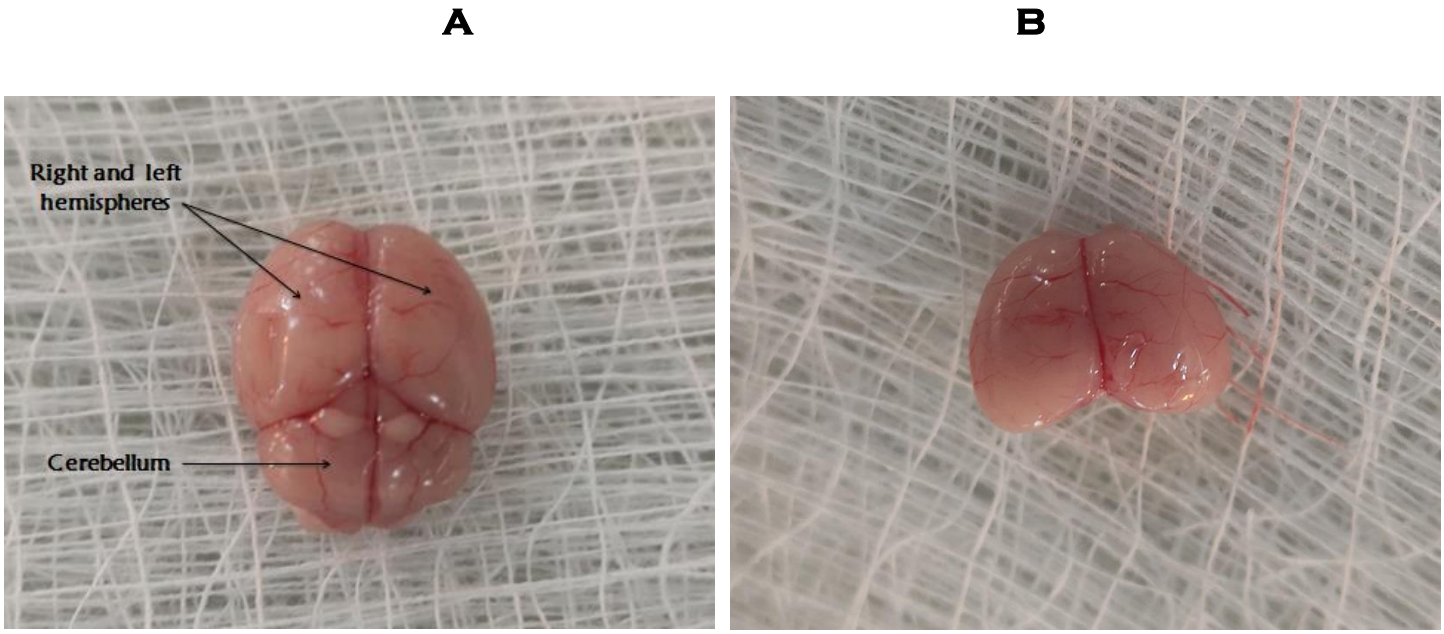

Figure 2.10. A: The entirety of the brain of the mouse; B: The separation of the brain into its two hemispheres

2.2.8. Tissue Homogenization

- Weighed 0.1 gm of the frozen brain and added it to Eppendorf tube contains PBS.
- Brain tissues in Eppendorf tube were homogenized via homogenizer and rotated at 4°C for 30 seconds.
- After homogenization, tubes were placed in centrifuge and rotated at 13000 rpm for 5 minutes at 4°C to separate the supernatants. Carefully collect the supernatant by micropipette.

2.2.9. Oxidative Stress estimation

2.2.9.1 Reduced Glutathione (GSH)

In accordance with the procedure developed by Murat Aliskit, the level of GSH in the tissue was analyzed (Murat *et al.*, 2019).

After mixing 100 µl of the serum with 100 µl of the trichloroacetic acid at a concentration of 25%, the mixture was cooled for a short period of time by being placed on ice. After that, the mixture was centrifuged at 3000 rotation per minute to sepatare the precipitate for a few minutes. A mixture consisting of 300 µl of the supernatant, 700 µl of a 0.2 M sodium phosphate buffer with pH 8, and 2 ml of 0.6 ml DTNB was created (5, 5-dithio-bis 2-nitrobenzoic acid prepared in 0.2 M buffer, pH 8). The yellow color that was developed was measured against a blank that had 0.1 ml of 5% TCA in place of the supernatant after 10 minutes of reaction time at a wavelength of 412 nm. The results were presented in micrograms per liter.

2.2.9.2. Malondialdehyde (MDA)

On a spectrophotometer, the malondialdehyde concentration was determined using the thiobarbituric acid (TBA) assay technique (Cristina Mas-Bargues *et al.*, 2021).

After adding 0.6 ml of TCA-TBA-HCl reagent to 0.4 ml of supernatant and after boiling, the final volume was 0.8 ml. The reagent contained 15% w/v trichloroacetic acid, 0.37% w/v thiobarbituric acid, and 0.25 N HCl. After a thorough mixing, it was placed in a boiling water bath for ten minutes. After the mixture had cooled, 1 ml of a freshly made solution of 1N sodium hydroxide was added. The blank was made by boiling 0.4 milliliters of distilled water and 0.6 milliliters of TCA-TBA-HCl reagent. Using a spectrophotometer, the absorbance of the supernatant with the clear pink color was measured in comparison to the standard blank at 535 nm. The findings were reported in millimoles per liter.

2.2.10. Anti-inflammatory estimation

2.2.10.1 Interleukin-6

Using an enzyme linked immunosorbent assay (ELISA) kit, the IL-6 concentration in the tissue was determined (Aminuddin *et al.,* 2023).

2.2.10.2 Tumor necrosis factor-alpha

An enzyme linked immunosorbent test (ELISA) kit was utilized in order to obtain the tissue TNF- α reading. (Aminuddin *et al.,* 2023).

2.2.10.3 ELISA Procedure

1. The wells for the diluted standard, the blank, and the sample were determined. In the relevant wells, 100 µl of each dilution of the standard, the blank, and the sample was added. After applying the sealant that was included in the kit on the plate, the plate was finished. Incubation of Wells took place for a whole hour and a half at 37 degrees Celsius.

2. The liquid that was in each well was drained without being washed first. Soon after that, we added 100 µl of the working solution for the biotinylated detection Ab to each well. A fresh layer of sealer was applied over the plate. Incubated for a full hour at 37 degrees Celsius.

3. The solution in each well was drained, and 350 µl of wash buffer was added to each well before proceeding. After soaking for one minute, the solution was aspirated or decanted from each well, and the wells were pressed upon clean absorbent paper to dry. This phase of washing was repeated a total of three times. It is possible to utilize a microplate washer for this stage as well as the other wash procedures. After completing the washing procedure, the tested strips were immediately put into use. Wells were not allowed to run dry at any point.

4. In each well, add 100 µl of an HRP (horseradish peroxidase) conjugate working solution. A fresh layer of sealer was applied over the plate. 30 minutes of incubation at 37 degrees Celsius.

5. Drained the liquid from each well and carried out the washing procedure a total of five times in the same manner as described in step 3.

6. Poured 90 µl of substrate reaction mixture into each well. The plate was then coated with a fresh layer of sealant. A period of approximately 15 minutes was spent incubating at 37 degrees Celsius. The plate was shielded from the light. There is a maximum of thirty minutes that can be added on to or subtracted from the reaction time depending on the actual color change. Before measuring the optical density (OD), the Microplate Reader needed approximately 15 minutes of preheating.

7. Pour 50 µl of the stop solution into each well. The addition of the stop solution needs to follow the same sequence as the addition of the substrate solution.

8. Using a micro-plate reader with the 450 nm setting, determined the optical density, also known as the OD value, of each well all at once.

2.3. Statistical analysis

Processing the data, inputting the data, and doing the analysis were all done using the 26th version of the Statistical Package for the Social Sciences (SPSS). A one-way analysis of variance (ANOVA) was used to look at the results of the study, accompanied by post hoc least significant difference testing. Results were displayed in the form of a mean and standard deviation (LSD). All of the aforementioned statistical tests were run with a significance level (*p*-value) set at 5%; a *p*-value of >0.05 indicates that the results are not significant, and a *p*-value of <0.05 indicates that the results are significant. The p-value that was reached should be as low as possible for the results to be considered significant.

.4. Ethical declarations:

All experimental procedures were performed in accordance with approved principles of laboratory animal care and ethical approval of College of Medicine/ University of Babylon/ Iraq. Also, all experimental procedures are performed in adherence for the guidelines for ethical conduct in the care and use of animals in research accordance to American Psychological Association, 2022.

CHAPTER THREE

Results

3. Results

3.1. Examinations of behavior

3.1.1 Social interaction test

3.1.1.1 Number of transition between chambers

Number of transition between chambers was extremely significant raised (p = <0.001) in low dose control-sitagliptin group as compared with other groups. Number of transition was extremely significant reduced (p = <0.001) in VPA-Saline group as compared with other groups.

Number of transition was extremely significant raised (p = <0.001) in VPA-risperidone group as compared with VPA-Saline group. Number of transition was extremely significant (p = 0.001) and significantly (p = 0.017) decreased in high and low dose VPA-sitagliptin groups respectively, as compared with VPA-risperidone group. All of the above details were mentioned in (figure 3.1).

3.1.1.2 Time spent with strange mouse

Time spent with strange mouse was extremely significant elevated (p = <0.001) in normal control-saline group as compared with other groups, and was extremely significant reduced (p = <0.001) in VPA-saline group in comparison with other groups while time spent was extremely significant elevated (p = <0.001) in both low and high dose VPA-sitagliptin and VPA-risperidone groups as compared with VPA-saline group. All of the above details were mentioned in (figure 3.2).

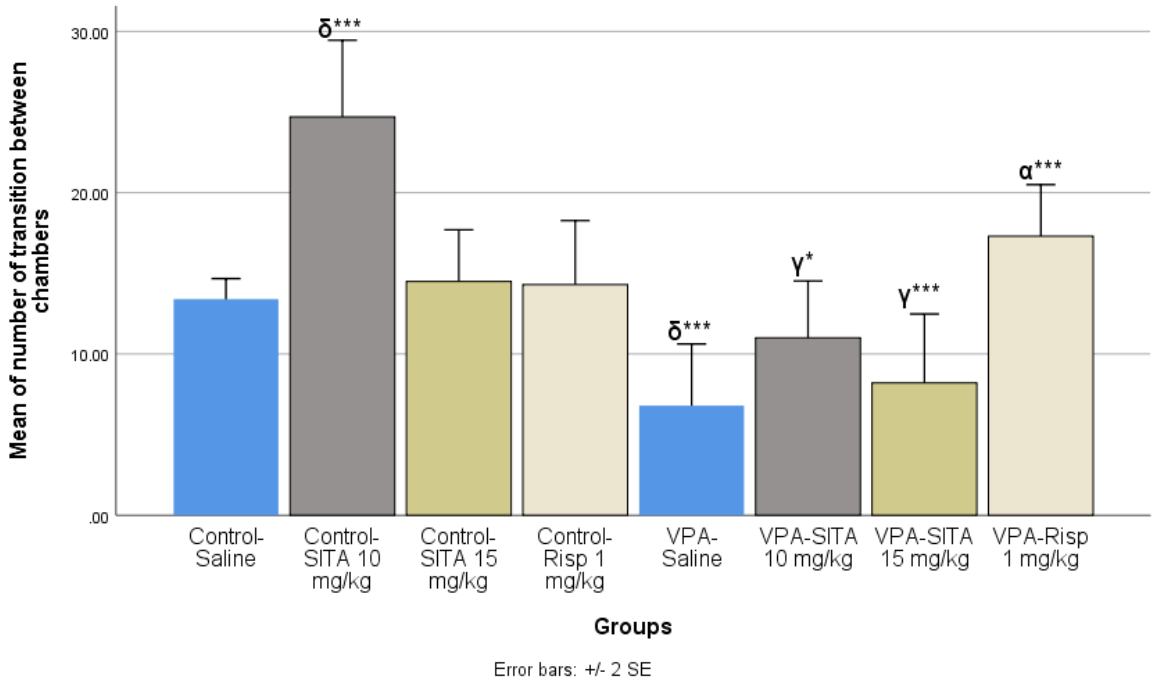

Figure 3.1: Effect of sitagliptin and risperidone on number of transition between chambers in social interaction test. *p = <0.05 (significant); ***p = <0.001 (extremely significant). δ: as compared with other groups. α: as compared to VPA-saline group, γ: in comparison with VPA-risperidone group.

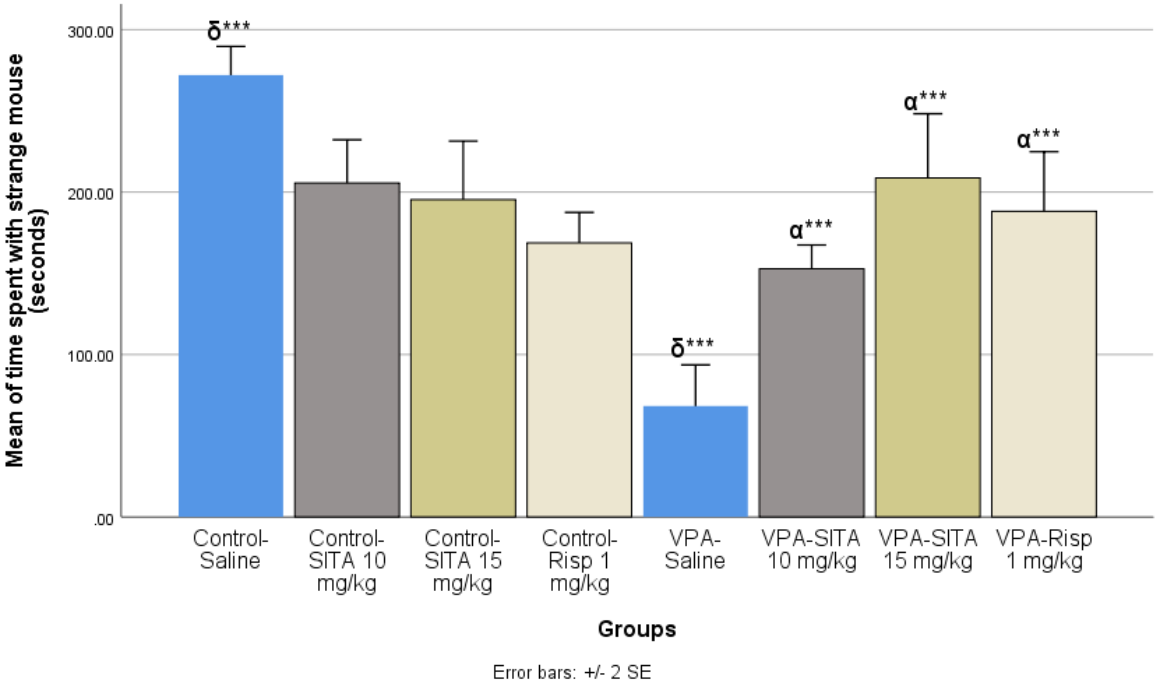

Figure 3.2: Effect of sitagliptin and risperidone on time spent with strange mouse in social interaction test. ***p = <0.001 (extremely significant). δ in comparison with other groups. α: in comparison with VPA-saline group.

3.1.2. Open field assessment

3.1.2.1 Line crossing

Number of line crossing was extremely significant raised (p = <0.001) in VPA-Saline group in comparison with other groups, and extremely significant diminished (p=<0.001) in both low and high dose VPA-sitagliptin and VPA-risperidone groups as compared to VPA-saline group.

Number of line crossing was extremely significant increased (p=<0.001) in high dose control-sitagliptin group and significantly increased (p=0.014) in low dose control-sitagliptin group in comparison with normal control-saline group while number of line crossing was extremely significant decreased (p = 0.001) in high dose VPA-sitagliptin group and highly significant decreased (p=0.009) in low dose VPA-sitagliptin group in comparison with VPA-risperidone group.

All of the above details were mentioned in (figure 3.3).

3.1.2.2 Rearing

Number of rearing was extremely significant elevated (p = <0.001) in VPA-saline group in comparison with other groups, and extremely significant reduced (p=<0.001) in both high and low dose VPA-sitagliptin groups in comparison with VPA-saline group.

Number of rearing was highly significant reduced (p = 0.002) in VPA-risperidone group as compared to VPA-saline group. Number of rearing was significantly declined (p=0.035) in low dose control-sitagliptin group and significantly decreased (p=0.043) in control-risperidone group (p = 0.006) in comparison with control-saline group.

Number of rearing was significantly reduced (p =0.018) in low dose VPA-sitagliptin group and extremely significant decreased (p = <0.001) in high dose VPA-

sitagliptin group as compared to VPA-risperidone group. All of the above details were mentioned in (figure 3.4).

3.1.2.3 Grooming

Number of grooming was extremely significant raised (p = <0.001) in VPA-saline group in comparison with other groups, and extremely significant declined (p= <0.001) in both low and high dose VPA-sitagliptin and VPA-risperidone groups as contrasted to VPA-Saline group.

Number of grooming was significantly decreased (p = 0.021) in high dose VPA-sitagliptin group as compared to VPA-risperidone group. All of the above details were mentioned in (figure 3.5).

3.1.2.4 Latency time on central zone

Latency time on central zone was extremely significant raised (p= <0.001) in high dose control-sitagliptin group as compared with other groups. Latency time was extremely significant reduced (p = <0.001) in VPA-saline group as opposed with other groups.

Latency time on central zone was extremely significant elevated (p = <0.001) in VPA-risperidone and both high and low dose VPA-sitagliptin groups as compared with VPA-Saline group. All of the above details were mentioned in (figure 3.6).

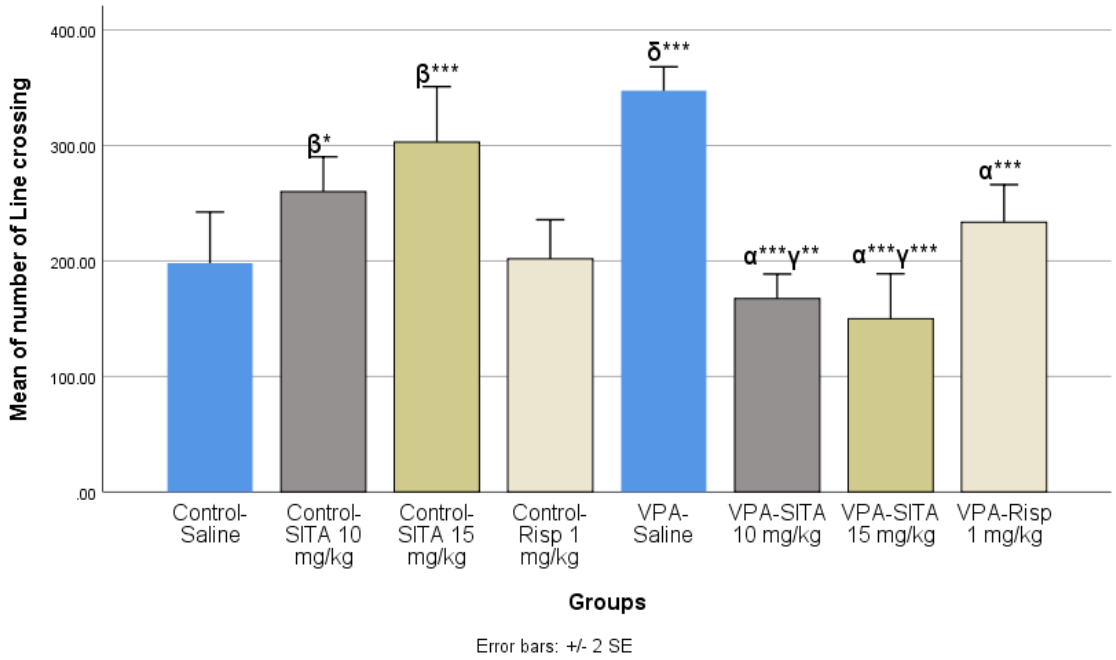

Figure 3.3: Effect of sitagliptin and risperidone on number of line crossing in open field test. *p = <0.05 (significant); **p = <0.01 (highly significant); ***p = <0.001 (extremely significant). δ: as opposed to other groups. α: in comparison with VPA-saline group, β: as opposed to control-saline group, γ: in comparison with VPA-risperidone group.

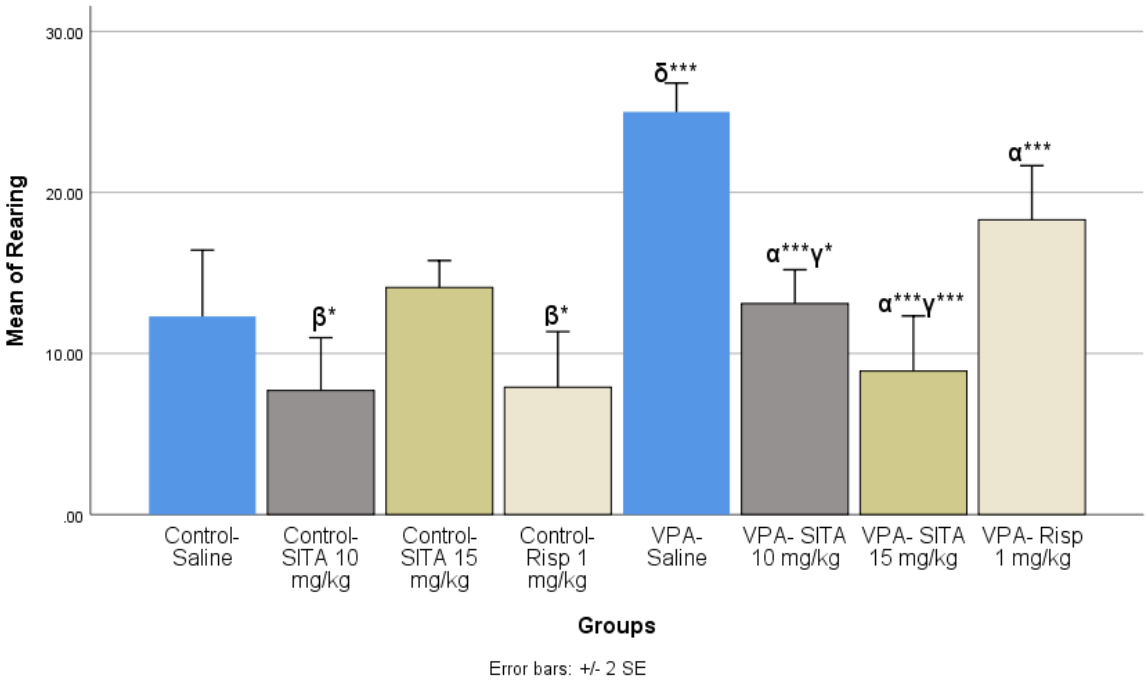

Figure 3.4: Effect of sitagliptin and risperidone on number of rearing in open field test. *p = <0.05 (significant); ***p = <0.001 (extremely significant). δ: as opposed to other groups. α: as compared to VPA-saline group, β: as opposed to control-saline group, γ: in comparison with VPA-risperidone group.

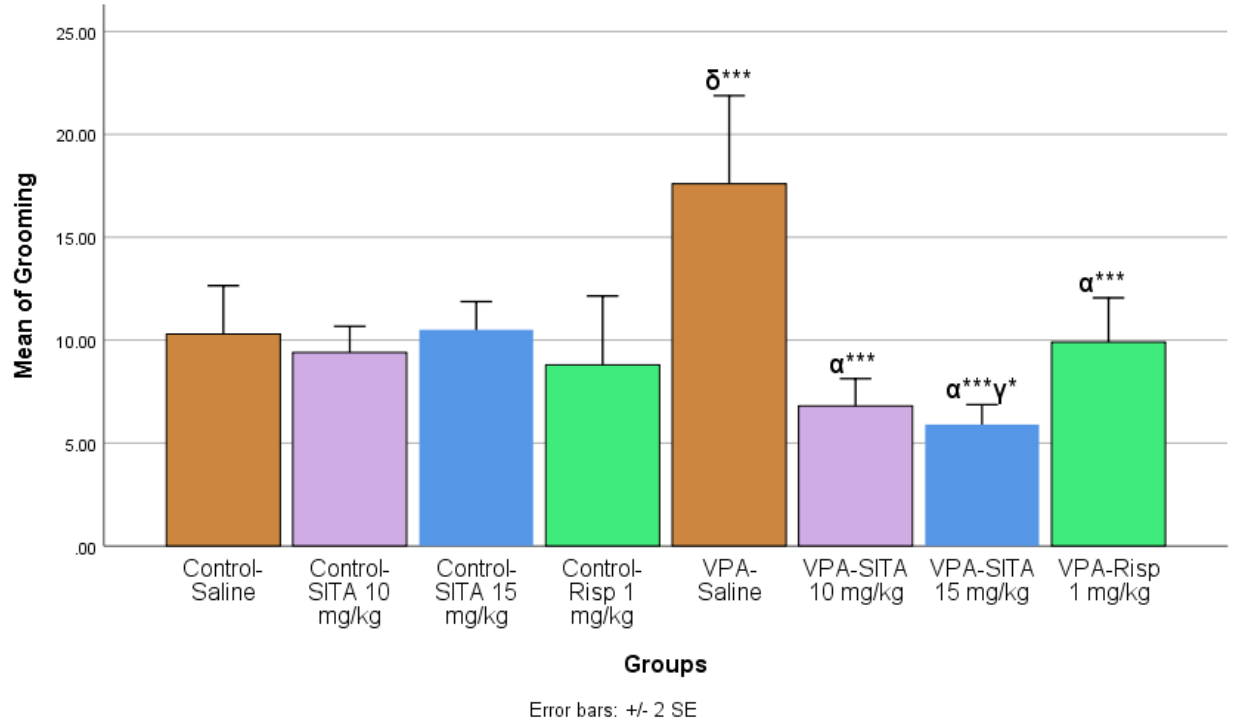

Figure 3.5: Effect of sitagliptin and risperidone on number of grooming in open field test. *p = <0.05 (significant); ***p = <0.001 (extremely significant). δ: as opposed to other groups. α: as compared to VPA-saline group, γ: as compared to VPA-risperidone group.

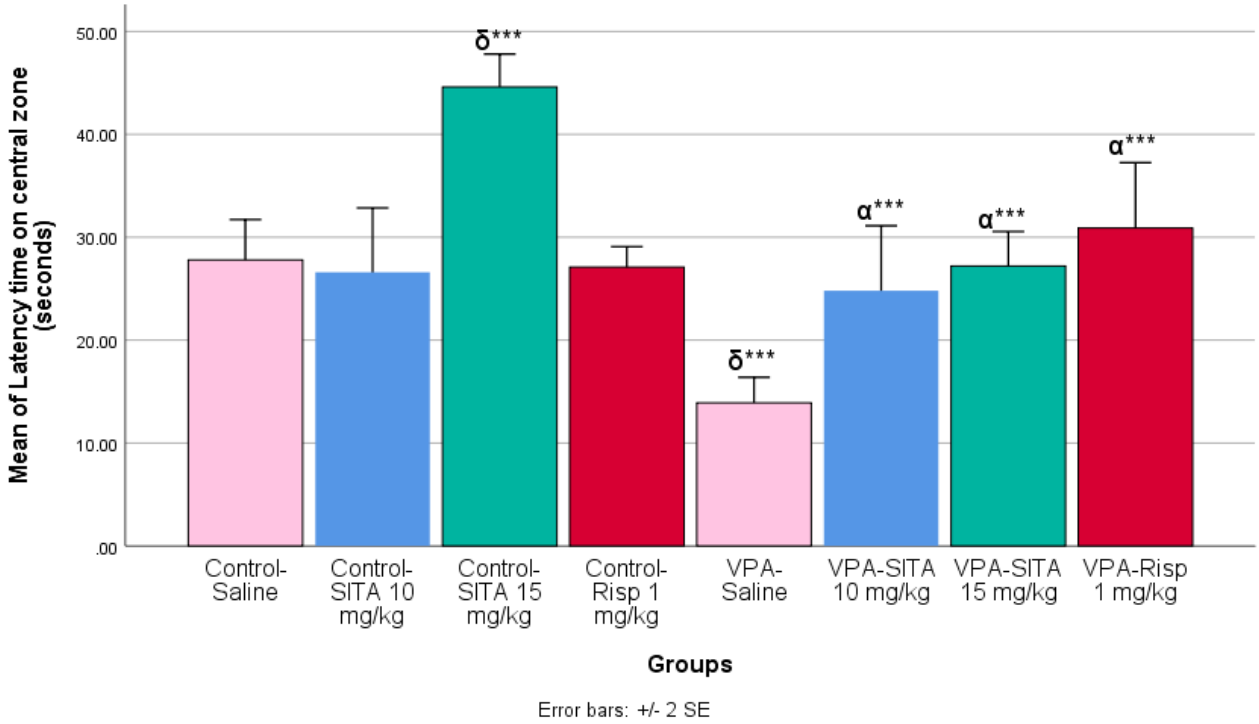

Figure 3.6: Effect of sitagliptin and risperidone on latency time on central zone in open field test. ***p = <0.001 (extremely significant). δ: as opposed to other groups. α: as compared to VPA-saline group.

3.2 Oxidative stress tests

3.2.1 Reduced glutathione

The concentration of GSH was extremely significant reduced (p = <0.001) in VPA-saline group as opposed with other groups while the level of reduced glutathione was extremely significant raised (p = <0.001) in both high and low dose VPA-sitagliptin and VPA-risperidone groups as compared with VPA-saline group.

The level of reduced glutathione was extremely significant increased (p=<0.001) in low dose control-sitagliptin group as compared with control-risperidone group. The level of reduced glutathione was extremely significant raised (p = <0.001) in high dose VPA-sitagliptin group as compared with VPA-risperidone group. All of the above details were mentioned in (figure 3.7).

3.2.2 Malondialdehyde

The level of malondialdehyde was extremely significant elevated (p = <0.001) in VPA-saline group as opposed to other groups. The level of MDA was extremely significant diminished (p=<0.001) in both low and high dose VPA-sitagliptin and VPA-risperidone groups in comparison with VPA-saline group.

The concentration of MDA was significantly decreased (p=0.05) in high dose control-sitagliptin group as opposed to control-saline group. The cocentration of MDA was extremely significant decreased (p=<0.001) in low dose VPA-sitagliptin group as compared to VPA-risperidone group. All of the above details were mentioned in (figure 3.8).

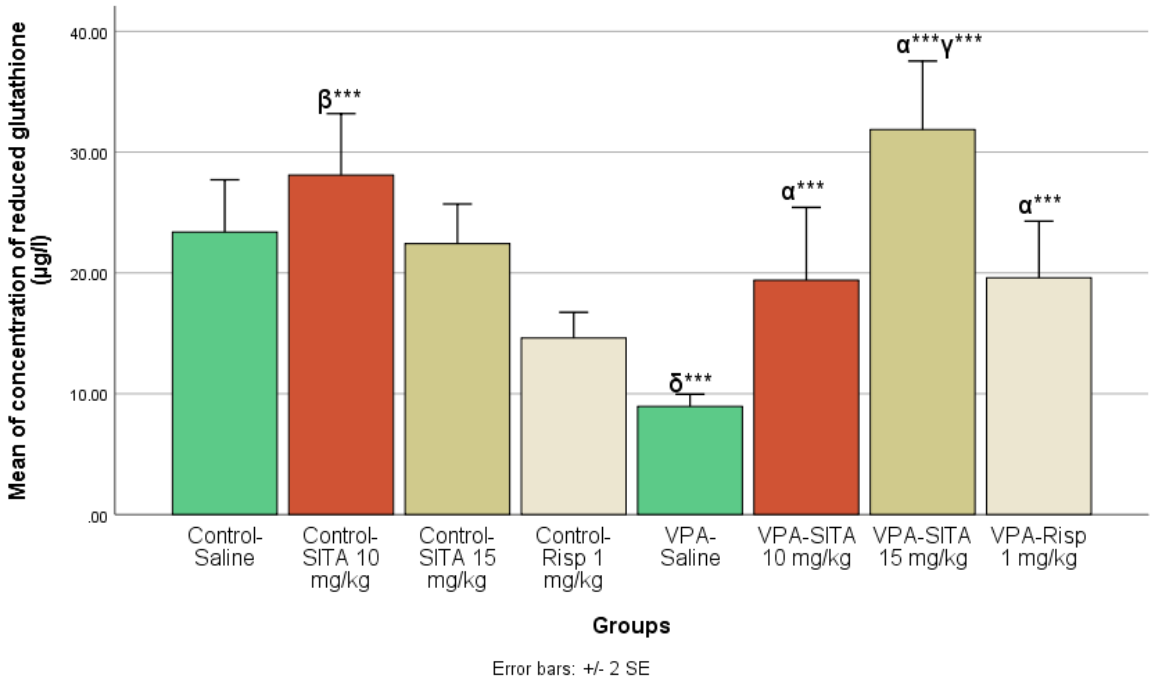

Figure 3.7: Effect of sitagliptin and risperidone on concentration of reduced glutathione. ****p*=<0.001 (extremely significant). δ: as compared to other groups. α: in comparison with VPA-saline group, β: as opposed to control-risperidone group, γ: in comparison with VPA-risperidone group.

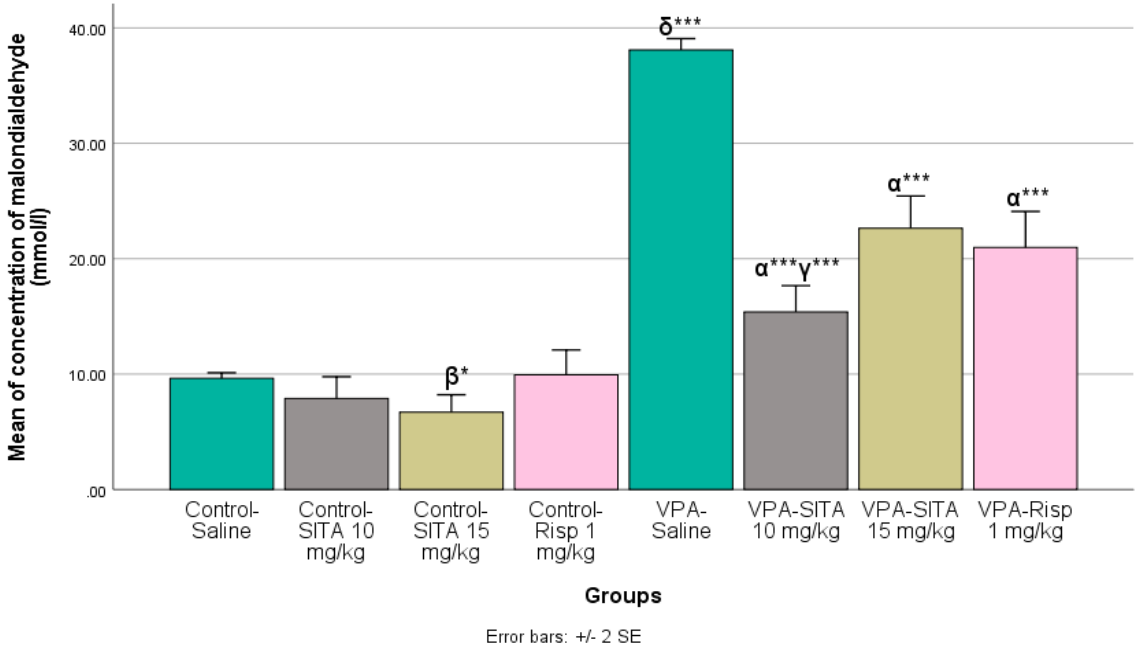

Figure 3.8: Effect of sitagliptin and risperidone on concentration of malondialdehyde. **p* = <0.05 (significant); ****p* = <0.001 (extremely significant). δ: as opposed to other groups. α: in comparison with VPA-saline group, β: as opposed to control-saline group, γ: in comparison with VPA-risperidone group.

3.3 Anti-inflammatory tests

3.3.1 Interleukin-6

The level of IL-6 was extremely significant raised (p=<0.001) in VPA-saline group as opposed with other groups. The level of IL-6 was extremely significant reduced (p=<0.001) in VPA-risperidone groups as opposed to other groups.

The level of IL-6 was extremely significant diminished (p=<0.001) in both low and high dose VPA-sitagliptin and VPA-risperidone groups as opposed to VPA-saline group while the concentration of IL-6 was extremely significant reduced (p = <0.001) in VPA-risperidone group as opposed with both low and high dose VPA-sitagliptin groups. The level of IL-6 was highly significant diminished in both low (p = 0.009) and high (p = 0.002) dose control-sitagliptin as compared with control-saline group. All of the above details were mentioned in (figure 3.9).

3.3.2 Tumor necrosis factor alpha

The level of TNF-α was extremely significant elevated (p = <0.001) in VPA-saline group as opposed to other groups. The level of TNF-α was extremely significant diminished (p = <0.001) in both low and high dose VPA-sitagliptin and VPA-risperidone groups as opposed to VPA-saline group.

The concentration of TNF-α was extremely significant and significantly diminished in high and low doses of control-sitagliptin (p = <0.001) and control-risperidone (p = 0.019) groups, respectively as compared with control-saline group. The concentration of TNF-α in low dose VPA-sitagliptin group was highly significant increased (p=0.005) as compared with high dose VPA-sitagliptin group, and was extremely significant elevated (p = <0.001) as opposed with VPA-risperidone group. All of the above details were mentioned in (figure 3.10).

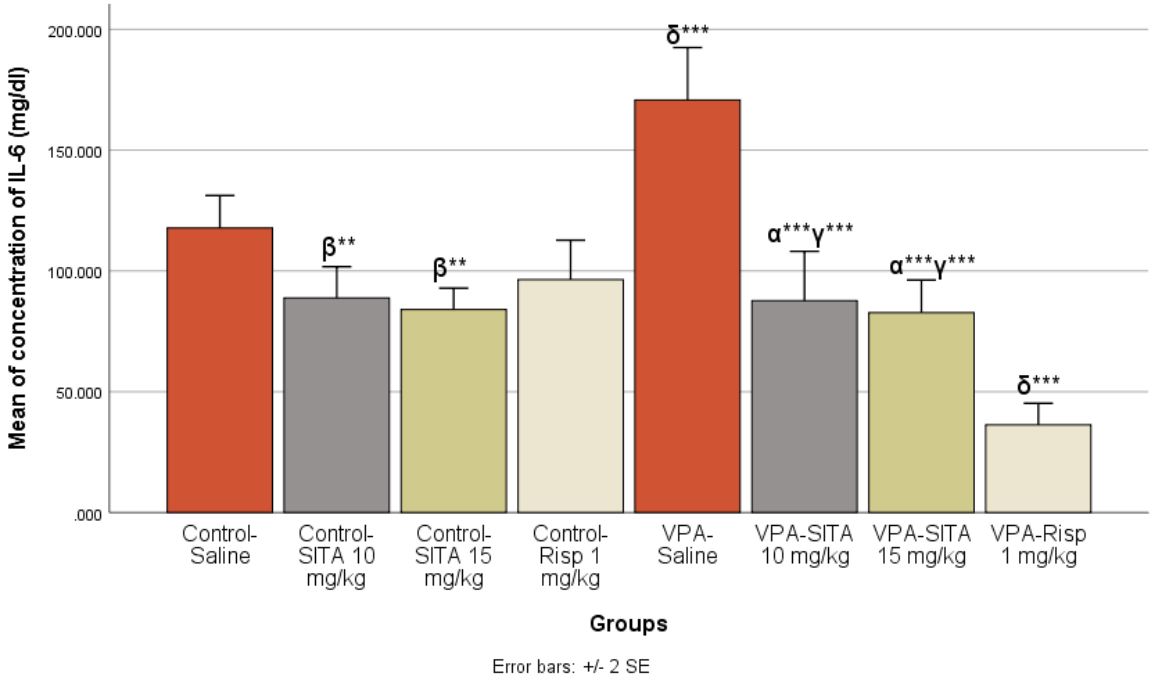

Figure 3.9: Effect of sitagliptin and risperidone on concentration of IL-6. ***p*=<0.01 (highly significant); ****p* = <0.001 (extremely significant). δ: as opposed to other groups. α: in comparison with VPA-saline group, β: as opposed to control-saline group, γ: in comparison with VPA-risperidone group.

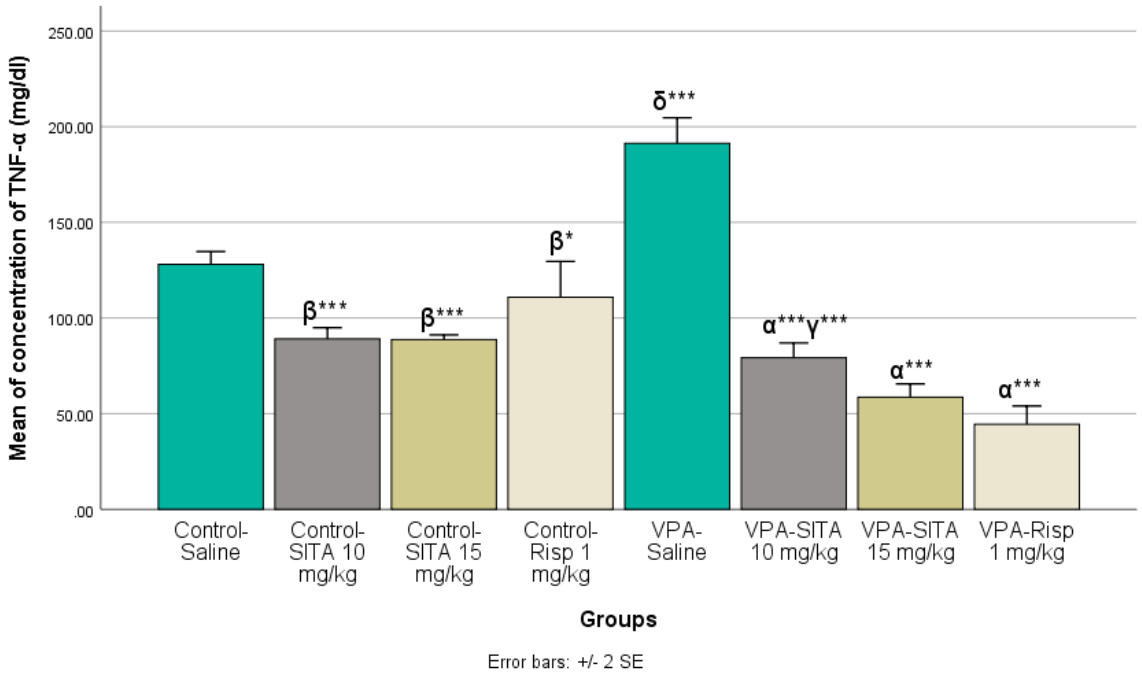

Figure 3.10: Effect of sitagliptin and risperidone on concentration of TNF-α. **p* = <0.05 (significant); ****p* = <0.001 (extremely significant).). δ: as opposed to other groups. α: as opposed to VPA-saline group, β: as opposed to control-saline group, γ: as compared to VPA-risperidone group.

CHAPTER FOUR

Discussion

4. Discussion

The present investigation is the first one of its kind whether or not sitagliptin has an effect on ASD. By injecting sodium valproate into the peritoneal cavity of pregnant mice on gestational day 12, the study hopes to be able to produce an animal model of autism. Mice that were given sodium valproate during their prenatal development exhibited the following behavioral changes: (i) decreased sociability and social creativity; (ii) elevated locomotor activity, including higher anxiety scores and reduced explorative action; (iii) increased oxidative stress; and (iv) increased release of inflammatory cytokines. In contrast, therapy with sitagliptin and risperidone enhanced the behavioral problems in the offspring, as well as reduced the oxidative stress and neuroinflammation.

Inbred mice with a consistent genotype are known to exhibit an albino characteristic. Albino mice not only consistently display ASD-like behaviors such as social communication impairment and repeatable stereotyped nature, in addition to this; however, they have aberrant cognitive development as well as altered immunological biochemical signs that are comparable to those that have been reported for patients with autism spectrum disorder (ASD). In comparison to rats, albino mice are therefore the superior choice for use as an animal model in research pertaining to autism (Hongmei Wu *et al.,* 2022).

4.1. Evaluation of sitagliptin and risperidone's impact on mice' capacity for social connection

One of the most prominent signs of ASD is a deficit in social interaction (Bruna *et al.,* 2021). The findings of this study revealed that the offspring of mice who had been exposed to VPA had the least positive social connections (number of transitions between chambers, time spent with stranger mice). It's possible that the mice's lack of social contact is due to worry, fear, or a decreased grasp of the communicative signals coming from the new animals. The therapy with sitagliptin and risperidone, on the

other hand, improved the impaired social interaction in the mice that had been prenatally exposed to VPA. These results are in line with those found in earlier research of other neurologic disorders (Mathieu *et al.,* 2022; Bruna *et al.,* 2021; Esraa *et al.,* 2022).

The findings of this study showed that sitagliptin at doses of 10 mg/kg and 15 mg/kg in the ASD-mice model were extremely significant raised ($p = <0.001$) as compared with the VPA-saline group in the time it took to interact with stranger mice. However, these doses did not significantly increase the number of transitions between compartments (there was no past research about the effect of sitagliptin on the social communication test); whereas, risperidone at a dose of 1 mg/kg showed extremely big improvements ($p = <0.001$) in both tests, which suggests that antagonizing dopamine receptors, particularly dopamine receptor D_2, and serotonin (5-hydroxytryptamine; 5-HT) receptors, particularly the 5-HT_{2A} receptor, which are the primary targets of atypical antipsychotics, plays a significant role in inhibiting the excitation and agitated behaviors. The results of this study, which are in agreement with the results of other studies, showed that inhibition of these receptors is necessary for the suppression of VPA-induced hyperlocomotion. (Tendilla-Beltran *et al.,* 2019; Chen *et al.,* 2022; Esraa *et al.,* 2022).

Comparing the low dose 10 mg/kg control-sitagliptin group with the control-saline group as well as the other groups resulted in an exceptionally substantial increase ($p = <0.001$) in the number of transitions between chambers. According to our knowledge, it was believed that this was because of the effect that sitagliptin had on neurotransmitters such as serotonin and GABA (gamma-aminobutyric acid), but there had been no studies done in the past regarding this effect.

Through the Blood Brain Barrier, insulin can attach to insulin receptor, mediating insulin signaling pathways to control energy metabolism and protect neurons. Impaired insulin signaling pathways could cause damage to the brain, which is suggested to be one of the etiologies of ASD, Glucagon like peptide-1 (GLP-1) binds and activates GLP-1Receptor (GLP-1R), regulating different neuronal functions. Sitagliptin attenuated neuronal apoptosis and preserved neurological function by stimulating GLP-1R, which is involved in activating insulin signaling pathways. GLP-1/GLP-1R signaling axis has recently been identified as a possible therapeutic target in CNS illness. Increased GLP-1R protein was accompanied by down regulation of apoptosis and enhancement of adenosine monophosphate-activated protein kinase (AMPK) activity as well as peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α) protein level in animals when treated with sitagliptin (Wen Han *et al.,* 2020). GLP-1R was shown to be highly expressed in the CNS and within neurons. However, endogenous GLP-1 is rapidly broken down by sitagliptin, shortening GLP-1's half-life. It is generally believed that neuronal apoptosis is a crucial process that is responsible for neurological impairment in mice with ASD. So, it is suggested that sitagliptin acts its roles, such as reducing blood glucose level, improving behavioral activities, anti-inflammatory activity and anti-oxidative stress in a GLP-1/GLP-1R dependent way.

4.2. The influence of sitagliptin and risperidone on the findings of the open field test

The findings of this study revealed an elevated level of anxiety in VPA-saline offspring when they were subjected to an open-field test. When compared with the control group, the offspring that were exposed to VPA had higher mean values for speed, grooming, and rearing. These findings are in agreement with the findings of the previous researches (Mathieu *et al*., 2022; Bruna *et al.,* 2021; Esraa *et al.,* 2022).

When compared with the VPA-risperidone group, the open field test of the VPA-sitagliptin group revealed a significant reduction in the amount of line crossings, rearing, and grooming as well as a significant increase in latency time in the central zone. On the other hand, the open field test of the control-sitagliptin group demonstrated a significant rise in the number of line crossings as well as latency time in the central zone when compared with the control-saline and VPA-risperidone groups. These findings are in agreement with the findings of the previous research, which showed that sitagliptin reduced anxiety in an open field test in other CNS disorders (Esraa *et al.*, 2022; Suzan *et al.*, 2022). Through indirect mechanisms for its antioxidant activity, sitagliptin highly expressed the nuclear factor erythroid 2-related factor 2 (Nrf2) (Hewedy, 2020), which is a transcription factor that regulates the cellular defense versus toxic and oxidative insults by controlling the expression of genes involved in oxidative stress response and drug detoxification. Cells that have been activated by Nrf2 are more resistant to the inflammatory and carcinogenic effects of chemicals. (He, 2020).

GLP-1 appears to be critical for the correct maintenance and growth of dopaminergic neurons in substantia nigra. Stimulation of GLP-1R by DPP-4 inhibitors such as sitagliptin has neuroprotective effects, which leads to improvements in motor and non-motor deficits (Marwa *et al.*, 2021). So that, according to the results of this study, it is suggested that sitagliptin has potent anxiolytic effect on individuals with ASD by decreasing its symptoms (fear, anxiety, nervousness and hyperactivity). In general, these findings point to the possibility that sitagliptin could be a promising new candidate medicine for the management of ASD in the near future.

4.3. The influence that sitagliptin and risperidone have on the oxidative stress

Damage to cells, particularly lipid peroxidation and modification of the fluidity of the cell membrane, is caused by reactive oxygen species, abbreviated as ROS. Hence, oxidative stress was caused by an imbalance between the generation of ROS and its removal from the system. Glutathione fights against lipid peroxidation through the reduction of per-oxidized phospholipids and cholesterols. Therefore, the level of GSH could be depleted during oxidative stress as observed in this study. The findings of this research indicated that the VPA-saline group was subjected to a greater amount of oxidative stress. In the VPA-saline group, there was a greater increase in lipid peroxidation. Also, this group had a decrease in their reduced glutathione levels. These findings indicated an increased degree of oxidative stress in the offspring that had been treated with VPA. These findings were in consistent with those of the earlier researches (Mathieu et al., 2022; Bruna *et al.,* 2021; Esraa *et al.,* 2022).

This is the first study that has been conducted to investigate the effect of sitagliptin on the amounts of glutathione and MDA in mice that have been induced with autism by VPA. In the VPA-sitagliptin and VPA-risperidone groups, the levels of reduced glutathione were higher and the levels of MDA were reduced as compared with VPA-saline group, which indicate that both drugs had potent antioxidant effects.

It was evidenced by an elevated level of glutathione ($p = <0.001$) in the 15 mg/kg VPA-sitagliptin group when compared with the VPA-risperidone group. This revealed that the effect of sitagliptin on the level of reduced glutathione developed in accordance to the dose of sitagliptin, as the dose increased as GSH levels increased. It was discovered that sitagliptin possessed powerful antioxidant and detoxifying properties as compared with rispridone according to the results of this study on GSH levels. This was consistent with the prior study's findings (Suzan *et al.,* 2022). The

nuclear factor eryhtoid 2-related factor 2 (Nrf2) expressions was elevated as a result of indirect pathways for the antioxidant action of sitagliptin (Hewedy, 2020).

It was found that both the VPA-sitagliptin and VPA-risperidone groups had considerably lower levels of MDA, which is an indicator of lipid peroxidation. Sitagliptin at the dose of 10 mg/kg demonstrated better antioxidant action on lipid peroxidation than VPA-risperidone group did. This is evidenced by the fact that the level of MDA was significantly reduced in the group that received 10 mg/kg sitagliptin in comparison to the group that received VPA-risperidone. The findings of this study were in agreement with those of the earlier research (Suzan *et al.*, 2022; Esraa *et al.*, 2022).

4.4. Anti-inflammatory activity of sitagliptin and risperidone as measured by IL-6 and TNF-α tests

Neuropathology is described by the generation of inflammatory cytokines, such as IL-6 and TNF-α, among other inflammatory cytokines. When the values of the VPA-saline group were compared to the values of the control group, the results showed that there was a significant rise in the TNF-α and IL-6 levels in the VPA-saline group's supernatants. This is consistent with the findings of the earlier research (Suzan *et al.*, 2022; Ahmed *et al.*, 2022).

By the activation of a cascade of events involving chemokines, cytokines, and growth factors, TNF-α is able to cause an inflammatory response. Inflammatory reactions can be caused by IL-6 acting as a trigger in the nuclear factor kappa B (NF-κB) signal transduction pathway, which stimulates the transcription and releasing of subsequent inflammatory mediators. It is possible for pro-inflammatory cytokines to trigger the production of reactive oxygen species (ROS), which in turn may activate the transcription factor NF-κB and result in an inflammatory response. NF-κB is then moved into the nucleus, where it activates a number of genes involved in the inflammatory response (Suzan *et al.*, 2022).

This is the first study that has been conducted to investigate the effect of sitagliptin on the levels of TNF-α and IL-6 in mice that have been induced with autism by VPA. The treatment of mice that had been prenatally exposed to VPA with sitagliptin and risperidone resulted in a reduction in the levels of the inflammatory cytokines TNF-α and IL-6. The levels of TNF-α and IL-6 were reduced significantly in the VPA-risperidone group when compared with the VPA-sitagliptin groups, and these amounts were also significantly decreased in the 15 mg/kg sitagliptin group in comparison with the 10 mg/kg sitagliptin group. These results were statistically significant ($p = <0.001$) in both of the comparison groups. This is in line with the findings of a previous study on dementia (Suzan A. Khodir *et al.,* 2022), which showed that the levels of proinflammatory cytokines were reduced with the use of sitagliptin. Sitagliptin is known to have powerful anti-inflammatory effects at both the nuclear and cytoplasmic levels (Wicinski *et al.,* 2018). It is also suggested that the neuroperotective effect of sitagliptin may be due to its stimulation of GLP-1R.

The effects that sitagliptin had on the innate immune system, such as stimulating macrophages and monocytes and activating T cells, contributed to the drug's powerful anti-inflammatory properties. According to the findings of another study (Hu *et al.,* 2017), sitagliptin has the potential to reduce the activation of NF-κB as well as cytokine expression. The elevation of transcription as well as the releasing of cytokines such as IL-6 and TNF-α result from the activation of the NF-κB pathway.

According to the findings of another study (Pansri *et al.,* 2021), the administration of sitagliptin significantly improved neurogenesis and neuronal plasticity. This improvement was demonstrated by a significant increase in the level of BDNF (Brain Derived Neurotrophic Factor) as well as an upregulation of Ki-67 gene immunoreaction in the hippocampal neurons. In rats with Parkinson's disease, sitagliptin improved intellectual disability via increasing BDNF expression, which led to an improvement in the animals' overall neurologic functions (Li *et al.,* 2018).

Conclusions

- The data from the results shows increased in social activity with stranger mouse after treatment with sitagliptin for 20 days in social interaction test.
- Sitagliptin can manage ASD-like symptoms (hyperactivity, anxiety and fearing), so it has potent anxiolytic activity in open field test.
- According to the results, it could be concluded that sitagliptin had strong antioxidant and free radical scavenger activities as compared with risperidone. At the dose 10mg/kg, sitagliptin had stronger effect on lipid peroxidation (MDA) than risperidone.
- Sitagliptin had potent anti-inflammatory effect by decreasing IL-6 and TNF-α in ASD mice, but this effect is less as compared with risperidone.

Recommendations

- Further studies are needed to study the effect of sitagliptin on level of neurotransmitters such as serotonin and GABA.
- Further studies must be made to show the influence of the combination of sitagliptin with risperidone on ASD mice and determine the type of drug interaction.
- Clinical trials are needed to study the effect of sitagliptin on autism in the patient.

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جمهورية العراق
وزارة التعليم العالي و البحث العلمي
جامعة بابل- كلية الطب
فرع الادوية

تأثير السيتاكليبتين على التوحد المستحث بفالبروات الصوديوم في نموذج الفئران

رسالة

مقدمة الى مجلس كلية الطب/ جامعة بابل كجزء من متطلبات نيل شهادة الماجستير في الادوية و السموم

من قبل

احمد سودان عباس محمد
بكلوريوس صيدلة (2014)

اشراف

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1445 هـ **2023** م

الخلاصة

اضطراب طيف التوحد هو اضطراب في النمو العصبي يوصف بتدهور التفاعل الاجتماعي والتواصل الذي يظهر خلال مرحلة الطفولة المبكرة.

هدفت هذه الرسالة إلى معرفة تأثير عقار السيتاكليبتين على اضطراب طيف التوحد في الفئران بعد استحداث هذا الاضطراب فيها بواسطة فالبروات الصوديوم. كانت الدراسة تهدف إلى تحديد التواصل الاجتماعي للفئران عن طريق اختبار التفاعل الاجتماعي والنشاط الحركي والقلق عن طريق اختبار الميدان المفتوح. أيضًا ، حددت هذه الدراسة النشاط المضاد للأكسدة لـ سيتاكليبتين عن طريق قياس الجلوتاثيون ، مالون دايالدهايد وتحديد النشاط المضاد للالتهابات عن طريق قياس انترلوكين-6 و عامل نخر الورم الفا (TNF-α).

استمرت فترة الدراسة من 2022/9/21 إلى 2023/2/5. أجريت هذه الدراسة على 60 فأرًا بالغًا سليمًا (20 ذكرًا و40 أنثى) تزن (26-39 غرام) ، مقسمة إلى 20 مجموعة تزاوج (كل مجموعة بها 1 ذكر و 2 أنثى). بعد تحديد اليوم صفر من خلال رؤية الحيوانات المنوية للفئران تحت المجهر الضوئي ، تم بعد ذلك تقسيم إناث الفئران إلى مجموعتين عند اليوم الثاني عشر. تم حقن مجموعة واحدة داخل الصفاق بالماء للحقن فقط ومجموعة أخرى تم حقنها بفالبروات الصوديوم 600 ملغم / كغم. و بعد الولادات، تم فصل نسل الفئران عن الأمهات في اليوم الأربعين وتم تقسيمهم إلى 8 مجموعات ، كل مجموعة تتكون من 10 حيوانات على النحو التالي:

- مجاميع النسل المسيطرة: المجموعة الاولى تلقت محلول ملحي طبيعي عن طريق الفم ، المجموعة الثانية تلقت سيتاكليبتين 10 ملغم / كغم عن طريق الفم ، المجموعة الثالثة تلقت سيتاكليبتين 15 ملغم / كغم عن طريق الفم والمجموعة الرابعة تلقت ريسبيريدون 1 ملغم / كغم عن طريق الفم.
- مجاميع النسل المستحثة بفالبروات الصوديوم: المجموعة الخامسة تلقت محلول ملحي طبيعي عن طريق الفم ، المجموعة السادسة تلقت سيتاكليبتين 10 ملغم / كغم عن طريق الفم ، المجموعة السابعة تلقت سيتاكليبتين 15 ملغم / كغم عن طريق الفم والمجموعة الثامنة تلقت ريسبيريدون 1 ملغم / كغم عن طريق الفم.

استمرت التجربة لمدة 20 يومًا ، ثم أجريت تفاعلات اجتماعية واختبارات ميدانية مفتوحة. بعد إجراء الاختبارات السلوكية ، تم التضحية بالفأر بقطع الرأس بعد 24 ساعة من آخر جرعة من العلاج وأخذ نصفي المخ لتقدير الإجهاد التأكسدي (الجلوتاثيون و مالون دايالدهايد) والنشاط المضاد للالتهابات (انترلوكين-6 و عامل نخر الورم الفا) بعد إجراء تجانس الأنسجة.

أظهرت نتائج هذه الدراسة أن سيتاكليبتين عند كلتا الجرعتين يزيد الوقت الذي يقضيه مع فأر غريب في اختبار التفاعل الاجتماعي. أيضًا ، كان له نشاط مزيل للقلق عن طريق تقليل أعراض اضطراب طيف التوحد (القلق والخوف وفرط النشاط) في اختبار المجال المفتوح. يحتوي سيتاكليبتين على أنشطة قوية لمضادات الأكسدة وأنشطة إزالة الجذور الحرة مقارنةً بالريسبيريدون. حددت نتائج هذه الدراسة أن 10 ملغم / كغم سيتاكليبتين له تأثير مضاد للأكسدة قوي على بيروكسيد الدهون مقارنة مع ريسبيريدون. في حين أن التأثير المضاد للالتهابات للسيتاكليبتين في كلا الجرعتين كان أقل من ريسبيريدون. على الرغم من أن سيتاكليبتين في كلتا الجرعتين كان له نشاط قوي مضاد للالتهابات في الفئران الصغيرة التي تسببها فالبروات الصوديوم.