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YEDİTEPE UNIVERSITY

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DEPARTMENT OF CLINICAL PHARMACY

IS HYPERHOMOCYSTEINEMIA A RISK FACTOR FOR COGNITIVE DEFECTS IN RATS EXPOSED TO METFORMIN

DEGREE OF MASTER OF SCIENCE

IN CLINICAL PHARMACY

PHARMACIST

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SUPERVISOR

Prof. Dr. M. Ece GENÇ

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THESIS APPROVAL FORM

DECLARATION

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which has been accepted for the award of any other degree except where due acknowledgment has been made in the text.

VUSALA ABDULLAYEVA

17 MARCH 2017

ACKNOWLEDGEMENTS

To begin with, I would like to express my gratitude to everyone who helped me in my graduate degree education.

First, I express my thanks to my dear advisor Prof. Dr. Ece Genç for her great motivation and guidance during this process.

Therewithal, I am very grateful to Dr. Çağlar Macit, Beril Kadıoğlu, Dr. Hatice Akkaya and Dr. Meltem Dağdelen for their priceless personal supports.

I would like to convey my sincere thanks to my dear instructor Prof. Dr. Feyza Arıcıoğlu for her special contributions on application of Passive Avoidance test.

I also offer my deepest gratitudes to Asst. Prof. Dr. E.Çiğdem Altunok for her contributions on statistics part.

Also, I am very glad to mr. Selim and Engin Sumer for all their contributions in my work with experimental animals.

Finally, I would like to give my best thanks to my mother, father, dear husband and mother-inlaw for their endless support in my academic life with their positive attitudes, my brother Mehdi Hagverdiyev for his supports and contributions and to my little baby for being with me throughout this challanging procedure

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LIST OF SYMBOLS AND ABBREVATIONS

AMP – Adenosine monophosphate 10

AMPK – Activated protein kinase 10

ATP – Adenosine triphosphate 10

BHMT – Betain homocysteine S-methyltransferase 14

BBB – Blood brain barrier 28

CHD – Coronal heart disease 8

CBC – Complete blood count 30

CSF – Cerebrospinal fluid 17

CNS – Central nervous system 17

DM – Diabetes Mellitus 1

DPP – Dipeptdyl peptidase 6

DNA – Deoxyribonucleic acid 14

ECF – Extracellular fluid 14

IDDM – Insulin dependent Diabetes mellitus 3

GDM – Gestational Diabetes mellitus 3

GLP – Glucagon like peptide 6

HHS – Hyperglycemic hyperosmolaris sendrome 8

HFD – High fat diet 28

Hcy – Homocystein xii

NTD- Neuronal tube defect 17

NIDDM – Non insulin dependent Diabetes Mellitus 3

MTHF – Metilen Tetra Hydrofolate reductase 15

MALT – Metformin associated lactic asidosis 8

MMA – Metil Malonil CoA 17

SAM – S- adenozyl methionine 14

STZ - Streptozotocin xiii

SGLT- Sodium glucose co - transport 7

SAH – S adenozyl homocystein 14

ROS – Reactive oxygen species 18

WHO – World health organization 4

ZDF – Zucker diabetic fatty rats 29

ABSTRACT

Is hyperhomocysteinemia a risk factor for cognitive defects in rats exposed to metformin. Yeditepe University Institute of Health Sciences Clinical Pharmacy Master Thesis. Istanbul, 2017

Aim: Diabetus mellitus currently is a very wide spread metabolic disorder. Metformin from Biguanides is a commonly used agent in DM treatment with side effects like diarrhea and Vitamin B12 deficiency. Aim of the study was to investigate Metformin's side effect, causing vitamin B12 deficiency on diabetic rats. Furthermore, it is known that deficiency of vitamin B12 leads to hyperhomocysteinemia. I examined how Hyperhomocysteinemia developes due to B12 deficiency due to metformin usage for a certain period of time, and whether cognitive changes might take place.

Material and Method:

This study is a prospectous and controlled work carried out between February and July 2015 on experimental animals. 24 female Sparque-Dawley rats were grouped in 4 different groups named Metformin-Control, STZ – Control, STZ – Metformin and Control-Control. Rats were daily given 250 mg/kg (p.o.) metformin and 2.5 cc (p.o.) distilled water as placebo treatment and control groups respectively for 21 days. At the end of this period, Passive avoidance test was applied to observe memory loss. After this application, blood was taken from animals, serum was isolated and checked for Homocystein levels.

Findings and Result:

In this study Passive Avoidance test did not result in a statistically significant change between groups. Homocystein (Hcy) data between treatment and control groups did not give statistically significant change either.

Key Words: diabetic rats, metformin, cognitive disease, passive avoidance test

ÖZET

Diyabetli sıçanlarda metformin kullanımına bağlı görülebilecek hiperhomosisteinemi kognitiv değişiklikler için risk faktörü müdür?

Amaç: Diyabet günümüzde yaygın olarak görülen metabolik bir hastalıktır. Metformin de DM tedavisinde çok fazla kullanılan ilaç olmakla beraber diyare, B12 eksikliği gibi yan etkilere sahip bir ajandır. Çalışmadaki amaç Metforminin vitamin B12 eksikliğine sebep olan yan etkisinin diabetli sıçanlar üzerinde araştırılmasıdır. 21 günlük deney sürecinde metforminle tedavi edilen diyabetli sıçanlarda B12 eksikliğinin ve buna bağlı olarak Hiperhomosisteineminin gelişip-gelişmediğinin ve kognitiv değişikliklerin test edilmesidir.

Materyal ve Metot:

Bu çalışma deney hayvanları üzerinde gerçekleştirilmiş kontrollü bir çalışma olup Şubat 2015 - Temmuz 2015 tarihleri arasında yapılmıştır. Streptozotocin(STZ) ajanıyla diyabet oluşturulmuş Spraque–Dawley 24 dişi sıçan Metformin - Kontrol, STZ-Kontrol, STZ–Metformin ve Kontrol-Kontrol grubu olarak 4 farklı gruba ayrılmışlardır. 21 günlük deney sürecinde tedavi gruplarına her gün 250 mg/kg dozda (p.o.) metformin, kontrol gruplarına ise her gün 2,5 cc (p.o.) distile su plasebo olarak uygulanmıştır. 21 günlük tedavi sürecinin sonunda deney gruplarında hafıza kaybının saptanması amacıyla Passiv Avoidance Testi yapılmıştır. Deney sonlandırılması dekapitasyon yoluyla yapılmış, kan örneyi alınarak Homocystein Elisa kitle serum homosistein düzeylerine bakılmıştır.

Bulgular ve Sonuç:

Bu çalışmada deney hayvanlarında hafıza kaybını gözlemlemek amacıyla yapılan PassiveAvoidance testi ve dekapitasyon yoluyla yaşamına son verilen hayvanlardan alınan kan örneklerinden Homocystein değerlerinin test edilmesi sonucunda çalışma ve kontrol grupları arasında istatiksel olarak anlam bulunmamıştır.

Anahtar kelimeler: diyabetik sıçanlar, metformin, bellek kaybı, pasif sakınma testi.

CONTENTS

1. INTRODUCTION

Nowadays, Diabetes Mellitus and its treatment are among the most widely studied topics. Moreover, a widely used drug in this treatment from Biguanides, Metformin hydrochloride causes a lot of conflicts with its unknown effects and side effects.

Before I decided on my thesis topic, I have observed that there have been lots of different research that resulted in various conclusions due to minor differences. I settled on metformin's side effect on vitamin B12. Namely, positive effects of metformin on memory loss which is observed in high fat diet induced insulin resistant people.

Furthermore, if we have a look at homocystein metabolism, serious folic acid and B12 deficiency may cause hyperhomocysteinemia and therefore cognitive changes, many kinds of disorders like Alzheimer, Autism and memory loss.

In most studies that I have analyzed, seperate hyperhomocysteinemia model has been created and further changes on cognition due to it was studied. Or high fat diet insulin resistance was developed in order to investigate the positive and negative effects of metformin treatment.

My aim in this study was to create a diabetes model on rats and after that to analyze how metformin used in DM treatment might cause hyperhomocysteinemia, memory loss and cognitive function disorder.

2. LITERATURE REVIEW

2.1 DIABETES MELLITUS

2.1.1 DEFINITION OF DM

Diabetes mellitus is a combination of number of metabolic disorders mainly characterized by increased blood glucose above the normal level caused by either decreased insulin secretion or inability of the body cells to respond sufficiently to normal insulin levels (1).

This chronic uncontrolled high level of blood glucose will definitely cause damage and impaired function of multiple body organs especially eyes , kidneys , nerves , heart and blood vessels which in turn will result in long-term complications (1).

The development of Diabetes mellitus primarily depends on insufficient insulin action either inadequate insulin secretion inappropriate with tissue needs due to autoimmune destruction of beta cells of the pancreas or due to resistance of the tissue cells to insulin action. Both ways lead to irregular and abnormal carbohydrates , fat and protein metabolism (1).

Patients of diabetes mellitus usually complain of frequent urination and drinking (polyuria and polydipsia) , marked weight loss and history of blurred vision. Chronic hyperglycemia causes also impairment or delayed growth and increase liability of getting infections. Long-term uncontrolled diabetes mellitus may result in serious complications such as hyperglycemia with ketoacidosis or the nonketotic hyperosmolar syndrome. (1)

Although different types of Diabetes mellitus share common pathological coarse after appearance of the disease symptoms, medical research has shown that different types of diabetes have different causes .(2)

2.1.2 Classification of DM

The classification DM summarized in Table 1. (2)

CLASS NAME	CHARACTERISTICS
Insulin dependent diabetes IDDM	Low or absent levels of circulating endogenous insulin and dependent on injected insulin to prevent ketosis Abnormal immune response and islet cell antibodies are frequently present at diagnosis Etiology probably only partially genetic, as only ~35% of monozygotic twins are concordant for IDDM
Non – insulin dependent diabetes NIDDM	Insulin levels may be normal, elevated, or depressed; hyperinsulinemia and insulin resistance characterize most patients; insulinopenia may develop as the disease progresses Not insulin-dependent or ketosis-prone under normal circumstances, but may use insulin for treatment of hyperglycemia
Gestational diabetes GDM	Glucose intolerance that has its onset or recognition during pregnancy Associated with older age, obesity, family history Conveys increased risk for the woman for subsequent progression to NIDDM Associated with increased risk of macrosomia
Other types of diabetes, including diabetes secondary to or associated with: Pancreatic disease Drug or chemical exposure Insulin receptor abnormalities	In addition to the presence of the specific condition, hyperglycemia at a level diagnostic of diabetes is also present Causes of hyperglycemia are known for some conditions, e.g., pancreatic disease; in other cases an etiologic relationship between diabetes and the other condition is suspected

2.1.3 Diagnostic criteria for DM

The criteria to diagnose diabetes were decided by NDDG1 and WHO2 in 1979-80.

Table 2. Diagnostic criteria for DM (2)

Two or more of the following glucose concentrations (fasting value and values at times after 100 –g oral glucose) must be met or exceeded:			
Blood	**Venous plasma**	**Venouse whole blood**	**Capillary**
Fasting	**105 mg/dl (5,8 mmol/L)**	**90 mg/dl (5 mmol/L)**	**90 mg/dl (5mmol/L)**
1 hour	**190 mg/dl (10,6 mmol/L)**	**170 mg/dl (9,4 mmol/L)**	**190 mg/dl (10,6 mmol/L)**
2 hour	**165 mg/dl (9,2 mmol/L)**	**145mg/dl (8,1 mmol/L)**	**165 mg/dl (9,2 mmol/L)**
3 hour	**145 mg/dl (8,1 mmol/L)**	**125 mg/dl (6,9 mmol/L)**	**145 mg/dl (8,1mmol/L)**

2.1.4 Pharmacotherapy for DM

Pharmacotherapy of Diabetes mellitus depends on the cause of insulin impairment function which is either due to insulin deficient secretion or insulin resistance or both . Then the treatment could be determined either be noninsulin therapies—insulin sensitizers, secretagogues, alpha glucosidase inhibitors, incretins, pramlintide and etc

Table 3. Noninsulin Therapy (3)

Subgroup	Generic name	Route	Adverse Effects
Insulin sensitizers			
Biguanides	Metformin (Glucophage)	Oral	Weight loss No hypoglycemia GI upset
Thiazilidinediones	Rosiglitazone (Avandia) Pioglitazone (Actos)	Oral	Weight gain Peripheral edema
Insulin secretagogues			
Sulfonylureas	Chlorpropamide Glibenclamide Glimepiride Glipizide Tolazamide Tolbutamide	Oral	Hypoglycemia Weight gain
Glinides	Nateglinide Repaglinide	Oral	Weight gain
Alpha – glucosidase inhibitors			
	Acarbose Miglitol	Oral	GI upset No hypoglycemia

Subgroup	Generic name	Route	Adverse effects
Incretins			
GLP-1 receptor agonists Short acting (4-6 hrs)	Exenatide (Byetta)	SC	Weight loss GI upset
GLP-1 receptor agonists Intermediate – acting (24hrs)	Liraglutide (Victuza)	SC	Weight loss Nausea
GLP-1 receptor agonists Long-acting (7 days)	Exenatide ER Albiglutide Dulaglutide	SC	Weight loss Nausea
DPP-4 inhibitors	Sitagliptin (Januva) Saxagliptin (Onglyza) Linagliptin (Tradjenita) Alogliptin (Nesina)	Oral	No hypoglycemia Nasopharyngitis Weight neutral
Pramlintide			
	Pramlintide(Symlin)	SC	Weight loss GI upset Adjunctive tx with insulin
Rapid-release bromocriptine			
	Bromocriptine quick-release (Cycloset)	Oral	Take with 2 hrs of awakening Nausea, stuffy nose
SGLT-2 inhibitors			
	Canagliflozin (Invokana) Dapagliflozin (Farxiga) Empagliflozin (Jardiance)	Oral	Polyuria UTIs

Table 4. Insulin therapy (3)

Insulin regimen	HbA1c (%)	Medication	Pattern	Diet	Monitoring
Basal-only	≥ 7.5-10	Oral medications adequately control postprandial glucose excurcions	High fasting glucose with minimal glucose rise during the day	Smal, regular meals	Fasting
Basal-bolus	≥ 7.5		Regimen can be matched to any pattern to achieve glycemic control	Regimen can be matched to any diet to achieve glycemic control	Frequent blood glucose monitoring
Once or	Twice	Daily premixed			
Rapid-acting analogue and intermediate acting	≥ 7.5	Oral agent failure	Any fasting glucose; glucose rises during the day	Large suppers, smalllunches	Fasting and pre-supper (if insulin is administered twice daily)
Regular and NPH	≥ 7.5	Oral agent failure	Any fasting glucose; glucose rises during the day	Isocaloric meals or larger lunches	Fasting and pre-supper (if insulin is administered twice daily)

2.1.5 Complications of DM

Acute and chronic complications

Acute	Chronic
-Diabetic ketoacidosis	- Neuropathy
-Hyperglycemic hyperosmolaris syndrome (HHS)	- Nephropathy
- Hypoglycemia	- Retinopathy
- Metformin associated lactic acidosis, MALT (4)	- Macrovascular disease (CHD)

Hemodynamic disturbances in diabetes

- Increased blood flow
- Increased permeability (4)

Hemorrheological and coagulation abnormalities

- increased plasma viscosity
- decreased red-cell deformability
- increased platelet aggregability

2.2.1 Metformin as a drug :

Metformin hydrochloride tablets

Description:

Metformin hydrochloride ($C_4H_{11}N_5$, mw 165.63 gram/mole) is a freely soluble white crystalline compound in water.

Pharmacokinetics: Absorption and bioavailability

The absolute bioavailability of a metformin hydrochloride 500 mg tablet given under fasting conditions is approximately 50-60% (5). The bioavailability of extended-release products was usually found to be less than that of the immediate-release products without alteration of elimination.

Metabolism and Elimination

Metformin is neither metabolized by the liver nor excreted in bile. It is excreted unchanged in urine.

Indication and usage:

Nowadays metformin is one of the most widely known drug used against diabetes with its beneficial effects on lipid profiles.

2.2.2 Metformin in DM therapy

The primary action of metformin is to reduce blood glucose level by decreasing glucose synthesis by the lever (inhibition of gluconeogenesis) . It also enhances insulin suppressor effect on endogenous production of glucose with reducing intestinal glucose absorption and enhance glucose utilization by the tissues especially muscles and adipose tissue.

It has been reported that metformin does not improve peripheral insulin sensitivity but the increase in insulin sensitivity in muscle may be due to the use of high doses metformin. Additionally, metformin may also improve glucose homeostasis by interacting with the incretin axis through the action of glucagon-like peptide 1 (GLP-1) (5).

2.2.3 Mechanism of action of Metformin：

As conclusion of several years of in vivo and in vitro research, people have come to the conclusion that action mechanism of metformin has been the anti-diabetic effect resulting from reduced glucose production. This reduction is caused from metformin's preclusive effect on gluconeogenesis.

For long years, this action mechanism was accepted ordinary and not studied well. However, it is reported after Zhou and his research group's studies that pleiotropic effect of metformin is strongly associated with AMPK activation (5,12).

Now it is evident that metformin in low concentrations suppresses production of glucose via AMPK activation (independent of increase in AMP/ATP ratio). What confused minds from that work is metformin's activation of AMPK with no change of energy charge. That is mainly because metformin indirectly triggers AMPK (secondary to the inhibition of the mitochondrial respiratory- chain complex 1, leading to ATP depletion and a rise in AMP levels)(5).

Gluconeogenesis is an anabolic process with high costs in terms of energy and it requires six ATP equivalents per molecule of synthesized glucose. It is supposed that the increase in AMP induced by metformin plays a major role in the flux control of hepatic gluconeogenesis by the drug. Actually, AMP is a potent allosteric inhibitor of a key enzyme in gluconeogenesis (fructose 1,6-bisphosphatase). Additionally, high AMP levels inhibits adenylate cyclase, thereby reducing cyclic AMP (cAMP) formation in response to glucagon and thus, fasting glucose levels (5,13)

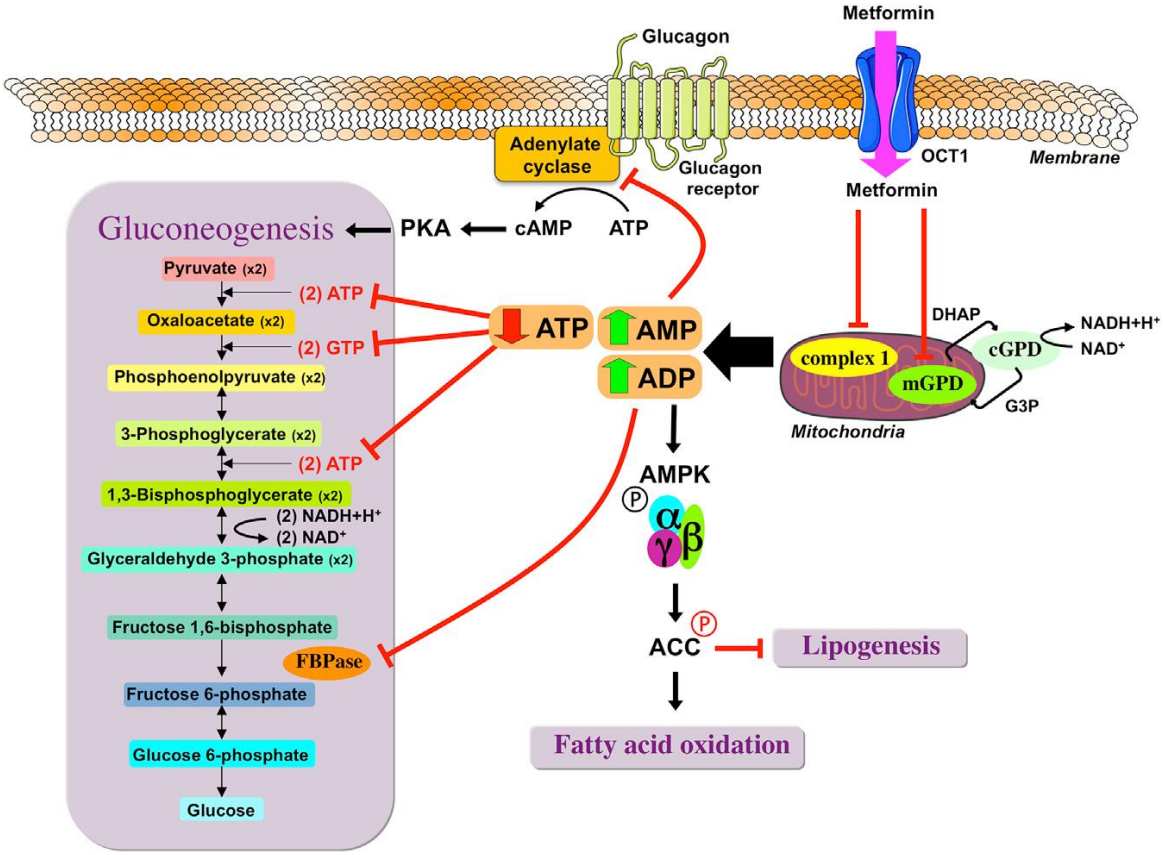


Figure 1. Mechanism action of Metformin

Dosage and Administration :

Daily maximum dosage is 2.5 g for adults, however, alternates due to some specifications of patient like age, weight, secondary diseases, progress of disease may be done.

OVERDOSAGE :

Although hypoglycemia has not been observed at ingestions of high dosage metformin, lactic acidosis has.

2.2.4 Precautions on Metformin therapy

Lactic acidosis

Lactic acidosis is one of the most known side effects of metformin. Increased lactate/pyruvate ratio, elevated blood lactate levels (>5 mmol/L), electrolyte disturbances with an increased anion gap and decreased blood pH; metformin plasma concentrations greater than 5 μg/mL are usually seen when metformin is implicated as the cause of lactic acidosis. It is fatal in approximately 30% of cases.

Some pathophysiologic conditions (including diabetes mellitus) may lead to lactic acidosis. Also hypo-perfusion and hypoxemia, dehydration, hepatic impairment, excess alcohol intake, sepsis are potential causes of acidosis. When suspected, immediate cessation of particular drug and hospitalizing of patient are first things to do.

Monitoring of renal function

As Metformin is totally excreted by the kidney , the risk for lactic acidosis increases with the degree of renal dysfunction and the patient's age due to metformin accumulation in the body especially with sustained released tablets (5). That's why continuous monitoring of serum creatinin level in patients receiving metformin is important . Patients should stop metformin if the level above the upper limit of normal for their age was exceeded.

Surgical procedures

Metformin therapy should be paused before surgical procedure. Doctors are convinced that resumption of metformin therapy after procedures requiring restricted food and fluid intake is risky (5). When patient starts to eat normally again with elevation of renal functions to normal level then it can be resumed.

Vitamin B12 levels

Metformin may interfere with vit B 12 absorption from B 12 and intrinsic factor complex (12). There is a decrease in vit B 12 serum level in 7 % of patient receiving

metformin. However, this decrease rarely causes anemia and can be easily reversed by stopping of Metformin tablets or with vit B 12 supply.

People with defects concerning vit. B 12 or calcium absorption or intake are at high risk of developing of subnormal levels of serum vit B 12 with metformin treatment. Those patient are advised with periodical measurement of hematological level of vitamins and minerals and they should consult their doctors with any abnormalities to be managed quickly. .

Hypoglycemia – Metformin therapy alone does not cause hypoglycemia with normal food intake and maintenance of normal glucose level. However, any condition that includes lowering of blood glucose level which can not be compensated with glucose or food intake will produce hypoglycemia as in severe exercise or during concomitant use of other glucose-lowering agents (such as sulfonylureas) or ethanol.

Contraindications of metformin treatment :

Metformin hydrochloride tablets are contraindicated in cases with significant tissue hypoperfusion and hypoxemia who are more likely to develop lactic acidosis as it mentioned before :

1. Renal disease

2. Congestive heart failure requiring pharmacologic treatment

3. Known hypersensitivity to metformin hydrochloride.

4. Acute or chronic metabolic acidosis, including diabetic ketoacidosis, with or without coma. Diabetic ketoacidosis should be treated with insulin (5).

Metformin should be discontinued at the time of or before an iodinated contrast imaging procedure or with patients undergoing radiological studies as these product may temporary affect renal functions.

Drug interactions

The following drugs may interact with metformin causing harmful effect : Furosemide, Glyburide, Nifedipine, Cationic drugs (amiloride, digoxin, morphine, procainamide, ranitidine, triamterene, trimethoprim, and vancomycin.) (5,13).

Adverse reactions

Diarrhea, nausea/vomiting, flatulence, asthenia, indigestion, abdominal discomfort, headache (6,13).

2.3 Homocystein

2.3.1 Role of homocystein in human body

Homocysteine is a sulfur - containing amino acid biosynthesized from methionine in the active methionine cycle after releasing of homocysteine. It has 3 fates : either remethylated to methionine or enter in cysteine biosynthesis or released into the ECF . The remethylation process of homocysteine occurs by methionine synthase enzyme which depends on both vitamin B12 and folate as essential cofactors in the methylation process , and deficiency of vitamin B12 which in turn will cause deficiency in folate, both will lead to elevated levels of homocysteine. On the other hand, low folate concentration will affect synthesis of SAM which is an important neurotransmitter whose deficiency will lead to neuropsychiatric defects.

It is an agonist for NMDA calcium channels, causing excitotoxicity and damaging neuronal DNA.

· It can start calcium efflux by having direct effects on glutamate receptors.

· It can potentiate its own pathogenic effects by inducing stress in the endoplasmic reticulum.

· It induces oxidative stress, leading to the formation of reactive oxygen species.

· It can activate apoptotic signal cascades, such as P53 and Bax.

· It can cause cytochrome *c* release and caspase activation. (6)

2.3.2 Homocysteine metabolism

Homocysteine is remethylated to methionine by the action of activated methionine synthase enzyme. This process requires both vitamin B12 as co-factor and 5-methyl tetra hydrofolate as source of one methyl group which is obtained from irreversible reduction of 5,10-methylene THF by MTHF reductase. This step requires continuous activation of methionine synthase by reducing it with methionine synthase reductase (MSR). On note , this step will result in transformation of 5-MTHF into THF. Another way of conversion of homocysteine into methionine is by betain homocysteine methylatransferase (BHMT) which doesn't depend on vitamin B12.

Methionine is then transformed into s-adenosyl methionine (SAM) by the action of methionine adenosyl transferase and in presence of ATP. SAM is an important general methyl donor needed by many important molecules and adrenaline, phosphatidylcholine and carnitine.

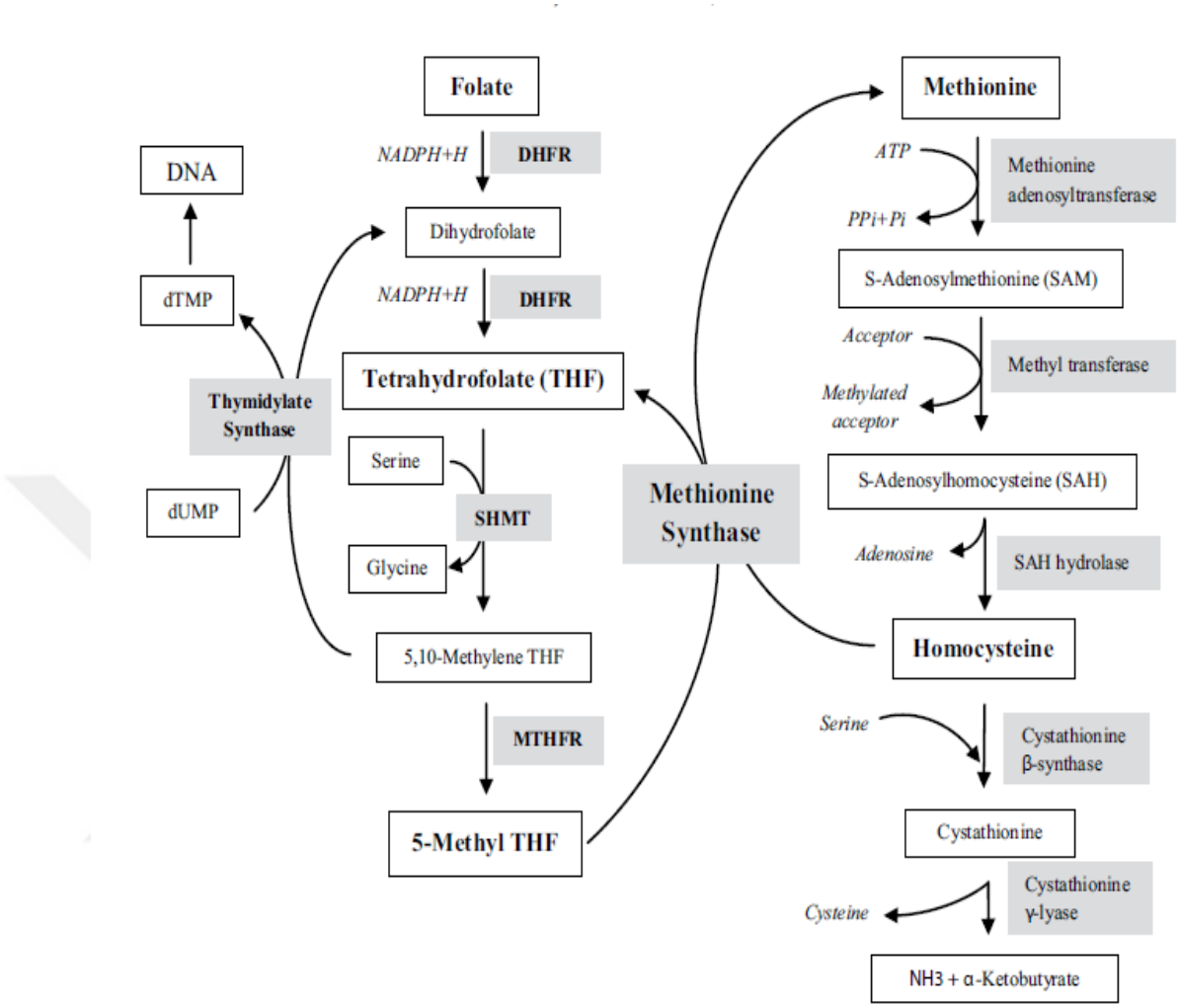


Figure 2. Homocysteine metabolism

The cycle is then completed by conversion of SAM into S- adenosyl homocysteine, later it is converted back into homocysteine

Another fate of homocysteine is entering in cysteine biosynthesis. In the beginning, homocysteine unites with serine producing cystathionine by the action of cystathionine- β -synthase (CBC). This reaction is irreversible. Vitamin B6 dependent synthesis of homocysteine is no longer available for remethylation back to methionine. Next cystathionine- γ -lyase (CGL) another Vit B6 dependent enzyme hydrolyse cytathionine into cysteine and α -ketobutyrate (6).

In vitamin B12 functional deficiency, homocysteine and methylmalonic acid (MMA) levels increase in plasma and urine. Because folate is another coenzyme of methionine synthase, MMA measurements are more selective for vitamin B12 status alone.

2.3.3 Homocysteine and cognitive disease

Vitamin B12 , folic acid and Vit B6 are essential cofactors in metabolism of homocysteine and methionine, and by knowing that folic acid which donates a methyl group for cobalamine to form methylcobalamin which remethylate homocysteine back to methionine, any deficiency in these cofactors will affect homocysteine metabolism that will result in a state of hyper-homocysteinuria.

Cases of hyperhomocysteinuria are usually found in elderly people due to dietary deficiency of these cofactors (vitamin B12 , B6 and folate) . These deficiencies are associated with decreased cognitive function and many neuropsychiatric disorders such as paresthesias, ataxia, sensory loss, and psychiatric disorders (7,14).

In fact, homocysteine can be transformed into cystathionine via cystathionine-β-synthase activity; as a result of this cystathionine-β-synthase action, the antioxidant glutathione can compensate for potential oxidative damage from excess homocysteine. Although there is no conversion of cystathionine into cysteine in the brain, the conversion of homocysteine into cystathionine does occur (8).

2.3.3

a) Alzheimer's disease

High level of homocysteine is now considered a risk factor in the development of Alzheimer's disease while advancing in age. Scientists consider high homocysteine levels as an early sign of cognitive impairment in geriatrics. Elderly with histologically confirmed Alzheimer's disease show low levels of folate in their CSF and in the contrary normal CSF : serum ratio of folate which is 3-4 :1 which makes a strong association between cerebral

cortex atrophy and low folate levels. Consequently decreased SAM concentrations in the CSF of Alzheimer patients compared to healthy individuals of the same age was found (8).

b) Autism

Pregnant women are encouraged to get plenty of folic acid in their diet or through vitamin supplements to protect their babies against birth defects of the brain and spinal cord as neural tube defect (NTD). However, a new study suggests that excessive amounts of folate (vitamin B9) and vitamin B12 in a mother's body might increase a baby's risk of developing an autism spectrum disorder (14). Especially after increasing folic acid fortification in the USA, The Autism Genome Project Consortium noted that both glutamate receptors and pathways and gene associated with glutamate are implicated in the pathologies of a number of neurodegenerative diseases due to their central role in neural development and their prevalence throughout the central nervous system. High folate levels which contain at least one glutamate residue in all species cause exaggerated glutamate action by many ways. It causes inhibition of neuronal uptake of glutamate in CNS, compete with inhibition of glutamate by inhibitor neurotransmitters, and slow glutamate decarboxylase activity. The kainic acid receptor is a subunit of glutamate receptor 6 and a mutation in the kainic acid receptor gene may be associated with autism.

3. MATERIALS & METHODS

3.1 METHOD

3.1.1 Experimental Animal

In this experiment, 24 female rat (weighting 200-250 g, Spraque – Dawley) provided by YÜDETAM were used.

3.1.2 Used drugs

Metformin (World Medicine, Science drug)
Streptozotocin (Santa Cruz 200719)

3.1.3 Preparation of solutions

a) Preparation of STZ solution for DM induction

147 mg sodium citrate was dissolwed in 50 mL distilled water to get a citrate bufer pH 4.5. 132 mg STZ was weighed and dissolved in 12 mL citrate buffer (1 mL for each animal, each animal received 11 mg of STZ -55mg/kg-). During this process, freshly prepared buffer was put in ice bath and used in maximum 30 minutes after STZ was dissolved. Since STZ is highly sensitive to light, solution wrapped to aluminum foil and waited in ice bath during this time. Because STZ has serious effects on human body, hand gloves and masks were used throughout the process.

b) Sucrosed water

200 g Sucrose was added to 1 L of drinking water to get a 20% solution, it was given to animals during 48 hours as drinking water.

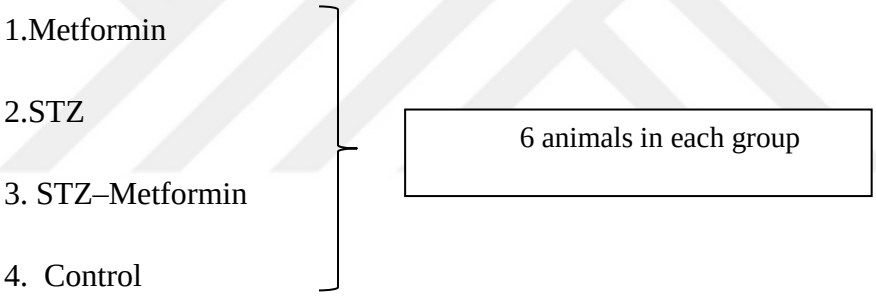
c) Preparation of Metformin solution

According to experimental conditions, fresh Metformin solution was prepared every day in order to give 12 animals in 2 groups in 2 mL proportions, via oral gavage method during 21 days of experiment (in a dose of 250 mg/ kg). 600 mg Metformin was dissolved in 24 ml distilled water to apply it via oral gavage method every day.

This study was a prospective and controlled work that carried out on experimental animals between February and July in 2015. Ethics committee permission number and date were 423/22.09.2014

3.1.4 Application of study

24 female Spraque-Dawley rats that have been used inthe study separated into 4 different groups that are:



55 mg/kg dose STZ applied to experiment animals that are left hungry a day before. After that, animals were left free to eat and drink water. 72 hours later, fasting blood sugar test was conducted. Results over 250 mg were considered as diabetic and included in the study. Control group animals tested for blood sugar levels as well.

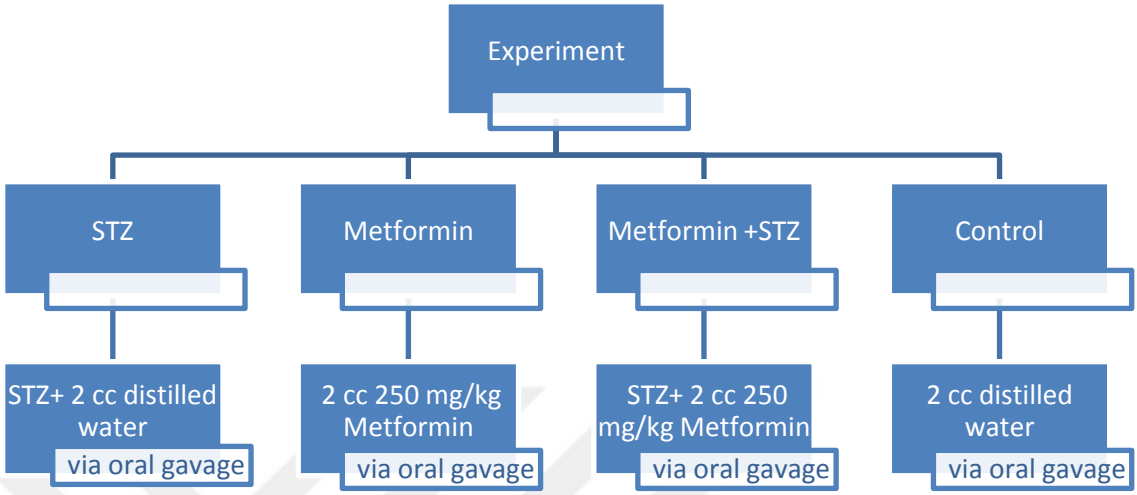


Figure 3. Applied sugar on the rest of the experiment are showed below in the scheme below:

Table 5. Blood glucose levels of the groups

1.STZ	2.STZ METFROMİN	3.METFORMİN-	4.KONTROL
1.650 mg/dL	1. 600 mg/dL	1. 120 mg/dL	1. 110 mg/dL
2.600 mg/dL	2. 450 mg/dL	2. 100 mg/dL	2. 115 mg/dL
3.High*	3. 150 mg/dL	3. 120 mg/dL	3. 120 mg/dL
4.High	4. High	4. 150 mg/dL	4. 110 mg/dL
5.350 mg/dL	5. High	5. 110 mg/dL	5. 120 mg/dL
6.550 mg/dL	6. High	6. 120 mg/dL	6. 130 mg/dL

*Blood glucose>650 mg/dL

During the 21 days of experiment, treatment groups and control group were given 250 mg/kg dosed (p.o.) metformin and 2cc dosed (p.o) distilled water (as placebo) respectively every day. At the end of this treatment process, Passive Avoidance Test was applied in order to observe memory loss of the treatment groups. Experiment was ended via decapitation as a result of thisapplication, blood was taken from experimental animals, gathered in serum and examined to determine Homocystein Elisa level.

3.1.5 Passive avoidance test

Since test was in a different laboratory, animals were brought there a day before.

Passive avoidance test was applied at the last two days (20^{th} and 21^{st}) of the experiment. Animals were brought to the lab as a group, however, test was carried out one by one to avoid others to get stressed from seeing and hearing the one that was subjected during the test.

Passive avoidance test application :

The device is one white (bright) and one black (dark) room that is seperated with a door. Test is applied 2 days, the first day is practice and the second is execution. Priorly, animal was taken from the cage and put into white room. In the first 20 seconds, the door between the rooms was closed, door opened after this 20 seconds automatically and animal was observed during 5 minutes to pass the other room. When animal passed to other room, it is exposed to a minor electro shock, in this way the time animal passes to the dark room was detected and recorded. All animals were exposed to the test, after the experiment eating and drinking water left free.

The test was repeated the next day at the same time. Aim of the test is to check if animals that do not have memory loss remember electro shock and pass to the dark room. They were put into device and observed 5 minutes again one by one, animals those have memory loss was waited to pass the dark room.

The time animals passed to the dark room was recorded. Data collected during 2 days of experiment is shown in the tab

4. RESULTS

Datas of Passive Avoidance test collected during two days of experiment is showen in the following table

Table 6. Practice procedure

STZ	STZ + METFORMİN	METFORMİN	KONTROL
1. 6 sec	1. 60 sec	1. 59 sec	1. 4 sec
2.102 sec	2. 8 sec	2. 44 sec	2. 10 sec
3. 250 sec	3. 19 sec	3. 120 sec	3. 29 sec
4. 182 sec	4. 24 sec	4. 2 sec	4. 14 sec
5. 14 sec	5. 58 sec	5. 31 sec	5. 69 sec
6. 34 sec		6. 72 sec	

Table 7. Experiment procedure

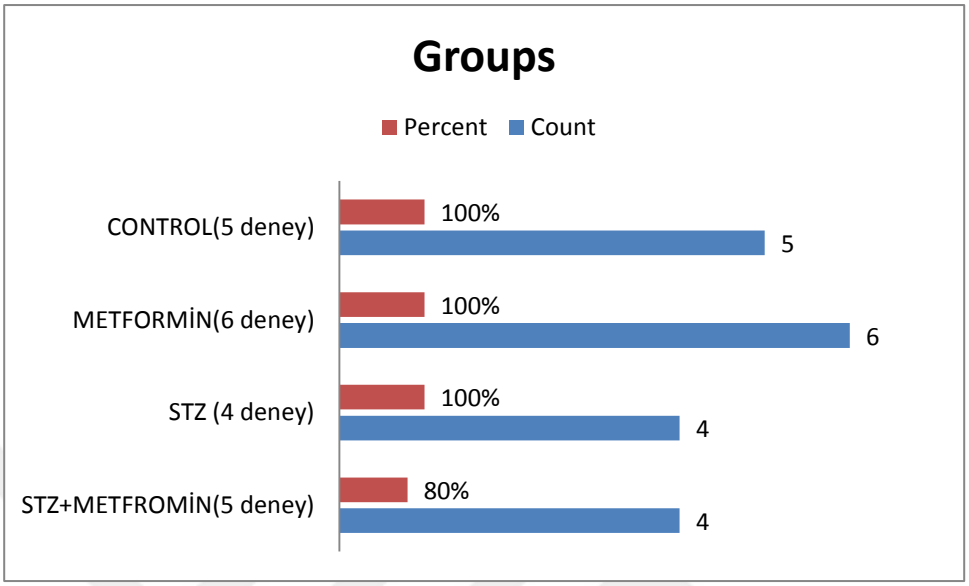
STZ	STZ+METFROMİN	METFORMİN	KONTROL
1. 300 sec	1.300 sec	1. 300 sec	1. 300 sec
2. 300 sec	2. 190 sec	2. 300 sec	2. 300 sec
3. 300 sec	3. 300 sec	3. 300 sec	3. 300 sec
4. 300 sec	4 . 300 sec	4. 300 sec	4. 300 sec
	5. 300 sec	5. 300 sec	5. 300 sec
		6. 300 sec	

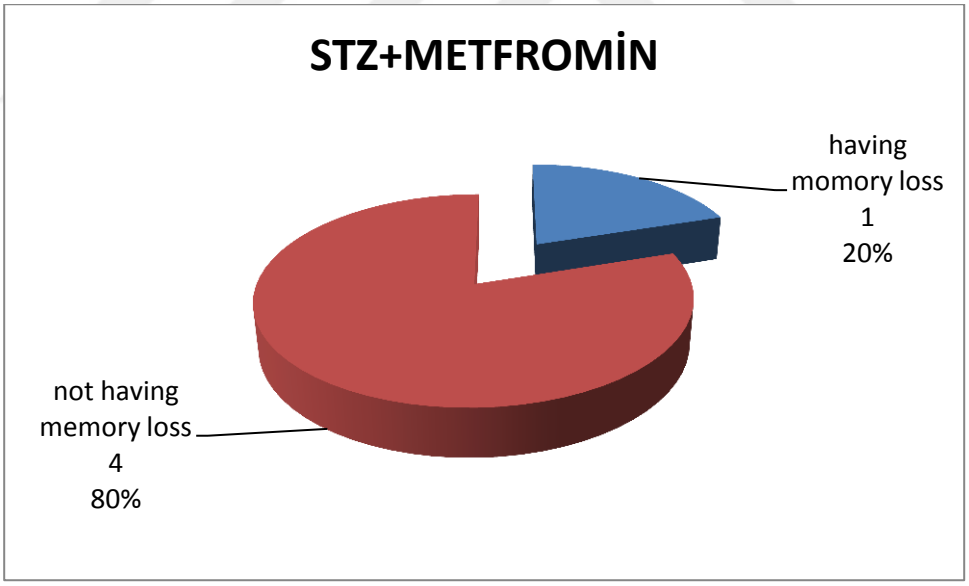
“Excel “software was applied to examine statistical difference of results of this test

According to obtained data’s, no statistical difference determined between groups

Comparison of groups is shown below in graphs

Figure 4. Comparison of groups





Statystical Analyses of Homocysteine levels

In order to determine if there is a meaningful relation among C (nmol/ml) mean values of all groups, Kruskal - Wallis test was applied. After the test p value found 0.716. Since p is greater than 0.05, there is no meaningful difference among groups statistically.

C (nmol/ml) mean values of other groups was compared to mean value of control group using Mann-Whitney U test. Results are below:

p=1.00 for Control-Met comparison (no meaningful difference statistically)

Table 8. Statistical analysis of control group

Control group

Statistics[a]

C(nmol/ml)

N	Valid		4
	Missing		1
Mean			.938861
Median			.803701
Std. Deviation			.3828470
Range			.8563
Minimum			.6459
Maximum			1.5022
Percentiles		25	.684869
		50	.803701
		75	1.328012

Table 9. Statistical analysis of metformin group

Statistics[a]

C(nmol/ml)

N	Valid		6
	Missing		0
Mean			.867199
Median			.845428
Std. Deviation			.2131971
Range			.4935
Minimum			.6604
Maximum			1.1538
Percentiles		25	.671263
		50	.845428
		75	1.055878

p=0.486 for Control-STZ comparison (no meaningful difference statistically)

Table 10. Statistical analysis of STZ group

Statistics[a]

C(nmol/ml)

N	Valid		4
	Missing		0
Mean			1.074020
Median			1.044993
Std. Deviation			.3512417
Range			.8563
Minimum			.6749
Maximum			1.5312
Percentiles		25	.765602
		50	1.044993
		75	1.411466

C(nmol/ml)

p=0.905 for Control-STZ+Met comparison (no meaningful difference statistically)

Table 11. Statistical analysis of STZ+ Metformin group

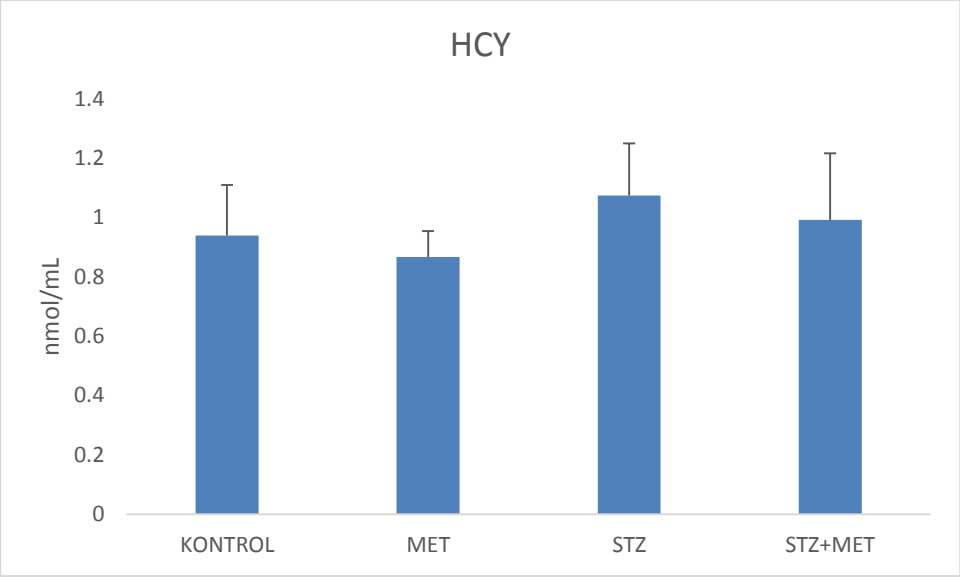
Statistics[a]

C(nmol/ml)

N		Valid	5
		Missing	0
Mean			.992743
Median			.812772
Std. Deviation			.4995780
Range			1.2482
Minimum			.6241
Maximum			1.8723
Percentiles		25	.711176
		50	.812772
		75	1.364296

C(nmol/ml)

Table 12. Homocysteine levels in the groups



5. DISCUSSION

The subject that I have worked on has been studied several times over humans and animals and contains lots of contradictions.

Metformin`s positive and negative effects on learning and memory, side effects of metformin treatment in diabetic rats, analysis of homocystein data reactions to insulin resistance, effect of B12 and folic deficiency on homocystein data, metformin`s association with cognitive disorders like Alzeihmer, Dementia, Autizm are among the most studied topics.

My aim while I was designing my study was to analyze contradictory effect of metformin on learning and memory.

In the previous studies, metformin`s effect on learning and memory was analyzed on diabetic rats (formed through high fat diet) and memory enhancing effects were observed. However, no change was detected on locomotor activity.

In this study, it is demonstrated that 12-week HFD consumption causes brain mitochondrial dysfunction. This is shown by a rise of brain mitochondrial ROS, brain mitochondrial depolarization and brain mitochondrial swelling. Metformin (N-1,1-dimethylbiguanide) is the drug of choice to treat T2DM (9).

Metformin’s mechanism of action is to increase the insulin mediated utilization of glucose and to reduce hepatic glucose output. Former studies indicated that metformin was able to cross the blood brain barrier (BBB) very rapidly. Also, anti-inflammatory and neuroprotective effects in the brain were among its beneficial effects . Over years, effects of metformin in insulin resistant model related to HFD consumption such as the spatial learning behavior and brain mitochondrial function have never been researched. The hypothesis that the administration of metformin can reverse the impairment of spatial learning behavior and brain mitochondrial dysfunction caused by 12-weeks of HFD consumption was tested in previous study (9).

After metformin treatment, they found that the mean time that the rats reached the platform during the acquisition test in the HFD group was significantly decreased, compared with the HFD group. Locomotor activity was determined by an open-field test. They found that the number of lines that the rats crossed during the test was not significantly different between the ND group (41.63±3.38 times/min) and the HFD group (44.14±2.81 times/min). These findings indicate that the locomotor activity of these rats were not different between the two diet groups (9).

As it is seen, 30 mg/kg dose metformin was applied in this study and showed positive impact. I have explored in my study how 50 mg/kg dose metformin will effect memory loss in experimental animals and how homocystein levels will differ.

In a different study, in leptin receptor– defective Zucker diabetic fatty rats with developed insulin resistance homocysteine levels were measured after 6 weeks of experiment and no difference occured (9).

Both types of diabetes are related with an increased risk of cardiovascular mortality, with the prevalence of atherosclerosis being two- to sixfold higher in diabetic patients than in people without diabetes. Insulin resistance, which immediately precedes the development of type 2 diabetes, is also associated with an increased risk of coronary artery disease, while hyperhomocysteinemia has been shown to be a stronger risk factor for cardiovascular disease and for mortality in patients with type 2 diabetes than in subjects without diabetes.

Plasma tHcy in diabetic patients is highly dependent on the presence or absence of nephropathy. Both type 1 and type 2 diabetic patients with nephropathy have elevated levels of tHcy, however, type 1 diabetic patients with no renal complications have plasma tHcy levels lower than controls. This decrease was also shown in an animal model of type 1 diabetes (10).

Therefore, effect of insulin resistance and type 2 diabetes on plasma tHcy and its metabolism in the liver were examined. An excellent model for type 2 diabetes, the leptin receptor– defective Zucker diabetic fatty rat (ZDF) was used in this work. Before developing Frank diabetes, a phase of insulin resistance has been investigated. That gave

them the opportunity to study the effects of both insulin resistance and of type 2 diabetes. (10)

When there is no renal damage, plasma tHcy levels decrease in both type 1 and type 2 diabetes as expected. This is true even at the prediabetic, insulin-resistant stage. However, literature reports on the phenomenon show a variety of responses of Hcy to insulin resistance. This variability is likely to result from different degrees of insulin resistance as well as other factors such as impaired renal function. The results which were obtained at 5 weeks in an animal model in which there was no evidence of impaired renal function as well as a consistent degree of insulin resistance, agree remarkably well with Rosolova's study of the relationship between Hcy and insulin resistance in healthy human subjects (10).

To summarize, this study shows a decreased plasma tHcy level in both insulin-resistant and type 2 diabetic rats, moreover, increased activities of BHMT and CBS in these states, as well as increased mRNA levels for these enzymes are also indicated together with the increased hepatic SAM levels which will activate CBSThese results highlight the importance of BHMT in regulating Hcy metabolism.

On the other hand, hyperhomocysteinemis due to B12 and folic acid deficiency is a discussion topic for many studies. Also, it is confusing that how metformin can have positive effects on memory while it is well known as causing B12 deficiency. So, results differ depending on experimental conditions. That is also main hypothesis of this study. Main purpose is to analyze the contradiction between metformin`s positive and side effects.

The methylation process may play an essential role in the biochemical bases of neuropsychiatry. Folate and vitamin B12 are crucial components of one carbon metabolism. The vitamin B12 deficiency is not only a cofactor for methionine synthase but also leads to a functional deficiency in folate and plays an important role as another cofactor of the same enzyme. Furthermore, low folate concentrations cause reductions in neurotransmitter synthesis, SAM synthesis, and the methylation of phospholipids in neuronal membranes.

The most important result of these deficiencies, and the most suitable biochemical sign of functional insufficiency of folate and vitamin B12 is elevated homocysteine levels.

Supplemental folic acid cannot override the methylation impairment, resulting in progressive neuropathology and neuronal death. Treating a combined folate and B12 deficiency with folic acid alone may correct hematological anomalies, but not neurological anomalies and can aggressively make worse B12-deficient neurological sequelae (11). Therefore, B12 deficiency should be ruled out before correcting folate deficiency. When folate and vitamin B12 are in balance, increased serum folate levels are related with decreased plasma Hcy and serum MMA levels. However, increased serum folate levels are related with increased plasma Hcy and serum MMA levels when vitamin B12 is underrepresented.

Pernicious anemia patients generally respond to supplemental B12 treatment favorably, especially if pernicious anemia is diagnosed early in the course of the disease. Some patients without pernicious anemia, but with B12 deficiency and either mild cognitive impairment or mild to moderate dementia, might show some degree of cognitive improvement with supplemental B12 treatment. Evidence that supplemental B12 treatment is beneficial for patients without pernicious anemia, but with B12 deficiency and moderately-severe to severe dementia is scarce. (11)

It is obvious from above that different results can be obtained with different approaches.

Taking similar works as reference, I designed my study to analyze statistical relation between homocystein data and memory loss in STZ induced diabetic rats applying high dosage of metformin (50 mg/kg).

Statistically, no meaningful conclusion obtained according to comparison of results of Passive Avoidance test and homocysteine levels. However, it should be underlined that animal number 2 in a main study group which is STZ + Metformin did not remember electroshock in the second day of experiment that it was exposed a day before and passed to the other side after 190 seconds. Highest homocystein data also observed at that animal.

Although one animal is not enough to explain contributions of this experiment to scientific world statistically, under different conditions like different dosage of drug, using improved analyses and tests, longer experimental processes might result in better conclusion.

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7. APPENDICES

7.1 Ethical approval

8.CURRICULUM VITAE

Person informations

Name	Vusala	Surname	Abdullayeva
Born place	Baku	Born date	09.04.1990
Nationality	Azerbaijan	Passaport No	99985329688
E-mail	vhaqverdiyeva@yahoo.com	Tel	

Education

Master degree	Clinical Pharmacy	Yeditepe University	2012 - 2017
Bachelor	Pharmacy	Azerbaijan Medicine University	2007 - 2011
School	-	158 no shcool	1996 - 2007

Language skills

English
Russian

Internship/ Work experience

Pharmacist	May pharmacy	04.2013 – 06.2013
Pharmacist	Saffron pharmacy	06.2011 – 07.2012