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Article

The Relationship Between Oxidative Stress and Infertility due to Antihypertensive Drugs in Rattus Norvegicus

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Abstract: The aim of this study was to investigate the effect of these drugs on reproductive function in Rattus norvegicuss and to show the role of oxidative stress in possible reproductive dysfunction. Rattus norvegicuss were selected as experimental animals and were divided into healthy (control group), Clonidine (CL), Rilmenidine (RLD), Methyldopa (MTL), Amlodipine (ALD) and Ramipril (RML) groups. Using blood obtained from rattus norvegicuss, serum malondialdehyde (MDA) levels; The high-temperature thiobarbituric acid. Was based on the spectrophotometric measurement of the adsorption of the pink colored complex formed by MDA at a wavelength of 532 nm. Total glutathione (tGSH) levels were determined by spectrophotometer.According to the study, the increase in MDA levels was not statistically significant in CL and RLD groups when compared with the control group. MDA was significantly increased in methyldopa, amlodipine and Ramipril groups. While total glutathione levels were only close to the control group in the CL group; RLD, MTL, ALD and RML groups showed statistically significant decrease. While CL and RLD did not cause infertility, infertility was observed in the groups treated with MTL-ALD-RML. Thus, it was found that MTL, ALD and RML, antihypertensive drugs, which were the aim of the study, had different effects on infertility and it was found that the use of such drugs decreased fertility due to increasing oxidative stress and decreasing antioxidant levels.

Keywords: antihypertensive drugs; antioxidant effect; infertility; oxidative stress

Introduction

Infertility is failing to establish a clinical pregnancy despite having regular sexual intercourse for at least a year without using contraceptive methods [1]. The leading causes of infertility are decreased ovarian reserve, ovulation, tubal, uterine, pelvic, male factors, and unexplained reasons [2].

In the literature, it is asserted that oxidative stress has a role in the pathogenesis of many diseases that cause infertility (ovarian ischemia-reperfusion injury, polycystic ovarian syndrome, infection, endometriosis, etc.) [3-6]. For example, long-term use of chemotherapeutic agents may cause oxidative stress associated with ovarian failure and infertility during childhood and the reproductive period [7, 8]. Likewise, it is reported that exposure to environmental factors such as organochlorine compounds, perfluorochemicals, and cigarette smoke causes infertility by inducing oxidative stress [6]. Similarly, it is argued that hypertension in chronic hypertensive women is associated with infertility [9, 10].

Several antihypertensive drugs are used to prevent hypertension and its complications. However, these drugs cause serious side effects like sedation, tremor (methyldopa), fetal development delay (atenolol, metoprolol), intrauterine developmental delay (labetalol), neonatal thrombocytopenia (hydralazine), edema, flushing (calcıum channel blockers) and fetal death [11-14]. In an experimental study with animals, antihypertensive drugs like clonidine and Rilmenidine did not cause a significant change in the oxidant and antioxidant parameters of ovarian tissue. However, methyldopa did cause mild oxidative damage, amlodipine caused moderate, and Ramipril caused

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severe oxidative stress ovarian damage [15]. In light of recent studies, it is seen that antihypertensive drugs causing oxidative stress in ovarian tissues may cause reproductive dysfunction [16-18]. No study was found in the literature on the effect of Clonidine, Rilmenidine, Methyldopa, Amlodipine, and Ramipril on animal reproductive function. For that reason, in our study, we aim to investigate the effect of Clonidine, Rilmenidine, Methyldopa, Amlodipine, and Ramipril on the reproductive functions of the rats and to show the role of oxidative stress in the probable reproduction dysfunction.

Materials and Methods

Determination of experimental animals: For the experiment, 36 albino Wistar species female rattus norvegicuss with a weight of 245-267 grams were used. Rattus norvegicuss were obtained from Atatürk University Medical Experimental Application and Research Center laboratories and kept in groups at normal room temperature (22° C).

Experiment Procedure: Experimental animals are grouped as healthy (control group), Clonidine (CL), Rilmenidine (RLD), Methyldopa (MTL), Amlodipine (AML), and Ramipril (RML) given groups. Rattus norvegicus in each group are marked with a number from one to six. Doses of Clonidine 0.075 mg/kg, Rilmenidine 0,5mg/kg, methyldopa 100mg/kg, amlodipine 2mg/kg and Ramipril 2mg/kg was given with a gavage to stomach orally to the rattus norvegicuss grouped as CL(n=6), RL (n=6), MTL(n=6), ALD (n=6) and RML (n=6). Drug dosage adjustment was determined according to the weight of the rats and the literature. [17]. The control group (n=6) is given distilled water in the same volume. This procedure is repeated each day once for thirty days. In the end, blood samples were taken from tail veins to analyze serum malondialdehyde (MDA) and total glutathione levels in the serum of all rattus norvegicus. After sampling, two mature male rattus norvegicuss are added to every group of six female rattus norvegicuss and kept in a proper laboratory environment for two months. Pregnant rattus norvegicuss are taken to the solitary cage with the proper setting during this time. Rattus norvegicuss that did not get pregnant and did not give birth during this period are considered infertile. The results were evaluated by comparison among the groups.

Biochemical Analyses

Preparations of the samples: Blood samples were drawn from all rattus norvegicuss and taken into serum tubes with separation gels. All the blood samples were incubated for 15 minutes at room temperature. The serum is separated by centrifuging with 1500x G for 10 minutes. All serum samples were kept at -80° C until the biochemical analysis.

Serum MDA levels: Serum MDA levels were evaluated in Atatürk University Medical Biochemistry Department Research Laboratory. The MDA level evaluation method used by Okhawa et al. is based on spectrophotometric measurement of the absorbance of the pink-colored complex formed by high temperature (95°C thiobarbituric acid (TBA) and MDA at a wavelength of 532 nm. [16].

Experiment Procedure: 0.1 ml serum sample was added to a 0.2mL solution of 80 g/L Sodium dodecyl sulfate, 1.5mL 200g/L acetic acid, 1.5mL 8 g/L 2-thiobarbiturate and 0.3 mL distilled water. The solution was incubated for one hour at 95° C. After cooling down, 5mL of n-butanol: pyridine (15:1) was added. The solution was vortex mixed and centrifuged for 30 minutes at 4000 rpm. The absorbance of the supernatant was measured at 532 nm. A standard curve was obtained by using 1,1,3,3- tetra methoxy propane.

Serum tGSH levels: Serum GSH levels were evaluated according to the method of Sedlak and Lindsay with a spectrophotometer device. DTNB (5 5'-dithiobis [2-nitrobenzoic acid]) disulfide is a chromogen and is reduced easily by sulfhydryl grouped compound, and the yellow color produced during reduction is measured with spectrophotometry at 412 nm wavelength.

Experiment Procedure: For measurement a cocktail solution (measurement tampon) (5.85 mL 100 mM Na-phosphate tampon, 2.8 mL 1 mM DTNB, 3.75 mL 1 mM NADPH and 80 uL 625 U/L Glutathione reductase) was prepared. Before measurement, 0.1 mL metaphosphoric acid was added to 0.1 mL serum for deproteinization and centrifuged for 2 minutes at 2000 rpm. 0.15mL cocktail solution was added into 50ul supernatant. A standard curve was obtained using oxidized glutathione

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(GSSG). The yellow color formed was read against distilled water at 412 nm wavelength in a spectrophotometer.

Statistical Analysis: Experiment results are given as "mean value± standard deviation." The conformity of the parameters to the normal distribution was evaluated with the Kolmogorow-Smirnow test. The degree of importance of intergroup difference was determined using a one-way ANOVA test and independent samples-t-test. All the statistical analysis was made with "IBM SPSS Statistics Version 20" (SPSS, Chicago, IL, United States) statistic program, and $p<0.05$ value is accepted as significant.

Results

Experiment Procedural Findings

The results obtained by the procedures mentioned in the material and method section are as follows.

No infertile rattus norvegicuss was found in the control group and six offspring was born from each in average. Mean pregnancy duration of the rattus norvegicuss in this group was 30 days. Mean pregnancy duration for rattus norvegicuss given clonidine (CL group) was 33 days with a similar number of offspring as the control group. Mean pregnancy duration for RDL group was 32 days. Mean offspring number for this group from each rattus norvegicus was 6 in average. All rattus norvegicuss in control, CL and RLD groups were fertile. One rattus norvegicus from MTL group was infertile. Other rattus norvegicuss in this group had 34 day pregnancy duration. Average number of offspring from MTL group was 6 excluding the infertile one. Number of pregnant rattus norvegicuss in ALD group was 4. Number of infertile in the same group is 2. Fertile rattus norvegicuss in this group had pregnancy duration of 38 days and had 5 offspring in average. RML group is the group with most infertility. In this group, 3 rattus norvegicuss were infertile and the other 3 had pregnancy duration of 45 days. Number of offspring in average was 4. (Table 1)

Table 1. Distribution of the reproduction test results of the groups.

Reproductive results	Control $\bar{X}$±SS	CL $\bar{X}$ ±SS	RLD $\bar{X}$±SS	MTL $\bar{X}$±SS	ALD $\bar{X}$±SS	RML $\bar{X}$±SS
Number of pregnant	6.00±1.02	6.00±1.12	6.00±1.05	5.01.08	4.00±0.9	3.00±0.8
Pregnancy duration(Days)	29.83±4..21	33.83±5.21	32.16±5.01	34.8±5.33	38.5±5.92	44.66±6.01
Infertil rats	0	0	0	1.00±0.01	2.00±0.22	3.00±1.02
Number of puppies born	6.83±1.5	6.33±1.4	6.16±1.1	6.20±1.5	5.00±1.0	4.66±0.9
Pup sex						
Male	24	20	21	18	8	5
Female	17	18	16	13	12	9

Results of Biochemical Analysis

Results of serum MDA levels: MDA and tGSH levels were measured from serums obtained from the rattus norvegicuss. According to the results in Table 2, RML group had the highest level of serum MDA. The lowest level of serum MDA was seen in control group.. According to the results in Table 2, control group had the highest level of tGSH among all groups and the lowest level of tGSH was seen in RML group.

Table 2. Comparison of MDA and tGSH results of each group with the control group.

Groups	Parameters	
	MDA	tGSH

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	$\bar{X}$±SS	$\bar{X}$±SS
CL	1.78±0.40	8.66±0.40
Control	1.55±0.33	9.28±0.45
p value	p=0.9	p=0.2
RLD	1.93±0.40	8.40±0.30
Control	1.55±0.33	9.28±0.45
p value	p=0.6	**p=0.02**
MTL	2.85±0.52	5.31±0.75
Control	1.55±0.33	9.28±0.45
p value	**p=0.001**	**p=0.001**
ALD	4.30±0.51	3.21±0.43
Control	1.55±0.33	9.28±0.45
p value	**p=0.001**	**p=0.001**
RML	6.18±0.55	1.76±0.30
Control	1.55±0.33	9.28±0.45
p value	**p=0.001**	**p=0.001**

Table 3. Comparison of serum MDA and tGSH levels between fertile and infertile rattus norvegicuss.

Parameters	**Fertility**		**Test and p value**
	Infertile (n=6)	**Fertile (n=30)**	
MDA(µmol/gr)	4.78±1.30	2.76±1.60	**t=2.85, p=0.007**
tGSH(nmol/gr)	2.86±1.45	6.75±2.75	**t=3.35, p=0.002**

Serum levels of MDA and tGSH of rattus norvegicuss were compared according to their fertility in table 3. In terms of both MDA and tSGH levels there is significant difference among fertile and infertile groups. (Consecutively p=0.007, p=0.002)

Discussion

Nowadays desire to have children is shifted towards the end of the reproductive period due to changes in socioeconomic circumstances, much more available university education and career plans etc. As a result both the prevalence of infertility and hypertension increase therefore the need for antihypertensive medication. In this study, we used common antihypertensive medication such as Clonidine, Rilmenidine, Methyldopa, Amlodipine and Ramipril. Prior studies investigated the effects of these medications on the oxidant and anti-oxidants parameters of uterine and ovarian tissues and found that these medications cause varying degrees of oxidative stress [17, 18].

Oxidative stress is the breaking of the equilibrium between free radicals and antioxidants towards free radicals. Free radicals are molecules that contain uneven number of electrons. Uneven number makes reactions between free radicals and other molecules easy therefore starting large chain chemical reactions and damaging the cell constituents such as proteins, lipids, DNA etc. [19]. Oxidative stress compromises female reproductive system by changing the efficiency of the individual's immune system. A lot of studies determined that toxic substances and medications affect fertility by increasing oxidative stress [20]. If we take increased incidence of hypertension and infertility into consideration, it is important to enlighten the mechanisms and the causes of oxidative stress in patients taking antihypertensive medication. For this reason, in our study we investigated the relationship between oxidative stress and infertility due to antihypertensive medication in rattus norvegicuss. If we evaluate the serum levels of oxidant MDA among groups, it is seen that MTL, ALD and RML increase serum MDA levels significantly when compared to control group and other antihypertensive medication in the study. However in CL and RLD groups serum MDA levels were close to control group levels. When we compare the serum level of antioxidant tGSH, CL group levels were close to control group levels, however, antioxidant tGSH serum level was significantly

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decreased in RLD, MTL-ALD and RML groups. It is stated in various studies that clonidine can alleviate the symptoms of withdrawal in some addictions, including smoking, by acting on the central nervous system apart from its antihypertensive usage [21]. According to the study performed by Reem et al., it was shown that Clonidine having depression like an effect increases oxidative stress (decreasing GSH and antioxidant enzyme superoxide dismutase, increasing MDA levels) in rattus norvegicuss brains [22]. In spite of that, according to the study of Yusoff et al. performed with experimental hypertension-induced rattus norvegicuss models, clonidine decreased oxidative stress and increased antioxidant levels [23]. The difference between the results of the studies performed by Reem et al. and Yusoff et al. is thought to be due to different experimental hypertensive models. The study carried out by Elkomy et al. showed that clonidine improves kidney functioning and decreases the inflammation and fibrosis of the kidney in rattus norvegicuss with induced chronic alcoholism. Clonidine decreased renal oxidative stress by decreasing myeloperoxidase (the enzyme producing hydrogen peroxide in phagolysosomes), malondialdehyde, inducible nitric oxide synthase- total nitric oxide levels and increase in superoxide dismutase level [24]. According to the data gathered from this study, an increase in MDA levels and a decrease in GSH levels are close to the control group in the clonidine-given group, and there was no statistically significant difference thus, no effect on infertility was noted. In a study investigating the effect of antihypertensive drugs on oxidant/antioxidant parameters of ovarian tissue by Salman et al., no prominent negative effect was seen with clonidine and Rilmenidine [18]. These findings support our findings while concluding the effect of clonidine on oxidative stress is debatable. Rilmenidine is an antihypertensive drug that stimulates sympathetic system effects on the central nervous system. According to the study of Malkoç et al., Rilmenidine increased serum levels of MDA and myeloperoxidase (MPO) in rattus norvegicuss kidney tissue samples more than methyldopa and Ramipril and less than clonidine and amlodipine. Therefore Rilmenidine had a nephrotoxic effect due to increased oxidative stress [25, 26]. In a study performed by Mercer et al., it is shown that 3, 4-metilendioksimetamfetaminin (MDMA) affects serotonin (5-HT) neurons primarily in the primitive brain. It causes degeneration of 5-HT axons and nerve fibers due to mitochondria-mediated oxidative stress, and it is shown that Rilmenidine protects 5-HT neurons against MDMA-mediated oxidative stress completely and selectively [27]. In a study investigating the effect of antihypertensive drugs, including Rilmenidine on the uterus, Rilmenidine was in the group with moderate negative effects [17]. In this study, MDA serum levels were close to the control group as in clonidine, and tGSH serum levels were significantly decreased. However, no infertile rattus norvegicuss was seen in this group. Methyldopa is one of the first-line antihypertensive drugs and is still one of the most commonly used antihypertensive drugs in the world [28]. Mahmud H. et al. investigated the side effect of methyldopa by reducing erythrocyte production or causing hemolysis resulting in anemia. Methyldopa caused oxidative stress by reducing GSH/GSSG ratio and thus causing anemia [29]. Methyldopa increased levels of MDA and decreased levels of GSH in uterine and ovarian tissue in a study by Salman et al. In our study increase in MDA serum levels and a decrease in serum tGSH level were found statistically significant, and one of the rattus norvegicuss became infertile. Amlodipine has antihypertensive and antioxidant activity in vivo. It inhibits oxidative stress-mediated cardiovascular damage due to angiotensin-II effectively. According to the study performed by Zhou, M. S. et al., amlodipine decreases blood pressure and aorta hypertrophy, has a significant antioxidant effect, and preserves endothelium function in angiotensin-II induced hypertension [30]. In a study by Ganafa et al. performed with hypertension-induced rattus norvegicuss by oxidative stress due to glutathione inhibition, it was shown that the antihypertensive effect of amlodipine was decreased by oxidative stress mediated by partial prostanoid endothelium-based factors and nitric oxide [31]. Amlodipine inhibited excessive MDA production and therefore reduced oxidative stress in atherosclerosis-induced rattus norvegicuss in another study. Amlodipine accelerates erythrocyte glutathione redox cycle activity and therefore increases the efficacy of the glutathione system [32]. In a study performed, it was shown that lipophilic calcium channel antagonists inhibit lipid peroxidation by changing the physicochemical features of the lipid bilayer of cell membranes independent of calcium channel inhibition. It was seen that amlodipine was the most powerful antioxidant among calcium channel

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blockers as a result of its different biophysical interactions with the lipid bilayer of the cell membrane [33]. In our study, Amlodipine caused a statistically significant increase in MDA levels and a decrease in GSH levels when compared with the control group and caused infertility in 2 rattus norvegicuss out of 6. In a study by Salman et al. investigating biochemical side effects of antihypertensive drugs on ovarian tissue, they classified amlodipine in the group of moderately negative effect. In this study, oxidative stress was increased with amlodipine in contrast to significant antioxidant effects stated in literature [34-38]. In our study Amlodipine decreased fertility more than Methyldopa and less than Ramipril. This clinical finding confirmed our results and shows that oxidative stress might be an indirect parameter. Ramipril is a strong antihypertensive drug. It was shown that Ramipril increases endothelium-dependent vasodilation in type 2 diabetic rattus norvegicuss probably by decreasing ROS serum levels (39]. It was shown that Ramipril decreases blood pressure and oxidative stress in post-transplant hypertensive patients [40]. In a study investigating Ramipril's neuroprotective efficacy in decreasing white matter lesions due to chronic hypo perfusion and inhibiting oxidative stress, the Ramipril-taking group had significant neuroprotection. Malondialdehyde (MDA) and oxidized glutathione (GSSG)/total glutathione (GSH t) ratios were significantly decreased in the Ramipril group. These results show that Ramipril can protect against white matter lesions caused by chronic ischemia due to its antioxidant features [41,42]. In our study, Ramipril was the drug with the highest MDA serum level and lowest GSH serum level. In our study, Ramipril had the most oxidative effect in contrast to its antioxidant effect in literature [39, 40] and in our opinion, this oxidative stress decreased fertility more than other antihypertensive medications. This is the group with three infertile rattus norvegicuss out of 6 with the highest ratio of infertility among groups. When you look at the literature for previous studies [17, 18] investigating the effect of chronic usage of antihypertensive medications on oxidant and antioxidant parameters of ovarian and uterine tissues, Ramipril had severe negative effects on both groups. These findings are consistent with our findings.

Conclusion and Recommendations

Experimental animals are divided into groups of healthy, Clonidine, Rilmenidine, Methyldopa, Amlodipine, and Ramipril. All the animals in each group were marked from 1 to 6. Clonidine 0.075mg/kg, Rilmenidine 0,5mg/kg, methyldopa 100mg/kg, amlodipine 2 mg/kg and Ramipril 2mg/kg were given orally with gavage into the stomach to the groups of CL (n-6), RL (n-6), MTL (n-6) and RML (n-6). The distilled water of the same volume is given to the control group with the same route. . This procedure is repeated each day once for thirty days. In the end, to analyze serum malondialdehyde (MDA) and total glutathione levels in the serum of all rattus norvegicuss, blood samples were taken from tail veins. After sampling, two mature male rattus norvegicuss are added to every group consisting of six female rattus norvegicuss and kept in a proper laboratory environment for 2 months. During this time, pregnant rattus norvegicuss are taken to the solitary cage with the proper setting. Rattus norvegicuss that did not get pregnant and did not give birth during this period are considered infertile. We evaluated the results by comparing amongst groups and the data obtained by those results as follows. According to the study we performed, in rattus norvegicuss given clonidine, oxidant and antioxidant parameters were close to the control group, and also reproduction rest results were similar to the control group. Statistically speaking, no significant difference in MDA levels was seen in CL and RLD groups ($p>0.05$), but a significant difference was seen in MTL, ALD, and RML groups ($p<0.05$). The highest level of MDA was seen in control group, with CL group coming close. It was found that methyldopa, amlodipine, and Ramipril have negative effects on fertility due to increased oxidative stress and decreasing antioxidant levels, thus causing varying degrees of infertility. One rattus norvegicus from MTL group was infertile and did not get pregnant. Four rattus norvegicuss were pregnant, and 2 rattus norvegicuss were infertile in ALD group. RML group is the group with the most infertile rattus norvegicuss with a number of 3. In light of those data, the following recommendations are considered appropriate. Oxidative stress is increased in women with infection; thus, antioxidants are used to treat infertility caused by oxidative stress and succeeded. L- Carnitine (LC) and acetyl L-carnitine (ALC) have massive

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functional features to regulate oxidative and metabolic statements of a woman's reproductive system. The vulnerability of a woman's reproductive system to free radicals prompts new advanced treatments. For this purpose, “half vitamins” LC and ALC can be used separately or together or with other antioxidants. The mean marriage age and mean age for conception are increasing in our country as it increases in the whole world. This situation increases the probability of hypertension and the usage of antihypertensive medication as a comorbidity in women with the desire to have children. We are of the opinion that public medicine and infertility experts should be aware of this fact. There is very little information in the literature about the association between antihypertensive medication and female infertility treatment. For this reason, larger randomized trials are needed. It is a new field of research to develop a new group of antihypertensive drug that decreases oxidative stress and nitric acid, thus preventing hypertension and complications developed by hypertension.

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