Chapter

ZDF Rats: A Suitable Model to Study Male Reproductive Dysfunction in Diabetes Mellitus Type 2 Patients

Filip Benko, Mária Chomová, Oľga Uličná and Eva Tvrdá

Abstract

This chapter examines the impact of diabetes mellitus type 2 (DM 2) on the vitality of male reproductive cells collected from Zucker diabetic fatty (ZDF) rats which could be a suitable experimental model for simulating this metabolic disorder. Epididymal spermatozoa were subjected to the assessment of motility, membrane integrity, mitochondrial activity, DNA fragmentation, and oxidative profile. Our results show that DM 2 in combination with obesity negatively affects the sperm vitality and increases the chances of oxidative damage to male gametes. In conclusion we may state that DM 2 has a negative impact on the spermatogenic aspect of male fertility and decreases the sperm quality.

Keywords: diabetes mellitus type 2, obesity, ZDF rats, male reproduction, spermatozoa

1. Introduction

Diabetes mellitus is a chronic metabolic dysfunction which involves alterations in insulin production. Pancreatic β cells are primarily responsible for insulin secretion. There is a variety of complications associated with this disease such as chronic hyperglycemia, neuropathy, retinopathy, and cardiovascular diseases. Diabetes mellitus may be divided into three types: diabetes mellitus type 1 (DM1), type 2 (DM2), and type 3 (DM3)—also known as gestational diabetes mellitus. DM1 is based on an immune-mediated destruction of β cells. It is an autoimmune disease, most common in children and young adults. The treatment includes monitoring of blood glucose levels and insulin therapy [1, 2]. DM2 is associated with insulin resistance. The main cause is a reduced sensitivity of affected tissues to the metabolic effects of insulin. The development of the disease is often associated with obesity, which, on the other hand, causes health complications, such as fertility issues. Contraindications associated with DM2 include disorders of the male reproductive system because metabolism of glucose is essential for a correct process of spermatogenesis. DM2 has a negative impact on the sperm quality markers such as motility, DNA integrity, and seminal plasma composition [3, 4]. Development of DM3 is mostly common among women who are overweight or obese in comparison with thin or normal-weight women. This type of diabetes

may cause higher levels of adipose tissue in the fetus and an increased child birth weight. Also a few studies reported that DM3 is associated with higher levels of abdominal fat and an increased risk for visceral adiposity which could be linked to other consequences following birth such as future development of DM2 and cardiovascular diseases [5, 6].

Another factor that negatively affects male fertility is obesity. Obesity has been shown to negatively affect the male reproductive potential not only by decreasing sperm quality but mainly by altering the germ cell molecular structure in the testes [7]. Animal models play a pivotal role in monitoring and understanding DM pathogenesis due to a combination of their genetic and functional characterization [8]. Suitable models to demonstrate the effects of DM2 on the organism are Zucker diabetic fatty (ZDF) rats. This type of rats had been discovered in 1961 following cross-breeding of Merck (M-strain) and Sherman rats. ZDF rats have the unique ability to simulate symptoms and contraindications of DM2. These rats have insulin resistance caused by the presence of homozygous mutation of the leptin hormone receptor (*fa gene*) which causes obesity and an increased insulin secretion [9, 10]. The aim of this chapter was to evaluate the effect of diabetes mellitus type 2 on the vitality of ZDF rat reproductive cells.

2. Material and methods

2.1 Biological material

For the experiment three separate groups (**Figure 1**) of adult ZDF and Wistar rats at the age of 120 days were used. The animals were obtained from the Institute of Experimental Pharmacology (Slovak Academy of Sciences, Slovakia) and kept in plastic cages at 24 ± 1°C and 12 h light/12 h dark photoperiod. The animals were provided with water ad libitum. Institutional and national guidelines for the care and use of laboratory animals were followed, and all procedures were approved by the State Veterinary and Food Institute of the Slovak Republic (no. 493/18-221/3) and Ethics Committee.

The first group consisting of 15 Wistar rats (15) was the healthy control group. The second group consisted of 15 ZDF rats on a normal diet (ZDN), while the third group comprised 16 ZDF rats on a special high-caloric diet (Purina 5000; ZDF).

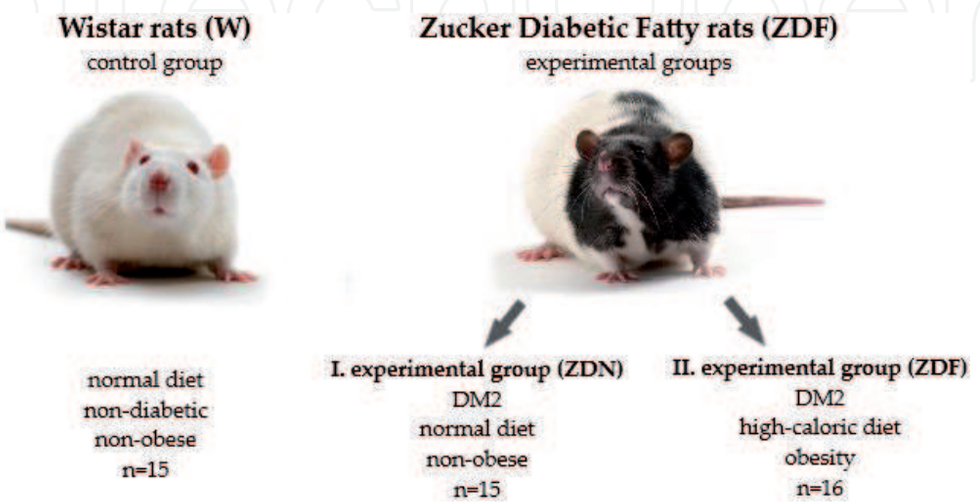

Figure 1.
Distribution of the tested animals (source: Author).

All ZDN and ZDF rats had diabetes confirmed by a blood test. Overall, we analyzed samples from 46 rats.

Following anesthesia and decapitation, epididymes were collected from the rats, cut with a scalpel into smaller fragments, and incubated in phosphate buffer saline (PBS, with Ca^{2+} and Mg^{2+}, Sigma-Aldrich, St. Louis, USA) at 37°C for 15 minutes. After incubation we used the epididymal spermatozoa for further assessment of sperm quality, including motility, membrane and acrosome integrity, DNA fragmentation, mitochondrial activity, lipid peroxidation (LPO), and production of superoxide radicals.

2.2 Sperm motility

Sperm motility is one of the most important parameters examining the fertilizing ability of male gametes. This parameter has an important role in the fertilization process. We examined the motility manually. Rats are known for a high level of motile spermatozoa, generally within a range of 85–96% [11]. After sample collection we applied 10 μl of the epididymal sperm suspension into Makler's counting chamber and counted motile spermatozoa using a light microscope (Olympus, Tokyo, Japan) and a magnification of 40×.

2.3 Membrane integrity

Membrane integrity is a parameter which defines the quality and condition of the reproductive cells. For the determination of membrane integrity, we used a combination of eosin and nigrosin dye. This protocol follows a differential staining method for the analysis of vital and damaged cytoplasmatic membrane in sperm cells of mammals and birds. Eosin is one of the most common dyes to stain the cytoplasm and cytoplasmic proteins of cells. After application of eosin, damaged cytoplasmatic membrane of sperm cells absorbed the dye and changes color into light-red or light-pink. The vital spermatozoa stayed without any change of color. Nigrosin (Sigma-Aldrich, St. Louis, USA) provides background for the smear as a contrast dye for a better differentiation [12–14]. The slides for the analyses were prepared as follows: we applied a drop of semen on the slide and dyed it with 4 μl of eosin solution (Sigma-Aldrich, St. Louis, USA). After application of eosin, we did the same with the nigrosin solution (Sigma-Aldrich, St. Louis, USA), and using another slide glass, we did a smear on the slide. The samples were air-dried at room temperature and observed using a light microscope (Olympus, Tokyo, Japan) and a magnification of 40×. At least 100 cells were evaluated in each slide.

2.4 Acrosome integrity

For the analysis of the acrosome integrity, we used a double fast green–rose bengal stain. After application of 10 μl of semen in a glass slide, we added the same volume of fast green–rose bengal mixture (Sigma-Aldrich, St. Louis, USA), incubated for 60 seconds, and used another slide glass to prepare a smear which was subsequently air-dried at room temperature. With the help of a light microscope (Olympus, Tokyo, Japan), we observed the integrity and compactness of the sperm acrosome. Damage to the acrosome was observed as a disruption of the membrane and cluster of stain localized in the sperm head. We evaluated at least 100 cells in each slide and calculated the percentage of cells with a normal or a damaged acrosome.

2.5 Test of metabolic activity

The mitochondrial toxicity test (MTT test) is a colorimetric test often used for the determination of cell metabolic activity. The main principle of the test is the application of yellow tetrazolium bromide (Sigma-Aldrich, St. Louis, USA) and its subsequent reaction with insoluble mitochondrial succinate dehydrogenase produced by cell mitochondria. This reaction ends with the formation of a blue-violet formazan [15]. The amount of formed formazan is directly proportional to the level of metabolic activity of the cells. We evaluated this test spectrophotometrically at 570 against 620 nm (Multiscan reader, Thermo Fisher, Vantaa, Finland).

2.6 Production of superoxide radicals

For the detection of the superoxide radical concentration, we used the nitroblue tetrazolium or NBT test. The principle of this method is the application of nitroblue tetrazolium (Sigma-Aldrich, St. Louis, USA) to the semen sample. This substance reacts with cellular superoxide to form derivates of formazan. Following washing with PBS and centrifugation (1250 rpm, 5 min), the concentration of formasan derivates was evaluated spectrophotometrically [16] at wavelengths of 620 against 570 nm (Multiscan reader, Thermo Fisher, Vantaa, Finland).

2.7 DNA fragmentation

This type of DNA damage is characterized by both single and double DNA strand breaks. Several types of DNA damage may be observed in mammalian germ cells and are often associated with male infertility. Nowadays various tests are available to detect sperm DNA damage. DNA fragmentation arises from various reasons such as deficiencies in recombination during spermatogenesis, abnormal sperm maturation, abortive apoptosis, or oxidative stress [17, 18]. In our research we used the chromatin-dispersion test by using the Halomax diagnostic kit (Halotech, Madrid, Spain). This kit can analyze the integrity of the DNA molecule and is based on a controlled DNA denaturation process to facilitate the subsequent removal of the proteins contained in each spermatozoon. The main principle of this method is that damaged spermatozoa create halos formed by loops of fragmented DNA at the head of the sperm which are not present in normal spermatozoa. For the evaluation we used a fluorescent microscope (Leica, Holzheim, Germany) and a magnification of 40×. At least 300 cells were evaluated for DNA fragmentation in each slide containing agarose with processed spermatozoa.

2.8 Lipid peroxidation

Lipid peroxidation is a process of membrane lipid degeneration caused by free radicals [19]. The extent of lipid peroxidation in our samples was expressed as the amount of malondialdehyde (MDA) production following the addition of thiobarbituric acid (Sigma-Aldrich, St. Louis, USA) and exposure to heat (100°C, 1 h). The MDA concentration in the samples was evaluated spectrophotomerically at a wavelength of 540 nm (Multiscan reader, Thermo Fisher, Vantaa, Finland).

3. Statistical analysis

The data we obtained were statistically processed using GraphPad Prism (version 6.0 for Windows, GraphPad Software incorporated, San Diego, California,

USA, http://www.graphpad.com/). Differences between the compared groups were statistically evaluated by one-way analysis of variance (ANOVA) and the Tukey comparative test. Statistical significance was assessed at levels ***($P < 0.001$), **($P < 0.01$), and *($P < 0.05$).

4. Results and discussion

When we compared the sperm motility of individual groups (**Figure 2**), we found statistically significant differences ($P < 0.001$) when comparing the experimental group ZDF with the control W group as well as with the ZDN experimental group. Similarly to our results, Ohta et al. [20] reported a reduction in the sperm motility in rats with obesity in comparison with the control group. They tried to demonstrate the potential link between obesity and a reduction of sperm motility, which ultimately affects the fertility of rats. Simas et al. [21] also reported a decrease of morphologically normal spermatozoa in rats with diabetes. The most common abnormalities found were sperm head deformations and flagella deformities. There is a very close connection between the sperm volume and percentage of motile spermatozoa. Both types of diabetes have a serious impact on the sperm quality, and a lot of studies examine the effect of diabetes on the male fertility. Condorelli et al. [22] showed different pathophysiological effects in type 1 and type 2 of diabetes on the sperm function and quality. The aim of their experiment was to compare DM1 and DM2 patients with healthy and fertile subjects. Male patients suffering from diabetes mellitus type 2 have several contraindications. Among these, the most prominent include low sperm volume, higher concentration of mitochondrial superoxide anions, an increased reactive oxygen species production in the seminal fluid, and lipoperoxidation. The study showed that DM2 caused an inflammatory condition and increased the level of oxidative stress which led to an increased sperm DNA fragmentation and a decreased vitality of spermatozoa. In our experiments we also observed several contraindications in the rats which suffered from diabetes mellitus type 2 including a decreased motility and vitality of sperm cells, a lower mitochondrial activity, followed by higher levels of superoxide production and lipid peroxidation. On the other hand, a combination of DM2 and obesity has more serious consequences to male fertility.

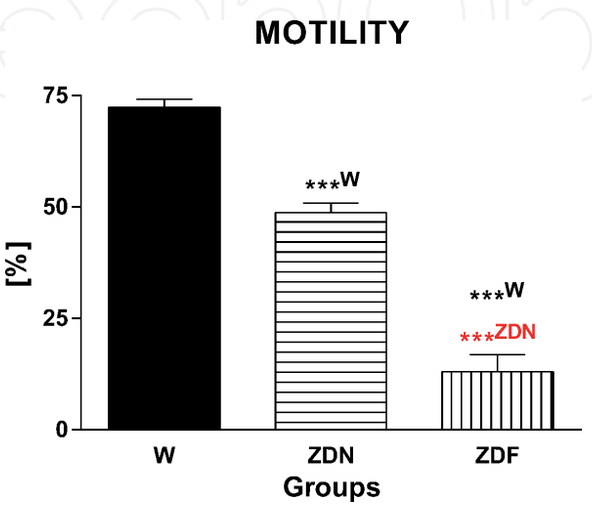

Figure 2.
*Differences in the sperm motility among the observed groups. W, Wistar rats, control group; ZDN, nonobese ZDF rats with diabetes mellitus type 2; ZDF, ZDF rats with obesity and diabetes mellitus type 2. ***($P < 0.001$). ^{W}, compared to the Wistar group; ^{ZDN}, compared to the ZDN group.*

Numerous studies have shown the negative impact of obesity on the semen quality. Obesity causes a decrease of sperm concentration and motility and an increased DNA fragmentation index, and it is often associated with erectile dysfunction. Fernandez et al. [23] examined the impact of obesity on the fertility in male Wistar rats which were fed a high-fat diet and compared these with the control group consisting of nonobese rats. Similarly to our study, their results reported that obese animals have a low sperm quality caused by a decreased percentage of spermatozoa with progressive motility which may lead to the development of subfertility. We also recorded higher levels of non-motile spermatozoa in obese rats which were fed with a high-caloric diet.

The evaluation of the membrane integrity of spermatozoa (**Figure 3**) showed statistically significant differences ($P < 0.001$) among the groups. The sperm vitality of the ZDF experimental group was significantly lower than the control W group as well as with the ZDN experimental group. Sabeti et al. [24] also observed statistically significant differences ($P < 0.05$) in the viability between the experimental and control group of rats suffering from diabetes.

The membrane integrity is also affected by the level of cholesterol in the organism. Normal levels of cholesterol are highly important for the membrane fluidity and sperm motility. If there is an increase in the cholesterol levels because of the presence of obesity, then more cholesterol is incorporated into the lipid bilayer of the cells, leading to its adverse effects on the membrane integrity. These facts support the theory that changes in the levels of systemic cholesterol, triglyceride, and free fatty acids are associated with alterations of the cell membrane dynamics, sperm motility, morphology, and susceptibility to DNA damage [25].

As the graph shows, there were no statistically significant differences between any of the studied groups (**Figure 4**). In our opinion this condition could show that diabetes and obesity affect other semen parameters, but the integrity of the acrosome remains unchanged. It is important to know that diabetes mellitus type 2 in combination with obesity does not cause acrosome deformations. This theory was supported by Ding et al. [3] who studied differences between diabetes mellitus type 1 and type 2. In their study, they found that diabetes mellitus type 1 has a markedly impaired glycation process and synthesis of proteins, leading to the development of acrosome integrity disorders, while diabetes mellitus type 2 has less serious consequences to the acrosome. The acrosome integrity was the only parameter which remained stable. It could be caused by the presence of acid hydrolases

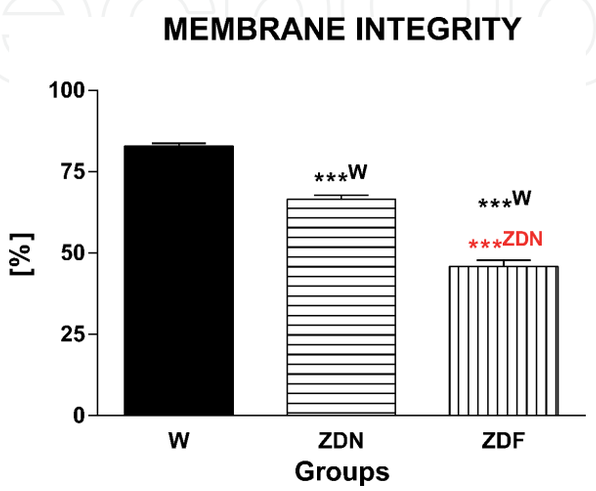

Figure 3.
*Differences in the membrane integrity or viability among the observed groups. W, Wistar rats, control group; ZDN, nonobese ZDF rats with diabetes mellitus type 2; ZDF, ZDF rats with obesity and diabetes mellitus type 2. ***($P < 0.001$). ^{W}, compared to the Wistar group; ^{ZDN}, compared to the ZDN group.*

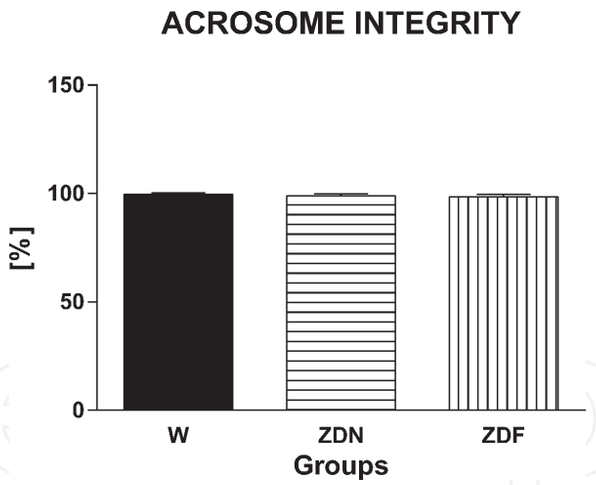

Figure 4.
Acrosome integrity among the observed groups. W, Wistar rats, control group; ZDN, nonobese ZDF rats with diabetes mellitus type 2; ZDF, ZDF rats with obesity and diabetes mellitus type 2. ^{W}, compared to the Wistar group; ^{ZDN}, compared to the ZDN group.

and lysosomal proteins because the acrosome does not need carbohydrates for its function. The presence of polyunsaturated fatty acids in the plasma membrane could exhibit protective effects against the negative activity of ROS and prevent alterations to the acrosome. This theory was also supported by Reddy et al. [26] who tested diabetic-induced reproductive toxicity in male Wistar rats.

The evaluation of the MTT test (**Figure 5**) showed statistically significant differences ($P < 0.5$) between the experimental group ZDF and the experimental group ZDN as well as between the experimental group ZDN and the control group W. Another statistically significant difference ($P < 0.01$) was observed between the ZDN group and the W group. Our results show that the lowest values of mitochondrial activity were observed in the experimental ZDF group. Mitochondrial activity is closely related to the motility as mitochondria are the energy-metabolic center of the cell. We also observed the lowest sperm motility values in ZDF group of rats (**Figure 1**). In the analysis of diabetic rats, Simas et al. [21] reported a significant decrease in the metabolic activity of these rats compared to the control group. Experimental animals had an induced DM1. In all group of rats with diabetes, they observed an increase of inactive mitochondria which caused a

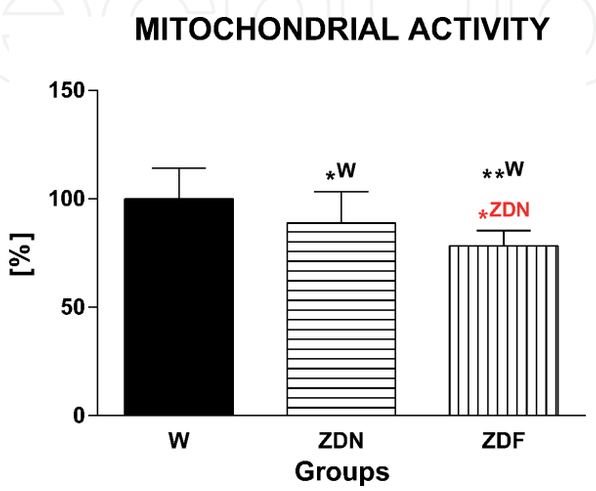

Figure 5.
*Mitochondrial activity among the observed groups. W, Wistar rats, control group; ZDN, nonobese ZDF rats with diabetes mellitus type 2; ZDF, ZDF rats with obesity and diabetes mellitus type 2. **($P < 0.01$); *($P < 0.5$). ^{W}, compared to the Wistar group; ^{ZDN}, compared to the ZDN group.*

decrease of mitochondrial activity. Like Simas et al., we also observed low levels of mitochondrial activity in the ZDF group when compared to other groups. A decrease of mitochondrial activity was also observed in the experimental ZDN group. This phenomenon could be because diabetes mellitus had a negative impact on the metabolism of carbohydrates, which are the source of energy for a proper mitochondrial function.

Results of the superoxide radical production (NBT test) (**Figure 6**) showed statistically significant differences ($P < 0.05$; $P < 0.01$) when we compared both experimental groups with the control group W. In the experimental ZDF group, we recorded the highest superoxide radical production. This finding suggests that the group of ZDF rats is more at risk for the onset of oxidative stress, which negatively affects the viability of spermatozoa produced by these rats. Sabeti et al. [24] demonstrated that the abnormal production and presence of the superoxide radicals in combination with obesity leads to a reduced semen quality and sperm viability. Overproduction of superoxide may also be due to an increase in metabolic functions in order to maintain the homeostasis in obese individuals. Amaral et al. [27] reported no significant differences in the testicular cell concentration between diabetic and nondiabetic rats, but there was a massive decline in the sperm cell concentration due to the influence of hyperglycemia in late stages of spermatogenesis and increased production of reactive oxygen species (ROS) that may lead to the development of oxidative stress. The consequences of oxidative damage include the loss of motility due to lipid peroxidation, induction of DNA damage of the sperm nucleus, and defects in spermatogenesis affecting the final fertilizing potential of sperm cells.

The oxidative environment in testicular tissue of patients with DM2 may result in cellular damage, including lipid peroxidation, formation of carbonyl groups, and DNA damage which causes sperm abnormalities. In fact, this negative condition contributes to a reduction in the sperm motility and viability followed by an increased percentage of abnormalities in the sperm cells of rats [28].

Figure 7 points out the levels of DNA fragmentation between the experimental groups and the control. After evaluation of our results, we found statistically significant differences between the DNA fragmentation following the presence of diabetes mellitus type 2 and obesity. Statistically significant differences ($P < 0.001$) were observed when we compared the experimental ZDF group with the ZDN group and the control W group and also when we compared the experimental ZDN group with the control W group. As seen from our results, a continuous increase in

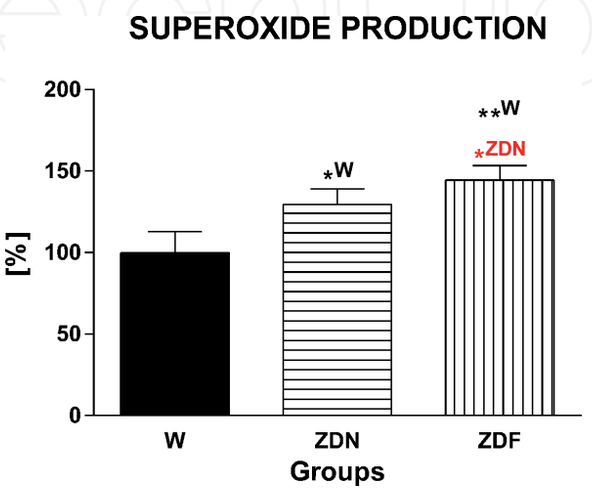

Figure 6.
*Production of superoxide among the observed groups. W, Wistar rats, control group; ZDN, nonobese ZDF rats with diabetes mellitus type 2; ZDF, ZDF rats with obesity and diabetes mellitus type 2. **(P < 0.01); *(P < 0.5). ^{W}, compared to the Wistar group; ^{ZDN}, compared to the ZDN group.*

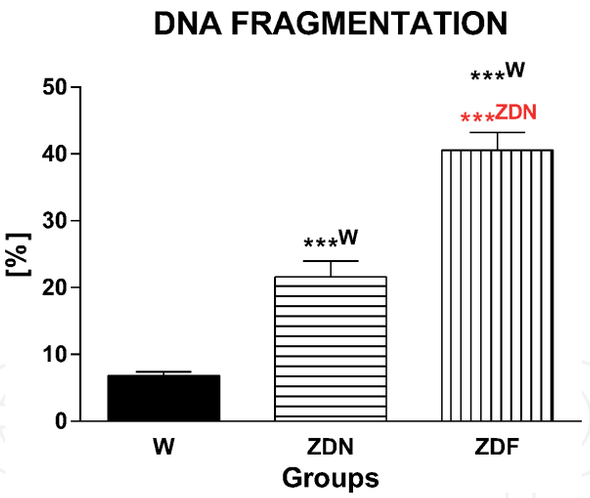

Figure 7.
*Levels of DNA fragmentation among the observed groups. W, Wistar rats, control group; ZDN, nonobese ZDF rats with diabetes mellitus type 2; ZDF, ZDF rats with obesity and diabetes mellitus type 2. *** ($P < 0.001$). ^{W}, compared to the Wistar group; ^{ZDN}, compared to the ZDN group.*

DNA fragmentation was present, depending on the presence of diabetes mellitus type 2, obesity, or both. Abbasihormozi et al. [29] found the link between leptin receptor mutation and higher levels of DNA fragmentation as a consequence of a higher production of reactive oxygen species. Leptin is an adipose tissue-derived hormone which has an important role in the metabolism and energy homeostasis and ensures a proper function of the neuroendocrine and reproductive system. A direct administration of leptin could affect male infertility by ROS overproduction or hormone profile modulation, but the exact mechanism of the effect of leptin on spermatogenesis is still not understood.

As with the evaluation of the superoxide radical production, we found statistically significant differences ($P < 0.001$) in the case of the concentration of malondialdehyde (**Figure 8**) among both experimental groups (ZDN and ZDF) with the control group (W) and also when comparing the experimental group ZDF with the second experimental group ZDN. Like us, Simas et al. [21] evaluated that there was an increase in the MDA concentration in diabetic rats when compared to all other groups. They also recorded a direct correlation between the superoxide radical production values and MDA concentration. Vignera et al. [30] found a link between

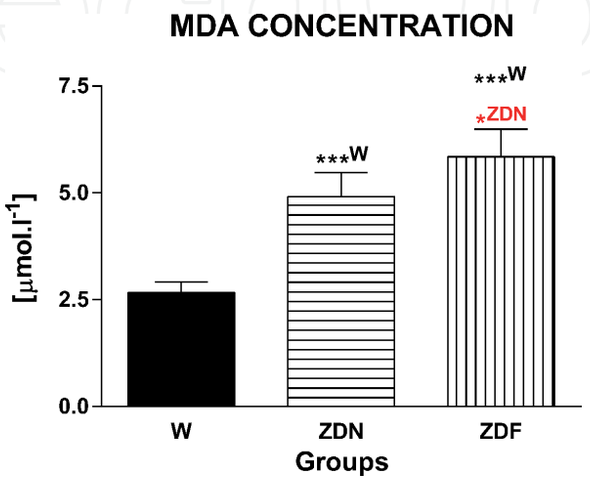

Figure 8.
*Concentration of malondialdehyde (MDA) among the observed groups. W, Wistar rats, control group; ZDN, nonobese ZDF rats with diabetes mellitus type 2; ZDF, ZDF rats with obesity and diabetes mellitus type 2. ***($P < 0.001$); *($P < 0.5$). ^{W}, compared to the Wistar group; ^{ZDN}, compared to the ZDN group.*

production of MDA and regression of enzymatic activity. The activity of malondialdehyde and caspase-3 were significantly higher, whereas the enzymatic activities of superoxide dismutase and glutathione peroxidase were significantly lower in the diabetic rats than in the control group of healthy rats. Supportive antioxidant treatment could help to increase the antioxidant enzymatic activity and to decrease the activity of malondialdehyde and caspase-3. This treatment also could reduce the apoptosis of germ cells. These facts support the theory about testicular oxidative damage caused by diabetes and subsequent protective effects of antioxidant treatment.

In our opinion the decrease of rat sperm vitality was associated with high levels of body fat because adipose tissue works as an individual endocrine system affecting spermatogenesis and steroidogenesis. On the other hand, diabetes mellitus type 2 affected correct mechanisms of spermatogenesis as carbohydrates are the main source of energy in spermatozoa.

5. Conclusion

The results of our analysis show that diabetes mellitus type 2 has a negative effect on the vitality of rat male reproductive cells. We may conclude that the disease affects sperm motility, viability, and DNA stability negatively. The disease increases the production of superoxide radicals and the concentration of malondialdehyde and also affects the mitochondrial activity of spermatozoa. However, diabetes mellitus type 2 has no effect on the acrosome integrity. At the same time, we observed visible differences in the experimental group of ZDF rats suffering from diabetes mellitus type 2 in combination with obesity in all evaluated parameters. However, differences were also observed in the experimental group of ZDN rats who developed type 2 diabetes mellitus but did not have obesity.

Acknowledgements

This research was created with financial support of the projects APVV-15-0544 and VEGA 1/0314/19.

Conflict of interest

None declared.

Abbreviations

CASA	Computer-assisted sperm analysis
DM1	Diabetes mellitus type 1
DM2	Diabetes mellitus type 2
GDM	Gestational diabetes mellitus
MDA	Malondialdehyde
MTT	Mitochondrial toxicity test
NBT	Nitroblue tetrazolium
ROS	Reactive oxygen species
ZDF	Zucker diabetic fatty rats with high-caloric diet
ZDN	Zucker diabetic fatty rats with normal diet
W	Wistar rats

Author details

Filip Benko[1], Mária Chomová[2], Oľga Uličná[3] and Eva Tvrdá[1]*

1 Department of Animal Physiology, Faculty of Biotechnology and Food Sciences, Slovak University of Agriculture in Nitra, Slovakia

2 Faculty of Medicine, Institute of Medical Chemistry and Clinical Biochemistry, Comenius University in Bratislava, Slovakia

3 Third Intern Clinic, Faculty of Medicine, Comenius University in Bratislava, Slovakia

*Address all correspondence to: evina.tvrda@gmail.com

References

[1] Al-awar A, Kupai M, Veszelka M, Szűcs G, Attieh Z, Murlasits Z, et al. Experimental Diabetes Mellitus in Different Animal Models. Journal Diabetes Research. 2016;**2016**:1-12. DOI: 10.1155/2016/9051426

[2] Siwy J, Zoja C, Klein J, Benigni A, Mullen W, Mayer B, et al. Evaluation of the Zucker diabetic fatty (ZDF) rat as a model for human disease based on urinary peptidomic profiles. PLoS One. 2012;**7**(12):e51334. DOI: 10.1371/journal.pone.0051334

[3] Ding G, Liu Y, Liu M, Pan J, Guo M, Sheng J, et al. The effects of diabetes on male fertility and epigenetic regulation during spermatogenesis. Asian Journal of Andrology. 2015;**17**:948-953. DOI: 10.4103/1008-682X.150844

[4] Temidayo O, du Plessis S. Diabetes mellitus and male infertility. Asian Pacific Journal of Reproduction. 2018;**7**:6-14. DOI: 10.4103/2305-0500.220978

[5] Chu S, Callaghan W, Kim S, Schmid C, Lau J, England L, et al. Maternal obesity and risk of gestational diabetes mellitus. Diabetes Care. 2007;**30**:2070-2076. DOI: 10.2337/dc06-2559a

[6] Page K, Romero A, Buchanan T, Xiang A. Gestational diabetes mellitus, maternal obesity, and adiposity in offspring. The Journal of Pediatrics. 2014;**164**(4):807-810. DOI: 10.1016/j.jpeds.2013.11.063

[7] Palmer N, Bakos H, Fullston T, Lane M. Impact of obesity on male fertility, sperm function and molecular composition. Spermatogenesis. 2012;**2**:253-263. DOI: 10.4161/spmg.21362

[8] Teerds KJ, De Rooij DG, Keijer J. Functional relationship between obesity and male reproduction: From humans to animal models. Human Reproduction Update. 2011;**17**:667-683. DOI: 10.1093/humupd/dmr017

[9] Griffen S, Wang J, German M. A genetic defect in beta-cell gene expression segregates independently from the fa locus in the ZDF rat. Diabetes. 2001;**50**:63-68. DOI: 10.2337/DIABETES.50.1.63

[10] Phillips M, Liu Q, Hammond H, Dugan V, Hey P, Caskey C, et al. Leptin receptor missense mutation in the fatty Zucker rat. Nature Genetics. 1996;**13**:18-19. DOI: 10.1038/ng0596-18

[11] Creasy D, Chapin R. Sperm motility. In: Toxicologic Pathology. 3rd ed. Cambridge, Massachusetts, USA: Academic Press; 2013. pp. 2859-2963. DOI: 10.1016/C2010-1-67850-9

[12] Anthony A, Leslie G. Membrane integrity monitoring tests. In: Advanced Membrane Science and Technology for Sustainable Energy and Environmental Applications. Sawston, Cambridge, UK: Woodhead Publishing; 2011. pp. 792-818

[13] Knebel Doeberitz M, Wentzensen N. Cytoplasmatic stain. In: Comprehensice Cytopathology. 3rd ed. Philadelphia, Pennsylvania, USA: Saunders; 2008. pp. 1091-1120. DOI: 10.1016/B978-1-4160-4208-2.X0041-3

[14] Kondracki S, Wysokińska A, Kania M, Górski K. Application of two staining methods for sperm morphometric evaluation in domestic pigs. Journal of Veterinary Research. 2017;**61**:345-349. DOI: 10.1515/jvetres-2017-0045

[15] Bahuguma A, Khan I, Bajpai V, Kang S. MTT assay to evaluate the cytotoxic potential of a drug.

Bangladesh Journal of Pharmacology 2017;**12**:115-118

[16] Esfandiari N, Sharma R, Saleh R, Thomas A, Agarwal A. Utility of the Nitroblue Tetrazolium reduction test for assessment of reactive oxygen species production by seminal leukocytes and spermatozoa. Journal of Andrology. 2013;**24**:862-870. DOI: 10.1002/j.1939-4640.2003.tb03137.x

[17] González-Marín C, Gosálvez J, Roy R. Types, causes, detection and repair of DNA fragmentation in animal and human sperm cells. International Journal of Molecular Sciences. 2012;**13**:14026-14052. DOI: 10.3390/ijms131114026

[18] Enciso M, Sarasa J, Agarwal A, Fernández JL, Gosálvez J. A two-tailed comet assay for assessing DNA damage in spermatozoa. Reproductive BioMedicine Online. 2009;**18**:609-616

[19] Ayala A, Muñoz F, Argüelles S. Lipid peroxidation: Production, metabolism and signaling mechanisms of malondialdehyde and 4-Hydroxy-2-Nonenal. Oxidative Medicine and Cellular Longevity. 2014;**2014**:360438. DOI: 10.1155/2014/360438

[20] Ohta T, Katsuda Y, Miyajima K, Sasase T, Kimura S, Tong B, et al. Gender differences in metabolic disorders and related diseases in spontaneously diabetic Torii-Lepr (fa) rats. Journal Diabetes Research. 2014;**2014**:841957. DOI: 10.1155/2014/841957

[21] Simas J, Mendes T, Paccola C, Vendramini V, Miraglia S. Resveratrol attenuates reproductive alternations in type 1 diabetes-induced rats. Journal of Experimental Pathology. 2017;**98**:312-328. DOI: 10.1111/iep.12251

[22] Condorelli R, La Vignera S, Mongioi L, Alamo A, Calogero A. Diabetes mellitus and infertility: Different pathophysiological effects in type 1 and type 2 on sperm function. Frontiers in Endocrinology. 2018;**9**:268. DOI: 10.3389/fendo.2018.00268

[23] Fernandez C, Bellentani F, Fernandes G, Perobelli J, Favareto A, Nascimento A, et al. Diet-induced obesity in rats leads to a decrease in sperm motility. Reproductive Biology and Endocrinology. 2011;**9**:32. DOI: 10.1186/1477-7827-9-32

[24] Sabeti P, Pourmasumi S, Rahiminia T, Akyash F, Talebi A. Etiologies of sperm oxidative stress. International Journal of Reproductive BioMedicine. 2016;**14**:231-240

[25] Palmer N, Bakos H, Owens J, Setchell B, Lane M. Diet and exercise in an obese mouse fed a high-fat diet improve metabolic health and reverse perturbed sperm function. American Journal of Physiology - Endocrinology and Metabolism. 2012;**302**:768-780. DOI: 10.1152/ajpendo.00401.2011

[26] Reddy K, Narayana Rao M, Murthy J, Reddy P. Lead aggravates the diabetic-induced reproductive toxicity in male Wistar rats. Toxicology Research. 2016;**5**:1465-1476. DOI: 10.1039/c6tx00099a

[27] Amaral S, Moreno A, Santos M, Seica R, Ramalho-Santos J. Effects of hyperglycemia on sperm and testicular cells of Goto-Kakizaki and streptozotocin-treated rat models for diabetes. Theriogenology. 2006;**66**:2056-2067. DOI: 10.1016/j.theriogenology.2006.06.006

[28] Oliviera P, Tomás G, Dias T, Martins A, Rato L, Alves M, et al. White tea consumption restores sperm quality in prediabetic rats preventing testicular oxidative damage. Reproductive Biomedicine Online. 2015;**31**:544-556. DOI: 10.1016/J.RBMO.2015.06.021

[29] Abbasihormozi S, Shahverdi A, Kouhkan A, Cherargi J, Akhlaghi A, Kheimeh A. Relationship of leptin administration with production of reactive oxygen species, sperm DNA fragmentation, sperm parameters and hormone profile in the adult rat. Archives of Gynecology and Obstetrics. 2013;**287**:1241-1249. DOI: 10.1007/s00404-012-2675-x

[30] Vignera S, Condorelli R, Vicari E, D'Agata R, Calogero A. Diabetes mellitus and sperm parameters. Journal of Andrology. 2013;**33**:145-153. DOI: 10.2164/jandrol.111.013193