SHORT COMMUNICATION

THE IMPACT OF *DIABETES MELLITUS* TYPE 2 ON THE STEROIDOGENESIS OF MALE ZUCKER DIABETIC FATTY RATS

Filip BENKO[1], Mária CHOMOVÁ[2], Oľga ULIČNÁ[3], Michal ĎURAČKA[1], Ján KOVÁČ[1], Eva TVRDÁ[1]

[1]Institute of Applied Biology, 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

Corresponding author: Filip Benko, Institute of Applied Biology, Faculty of Biotechnology and Food Sciences, Slovak University of Agriculture in Nitra, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia. E-mail: xbenkof@uniag.sk

SUMMARY

The aim of this study was to evaluate the impact of *diabetes mellitus* type 2 (DM2) on the male endocrine system of Zucker Diabetic Fatty (ZDF) rats. Sexually mature ZDF rats were divided to a lean (control) and obese group, and had diabetes confirmed by blood tests. For the *in vivo* experiment, fasting blood was collected to obtain blood plasma. In case of the *in vitro* experiments, testicular fragments were cultured for 24 h, and the culture medium was collected. The concentrations of testosterone (T), androstenedione (A4), dehydroepiandrosterone (DHEA-S), estradiol (E2), follicle-stimulating hormone (FSH) and luteinizing hormone (LH) were quantified in the blood plasma and the medium by the ELISA method, while cholesterol (CHOL) was assessed spectrophotometrically. A significant decline of T (36.31%), A4 (25.11%) and FSH (26.99%) as well as a significant increase of CHOL and E2 (36.17%) was observed in the blood plasma of obese ZDF rats in comparison to the control. Under *in vitro* conditions, a significant decrease of FSH (23.35%) accompanied by an increase of E2 was observed in the obese group compared to the control. In the case of CHOL, LH, T, DHEA and A4 no significant differences were observed. Our results suggest that except for FSH and E2 all steroid biomolecules were synthetized normally by the testicular tissue, however a dramatic endocrine disturbance was observed at the system level. We may conclude that DM2 has negative effects on systemic hormone secretion and these alterations are more pronounced in combination with obesity.

Key words: *diabetes mellitus* type 2, ZDF rats, obesity, steroidogenesis, reproductive hormones

TYPE 2 DIABETES AND STEROIDOGENESIS OF MALE ZDF RATS

Diabetes mellitus type 2 (DM2) is a metabolic disorder which develops when the feedback loops between insulin action and secretion do not function properly. Diabetes accelerates dyslipidaemia and chronic hyperglycaemia, ultimately disturbing the blood circulation **[1,2]**. The progress of DM2 is closely related to obesity, which may affect spermatogenesis by reducing the sperm quality and decreasing the synthesis of steroid biomolecules required for a proper function of the male reproductive system **[3-5]**. A commonly used experimental model for the study of DM2-associated pathogenesis are Zucker diabetic fatty (ZDF) rats. These are characterized by a reduced action of the leptin receptor, which controls satiety. As such, these rats develop obesity and hyperglycaemia **[6]**. In general, DM2-associated male subfertility is largely studied in the context of sperm function and testicular structure, nevertheless specific mechanisms of action on the male endocrine system have not been elucidated in detail yet. Hence, in the present research, we evaluated the consequences of DM2 and/or obesity on the male steroidogenesis of ZDF rats under *in vivo* and *in vitro* conditions.

The experiment comprised 31 sexually mature male rats (age of 270 days). The experimental group consisted of 15 obese ZDF rats, while the control group included 16 lean ZDF rats. Fasting blood glucose concentration was monitored using a FreeStyle Optium Neo Glucose and Ketone Monitoring System (Abbott Diabetes Care Ltd., UK). *Diabetes* was acknowledged when the concentration of blood glucose was equal to or higher than 16 mmol/l **[7]**. By week 8, all animals developed a persistent hyperglycaemia. The animals were obtained from the Institute of Experimental Pharmacology (Slovak Academy of Sciences, Slovakia). All producers were approved by the State Veterinary and Food Institute of the Slovak Republic (no. 493/18-221/3) and Ethic Committee. The control group had not a continual access to the food, while obese rats had unrestricted food reservoir (Purina Rodent LabDiet 5008, IPS Product Supplies, UK) with a fat content of 6.50%, which lead into overeating and obesity development.

Following anaesthesia by sevoflurane and decapitation, blood was collected into test tubes (S-Monovette® K3; Sarstedt, Nümbrecht, Germany), with EDTA (ethylenediaminetetraacetic acid) to prevent coagulation and subsequently centrifuged at 3000 RPM for 20 min (20 °C) to obtain blood plasma. The testes were surgically removed, cleaned, and cut into smaller pieces. The resulting fragments of equal size and weight were cultured in Dulbecco's modified Eagle Medium (Sigma-Aldrich, St. Louis, USA), 1% antibiotic/antimycotic (Sigma-Aldrich, St. Louis, USA), and 10% fetal bovine serum (Sigma-Aldrich, St. Louis, USA) at 37 °C and 5% CO_2 for 24 h. Subsequently, the culture medium was transferred into cryotubes and kept at -80 °C for further assessment.

The concentration of cholesterol (CHOL) was evaluated using the CHOD-PAP commercial kit (DIALAB, Vienna, Austria) and the Rx Monza (Randox Laboratories, Ltd., Crumlin, United Kingdom) automatic spectrophotometer. Concentrations of selected steroid molecules (testosterone-T; androstenedione-A4; dehydroepiandrosterone-DHEA-S; estradiol-E2; follicle-stimulating hormone-FSH; luteinizing hormone-LH) were assessed using ELISA-based commercial kits (My BioSource, San Diego, California, USA). The reaction was evaluated with a plate spectrophotometer at a wavelength of 450 nm (Glomax, Promega, Madison, Wisconsin, USA) **[8]**.

The GraphPad Prism program (version 8.1 for Mac; GraphPad Software Inc.; San Diego, California, USA) was used for statistical analysis. All data were subjected to the Shapiro-Wilk normality test. Subsequently, differences between the groups were evaluated using an unpaired t-test. Statistical significance was set at ***P<0.001; **P<0.01 and *P<0.05.

The results in Table 1 indicate that the concentration of blood plasma (*in vivo*) CHOL and E2 were significantly increased (P<0.001; P<0.05) in ZDF obese rats when compared to the ZDF lean rats. In case of FSH, T and A4 statistical decrease (P<0.05) was observed in the ZDF obese group against the control. No significant differences were recorded in the concentrations of LH or DHEA among the groups. Under *in vitro* conditions significant differences (P<0.05) were observed only in case of FSH and E2. FSH was significantly lower (P<0.05) in ZDF obese rats, while the concentration of E2 was significantly higher (P<0.01) in comparison with ZDF lean rats. However, non-significant differences were observed in the concentrations of CHOL, LH, T, DHEA and A4.

One of the main causes underlying alterations to the steroid biosynthesis may lie in obesity, which is closely connected with DM2, both negatively affecting proper metabolic functions as well as cellular homeostasis. An already moderate obesity and hyperinsulinemia may dramatically decrease total T due to an enhanced activity of aromatases from the cytochrome P450 family, which will increase the conversion of androgens (T, A4) to E2 in adipose tissue **[9-11]**. The presence of DM2 reduced the concentration of serum T which was not the case of intratesticular T. A significant decrease of serum T may be associated with a reduction of sex hormone binding globulin (SHGB), which is essential for T transportation in blood **[12].**

Elevated concentration of E2 as a result of peripheral aromatization of androgens may lead to an inhibition of the reproductive axis and a subsequent lower T synthesis. However, a partial reduction of T concentration in serum did not affect intratesticular T synthesis or spermatogenesis **[13].** According to previous studies, the secretion of serum LH in diabetic rats

was elevated, which may be explained by decreased feedback of the LH subunit mRNA expression due to low T concentration **[14].** There is a strong connection between a lack of insulin and modulation of FSH concentration in the serum. Insulin or glucose may have an impact on the pituitary biosynthesis and secretion of FSH accompanied by a decrease in the response of tubular FSH receptors **[15]**.

Kelly and Jones **[16]** hypothesize that the concentration of T may affect the process of adipogenesis by inhibiting the differentiation of new adipocytes. Accordingly, low T concentration may increase the fat mass and the risk of obesity development. Adipose tissue presents with an individual active secretory function by producing adipocytokines and converting stored or circulating sex steroids precursors (A4, DHEA) to T and E2 with the help of 17β-hydroxysteroid dehydrogenases (17β-HSD) and aromatase, thus modulating the lipid metabolism and steroid synthesis **[17].**

Diabetes may affect several enzymatic pathways of steroidogenesis by downregulating the expression of testicular mRNA transcripts for the androgen receptor, LH receptor, cytochrome P450 enzyme (CYP17A1), 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-HSD, which may cause a decreased synthesis of DHEA from pregnenolone regulated by CYP17A1, while 3β-HSD and 17β-HSD control T synthesis from DHEA, possibly leading to a reduced concentration of serum and intratesticular T **[18]**.

Generally, T synthesis is mediated through two pathways (Figure 1), the Δ-4 pathway (via A4) and Δ-5 pathway (via DHEA). We may speculate that the Δ-4 pathway failed because of the decline of A4 in the blood plasma of obese ZDF rats. This may activate the Δ-5 pathway in order to ensure normal concentration of T, however our data suggest that the Δ-5 pathway may not have fully compensated for the Δ-4 pathway failure, which could lead to a significant decrease of T in the blood plasma, as previously postulated by Ohta et al. **[19]**.

Under *in vitro* conditions, testicular tissue revealed to carry out a proper synthesis of T, A4 as well as DHEA-S. This could be associated with the absence of adipose tissue, which originally surrounded the testicular tissue in the animal. The subsequent culture included the testicular fragments without additional adipose tissue, which might have acted as a barrier for a subsequent distribution of androgens into the blood. What is more, leptin is able to cross the blood-testis barrier, interact with testicular receptors of Leydig cells and subsequently inhibit T synthesis by disrupting the testicular leptin transduction pathway **[20].**

In summary, we may conclude that DM2 has a negative impact on the concentration of steroid biomolecules, especially in the blood plasma at the system level. Diabetes combined with obesity most likely disrupted the functions of specific receptors of the hypothalamic-

pituitary-testicular axis, since the *in vitro* endocrine function of testicular tissue was affected by the presence of DM2 and obesity to a lower extent. Nevertheless, this study has potential limitations. The evaluation of the concentration of SHGB could further illustrate alterations to the transport pathways of steroid biomolecules in diabetic and/or obese males.

Conflict of interest

There is no conflict of interest.

Acknowledgements

This study was supported by the Slovak Research and Development Agency (APVV-15-0544), as well as by the KEGA 008SPU-4/2021 and VEGA 1/0314/19 projects.

References

1. LIU Z, FU CH, WANG W, XU B. Prevalence of chronic complications of type 2 diabetes mellitus in outpatients – a cross – sectional hospital based survey in urban China. Health Qual Life Outcomes 2010;8:62. https://doi.org/10.1186/1477-7525-8-62
2. ZHENG Y, LEY SH, HU FB. Global etiology and epidemiology of type 2 diabetes mellitus and its complications. Nat Rev Endocrinol 2018;14:88-98. https://doi.org/10.1038/nrendo.2017.151
3. PALMER NO, BAKOS HW, FULLSTON T, LANE M. Impact of obesity on male fertility, sperm function and molecular composition. Spermatogenesis 2012;2:253-263. https://doi.org/10.4161/spmg.21362
4. SIWY J, ZOJA C, KLEIN J, BENIGNI A, MULLEN W, MAYER B, MISCHAK H, JANKOWSKI J, STEVENS R, VLAHOU A, KOSSIDA S, PERCO P, BAHLMANN FH. Evaluation of the Zucker diabetic fatty (ZDF) rat as a model for human disease based on urinary peptidomic profiles. PLoS ONE 2012;7:513-534. https://doi.org/10.1371/journal.pone.0051334
5. DING GL, LIU Y, MIAO-E L, PAN JX, GUO MX, SHENG JZ, HUANG HF. The effects of diabetes on male fertility and epigenetic regulation during spermatogenesis. Asian J Androl 2015;17:948-953. https://doi.org/10.4103/1008-682x.150844
6. GRIFFEN S, WANG J, GERMAN M. A genetic defect in beta-cell gene expression segregates in dependently from the fa locus in the ZDF rat. Diabetes 2001;50:63-68. https://doi.org/10.2337/diabetes.50.1.63
7. CHOMOVA M, BALAZOVA M, MUCHOVA J. Diabetes-induced abnormalities of mitochondrial function in rat brain cortex: the effect of n-3 fatty acid diet. Mol Cell Biochem 2017;435;109-131. https://doi.org/10.1007/s11010-017-3061-6
8. JAMBOR T, TVRDÁ E, TUŠIMOVÁ E, KOVÁČIK A, BISTÁKOVÁ J, FORGÁCS Z, LUKÁČ N. In vitro effect of 4-nonylphenol on human chorionic gonadotropin (hCG) stimulated hormone secretion, cell viability and reactive oxygen species generation in mice Leydig cells. Environ Pollut 2017;222:219-225. https://doi.org/10.1016/j.envpol.2016.12.053
9. ALMON R, WANG X, DUBOIS DC, SUKUMARAN S, AYYAR V, JUSKO WJ. Variability in Zucker diabetic fatty rats: differences in disease progression in

hypergylcemic and normoglycemic animals. Diabetes Metab Syndr Obes 2014;7:531-541. https://doi.org/10.2147/dmso.s69891

10. FUI MN, DUPUIS P, GROSSMANN M. Lowered testosterone in male obesity: mechanisms, morbidity and management. Asian J Androl 2016;16:223-231. https://doi.org/10.4103/1008-682x.122365
11. BAKHTYUKOV A, DERKACH K, SOROKOUMOV V, STEPOCHKINA A, ROMANOVA I, MORINA I, ZAKHAROVA I, BAYUNOVA L, SHPAKOV A. The Effects of Separate and Combined Treatment of Male Rats with Type 2 Diabetes with Metformin and Orthosteric and Allosteric Agonists of Luteinizing Hormone Receptor on Steroidogenesis and Spermatogenesis. Int J Mol Sci 2022;23;198. https://doi.org/10.3390/ijms23010198
12. MANSOUR M, COLEMAN E, DENNIS J, AKINGBEMI B, SCHWARTZ D, BRADEN T, JUDD R, PLAISANCE E, STEWART L, MORRISON E. Activation of PPARγ by Rosiglitazone Does Not Negatively Impact Male Sex Steroid Hormones in Diabetic Rats. PPAR Research 2009. https://doi.org/10.1155/2009/101857
13. VIGUERAS-VILLASENOR RM, ROJAS-CASTANEDA JC, CHÁVEZ-SALDANA M, GUTIÉRREZ-PÉREZ O, GARCIÁ-CRUZ ME, CUAVES-ALPUCHE O, REYES-ROMERO MM, ZAMBRANO E. Alterations in the spermatic function generated by obesity in rats. Acta Histochem 2011;113:214-220. https://doi.org/10.1016/j.acthis.2009.10.004
14. OLIVARES A, MÉNDEZ J, CÁRDENAS M, OVIEDO N, PALOMINO M, SANTOS I, PERERA-MARÍN G, GUTIÉRREZ-SAGAL R, ULLOA-AGUIRRE A. Pituitary-testicular axis function, biological to immunological ratio and charge isoform distribution of pituitary LH in male rats with experimental diabetes. Gen Comp Endocrinol 2009;161;304-312. https://doi.org/10.1016/j.ygcen.2008.12.021
15. BALLESTER J, MUNOZ C, DOMÍNGUEZ J, RIGAU T, GUINOVART J, RODRÍGUEZ-GIL J. Insulin-Dependent Diabetes Affects Testicular Function by FSH- and LH-Linked Mechanisms. J Androl 2013;25;706-719. https://doi.org/10.1002/j.1939-4640.2004.tb02845.x
16. KELLY DM, JONES TH. Testosterone and obesity. Obes Rev 2015;16:581-606. https://doi.org/10.1111/obr.12282
17. SHOELSON SE, LEE J, GOLDFINE AB. Inflammation and insulin resistance. J Clin Invest 2006;116:1793-1801. https://doi.org/10.1172/jci29069

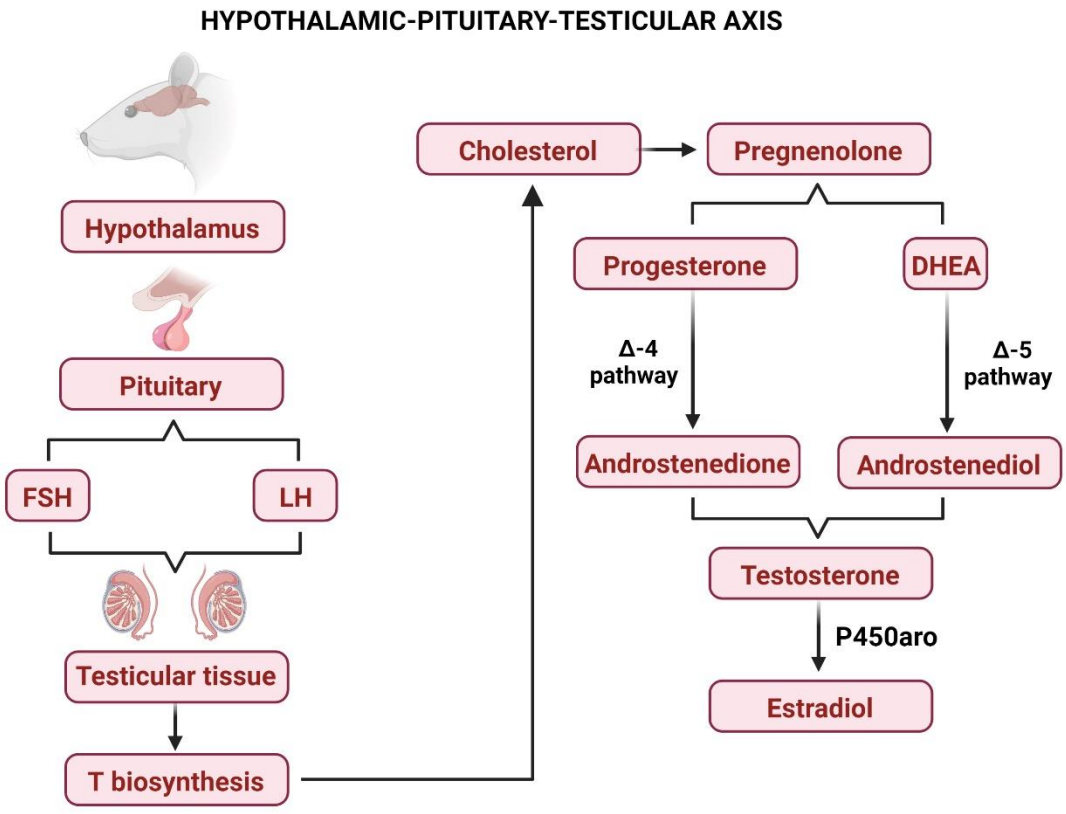
18. NNA V, BAKAR B, AHMAD A, MOHAMED M. Down-regulation of steroidogenesis-related genes and its accompanying fertility decline in streptozotocin-induced diabetic male rats: ameliorative effect of metformin. Andrology 2018;7;110-123. https://doi.org/10.1111/andr.12567
19. OHTA T, KATSUDA Y, MIYAJIMA K, SASASE T, KIMURA S, TONG B, YAMADA T. Gender Differences in Metabolic Disorders and related Diseases in Spontaneously Diabetic Torii-Lepr (fa) rats. J Diabetes Res 2014;1-7. https://doi.org/10.1155/2014/841957
20. YI X, GAO H, CHEN D, TANG D, HUANG W, LI T, MA T, CHANG B. Effects of obesity and exercise on testicular leptin signal transduction and testosterone biosynthesis in male mice. Am J Physiol Regul Integr Comp Physiol 2017;312:501-510. https://doi.org/10.1152/ajpregu.00405.2016

247 **Table 1.** *In vivo* and *in vitro* concentrations of selected steroid biomolecules of ZDF-lean and
248 ZDF-obese rats.

IN VIVO	ZDF-lean	ZDF-obese	*IN VITRO*	ZDF-lean	ZDF-obese
CHOL (mg/dL)	69.16±9.46	515.90±48.68***	**CHOL (mg/dL)**	6.44±0.27	5.48±0.55
FSH (ng/mL)	30.31±3.26	22.13±4.06*	**FSH (ng/mL)**	38.56±2.71	29.56±2.66*
LH (ng/mL)	3.08±0.37	2.38±0.31	**LH (ng/mL)**	7.17±0.56	5.08±0.83
T (ng/mL)	5.04±0.43	3.21±0.36*	**T (ng/mL)**	89.27±0.87	87.94±1.95
E2 (pg/mL)	2.46±0.65	3.35±0.72*	**E2 (pg/mL)**	2.13±0.26	4.66±0.58**
DHEA (ng/mL)	4.09±0.31	4.07±0.33	**DHEA (ng/mL)**	0.87±0.04	0.86±0.03
A4 (ng/mL)	9.48±0.54	7.10±0.52*	**A4 (ng/mL)**	6.28±0.07	6.24±0.04

249 Mean±SD. ***P<0.001; **P<0.01; *P<0.05.

250



251

252 **Figure 1.** Hypothalamic-pituitary-testicular axis and steroidogenesis via Δ-4 and Δ-5 pathway