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DIABETES-MEDIATED CHANGES IN RAT TYPE I COLLAGEN AND SPERMATOGENESIS INDICES

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Abstract

Objectives: *To investigate the effects of diabetes on the reproductive system and extracellular matrix proteins of diabetic rats.* ***Materials and Methods:*** *Wistar albino male rats, body weight (BW) 160–200 g, were divided into two groups: I – streptozoticin diabetes, II – normal non-diabetic animals. The content of amino acids in rat type I collagen was determined using an amino acid analyzer. Morphological analyses of gonadic structures were carried out by an optic microscope.* ***Results:*** *The study of the effects of diabetes on type I collagen amino acid content, testis cells morphologic and morphometric parameters and spermatogenesis demonstrated the presence of diabetes-mediated quantitative and qualitative changes in male rat reproductive organs, spermatogenetic epithelial cells and extracellular matrix proteins in comparison with normal.* ***Conclusions:*** *Observed collagen molecules changes could hence affect the properties and correct functioning of spermatogenetic epithelium and of other tissues of reproductive organs. They could be caused by diabetes via deficiency of insulin which is involved in collagen synthesis regulation at different stages of this process, cytochrome P450-2E1 induction and reactive oxygen species effects on protein biosynthesis processes.*

key words: *diabetes, spermatogenesis, type I collagen, rats*

Objectives

According to WHO data, diabetes is on the third place among diseases which cause the highest levels of morbidity and mortality [1]. Results of numerous investigations clearly demonstrate that modern diabetes pharmacological treatment is not able to prevent completely diabetes complications, among which cardiovascular disease and diabetic nephropathy have the highest levels of incidence and the poorest prognosis [2]. The adverse effects of diabetes on female and male reproductive function are often a major patient concern [3]. Diabetes may affect male reproductive function at multiple levels as a result of its effects on the endocrine control of spermatogenesis itself or by impairing penile erection or ejaculation [4].

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Connective tissue disturbances and especially qualitative changes of collagen synthesis always accompany the diabetic pathologic process [5]. Thus, diabetes induces changes in the structure and function of the extracellular matrix proteins in many tissues [6].

It was shown that processing and secretion of collagen is controlled by insulin at a post-transcriptional site [7]. Other authors demonstrated that the α2 (I) collagen gene contains two functional promoters, and its expression in cells is regulated at both trans-criptional and post-transcriptional levels [8].

Diabetes-mediated dermal connective tissue changes, impaired wound healing, osteopenia and decreased bone strength may be associated with altered metabolism of Type I collagen. It is assumed that alterations in Type I collagen amino acids as a result of gene variation may strongly affect protein properties and physiological function; however, very limited evidence exist regarding amino acid composition of Type I collagen.

Taking into account that quantitative changes of collagen structure correlated with parameters of male reproductive system such as percentage of normal sperm and sensitivity to testicular degeneration [9], it becomes obvious how important is the analysis of diabetes simultaneous effects on collagen and reproductive system.

The aim of the study was to estimate the putative changes induced by diabetes in rat type I collagen amino acid content, testis cells morphologic and morphometric parameters and spermatogenesis.

Material and methods

Wistar albino male rats, body weight (BW) of 160-200 g, were used in the study. Animals were kept in standard conditions of nutrition, water and light regimes.

All animal studies were performed in accordance with the recommendations of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes and approved by the Institutional Animal Care and Use Committee.

In the experiments, we used streptozotocin produced by Sigma-Aldrich, USA (lot 018K1566). Rats were kept in quarantine for 10 days, then they were randomized into experimental and control groups. Each group included 6 animals. Diabetes was induced by a single intraperitoneal injection of freshly prepared solution of streptozotocin in citrate buffer (*p*H 4,5) at 60 mg/kg of body weight [10]. The rats with blood glucose level over 16.5±0.8 mmol/l were taken into experiment. Blood glucose level was determined by Smartest Optima (Biotest T Medical Corporation, Germany). Body weight was measured weekly. The control group received only citrate buffer in corresponding volumes.

After 105 days of the experiment, rats of the experimental and control groups were sacrificed under mild ether anesthesia via decapitation. The rats' skin, bones, testis and epididymis were used for investigation.

Skin and bone type I collagens were extracted and purified according to [9]. All procedures were carried out in cold regime (+4°C). Fractionation of pure type I collagen was carried out with NaCl according to [11, 12]. Collagen preparations purity was controlled electrophoretically [13]. Collagen fractions were hydrolyzed for 24 h with 6N HCl at 105°C [14]. Their amino acid compositions were analyzed by ion exchange

chromatography on the amino acid analyzer AAA-881 (Czech Republic).

For investigation of morphologic and morphometric parameters of germ cells and spermatogenesis processes the right testicle was used. Its radiuses, volume and weight was measured. Then it was fixed in 10% solution of neutral formalin, dehydrated in ethanol solutions and embedded in paraffin. Histologic sections (6mm) were stained by hematoxilin and eosine. Microscopic studies were carried out with microscope Cytophan (Leica Microsystems Wetzlar GmbH). Determination of the spermatogenetic index in testicles was carried out according to four points system [15]. Simultaneously with the determination of the above mentioned quantitative parameters of spermatogenesis, we also investigated qualitative changes of spermatogenetic epithelium: cells desquamation (shedding of epithelial elements), epithelium exfoliation (detachment) from tubule basal membrane, and presence of cell-free regions ("windows").

The obtained data were calculated by one-way analysis of variance (ANOVA) and expressed as the mean ± standard error of the mean (M±S.E.M.). Data were compared using Tukey test. Differences were considered to be statistically significant at $p < 0.05$.

Results

Everyday observation of animals with streptozotocin diabetes testified some changes in their sexual behaviour in comparison with the control group (lowering of motivation and mating activity) which are in good correspondence with other authors' results [16].

Testicles external examination at rat's autopsy did not mark any visible pathology: organs were without any traces of edema, with normal blood filling. However, differences were recorded regarding some morphometric indices and spermatogenesis indices as presented in Table 1.

Table 1. Indices of rat testis in control and diabetic rats (M ± S.E.M., n = 6).

Indices	Animal groups	
	Control	Diabetes
Testis weight (g)	3.60±0.08	3.16±0.22
Testis relative weight (g/100g b.w.)	0.90±0.022	1.04±0.048*
Testis volume (mm^3)	1915±0.61	1816±0.79
Number of spermatozoids (10^6/ml)	78.25±7.69	48.13±5.48*

*- P<0.05 in comparison with control

According to Table 1 data, rat testes absolute weights and volumes in the experimental group did not greatly differ from control but testes relative weights were increased (+15.6 %). Microscopic investtigation of epididymal suspension demonstrated inhibition of spermatogenetic function and lowering of sperm production (–39%) with diabetes in comparison with control.

Morphometric changes in reproductive organs were accompanied by changes in spermatogenetic epithelium. Results of rat testis spermatogenetic epithelium microscopy are presented in Table 2.

Table 2. Rat spermatogenetic epithelium morphometric indices in control and diabetic rats (M±S.E.M., n = 6).

Indices	Animal groups	
	Control	Diabetes
Spermatogenetic index (stages of spermatogenesis total / number of examined tubules)	3.57±0.01	3.50±0.01*
Number of spermatogonia (per tubular cross section)	75.16±0.74	65.40±0.81*
12-th stage of meiosis, %	4.8±0.49	3.25±0.63
Desquamated epithelium, %	1.2±0.58	2.25±1.03*
Exfoliation of epithelium, %	0	3.5±0.65*
"Windows",%	0	6.75±1.11*

*- P<0.05 in comparison with control

According to the data of Table 2, the spermatogenetic index in the experimental group was decreased in comparison with controls (simultaneously with mitotic activity and number of spermatogonia).

Beside the above mentioned quantitative changes, qualitative changes of spermatogenetic epithelium were also present in the convoluted seminiferous tubules. Increased level (by 1.88 times) of epithelial cells desquamation was observed in the diabetes group (Table 2). Great degenerative changes in testes such as epithelium exfoliation from tubule basal membrane, vessels thrombosis and presence of cell-free regions ("windows") were also present in the experimental group (Figures 1, 2).

We also analyzed the effect of diabetes on collagen amino acid content. Changes in rat skin and bone type I collagen amino acid content induced by streptozotocin diabetes as compared to control are shown in Tables 3 and 4. Statistically significant changes were registered in bone collagen for 16 amino acids and in skin collagen – for 15 amino acids.

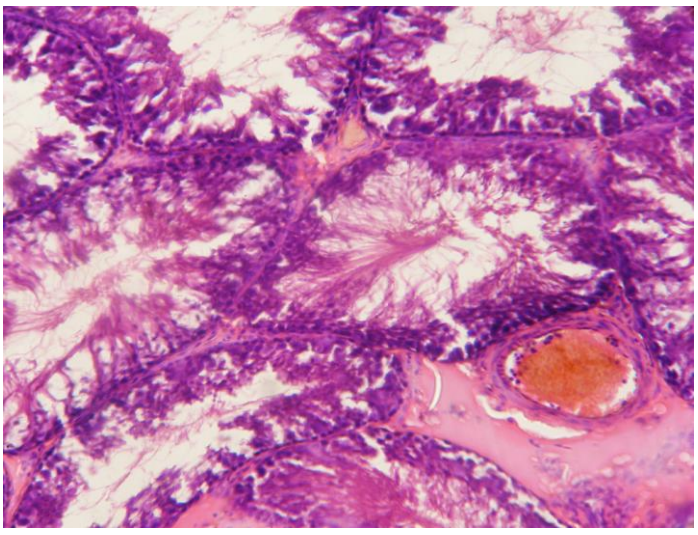

Figure 1. Dystrophic changes and loss of spermatogenetic epithelium, vessels thrombosis and interstitial edema in rat testis with diabetes. Hematoxilin and eosine, x 400.

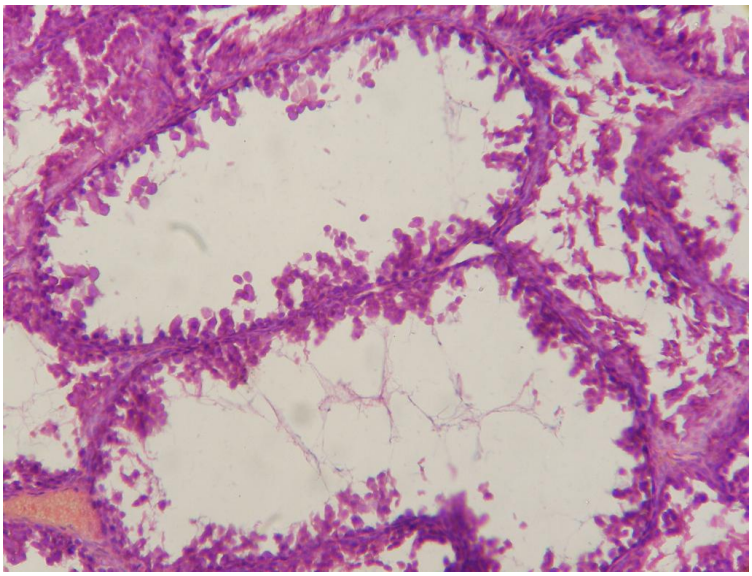

Figure 2. Desquamation of epithelial cells, observed in the group of animals with diabetes. Hematoxilin and eosine, x 400.

Table 3. Rat bone type I collagen amino acid content (residues/1000 residues) in control and diabetic animals (M ± S.E.M., n = 6).

Amino acid	Control (norm)	Diabetes
Hydroxylysine	6.70±0.10	1.70±0.11*
Lysine	39.1±0.40	56.5±1.26*
Histidine	4.70±0.20	10.65±1.76*
Arginine	48.30±0.60	79.90±7.13*
Hydroxyproline	99.00±1.40	12.40±3.64*
Aspartic acid	38.80±0.80	116.30±3.03*
Threonine	24.00±0.60	40.20±1.52*
Serine	38.20±1.40	60.00±2.35*
Glutamic acid	97.50±1.30	195.00±0.67*
Proline	101.90±1.80	70.10±2.10*
Glycine	305.00±2.50	149.10±6.32*
Alanine	109.00±1.10	81.20±1.27*
Valine	23.00±0.50	27.90±4.31
Methionine	6.20±0.10	13.50±1.34*
Isoleucine	13.70±0.30	23.40±3.05*
Leucine	27.90±0.20	62.50±2.52*
Tyrosine	4.80±0.20	9.70±2.00
Phenylalanine	12.00±0.20	37.10±3.99*

*- P<0.05 in comparison with control

Bone type I collagen of diabetic rats contains significantly lower contents of hydroxylysine (–75.0 %), hydroxyproline (–87.5%), proline (–31,3 %), glycine (–51,2%), alanine (-25,5 %), all being amino acid residues with a strong effect on collagen helix structure (its structure presumably consisting in Gly-X-Pro or Gly- X-Hyp triplets), rigidity and cross-linking [17]. In our experiment, the content of arginine (+65.4%), aspartic acid (+200.0%), threonine (+67.5%), serine (+57.1%), glutamic acid (+100,0 %), methionine (+117,7 %), isoleucine (+70,8%), leucine (+124,0%), phenylalanine (+209,0%)

were increased in diabetic compared to normal rats. Our results are in agreement with data of other authors which demonstrated disturbances of collagen ultra-structure in streptozotocin-induced diabetes [18, 19].

Skin type I collagen of diabetic rats also contains significantly lower contents of hydroxyproline (–74.4%), proline (–16.5%), glycine (–49.0%), alanine (–12.0%) and valine (+19.7%) residues, simultaneously with higher contents of lysine (+78.2%), histidine (+60.4%), arginine (+51.0%), aspartic acid (+99.0%), threonine (+75.0%), serine (+34.6%), glutamic acid (+86.7%), methionine (+117.6%), leucine (+68.0%), tyrosine (+218.0%) and phenylalanine (+162.3%) residues. Thus, in this tissue, diabetes caused similar quantitative changes in collagen molecule as it was in bone.

Table 4. Rat skin type I collagen amino acid content (residues/1000 residues) in control and diabetic animals (M ± S.E.M., n = 6).

Amino acid	Control (norm)	Diabetes
Hydroxylysine	4.30±0.10	4.60±1.03
Lysine	29.80±1.30	53.10±3.62*
Histidine	4.80±0.20	7.70±0.62*
Arginine	50.20±0.60	75.80±3.24*
Hydroxyproline	92.70±2.30	23.70±2.64*
Aspartic acid	44.70±1.10	88.90±2.96*
Threonine	17.80±0.70	31.10±1.53*
Serine	35.80±1.00	48.20±0.80*
Glutamic acid	75.20±1.50	140.40±3.63*
Proline	130.60±3.00	109.50±8.68
Glycine	324.40±3.70	165.20±9.02*
Alanine	105.20±2.10	92.40±2.36*
Valine	26.40±0.60	21.20±1.03*
Methionine	6.80±0.10	14.80±2.07*
Isoleucine	12.80±0.80	18.80±1.87
Leucine	29.20±1.70	49.10±4.25*
Tyrosine	3.70±0.20	11.80±2.32*
Phenylalanine	12.20±0.50	32.00±4.60*

*- P<0.05 in comparison with control

Discussions

Diabetes-mediated changes in rat reproductive system (Tables 1, 2) could be a result of degenerative processes in rat reproductive organs caused by hyper-glycation of key proteins (with subsequent changes of their functions) and great disturbances in metabolisms of amino acids, peptides, lipids [4,6,7,10,16,18,19].

An important aspect of spermatogenesis involves the detachment of germ cells from the basement membrane and their subsequent migration towards the tubule lumen. Pro-collagen I, a precursor of type I collagen, is a trimer consisting of two α1 chains and one α2 chain whose sequences are encoded by two different genes, namely COL1A1 and COL1A2 respectively [20]. Distribution of pro-collagen I within the seminiferous tubules of immature and adult mice correlate with the process of germ cells attachment to and detachment from the basement membrane. The unique distribution pattern of pro-collagen I in

adult mouse testis implies possible roles of COL1A1, COL1A2 and pro-collagen I in regulating the adhesion of spermatogonia and preleptotene spermatocytes to the basement membrane and the detachment and migration of later spermatocytes and spermatids towards the lumen during spermatogenesis [21].

The profound diabetes-mediated changes in collagen amino acid composition reported by us in Tables 3 and 4 might induce disturbances in its physicochemical behavior. Hydroxylysine residues along with lysine and histidine [17] participate in collagen cross-linking. Changes in the ratio of hydroxylysine : lysine : histidine residues could seriously influence the number and type of cross-links in collagen fibrils**.** This might induce changes in the mechanical strength, elasticity/rigidity of the extracellular matrix. Our results reporting changes of hydroxylysine, lysine and histidine residues are in accordance with data of other authors on changes of collagen cross-links in diabetic animals [22].

In diabetes, non-enzymatic glycosylation of collagen occurs as its turnover decreases during maturation, with complex carbohydrates accumulating slowly and the end-products of these reactions becoming permanent. This can lead to: 1) cross-linking: intermolecular cross-linking may occur between two adjacent molecules and involve lysine to lysine or lysine to arginine residues [23]. 2) Modification of arginine within the sites RGD and GFOGER recognized by the two specific integrins (alpha1beta2 and alpha2beta1) for collagen. These changes can ultimately affect repair of vascular damage and dermal wound healing in diabetes mellitus. Both types of modifications are deleterious to the optimal properties of collagen as a supporting framework structure and as a controlling factor in cell matrix interactions. Glycosylation during diabetes is therefore responsible for malfunctioning of the diverse collagen tissues throughout the body [23]. Changes in the number of arginine, aspartic acid, threonine, serine and glutamic acid residues could cause great changes in the surface charge of collagen molecule [17,24], whereas changes in quantity of isoleucine, tyrosine and phenylalanine residues could influence the level of collagen helix rigidity [17]. In addition, changes in arginine, glycine and aspartic acid residues could affect the number of domains Arg-Gly-Asp which are responsible for the processes of cell adhesion on collagen structures [25-28]. Arginine and glycine residues in collagen molecule are also part of special loci responsible for interactions with chaperones and for pro-collagen to collagen processing [29].

Our experiments indicated the presence of qualitative changes in diabetic rat skin and bone type I collagen. These changes could lead to alterations of the helix structure, surface charge, rigidity, number and types of cross-links and specific loci responsible for cell adhesion, interaction with chaperons and procollagen processing to collagen. Such collagen molecules changes could hence affect the properties and correct functioning of a number of tissues.

We can hypothesize that such changes could be caused by insulin deficiency which is involved into collagen synthesis regulation at different stages of this process [7,8,30,31]. Beside the direct influence of insulin deficiency on collagen synthesis, pathologic changes in amino acid metabolism in the whole organism [32] could also influence the

collagen metabolism. Ability of the majority of amino acids to regulate protein biosynthesis at the level of translation via stimulation of 70kD-ribosomal proteinS6-kinase has been established in vitro [33].

On the other hand, taking into account the existence of collagen gene polymorphisms [34,35], changes could be also a result of disturbances in rates of transcription of different genes from the same collagen type I superfamily as it was previously demonstrated for osteogenesis imperfecta [36]. Such collagen molecules changes could hence affect the properties and correct functioning of spermatogenetic epithelium and other tissues of reproductive organs.

It could be also hypothesized that diabetes-mediated changes could be partially caused by oxygen reactive forms produced via cytochrome P450 2E1 [37]. These reactive oxygen species were reported to mediate paracrine stimulation of type I collagen synthesis on different stages of this process [5] and caused genotoxic effects on rodents' germ cells [25].

Conclusion

Our data add to the understanding of the mechanisms involved in male infertility which often accompanies diabetes. The reported collagen molecules changes could affect the properties and correct functioning of spermatogenetic epithelium and other tissues of reproductive organs. They could be caused by diabetes via deficiency of insulin which is involved into collagen synthesis regulation on different stages of this process, cytochrome P450 2E1 induction and reactive oxygen species effects on protein biosynthesis processes.

REFERENCES

1. **Mladovsky P, Allin S, Masseria C et al.** Health in the European Union. Trends and Analysis. Observatory Studies Series № 19**.** *Publications, World Health Organization Regional Office for Europe*, Copenhagen, pp 200, 2009. (http://www.euro.who.int/pubrequest).

2. **American Diabetes Association.** Diabetic nephropathy. *Diabetes Care* 24[Suppl 1]:S69-S72, 2001.

3. **Ramalho-Santos J, Amaral S, Oliveria PJ.** Diabetes and the impairment of reproductive function: Possible role of mitochondria and reactive oxygen species. *Curr Diabetes Rev* 4:46-54, 2008.

4. **Agbaje IM, Rogers DA, McVicar CM et al.** **I**nsulin dependent diabetes mellitus: implications for male reproductive function. Hum Reprod *22: 1871-1877, 2007.*

5. **Nieto N, Friedman SL, Cederbaum AI.** Cytochrome P450 2E1-derived reactive oxygen species mediate paracrine stimulation of collagen I protein synthesis by hepatic stellate cells. *J Biol Chem* 277: 9853-9864, 2002.

6. **Lehti T M, Silvennoinen M, Kivela R et al.** Effects of streptozotocin-induced diabetes and physical training on gene expression of extracellular matrix proteins in mouse skeletal muscle. *Am J Physiol Endocrinol Metab* 290:E900-E907, 2006.

7. **Bembenk ME, Liberti JP.** The anabolic effects of insulin on type II collagen synthesis of Swarm rat chondrosarcoma chondrocytes. *Arch Biochem Biophys* 233: 203-211, 1984.

8. **Pallante KM, Niu Z, Zhao Y et al.** The chick α2 (I) collagen gene contains two functional promoters, and its expression in chondrocytes is regulated at both transcriptional and post-transcriptional levels. *J Biol Chem* 271: 25233-25239, 1996.

9. **Bondarenko LB, Shayakhmetova GM, Byshovets TF, Kovalenko VM**. Pyrazinamide-mediated changes in rat type I collagen and

spermatogenesis indices. *Acta Pol Pharm* 68: 285-290, 2011.

10. Sricharoenvej S, Tongpob Y, Lanlua P, et al. Renal microvascular changes in streptozotocin-induced, long-termed diabetic rat. *J Med Assoc Thai* 90: 2677-2682, 2007

11. Trelstad RL, Catanese VM, Rubin DF. Collagen fractionation: separation of native types I, II and III by differential precipitation. *Anal Biochem* 71:114–118, 1976

12. Rubin AL, Drake MP, Davison PF et al. Effect of pepsin on the interaction properties of tropocollagen macromolecules. *Biochemistry* 4: 181-190, 1965.

13. Maurer G. The Disk-electrophoresis, Mir, Moscow, p. 247, 1971 (in Russian).

14. Deveni T, Gherghey J. The amino-acids, peptides and proteins, Mir, Moscow, p. 364, 1976 (in Russian).

15. Boekelheide K, Chapin R. Male reproductive toxicology**.** In *Current Protocols in Toxicology***,** Costa LG, Hodgson E, Lawrence DA, Ozolins TR, Reed DJ, Greenlee WF Eds., John Wiley & Sons, Inc, pp. 16.0.1-16.0.2, 2005.

16. El'tseva TV, Adamskaya EI, Peryshkova TA, Babichev VN. Disturbance of neuroendocrine regulation of sexual behavior of male rats with streptozotocin diabetes. *Neurosci Behav Physiol* 23: 538-544, 1993.

17. Ramachandran GN. Biochemistry of collagen. Plenum Press, New York, London pp. 536, 1976.

18. Yavuz D, Tugtepe H, Cetinel S et al. Collagen ultrastructure and TGF-beta1 expression preserved with amino guanidine during wound healing in diabetic rats. *Endocr Res* 31: 229-243, 2005.

19. Kim BM, Eichler J, Reiser KM et al. Collagen structure and nonlinear susceptibility: effects of heat, glycation, and enzymatic cleavage on second harmonic signal intensity. *Lasers Surg Med* 27: 329-335, 2000.

20. Chamberlain JR, Schwarze U, Wang PR et al. Gene targeting in stem cells from individuals with osteogenesis imperfect. *Science* 303: 1198-1201, 2004.

21. He Z, Feng L, Zhang X et al. Expression of Col1a1, Col1a2 and procollagen I in germ cells of immature and adult mouse testis. Reproduction *130: 333-341, 2005.*

22. Nakamura F, Suyama K. An amino acid derived from aldol crosslink of elastin and collagen: structure, distribution, aging, and two models of hyperglycemia. *Arch Biochem Biophys* 325: 167-173, 1996.

23. Avery NC, Bailey Aj. The effects of the Maillard reaction on the physical properties and cell interactions of collagen. *Pathol Biol (Paris)* 54: 387-395, 2006.

24. Hadley JC, Meek KM, Malik NS. Glycation changes the charge distribution of type I collagen fibrils. *Glycoconj J* 15: 835-840, 1998.

25. Pedchenko VK, Chetyrkyn SV, Chuang P, Ham AJ. Mechanism of perturbation of integrin-mediated cell-matrix interactions by reactive carbonyl compounds and its implication for pathogenesis of diabetic nephropathy. *Diabetes* 54: 2952-2960, 2005.

26. Kornblihtt AR, Gutman A. Molecular biology of the extracellular matrix proteins**.** *Biol Rev Camb Philos Soc* 63: 465-507, 1988.

27. Van der Rest M, Garrone R. Collagen family of proteins*. FASEB J* 5: 2814-2823, 1991.

28. Loeser RF, Wallin R. Cell adhesion to matrix Gla protein and its inhibition by an Arg-Gly-Asp-containing peptide. *J Biol Chem* 267:9459-9462, 1992.

29. Koide T, Takahara Y, Asada S, Nagata K. Xaa-Arg-Gly triplets in the collagen triple helix are dominant binding sites for the molecular chaperone HSP47 *J Biol Chem* 277: 6178-6182, 2002.

30. Kato M, Zhang J. Wang M et al. MicroRNA-192 in diabetic kidney glomeruli and its function in TGF-beta-induced collagen expression via inhibition of E-box repressors. *Proc Natl Acad Sci USA* 104: 3432-3437, 2007.

31. Knoll KE, Pietrusz JL Liang M. Tissue-specific transcriptome responses in rats with early streptozotocin-induced diabetes. *Physiol Genomics* 21: 222-229, 2005.

32. Bondarenko LB, Saprykina NA, Kovalenko VM. Lung and spleen contents of free amino acids after

pyrazinamide treatment. *Acta Toxicologica* 14:79–86, 2006.

33. Fox HL, Kimbell SR, Jefferson LS, Lynch CJ. Amino acids stimulate phosphorylation of $p70^{s6k}$ and organisation of rat adipocytes into multicellular clusters. *Am J Physiol* 274: C206-C213, 1998.

34. Inamasu J, Guiot BH, Sachs DC. Ossification of the posterior longitudinal ligament: an update on its biology, epidemiology, and natural history. *Neurosurgery* 58: 1027-1039, 2006.

35. Lee B, D'Alessio M, Vissing H et al. Characterization of large deletion associated with polymorphic block of repeated dinucleotide in the type III procollagen gene (COL 3A1) of patient with Ehlers-Dahnlos syndrome type IV. *Amer J Hum Genet*. 48: 511-517, 1991.

36. Wirtz MK, Rao VH, Glanville RW et al. A cysteine for glycine substitution at position 175 in an α1(I)-chain of type I collagen at clinically heterogenous form of osteogenesis imperfecta. *Connect Tissue Res* 29: 1-11, 1993.

37. Sapone A, Affatato A, Canistro D et al. Induction and suppression of cytochrome P450 isoenzymes and generation of oxygen radicals by procymidone in liver, kidney and lung of CD1 mice. *Mutat Res* 527: 67-80, 2003.