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Effects of Streptozotocin Induced Diabetes on One-Carbon Cycle and Sperm Function

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Abstract

Objective: Diabetic men suffer an increased risk of infertility associated with signs of oxidative damage and decreased methylation in sperm pointing to a deficit of the one-carbon cycle (1CC). We aimed to investigate this deficit using mice models (type 1 and 2) of streptozotocin-induced diabetes.

Materials and Methods: In this experimental study, 50 male mice, aged eight weeks, were divided randomly into four groups: sham, control, type 1 diabetes mellitus (DM1), and DM2. The DM1 group was fed a normal diet (ND) for eight weeks, followed by five consecutive days of intraperitoneal administration of Streptozotocin (STZ, 50 mg/kg body weight). The DM2 group was fed a high-fat diet (HFD) for eight weeks, followed by a single intraperitoneal injection of STZ (100 mg/kg). After twelve weeks, all the mice were euthanized, and study parameters assessed. In the sham group, citrate buffer as an STZ solvent was injected.

Results: Both types of diabetic animals had serious impairment of spermatogenesis backed by increased DNA damage (P=0.000) and decreased chromatin methylation (percent: P=0.019; intensity: P=0.001) and maturation (P=0.000). The 1CC was deeply disturbed with increased homocysteine (P=0.000) and decreased availability of carbon units [methionine (P=0.000), serine (P=0.088), folate (P=0.016), B12 (P=0.025)] to feed methylations.

Conclusion: We have observed a distinct impairment of 1CC within the testes of individuals with diabetes. We speculate that this impairment may be linked to inadequate intracellular glucose and diminished carbon unit supply associated with diabetes. As a result, interventions focusing on enhancing glucose uptake into sperm cells and providing supplementary methyl donors have the potential to improve fertility issues in diabetic patients. However, additional clinical testing is required to validate these hypotheses.

Keywords: Chromatin, Diabetes, Glucose, Methylations, Spermatogenesis

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Introduction

Diabetes is a chronic metabolic disorder characterized by high blood sugar levels, resulting from impaired insulin secretion and/or action. There are three main types of diabetes: type 1, type 2, and gestational diabetes. Type 1 diabetes is an autoimmune disease in which the immune system attacks and destroys insulin-producing beta cells in the pancreas, leading to insulin deficiency. Type 2 diabetes is a metabolic disorder characterized by the pancreas being unable to secrete adequate insulin due to the development of insulin resistance (1). There is a well-established association with oxidative stress in the context of diabetes, characterized by an imbalance between the production of reactive oxygen species (ROS) and the body's antioxidant defense mechanisms. This imbalance in oxidative homeostasis can contribute significantly to the development and progression of diabetes-related complications. Hence, maintaining a harmonious equilibrium between ROS generation and elimination, known as redox homeostasis, is crucial for cellular functionality and overall health. Disturbances in redox homeostasis, such as oxidative stress and reductive stress, can lead to heightened levels of ROS, resulting in cellular damage and compromised physiological function (2-4). Previous studies have shown a correlation between all types of diabetes and increased ROS, which can damage cellular proteins, lipids, DNA, and ultimately, cell death (4, 5). Additionally, excessive ROS can directly disrupt the insulin signaling pathway and hinder glucose uptakes in peripheral tissues, such as adipose tissue and muscles

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(6, 7), in part mediated by the release of inflammatory cytokines like tumor necrosis factor-alpha (TNF-α).

The one-carbon cycle (1CC) is a crucial metabolic cycle playing a vital role in maintaining the cellular redox state. This metabolic pathway, responsible for the synthesis and metabolism of various biomolecules such as amino acids, nucleotides, and lipids, is a complex network of interconnected reactions and necessitates adequate availability of several enzymes, cofactors, and vitamins, including folate, vitamin B12, and betaine, to ensure proper functioning (8, 9). The 1CC plays a vital role in generating the antioxidant glutathione protecting cells from oxidative damage (10). Briefly, the 1CC product S-adenosylmethionine (SAM), besides acting as the universal methyl donor for DNA methylation and other cellular processes, is also the activator of cystathionine beta-synthase (CBS), the regulating enzyme for de-novo Glutathione (GSH) synthesis (11). In turn, oxidative damages can hamper DNA methylation, making the 1CC a pivotal pathway connecting epigenetic programming and redox regulation (9, 12).

Previous studies have demonstrated that infertile men exhibit lower levels of sperm DNA methylation, increased oxidative stress, and elevated DNA damage compared to fertile men (13, 14). Dattilo et al. (15) further emphasized that impaired DNA methylation and/or the oxidation of methyl imprints could result in abnormal sperm chromatin condensation. The disruption of redox homeostasis and DNA methylation, which are characteristic of sperm damage in both clinical and experimental diabetes (16), may also be influenced by disturbances in the 1CC. In this study, we aimed to investigate the involvement of the 1CC in male reproductive impairments using a mouse model of both type 1 and type 2 diabetes.

Material and Methods

Ethical committee approval

This experimental study was conducted in compliance with ARRIVE guidelines (17) and received approval from the Scientific Ethics Committee of Royan Institute (IR.ACECR.ROYAN.REC.1399.072). The mice used in the study were housed at the Royan Institute for Animal Biotechnology in Isfahan, Iran.

Study design

A total of 50 male C57 mice (8-week-old, 25-30 g) were housed in special cages under normal light conditions with a 12-hour light-dark cycle, a temperature of 23 ± 1ºC, and a moisture level of 45-60%. The mice were provided with ad libitum access to pellet rodent diet and water throughout the study. The entire study was conducted by a single person. After a one-week acclimatization period, the mice were randomly assigned to one of four groups: sham (n=10), control (n=10), type 1 diabetes mellitus (DM1, n=15), or DM2 (n=15). The allocation to each group was done randomly.

The sham group received a normal diet (ND) and a single intraperitoneal (i.p) injection of sodium citrate buffer dissolved in double distilled water (1.47 g/kg in double distilled water, pH=4.5) after 8 weeks from the start of the study. The control group was fed an ND without any injection. The DM1 group was fed an ND for 8 weeks and then intraperitoneally administered Streptozotocin (STZ, Sigma-Aldrich, St Louis, MO) dissolved in citrate buffer (pH=4.5) at a dose of 50 mg/kg body weight (b.w.) for 5 consecutive days. Afterwards, the mice were fed an ND for 4 weeks. The DM2 group was fed a high-fat diet (HFD-60% kcal from fat) for 8 weeks and then received a single intraperitoneal injection of STZ (100 mg/kg, dissolved in 0.1 M sodium citrate buffer). The mice were then fed an HFD for 4 weeks.

After twelve weeks from the start of the study, a glucose tolerance test (GTT) was performed in all groups to confirm diabetes induction. Subsequently, all mice were euthanized, and blood samples collected via cardiac puncture. The serum was then separated from the blood and evaluated for sex hormone factors [luteinizing hormone (LH), follicle-stimulating hormone (FSH), and testosterone] as well as methionine, homocysteine, ferritin, and amino acids involved in the one-carbon cycle. The testes, epididymides, pancreas, liver, and kidneys were removed, dissected, and weighed. Testicular morphometric parameters, such as width, length, and thickness, were also measured. Furthermore, the testicular volume was calculated using the formula: length×width²×0.52 (18).

Portions of all organ tissues were fixed in 10% formalin solution and stained with hematoxylin and eosin (H&E) after preparing paraffin sections. The spermatogenesis process in the testicular tissue sections was evaluated based on three criteria: Johnson scores, tubular differentiation index (TDI), and spermatogenesis index (SPI) (19). The cauda epididymis was dissected and cut into small pieces. These pieces were incubated in VitaSperm washing medium (Inoclon, Iran) supplemented with 10% serum for 30 minutes at 37°C, and the extracted sperm was used to assess the study parameters.

Glucose tolerance test

A GTT was performed by fasting the mice for 4-5 hours, followed by an intraperitoneal injection of 2 g/kg D-glucose in 30% phosphate-buffered saline (PBS). Blood glucose levels were measured at 0, 30, 60, 90, and 120 minutes after injection (20) using a glucometer (EASY Gluco Auto-coding TM).

Serum analyses

To evaluate the main components of the 1CC and testosterone levels, high-performance liquid chromatography (HPLC) and ELISA techniques were used. All parameters were assessed by an expert in a clinical laboratory following standard procedures. ELISA kits (Elecsys 2010 and Cobas e411 analyzers) were used

to assess the levels of testosterone (Testosterone II, Roche, Switzerland), folate (Folate III, Roche, Switzerland), and vitamin B12 (Vitamin B12 II, Roche, Switzerland) by the manufacturer's protocol. The serum levels of methionine, homocysteine, serine, lysine, glycine, threonine, aspartic acid, and other factors were analyzed using HPLC. In addition, LH (Luteinizing hormone, Roche, Switzerland) and FSH (Follicle stimulating hormone, Roche, Switzerland) were evaluated by an ELISA kit (Elecsys 2010 and Cobas e 411 analysers).

Assessment of sperm parameters, and sperm function

Briefly, sperm concentration and sperm motility were by evaluated using a sperm counting chamber (Sperm meter, Sperm Processor, Aurangabad, India), under a light microscope with 40x magnification. Sperm abnormal morphology was assessed through Eosin/Nigrosine staining. In addition, sperm functional tests were conducted including aniline blue staining to assess sperm retained histones, chromomycin A3 staining to determine protamine deficiency, acridine orange staining to evaluate sperm DNA damage, BODIPY C11 Probe to measure lipid peroxidation, and Dichloro-dihydro-fluorescein diacetate (DCFH-DA) to assess cytosolic ROS, based on the methodology described by Pouriayevali et al (21).

Assessment of sperm chromatin maturation (protamine deficiency, and sperm persistent histones)

The assessment of excessive retained histones was conducted by using aniline blue staining, which selectively binds to lysine residues on histones. To perform the test, a smear of 20 microliters of extracted epididymal spermatozoa was air-dried and fixed for 2 hours in a 3% glutaraldehyde solution in 0.2 M phosphate buffer at pH=7.2. Subsequently, the prepared slides were dried and subjected to staining with a 5% aqueous solution of aniline blue in 4% acetic acid at pH=3.5 for 90 minutes. Under a light microscope (100x magnification, CX31 OLYMPUS, Japan), a random count of 200 spermatozoa was performed for each sample, and those exhibiting a dark blue stain were considered positive for aniline blue (AB+), indicating abnormal chromatin compaction (21).

The effectiveness of protamine substitution was evaluated using CMA3 staining competing with protamines for DNA binding. Briefly, extracted epididymal spermatozoa in PBS buffer (20 microliters) smeared on slides and fixed with Carnoy's solution (methanol/acetic acid, 3:1, Merck, Darmstadt, Germany) for 5 minutes at a temperature of -4°C. Subsequently, the slides stained with 100 µl of a 0.25 mg/ml chromomycin A3 solution (Sigma Chemical Co., St. Louis, USA) for 1 hour. Following staining, the slides washed twice with PBS, air-dried, and covered with a coverslip. A minimum of 200 spermatozoa on each slide were examined by using a fluorescent microscope (Olympus, BX51, Tokyo, Japan) equipped with the appropriate filters (460-470 nm). Spermatozoa displaying bright yellow staining (CMA3 positive or indicative of protamine deficiency) were recorded (21).

Assessment of sperm DNA methylation

Sperm DNA methylation was evaluated by using a 5-methylcytosine (5-mc) antibody by flowcytometery. Briefly, two million sperm cells mixed with PBS, and fixed with 96% acetone-alcohol. The mixture then kept at 4°C for 30 minutes. After that, the samples were washed with PBS and centrifuged at 500 g for 5 minutes. The supernatant then discarded. In order to decondense the sperm head, a mixture of Triton x-100 and dithiothreitol (DTT) in PBS slowly added to the sperm pellet and the mixture kept in the dark room for 20 minutes. The samples were then washed with PBS and centrifuged at 500 g for 5 minutes.

To block non-specific binding, a solution of 6% normal goat serum (NGS+PBS) was added directly to each sample, and incubated at 25°C for 30 minutes. The samples were then centrifuged at 500 g for 5 minutes, and the primary antibody [5-methylcytosine Antibody (5-mc); Eurogentec; Belgium; Cat. No. BI-MECY-0100, 1:400] added to the sperm pellet in PBS containing 3% NGS and 0.1% Triton x-100 followed by overnight incubation at 4°C. On the following morning, the samples were incubated for one hour at 37°C, then washed and incubated in a darkroom with the secondary antibody (Goat Anti-Mouse IgG (H&L) FITC; Chemicon; USA; Cat. No. AP124F; Lot number: 0605029979) at a dilution of 1:200 for two hours. Afterwards, the samples were rinsed twice with PBS and analyzed using a flow cytometer (FACS Caliber; Becton Dickinson, San Jose, CA, USA). The same method was employed for the isotype control tube of each sample with an isotype antibody (Mouse IgG Negative Control Antibody; Chemicon; Japan; Cat. No. CBL600, 1:300) as the primary antibody. For each sample, the percentage and intensity of sperm DNA methylation was reported.

Statistical analysis

Results were reported as mean ± standard error, and the significance between study groups was determined using Tukeys post hoc multiple comparison test, following a one-way analysis of variance. A $P<0.05$ was considered highly statistically significant.

Results

Confirmation of diabetic models

After a 12-hour fasting period, mice were injected intraperitoneally with 2 g/kg D-glucose in 30% PBS, and their glucose tolerance was tested at 0, 30, 60, 90, and 120 minutes. Figure 1 shows that the blood sugar levels in both type 1 and type 2 diabetic mice were higher than in the control and sham groups. The mean fasting blood sugar (FBS) was significantly higher in both the type 1 (DM1) and type 2 (DM2) diabetic groups compared to the control and sham groups ($P<0.05$). Thereafter, a significant difference was observed in diabetic mice at 60,

90, and 120 minutes compared to control and sham mice.

Spermatogenesis indexes were reduced in the type 1, and type 2 diabetic mice in the testicular tissue

We evaluated the spermatogenesis process in mice testis using the Johnsen score system, TDI, and SPI criteria, and compared the results within the control, sham, type 1, and type 2 diabetic groups. As shown in Figure 2, the mean Johnsen score was significantly lower in type 1 and type 2 diabetic mice compared to the control (P=0.002) whereas the means of TDI (P=0.001) and SPI (P=0.007) were significantly lower in type 1 and type 2 diabetic mice compared to both control and sham groups. We illustrated these results schematically in a section of the testicular tissue of all studied groups using H&E staining.

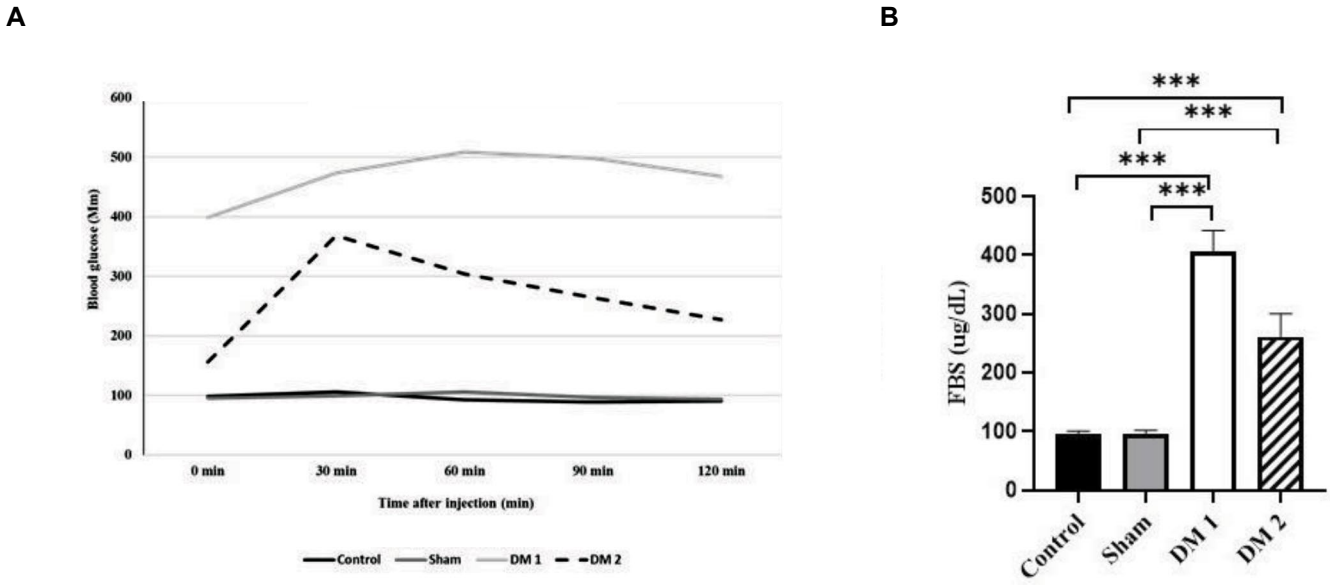


Fig.1: Assessment of glucose tolerance test (GTT) and fasting blood sugar (FBS) to confirm the type 1 and type 2 diabetic models. **A.** GTT was evaluated by intraperitoneally injecting 3% glucose at 0, 30, 60, 90, and 120 minutes. **B.** FBS was assessed in the control (n=10), sham (n=10), type 1 (DM1, n=15), and type 2 (DM2, n=15) diabetic groups. ***; P<0.001.

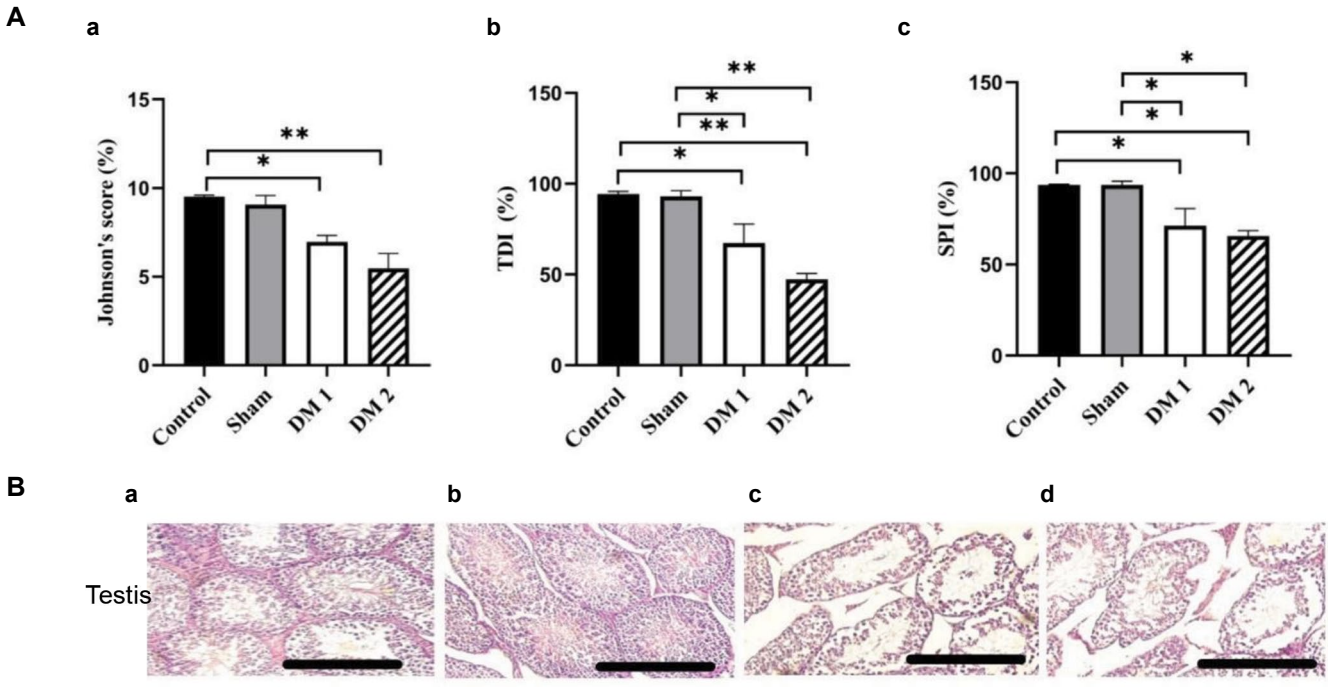


Fig.2: Assessment of testicular morphometric parameters in study groups. **A.** Comparison of spermatogenesis results using the Johnsen score system (a), Z tubular differentiation index (TDI, b), and spermatogenesis index (SPI, c) within the testicular tissues of control, sham, type 1 (DM1), and type 2 (DM2) diabetic mice. **B**. The pattern of these results was illustrated in the section prepared from the testicular tissue of each group using H&E staining (a-d, scale bar: 200 µm). *; P<0.05 and **; P<0.01 [Sham (n=3), Control (n=3), DM1 (n=3), and DM2 (n=3)].

Body weight, and the weight of organs such as the liver, pancreas and kidneys reduced in the type 1, and type 2 diabetic mice.

As shown in Figure 3, the body weight was decreased in type 1 and type 2 diabetic groups compared to control and sham groups, but this decrease was significant only between type 1 diabetic mice and the control group (P=0.000). The weight of organs such as the liver (P=0.005), pancreas (P=0.000) and kidneys (left kidney: P=0.000; right kidney: P=0.001) were also significantly decreased compared to either controls or sham groups.

Testicular weight reduced in the type 1, and type 2 diabetic mice

The mean testicular volume did not show any significant difference between the groups. However, the mean testicular weight was significantly lower in the diabetic groups compared to the control group. The epididymal fat, as shown in Figure 3, was significantly lower in the type 1 diabetic mice compared to controls (P<0.001) and sham (P<0.05) and to type 2 diabetes (P<0.001).

The quality of sperm parameters and sperm function reduced in the type 1, and type 2 diabetic mice

Sperm parameters (Fig.4): Sperm concentration was significantly lower in type 1 and type 2 diabetic mice compared to the control and sham groups (P=0.000). Sperm motility was not different among groups whereas we observed a significant reduction in the mean percentage of progressive motility in the type 1 and type 2 diabetic groups as well as in the sham group compared to the control group (P=0.017). Furthermore, there was a significant increase in the percentage of sperm abnormal morphology in type 1 and type 2 diabetic mice compared to the control group (P=0.003).

Sperm functional tests (Fig.4): The mean values of sperm lipid peroxidation and intracytoplasmic ROS were significantly higher in type 1 and type 2 diabetic groups compared to control and sham groups (P=0.000). In addition, the means of sperm DNA damage as well as sperm chromatin maturation (protamine deficiency, and sperm persistent histones) were significantly higher in type 1 and type 2 diabetic groups compared to the control and sham groups (P=0.000).

The level of sex hormonal factors altered in the type 1, and type 2 diabetic mice

Unlike LH which only showed a significant decrease in type 2 diabetic mice compared to the control group (P=0.028), the mean levels of FSH (P=0.003) and testosterone (P=0.000) were significantly lower in both type 1 and 2 diabetic mice compared to the control and sham groups (Fig.5).

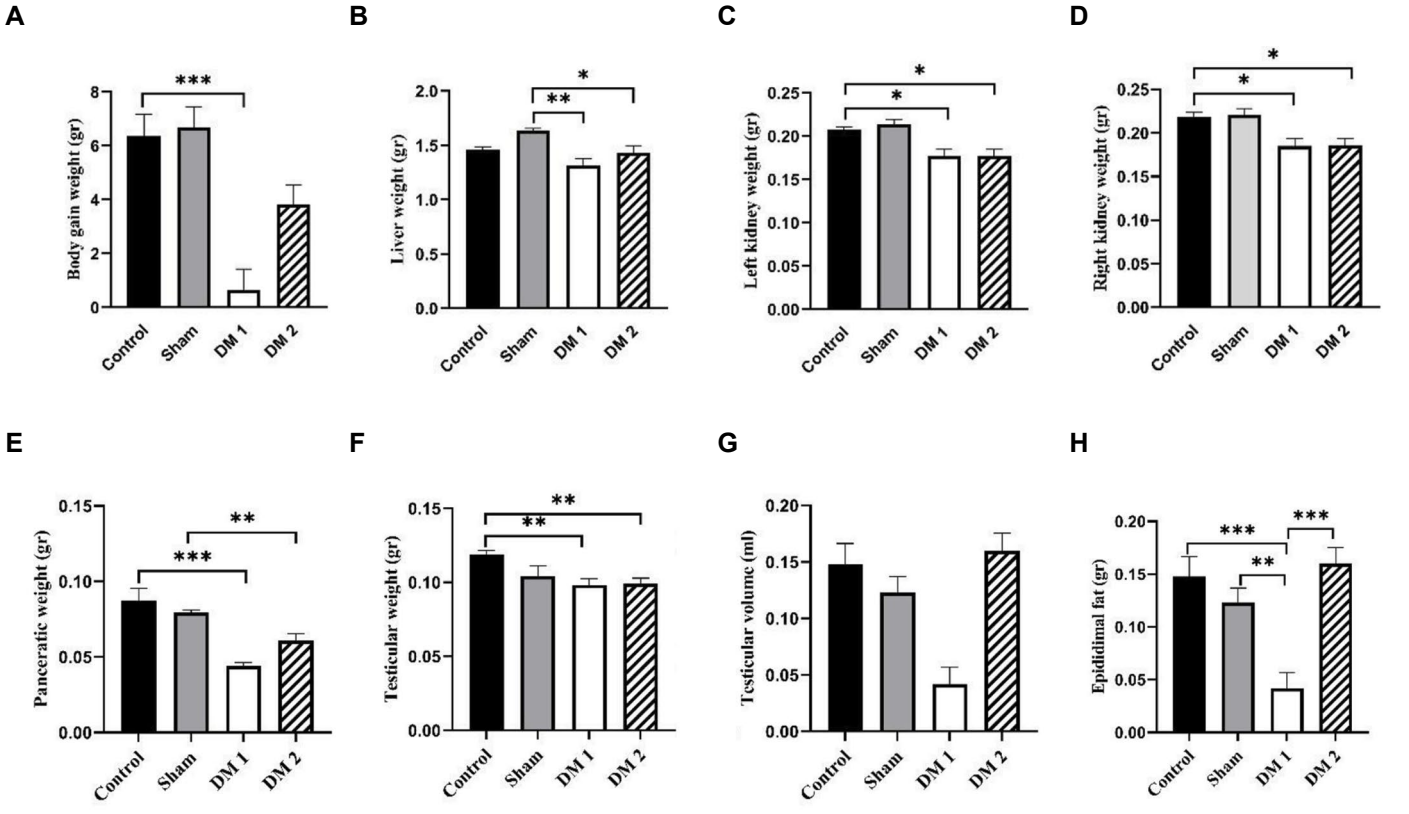


Fig.3: Comparison of body weight, and organs weight within groups. **A.** Body weight, **B**. Liver weight, **C, D**. Right and left kidney weight, **E.** Pancreas weight, **F**. Testicular weight, **G.** Testicular volume, and **H.** Epididymal fat. Control (n=10), Sham (n=10), Type 1 (DM1, n=15), and Type 2 (DM2, n=15) diabetic groups. *; P<0.05, **; P<0.01, and ***; P<0.001.

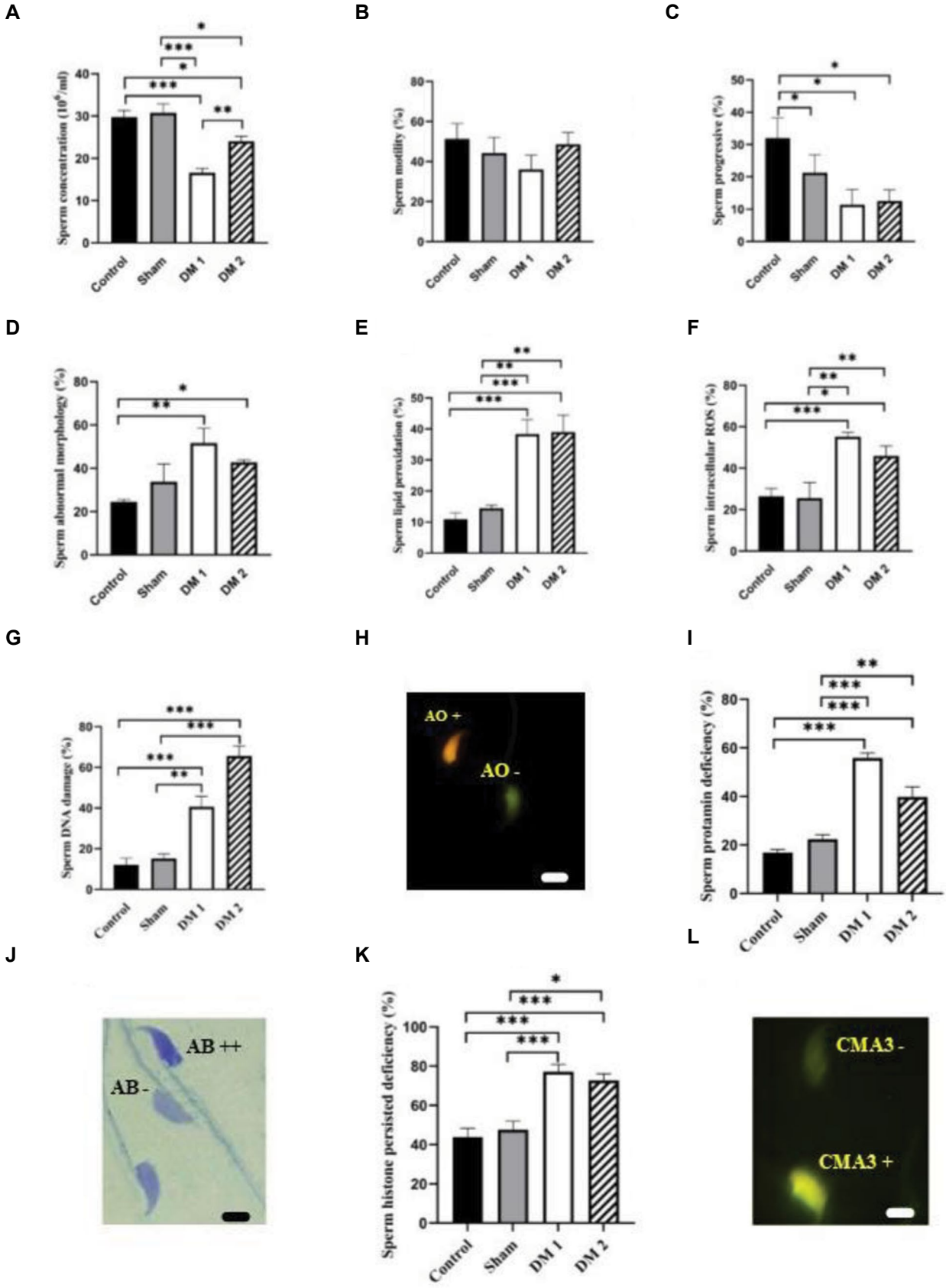


Fig.4: Comparison of sperm parameters and sperm functional tests within groups. **A-D.** Comparison of sperm parameters, and **E-L.** Sperm functional tests within the control, sham, type 1 (DM1), and 2 (DM2) diabetic groups. Sperm lipid peroxidation and sperm intracytoplasmic ROS were assessed by BODIPY and dichlorofluorescin diacetate (DCF-DA) staining, respectively by flow cytometry. In addition, sperm DNA damage, protamine deficiency, and sperm persistent histones were evaluated by acridine orange (orange and yellow sperm as DNA damaged sperm while green sperm were considered to have intact DNA), chromomycin A3 staining (CMA3 positive sperm as sperm with protamine deficient while negative sperms were ranked as sperm with normal protamine content), and aniline blue staining (depending on the intensity of the blue color, dark blue sperm have persistent histones, while pale blue sperm or colorless have a normal level of histone remaining in the sperm), respectively. *; P<0.05, **; P<0.01, and ***; P<0.001 [Sham (n=10), Control (n=10), DM1 (n=15), and DM2 (n=15) (scale bar: 50 µm)].

The level of the main factors involved in the one-carbon cycle altered in the type 1, and type 2 diabetic mice

The levels of the main factors involved in the 1CC such as methionine, homocysteine, serin, lysin, folate, ferritin, and vitamin B12 were compared among study groups. As shown in Figure 6, the mean serum levels of methionine were significantly lower in both type 1 and 2 diabetic groups compared to the control and sham groups (P=0.000). The mean levels of serum homocysteine and ferritin were significantly higher in both diabetic groups compared to the control and sham groups (P=0.000). Vitamin B12 was significantly lower only in the type 1 diabetic group compared to control and sham groups (P=0.025). Serum folate and serine were significantly reduced in the type 1 diabetic group when compared, respectively, to the control group (P=0.030) and the sham (P=0.048) group. Additionally, the mean levels of lysine were significantly lower in both diabetic groups compared to the sham group (P=0.006). Changes in other metabolites involved in the 1CC are reported in Fig.S1 (See Supplementary Online Information at www.celljournal.org).

Sperm DNA methylation was reduced in the type 1, and type 2 diabetic mice

As illustrated in Figure 6, the mean percentages and intensities of sperm DNA methylation were significantly lower in both type 1 and 2 diabetic groups compared to the control and sham groups (P<0.001).

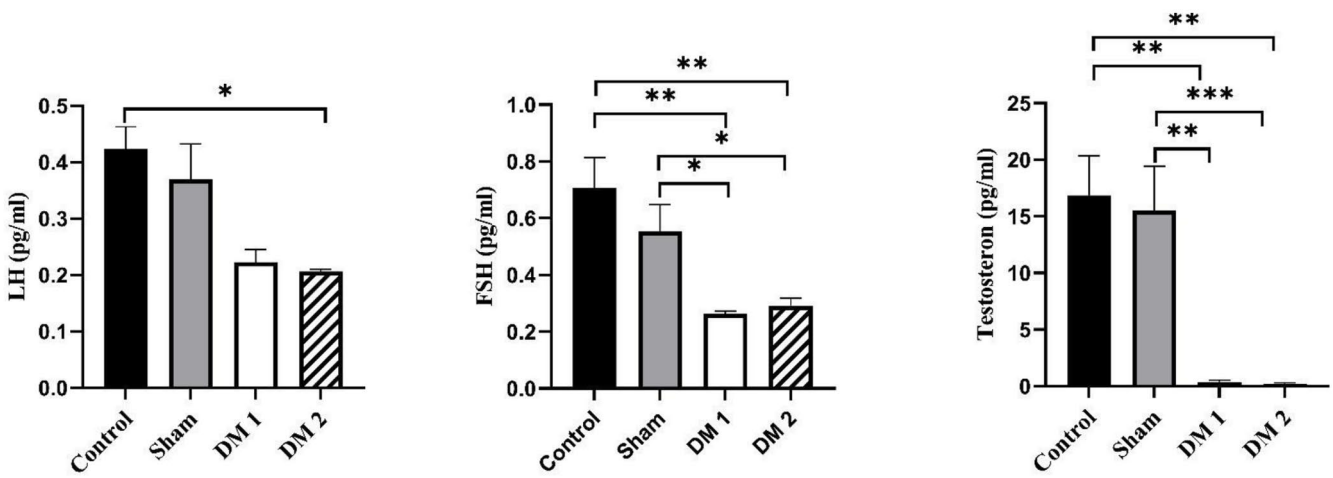
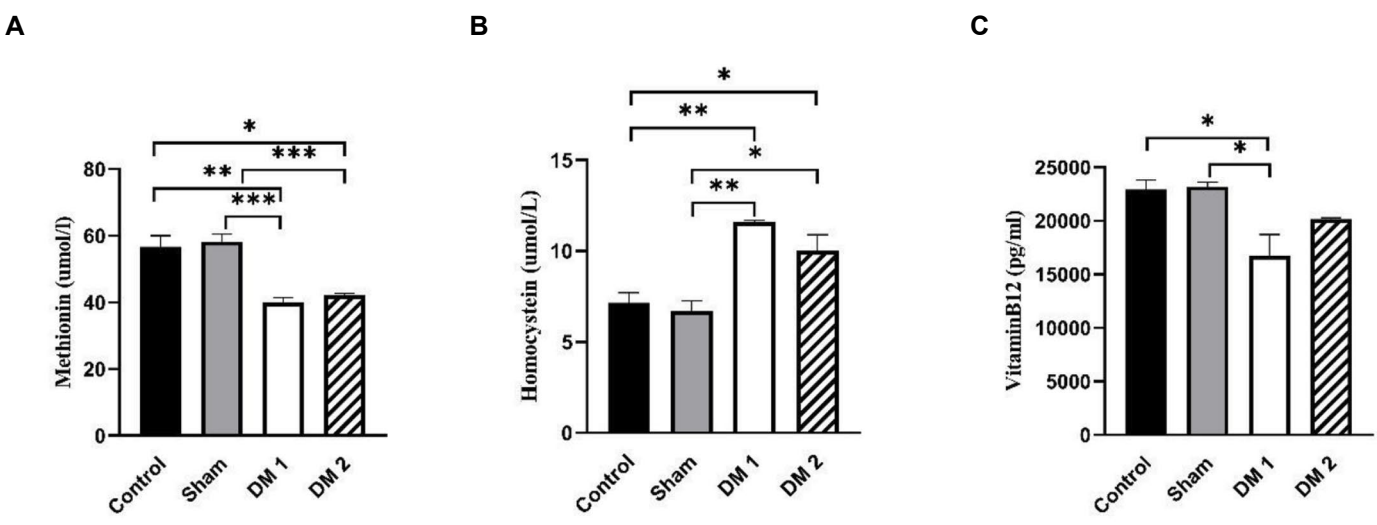


Fig.5: Comparison of serum sex hormonal factors (LH, FSH, and testosterone) among control (n=3), sham (n=3), type 1 (DM1, n=3), and 2 (DM2, n=3) diabetic groups. LH; Luteinizing hormone, FSH; Follicle-stimulating hormone, *; P<0.05, **; P<0.01, and ***; P<0.001.



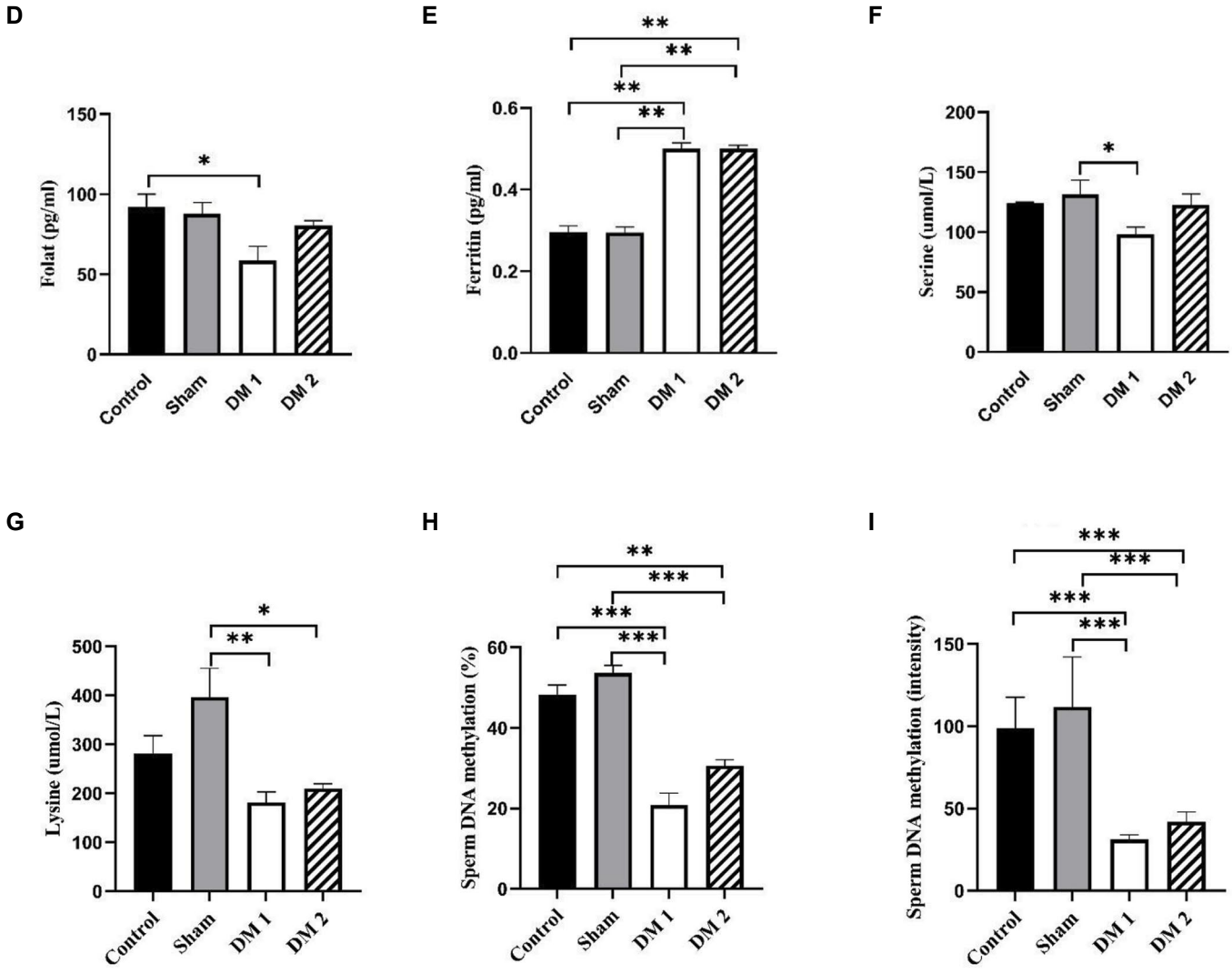


Fig.6: Comparison of the main serum factors involved in One-carbon cycle, and sperm DNA methylation among the various study groups. **A.** Methionine, **B.** Homocysteine, **C.** Vitamin B12, **D.** Folate, **E.** Ferritin, **F.** Serine, **G.** Lysin, **H.** and **I.** Sperm DNA methylation. For assessment of folate, ferritin, serine, and lysine, we used three samples for each group while, for assessment of sperm DNA methylation (% and and intensity), 10 samples were used for each of the control and sham groups and 15 samples were used for each of DM1, and DM2 groups. DM1; Diabetes mellitus type 1, DM2; Diabetes mellitus type 2, *; P<0.05, **; P<0.01, and ***; P<0.001.

Discussion

Diabetes has been shown to have adverse effects on male fertility, with diabetic men experiencing lower sperm quality and higher rates of infertility (22, 23) and such damage is linked to a perturbation of both redox homeostasis and DNA methylation (16). The above functions strictly depend on the correct functioning of the 1CC, therefore we aimed to investigate the perturbations of the 1CC using both type 1 and type 2 diabetes models in mice.

As already shown with similar models (24), both our type 1 and type 2 diabetes mice suffered testicular alterations, defective spermatogenesis and sperm motility, sperm oxidative damage, and defective nuclear maturation baked by a deep suppression of testosterone and FSH. These damages were associated in our animals with significantly increased ROS and oxidative damage to both lipids and DNA together with defective DNA methylation, all pointing to an improper function of the 1CC.

Accordingly, the 1CC was deeply disturbed in our diabetic animals. In their serum we found a sharp increase of homocysteine, likely reflecting defective re-methylation to methionine, which was indeed decreased together with the other source of carbon units, serine, and with a discrete decrease of the methyl donors necessary for homocysteine re-methylation, folate and B12. Chen et al. (25), working on hepatic tissue samples, had already shown that STZ-induced diabetes is associated with deep perturbations of the 1CC within the liver, therefore changes in liver metabolism might as well explain our findings in serum. However, the above 1CC-defective metabolic fingerprint was associated in our animals with decreased sperm methylation, which is a strictly intra-testicular process, proving that the 1CC defect occurred

directly within the testes. This might be dependent on the lower availability of folates and B12 in serum, however, their decrease was modest compared to very evident defects in sperm methylation, so a direct interference of streptozotocin diabetes with the 1CC within the testicular metabolism was likely in place.

The derangement of the 1CC in our animals is unlikely to depend on a direct effect of STZ. Indeed, we found 1CC defect, i.e. lower DNA methylation, within the testes whereas STZ enters the cells via GLUT2 (26) and therefore affects tissues with a high density of GLUT2, like the pancreas and liver, which is not the case of testes that mostly rely on GLUT8 (27). More importantly, in STZ-induced diabetes, the administration of insulin reverts the increase of homocysteine (28), which points to a direct role of glucose in the impairment of the 1CC. However, the sperm was shown to be very resistant to extra-cellular glycemia with no negative effects up to 50 millimolar D-glucose whereas the shortage of intracellular glucose was more likely to hamper (29).

Indeed, the specific importance of glucose for sperm metabolism and function is well known (30). Whether it is due to a lack of insulin (type 1) or defective insulin signaling (type 2), diabetes may have caused a defect in intracellular glucose in our mice. Within cells, besides being oxidized to produce energy, glucose can also be used by the serine synthesis pathway (SSP) to generate serine and thereafter methionine, at least in conditions of low methionine (31), thus feeding carbon units to the 1CC. This would not happen in diabetes where intracellular glucose is in shortage and unlikely to feed the above pathway. Thus, the impairment of the 1CC in our animals may directly depend on the shortage of intracellular glucose and the significant reduction of serum serine in our type 1 diabetes group seems to endorse this interpretation.

Whatever the mechanisms, the defect in methylations may easily explain the oxidative damage to the sperm of our diabetic animals. First, the defective homocysteine re-methylation leads to a shortage of SAM, necessary to activate GSH de novo synthesis (11) and antioxidant defences, which may explain the increase of ROS and the activation of lipoperoxidation. Second, the defect in methylation may cause histone retention and protamine defect (32), both reducing the efficacy of the protamine shield and thus increasing the exposure of sperm DNA to ROS insult explaining the increased DNA damage.

The present study reveals a significant difference in the mean levels of valine and glycine between mice with type 1 and type 2 diabetes. This distinction may be attributed to various potential causes and mechanisms. These include dysregulation of specific metabolic pathways involved in amino acid metabolism, such as the branched-chain amino acid (BCAA) pathway, as well as impaired insulin signaling and insulin resistance observed in type 2 diabetes (33, 34). These factors can impact the metabolism of amino acids, including valine and glycine. A study by Vangipurapu et al. (34) demonstrated that nine amino acids (phenylalanine, tryptophan, tyrosine, alanine, isoleucine, leucine, valine, aspartate, and glutamate) were significantly associated with decreased insulin secretion (disposition index) and elevated fasting or 2-hour glucose levels. Among these amino acids, five (tyrosine, alanine, isoleucine, aspartate, and glutamate) were also significantly associated with an increased risk of developing type 2 diabetes. However, no studies have compared these amino acids between type 1 and type 2 diabetes, necessitating further investigation.

In summary, we are showing that both type 1 and type 2 STZ diabetes models in mice result in deep disturbance of the 1CC within the testes leading to defective sperm nuclear maturation and increased oxidative damage. Based on our findings, the most likely reason for these damages is the shortage of intracellular glucose that, besides bioenergetic problems, may generate a reduced flux of carbon units to feed the methylation metabolism. Due to the high relevance of methylations in sperm maturation, the impairment of the 1CC is likely involved in the decreased fertility of diabetic patients.

One of the notable innovations of this research is the concurrent assessment of the fundamental components of the 1-carbon cycle and sperm function investigated in both type 1 and type 2 diabetes conditions. The main limitation of the present study is that we could not measure GSH, therefore we cannot confirm that the methylation defect resulted in a shortage of antioxidant defences in our animals. Moreover, we neither measured intracellular glucose nor did we assess the markers of glycolysis to show a reduced activity, therefore the shortage of intracellular glucose that we claim, although very likely to be in place, remains speculative.

However, our findings may have wider implications. Based on our interpretation, the well-known activation of oxidative damage in diabetes seems to be a secondary phenomenon linked to a deficit of the 1CC and may have little chance of being corrected by oral antioxidants that, moreover, cannot remedy the defect of methylations. These concerns may explain the conflicting and non-conclusive clinical benefits of dietary or supplemented antioxidants in clinical diabetes (35). The same concerns apply to the effect of metformin on sperm function. Metformin mainly works by decreasing the synthesis of glucose and, although decreasing glycemia, may therefore further decrease the feed of carbon units from glucose and, hence, the sperm methylation defects. Metformin assumption by fathers in the three months before conception was reported to increase by 40% the risk of birth defects in the offspring (36) fitting with a methylation deficit and epigenetic derangement.

Conclusion

We have observed a distinct impairment of 1CC within the testes of individuals with diabetes. We speculate that this impairment may be linked to inadequate intracellular glucose and diminished carbon unit supply associated with diabetes. As a result, interventions focusing on enhancing glucose uptake into sperm cells and providing supplementary methyl donors have the potential to improve fertility issues

in diabetic patients. However, additional clinical testing is required to validate these hypotheses.

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Authors' Contributions

F.P.; Sample preparation, Conduction of experiment, and Data collection. F.K.; Induction of diabetes. M.T., M.D., M.H.N.-E.; Study design, Statistics analysis, Writing the original draft Review, and Editing. All authors have read and approved the final manuscript.

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