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Young plasma transfer recovers decreased sperm counts and restores epigenetics in aged testis

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ABSTRACT

Background and aim: Aging is one of the causes of male infertility, and abnormal global DNA methylation and imprinting defects have been characterized in testis during biological aging. One of the important emerging approaches aims to take advantage of the healing properties of young blood plasma to limit the progression of aging in various organs in the body. We aimed to show whether blood plasma transfer has an effect on DNA methylation and spermatogenetic cell development. In addition, we aimed to show whether the young plasma transfer to old mice has an effect on the rejuvenation of the old and whether the impaired DNA methylation and PCNA expression in old age can be restored.

Methods: Groups were (i) young control, (ii) young plasma transfer to aged, (iii) aged control, (iv) aged plasma transfer to young. We utilized IHC and WB in protein level of Dnmts. For the global DNA methylation level, we used 5-methylcytosine staining. We also analyzed PCNA protein expressions in all groups by IHC.

Results: We found that transfusion of young plasma into the old animal restored DNA methylation and PCNA expression as it did in the young animal. Most importantly, we observed an increase in spermatogonia and spermatid counts in older animals after young blood plasma transfer.

Conclusions: Our findings show that young plasma transfer can restore epigenetic disorders that occur with aging and solve infertility problems by increasing the sperm count that decreases. It needs to be supported by different studies, especially human studies.

1. Introduction

Aging causes irreversible changes in all eukaryotic organisms at the level of molecules, cells, tissues, organs, and systems. Telomere shortening, epigenetic and genomic instabilities, damaged protein accumulations also cause degenerations in tissues and physiological disorders with aging (Gonskikh and Polacek, 2017; Krisko and Radman, 2019). Both male and female reproductive capacities decline with age. Generally, paternal age causes increased oxidative stress, lipid peroxidation and mitochondrial ROS generation. Increased ROS generation causes apoptosis and decreased antioxidant capacity. Decreased total antioxidant capacity causes oxidative DNA damage in the germ line. If fertilization occurs, DNA mutations in the offspring increases and in offspring have increased miscarriages, childhood cancers, metabolic diseases and autosomal and X linked dominant genetic diseases (Gunes et al., 2016). Paternal aging can cause infertility as well as these. Daily sperm production, total sperm count, and sperm viability are negatively correlated with age. Daily sperm production decreases >30 % in men over the age of 50 and is negatively correlated with age in men in general (Homonnai et al., 1982; Johnson et al., 1984; Neaves et al., 1984). Recently, a meta-analysis was conducted to evaluate the effects of aging on semen parameters including semen volume, sperm concentration, total sperm count, morphology, total motility, progressive motility, and DNA fragmentation. The results have demonstrated that paternal aging leads to a decrease in sperm parameters and increased sperm DNA fragmentation (Gunes et al., 2016). A study examining paternal age effect on 3287 couples with natural fertilization demonstrated an increased delay in pregnancy men were older than 40 years (de La Rochebrochard and

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Thonneau, 2003).

Various approaches have emerged to meet the clinical need for treating aging and related diseases, and aimed the slowing the aging process and improving health. One of the important emerging approaches aims to take advantage of the healing properties of young blood plasma to limit the progression of aging in various organs in the body. Early studies investigating the effect of systemic factors in blood plasma on aging and lifespan showed positive effects of young plasma on quality of life in aged animals (Kennedy et al., 2014). For instance, in an injury model, it has been stated that there are significant differences between the regeneration levels of skeletal muscle in an elderly individual receiving young blood plasma compared to an elderly individual not receiving plasma (Conboy et al., 2005). In another study, it was reported that age-related reductions in pancreatic beta cell proliferation returned to youthful levels only 2–3 weeks after sharing young plasma, and improvements in renal aging parameters were observed in elderly individuals whose young plasma was shared (Huang et al., 2018). In a study conducted to improve the age-related brain dysfunction, it has been observed that young blood plasma affected the increasing adult neurogenesis and enhanced hippocampal synaptic plasticity in cognitional levels and also has curative effect (Villeda et al., 2014). In liver damage due to age-related impaired autophagy, it has been suggested that application of young plasma moderately reduces liver damage by restoring the dysfunctional autophagy process in aged rats; therefore, young plasma has a beneficial property in ameliorating aging-related organ damage (Liu et al., 2018). Many studies similar to the above have shown the healing properties of youth plasma in different tissues/organs by certain cellular processes/paths in favour of healthy aging but it is not known how the epigenetic mechanisms are affected by these transfers. It is very important point because aging causes abnormality in epigenetic mechanisms which end up with epigenetic disorders.

DNA methylation is an epigenetic mechanism plays important roles in the transcriptional repression/activation of development-related genes, X-chromosome inactivation, cell differentiation, tumorigenesis and genomic imprinting. In the process of DNA methylation, DNA methyltransferase (Dnmt) enzymes specifically add a methyl group to the fifth carbon atom of the cytosine residues using S-adenosyl-L-methionine (AdoMet) as a methyl donor (Turek-Plewa and Jagodzinski, 2005). DNA methylation commonly occurs in cytosine-phosphate-guanine (CpG) dinucleotide sites and rarely in the non-CpG sequences. Two different DNA methylation mechanisms have been identified: de novo and maintenance methylation (Turek-Plewa and Jagodzinski, 2005). De novo methylation is responsible for methylating unmethylated DNA sequences whereas maintenance methylation provides methylation of hemimethylated DNA sequences during DNA replication. The DNA methylation processes are catalyzed by DNMT enzymes. Up to date, structurally and functionally six different Dnmts have been characterized: Dnmt1, Dnmt2, Dnmt3a, Dnmt3b, Dnmt3c and Dnmt3l. Dnmt1 is primarily implicated in maintenance methylation and also contributes to de novo methylation process (Fatemi et al., 2002). On the other hand, Dnmt3a and Dnmt3b have essential roles in establishing de novo methylation and in creating new genomic imprints (Turek-Plewa and Jagodzinski, 2005). Although Dnmt3l does not have any catalytic domain, it is capable of inducing Dnmt3a and Ddnmt3b activities, so it participates in de novo methylation indirectly (Deplus et al., 2002; Margot et al., 2003). Interestingly, Dnmt2 methylates the cytosine 38 localized in the anticodon loop of aspartic acid transfer RNA instead of methylating DNA (Goll et al., 2006). Dnmt3c is recently found and prevents male germ cell genome from potential transposon activity, providing epigenetic control of retrotransposons (Barau et al., 2016). In the published review article, we have comprehensively evaluated the spatial and temporal expression levels and localizations of the Dnmts and their mRNA expression patterns in the mouse and human spermatogenetic cells during spermatogenesis (Uysal et al., 2016). It is important to note that establishment of maintenance and de novo DNA methylation, and genomic imprinting in the spermatogenic cells during spermatogenesis is essential for producing competent spermatozoa to fertilize mature oocytes, and for subsequent embryonic and fetal development (Saitou et al., 2012). Consistently, Dnmt knockout studies have shown that lack of Dnmts lead to infertility, imprinting disorders, epigenetic anomalies, or embryonic lethality (Uysal et al., 2016). Also, abnormal DNA methylation in the imprinting genes of spermatogenic cells is found to be related to development of male infertility (Omisanjo et al., 2007; Marques et al., 2008; Nasri et al., 2017). We aimed to show whether blood plasma transfer has an effect on DNA methylation, PCNA (proliferative cell nuclear antigen) expression and spermatogenetic cell development. In addition, we aimed to show whether the young plasma transfer to old mice has an effect on the rejuvenation of the old and whether the impaired DNA methylation and PCNA expression in old age can be restored. We found that transfusion of young plasma into the old animal restored DNA methylation as it did in the young animal. Most importantly, we observed an increase in spermatogonia and spermatid counts in older animals after transfer. The results of our study show that young plasma transfer can restore epigenetic disorders that occur with aging and solve infertility problems by increasing the sperm count that decreases with aging.

2. Materials and methods

2.1. Collection of rat testes

The testes were collected from Male Sprague Dawley rat species with standard animal conditions. For plasma collection; pooled rat plasma was collected by terminal cardiac puncture at the time of euthanasia. Plasma was prepared from blood collected with EDTA followed by centrifugation at 1000*g*. For plasma denaturation, plasma was heated for 2–3 min at 95 °C, followed by a short spin at 1000 g. All plasma aliquots were stored at −80 °C until use. Before administration, plasma was dialyzed using 3.5-kDa D-tube dialyzers (EMD Millipore) in PBS to remove EDTA (Villeda et al., 2014; Ceylani and Teker, 2022). The aged rats (24 months n = 7) were treated with pooled plasma (0.5 ml per day for 30 days, intravenously into the tail vein) collected from young (5 weeks, n = 51) rats. The young rats (5 weeks, n = 7) were treated with pooled plasma (0.25 ml per day for 30 days, intravenously into the tail vein) collected from aged (24 months n = 16) rats. All animals were housed under standard animal care conditions and had free access to food and water. Our study was carried out with the approval of the Ethics Committee (approval number: 2021/03) from the Saki Yenilli Experimental Animal Production and Practice Laboratory.

2.2. Paraffin embedding

The rat testes at the different groups were immersed in Bouin's solution (Sigma-Aldrich, St. Louis, MO, USA) at +4 °C for 12 h, and then they were dehydrated through a graded ethanol series. Following that the testes were cleared in xylene and subsequently embedded in paraffin. Serial cross sections at 5 μm thickness were cut from the paraffin blocks using a rotary microtome (Leica, Nussloch, Germany), and mounted on the *superfrost plus* glass slides (Thermo Scientific, Rockford, IL, USA) which were later used for hematoxylin-eosine (HE) and immunohistochemical staining. The HE-stained slides were analyzed to determine their histopathological status.

2.3. Immunohistochemistry

Expression of the Dnmt1, Dnmt3a, Dnmt3b, and Dnmt3l proteins as well as 5-methylcytosine (5mC) staining in the rat testes were evaluated by using immunohistochemistry. The paraffin sections at 5 μm thickness were deparaffinized in fresh xylene, and then rehydrated in a series of decreasing ethanol concentrations. After deparaffinization and rehydration processes, citrate buffer (pH 6.0) was treated for antigen retrieval using microwave (2 × 5 min at 750 watts). The endogenous

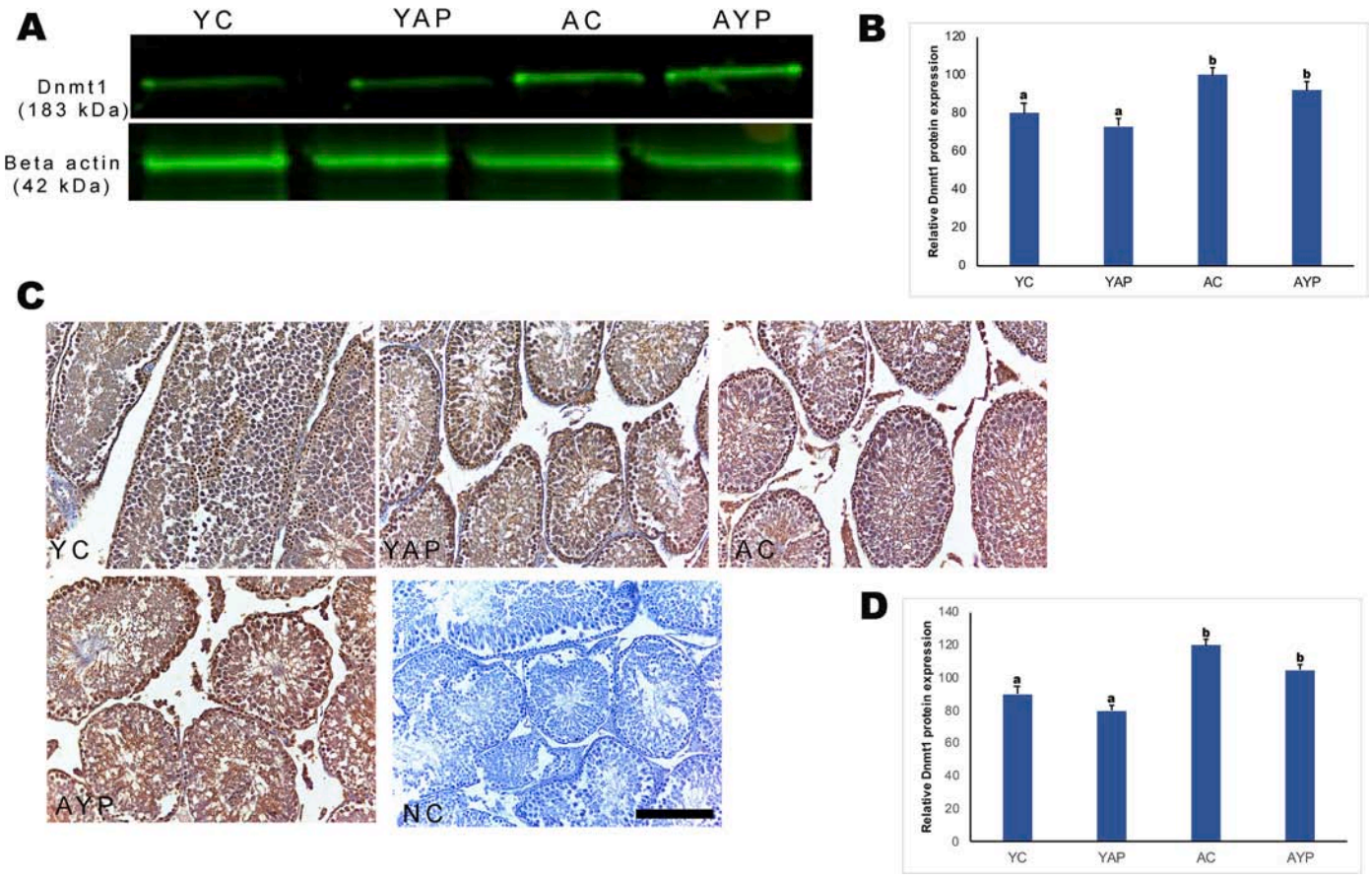

Fig. 1. Dnmt1 expression in controls and blood transfer groups (A) WB assays show the fluorescent-labeled 183 kDa band specific to Dnmt1, and 42 kDa band to ß-actin used as the internal control. (B) Relative Dnmt1 band intensity level in WB analysis. Dnmt1 expression was significantly increased in aged control group, but it significantly decreased after young plasma transfer in aged group. (C) Dnmt1 expression in all groups. (D) Relative Dnmt1 protein expression level in IHC analysis. The statistical significance was determined by using one-way ANOVA followed by Dunn's post hoc test. The $P < 0.05$ was considered statistically significant, shown different letters on the columns. Bars in graphs are represented as mean ± SD. YC, young control; YAP, young plasma transfer to aged; AC, aged control; AYP, aged plasma transfer to young. Scale bar: 20 μm.

peroxidase activity in the sections was ceased by 3 % hydrogen peroxide prepared in methanol for 30 min at room temperature (RT). Following several washes with 1× phosphate buffered saline (PBS), sections were blocked with blocking buffer (Thermo Scientific) at RT for 7 min in order to prevent non-specific binding. Then, sections were incubated with the following primary antibodies: Dnmt1 (1:300; catalog no. ab87654, Abcam), Dnmt3a (1:200; catalog no. ab23565, Abcam), Dnmt3b (1:300; catalog no. ab2851, Abcam), Dnmt3l (1:50; catalog no. 194094, Abcam) and 5mC (1:200; catalog no. MABE146, Millipore) at +4 °C overnight. One of three sections on each slide was incubated with antibody isotype (catalog no. ab172730, Abcam) for testing the primary antibodies specificity. After that, sections were washed three times in PBS for 15 min each, and subsequently incubated with anti-rabbit secondary antibody (1:400; catalog no. BA-1000, Vector) for Dnmt1, Dnmt3a, Dnmt3b, and Dnmt3l expression, and incubated with anti-rat secondary antibody (1:500; catalog no. BA-9200, Vector) for 5mC staining at RT for 1 h. Finally, sections were incubated with streptavidin-horseradish peroxidase (HRP) complex (catalog no. TS-125, Thermo Scientific) for 20 min at RT. The immune reactions were visualized using 3,3′-diaminobenzidine (DAB) chromogen (catalog no. D4168, Sigma-Aldrich) under a light microscope. Then, sections were washed in tap water and counterstained with Mayer's hematoxylin. The expressional distributions of the Dnmt1, Dnmt3a, Dnmt3b, and Dnmt3l proteins, and 5mC staining in all groups were evaluated and captured under a bright-field microscope (Carl Zeiss, Oberkochen, Germany). The relative immunostaining intensity of these proteins and 5mC staining in all ages were analyzed by using ImageJ software (National Institutes of Health, Bethesda, Maryland, USA). Seven rats and 21 slides were evaluated for each group by evaluating totally 500–600 different cell types for stage by stage for individual section.

2.4. Western blottting

Total protein, Dnmt1, Dnmt3a, Dnmt3b, Dnmt3l and β-actin quantities in four groups using WB. All groups were dissolved in lysis buffer [1 % sodium dodecyl sulphate (SDS), 1.0 mM sodium ortho-vanadate,10 mM Tris pH 7.4] supplemented with 1× protease inhibitor cocktail (Amresco, USA). The protein concentration was measured using the BCA (bicinchoninic acid) method. Fifty micrograms of protein from each group were separated on 10%Tris- HCl gel using protein electrophoresis (BioRad, USA). Then electrotransferred to a polyvinylidene difluoride (PVDF) membrane (Roche, UK) o/n at +4 °C. Then, the membrane was blocked with 5 % (w/v) bovine serum albumin (BSA) prepared in TBS-T (20 mM Tris/HCl and 150 mM NaCl plus 0.05 % Tween-20 at pH 7.4) at RT for 1 h. Membranes were incubated with primary antibodies specific to Dnmt1 Dnmt3a, Dnmt3b, Dnmt3l or β-actin (Abcam; 8227) [1:1000 in 5 % (w/v) BSA containing TBS-T] for 2 h at RT. Following a triple-wash in TBS-T for 15 min each, membranes were incubated with goat anti-rabbit secondary antibody (800 nm) (1:2000 in TBS-T) (Licor Biosciences, USA) at RT for 1 h on a shaker. Protein band intensities were measured using a Li-Cor Odyssey CLx infrared detection system (LICOR Biosciences, USA) following the

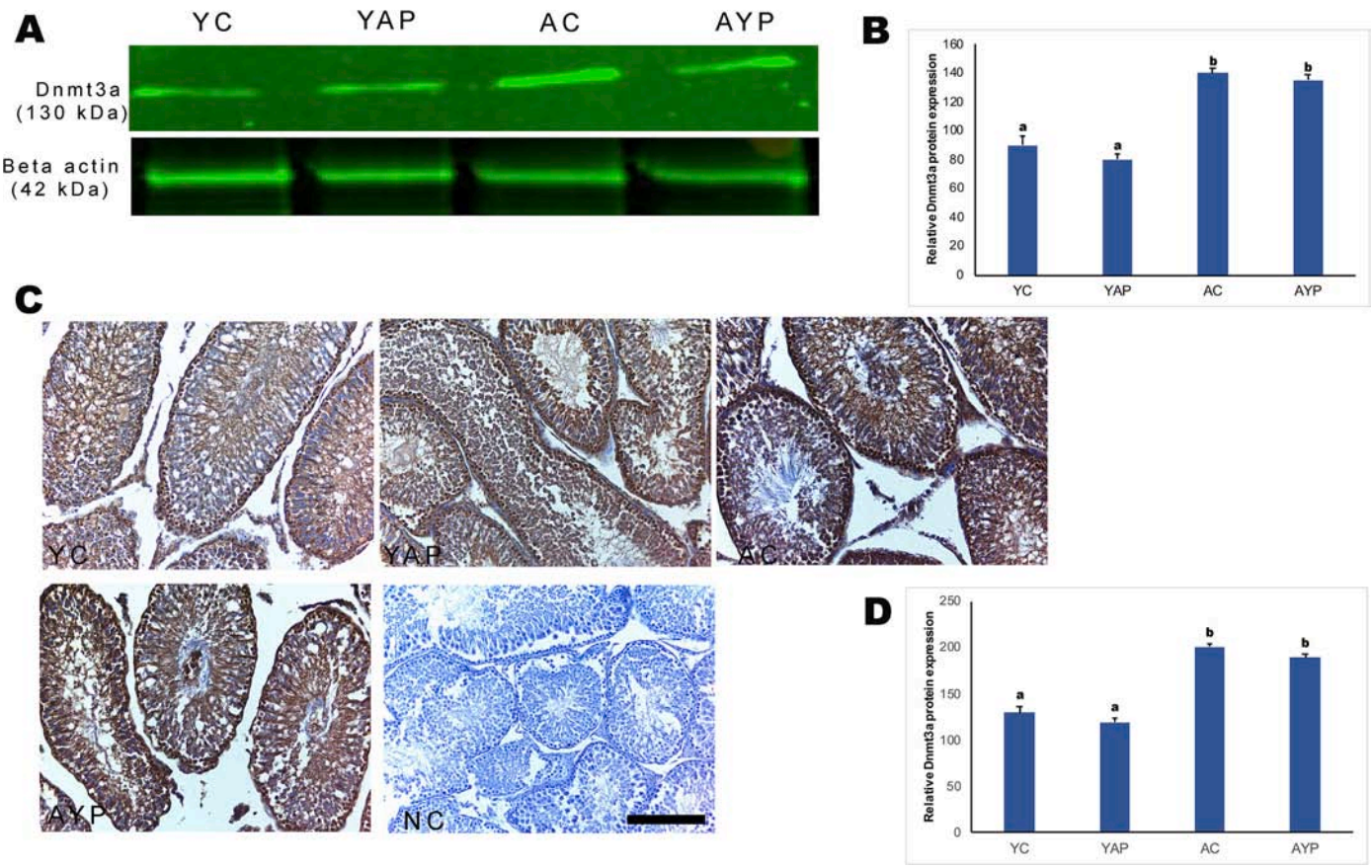

Fig. 2. Dnmt3a expression in controls and blood transfer groups (A) WB assays show the fluorescent-labeled 130 kDa band specific to Dnmt3a, and 42 kDa band to ß-actin was used as the internal control. (B) Relative Dnmt3a band intensity level in WB analysis. Dnmt3a expression was significantly increased in aged control group, but it significantly decreased after young plasma transfer in aged group. (C) Dnmt3a expression in all groups. (D) Relative Dnmt3a protein expression level in IHC analysis. The statistical significance was determined by using one-way ANOVA followed by Dunn's post hoc test. The $P < 0.05$ was considered statistically significant, shown different letters on the columns. Bars in graphs are represented as mean ± SD. YC, young control; YAP, young plasma transfer to aged; AC, aged control; AYP, aged plasma transfer to young. Scale bar: 20 μm.

manufacturer's guidelines. Relative band intensities were measured and analyzed using ImageJ v.3.91 software.

2.5. Statistical analysis

The data from all experiments were examined by using one-way analysis of variance (one-way ANOVA) followed by Dunn's post hoc test. We conducted the statistical calculations by using SigmaStat for Windows, version 3.5 (Jandel Scientific Corp). $P < 0.05$ was considered to be statistically significant.

3. Results

In the current study, histopathology of testicular cells, distribution and signal intensity of the Dnmts protein levels and PCNA were evaluated in the rat testes and spermatogenetic cell stages as well as Sertoli cells. In addition, the global DNA methylation levels were analyzed. We found that Dnmts expression and global DNA methylation levels were significantly increased in the aged group compared to young counterparts. Interestingly, Dnmts expression and global DNA methylation decreased when young plasma transfer to aged group. Otherwise, Dnmts expression and global DNA methylation increased when old plasma transfer to young group. On the other hand, it is well-known that DNA methylation plays important role in transcriptional repression. PCNA expression decreased in aged group and increased in young groups but when the young plasma transfer to aged group, PCNA expression significantly increased.

3.1. Dnmt1, Dnmt3a, Dnmt3b, and Dnmt3l protein expressions

When we have further evaluated the relative Dnmt1, Dnmt3a and Dnmt3l intensity levels significantly increased in the aged group compared to young counterparts (Figs. 1, 2, 4). Interestingly, Dnmt1, Dnmt3a and Dnmt3l expression decreased when young plasma transfer to aged group. Otherwise, Dnmt1, Dnmt3a and Dnmt3l expression increased when old plasma transfer to young group. However, there were no differences for Dnmt3b expression between groups (Fig. 3). We secondly analyzed the distribution and signal intensity patterns in IHC images. Dnmt1, Dnmt3a and Dnmt3l signal intensity levels significantly increased in the aged group compared to young counterparts and decreased when young plasma transfer to aged group. In addition, Dnmt1, Dnmt3a and Dnmt3l expression increased when old plasma transfer to young group like in WB results. There were no differences for Dnmt3b signal intensity between groups. Thirdly, we evaluated the distribution and signal intensity patterns in spermatogenic series including spermatogonium, spermatocyte, round spermatid, elongated spermatid and Sertoli cells. Dnmt1 protein distribution was high level spermatogonia and round spermatid and low level in spermatocyte. Dnmt3a protein distribution was high level spermatogonia and low level in Sertoli cell. Dnmt3b protein distribution was high level spermatogonia and spermatocyte and low level in round spermatid. Dnmt3l protein distribution was high level spermatocyte and low level in spermatogonia. No Dnmt1, Dnmt3a, Dnmt3b and Dnmt3l signal was detected in elongated spermatid.

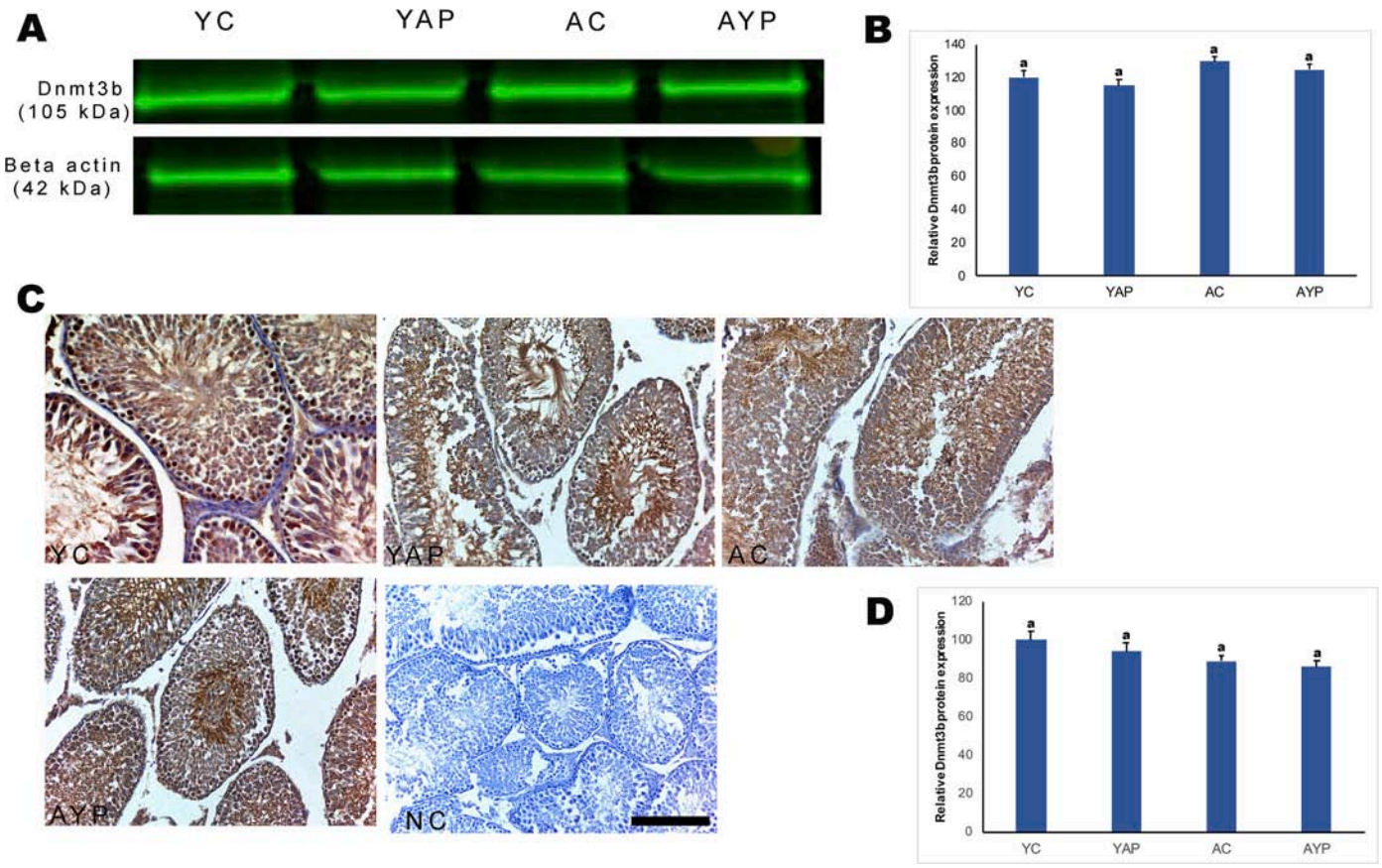

Fig. 3. Dnmt3b expression in controls and blood transfer groups (A) WB assays show the fluorescent-labeled 105 kDa band specific to Dnmt3b, and 42 kDa band to ß-actin was used as the internal control. (B) Relative Dnmt3b band intensity level in WB analysis. There were no significant differences between groups. (C) Dnmt3b expression in all groups. (D) Relative Dnmt3b protein expression level in IHC analysis. The statistical significance was determined by using one-way ANOVA followed by Dunn's post hoc test. The $P < 0.05$ was considered statistically significant, shown different letters on the columns. Bars in graphs are represented as mean ± SD. YC, young control; YAP, young plasma transfer to aged; AC, aged control; AYP, aged plasma transfer to young. Scale bar: 20 μm.

3.2. Global DNA methylation

When we have further evaluated the relative global DNA methylation levels, significantly increased DNA methylation in the aged group compared to young counterparts was observed. Global DNA methylation level decreased when young plasma was transferred to aged group. Global DNA methylation was at the highest level in elongated spermatid, then the level was high in spermatogonia and spermatocyte, it decreased in round spermatid and was at the lowest level in Sertoli cells (Fig. 5).

3.3. PCNA expression

When we have further evaluated the relative PCNA protein intensity levels, PCNA expression significantly decreased in the aged group compared to young counterparts. When we transferred then young plasma to aged rats, PCNA expression significantly increased similar to young counterparts. On the other hand, when we transferred to aged plasma to young rats, PCNA expression significantly decreased similarly aged group. It is compatible with our DNA methylation and Dnmts expressions results. In young and young plasma transfer to aged groups, DNA methylation and Dnmts expression levels significantly decreased, so they cause increased PCNA expression level (Fig. 6). Because it is well-known that DNA methylation plays crucial role in transcriptional repression of developmental related genes.

3.4. Developmental dynamics upon blood plasma transfer

To analyze further, whether blood plasma transfer play any role in the kinetics of development, we counted cells stage by stage (Fig. 7). The cells in the young group (YC) were normalized to 100 %. The spermatogonium ratios in aged group (AC), were found 60 % that was very low compared to the young control indicating that aging impairs spermatogonium development. It is very important that the spermatogonium ratios in young blood plasma transfer to aged group (YAP) was found 94 %. The spermatogonium ratios in aged blood plasma transfer to young (AYP) was found 90 %. In order to determine the maturation success of developing cells in transfer groups, we counted the number of spermatogenic cells at each developmental stage. The spermatocyte ratios in aged group (AC), were found 50 %, 60 % in young blood plasma transfer to aged group (YAP), 90 % in aged blood plasma transfer to young (AYP). There was no prominent difference between aged and young plasma transfer to aged groups in terms of spermatocyte counts. The round spermatid ratios in aged group (AC), were found 45 %, 85 % in young blood plasma transfer to aged group (YAP), 87 % in aged blood plasma transfer to young (AYP). The elongated spermatid ratios in aged group (AC), were found 40 %, 70 % in young blood plasma transfer to aged group (YAP), 95 % in aged blood plasma transfer to young (AYP). The Sertoli cell ratios in aged group (AC), were found 86 %, 90 % in young blood plasma transfer to aged group (YAP), 92 % in aged blood plasma transfer to young (AYP).

4. Discussion

DNA methylation is one of the epigenetic mechanisms that play crucial roles in the expressional regulation of the development-related genes, which are required during spermatogenesis. DNA methylation

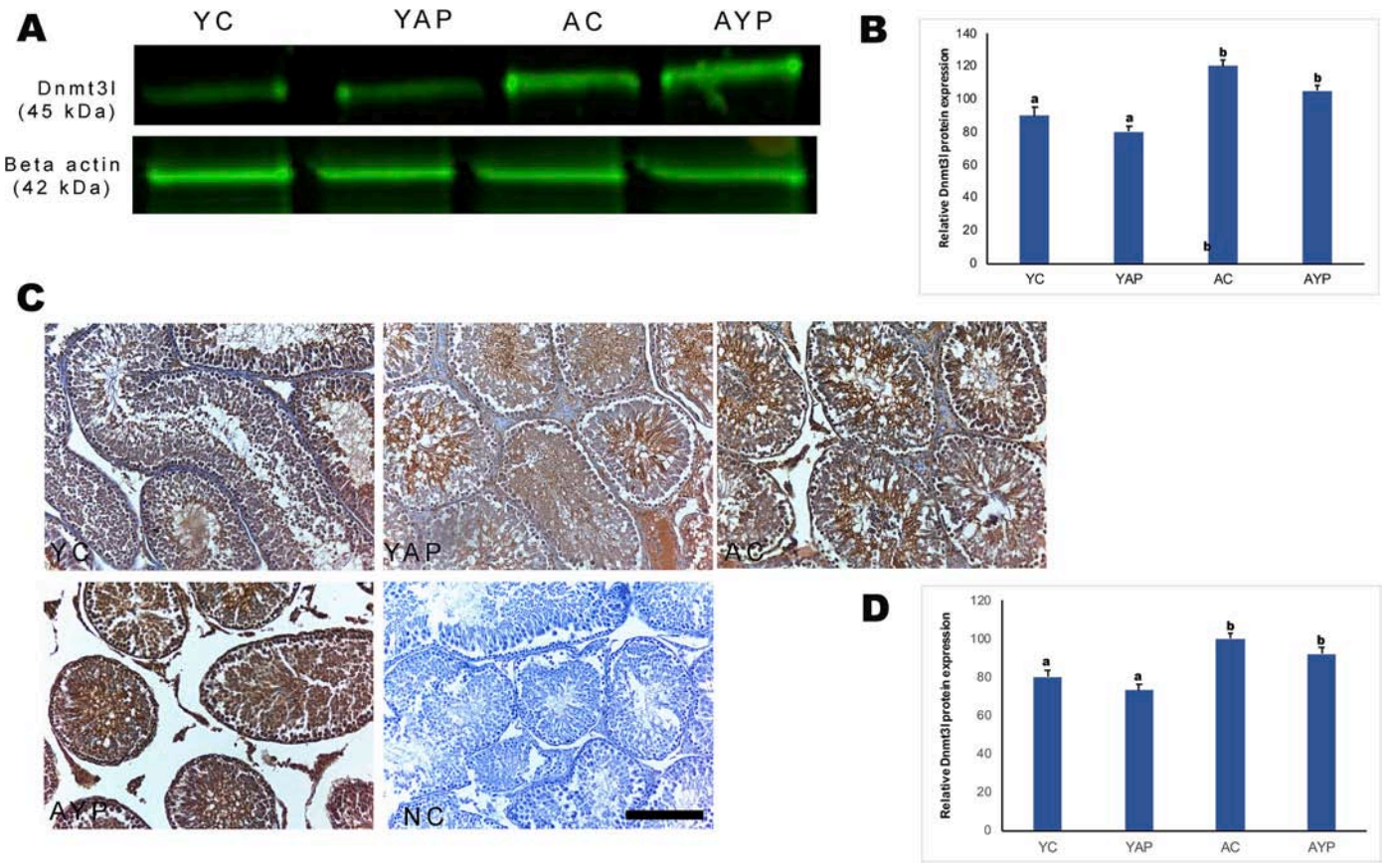

Fig. 4. Dnmt3l expression in controls and blood transfer groups (A) WB assays show the fluorescent-labeled 45 kDa band specific to Dnmt3l, and 42 kDa band to ß-actin was used as the internal control. (B) Relative Dnmt3l band intensity level in WB analysis. Dnmt3l expression was significantly increased in aged control group, but it significantly decreased after young plasma transfer in aged group. (C) Dnmt3l expression in all groups. (D) Relative Dnmt3l protein expression level in IHC analysis. The statistical significance was determined by using one-way ANOVA followed by Dunn's post hoc test. The $P < 0.05$ was considered statistically significant, shown different letters on the columns. Bars in graphs are represented as mean ± SD. YC, young control; YAP, young plasma transfer to aged; AC, aged control; AYP, aged plasma transfer to young. Scale bar: 20 μm.

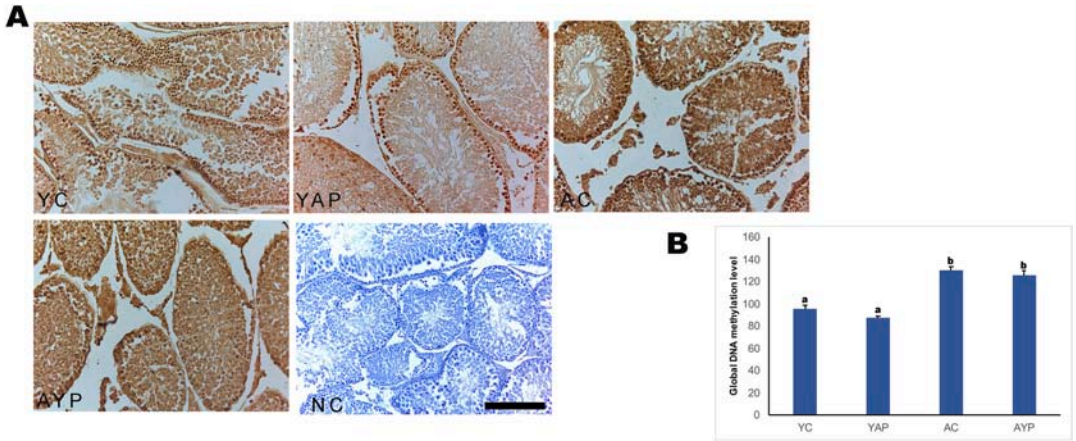

Fig. 5. Global DNA methylation (5mC staining) level in controls and blood transfer groups (A) Localization of global DNA methylation in all groups. (B) Relative global DNA methylation level in IHC analysis. Global DNA methylation was significantly increased in aged control group, but it significantly decreased after young plasma transfer in aged group. The statistical significance was determined by using one-way ANOVA followed by Dunn's post hoc test. The $P < 0.05$ was considered statistically significant, shown different letters on the columns. Bars in graphs are represented as mean ± SD. YC, young control; YAP, young plasma transfer to aged; AC, aged control; AYP, aged plasma transfer to young. Scale bar: 20 μm.

begins to progressively increase after birth in primordial germ cells, and reaches to the highest levels in the spermatogonia at puberty by de novo methylation mechanism and continues from spermatocyte to spermatozoa stage by the maintenance methylation process, both of which are catalyzed by Dnmts (Saitou et al., 2012; Boissonnas et al., 2013). We nearly published that Dnmts expression and global DNA methylation levels were significantly differed in total testes and spermatogenetic cells in a stage-dependent manner. Dnmt3b and Dnmt3l were more

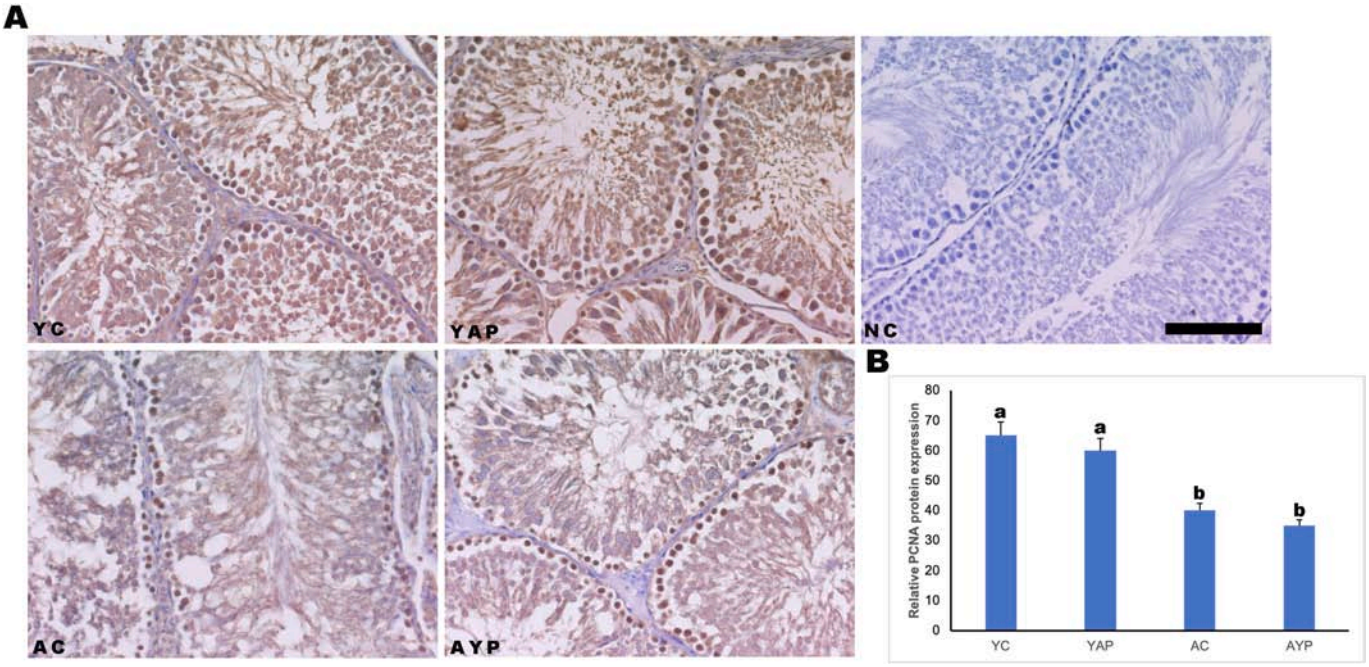

Fig. 6. PCNA expression in controls and blood transfer groups (A) PCNA expression in all groups. (B) Relative PCNA protein expression level in IHC analysis. PCNA expression was significantly decreased in aged control group, but it significantly increased after young plasma transfer in aged group. The statistical significance was determined by using one-way ANOVA followed by Dunn's post hoc test. The $P < 0.05$ was considered statistically significant, shown different letters on the columns. Bars in graphs are represented as mean ± SD. YC, young control; YAP, young plasma transfer to aged; AC, aged control; AYP, aged plasma transfer to young. Scale bar: 20 μm.

abundant in testes, while Dnmt1 and Dnmt3a were comparatively low (Uysal et al., 2022). During spermatogenesis, DNA methylation are responsible for activation or repression of genes for maternal and paternal imprints establishment (Saitou et al., 2012). It has been reported that abnormal DNA methylation in imprinting genes causes male infertility (Boissonnas et al., 2010; Aston et al., 2015; Urdinguio et al., 2015). In addition, the lack of Dnmt enzymes causes infertility, epigenetic anomalies or embryonic lethality (Uysal et al., 2016).

It is the first time; we have shown that the plasma transfer between young and aged rats led to significant changing in the DNA methylation and Dnmts expressions. We found that Dnmts expression and global DNA methylation levels were significantly increased in the aged group compared to young counterparts. Interestingly, Dnmt1, Dnmt3a, Dnmt3l expressions and global DNA methylation decreased when young plasma transfer to aged rats similar to young counterparts. Otherwise, Dnmts expression and global DNA methylation increased when old plasma transfer to young group. These results shows that blood plasma transfer control epigenetic changes and it restores impaired DNA methylation in old age to a youthful state. Our study is very concise and valuable in that it has the first and striking results in the literature and can be used especially in the treatment of male infertility. Because, in the literature, many studies in the field of reproduction are on platelet rich plasma transfer and it has been associated with female infertility. Most of the studies are on the poor ovarian reserve and endometrium. It has been nearly reviewed that platelet rich plasma transfer increase endometrial thickness, AMH, and decrease FSH levels and also increase pregnancy rates (Sharara et al., 2021). Studies show that platelet rich plasma transfer can improve female infertility.

As is known, daily sperm production, total sperm count, and sperm viability are negatively correlated with age, also aging is one of the reasons for infertility. Normal DNA methylation and Dnmts expression are required for male fertility. Abnormal DNA methylation and DNMT1 expression cause impairment during spermatogenesis (Hartmann et al., 2006; Takashima et al., 2009). Abnormal DNA methylation in the imprinting genes results in oligospermia and azoospermia (Kelly et al., 2003; Marques et al., 2008; Filipponi and Feil, 2009; Boissonnas et al., 2010; Hammoud et al., 2010; Aston et al., 2015). Abnormal DNA methylation patterns in the sperm cells cause unexplained infertility (Urdinguio et al., 2015). So, DNA methylation in this process is critical for normal development and fertility. We found that methylation and its responsible enzymes, which increase with age, become youthful after young plasma transfer.

We noticed that the spermatogenetic cell rations increased in aged group after young blood plasma transfer. Such an increase in spermatid count when plasma transfer is made from young to old is a promising result for increasing the number of sperm that decreases with aging.

We also evaluated the PCNA expression which is important gene for spermatogenesis. In a study, it was shown that the more impaired sperm production in the testis, the lower the PCNA expression (Li et al., 2014). In young group, PCNA expression is significantly increased and decreased in aged groups. After young plasma transfer, PCNA expression significantly increased in aged group. This result shows that high methylation can suppress PCNA expression in old animals, but this condition can be restored after transfer from young animals. Specific genes or transcription factors need to be identified that are targeted by the blood plasma transfer in further studies.

5. Conclusions

In the current work demonstrated that young blood plasma transfer is critical for DNA methylation and spermatogenesis in aged rats. Additional studies, such as gene sequencing after the blood plasma transfer, may help to clarify which pathways are affected and thus essential for the spermatogenetic cell stages. Finding these genes may contribute to the restoration of expression levels that deteriorate in old age and to regain fertility. Although this point seems one of the limitations of the present study, we believe that our results after young blood plasma transfer represent the initial findings in the literature and will lead to further studies being performed.

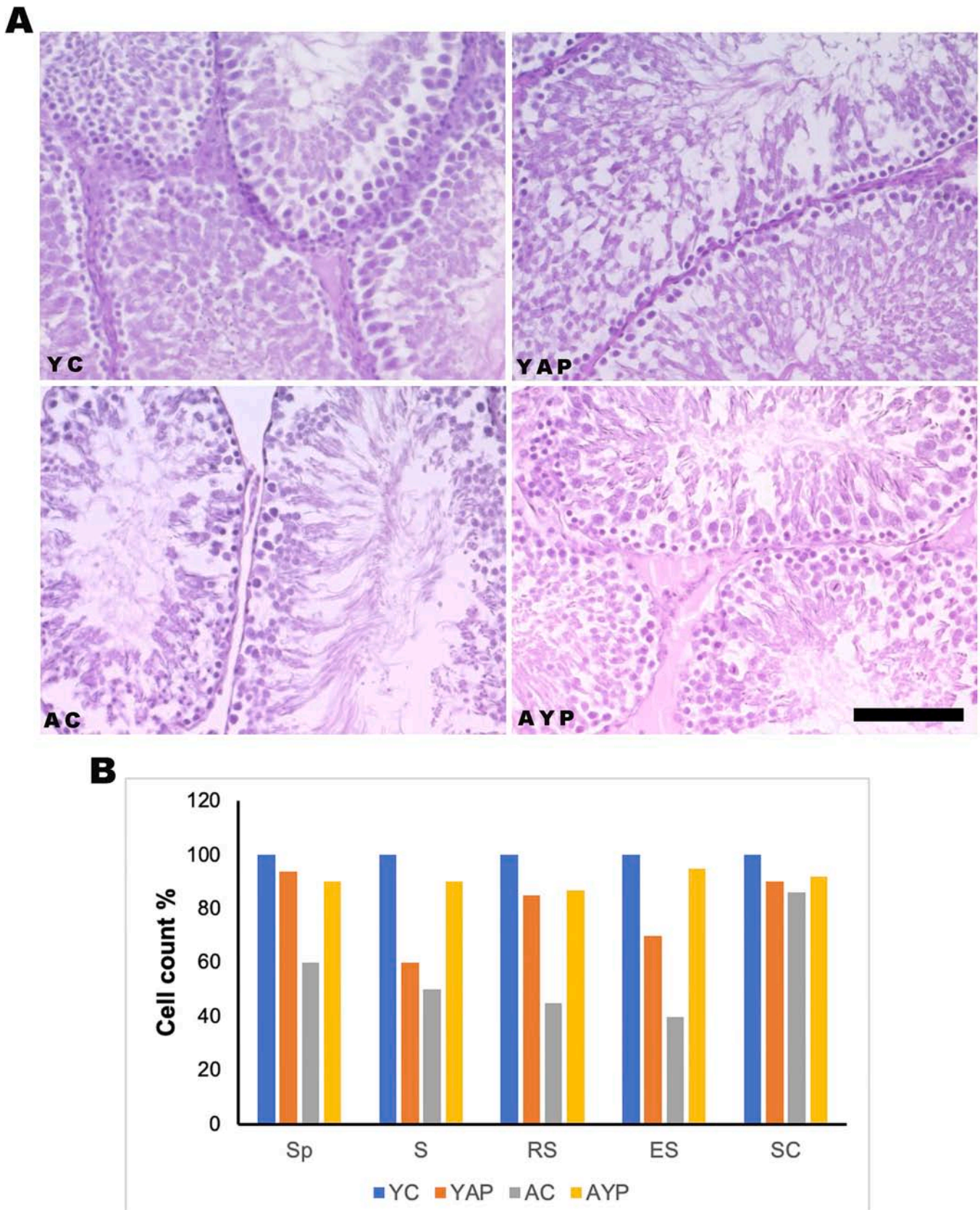

Fig. 7. Spermatogenetic cell stages ratio from spermatogonium to spermatid. (A) Representative HE images from testis section in controls and blood plasma transfer groups. (B) Percentage of spermatogenetic cells in each group as spermatogonium, spermatocyte, round spermatid, elongated spermatid and Sertoli cells. Especially spermatogonium and spermatid ratio were significantly decreased in aged control group, but it significantly increased after young plasma transfer in aged group. YC, young control; YAP, young plasma transfer to aged; AC, aged control; AYP, aged plasma transfer to young; Sp, spermatogonium; S, spermatocyte; RS, round spermatid; ES, elongated spermatid; SC, Sertoli cell. Scale bar: 20 μm.

CRediT authorship contribution statement

Kadriye Erdogan: Conceptualization; Data curation; Investigation; Writing-Original draft preparation. **Taha Ceylani:** Conceptualization; Methodology; Data curation. **Hikmet Taner Teker:** Methodology; Data curation. **Ahmet Zeki Sengil:** Resources; Data curation. **Fatma Uysal:** Conceptualization; Resources; Data curation; Writing - Reviewing and editing.

Declaration of competing interest

The authors do not declare any conflict of interest.

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