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ORIGINAL PAPER

Effect of middle-age plasma therapy on ileum morphology, immune defense (IgA) and cell proliferation (Ki-67) of female aged rats

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Abstarct

Blood plasma therapy, a new treatment method to eliminate the damage and deterioration caused by aging in many organ systems, has attracted increasing attention. The digestive tract, which cooperates with many different systems, has strong effects on our health. In the present study, the effects of plasma therapy on the ileum of elderly rats were investigated. Wistar rats ($n = 7$; 12–15 months old) were given pooled plasma collected from middle-age rats (6 months, $n = 28$) (for 30 days, 0.3 ml daily, intravenously into the tail vein). At the end of the experiment, villus height, crypt depth, total mucosal thickness and surface absorption area were evaluated. In addition, the effects of IgA, which plays a role in the digestive system's defense against microorganisms, were examined. Both the cell proliferation intensity and proliferation index were evaluated in crypt cells. An increase was determined in all morphological parameters in the experimental group. Similarly, plasma application decreased IgA expression and numbers in the experimental groups. Contrarily, cell proliferation parameters showed a significant increase in the experimental groups' crypt cells. Therefore, we found that the treatment supports the digestive system in terms of both nutrient utilization and absorption-related parameters and has a protective effect on intestinal immune system parameters.

Keywords IgA · Ileum · Blood plasma treatment · Ki-67 · Wistar rat

Introduction

Recent research on plasma exchange has garnered significant attention, particularly concerning the rejuvenating effects of young blood plasma on aging. Young plasma has been shown to facilitate tissue repair and restore functionality, highlighting its potential in anti-aging therapies (Scudellari 2015). It is known that changes in plasma proteins have an effect on the aging process and age-related diseases. Parabiosis models, which aim to explain the effect of young or old blood on aging, have presented studies showing that young blood rejuvenates old tissues in multiple organs including the musculoskeletal, circulatory and central nervous systems (Conboy et al. 2005; Baht et al. 2015). Initial studies in parabiosis models identified GDF11 as a molecule that can rejuvenate cerebral, cardiac and skeletal muscle functions (Katsimpardi et al. 2014; Loffredo et al. 2013; Sinha et al. 2014). Similarly, C–C motif chemokine 11 (CCL11) and β2 microglobulin (B2M) have been reported to negatively affect neurogenesis and cognitive function in the hippocampus. In addition, various plasma proteins have been identified as rejuvenating factors that provide beneficial effects in various tissues. With the identification of these circulating proteins, anti-aging interventions have paved the way for new treatment methods targeting age-related diseases (de Magalhães et al. 2017). Circulating factors have been identified in the studies conducted, and appropriate approaches have been developed. Among the circulatory factors, apelin, which supports mitochondriogenesis and protein synthesis, Cadherin13, which prevents osteoclast differentiation and improves age-related bone loss, forkhead

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transcription factor GDF11, which reverses age-related cardiac hypertrophy by suppressing phosphorylation, eNAMPT, which improves physical activity by promoting NAD + biosynthesis, oxytocin, which prevents skeletal muscle aging by promoting the proliferation of myogenic progenitor cells, SPARCL1, which plays a role in improving neurotransmission, and TIMP2, which increases synaptic plasticity and hippocampus-dependent cognition, were found to have low plasma expressions, while β2-microglobulin, which disrupts hippocampus-dependent cognitive function and neurogenesis, and CCL11, which plays a role in decreased synaptic plasticity, contextual fear conditioning, and spatial learning and memory impairment, were found to have low plasma expressions (Kang and Yang 2020). Studies are shedding light on factors whose expression intensity and amount are determined in aged and young plasma.

However, while these factors demonstrate partial anti-aging effects, extensive preclinical studies are required before plasma therapies can be considered clinically viable (Conese et al. 2017). Notable animal studies indicate that young plasma injections enhance synaptic plasticity and cognitive functions in aged mice (Villeda et al. 2014) and promote neurogenesis, thereby mitigating age-related decline in the brain (Katsimpardi et al. 2014). Additionally, plasma exchange with young plasma has been found to reduce liver fibrosis and reverse age-associated cellular and molecular alterations (Rebo et al. 2016), further enhancing hippocampal neurogenesis and cognitive function (Castellano et al. 2017). Young plasma has been demonstrated to reverse age-related liver dysfunction by restoring autophagy (Liu et al. 2019), and its therapeutic potential in Alzheimer's disease has been explored, with findings suggesting it may decelerate or arrest disease progression (Aicardi 2018).

More recent studies continue to support these findings, revealing that young plasma can reverse age-related protein changes and enhance cellular function (Allahverdi 2024; Lehallier et al. 2019). Other research corroborates this by demonstrating that young plasma reinstates autophagic activity and reduces ischemia-reperfusion injury in aged livers (Liu et al. 2019). Improvements in glial cell function and cognitive abilities following young plasma administration in aged mice have also been observed (Horowitz et al. 2020). Additionally, young plasma exhibits broad-spectrum benefits, including enhanced cognitive function, physical activity and overall health in aged rats, along with reductions in inflammation and oxidative stress (Tripathi et al. 2021). The rejuvenating effects of heterochronic parabiosis between young and aged rats have also been confirmed, leading to stem cell activation and tissue revitalization across multiple organs (Ma et al. 2022). Studies on plasma exchange's effects on liver tissue reveal significant impacts on liver biomolecular composition and tissue architecture. Young plasma has been shown to improve cellular architecture, reduce oxidative stress and inflammation and alter hepatocyte metabolic activities, underscoring its rejuvenation potential (Teker et al. 2023). Histopathological and immunohistochemical analyses also indicate that young plasma exerts a protective effect on intestinal tissue structure, increasing the density of goblet and Paneth cells while reducing inflammation as well as significantly decreasing the expression of inflammatory mediators TNF-α and COX-2 in aged rats (Ceylani et al. 2023a, b).

The digestive system plays a critical role in maintaining overall health and well-being by facilitating the intake and absorption of essential nutrients and water, which are vital for energy production, hormone balance, skin health and elimination of harmful substances and metabolic waste (Guler et al. 2022). The digestive process provides the necessary building blocks for the body to function normally and remain healthy. Recent studies have shown that intestinal health is influenced by various factors, including age-related changes in plasma composition. For instance, Ceylani et al. (2023a, b) examined the impact of these changes on intestinal bacterial diversity and found that the bacterial diversity within the intestinal microbiota tends to decrease with age. However, their findings suggest that young plasma may enhance bacterial diversity and improve the presence rates of beneficial bacteria in the intestinal microbiota of aged rats. Additionally, the intestinal epithelium, which lines the digestive system, undergoes perpetual renewal, driven by adult stem cells located at the base of the intestinal crypts, which proliferate and migrate upward toward the villus tips, ultimately shedding into the intestinal lumen. Increased crypt depth, villus height, local angiogenesis and enhanced blood flow are associated with improved tissue oxygenation, which further supports mucosal growth and absorptive functions (Bonis et al. 2021). Therefore, these processes must be coordinated in a healthy way to ensure the functional integrity of the intestinal epithelial barrier. Otherwise, epithelial cells escape from the control points, inflammatory diseases develop, and even tumor-like tissues are formed (Barker et al. 2007).

The morphological structure of the small intestine, where nutrient absorption takes place in the body, is important in terms of nutrient utilization because of the increased absorption surface and absorption. While most nutrients are absorbed by the intestinal surface epithelium in the digestive system, the difference in the nutritional composition causes some changes in the intestinal epithelium or villi (Asmaz and Seyidoglu 2022). Changes in the digestion of food and metabolic activity are caused by the length of intestinal villi, whether they are short or long, and by differences in the cells that form the mucosa and crypts. Histomorphologically, the increase in intestinal parameters increases nutrient utilization, and the increased proliferation of mitotic activity,

especially in the intestinal crypts, directly supports the morphological parameters (Asmaz et al. 2022).

The weakening of the immune system that occurs with age contributes to the gradual decrease in resistance to pathogens in individuals and leads to an increased risk of sickness and death from infections among the elderly (Beharka et al. 2001). The immune system's association with the digestive system is its most important part. The gastrointestinal-associated mucosal immune response (GAIR) plays a role in ensuring harmony between the environment and the organism by developing different structural and functional characteristics. However, although GAIR can mainly generate a humoral immune response, the predominant immunoglobulin (Ig) isotype produced is IgA (Beharka et al. 2001). Little is known about GAIR in terms of age-related changes. Although several studies have investigated age-related changes in cellular immunity, the results have been inconsistent. In general, a decrease or no change in cellular immunity with age has been reported (Gupta et al. 2023; Kawanishi et al. 1990). Although young plasma treatments have been important in recent years, young plasma application in the clinic may sometimes not be possible because of the difficulty of the plasma collection process. The facts that young plasma treatment has clinical limitations and that middle-age plasma is easier and more applicable in the clinic led us to investigate the effects of middle-age plasma treatment on aged tissues.

This study investigates the rejuvenating effects of middle-age plasma treatment on the aged rat ileum, hypothesizing that it can reverse age-related declines in intestinal structure and immune function. By examining morphological improvements and changes in IgA levels, the research aims to uncover potential therapeutic benefits for aging-related gastrointestinal issues.

Materials and methods

Animal studies: Female Wistar rats were used as a model organism in the study. Wistar rats (aged 12–15 months, $n = 7$) were given pooled plasma collected from middle-age rats (6 months old, $n = 28$) for 30 days, 0.3 ml daily, intravenously into the tail vein. After plasma application, the rats in the experimental group ($n = 7$), control group ($n = 7$) and middle-aged positive control group (MPC) (6 months old, $n = 7$) were slightly stunned with ether and killed by cervical dislocation. All animals were housed under standard animal care conditions and had free access to food and water (Ceylani and Teker 2022; Villeda et al. 2014). Our study was conducted with the approval of the Ethics Committee of the Saki Yenilli Experimental Animal Production and Application Laboratory (approval no. 2022/05/06/21) and was conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

Plasma collection: Prior to extracting plasma from the experimental cohort, vaginal smears were conducted to ascertain whether the 6-month-old subjects from whom plasma was to be obtained were in the diestrus, proestrus, estrus or metestrus phase. Thereafter, seven subjects from each phase were killed, and their blood plasma was collected (Westwood, 2008). Pooled rat plasma was collected by terminal cardiac puncture, centrifuged at 1000 g and heated at 95 °C for 3 min for denaturation. Then, it was centrifuged again at 1000 g and stored at − 80 °C until use.

Histological analyses: Ilia from sacrificed animals were collected. Ileum tissues were collected from animals and fixed with 10% buffered neutral formalin for 24 h and were then processed routinely using paraffin. The paraffin sections were stained using Mallory's triple staining technique modified by Crossmon (1937) for general histological examination. For morphometric parameters, villus height (µm), total mucosal thickness (µm), surface absorption area (mm²) and crypt depth (µm) were measured using Carl Zeiss-GmbH ZEN 3.5 software (Graphics adapter: support for resolutions of at least 1920 × 1200 pixels, 32-bit true color, DirectX® 11.0 or higher, e.g., integrated Intel® UHD Graphics 630 or dedicated graphic card NVIDIA Quadro P400 2 GB, monitor 24" TFT display, vertical resolution ≥ 1200 pixels) (Yesilbag et al. 2022). *Surface absorption area:* Villus absorptive surface area = 2π × (average villus width/2) × villus height was calculated (Guler et al. 2022). Some of the sections were reserved for immunohistochemistry to determine the IgA and Ki-67 expression and proliferation index (PI).

Immunohistochemical Analysis: After the sections were deparaffinized, they were passed through an alcohol series. For the antigen retrieval stage, 2.1 g citric acid was dissolved in 1 l distilled water, and the pH was adjusted to 6. The tissues were treated in a 750-W microwave (Arçelik MD 524) for 3 × 5 min. To block endogenous peroxidase activity, 3% hydrogen peroxide solution was used; to prevent nonspecific protein binding, secondary blocking serum (MP7401; ImmPRESS reagent Vector Laboratories, Inc.) was used. Then, primary antibodies (Abcam Goat Anti-Rat IgA ab97185, dilution: 1/200, Abcam Anti-Ki67 antibody [SP6] ab16667, dilution: 1/200) were applied to the sections and kept at + 4 °C overnight. Negative controls were incubated with the antibody diluent without using the primary antibody. Sections were incubated with the secondary antibody in the kit (MP7401; ImmPRESS reagent Vector Laboratories, Inc.), and 3,3'-diaminobenzidine (DAB-Zymed

Laboratories, USA) chromogen was applied. The preparations were counterstained with hematoxylin and covered with Entellan.

To evaluate the IgA and Ki-67 expression made according to the scoring system, 0 means no immune reaction; 1, weak immune reaction; 2, moderate immune reaction; 3, strong immune reaction (Özgüden-Akkoç et al. 2018).

To calculate the mean values of IgA + cells, IgA + cells were counted in five randomly selected areas from five sections per group, and the mean IgA + cell numbers per view (over areas of 0.01 mm^2) were calculated for each group (Yang et al. 2021).

The proliferative index (PI) was calculated by determining the ratio of the number of Ki-67-positive cells to the total number of crypt cells in the crypt glands of the ileum. It was defined as the average of the proliferating cell numbers in seven randomly selected crypts from the groups (Asmaz and Seyidoglu 2022).

Statistical analysis: After the normality test, differences between groups were analyzed using SPSS 27's Kruskal-Wallis and Mann-Whitney U-test. Results are presented as mean ± SE (standard error of the mean).

Results

Histomorphological analysis

The histomorphological parameters such as villus height, crypt depth, total mucosal thickness and surface absorption area were evaluated under the microscope (Table 1; Fig. 1). Examining villus height showed that the experimental and MPC groups had increased villus height compared to the control group ($p < 0.001$). A statistical difference was determined between the MPC and experimental groups at the $p < 0.05$ level. The highest villus height was observed in the experimental group (Table 1, Fig. 1).

No statistical difference was determined between the experimental group and MPC in crypt depth measurements. However, a statistical difference was determined between the control and experimental groups and MPC at the $p < 0.001$ level. The highest crypt depth after treatment was determined in the MPC group (Table 1, Fig. 1).

Compared with other groups, total mucosal thickness was statistically lower in the control group (Table 1, Fig. 1). A statistical difference was determined between the control group and both the experimental and MPC groups in terms of surface absorption area ($p < 0.05$, Fig. 1, Table 1).

Immunohistochemical analysis: IgA was expressed in the villi of the ileum and in the crypt glands. IgA expression in the control group was determined at a medium-strong level. Statistically, a more intense IgA expression was detected in the control group than in the experimental group with low-medium expression intensity at $p < 0.001$ level and in the MPC group with medium expression intensity at $p < 0.05$ level. However, no statistical difference was observed between the experimental group and the MPC group (Table 2, Fig. 2a, b, c, g, h). The most IgA cells were seen in the control group, just like the expression intensity. The number of IgA + cells determined in the control group was found to be higher than in both the control and MPC groups and was statistically significant in both groups at $p < 0.001$ level (Table 2, Fig. 2a, b, c, g, h).

Ki-67 expression was weakest in the control group. The experimental and MPC groups showed an increase in cell proliferation intensity with higher Ki-67 expression levels compared to the control group showing weak immunoreaction ($p < 0.001$; Fig. 2d, e, f, g, h, Table 3). However, no statistical significance was observed between the experimental and MPC groups. Proliferation index was found to be higher in the plasma-treated experimental group and MPC group than in the control group ($p < 0.001$, Fig. 2d, e, f, g, h, Table 3).

As expected, no immunoreaction was observed in the negative control groups for either IgA or Ki-67 (Fig. 3).

Table 1 Morphometric analysis of the villus height, crypt depth, total mucosal thickness and villus surface absorption area of the control and experimental groups' ileum. MPC: middle-age positive control. Different letters in the same column show statistical significance ($^{x, y, z}$). In terms of villus height, significance was found at the 0.05 level between the control and MPC, at the 0.05 level between the experimental group and MPC, and at the 0.001 level between the control and experimental groups

Groups	*N*	Villus height (Mean ± SE)	Crypt depth (Mean ± SE)	Total mucosa (Mean ± SE)	Surface area (Mean ± SE)
Control	7	812.02 ± 15.1^{x}	198.2 ± 22.3^{x}	1011.6 ± 33.8^{x}	1.12 ± 0.1^{x}
Experimental	7	1214.65 ± 24.5^{y}	302.4 ± 26.2^{y}	1512.4 ± 40.2^{y}	1.67 ± 0.02^{y}
MPC	7	1020.24 ± 12.1^{z}	319.2 ± 21.4^{y}	1421.2 ± 37.1^{y}	1.44 ± 0.2^{y}
P value		0.05, 0.001	0.001	0.001	0.05

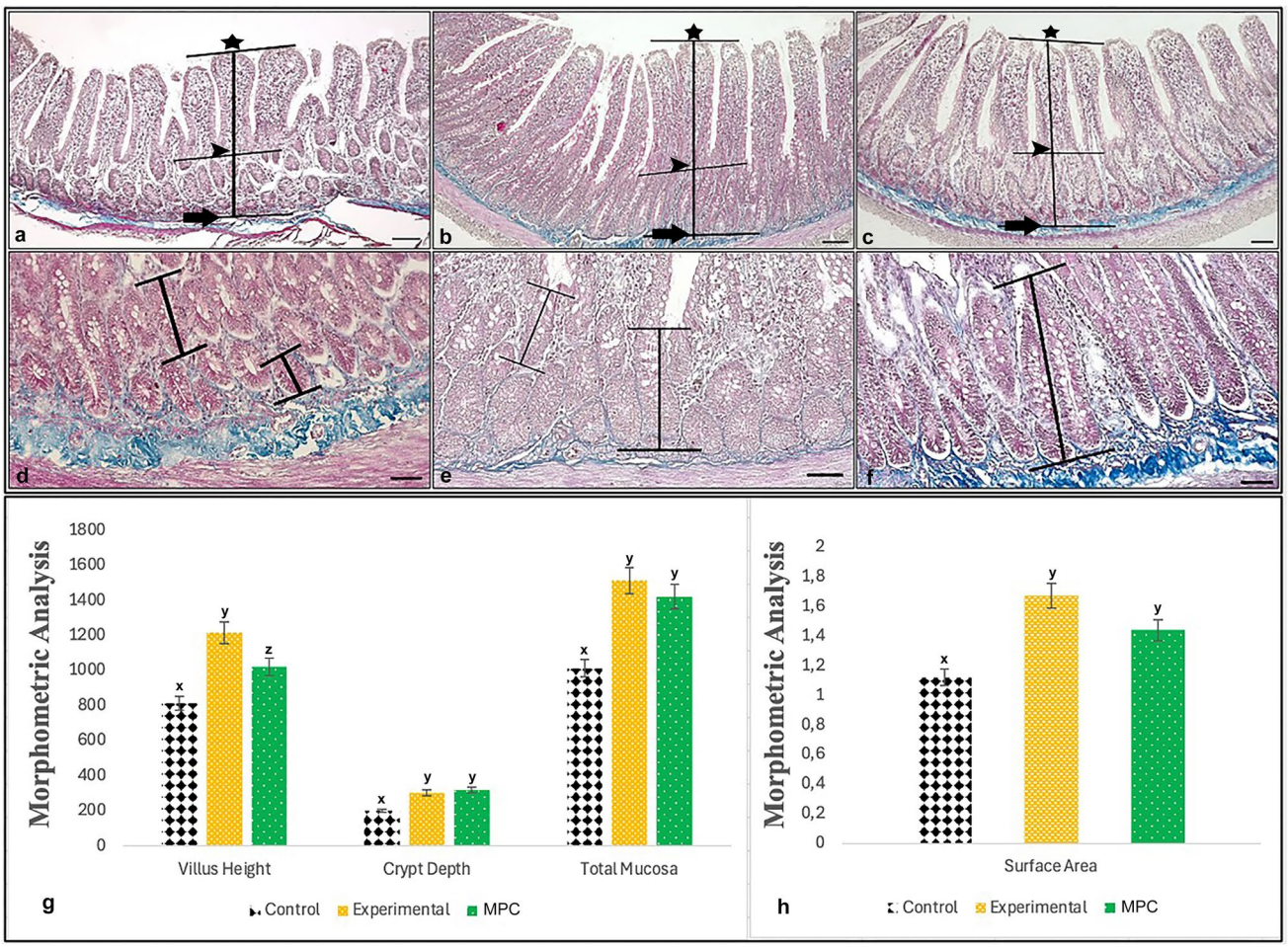

Fig. 1 Morphometric measurement of the ileum in control and experimental groups. **a–d** Control group, **b–e** experimental group, **c–f** MPC group. **d–f** Crypt depth measurement. Star to arrowhead: villus height; arrowhead to thick arrow: crypt depth; star to thick arrow: total mucosal thickness. **g–h** Morphometric analysis of control and experimental groups, **x, y, z** statistical difference (bar: 50 µm)

Table 2 IgA expression intensity and cell count in control and experimental groups. MPC: Middle-aged positive control. Different letters in the same column show statistical significance ($^{x, y}$). For IgA expression intensity, significance was found at the 0.05 level between the control and MPC, at the 0.05 level between the experimental group and MPC, and at the 0.001 level between the control and experimental groups

Groups	*N*	IgA expression intensity	IgA cell count
Control	7	2.45 ± 0.12^{x}	162.20 ± 0.21^{x}
Experimental	7	1.77 ± 0.11^{y}	99.2 ± 0.10^{y}
MPC	7	2.00 ± 0.10^{y}	108.60 ± 0.14^{y}
P value		0.05, 0.001	0.001

Discussion

The results of this study substantiate our hypothesis that plasma treatment can reverse the age-related deterioration in both intestinal morphology and immune functionality. This finding expands on the existing body of gerontological research by demonstrating that younger plasma improves the structural integrity of the gastrointestinal tract, supports cell renewal in the intestinal crypt gland and regulates immune responses, particularly through changes in IgA levels. Such evidence suggests potential therapeutic applications of younger plasma to mitigate aging-related declines in intestinal health. Such a dramatic decrease in IgA is unexpected and requires further investigation of the complex interplay between the aging immune system and microbiota dynamics and potentially offers new insights into age-related immune adaptation.

The duodenum, jejunum and ileum are important in the hydrolysis and absorption of nutrients and can be considered

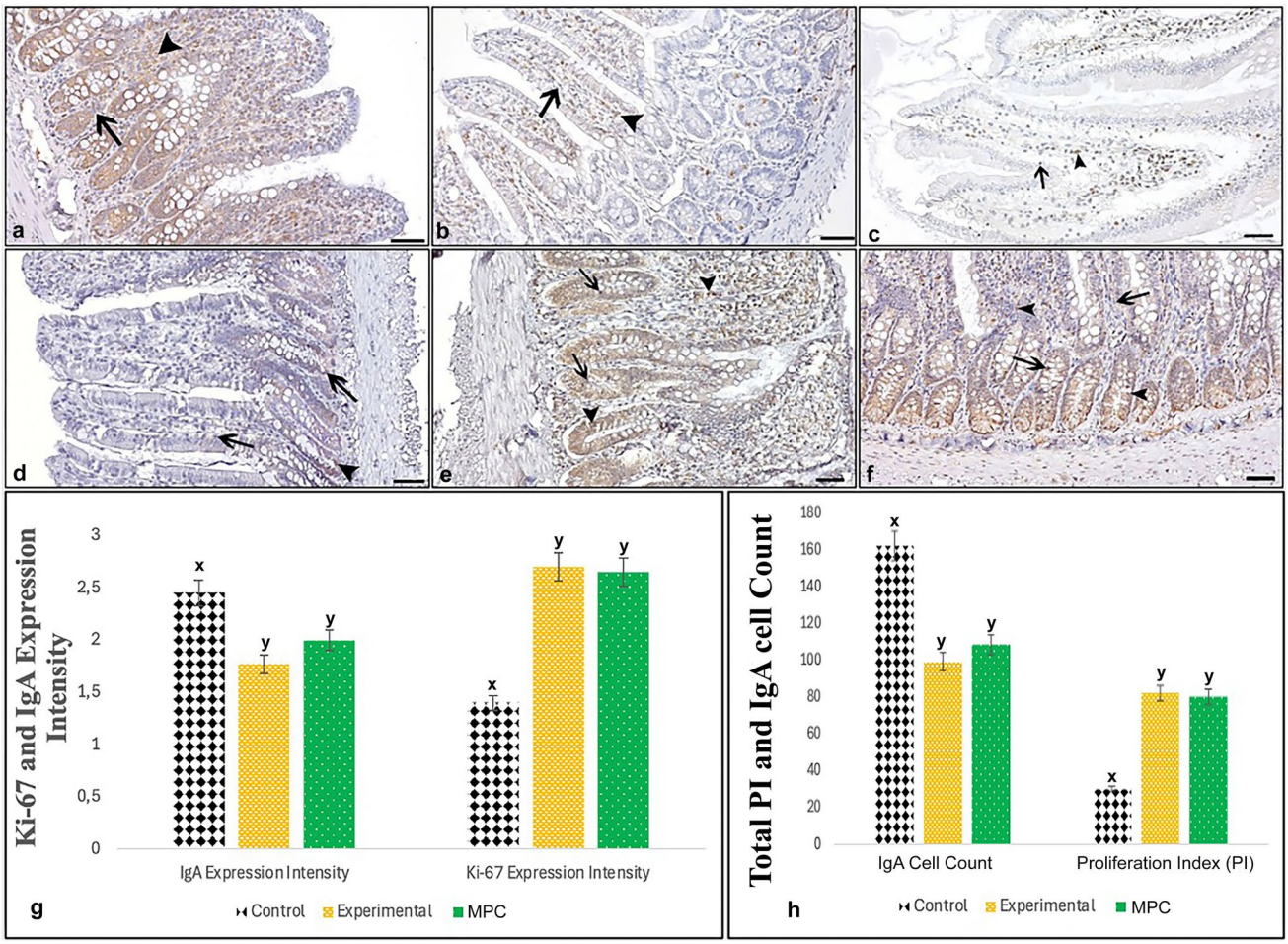

Fig. 2 IGA and Ki-67 evaluation in the ileum of control and experimental groups. **a** IgA expression in control group, **b** IgA expression in experimental group, **c** IgA expression in MPC group, **d** Ki-67 expression in control group, **e** Ki-67 expression in experimental group, **f** Ki-67 expression in MPC group. Arrowhead: IgA and Ki-67 positive cells, arrow: IgA and Ki-67 negative cells. **g** Ki-67 and IgA expression intensity in control and experimental groups, **h** IgA cell count and proliferation index in control and experimental groups. **x, y, z** statistical difference. (Bar: 50 µm)

Table 3 Ki-67 expression intensity and proliferation index (PI) in control and experimental groups. MPC: Middle-age positive control. Different letters in the same column show statistical significance ($^{x, y}$)

Groups	*N*	Ki-67 expression intensity	Proliferation index (PI)
Control	7	1.40 ± 0.15^{x}	30.10 ± 1.16^{x}
Experimental	7	2.70 ± 0.20^{y}	82.20 ± 1.87^{y}
MPC	7	2.65 ± 0.18^{y}	80.12 ± 1.72^{y}
P value		0.001	0.001

an inter-environment barrier (Sobolewska et al. 2011, 2017). Morphological indexes such as villus height and crypt depth in the small intestine, which is the main organ involved in absorption and nutrient utilization, are important indicators of its health and functional status (Calik and Ergün 2015). The enterocyte proliferation process in the intestine can cause changes in mucosal surface absorption area, crypt depth, villus height and total mucosal thickness, all of which indicate morphological adaptation of the intestine (Puzio et al. 2021). This suggests that intestinal adaptation involves not only changes in cell number but also in the quality of enterocytes (Shaw et al. 2012). After adaptation, intestinal enterocytes are more functional and have a higher capacity to digest and absorb nutrients. This can be confirmed by the increase in enzymatic activity within the intestine, which can be achieved at later stages. In the present study, we determined an increase in crypt depth and villus height in the ileum of aged rats treated with young plasma. We also found an increase in cell renewal and proliferation in crypt glands, considering studies on the functionality of human and mouse stem cells report that the activity of these stem cells decreases with aging; therefore, their ability to maintain homeostasis due to aging decreases (Florian et al. 2013;

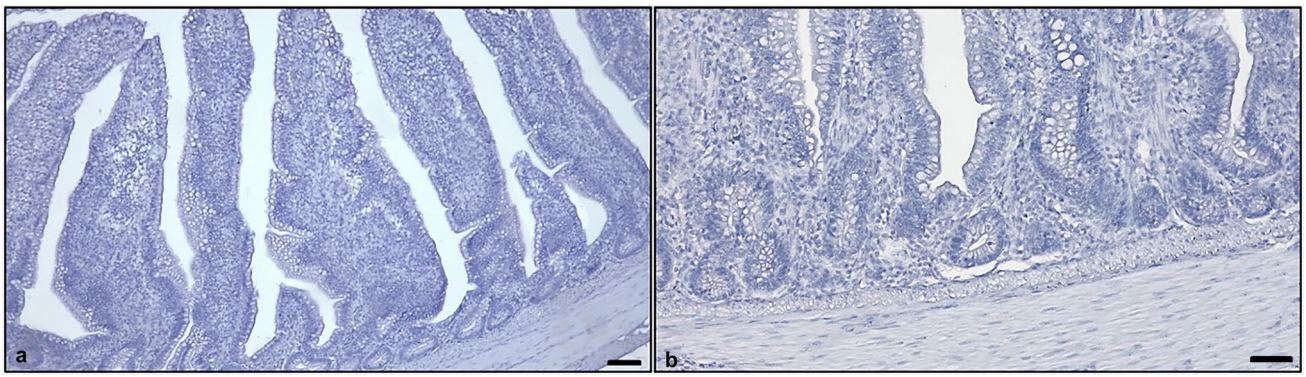

Fig. 3 Negative control groups. **a** Negative control group for IgA. **b** Negative control group for Ki-67. (Bar: 50 µm)

Sinha et al. 2014). Our treatment supports intestinal adaptation in particular and shows that the age-related decrease in cell renewal is stopped and at some point reversed. This shows that the treatment improved the morphological development and cell proliferation of the small intestine and that our treatment supports the digestive system positively.

Aging has a negative impact on the regeneration of the intestinal epithelium and the integrity of the mucosal barrier, which is compounded by a decline in the intestinal microbiota. This decline is associated with the onset of age-related diseases (Parrish 2017). The mucosal barrier, composed of goblet cells, Paneth cells, antimicrobial peptides and immunoglobulin A (IgA), is critical in maintaining the separation between the microbiota and intestinal epithelium (Ceylani 2023; Teker and Ceylani 2022). IgA plays a crucial role in preserving this barrier and regulating bacterial populations to prevent systemic immune reactions (Golby and Spencer 2002; Pietrzak et al. 2020; Teker et al. 2024).

Our research shows that reduced levels of IgA in aged rats treated with middle-age plasma are essential for maintaining the integrity of the intestines and fortifying the mucosal defense against microbial invasion. IgA's role in controlling bacterial populations is vital; without adequate IgA, these populations expand, potentially breaching the mucosal barrier and triggering both local and systemic immune responses (Fernandez et al. 2003; Suzuki et al. 2004). It also leads to physiological inflammation and an increase in IgA plasma cells, characterized by increased activation of the intestinal immune system in response to dietary antigens, pathogens and commensal flora. Notably, most of this response is triggered by normal flora rather than pathogens or dietary antigens (Pickard et al. 2004).

Interestingly, while high mutation frequencies in the genes of human intestinal plasma cells in the elderly suggest chronic antigenic exposure to microflora, indicative of an adaptive response to longstanding microbial presence (Boursier et al. 1999), the decreased IgA expression and cell counts observed in our experimental group do not appear to be directly related to pathogenic interactions but rather to an age-induced decline in mucosal integrity. This nuanced understanding underscores the complexity of aging's impact on intestinal immune functions and the potential therapeutic role of young plasma in moderating these effects.

Recent studies have also highlighted the role of younger plasma therapy in modulating gut microbiota. Ceylani et al. (2023a, b) showed that plasma exchange between young and old rats significantly altered gut microbiota composition, increasing alpha diversity indices and reducing the firmicute-to-bacteroidete ratio in aged rats. This microbiota modulation is indicative of a healthier and more balanced gut environment, which is critical for optimal nutrient absorption and immune function. Our study's findings of improved villus height and crypt depth further support the notion that young plasma therapy can enhance the functional capacity of the gut, potentially through similar mechanisms of microbiota modulation.

In conclusion, the results of our study suggest that the administration of middle-age plasma leads to an improvement in intestinal structure, promotes intestinal cell regeneration and modulates immune responses in aged rats, thereby increasing mucosal integrity and reducing IgA levels. These results support the potential of younger plasma to treat gastrointestinal aging and underscore its therapeutic potential. Future research should explore the underlying mechanisms responsible for these effects and their broader implications for age-related conditions, with the ultimate goal of improving the health of elderly individuals.

Author contributions EDA, TC and HTT contributed to all aspects of this study, from conceptualization to writing and editing. HTT and TC conceived and designed the project/study. EDA and ZTS carried out the experiment and analyzed the sera and tissue samples. EDA analyzed the data. EDA and ZTS performed the histopathological analysis, interpreted results and contributed to writing and editing. AIG was involved in the investigation, methodology, validation and visualization. All authors have approved the final manuscript.

Data availability No datasets were generated or analyzed during the current study.

Declarations

Conflict of interest The authors declare no competing interests. The authors have no conflicts of interest to declare.

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