BioResearch Open Access
Volume 3, Number 5, October 2014

DOI: 10.1089/biores.2014.0043

Aged Mice Repeatedly Injected with Plasma from Young Mice: A Survival Study

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Abstract

It was reported using various biological models that the administration of blood factors from young animals to old animals could rejuvenate certain functions. To assess the anti-aging effect of young blood we tested the influence of repeated injections of plasma from young mice on the lifespan of aged mice. One group of 36 CBA/Ca female mice aged 10–12 months was treated by repeated injections of plasma from 2- to 4-month-old females (averaging 75–150 μL per injection, once intravenously and once intraperitoneally per week for 16 months). Their lifespan was compared to a control group that received saline injections. The median lifespan of mice from the control group was 27 months versus 26.4 months in plasma-treated group; the repeated injections of young plasma did not significantly impact either median or maximal lifespan.

Key words: aging; immunology

Introduction

The deterioration of various organs and system functions with age is well documented. Numerous dysfunctions in metabolism, the cardiovascular system, and the immune system lead to the development of specific diseases associated with aging that are the main cause of death in older age groups.

Various studies have reported health improvements in old mice that were treated with blood factors from young mice, either through heterochronic parabiosis, plasma, or mesenchymal stem cell engraftment.[1,2] This potentially life-extending therapy could occur in humans with blood transfusions, so gathering statistics in humans and identifying the ''good'' factors in plasma could be key for improving health in an aging population. Positive effects may be seen during injections of plasma from young donors to old recipients. In particular, injections of the platelet-rich plasma (including autologous injections) seemed to have positive effects in countering cell senescence, promoting skin rejuvenation, and improving several pathologic conditions.[3–6]

However, opposing data have also been reported. In a classical work by Carrel and Ebeling,[7] serum from old animals was found to inhibit the growth of cell cultures. More recently, Villeda et al.[8] showed that injection of plasma from older animals to young animals can cause age-related changes in the nervous system of the young recipients. Effects of contact with systemic environments between young and old animals have also been studied.[8] This and other works suggest that transferring of plasma from old animals can induce age-related changes in different organs in young animals.

Such reports suggest that the old animals contain factors that control the rate of aging, and that transferring plasma from young animals can be beneficial for aging mice and induce some type of rejuvenation.

Here, aged mice were repeatedly injected with plasma from young mice, and we observed the effects on lifespan and on markers of aging (CD4/8 ratio in peripheral blood and plasma thyroxine level).

Materials and Methods

Two groups of aged mice followed for survival

Long-lived CBA/Ca mice were selected for the experiment to eliminate the effect of young plasma injections on the development of certain types of age-related pathologies and to assess the impact on the species life span of mice generally.

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Seventy-two CBA/Ca female mice aged 10 months ($n=7$), 11 months ($n=34$), and 12 months ($n=31$) were randomly divided into two groups of equal age distribution and size ($n=36$). The treatment group was repeatedly injected with young heparinized plasma, and the control group was repeatedly injected with saline with the same heparin concentration. All manipulations were carried out in accordance with the guidelines established by the D. F. Chebotaryov State Institute of Gerontology Administrative Panel for Lab Animal Care. The mice were housed in nine cages, with each cage containing approximately the same number of treated and control mice, for better comparability. Mice received food and water *ad libitum*. Temperature was $21 \pm 1°C$.

Deaths were checked every 1–3 days. Necropsies of dead animals were made to assess the presence of visible pathologies, with a particular focus on the following internal organs: lungs, liver, kidneys, thymus, spleen, and intestine. Any deviation from the norm was recorded, such as the expansion of tissue, tumors, cysts, tissue atrophy, and hemorrhage. Posthumous investigations were not done in cases of great body damage or prolonged time at room temperature.

Injections of young plasma or saline

Design. The two groups were treated by injections on the same days, with the same method of administration (intravenous or intraperitoneal); the two methods were used to minimize injury to veins and to maximize the chance of delivering possible ''youth factors.'' On average, there was one intravenous (75 μL) and one intraperitoneal (150 μL) injection per mouse per week for 16 consecutive months. For each injection of plasma in the treatment group, the control group was injected with an equal volume of heparinized saline. The injection volume was 225 μL of plasma per mouse per week, or 900 μL per mouse per month in total. According to generally accepted estimates, 80%–100% of the blood plasma volume of the whole organism was injected monthly.

Plasma. Blood plasma is composed of a complex mixture of transport proteins, growth factors, and small molecule compounds as well as the transport vesicles, which have a wide range in terms of their biological effects and half-lives. This makes the search for rejuvenation factors extremely difficult and requires a careful approach to selecting the method of blood sample isolation. For this purpose, we used blood plasma for injection since the process of blood coagulation leads to a sharp increase in the amount of transport vesicles with blood coagulation factors in the serum, changes the levels of some amino acids and proteins, and lowers blood serum composition stability. [9] To obtain plasma, we used the heparin, which is suitable for use *in vivo*, in contrast to anticoagulant citrate dextrose and EDTA. Heparin has several side effects during chronic use, but in this experiment, we used concentrations that were 1/10 of the therapeutic dose. This approach was expected to negate the occurrence of side effects and to minimize binding with blood proteins in the mice of control group. We injected freshly isolated blood to minimize the loss of short-lived blood factors that are destroyed during dialysis and long-term storage.

Plasma was aseptically prepared on the day of injection. Blood from 2- to 4-month-old CBA/Ca female mice was collected retro-orbitally and pooled in one standard heparinized BD Vacutainer® (90 USP units/7 mL of blood; the final concentration of sodium heparin was 12.9 USP units/mL). Plasma was obtained via centrifugation (1500 g for 15 minutes). The control group was injected with saline (NaCl, 9 g/L) with the same concentration of heparin as in the plasma-treated group.

Injections. This experiment involved a chronic course of injections, which can be a traumatic procedure. To minimize the traumatic effect and stabilize the route of administration in case of venous thrombosis, we used a combined approach for injecting plasma: one injection intravenously and one injection intraperitoneally weekly. Intravenous and intraperitoneal injections are the two most widely used administration routes. Intraperitoneal injection has several disadvantages, including a lower rate of absorption compared with the intravenous injection. On the other hand, the intraperitoneal route is often considered to be effective for tumor therapy.[10,11] Some studies even concluded that this route was slightly preferable to others.[12,13]

Prior to injection, the tail was warmed, and tail vein injections were performed with 30-gauge needles. To minimize potential bias of injury, subsequent injections were made to the alternate tail vein, and starting from the 13th month of the experiment, the mice received only intraperitoneal injections, twice a week. After the 16th month of the experiment, no further injections were given. Injections were done in parallel for control and experimental animals by a single operator at the same time of the day.

Throughout the experiment, investigators carrying out procedures and making observations were blinded with regard to whether animals belonged to the treatment or control group.

CD4/CD8 measures by flow cytometry

To assess changes in peripheral T-cell composition, the $CD4^+/CD8^+$ ratio was measured with flow cytometry. For this study we used eight animals from each group at each time point. For the first two time points, the same animals were used. However, after some animals died, we used additional animals for studies of this parameter.

Peripheral blood for analysis was taken five times during the experiment, at 3.2, 5.9, 7.7, 8.9, and 13.5 months. Twenty-five microliters of blood was taken from eight mice of each experimental group from the tail vein and placed in phosphate-buffered saline (PBS) with 1% fetal calf serum and 1.9 mM EDTA. Blood samples with EDTA were kept cold until staining with antibodies: anti-CD8α/Lyt-2 (Texas Red conjugate, clone 53-6.7) and anti-CD4/L3T4 (PE-Cy7, GK1.5) (PickCell Laboratories). Briefly, single-cell suspensions were resuspended in PBS with 2% fetal bovine serum (Sangva) at 2×10^7 cells/mL. Cells were incubated with antibodies for 30 min on ice in the dark. After incubation red blood cells were lysed with RBC Lysing Solutions. After lysis, cells were washed with PBS and fixed with 1% paraformaldehyde in PBS (pH 7.0). Events were analyzed using a BD FACSAria™ (BD Biosciences). Dead cells were excluded based on forward and side light scattering.

Thyroxine measures by enzyme-linked immunosorbent assay

Twenty-five microliters of blood was taken from the tail vein of eight mice from each experimental group in heparinized capillaries four times during the whole experiment, at

5.9, 7.7, 8.9, and 13.5 months. After centrifugation, 10 μL of plasma was frozen at −20°C until analyses. Total thyroxine (T_4) level in the plasma of experimental animals was measured by enzyme-linked immunosorbent assay (ELISA; Thyroxine ELISA kit, Diagnostic Systems) according to the manufacturer's instructions.

Statistical analysis

Comparison of changes in $CD4^+/CD8^+$ ratio and T_4 level between saline- or plasma-treated groups were done by Mann–Whitney *U*-test (U). Analysis of animals' survival was done by Kaplan–Meier estimates and the logrank statistic. Analysis of pathology frequencies was done by Fisher exact test. All operations were done with the help of STATISTICA 7 software (StatSoft Inc.).

Results

Control group survival

The median lifespan of the control group was 27 months. This is inline with a reported median lifespan of 27.5 months for CBA/Ca females (Harlan Laboratories) and suggests that the multiple injections with heparinized saline did not affect lifespan.

Treatment does not impact lifespan

The lifespans of the two experimental groups were very similar (Fig. 1), including median and maximal lifespan ($p > 0.05$). We had hoped to observe a lifespan increase because available scientific evidence suggests that the treatment with some factors from young systemic environments may improve stem cell functioning in old animals and should have some beneficial effect in them. Therefore, we wondered if frequent injections of plasma from young animals would provide some ''youth factors'' that would increase the lifespan of aged animals. However, we did not observe such an effect.

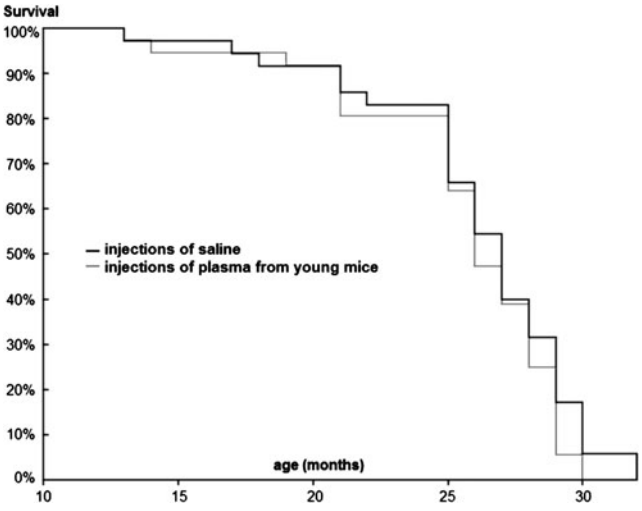

FIG. 1. Survival rate of CBA/Ca female mice treated either heparinized plasma from young animals or heparinized saline. Injections were started while mice were 12.6 ± 0.7 months old and lasted for 16 months of the experiment. After 16 months, injections were stopped as we considered harm from the injection itself. Survival in both control and experimental groups was assessed during the whole experiment.

The $CD4^+/CD8^+$ ratio is decreased in plasma-treated group

According to existing evidence (see Discussion), a positive effect of young plasma injections could be expected for some immune parameters in plasma-treated mice; in particular, the $CD4^+/CD8^+$ ratio in the blood. It is known that T-cell immunity is subject to significant changes during aging, and the $CD4^+/CD8^+$ blood ratio is one of the key markers of immune status assessment.[14] This parameter is routinely used by different laboratories and is easily reproducible. Therefore, the aim of this study was to evaluate the change in the ratio relative to a control group of animals of the same age. Data are reported as percent change versus the control group. In young animals, the typical $CD4^+/CD8^+$ ratio usually exceeds 2 and is sharply reduced with age (Fig. 2B). We assessed this ratio at 3.2, 5.9,

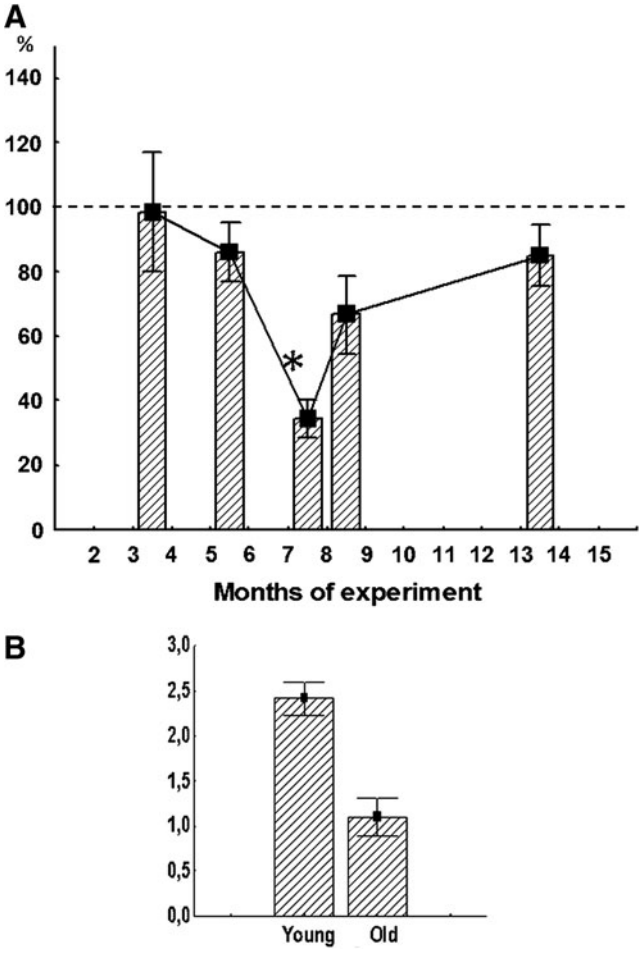

FIG. 2. Changes in the $CD4^+/CD8^+$ ratio in peripheral blood of CBA/Ca female mice treated either with heparinized plasma from young animals or heparinized saline. Measurements were performed five times during the experiment. Dashed line: the $CD4^+/CD8^+$ ratio of control animals taken as 100%. Data are reported as percent change versus the control group. Each group had eight animals. **(A)** Typical ratio of $CD4^+/CD8^+$ in young (2–3 months) and aged (22–24 months) females mice CBA/Ca strain. **(B)** Central marker is the mean value and whiskers are the standard error from the mean value. $*p_{(U)} < 0.001$ comparing to saline-treated group.

7.7, 8.9, and 13.5 months of treatment (Fig. 2A) and did not find any significant differences in the first two measures. Conversely we observed a decrease in the blood $CD4^+/CD8^+$ ratio in the plasma-treated mice compared with the saline-treated group at 7.7 and 8.9 months. These changes were statistically significant only at 7.7 months ($p_{(U)}<0.001$ and $p_{(U)}=0.1$, respectively). The late gradual restoration of the $CD4^+/CD8^+$ ratio to the level of the control group appeared in peripheral blood at 8.9 and 13.5 months (Fig. 2A).

Changes in the T_4 level

We wondered if the transfer of hormones or other young blood-borne factors could have explained the lower $CD4^+/CD8^+$ ratio. T_4 is one of the factors that are the most impacted by aging. It is well documented that its concentration becomes lower with age.[15] Clinical consequences are generally controversial. It is thought that a low T_4 level is connected to multiple age-related metabolic and immune system disorders.[16,17] Further, considerable evidence suggests a beneficial effect of additional thyroid hormones to the health of elderly individuals.[18]

We measured the total T_4 level by ELISA at 5.9, 7.7, 8.9, and 13.5 months (Fig. 3). As expected the T_4 level was first stable and then gradually decreased with age in the control group (the time point at 8.9 months was not significant). The plasma group appeared to have a different trajectory, despite the low statistical significance. At 5.9 months the plasma-treated group had a slightly lower total T_4 level compared to the saline-treated groups ($p_{(U)}=0.52$). At 7.7 and 8.9 months the T_4 level was higher but did not reach statistical significance compared to the control group ($p_{(U)}=0.23$ and $p_{(U)}=0.14$, respectively).

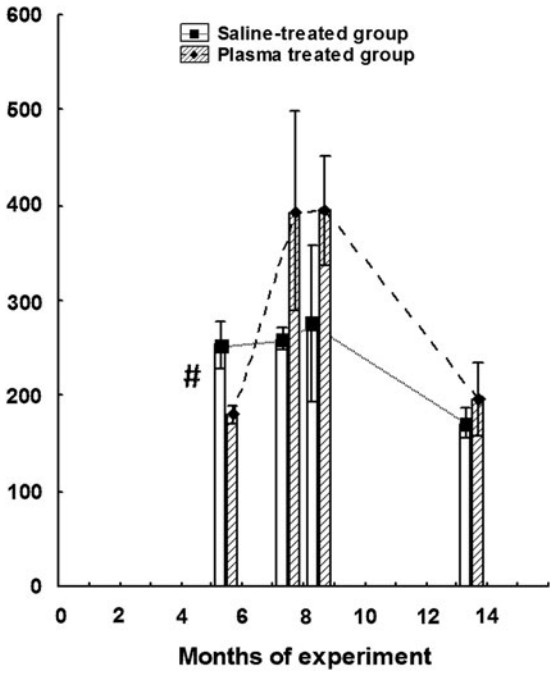

FIG. 3. Changes in the total thyroxine level (nmol/L) in peripheral blood of CBA/Ca female mice treated either with heparinized plasma from young animals or with heparinized saline. Measurements were performed four time during the experiment. Each group had eight animals. Central marker is the mean value and whiskers are the standard error from the mean value. $^{\#}p_{(U)}<0.1$ comparing to the saline-treated group.

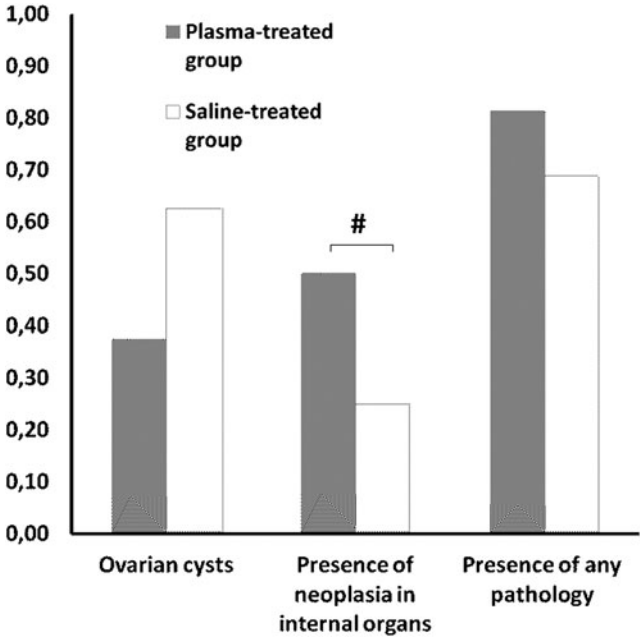

FIG. 4. Frequency of different pathologies in CBA/Ca female mice treated either heparinized plasma from young animals or heparinized saline. Dead animals were investigated only if their bodies were undamaged and they had not been dead for more than 24 h. During necropsy, visual investigations for presence of any type of pathology were made. Each group had 16 animals. $^{\#}p=0.067$ (Fisher's exact test).

Necropsies

The CBA/Ca strain is well known for its longevity, which is due to a low tumor frequency in females.

We performed necropsies on undamaged mice no more than 24 h after death, which accounted for half of the animals in each group ($n=16$). We limited our analysis to visible organ pathologies in the peritoneum and the thoracic chest. Each necropsy was analyzed with an anatomo-pathologist to ensure correct conclusions.

Results of necropsies are shown in Figure 4. Given the relatively small number of necropsies, statistical significance was difficult to reach. We observed a tendency for a slightly higher frequency of oncologic pathologies (total number of visible tumors in organs; Fisher exact test, $p=0.067$) in the plasma-treated group; differences between both groups were in the 90% confidence interval (90% CI), 0.036–0.932. There was a tendency for a slightly lower frequency of ovarian cyst (Fisher exact test, $p=0.144$, differences between both groups were in 90% CI 0.54–0.041) in the plasma-treated group.

Discussion

Aging is for now an irreversible process that affects multiple organs and is the leading cause of age-associated mortality and morbidity. The search for an efficient way to counter age-related changes in an organism is a task of high importance.

Recently, scientific evidence of a rejuvenating effect of young blood on different tissue and organ functions was published. Among these studies, heterochronic parabiosis was particularly interesting: the model demonstrates the possibility of constant exchanges of cellular and humoral factors through the blood between animals of different ages.[19,20]

The coexistence of animals for 5 weeks in parabiosis was shown to lead to improved cognitive function in old heterochronic partners and positive effects on regeneration in the brain.[21] Reverses in age-related cardiac hypertrophy were reported by Loffredo et al.,[22] Ruckh et al.[23] as well as Villeda et al.[8,24] Irina Conboy's laboratory found some evidence of possible positive effects on aged cells *in vitro* and on several organism's systems, including muscle and liver regeneration.[1] Mesenchymal stem cell transplantation was reported to have rejuvenating effects on aged hosts, but the results of such studies of stem cell transplantation in aging are quite contradictory.[2] Furthermore, a number of clinical studies on autologous platelet-rich plasma injection to correct some health conditions can fall in the category of introducing young factors.[25]

Still, after such mostly positive reports, the question of the overall beneficial effect becomes extremely intriguing: Instead of looking at a specific parameter or a short period of time, is maintaining a young milieu globally beneficial over time? This can be asked through a simple measure: Does it increase lifespan? In previous experiments, we looked at the survival of mice following temporary isochronic and heterochronic parabiosis (unpublished data). It was found that aged mice tended to live longer after a period of heterochronic parabiosis than isochronic parabiosis, suggesting a globally beneficial effect of the young milieu. However, the difference was not statistically significant, and lifespans were not in the range of those of untreated animals, possibly due to the traumatic condition of parabiosis. Therefore, general conclusions were very uncertain regarding ''anti-aging'' effects, and another model for long-term effects was sought, namely, plasma injections.

Injection of plasma from one animal to another animal of a different age is a particularly interesting model. Unlike heterochronic parabiosis, it is unidirectional, and it avoids the risk that arise from bidirectional partners. It is less traumatic. Further, it is realistically applicable to humans—it is happening every day with blood transfusions, making its study potentially particularly meaningful for current patients.

Continuous perfusions—modeled by parabiosis—could have much stronger effects than occasional injections—modeled by plasma injections. However, Wyss-Coray's laboratory reported that in addition to neurological findings from heterochronic parabiosis, negative results were obtained by injecting plasma from old animals to young ones. Positive results were obtained by injecting plasma from young animals to old ones, and injecting, GDF11, a specific protein present in high concentration in young plasma, also yielded positive results.[8,21]

In our study, we used a similar scheme and dosage for young plasma injection, but with some differences. To maximize preservation of the young blood composition, we used freshly isolated heparinized plasma and, accordingly, heparinized saline in the control. The control group that received saline injections had a very normal lifespan curve. Certainly, heparin has a number of side effects during chronic use. However, we used a low concentrations of the drug, and this approach should negate the occurrence of side effects. It is disappointing that administration of young plasma to middle-aged female mice did not affect their lifespan in comparison with the control group.

While young blood may not extend lifespan, the lack of life extension in our study could be specific to factors within it. We do not suggest it could be a (nondetected) disease, as both groups (saline and plasma) were represented in every cage. We wondered if the difference in plasma collection could explain the positive short-term findings from Wyss-Coray's laboratory, while we did not find positive long-term results. Contrary to them, we collected the plasma on heparin and not on EDTA, and we injected plasma on the same day rather than after freezing it.[8] The reason for using heparin is simply that, in contrast to EDTA, it is suitable for use *in vivo*. We also injected freshly isolated blood to minimize the loss of short-lived blood factors that are destroyed during dialysis and long-term storage. Of course, differences in strain, sex, and exact ages could be such that some beneficial effects are not observed. Short-term beneficial effects were reported by various teams with various models, so we would be surprised if these differences had an effect; here again, testing a short-term effect on our commonly used model of mice would be reassuring.

Given current results, our best hypothesis is that administering young plasma simply does extend lifespan in the proposed design of the experiment. It is possible that the reported short-term beneficial effects may well happen but are insufficient to affect the lifespan. It is also possible that the administration of heparinized young plasma introduces some disequilibrium as well.

To analyze the possible changes that occur in an old organism receiving young plasma, we tested some blood parameters. One was the $CD4^+/CD8^+$ ratio in the peripheral blood. Indeed, age-related changes in the immune system play important roles in pathogenesis of many disorders associated with age.[22] It is well known that T cells are one of the most affected units during aging, and age-related changes in T cells have been amply documented, with an increase in the number of $CD8^+$ cells and a decrease in number of $CD4^+$, causing the $CD4^+/CD8^+$ ratio to shift with age. Further, there is a decrease in the proliferative capacity of T cells from aged persons as well as an increase in the number of T cells with memory phenotype, which have defective functional properties.[26,27] All these changes are seen as negative predictive factors. However, the $CD4^+/CD8^+$ ratio is commonly used as a marker of immune system aging and as a prognostic factor for human longevity.[14]

We found that the $CD4^+/CD8^+$ ratio decreased in the peripheral blood of experimental mice in comparison to the control group at 8 months of plasma injection (Fig. 2A). This reduction was due to an increase in the number of $CD8^+$ cells in the blood. Such changes in the subpopulation of T lymphocytes have been found in our previous experiments on the model of heterochronic parabiosis.[28] We suggest that these changes are due to the ability of young plasma to stimulate the homeostatic proliferation of CD8 cells in the old organism, which normally should be suppressed. However, subsequent measurements revealed no significant differences for this parameter as compared with the control group at 9 and 13 months. This fact implies the absence of a stable positive effect

of young plasma injection to maintain a balance between CD4 and CD8 cell populations in the blood of aging animals in our experiment design.

The total T_4 level in plasma-treated mice was not statistically significant in comparison with the control group (Fig. 3).

Analysis of pathologies occurring in experimental animals upon aging shows a double effect. On the one hand, a slightly higher, but nonsignificant prevalence of cancer pathology was identified in the plasma-treated mice, which suggests the possibility that youth factors may perturb the aged host. The development of malignancies due to the uncontrolled influence of some enrichment in ''youth stimuli'' in an aged systemic environment have been previously suggested.[29] Such stimuli seem to exist: Conboy et al.[1] reported an increase in the stem cell proliferation from old donors when the cells were cultured in media containing serum from younger animals; however, such stimuli may have some undesired effects for the whole body *in vivo*. On the other hand, we observed the opposite effect when assessing the occurrence of ovarian cysts—their frequency tended to be lower in plasma-treated mice (Fig. 4). These facts suggest no effect of prolonged administration of young blood plasma on the development of age-related pathologies in female mice CBA/Ca in our study design.

In the present study, we tested the possible positive effect of humoral factors present in young plasma on middle-aged female CBA/Ca mouse lifespan. In our study design, the administration of young plasma did not affect lifespan. We did not find any significant effect of young plasma on the blood CD4/CD8 ratio, plasma T_4 level, and the development of age-related pathology of internal organs. The absence of any effect of young plasma in our study may be due to the peculiarities of the mouse strain, sex, or dose and regimen of plasma injection. Given the potential importance of the question we addressed, and of the beneficial short-term effects reported in the literature, further research is planned to investigate the question with another design.

Author Disclosure Statement

No competing financial interests exist.

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Abbreviations Used

ELISA = enzyme-linked immunosorbent assay
PBS = phosphate-buffered saline
T_4 = thyroxine