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Overview of parabiosis and heterochronic parabiosis research in the context of aging

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Abstract: The parabiosis model has become extremely popular in recent years, mainly due to research in the field of aging. It has been shown that due to the general circulation between animals of different ages, many cells and biological factors are exchanged, some of which are considered potential targets for preventing aging. However, behind the external simplicity of the model lie complex interactions that are not always possible to analyze based on the results obtained. The multidirectional effects of heterochronic parabiosis in different publications vary from demonstrating pronounced phenotypic signs of rejuvenation of old partners to induced accelerated aging of young ones. This review analyzes the basic physiological characteristics of the model, as well as the possible contribution of the physiological features of partners of different ages to them. Particular attention is paid to the rate of mutual circulation between partners and the age-related influence of soluble factors (hormones, growth factors, *etc.*) and extracellular vesicles; as well as the migration of stem, lymphoid, and non-lymphoid cells, including the effects of its fusion with various tissues of parabiotic partners. Since the decrease in the lifespan of a young partner in heterochronic parabiosis after separation was more pronounced than the prolongation of the life of the old one the need to study the soluble factors, extracellular vesicles, or cells which capable of inducing accelerated aging in young animals is emphasized. It is assumed that the elimination/inhibition of such "aging" factors in old animals may become one of the approaches to prolong life.

Keywords: parabiosis; aging; rejuvenation; blood exchange

1. Introduction

The mammalian organism is a complex organization of cells that interact with each other through various types of signals-intercellular interactions, humoral factors, extracellular vesicles, migrating cells, and peripheral neuronal regulation. The development of most pathological processes, including aging, involves almost all possible interactions that complicate the analysis of the mechanisms of disease development. The parabiosis model

was developed specifically for the analysis of humoral and cellular pathways of interaction between cells or organs that influence the development of pathology and aging.

A parabiotic pair is a chimeric organism created by surgery and consists of two individuals with a unified circulatory system. Blood circulation between partners in parabiosis is formed by the reciprocal germination of capillaries and peripheral nerves and provides a circulatory exchange of biological fluids, soluble factors, extracellular vesicles, and hematopoietic cells between them. Thus, parabiosis model of physiological and pathophysiological processes, including aging [1,2]. However, studies that describe the basic characteristics of the model are limited.

Since the first parabiosis surgery by Paul Bert in 1864 [3], according to Pubmed, at least 2160 articles have been published using this model [4]. The most significant results with parabiosis model were obtained using only young animals. In particular, the most famous work was the study of Coleman, who demonstrated the presence of a satiety factor in the circulation, which led to a decrease in the food intake of wild mice paired with diabetic animals [5], and which was identified as leptin subsequently [6]. In the following years, it was shown that the general circulation contributes to the hormonal exchange between the pituitary gland and gonads [7], exchange by extracellular vesicles of different origins [8,9], migration of liver and intestinal stem cells to areas of muscle damage, as well as their integration into vessels in the process of neovascularization [10], migration of hematopoietic stem cells to the bone marrow [11], and affected areas of organs [12], fusion between HSCs, macrophages, and intestinal epithelial cells [13] and many others phenomenon were demonstrated using this model.

If the biological effects identified using the parabiosis model in young animals showed unambiguous and reproducible results, the studies conducted on animals of different ages were extremely multidirectional. Following the successful work of Irina Conboy, who demonstrated the powerful rejuvenating effect of heterochronic parabiosis on the progenitor cells of the old partner [14], age-dependent inhibition of adult neurogenesis in young heterochronic partners was demonstrated because of parabiosis in the studies of Villeda *et al.* [15]. Following an earlier publication by Tauchi *et al*, who demonstrated a decline in the liver function of young heterochronic partners after 3 months of coexistence with old ones [16], the rejuvenating effect of 5 weeks of parabiosis on the liver function of an old heterochronic partner was demonstrated because of parabiosis in a study by Liu *et al.* [2]. Published articles testify that young heterochronic partners separated after 3 months of parabiosis can restore their biological age [17], and vice versa, they are unable to do this, since their life expectancy is significantly reduced [18].

The model of heterochronic parabiosis turned out to be rather complicated for an unambiguous analysis of the obtained results. This review is aimed at considering the main physiological characteristics of the model, as well as its changes with aging that may affect the conclusions of ongoing studies, and at least a little closer to understanding the aging process.

2. Basic physiological characteristics of the parabiosis model

2.1. Parabiosis model from a biological point of view

The protocol for creating a parabiosis model is well presented in many publications [1,19–24] and consists of surgical steps common to all laboratories, which, however, may differ in detail. In general, the parabiosis procedure is aimed at creating common anastomoses in animals that can provide common blood circulation between parabiosis partners due to the mutual sprouting of capillaries. The main stages of the surgical connection of animals include the suturing of massive volumes of skin. The suture runs almost along the entire body of the animal (starting from the knee joint of the lower limb and up to the knee joint of the upper limb), which is about 8 cm on both dorsal and ventral sides of the mouse body. It should be noted that with an average mouse body length of 10 cm, the approximate estimated skin area is about 83 cm^2. In this case, the suture area is 8 cm × 0.3 cm + 8 cm × 0.3 cm = 4.8 cm^2 of skin and occupies about 5%–6% of the body surface for each parabiotic partner. In most publications, the operation involves limited suturing of the walls (muscles) of the peritoneal cavity (of the order of 1 cm) and pairing of the joints of the upper and lower extremities, or shoulder blades. At the same time, the question remains whether this procedure leads to the occurrence of anastomoses between the pressed muscles of the limbs or not.

Conducting a surgical connection between two animals is accompanied by tissue damage, which is subsequently replaced by the process of wound healing and regeneration of damaged tissues. The mechanism of wound healing is a well-studied process that includes at least four phases. 1) Hemostasis stage, which provides blood vessels constrict to restrict the blood flow in damage, and the formation of a clot. 2) Inflammatory acute response, which occurs immediately after injury and lasts for the first 48 h. 3) Proliferative stage, which is directly responsible for the regeneration of damaged areas and includes the processes of angiogenesis and reinnervation of damaged tissues, fibroplasia, and re-epithelialization of the skin. These processes can last up to 5–14 days after surgery. 4) The maturation (or remodeling) stage is when collagen is remodeled from type III to type I, and the wound fully closes [25–28]. From the point of view of evaluating the effects of the parabiosis model, the most interesting is the proliferative stage, which includes the processes of angiogenesis and reinnervation, and which can affect the studied parameters during the first 14 days of parabiosis in young animals.

It should be noted also, that despite the use of highly inbred mouse strains, in some cases, a sluggish graft-versus-host immune response to minor antigens still occurs, which can lead to up to 20%–30% mortality up to 6 weeks after surgery [1,29], more so in heterochronic couples. The reason can be either genetic drift (leading to the occurrence of point mutations of individual proteins), or age-related structural or post-transcriptional modification of proteins in old animals, or other changes that can activate the immune system and provoke a graft-versus-immune reaction. Therefore, when creating isochronic pairs, some researchers recommend using littermates [22,30,31]. However, this approach does not allow an objective comparison of the results of isochronic and heterochronic pairs.

Another aspect of parabiosis is the chronic stress that animals are subjected to. Chronic stress is closely related to the development of various diseases by affecting the diverse mechanisms of tissue homeostasis. Thus, it has been shown that pre-exposure to chronic stress enhanced lung metastasis in mice with tumors by enhancing the infiltration of macrophages and modulating the pre-metastatic niche for lung colonization by tumor cells [32]. Chronic stress impacts the cardiovascular system in different animal models [33], induces impaired hematological parameters profile [34], increases inflammation, and affects immune function [35], a link between chronic stress and accelerated biological age was found using DNA methylation-based estimates of cellular age [36].

In the parabiosis model stress can be provoked not only by the surgery but also by intimidation of the parabiosis partner by the dominant partner. In several publications, it is noted that one should pay attention to the equality of the body weight of partners, which is extremely difficult when creating a heterochronic pair — since the weight of an old animal, as a rule, is always greater than that of a young one. However, parabiosis did not drastically affect the physical health or longevity of the paired mice [20,37].

Stress is known to activate the hypothalamic-pituitary-adrenal axis, which leads to increased secretion of several glucocorticoids by the adrenal glands. The most studied of these is cortisol (or its analog corticosterone, which mediates the effects of stress in rodents), the level of which increases many times in stressful situations [33]. Examination of fecal metabolites of stress-hormone corticosterone showed that their levels were extremely elevated 48 h after surgery but dropped to two to four times the control values within 72 h after surgery. The levels of corticosterone metabolite in the feces of young isochronic parabionts decreased almost to control values only on the 75th day after surgery [38]. In young isochronic parabionts, levels of corticosterone tended to increase compared with single ones. In old isochronic mice, the level of corticosterone did not differ from that in single old animals [18]. Therefore, despite the strong influence of stress on many physiological parameters, it is extremely difficult to assess their contribution to the effects of heterochronic parabiosis.

2.2. Organ size control

It may be somewhat unexpected to look at parabiosis from the point of view of combining two organisms, following the example of Siamese twins. In such a model, mechanisms for regulating the size of organs can be included. Examples include such well-known mechanisms as compensatory renal hypertrophy following nephrectomy of a paired kidney [39], and compensatory liver regeneration following acute liver injury in humans [40], spleen transplantation in mice which growth is suppressed in the presence of the host's own spleen, and sharply increases after splenectomy [41,42]. The control of organism development in general plays a fundamental role in mammalian biology. Recent studies have identified various genes and signaling pathways that are involved in the formation of organ sizes in normal and pathological conditions [43,44]. It is known that the coordination of growth between organs has both autonomous and non-autonomous mechanisms. At the same time,

organ size control is the result of a complex integrated interaction of internal genetic programs for growth, development, and environmental influences [44]. Activation of the transcription co-activator YAP in the Hippo signaling pathway leads to an increase in the organs size and underlies oncogenesis in many human cancers. Shen *et al.* demonstrated a positive feedback mechanism underlying the robustness of the Hippo signaling pathway in organ size control and tumorigenesis [45]. Recent studies show that the Hippo signaling pathway is also involved in many mechanisms that mediate the aging process of the organism [46,47]. In particular, the size of internal organs can be regulated by feedback factors such as TGFβ, GDF11, mTOR, and others, production of which changes significantly with age [44].

In the model of parabiosis, the mechanism of organ size, which regulated by factors circulating in the blood, was first demonstrated by Wenneker *et al.* [48]. In his study, one rat was subjected to partial hepatectomy, while another isochronic parabiotic partner was found to have an increased number of hepatocytes with mitoses in the liver, as well as increased organ mass [48]. Detailed studies of the involvement of mechanisms regulating organ size in the effects of heterochronic parabiosis have not been carried out yet. However, in a study of Loffredo *et al.*, a similar mechanism could potentially be responsible for the decrease of cardiac hypertrophy during heterochronic parabiosis, and the regulation of which is mediated by GDP-11, one of the factors described above, and involved in the mechanism of regulation of organ size [49]. But this field of investigation needs further research.

2.3. Features of the common blood circulation and mutual exchange of soluble factors in parabiosis

The average blood volume (total, including tissues) for a mouse is approximately 0.077–0.080 mL/g body weight, or an average of 2.0 mL per 25 g mouse [50], and cardiac output in mice averaged 20.4 ± 3.4 mL/min [51], which is an average of 1224 mL, or 61,200% of the circulating blood volume per hour for the intact mouse. Blood exchange between parabiont partners is 16 μL/hr, or 0.66% of the circulating blood volume per hour based on lymphoid cell exchange [52], which is quite a low parameter compared with cardiac output. Cross-circulation equilibrium, calculated by authors for blood cell exchange, was established within 12 days [52]. Summarized characteristics of blood exchange are presented in Table 1.

Somewhat different from the above results were obtained by Zhang *et al.* [38]. They showed that after 12 weeks of parabiosis the total blood exchange between both partners is in diapason of 63 to 107 min, or 100%–170% of the circulating blood volume per hour based on Evans Blue exchange. Huff *et al.* published result of 0.66% of the circulating blood/min (or 39.6% per hour) in mice based on Fe59 tagged erythrocytes exchange [53]. And Harris *et al.* demonstrated result of 1.0%–2.0% of total blood volume exchanges per minute (or 60%–120% per hour) in rats [54], and mice [55] based on Evans Blue exchange.

Table 1. The basic characteristics of parabiotic pairs.

Parameters	Characteristics for parabiotic pairs		References
	Young, isochronic	Heterochronic	
% of surgically united body surface (skin, muscles)	~ 5%–6% of body surface	NA	
Time of complete tissue regeneration after surgery in intact mice	~ 2 weeks	NA	[26]
Predicted density of capillaries of mutual blood circulation	1274 capillaries/anastomosis with a total cross-sectional area of 22,914μm^2	NA	[52]
Blood exchange rate between pairs	0.66% of the circulating blood/hr (blood microfluorimetry)	NA	[52]
	0.66% of the circulating blood/min (Fe59 tagged erythrocytes exchange)	NA	[53]
	1%–2% of the circulating blood/min (Evans blue exchange)	NA	[54]
Cross-circulation equilibrium for lymphoid cells in the blood	CD11c, Gr-1^{hi}, CD4, CD8, CD220 and Gr-1^{lo} lymphocytes within 14 days	NA	[52]
	Within 12 days	NA	[56]
Donor-derived lymphoid cells in the lymphoid organs	Equilibrium (~50%) of CD8+ and CD11c+ were in the lymph nodes, and in the spleen of the partner 28 days after parabiosis	NA	[52]
	None of lymphoid cell populations has reached equilibrium in the bone marrow	NA	[52]
Presence of donor-derived lymphoid cells in the tissues	Almost every tissue in the body: liver, kidney, lung, skin, femur, cranial dura dental pulp, lacrimal gland, intestinal mucosa	Almost every tissue in the body	[12,57–64]
	Absent in brain	Absent in brain	[15]
Presence of donor-derived cells of non-lymphoid type in the tissues	Ischemic hind limb vessels	NA	[10]
	Liver	NA	[10]
	Brain, fusion with Purkinje neurons in cerebellum	NA	[65]
	Damaged tissue during regeneration		
	Sites of chronic inflammation in the brain, muscles, liver, heart, intestine	NA	[66]
		NA	[65,67,68]

The rate of blood exchange between partners directly influences the rate of clearance of biologic factors from the bloodstream and is well described in review of Harris RB [69]. Such close attention to the clearance of substances is because some factors with a short half-life are removed from the bloodstream faster than they can reach equilibrium between partners in parabiosis, and which was confirmed in the model of unilateral kidney removal [69]. A classic example of the high clearance of substances is presented in the work of Van Dyke *et al.*, who clearly demonstrated that the half-life of ACTH from the bloodstream is approximately 5.5 minutes [70], and the evidence of presence of glucose concentration gradient between parabionts [71,72]. The study of Harris *et al.* demonstrated that recombinant leptin exchanges does not reach equilibrium between parabiotic partners due to short half-live of leptin (36 min) [73]. Most types of tissue-derived, and some bloodborne exosomes which have half-lives ranging from 2 to 30 min [74], have such disadvantage in efficacy as soluble factors with a short half-life in the parabiosis model.

In general, the estimate of total blood exchange between parabiotic partners per hour ranges from 0.001% to 0.3% of the physiological blood exchange rate of an intact animal according to various references. However, it should be noted that all studies of blood flow velocity between parabionts were carried out exclusively on young animals. At the same time, it is known that with age, microvascular growth and remodeling disorder are common denominators for most age-related pathologies, while for several pathologies, the key is the decrease in angiogenesis with age (myocardial infarction, stroke, hypertension), and for others — microvascular overgrowth (tumor growth, retinopathies, rheumatoid arthritis) [75,76]. In a skin wound model, it was shown that in healing wounds of old animals there was an increased area of stained blood vessels compared to young ones [77]. Therefore, it can be assumed that the rate of blood exchange between partners of isochronic couples and heterochronic couples may differ in the direction of increasing the latter, which can significantly affect the results. However, this assumption requires further research.

There is also an opinion that older animals with greater body mass may have more blood volume than their younger partners in heterochronic parabiosis [20]. However, it should be borne in mind that adipose tissue makes a large contribution to body weight with age. Numerous blood volume studies in obese animals have shown that total blood volume was comparable between obese and lean Zucker rats [78,79]. Similar results were obtained in a study by Yen *et al.* using blood labeled with ^{51}Cr. They compared blood volumes in mutant obese and diabetic mice, which were twice as heavy as wild-line mice. Despite significant differences in body weight, the blood volume of the mutant mice was only 25% greater than that of the wild line [80]. It is this approach (considering the volume of blood, not body weight) that is used to calculate the dose of drugs *in vivo* in the development of drugs for obesity. It was not possible to find out how blood volume changes with age in the available sources, but such a change may affect the rate of blood exchange, as well as the interpretation of the biological effects of parabiosis.

2.4. Features of the extracellular vesicles (EVs) exchange between parabiotic partners

EVs are one of the main mediators of intercellular communication transmitted between cells, and with blood, along with growth factors and hormones. EVs can contain a wide range of biologically active molecules, which includes specific proteins, lipids, and small DNA or RNA [81–83]. The presence of marker proteins on their surface makes it possible to deliver the content of EVs to a specific tissue or organ, as well as to determine their origin [84]. The listed characteristics of EVs make these mediators promising targets for studying changes in the aging rate in various models, including heterochronic parabiosis [85,86].

EVs are produced by almost all types of cells of the hematopoietic system, as well as internal organs and tissues, therefore a wide range of vesicles are found in the circulation [74]. A study based on the analysis of plasma EV-RNA data from healthy people showed that the bulk of EVs are derived from lymphocytes (B, and T cells, monocytes, NK cells), neutrophils, platelets, and erythrocytes in the blood, and constitute a total of 99% of the plasma EV pool. The remaining human plasma EVs (1%) are derived from solid organs [87,88]. However, the difficulty of analyzing the exchange of EVs with the bloodstream between animals is that some of them have an extremely short half-life. The half-life of EVs can range from a few minutes to four hours of complete elimination after intravenous injection [89]. According to Lai *et al.*, more than half of the intravenously administered EVs are cleared from the blood of mice within 30–60 minutes after injection [90]. A study of the distribution of exogenous vesicles showed that after systemic administration they are distributed in blood and various organs — in the adipose tissue, liver, spleen, lungs, kidneys, muscle, heart, brain, gastrointestinal tract, and other, while their uptake occurs mainly through tissue macrophages [74,91]. In addition, when analyzing the consequences of heterochronic parabiosis, it is necessary to consider that the main part of EVs exchanged with blood comes from blood cells [87], and its composition can be significantly influenced by EVs that are produced by donor blood cells migrating between parabionts. The latter fact significantly complicates the analysis of the effects that EVs mediate in the model of heterochronic parabiosis.

It should be noted that there is no direct evidence of the influence of specific EVs of donor origin on the studied functions in heterochronic parabiosis. Nevertheless, parenteral administration of young EVs to old animals led to several rejuvenating effects. For example, it was suggested, that exosomes from young blood serum may contribute to the amelioration of Huntington's disease in the parabiosis model with r6/2 mouse strain [9]. Intravenous injection of mesenchymal stem cell-derived extracellular vesicles may upregulate pro-angiogenic signaling pathways in the chronically ischemic myocardium of swine [92]. Treatment of old animals with EVs derived from adipose mesenchymal stem cells of young mice (in the form of two sequential intravenous injections with a dose of 20 μg) led to improvements in motor coordination parameters, kidney function, and decreased markers of inflammation and aging in the muscles and kidneys of old mice [93].

Age-related changes in the composition and function of circulating EVs have been demonstrated by many studies. Study of muscle-derived EVs have shown that the concentration of vesicles containing the aging-associated miRNA-34a increases in the serum

of aged mice. In addition, *in vitro* study has demonstrated that EVs enriched with microRNA-34a suppress the level of sirtuin 1 and induce senescence of bone marrow stem cells [94,95]. Also, an increased concentration of EVs containing microRNA-166a, microRNA-21, microRNA-223 and let-7a was found in the blood of old mice, the level of which decreased when treated with senolytics in mice [96]. With age, the content of EVs in the blood increases, which contributes to aging. Exosomes contribute to DNA damage, activation of mTOR, increasing the spread of pro-inflammatory factors SASP [97], and can disrupt the function of stem cells [86]. Thus, the pool of EVs circulating in the blood of heterochronic parabionts may represent a combination of aging-promoting and aging-slowing EVs in a wide spectrum, which reflects the age-related status of paired organisms.

In general, EVs are extremely difficult to analyze and at the same time extremely promising factors capable of mediating the consequences of heterochronic parabiosis. Moreover, EVs from healthy, non-aging cells (such as stem cells) can serve as mediators of a wide range of anti-aging effects; and vesicles produced by a network of cells with a senescence-associated secretory phenotype may serve as mediators of senescence-inducing effects of heterochronic parabiosis.

2.5. Features of cell migration between parabiotic partners

2.5.1. Hematopoietic stem cells

The migration pathways of BM-derived hematopoietic cells have been best studied in young parabionts, since this model was used both to assess the presence of blood circulation between partners and to study various types of pathology. It has been shown that the intensity of mutual migration of blood cells does not depend on their size [85]. Blood cell equilibrium occurs already after 14 days for most lymphocytes, however, with some variations for different cell phenotypes [52]. Equilibrium is also achieved in the lymphoid organs such as the spleen, thymus, and lymph nodes, but not in the bone marrow after 14 days parabiosis of young mice [52].

The presence of circulating HSCs in the blood and their exchange between parabiotic partners was shown in studies by Tyler *et al.* [56]. They showed that after 7 weeks of parabiosis in young isochronous animals, approximately 5%–10% of HSCs are mate-derived [11]. These data have been confirmed in study of transgenic mice with deficits in hematopoietic stem cell — HSCs mobilization recover normal functions when transgenic mice were parabiotically paired with wild-type mice [98].

Studies by E. Donskoy *et al.* [99] showed that the maintenance of thymocytopoiesis in adult animals depends on the migration of prothymocytes from the bone marrow to the thymus. They demonstrated that intrathymic progenitors are replaced on average 2% to 3% per day between parabionts. At the same time, the level of chimerism did not exceed 25% 6–7 weeks after the parabiotic surgery and decreased by half 9 weeks after the separation of the parabiotic partners [99].

2.5.2. Blood leukocytes

The study of leukocyte migration between partners is a standard procedure for evaluating the model of parabiosis and is used to confirm the presence of general circulation. Therefore, there are a lot of publications with similar data. The already classic work by Gibney *et al.* [52] showed that the populations of leukocytes with the CD11c, Gr-1^{hi}, and Gr-1^{lo} phenotype approached equilibrium in the blood of partners during the first 2 weeks of parabiosis. The same dynamics in the blood was observed for the subpopulation of CD4, CD8, CD220 lymphocytes. However, lymphocytes with the CD4 phenotype showed a subsequent decline by day 28 of parabiosis. At the same time, the authors did not establish a significant difference in the calculated exchange rate between the populations of leukocytes in the blood of parabiosis partners [52]. A study of the distribution of donor leukocytes in peripheral organs showed that cells of donor origin with the phenotype of CD8+ and CD11c+ were in equilibrium in the lymph nodes, and in the spleen of the partner 28 days after parabiosis surgery, the proportion of B220+ and CD4+ cells were significantly less in these lymphoid organ than expected, and none of the studied cell populations has reached equilibrium in the bone marrow [52].

Lymphocytes of donor origin were found in almost all organs and tissues of the parabiosis partner. These are Kupffer cells in the liver [57], CD3, CD4, CD8, NK1.1, NK1.1+CD3+, CD19+MCHII+ cells, neutrophils, F4/80^{lo} macrophages, CD11c^{hi} dendritic cells in the kidney [58,59], was demonstrated a minor migration donor cells into femur and skull bone marrow, cranial dura [60], dental pulp [61], lacrimal gland [62], and others. However, it has been shown in several cases that lung resident macrophages [63], F4/80^{hi} macrophages in the kidney [58], and other tissue resident cells are not replaced by donor cells.

It should be noted that such close attention to the migration of lymphocytes is due to the presence of pathology, which is accompanied/potentiated by the migration of immunocompetent cells, including those of donor origin. For example, during the development of inflammatory processes, the number of cells migrating to the area of inflammation can increase sharply with experimental unilateral IRI. At the same time, the absolute number of peripheral GFP+ T cells in the kidneys of injured separants was significantly higher than that of injured parabionts [59]. The same effect is observed in radiation-induced injury and subsequent lung regeneration —5% to 20% of lung fibroblasts in a parabiosis partner have a donor phenotype for cells of interstitial monocytes/macrophages, subepithelial fibroblast-like interstitial cells, and additionally type I alveolar epithelial cells [12]. In heterochronic young male lacrimal glands were significantly more infiltrated with immune B-cells compared to isochronic ones. The authors suggest that chronic exposure with old blood including soluble factors and inflammatory cells may induce inflammation in the young partners [62]. The exchange of blood cells between healthy and pathological parabionts leads to extra-intestinal incorporation of bone marrow-derived cells into the injured intestinal mucosa, which contributes to its recovery [64].

It should be taken into account that immune cells such as macrophages, T- and B-cells, neutrophils and mast cells migrate to areas of tissue damage or pathology under the influence of

chemokines, and locally produce various inflammatory cytokines and growth factors that increase inflammation and can affect the processes of tissue regeneration and remodeling [100–102]. Excessive migration/activation/proliferation of young cells into an old organism during parabiosis can be provoked by the immunogenicity of aggregated, misfolded proteins in old tissues as auto-antigens (amyloid-beta, glycosylated and cross-linked proteins, circulating immune complexes, infections, *etc.*) suggests a possible connection between pathological processes and the development of autoimmunity. In turn, a greater propensity for autoimmune responses of cells of the old organism is reflected in the expansion of autoimmune B cells, an increased prevalence of autoantibodies, an increased frequency of pro-inflammatory T cells, and other phenomena that can affected the studied parameters in young heterochronic partners [103,104].

2.5.3. Cells of non-lymphoid type

Cells of a non-lymphoid type of donor origin are also detected in partners during parabiosis. Circulating progenitor cells of non-bone marrow origin can take part in tissue regeneration processes neovascularization. A recent study by Aicher *et al.* demonstrated that circulating non-bone marrow-derived cells represent 74 ± 13% of donor cells that are recruited into ischemic hind limb of mice in parabiosis combined with reverse bone marrow transplantation model [10]. This fact suggests that internal organs with a high remodeling rate and a high content of tissue resident progenitor cells [e.g., skin, small intestine, liver] are potential sources of progenitor cells that can compete with bone marrow cells in tissue regeneration processes. In the same work, Aicher *et al.* showed that intestinal progenitor cells represent 4.7% of cells incorporated into vessels after hind limb ischemia, liver cells – 6.3% [10]. However, it was not possible to detect donor incorporated cells in physiologically intact vessels.

The presence of Purkinje neurons of donor origin was shown in young isochronic parabionts [65]. However, it was found that the appearance of donor neurons may be due not to the migration of progenitor cells from a partner in parabiosis, but by the fusion of host neurons with donor hematopoietic bone marrow cells and the formation of heterokaryons. It has also been shown that in the nuclei originating from bone marrow cells endogenous genes specific to Purkinje neurons are activated, and hematopoietic proteins are absent after fusion. Neurons with the donor phenotype appeared only after 12 weeks of parabiosis and their number increased tenfold in the presence of inflammatory pathology (such as ulcerative dermatitis for example) in the host partner [65]. A study conducted in the earlier term of parabiosis did not reveal cells of donor origin in the brain [15].

It has been shown that bone marrow cells are involved in the regeneration of tissue damage through the mechanism of fusion with various types of somatic cells, including cardiomyocytes [66], Purkinje neurons and hepatocytes [105,106], muscle cells [107], lung epithelial cells [108], intestinal epithelium cells [109], ectodermal keratinocytes [110], renal tubules [111]. Fusion cells are found in various tissues and organs under various physiological or pathophysiological conditions such as growth, cancer, aging, and others [112]. Parabiosis

model was used for multiple studies of involvement of circulation cells in the processes of healing and regeneration of different tissues after injury. Skinner *et al.* [66] have shown that intra-hematopoietic cell fusion occurs not only under conditions of radiation-induced injury of the host animal, but also under physiological conditions in a parabiosis model. Fusions with donor cells have been identified in clonogenic progenitors as well as differentiated myeloid and lymphoid host cells, although in trace amounts — less than 1% in the spleen, and less than 0.01% in the bone marrow of mice. However, the frequency of fused cells can increase many times under X-ray radiation damage to the host [66]. The low frequency of cell fusion described in the studies above has led to the assumption that cell fusion does not occur under physiological conditions or occurs extremely rarely. However, several publications have reported that chronic inflammation can potentiate this process in the brain, muscles, liver and heart, intestine [65,67], suggesting that physiological inflammatory mediators can influence cell fusion during tissue regeneration [67]. Since the aging is accompanied with a process of chronic inflammation, the frequency of cell fusion in the tissues of old parabiosis partners can increase many times over. Unfortunately, such studies have not been conducted, so this statement requires verification.

3. The biological effects of heterochronic parabiosis

The model of heterochronic parabiosis has been used in many studies, some of which have demonstrated multidirectional effects on the functions of cells and tissues of heterochronic parabionts of both ages. In particular, recovery of certain parameters in old animals has been demonstrated in studies of skeletal muscle regeneration, and improvement in liver progenitor cell function [14], restoration of age-related cardiac hypertrophy [49], improvement in cerebral vascular remodeling, increased neurogenesis, and improved olfactory discrimination was found in aging partners [113], wound healing process was restored in the old animal to the level of to a young mice [114], blood exchange between heterochronic parabiosis improved the quality of aged fracture repair, reduced osteoblast differentiation in aged partners [115], and reduced tubule and interstitial tissue scores in the kidneys of old heterochronic mice [116].

At the same time, numerous publications have shown that heterochronic blood exchange can have mutually opposite effects — along with the identification of signs of rejuvenation of old parabiosis partners, signs of accelerated aging of young ones appear. The study of transcriptomes of different tissues after heterochronic blood exchange showed that senile blood, as a rule, accelerates the “age-related changes” in the erythrocytes of young partners, and young blood simultaneously “rejuvenates” in old mice [117], in the studies of Tauchi *et al.* the hepatic cells of the young partners were like those in the old controls [16]. Several studies have shown that heterochronic blood circulation leads to accelerated aging of immune function in young heterochronic partners without any significant changes in old ones [118–120]. Other biological effects of heterochronic parabiosis [121–123] are summarized in Table 2.

Table 2. The biological effects of heterochronic parabiosis.

Mice strain, sex	Parabiosis duration	Test system	Results	Effect	References
C57BL/6, F	8 weeks	Wound healing	The wound healing process in the old animal proceeds according to a young mechanism.	Rejuvenation	[114]
CBA/Ca	12 weeks	Immune system	Immune system aging in young partners	Pro-aging effect	[118]
CBA/Ca	12 weeks	Immune system	Immune system aging in young partners	Pro-aging effect	[119]
C57BL/6	5 weeks	Liver	Reversed age-related changes in liver	Rejuvenation	[2]
C57BL/6	5 weeks	Liver	Increased aged hepatocyte proliferation	Rejuvenation	[14]
C57BL/6	3 to 9 months	Liver	The hepatic cells of the young partners were similar to those in the old controls. No changes in old partners.	Pro-aging effect	[16]
C57BL/6J	4–5 weeks	Brain	Age-related changes in the transcriptome	Rejuvenation	[121]
C57BL/6J	4–5 weeks	Brain	Inhibition of adult neurogenesis	Pro-aging effect	[15]
C57BL/6J	4–5 weeks	Brain	Restored remyelination after lesion	Rejuvenation	[122]
C57BL/6J	6 weeks	Intestinal function and inflammation	Plasma LPS levels decrease	Rejuvenation	[124]
C57BL/6J	6 weeks	Intestinal function and inflammation	Mucus-containing goblet cell density in the colon	No changes	[124]
C57BL/6	3 weeks	Pancreas	Increased β-cell replication	Rejuvenation	[123]
C57BL/6	4 weeks	Microbiome	Gut microbiome patterns	No changes	[124]
C57BL/6J	8 weeks	Lacrimal gland	Increased inflammatory infiltration in the lacrimal gland of male heterochronic young partners.	Pro-aging effect	[93]
C57BL/6	4-5 weeks	Muscles	Restored the proliferation and regenerative capacity of aged satellite cells	Rejuvenation	[14]
C57BL/6	10 weeks	Heart	Regression of cardiac hypertrophy	Rejuvenation	[49]
C57BL/6	8 weeks	Behavior	Impaired learning and memory	Pro-aging effect	[15]
CBA/Ca, M	3 months, following disconnection	Lifespan	Without change for old partner, decreased lifespan in young partners	Pro-aging effect	[18]
C57BL/6	3 months, following disconnection	Biological age	Reversible increase in biological age	Pro-aging effect of young partners with rejuvenation after disconnection	[17]
C57BL/6	3 months, following disconnection	Lifespan	Prolong lifespan for old heterochronic partner.	Rejuvenation	[125]
C57BL/6	2 months	Lymph nodes cellularity	Without change for old partner. Age-related changes for young partners.	Pro-aging effect of parabiosis	[120]
C57BL/6	2 months, following disconnection	Lymph nodes cellularity	Reversible increase in tested parameters.	Rejuvenation young partners after disconnection	[120]

Indeed, the organism of mammals is a complex self-regulating system, intervention in which can lead to multidirectional results depending on the conditions of maintenance and external influences of animals (strain, sex of animals, type of anesthesia, choice of anti-inflammatory and analgesic drugs for post-surgery maintenance, feed composition, microflora of premises for keeping animals, and much more). With such a variety of external influences, how to assess whether we really register the processes of "rejuvenation" or "accelerated aging" on the model of heterochronic parabiosis? What options to choose? Different laboratories show different results. Assessing these outcomes is extremely difficult, just as it is difficult to evaluate the contribution of each test parameter to life expectancy. For example, a study of Poganik *et al.* demonstrated that young heterochronic partners after three months of parabiosis increased in biological age and recovered main tested parameters to young level for two months after subsequent pair separation [17]. The authors made this conclusion using a preliminary developed algorithm for biological age calculation based on the analysis of DNA methylation sites [126–128]. Similar results were demonstrated in the Davies *et al.* study — they showed that T-cell and stromal cellularity of the peripheral lymph nodes of young heterochronic parabionts decreased to the level of old animals after 8 weeks of parabiosis, and then recovered after separation of the parabionts [120]. At the same time, a study of the life expectancy of heterochronic parabionts after 3 months of coexistence and subsequent separation showed a significant decrease in the average and maximum life expectancy of young partners [18]. What is the reason for this discrepancy in results? Obviously, approaches to determining biological age have not yet reached perfection and cannot guarantee that the trends in the biological clock data were highly consistent with predictable lifespan. This makes the results of biological tests extremely difficult to interpret, including for the model of heterochronic parabiosis.

The results obtained indicate that researchers in the field of gerontology have not yet come to a consensus — what is an aging marker (or biological age), and how effectively can it predict life expectancy? Do we really need a marker of aging to study the effectiveness of anti-aging drugs, or would it be more correct to develop a panel of markers of increased risk of mortality? In clinical research, this area is being intensively developed, and the results are published in many studies [128–131]. In the field of experimental biology of aging, this field has also begun to be actively developed [132–135] and is already used in research [124,136].

Another intriguing aspect of the heterochronic parabiosis model emerged from the study of the lifespan of old parabiosis partners. Studies conducted by two independent groups showed that the lifespan of old partners increased for 2–6 weeks (1.5%–4.5% of the maximum lifespan of animals in this study) after 3 months of parabiosis and subsequent separation [125]. Or did not change — in another similar study [18]. This is a very weak effect of life extension against the backdrop of high expectations, and prerequisites from many studies with a confirmed rejuvenating effect of young blood.

On the other hand, blood factors, EVs, or cells that were transmitted with blood from an old heterochronic partner to a young one can irreversibly shorten the lifespan of young parabiosis partners after three months of parabiosis and subsequent separation (17%–19% of the maximum lifespan of animals in this study) [18].

Perhaps there are factors in the old blood that induce age-related changes. Moreover, will their removal be more effective in prolonging a healthy life than replacing them with young blood? The effectiveness of this approach has already been confirmed in an experiment on a neutral blood exchange model [137] and is being tested in clinical studies [138]. However, evidence of life extension using this approach is not yet available. In addition, it would like to focus on the fact that the procedure for removing part of the protein fractions from the blood (plasmapheresis, hemosorption, or therapeutic plasma exchange), resembles the work of an artificial kidney – it removes the consequences of diseases, but not the cause of its development, or aging. Therefore, the search for the cause (or inducer) of aging is still open.

4. Conclusion

The parabiosis model represents two biological systems combined into one and in complex interaction. A study of the physiological characteristics of the model, such as the area of capillary sprouting, the rate of blood exchange, the percentage of mutual population with donor cells of lymphoid and non-lymphoid origin, was carried out exclusively on young animals without any studies in old ones, which requires additional research for an objective interpretation of the results obtained.

In the study of blood factors exchanging with the bloodstream in a model of parabiosis, much more attention is paid to the analysis of soluble blood factors, such as GDF11, Apelin, CCL11 and other [14,37,49,138–140]. Moreover, in contrast to soluble blood plasma factors, many of which do not reach equilibrium between partners due to a short half-life, most leukocytes and lymphoid cells achieve balance between partners after 14 days of coexistence [52], and able to actively participate in the processes of reciprocal regeneration. The participation of lymphocytes and circulating HSCs in the processes of regeneration of tissues of internal organs in case of injury (including the fusion of cells of lymphoid origin into various tissues) has been shown mainly in studies on young animals and has been little studied in heterochronic parabionts. Therefore, the analysis of many the effects of heterochronic parabiosis may be insufficient and needs to be revised, as well as markers used to assess the rejuvenation/pro-aging effects of the organism — for example, extracellular vesicles, which can both be exchanged with the blood and produced by donor cells in the body of partners in parabiosis.

The absence of the effect of parabiosis on life extension in old heterochronic partners casts doubt on the presence in the blood of young animals of factors that can rejuvenate the old organism. And the shortening of the life of young partners opens a new direction in the search for aging inducers that can irreversibly induce aging and shorten life.

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Conflicts of interests

The author declares no conflict of interest.

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