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Original Article

Fetal progenitor cells for treatment of chronic limb ischemia

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Abstract: Objectives: This study investigated the therapeutic potential of fetal progenitor cells (FPCs) in the treatment of chronic non-healing wounds and ulcers associated with chronic limb ischemia (CLI). The research aimed to elucidate the mechanism of action of FPCs and evaluate their efficacy and safety in CLI patients. Methods: The researchers isolated FPCs from aborted human fetal liver, brain, and skin tissues and thoroughly characterized them. The preclinical phase of the study involved assessing the effects of FPCs in a rat model of CLI. Subsequently, a randomized controlled clinical trial was conducted to compare the efficacy of FPCs with standard treatment and autologous bone marrow mononuclear cells in CLI patients. The clinical trial lasted 12 months, with a follow-up period of 24-36 months. The primary outcomes included wound healing, frequency of major and minor amputations, pain reduction, and the incidence of complications. Secondary outcomes involved changes in local hemodynamics and histological, ultrastructural, and immunohistochemical assessments of angiogenesis. Results: In the animal model, FPC treatment significantly enhanced angiogenesis and accelerated healing of ischemic wounds compared to controls. The clinical trial in CLI patients demonstrated that the FPC therapy achieved substantially higher rates of complete wound closure, prevention of major amputation, pain reduction, and improvement in ankle-brachial index compared to control groups. Notably, the study reported no serious adverse events. Conclusions: FPC therapy exhibited remarkable efficacy in promoting the healing of ischemic wounds, preventing amputation, and improving symptoms and quality of life in patients with CLI. The proangiogenic and provasculogenic effects of FPCs may be attributed to their ability to secrete specific growth factors. These findings provide new insights into the development of cellular therapeutic angiogenesis as a promising approach for the treatment of peripheral arterial diseases.

Keywords: Chronic limb ischemia, fetal progenitor cells, bone marrow mononuclear cells, regenerative medicine

Introduction

Chronic limb ischemia (CLI), the most advanced stage of peripheral arterial disease (PAD), represents a significant unmet medical need with high rates of morbidity, mortality, and limb loss. Atherosclerosis causes blockages in the arteries supplying blood to the legs and arms, which can worsen over time, leading to significantly reduced blood flow and the development of CLI [1]. Key mechanisms underlying this progression include atherosclerotic plaque build-up, inflammation, thrombosis, collateral artery failure, and tissue damage [2].

The prevalence of CLI remains challenging to determine due to the heterogeneity of available data. However, estimates suggest that CLI affects approximately 10% of all PAD patients, who are at high risk of death, with reported 4-year mortality rates ranging from 18.9% to 63.5%, depending on the Rutherford classification [3]. Most CLI patients require hospitalization for surgical or endovascular interventions, while others need frequent outpatient visits to assess disease stability or progression and require dressing changes for non-healing ulcers. In 2014, the annual cost burden for the hospitalization of PAD patients in the United States was approximately USD 6.31 billion [4].

Despite advances in revascularization techniques, surgical or endovascular revascularization may not be feasible in some patients due to

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significant comorbidities which leads to a reduction in life expectancy [5]. The Global Vascular Guidelines have improved the management of CLI patients with evidence-based revascularization (EBR), aiming to identify patients for whom revascularization would only increase the risks of adverse events rather than provide real benefits. The therapeutic choice should avoid unnecessary revascularization and focus on the patient as a whole, with improved risk-modifying therapy potentially being the key to future PAD management.

A non-negligible proportion of CLI patients are unsuitable for revascularization due to anatomical or physiological reasons, a subgroup defined as "no-option CLI" [6]. This concept is based on the assumption of potential successful revascularization in CLI patients without a suitable target arterial pathway and no visible arterial circulation in the foot (desert foot). This type of arterial disease is more common in patients with diabetes and end-stage renal disease and was previously the main criterion for performing major amputations. However, the development of new technologies and devices, especially in the endovascular field, has contributed to the re-emergence of no-option CLI as a new area of research before considering major amputations. The true incidence and prevalence of no-option CLI patients remain unknown due to the lack of epidemiological studies, but no-option CLI is known to be associated with ischemic heart disease, end-stage renal disease, and heart failure. The primary goals for these patients are relieving ischemic pain, healing ulcers, avoiding limb loss, improving quality of life, and prolonging survival.

Treatment for CLI primarily consists of risk factor mitigation, including control of cholesterol and glycemic levels, smoking cessation, and endovascular or open surgical revascularization when feasible. However, nearly 20-40% of patients may not respond to or have failed standard medical therapy or are unsuitable for revascularization [7-9]. Given the high economic burden, decreased quality of life, and poor survival associated with CLI, regenerative therapies aimed at promoting neovascularization to improve wound healing and limb salvage hold significant promise, highlighting the critical need for novel vascular regenerative therapies [10].

Cell therapy, particularly using mesenchymal stem cells (MSCs), has emerged as a promising regenerative strategy for stimulating therapeutic angiogenesis in CLI. MSCs play a crucial role in wound healing and angiogenesis due to their potential for multilineage cell differentiation, ability to secrete soluble factors and exosomes (paracrine functions), and immunomodulatory and antibacterial activity [11]. A study by Liotta et al. [12] introduced the concept of autologous cell therapy to treat no-option CLI, which has led to numerous investigations assessing the efficacy of bone marrow mononuclear cells (BM-MNCs) and peripheral marrow mononuclear cells (PM-MNCs). The findings suggest that these cells can reduce the rate of major amputations and promote wound healing, with PM-MNCs demonstrating superior efficacy as an autologous cell therapy due to their ease of collection and effectiveness in diabetic patients [10].

Despite the promising potential of MSCs several clinical trials have shown only minor benefits in amputation-free survival rates compared to placebo, possibly due to the mild potency of the administered MSCs [13, 14]. Furthermore, syngeneic MSC populations harvested from donors with CLI and/or concomitant diseases such as diabetes mellitus (DM) can suffer from reduced proliferative capacity and therapeutic potential compared to cells from healthy donors [15].

As a result, researchers worldwide have focused their attention on fetal progenitor cells (FPCs) as a potential therapeutic strategy for various diseases, including lower extremity ischemia. In a seminal review study, O'Donoghue and Fisk highlighted that fetal stem cells (FSCs) are cell lines derived from fetal tissues, possessing the ability to divide, proliferate, and differentiate into specialized cells. FSCs can be isolated in the forms of fetal hematopoietic stem cells, fetal mesenchymal stem cells, and neural crest stem cells, and exhibit higher pluripotency potentials and lower immunogenicity effects compared to adult stem cells [16].

Several articles have reported on the efficacy of FPCs in the treatment of a wide range of diseases [17-25]. Researchers have proposed FPCs as a potential therapeutic approach for autism spectrum disorders and wound healing

[26, 27], with several clinical trials currently underway [28-35].

Research on FPCs has been ongoing since the early 2000s, initially focusing on using hematopoietic cells derived from the fetal liver to treat various diseases [36-41]. Subsequently, researchers proposed the concept of organ-specific fetal cell therapy, whereby FPCs specific to a given organ/tissue are used to treat the corresponding affected organ/tissue of the patient, following the induction of central immunological tolerance [42-51]. The rationale for using each type of FPC is based on the pathogenesis of tissue ischemia and tissue defects observed in CLI patients, as described in previous studies [52-57].

Materials and methods

Fetal progenitor cell isolation and characterization

Cryopreserved fetal liver progenitor cells (FLPCs), fetal brain progenitor cells (FBPCs), and fetal skin progenitor cells (FSPCs) are populations of organ-specific fetal progenitor cells (FPCs) that were isolated from abortion material (liver, brain, and skin tissues of 13-15 weeks gestation) obtained as a result of medical termination of pregnancy (MTP). These organ specific FPCs were cryopreserved at -196°C for long term storage and future use.

Fetal progenitor cell (FPC) isolation protocol

1. Separation of fetal tissues: Under sterile conditions, the fetus is thoroughly washed three times in sterile Petri dishes containing 0.9% sodium chloride solution supplemented with antibiotics. Subsequently, the cranial and abdominal cavities are carefully opened, and the brain and liver are harvested. The skin is also collected from the body.

2. Preparation of FPCs: The harvested liver, brain, and skin tissues were dissected into small fragments, transferred to a homogenizer, where they were minced into a homogeneous mass. Cells were washed out from the homogenizer tube walls and pestle using phosphate-buffered saline (PBS) and collected in graduated test tubes after passing through a blood transfusion filter and a needle with a smaller diameter. FPCs were then isolated from the tissue homogenates using a sequential filtration and centrifugation method.

3. Preparation of cryo-protector: The cryo-protector solution was prepared in a sterile laminar box using dimethyl sulfoxide (DMSO) as the base. A 10% cryo-protector solution was prepared using 0.9% sodium chloride and stored at 4°C for a maximum of 10 days.

4. Addition of cryo-protector: The prepared FPC suspensions were mixed with an equal volume of 10% DMSO solution, resulting in a final DMSO concentration of 5% in the cell suspensions.

5. Packaging of FPCs into cryotubes: The cell suspensions were gently mixed and aliquoted into 1.8 ml cryotubes using disposable syringes. The cryotubes were tightly sealed, labeled, and transferred for cryopreservation. Two milliliters of each cell suspension were allocated for bacteriological control, 1 ml for PCR-based detection of contaminating infectious agents, and 2 ml for screening for the presence of hepatitis B, hepatitis C, treponema, and HIV1/HIV2. Cell concentrations are determined using a cell counter. The cell suspensions were aliquoted into 1.8 ml cryotubes, labeled, and transferred to the cryo-bank for freezing.

6. Programmed cryopreservation (Planer Kryo 560-16 controlled-rate freezer): Cryopreservation enabled long-term storage of cell suspensions. The cellular composition of the cryopreserved population did not differ significantly from that of the freshly prepared suspension. Storage in liquid nitrogen facilitated more extensive screening for cell contamination. The cryopreservation program ensured high cell viability and functional activity.

7. Cryo-bank data: FLPCs, FBPCs, and FSPCs suspensions were stored in the cryo-bank in liquid nitrogen at -196°C.

8. Thawing of cell suspensions: Frozen vials were removed from liquid nitrogen and immediately placed in a water bath maintained at 37-39°C. The vials thawed rapidly (1-2 min), and the contents were transferred to a 15 ml centrifuge tube. A special cell-support solution (9 ml per 1 ml of thawed cells) was added dropwise to the tube. The cells were gently mixed and centrifuged at 1500 rpm for 5 min. The cell

Table 1. Head-to-Head comparisons fetal skin progenitor cells manufactured in Ukraine and India (x±Sx)

Parameter	Ukraine data Suspensions of fetal skin cells: 13 (n=11), 14 (n=9), and 15 (n=7) weeks of gestation	India data Fetal skin cells suspensions at 13, 14 and 15 weeks of gestation after 11-24 months of storage in liquid nitrogen		
		13 weeks n=5	14 weeks n=5	15 weeks n=5
Viability, %	96.11 ± 0.39 97.53 ± 0.86 95.88 ± 0.74	95.96 ± 0.53 (P>0.05)	97.08 ± 0.71 (P>0.05)	96.88 ± 0.44 (P>0.05)
CD73, %	43.21 ± 5.25 44.79 ± 5.02 43.16 ± 4.98	43.98 ± 4.15 (P>0.05)	43.76 ± 4.44 (P>0.05)	43.28 ± 4.02 (P>0.05)
CD90, %	60.90 ± 4.53 62.35 ± 4.76 61.44 ± 4.39	61.72 ± 4.14 (P>0.05)	60.80 ± 4.24 (P>0.05)	61.98 ± 4.33 (P>0.05)
CD105, %	67.41 ± 5.10 66.92 ± 5.77 70.12 ± 5.80	66.68 ± 5.37 (P>0.05)	69.08 ± 5.59 (P>0.05)	67.28 ± 5.47 (P>0.05)
Cytokeratin 8, %	5.76 ± 1.13 5.91 ± 1.27 6.02 ± 0.99	5.86 ± 1.08 (P>0.05)	5.34 ± 1.09 (P>0.05)	6.04 ± 1.12 (P>0.05)

Remarks: p, significance of differences compared to Ukraine's data; n, number of cells suspensions.

Table 2. Head-to-Head comparisons fetal brain progenitor cells manufactured in Ukraine and India (x±Sx)

Parameter	Ukraine data Suspensions of fetal brain cells: 13 (n=11), 14 (n=9), and 15 (n=7) weeks of gestation	India data Fetal brain cells suspensions at 13, 14 and 15 weeks of gestation after 11-24 months of storage in liquid nitrogen		
		13 weeks n=5	14 weeks n=5	15 weeks n=5
Viability, %	96.60 ± 0.99 97.00 ± 1.02 96.39 ± 0.95	96.92 ± 0.64 (P>0.05)	96.14 ± 0.68 (P>0.05)	96.46 ± 0.71 (P>0.05)
Nestin, %	90.84 ± 1.32 91.77 ± 1.69 92.41 ± 1.57	90.78 ± 0.58 (P>0.05)	91.10 ± 1.29 (P>0.05)	90.58 ± 0.84 (P>0.05)
GFAP, %	23.94 ± 1.76 24.40 ± 2.15 23.71 ± 2.72	24.06 ± 2.63 (P>0.05)	23.60 ± 3.05 (P>0.05)	23.60 ± 3.05 (P>0.05)

Remarks: p, significance of differences compared to Ukraine's data; n, number of cells suspensions.

pellet was resuspended in fresh cell-support solution. Cell number and viability were determined by trypan blue or 7-Aminoactinomycin D exclusion test.

Comparative analysis of fetal progenitor cells derived from Ukrainian and Indian sources

The Indian study employed the technology for manufacturing FPCs developed in Ukraine, and the doses of FPCs were consistent with those used in the successfully completed clinical trials in Ukraine. A direct comparative analysis of Ukrainian and Indian FPCs revealed no significant differences in the number of FPCs and their phenotypic characteristics. The data presented in **Tables 1-3** demonstrate that the expression of specific markers on FPCs isolated from Ukrainian and Indian sources was statistically comparable. Specifically, the expression of CD34, CD45, and alpha-fetoprotein (AFP) on fetal liver progenitor cells (FLPCs), the

Table 3. Head-to-Head comparisons fetal liver progenitor cells manufactured in Ukraine and India (x±Sx)

Parameter	Ukraine data Suspensions of fetal liver cells: 13 (n=11), 14 (n=9), and 15 (n=7) weeks of gestation	India data Fetal liver cells suspensions at 13, 14 and 15 weeks of gestation after 11-24 months of storage in liquid nitrogen		
		13 weeks n=5	14 weeks n=5	15 weeks n=5
Viability, %	96.41 ± 1.01 95.89 ± 0.95 96.34 ± 1.60	96.22 ± 0.56 (P>0.05)	96.84 ± 0.81 (P>0.05)	96.36 ± 0.86 (P>0.05)
CD34, %	46.25 ± 3.11 46.38 ± 2.94 45.05 ± 2.62	46.48 ± 3.77 (P>0.05)	45.54 ± 1.07 (P>0.05)	46.92 ± 2.38 (P>0.05)
CD45, %	12.50 ± 2.46 12.00 ± 1.23 12.44 ± 1.75	12.36 ± 1.83 (P>0.05)	12.18 ± 2.15 (P>0.05)	12.26 ± 1.89 (P>0.05)
α-fetoprotein, %	10.88 ± 1.69 11.36 ± 1.55 11.87 ± 1.92	10.74 ± 2.31 (P>0.05)	11.58 ± 1.72 (P>0.05)	11.44 ± 2.27 (P>0.05)

Remarks: p, significance of differences compared to Ukraine's data; n, number of cells suspensions.

expression of NESTIN and Glial Fibrillary Acidic Protein (GFAP) on fetal brain progenitor cells (FBPCs), and the expression of CK-8, CD73, CD90, and CD105 on fetal skin progenitor cells (FSPCs) did not differ significantly between the two sources. These findings supported the use of the Ukranian FPC manufacturing technology and dosing regimen in the Indian study, ensuring consistency and comparability with the previous clinical trials.

Toxicology study

All animal experiments were conducted in compliance with the guidelines set forth by the National Research Council for the Care and Use of Laboratory Animals.

Single-dose toxicity

Rats: Single-dose toxicity studies were performed using three different routes of administration: intravenous fetal liver progenitor cells (FLPCs), multiple intramuscular injections of FLPCs and fetal brain progenitor cells (FBPCs), and fetal skin progenitor cells (FSPCs) application on the surface of wounds/ulcers. The study evaluated three dose levels: Dose 1 (1.80-2.10 × 10^6 FLPCs per kg body weight intravenously, 9.45-11.06 × 10^6 total FLPCs and FBPCs cells in 7 intramuscular injections, and 0.25-0.38 × 10^6 FSPCs per cm^2 of wound/ulcer area), Dose 2 (twice Dose 1), and Dose 3 (twice Dose 2). The study groups consisted of 24 rats (12 male, 12 female) for Dose 1 and Dose 2, 30 rats (15 male, 15 female) for Dose 3, and 22 rats (11 male, 11 female) for the control group (0.9% saline). Toxicity was assessed on day 14 post-administration.

Mice: Single-dose toxicity studies were conducted using two routes of administration: intraperitoneal FLPCs and multiple intramuscular injections of FLPCs and FBPCs. Three dose levels were tested: Dose 1 (1.80-2.10 × 10^6 FLPCs per kg body weight intraperitoneally and 6.75-7.90 × 10^6 total FLPCs and FBPCs cells in 5 intramuscular injections), Dose 2 (3.60-4.20 × 10^6 FLPCs per kg body weight intraperitoneally and 8.12-9.48 × 10^6 total FLPCs and FBPCs cells in 4 intramuscular injections), and Dose 3 (7.20-8.40 × 10^6 FLPCs per kg body weight intraperitoneally and 4.05-4.74 × 10^6 total FLPCs and FBPCs cells in 3 intramuscular injections). The study groups included 25 mice (13 male, 12 female) for Dose 1, 25 mice (12 male, 13 female) for Dose 2, 30 mice (15 male, 15 female) for Dose 3, and 20 mice (10 male, 10 female) for the control group (0.9% saline). Toxicity was evaluated on day 14 post-administration.

The studies assessed various parameters, including signs of intoxication, body weight, gross pathology of organs (brain, spinal cord, eye, middle ear, thyroid, parathyroid, spleen,

thymus, adrenal, pancreas, trachea, lung, heart, aorta, esophagus, stomach, duodenum, jejunum, terminal ileum, colon, rectum, liver, kidney, urinary bladder, epididymis, testis, ovary, uterus, skin, mammary gland, mesenteric lymph node, and skeletal muscle). Additionally, hematological, coagulation, urinalysis, and blood biochemical studies performed which included hemoglobin count, total RBC count, hematocrit, total WBC count, differential WBC count, platelet count, ESR, coagulation time, prothrombin time, activated partial thromboplastin time, urine color, appearance, osmolality, 24-hour urinary output, pH, albumin, sugar, acetone, bile pigments, urobilinogen, occult blood, blood glucose, cholesterol, triglycerides, bilirubin, SGPT (ALT), SGOT (AST), alkaline phosphatase (ALP), blood urea nitrogen, creatinine, total proteins, albumin, globulin, sodium, potassium, phosphorus, and calcium.

Repeated-dose toxicity

Repeated-dose toxicity studies were conducted in mice and rats using the same routes intended for human administration: intravenous FLPCs administration, multiple intramuscular FLPCs and FBPCs injections, and FSPCs application on the surface of wounds/ulcers. Additionally, in mice, repeated-dose toxicity was investigated using intraperitoneal FLPCs administration.

Rats: Repeated-dose toxicity was assessed following intravenous FLPCs administration, multiple intramuscular FLPCs and FBPCs injections, and FSPCs application on the surface of wounds/ulcers on days 0, 30, and 60. Three dose levels were evaluated: Dose 1 (total FLPCs dose of 5.40-6.30 × 10^6 per kg body weight, total FLPCs and FBPCs dose of 28.35-33.18 × 10^6, and total FSPCs dose of 0.75-1.14 × 10^6 per cm^2 of wound/ulcer area), Dose 2 (twice Dose 1), and Dose 3 (twice Dose 2). The study groups consisted of 30 rats (15 male, 15 female) for Dose 1, 34 rats (17 male, 17 female) for Dose 2, 38 rats (19 male, 19 female) for Dose 3, and 24 rats (12 male, 12 female) for the control group (0.9% saline). Toxicity was assessed on day 28 after the last FPCs administration.

Mice: Repeated-dose toxicity was evaluated following intravenous FLPCs administration and multiple intramuscular FLPCs and FBPCs injections on days 0, 30, and 60. Three dose levels were tested: Dose 1 (total FLPCs dose of 5.40-6.30 × 10^6 per kg body weight and total FLPCs and FBPCs dose of 20.25-23.70 × 10^6), Dose 2 (total FLPCs dose of 10.80-12.60 × 10^6 per kg body weight and total FLPCs and FBPCs dose of 24.36-28.44 × 10^6), and Dose 3 (total FLPCs dose of 21.60-25.20 × 10^6 per kg body weight and total FLPCs and FBPCs dose of 12.15-14.22 × 10^6). The study groups included 28 mice (14 male, 14 female) for Dose 1, 30 mice (15 male, 15 female) for Dose 2, 34 mice (17 male, 17 female) for Dose 3, and 22 mice (12 male, 12 female) for the control group (0.9% saline). Toxicity was assessed on day 28 after the last FPCs administration (FPCs were administered on days 0, 30, and 60).

Tumorigenicity

The tumorigenicity of fetal progenitor cells (FPCs) was investigated by intraperitoneal and ectopic introduction of FPCs to immunocompromised (SCID) mice (60 animals) at the maximum dose (Dose 3). The mice were immunosuppressed by administering busulfan (1 mg/kg body mass, once daily for 7 days). The FPCs were administered as follows: intraperitoneal administration of fetal liver progenitor cells (FLPCs) at a total dose of 7.20-8.40 × 10^6 cells per kg body weight, and ectopic administration of 0.3 ml of FPCs suspension containing 0.10 ml of FLPCs suspension (0.90-1.05 × 10^6 cells), 0.10 ml of fetal brain progenitor cells (FBPCs) suspension (0.23-0.27 × 10^6 cells), and 0.1 ml of fetal skin progenitor cells (FSPCs) suspension (0.10-0.15 × 10^6 cells), with a total FPCs dose of 1.23-1.47 × 10^6 cells. The ectopic introduction sites included the intraperitoneal cavity, intradermal (multiple injections, 0.05 ml of total FPCs suspension; 0.21-0.25 × 10^6 cells per injection), subcutaneous (multiple injections, 0.1 ml of total FPCs suspension; 0.41-0.49 × 10^6 cells per injection), intramuscular (multiple injections, 0.3 ml of total FPCs suspension; 1.23-1.47 × 10^6 cells per injection), anterior chamber of the eye (bilateral injections, 0.05 ml of total FPCs suspension; 0.21-0.25 × 10^6 cells per eye), under the kidney capsule (bilateral injections, 0.05 ml of total FPCs suspension; 0.21-0.25 × 10^6 cells per kidney), omentum (multiple injections, 0.1 ml of total FPCs suspension; 0.41-0.49 × 10^6 cells per injection), liver tissue (5 injections, 0.1 ml of total FPCs suspension; 0.41-0.49 × 10^6 cells

per injection), spleen tissue (5 injections, 0.1 ml of total FPCs suspension; 0.41-0.49 × 10^6 cells per injection), lung tissue (bilateral 6 trans-pleural injections, 0.1 ml of total FPCs suspension; 0.41-0.49 × 10^6 cells per injection), small intestine wall (multiple injections, 0.05 ml of total FPCs suspension; 0.21-0.25 × 10^6 cells per injection), large intestine wall (multiple injections, 0.05 ml of total FPCs suspension; 0.21-0.25 × 10^6 cells per injection), stomach wall (multiple injections, 0.05 ml of total FPCs suspension; 0.21-0.25 × 10^6 cells per injection), and sternum (3 intra-sternal injections, 0.05 ml of total FPCs suspension; 0.21-0.25 × 10^6 cells per injection).

Blood system toxicity

To investigate the potential toxic effects of FPCs on the blood system, experiments were conducted on Wistar rats and Grey Giant rabbits. The study consisted of two experimental groups and one control group, each containing 14 rats. All rats received intravenous FLPCs and multiple intramuscular FPCs injections (FLPCs + FBPCs) at Dose 3. The first group received a single-dose administration, while the second group received the thrice-dose administration. The intravenous administration of FLPCs consisted of a total cell dose of 7.20-8.40 × 10^6 per kg body weight, and the intramuscular administration of FLPCs and FBPCs consisted of a total cell dose of 27.00-31.60 × 10^6 delivered through 5 intramuscular injections. The control group received 0.9% sodium chloride administration. The investigation was performed on day 28 for all the groups.

Experiments were conducted on immunosuppressed Grey Giant rabbits to study the effects of FLPCs and FBPCs. The rabbits were given busulfan orally at a dose of 2 mg/kg body mass once daily for 5 days, with a total dose of 10 mg/kg body mass. This immunosuppressive treatment resulted in a 35% mortality, and the experiments were performed on the surviving animals. The experimental group consisted of 15 rabbits; 8 males, 7 females that received intravenous (ear vein) administration of FLPCs (0.8 ml of cell suspension; total cell dose of 7.20-8.40 × 10^6 per kg body weight) and intramuscular administration of FLPCs and FBPCs (0.4 ml of FLPCs suspension containing 3.60-4.20 × 10^6 cells; $CD34^+$ cells: 0.88-1.04 × 10^6 cells per injection; $CD45^+$ cells: 2.00-2.80 × 10^5 cells per injection; AFP^+ cells: 2.00-2.40 × 10^5 cells per injection, and 0.4 ml of FBPCs suspension containing 1.80-2.12 × 10^6 cells; Nestin-positive cells: 0.80-0.96 × 10^5 cells per injection; GFAP-positive cells: 2.40-2.80 × 10^7 cells per injection). A total of 15 intramuscular injections were administered (one injection per 1.0 cm, depth of 0.5 cm, in the projection of a. femoralis), with 5.40-6.32 × 10^6 cells per injection. The control group (15 animals; 8 males, 7 females) consisted of busulfan-immunosuppressed rabbits injected with saline instead of FPCs.

Male reproductive toxicity

Experiments were conducted on mature male Wistar rats, which were divided into four experimental groups and one control group. The control group consisted of 8 male rats that did not receive any treatment. The experimental groups were as follows: (1) Transplantation of FPCs in rats (n=5), with investigation conducted 30 days after FPCs introduction; (2) Administration of busulfan in rats (n=10), with experiments conducted 30 days after FPCs transplantation; (3) Administration of busulfan and transplantation of FPCs (n=16), with experiments conducted 30 days after FPCs introduction; and (4) Repeated introduction of FPCs in immunosuppressed rats (n=16), with experiments conducted 30 days after repeated FPCs introduction. The single FPCs administration dose (Dose #3) consisted of intravenous FLPCs (total cell dose of 7.20-8.40 × 10^6 per kg body weight) and intramuscular FLPCs and FBPCs (total cell dose of 27.00-31.60 × 10^6; 5 intramuscular injections). The repeated FPCs administration dose (Dose #3; thrice-dose administration) consisted of intravenous FLPCs (total cell dose of 22.0 × 10^6 per kg body weight) and intramuscular FLPCs and FBPCs with total cell dose of 84.0 × 10^6, delivered through 5 intramuscular injections. Busulfan was administered intraperitoneally at a dose of 1 mg/kg body mass once daily for 7 days, resulting in a total dose of 7 mg/kg body mass. The mortality rate after busulfan administration was 53%. The experiments were performed on the surviving animals.

Female reproductive toxicity and teratogenicity

Experiments were conducted on mature female Wistar rats. The control group consisted of 10 non-bred female rats that did not receive

any treatment. The experimental group included 25 female rats that received a single-dose FPCs administration and 5 non-bred male rats. The single FPCs administration dose (Dose #3) consisted of intravenous FLPCs with total cell dose of 7.20-8.40 × 10^6 per kg body weight and intramuscular FLPCs and FBPCs with a total cell dose of 27.00-31.60 × 10^6, delivered through 5 intramuscular injections. Two weeks after FPCs administration, female rats were divided into 5 groups, each containing 5 rats. Each group was housed in a cage with one male. After two weeks of cohabitation, the female rats were checked for pregnancy. To assess the teratogenic effects at different stages of pregnancy, one rat from each group was euthanized.

Preclinical study

CLI Rat Model Development: Rats exhibit some differences in the structure of the arterial bed compared to humans. The external iliac artery divides into the pudendal trunk and the femoral artery. The femoral artery, a direct continuation of the external iliac artery, passes along the medial surface of the thigh and distally into the popliteal artery. In addition to small branches, a rather large artery (a. saphena), analogous to the human deep femoral artery, departs from the femoral artery. This artery supplies the adductor magnus, semimembranosus, semitendinosus, and gracilis muscles and forms the patellar plexus. Near the semitendinosus muscle, two distal branches arise from the saphenous artery, which are involved in the blood supply to the leg and foot. The popliteal artery, a continuation of the femoral artery, divides distally into the anterior and posterior tibial arteries, supplying blood to the lower leg and foot. This structure of the arterial bed of the rat's lower extremities makes the effect of ischemic preconditioning possible. This effect consists of reducing the volume of the necrotic zone and activating the processes of regeneration and neoangiogenesis, which makes it difficult to assess the effectiveness of the studied stimulators of neoangiogenesis. This is particularly evident when modeling chronic leg ischemia in rats with an intact saphenous artery.

The experimental study was conducted on 150 nonlinear albino rats at room temperature, with an ordinary laboratory dietary intake. The average mass of the rats was 372.4 ± 8.2 g, and their age was 6.0 ± 1.2 months. Ketamine (30 mg/kg body weight) was used for anesthesia during surgical interventions. Under aseptic conditions, a linear incision was made in the area of the medial surface of the thigh from the inguinal fold to the knee joint. The neurovascular bundle was exposed, and the femoral artery was mobilized (first-order branches were cut off) along the entire length of the incision and placed on rubber clamps. The a. saphena was ligated and excised. The popliteal artery was ligated in the bifurcation area and cut off. A section of the great vessels, including the femoral, popliteal, and saphenous arteries, as well as the initial sections of the arteries of the shin, was removed. The wound was sutured with silk thread size 1.0 [58]. As ischemic onset was detected on the 3rd day of the experiment, human fetal progenitor cells (FPCs) were injected on this day.

The study included the following groups and dosing regimens: 1. Control group "S" (saline injections; n=20, 10 males and 10 females): received equivalent volumes of 0.9% sodium chloride solution instead of cells. 2. Control group "I" (ischemia without cell transplantation; n=10, 5 males and 5 females): untreated animals. 3. Control group "FLPCs" (FLPCs intramuscular administration without ischemia; n=20, 10 males and 10 females): received a total dose of 0.18-0.21 × 10^6 human fetal liver progenitor cells (FLPCs) per 100 g body weight. 4. Research group "IBM" (ischemia + human unsorted marrow mononuclear cell transplantation; n=40, 20 males and 20 females): received an intravenous injection of 1.8-3.6 × 10^6 human unsorted bone marrow mononuclear cells per 100 g body weight and 7 deep intramuscular injections of 0.90-1.80 × 10^6 cells per injection in the m. gastrocnemius. 5. Main research group "I + FLPCs + FLPCs/FBPCs" (ischemia + FLPCs intravenous administration + suspensions of FLPCs and fetal brain progenitor cells [FBPCs] multiple intramuscular injections): received intravenous FLPCs (total dose of 5.40-6.30 × 10^6 cells/kg body weight) and intramuscular FLPCs and FBPCs (total dose of 28.35-33.18 × 10^6 cells) on days 3, 33, and 63 after chronic leg ischemia modeling.

Animals were euthanized using ether overdose anesthesia at 1, 2, 3, 7, 10, 15, 20, and 25 days after the last cell administration, and m. gastrocnemius tissue samples were collected.

In vitro experimental study

An in vitro study was conducted to assess the cell processes occurring in wounds after transplantation of allogeneic progenitor cells and to investigate the mechanism of FPCs-stimulated angio- and vasculogenesis. Firstly, the reaction of fibroblasts from chronic ulcer-wound defects was analyzed in combined culturing with FSPCs to evaluate the in vitro cell processes. The influence of intravenous administration and deep wound layer injections of FPCs on local fibroblasts isolated from chronic ulcer-wound defects was experimentally assessed. The MTT-Cell Proliferation Assay results were compared among three patient groups: (1) standard conservative therapy, (2) single deep wound layer injections with FPCs, and (3) single intravenous administration and single deep wound layer injections with FPCs.

Secondly, to study the mechanism of FPCs-stimulated angio- and vasculogenesis, the production of 8 growth factors (EGF, FGF-7, HGF, IGF-1, VEGF, ANGPT1, VEGF-A, and bFGF) was examined in conditioned culture medium using ELISA (Bio-Rad). FPCs were isolated from the liver, skin, and brain tissues of 11 fetuses at gestational ages of 13-15 weeks (13 weeks: 4 fetuses; 14 weeks: 4 fetuses; 15 weeks: 3 fetuses). The cells were cultured in Biologix Culture Flask 25 with 7 ml of phenol red-free DMEM at a seeding density of 20,000 cells/cm^2 (total seeded cells: 5×10^5) for 24 hours in a CO_2 incubator (5% CO_2). A total of 33 flasks were used (FLPCs: 11; FSPCs: 11; FBPCs: 11), with 5 control flasks containing phenol red-free DMEM without FPCs. Conditioned culture medium samples were centrifuged at 1000 g for 5 min, and the supernatant was used to study growth factors by ELISA.

Muscle biopsy

In both experimental animal studies and clinical trials involving human participants, muscle tissue biopsies were performed to obtain samples for further analysis. In the experimental setting, animals were anesthetized with ether anesthesia. In clinical trials, after obtaining informed consent, muscle tissue biopsies were performed under local anesthesia (2% lidocaine solution). A 1-cm incision was made to expose the facial sheath, and the biopsy was obtained from the muscle tissue of the anterior-medial and lateral muscle groups of the affected limb. Post-hemostasis, the wound was closed with Prolen 3.0 sutures. The biopsy material was studied using immunohistochemical, histological, and electron microscopic techniques.

Immunohistochemical and electron microscopy study

General histological studies were performed using standard procedures. After fixation in formalin and alcohol, the study material was processed in paraffin. Sections were stained with hematoxylin-eosin and Van Gieson's picrofuchsin. Hematoxylin-eosin staining provided a general overview of tissue structures, allowing the identification of cellular and non-cellular elements. Van Gieson's picrofuchsin staining revealed changes in the connective tissue, with collagen fibers stained red, muscular and elastic fibers appearing brownish-yellow or yellow-green, and cell nuclei stained dark brown.

Immunohistochemical methods were used to investigate several markers of angiogenesis, including type IV collagen, von Willebrand factor, and vimentin, using the indirect streptavidin-peroxidase method. The prevalence and intensity of the reaction were evaluated semi-quantitatively on a scale from 0 to 3 points, considering the percentage of positively stained cells and the intensity of the staining.

Experimental and clinical electron microscopic studies were conducted to evaluate the effectiveness of fetal progenitor cells (FPCs) and autologous bone marrow unsorted mononuclear cells as stimulators of angiogenesis. The electron microscopic study assessed the state of intact capillary endothelial cells in muscle tissue (control) and changes in endothelial cells during ischemia and cell transplantation.

For electron microscopic examination, muscle tissue pieces were fixed in 2.5% glutaraldehyde solution in phosphate buffer (pH 7.2-7.4) and fixed with 1% osmium oxide solution. The material was dehydrated in alcohol with increasing concentrations and embedded in araldite. Morphological structures were contrasted during material dehydration using a saturated uranyl acetate solution, and sections were contrasted with lead citrate. Ultrathin sections (40-60 nm) were obtained using an LKB-III ultra-

tome and studied using a PM-125K electron microscope.

The histological, immunohistochemical, and electron microscopic studies were conducted to evaluate the effectiveness of FPCs and autologous bone marrow unsorted mononuclear cells as stimulators of angiogenesis.

Clinical trial design and patient groups

A prospective, interventional, open-label, comparative Phase II/III clinical trial (code BS 01/09) was conducted in Ukraine to evaluate the safety and efficacy of fetal progenitor cells (FPCs) and autologous mononuclear bone marrow cells (MNCs) administered intravenously, intramuscularly, and locally, compared to the standard of care in patients with chronic limb ischemia (CLI) and long-term non-healing ischemic wounds and ulcers associated with disorders of reparative regeneration.

The clinical trials were conducted in accordance with national laws, the requirements of the WMA Declaration of Helsinki, and the Office of Human Research Protections (OHRP) Guidelines. The clinical trial was conducted in accordance with the Law of Ukraine "On transplantation of organs and other human anatomical material" (July 16, 1999), the decision of the Cabinet of Ministers of Ukraine No. 1100 (September 5, 2007) "On measures on organizing the activities of health care facilities and research institutions associated with the transplantation of organs, tissues and cells", and the order of the Ministry of Health of Ukraine No. 630 (October 10, 2007) "On approval of arrangements for clinical trials of tissue and cell transplants and examination of materials of clinical trials".

Following toxicological and preclinical studies, FPCs were approved for use in clinical trials. Patients were randomized to three branches: standard therapy, treatment with autologous bone marrow MNCs, and treatment with FPCs. Within each branch, two groups were studied: CLI without wounds/ulcers and CLI with long-term non-healing wounds/ulcers. The study was initiated only after the approval of the clinical trial protocol by the ethics commissions of the Ministry of Health of Ukraine. Patients who were potential participants in the trial were informed about the nature of the clinical trial, the investigated products, and the possible risks associated with the use of FPCs. Each patient provided written consent to participate in the trial.

CLI patients with non-healing ischemic wounds and ulcers

The clinical trial in Ukraine enrolled a total of 264 patients, who were divided into three groups: Group of Conservative Treatment (GCT) with 98 patients, Fetal Progenitor Cells Group (GFPCs) with 102 patients, and autologous bone marrow Mononuclear Cells Group (GMNCs) with 64 patients. The study population consisted of 156 female patients (59.1%), and 108 male patients (40.9%), with a mean age of 58.5 years (range: 21 to 72 years). The duration of the disease varied from 6 weeks to 12 years, with an average of 2.4 years. At the beginning of the trial, the average area of the ulcers was 38.9 cm^2.

CLI patients without wounds and ulcers

In the CLI patients without wounds and ulcers, the GFPCs included 105 patients (mean age 57.4 ± 5.8 years) with IIb-III-degree chronic lower limb ischemia, consisting of 20 patients with obliterating endarteritis and 85 patients with obliterating atherosclerosis. The GMNCs comprised 56 patients (mean age 58.3 ± 6.0 years) with IIb-III-degree chronic lower limb ischemia, including 18 patients with obliterating endarteritis and 38 patients with obliterating atherosclerosis. The GCT, which served as a control group, included 49 patients matched by age and extremity ischemia stage with the patients in the GFPCs and GMNCs. The patients in the GCT received conservative therapy with prostaglandin E.

Clinical trials in India

Following the successful completion of the Ukrainian clinical trials, a multi-centric, Phase II clinical trial (CTRI/2023/05/052776) was planned in India to further evaluate the safety and efficacy of FPCs compared to autologous bone marrow MNCs and the standard of care in the treatment of CLI and long-term non-healing ischemic wounds and ulcers.

FPCs doses and routes of administration

Test Product "Fetal Progenitor Cells" for CLI patients without wounds and ulcer were inject-

ed intravenously (administration of FLPCs) and by deep intramuscular injections of FLPCs and FBPCs along the obliterated vessels of legs, once a day, at 2nd, 3rd and 4th visit.

Mode of intravenous administration: FLPCs were injected intravenously, 20-40 drops per 1 min, once a day at 2nd, 3rd and 4th visit. Dose - 0.2 ml of FLPCs suspension per 1 kg of body weight (1.8-2.1 × 10^6 FLPCs suspension per 1 kg of body weight) or exactly: $CD34^+$-cells - 0.88-1.20 × 10^6 cells per 1 kg of body weight; $CD45^+$-cells - 0.26-0.30 × 10^6 cells per 1 kg of body weight; AFP^+-cells - 0.22-0.24 × 10^6 cells per 1 kg of body weight.

Mode of administration along obliterated vessels: Content of cryotube with FLPCs and content of cryotube with FBPCs were injected intramuscular along obliterated vessels, once a day at 2nd, 3rd and 4th visits. Dose - 0.1 ml of cells suspension of FLPCs or exactly: $CD34^+$-cells - 0.44-0.51 × 10^6 cells per 1 injection; $CD45^+$-cells - 0.13-0.15 × 10^6 cells per 1 injection; AFP^+-cells - 0.11-0.12 × 10^6 cells per 1 injection; and 0.1 ml of suspension of FBPC or exactly: Nestin-positive cells - 0.41-0.48 × 10^6 cells per 1 injection; GFAP-positive cells - 0.12-0.14 × 10^6 cells per 1 injection. Number of injections depends of the length of the obliterated vessel (one injection per each 1 cm of obliterated vessel).

Test Product "Fetal Progenitor Cells" for CLI patients with non-healing wounds and ulcer were injected intravenous (introduction of FLPCs), and by deep injections of FLPCs and FBPCs suspensions under the wound bed, and by local application of FSPCs, once a day, at 2nd, 3rd and 4th visit.

Mode of intravenous administration: FLPCs were injected intravenously, 20-40 drops per 1 min, once a day at 2nd, 3rd and at 4th visit. Dose - 0.2 ml of FLPCs suspension per 1 kg of body weight (1.8-2.1 × 10^6 FLPCs per 1 kg of body weight) or exactly: $CD34^+$-cells - 0.88-1.20 × 10^6 cells per 1 kg of body weight; $CD45^+$-cells - 0.26-0.30 × 10^6 cells per 1 kg of body weight; AFP^+-cells - 0.22-0.24 × 10^6 cells per 1 kg of body weight.

Mode of under the wound/ulcer bed administration: Content of cryotube with FLPCs and content of cryotube with FBPCs were injected under the wound/ulcer bed, once a day at 2nd, 3rd and 4th visits. Dose - 0.1 ml of cells suspension of FLPCs or exactly: $CD34^+$-cells - 0.44-0.51 × 10^6 cells per 1 injection; $CD45^+$-cells - 0.13-0.15 × 10^6 cells per 1 injection; AFP^+-cells - 0.11-0.12 × 10^6 cells per 1 injection; and 0.1 ml of cells suspension of FBPCs or exactly: Nestin-positive cells - 0.41-0.48 × 10^6 cells per 1 injection; GFAP-positive cells - 0.12-0.14 × 10^6 cells per 1 injection. Number of injections depends on the wound/ulcer square (one injection per each 0.5 cm^2 of wound/ulcer bed).

Mode of administration by local application: 1. From cubital vein was collect blood in silicone syringes with sodium citrate at the ratio of 0.25 ml of blood per 1 cm^2 of wound/ulcer area. Sodium citrate (3.2% = 0.109 M) is added in the ratio "blood: sodium citrate" = 9:1. The blood is centrifuged at 150 g for 15 minutes. The blood plasma was transferred to sterile plastic test tubes (50-100 ml) by pipettes with disposable tips. 2. In the tube containing blood plasma was add the desired amount (see above) of progenitor cells of the fetal skin. Then was added distilled water and 0.277% solution of calcium chloride in the ratio "blood plasma: distilled water: calcium chloride" = 1:1:1.3. The contents of the tube were gently stirred for 5 seconds and poured onto a sterile gauze pad. After 2-3 minutes on the thick layered gauze, a fibrin gel was formed (containing the progenitor cells of the fetal skin). Thick layered gauze was applied on wound or ulcer. Above the thick gauze was apply tight bandage. The thick layered gauze was removed after 3 days. Following the latter, usual wound treatment was performed until the next term of application of the fibrin gel with the cells of the fetal skin. Suspension of FSPCs was added at the rate of 0.125 ml per each 1 cm^2 of wound/ulcer area at 2nd, 3rd and at 4th visit. In 0.125 ml of FSPCs number of $CD90^+$ cells were 1.58-2.36 × 10^5, number of $CD73^+$ cells - 1.0-1.5 × 10^4, number of $CD105^+$ cells - 1.63-2.44 × 10^5, and number of Cytokeratin-8^+ cells - 1.5-2.13 × 10^4.

For CLI patients without wounds and ulcers, FPCs were administered through two routes: intravenous injection of fetal liver progenitor cells (FLPCs) and deep intramuscular injections of FLPCs and fetal brain progenitor cells (FBPCs) along the obliterated vessels of the legs. The injections were given once a day during the 2nd, 3rd, and 4th visits (**Figure 1**).

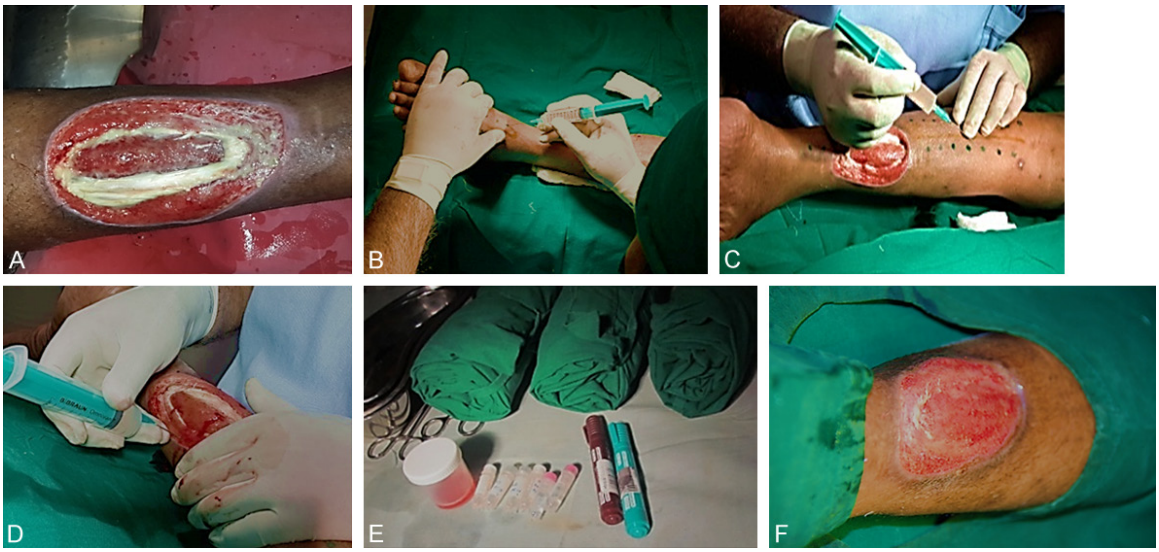

Figure 1. Clinical trial treatment of Patient K with chronic limb ischemia (CLI) and diabetic foot. A. Screening photo of the wound. B. Local injections of fetal progenitor cells (FPCs) around the ulcer and in the wound bed. C. Three months after FPCs treatment, accelerated wound epithelialization is observed. D. Six months after FPCs treatment, the wound shows a reduction in size and depth, along with active marginal epithelization.

In CLI patients with non-healing wounds and ulcers, FPCs were administered through three routes: intravenous injection of FLPCs, deep injections of FLPCs and FBPCs under the wound bed, and local application of fetal skin progenitor cells (FSPCs). The injections and local application were performed once a day during the 2nd, 3rd, and 4th visits.

Statistical analysis

Descriptive statistics were used to summarize all variables by treatment group, as appropriate. For continuous variables, the number of observations, mean, standard deviation, median, minimum and maximum were presented. Categorical variables were summarized using frequency counts and percentages for each category. A comprehensive Statistical Analysis Plan (SAP) was prepared before database lock to provide details about the statistical methods for the primary and secondary endpoints, adjustment for multiplicity, methods for handling missing data, and derivation and definitions required for the analysis of the study data.

Depending on the nature of variables, Pearson's Chi-square test or Fisher's exact test were used for categorical data, while the paired t-test was employed to test the differences in continuous variables. The following statistical values were calculated where appropriate: mean, standard deviation, minimum, lower quartile, median, upper quartile, maximum, frequency count, and percentage. *P*-values >0.05 were considered not significant, while *P*-values <0.05 were considered statistically significant.

One interim analysis was performed after half of the patients had completed the clinical period for the primary outcomes. The Mann-Whitney U test was used to compare the improvement in efficacy scores from baseline between two treatments. One-way ANOVA with treatment (A & B) as the factor was used to compare the change in scores from baseline for various parameters, including the efficacy score, Rutherford score, Wong-Baker FACES pain rating scale score, TWD, pain-free walking distance on a standardized treadmill, TBPI, ankle-brachial pressure index (ABI), and transcutaneous partial oxygen pressure (TcPO2). ANOVA was also used to compare changes in laboratory parameters between the two treatment groups.

Fisher's exact test was used to compare the number of patients showing improvement in blood flow after 12 months and the occurrence of adverse events between the two treatments. One-way ANOVA with treatment (C & D) as the first factor was employed to compare total wound healing with restoration of anatomic

structure between treatment groups C and D. ANOVA was also used to compare changes in laboratory parameters between treatment groups (A & B) and (C & D), while Fisher's exact test was used to compare adverse events between the two treatments.

The odds ratio (OR) and relative risk (RR) were estimated between treatments (A & B) and (C & D) for response and adverse events. All testing was done using two-sided tests at an alpha level of 0.05.

Safety evaluation

The safety analysis set was used to evaluate safety indicators, which included adverse events, injection site reactions, laboratory examinations, vital signs, physical examinations (including weight), and electrocardiograms.

Results

Toxicology study

Single-dose toxicity studies revealed no toxic effects, and the minimum lethal dose was not established in the repeated-dose toxicity study. In the repeated dose toxicity study, animals were observed for 28 days after the last intraperitoneal and multiple intramuscular FPCs administration on days 0, 30, and 60. No mortality or signs of intoxication were observed, and the animals displayed normal body mass growth. Pathological changes in organs were not observed, and hematological, coagulation, urinalysis, and blood biochemical parameters did not vary from control levels after thrice administration of FPCs.

Tumorigenicity results showed no macroscopic or microscopic signs of tissue malignancy in the research organs 3, 6, and 12 months after intraperitoneal and ectopic introductions of the FPCs suspension.

The administration of human FPCs did not show toxic effects on the blood system of Wistar rats or immunosuppressed Grey Giant rabbits. In both species, bone marrow displayed indications of weak expressive activation of erythropoiesis, and peripheral blood showed an insignificant increase in hemoglobin levels without reliable changes in erythrocyte quantity and a reduction in erythrocyte sedimentation rate. In Grey Giant rabbits, busulfan induced sharp depression of hemopoiesis in the bone marrow, characterized by pancytopenia and accompanied by erythropenia (anemia), hemoglobinopenia, thrombocytopenia, leukopenia, relative increase in neutrophil levels, absolute lymphopenia, and absolute monocytopenia in peripheral blood. The administration of human FPCs in immunosuppressed Grey Giant rabbits effectively restored bone marrow hemopoiesis and resolved peripheral blood disorders over 120 days.

Male reproductive toxicity studies showed that the administration of human FPCs in healthy male rats did not exhibit toxic effects on the spermatogenic epithelium of the testes. On the contrary, stimulation of spermatogenesis was observed, as evidenced by an increased spermatogenesis index and fructose content in the anterior prostate. Busulfan, on the other hand, showed pronounced toxic effects on the reproductive system of male Wistar rats, which were further exacerbated by the administration of human FPCs. However, repeated transplantation of FPCs completely restored both hormonal regulation of spermatogenesis and the functional condition of the spermatogenic epithelium of the testes.

In the female reproductive toxicity and teratogenicity study, no evidence of fetal malformation, miscarriage, or abnormal pregnancy was found. All newborn rat pups developed normally without developmental anomalies up to sexually mature age. These findings indicate that human FPCs do not have a toxic reproductive effect in female rats or a teratogenic effect on fetal development during pregnancy, or a toxic effect on the development of newborn rat pups in the postnatal period.

Preclinical study

In the ischemic tissues of animals in the "Ischemia" and "Ischemia + Bone Marrow Mononuclear Cells" groups, characteristic signs of ischemic cell damage were observed, which persisted for 3 weeks and were accompanied by weak activation of angiogenesis. In contrast, in the Main Research Group "Ischemia + FLPCs i/v + FLPCs/FBPCs i/m", a microcirculatory network of de novo-formed capillaries was already formed on day 22. These experimental data indicate that intravenous and intramuscular injections of FPCs into animals with an experimental model of CLI stimulate angio- and vasculogenesis in ischemic muscles.

In vitro study

The *in vitro* study consisted of two experiments: co-culture of fibroblasts from chronic wounds with FPCs and analysis of growth factor production by FPCs.

In the first experiment, co-culture of fibroblasts from the deep layer of chronic wounds with FPCs supported healing of chronic ulcer-wound defects. The results suggest that one possible mechanism of the positive effect of FPCs is the stimulation and restoration of the complete cell proliferation potential of the wound's fibroblasts in patients with chronic limb ischemia and non-healing wounds/ulcers.

The second experiment focused on the production of growth factors by FPCs in culture. No growth factors were detected in the control series flasks' conditioned media. Among the growth factors produced by FSPCs in culture, the concentrations of FGF-7, IGF-1, ANGPT1, and bFGF predominated, whereas FGF and VEGF-A were detected in significantly lower concentrations. In the conditioned media of fetal liver and fetal brain progenitor cells, the highest concentrations of growth factors such as HGF, IGF-1, ANGPT1, and VEGF-A were found, with fetal liver progenitor cells producing significantly higher levels than fetal brain progenitor cells: HGF by 1.5 times, IGF-1 by 3.3 times, VEGF-A by 3.0 times, and ANGPT1 by 15 times. The lowest concentrations of FGF, FGF-7, VEGF, and bFGF were found in the conditioned media of fetal brain progenitor cells (**Table 4**). Thus, the FPCs cultivation conditioned medium contained high concentrations of growth factors with a high regulatory potential for stimulating cell proliferation and angiogenesis.

Clinical trials

Clinical trials revealed the high efficiency of FPCs in treating CLI, which was associated with the activation of angio- and vasculogenesis. Ultrastructural, immunohistochemical, and histological examination of the patients' ischemic tissue biopsies confirmed *de novo* vascular formation in the treated limbs.

Anastomosis of new capillaries and formation of the vasculature was observed up to 3 months after FPCs transplantation. Capillary development occurred from proliferating marginal cells, and capillaries regenerated and anastomosed actively, forming an open-loop intraorganic capillary network. The nuclei of young endotheliocytes exhibited irregular shapes, swelling, and lightened nucleoplasm; coarse granular chromatin gathered in clusters locating eccentrically around the internal membrane and a certain part distributed diffusively. In some areas, the perinuclear space was dilated, and the nuclei had a compact or spongious structure. Newly formed endotheliocytes were characterized by the presence of cytoplasmic processes, large mitochondria, and vacuole structures. The membrane of the endotheliocytes of the new capillaries had microvilli, invaginations, and enlarged processes oriented inside the capillary lumen. The luminal surface was covered by a lamellar substance and had an irregular shape. The young endotheliocytes displayed, which contained polysomes, a moderately developed endoplasmic reticulum, and mitochondria with a thick matrix. The apparent expression of von Willebrand factor 1 month after FPCs transplantation proved the quick activation of angiogenesis. Six months after transplantation, only weak isolated foci of perimysium edema and perivascular sclerosis were found against the background of areas of myosimplast regeneration.

Clinical trials demonstrated that in patients with CLI without chronic non-healing ischemic wounds/ulcers, treatment with FPCs (n=105) was significantly more effective than standard conservative treatment (n=49) and treatment with autologous bone marrow mononuclear cells (n=56). The superiority of FPCs treatment was observed in several aspects including prevention of CLI development, reduction in the incidence of lesions in the second limb, promotion of neovascularization and new microvessel network formation, acceleration of small wound/ulcer healing, improved social rehabilitation, decreased rate of small amputation or major amputation, reduced infection rates in small wound/ulcer, lower relapse rates of CLI, and decreased mortality from cardiovascular causes. These findings suggest that FPCs treatment is a promising approach for managing CLI in patients without chronic non-healing ischemic wounds/ulcers, offering better outcomes than standard conservative treatment and autologous bone marrow mononuclear cell therapy.

Table 4. Concentrations of FPCs-producing growth factors in conditioned culture medium (x±Sx)

Progenitor cells/Growth Factor	EGF pg/ml	FGF-7 pg/ml	HGF pg/ml	IGF-1 pg/ml	VEGF pg/ml	ANGPT1 pg/ml	VEGF-A pg/ml	bFGF pg/ml
Fetal Skin Progenitor Cells, n=11	9.36 ± 0.98	**161.9 ± 13.13**	60.83 ± 25.80	**552.0 ± 139.0**	76.96 ± 8.14	**578.9 ± 60.4**	7.58 ± 1.26	**126.42 ± 9.52**
Fetal Liver Progenitor Cells, n=11	8.19 ± 4.43 (p1>0.7)	140.0 ± 28.04 (p1>0.4)	**564.1 ± 221.0** (p1<0.05)	**1266.0 ± 303.9** (p1<0.05)	145.30 ± 64.98 (p1>0.3)	**23054.0 ± 981.5** (p1<0.001)	**2239.0 ± 176.9** (p1<0.001)	75.67 ± 10.11 (p1<0.01)
Fetal Brain Progenitor Cells, n=11	1.28 ± 0.80 (p1<0.01) (p2<0.001)	25.97 ± 11.09 (p1<0.001) (p2<0.01)	**375.2 ± 70.30** (p1<0.001) (p2>0.4)	**382.2 ± 26.26** (p1>0.2) (p2<0.01)	4.85 ± 1.29 (p1<0.001) (p2<0.05)	**1534.0 ± 367.1** (p1<0.02) (p2<0.001)	**738.4 ± 86.3** (p1<0.001) (p2<0.001)	46.31 ± 8.20 (p1<0.001) (p2<0.05)

Remarks: p1, comparison with FSPCs; p2, comparison with FLPC; n, number of observations. The highest concentrations of growth factors are highlighted in bold.

In patients with CLI and long-term non-healing wounds/ulcers, the introduction of FPCs demonstrated several beneficial effects on wound healing and fibroblast proliferation. Intravenous administration of FPCs increased the proliferation of fibroblasts isolated from chronic wound defects by 4.4 times, while introduction of FPCs in the deep layer of wounds increased fibroblast proliferation by 2.1 times.

Moreover, FPCs treatment (n=102) compared with standard conservative treatment (n=98) and treatment with autologous bone marrow mononuclear cells (n=64), in several aspects of wound healing including Wound size reduction to 25%, 50%, and 75% of the original size, Total wound closure, Wound bed neovascularization.

Social rehabilitation in terms of wound size reduction to 25%, 50%, and 75%; total wound closure; wound bed neovascularization; and social rehabilitation.

In addition to wound healing, patients with CLI and non-healing wounds/ulcers experienced a significantly greater reduction in pain, as measured by visual analogue score when treated with FPCs therapy compared to those receiving standard conservative treatment or bone marrow mononuclear cells. These findings suggest that FPCs therapy is a promising approach for managing CLI patients with long-term non-healing wounds/ulcers, offering better outcomes in terms of wound healing, fibroblast proliferation, and pain reduction compared to standard conservative treatment and autologous bone marrow mononuclear cell therapy.

Clinical trials demonstrated that treatment with FPCs was well tolerated by patients. The incidence of adverse events was significantly lower in patients receiving FPCs therapy compared to those undergoing standard conservative treatment and autologous bone marrow mononuclear cell therapy groups.

The results of the clinical trials provide evidence that intravenous administration of FPCs and their injections in the deep layer of wounds contribute to the healing of chronic ulcer-wound defects in patients with CLI. One of the possible mechanisms of the positive clinical effect of FPCs is their ability to stimulate and restoration of the complete cell proliferation potential of the wound's fibroblasts in these patients.

In summary, FPCs therapy appears to be a safe and effective approach for treating CLI patients with chronic non-healing wounds/ulcers, offering better outcomes in terms of wound healing and lower rates of adverse events compared to standard conservative treatment and autologous bone marrow mononuclear cell therapy. The beneficial effects of FPCs may be attributed to their ability to promote fibroblast proliferation and regeneration in the wound bed.

Long-term follow-up observation

During the long-term follow-up period of 24-36 months, patients with CLI without wounds/ulcers who received FPCs demonstrated statistically significantly higher (better) quality of life indicators on the SF-36 scale compared to patients who received standard conservative therapy or autologous bone marrow mononuclear cells. Moreover, the ankle-brachial pressure index in patients treated with FPCs exceeded that of patients who received standard conservative therapy or autologous bone marrow mononuclear cells, indicating a long-term and more effective positive effect of FPCs on the arterial vessels of the legs. Data from the Efficacy Score and Rutherford scale also demonstrated the maximum efficacy rates and long-term preservation of efficacy in patients who received FPCs.

In CLI patients with non-healing wounds/ulcers during the long-term follow-up period, standard conservative therapy contributed to a reduction in the average wound area by 2 times at the end of clinical trials (12 months). However, during the long term follow up period, the average wound area increased again by 69.3% compared to the data at the end of clinical trials. In patients who were injected with autologous bone marrow mononuclear cells, the average wound area increased, albeit not statistically significantly, in the long-term follow-up period. In contrast, patients treated with FPCs not only did not increase in the average wound area but also showed a tendency towards further decrease relative to the data at the 12th month of clinical trials. The Photographic Wound Assessment Tool (PWAT) revealed that in the long-term follow-up period, active healing of chronic wounds continued effectively in patients receiving FPCs, while the chronic nature of the wounds persisted in patients receiving standard conservative therapy or

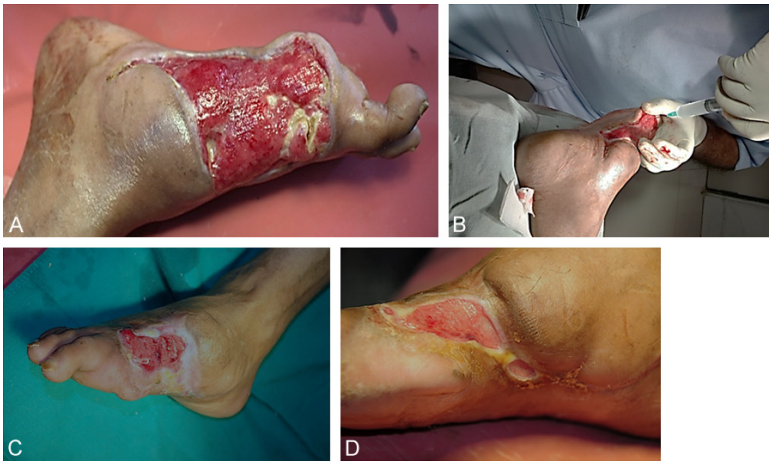

Figure 2. Clinical trial treatment of Patient K with chronic limb ischemia (CLI) and diabetic foot. A. Screening photo of the wound. B. Local injections of fetal progenitor cells (FPCs) around the ulcer and in the wound bed. C. Three months after FPCs treatment, accelerated wound epithelialization is observed. D. Six months after FPCs treatment, the wound shows a reduction in size and depth, along with active marginal epithelization.

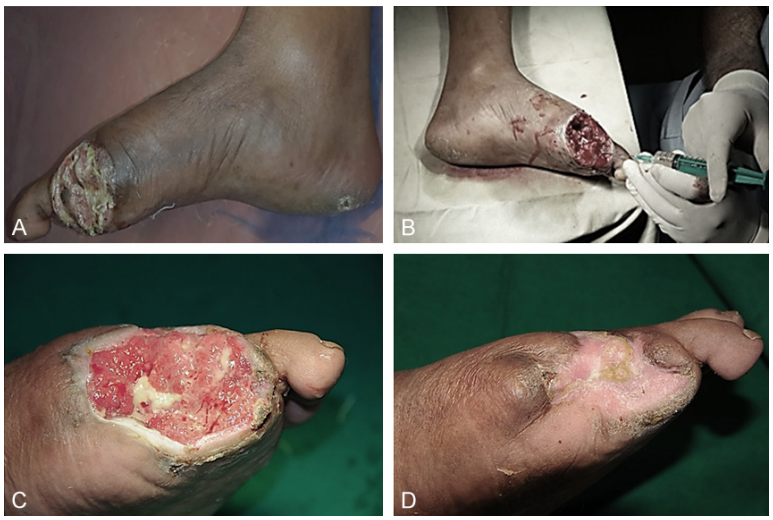

Figure 3. Clinical trial treatment of Patient Ch, a male with Thromboangiitis obliterans (Buerger's disease). A. Screening photo of the wound. B. Local injections of fetal progenitor cells (FPCs) around the wound and in the wound bed. C. Three months after FPCs treatment, the wound is covered with granulation tissue, and active marginal epithelization has begun. D. Six months after FPCs treatment, the wound has completely healed.

autologous bone marrow mononuclear cells (**Figures 2**, **3**).

In summary, FPCs demonstrated safety, the absence of long-term adverse reactions, good tolerability, and the highest long-term preservation of efficacy in both CLI patients with and without non-healing wounds/ulcers during the long-term follow-up period.

First results of clinical trials in India

The first results of clinical trials in India, conducted during the period from June 15, 2023, to February 15, 2024 (8 months), demonstrated the high efficiency of FPCs in treating chronic long-term non-healing ischemic wounds in patients with CLI. The effectiveness of FPCs in stimulating angio- and vasculogenesis was confirmed by angiography data, which showed the appearance of multiple collaterals around obliterated arteries 6 months after FPCs treatment (**Figures 4**, **5**). Following FPCs therapy, patients experienced significant improvements in their condition, including decreased pain in the affected leg, improved sleep quality, and increased walking distance.

Discussion

Angiogenesis, vasculogenesis, and arteriogenesis are considered three distinct processes underlying the development of new blood vessels [59]. Angiogenesis is involved in the development of the vasculature from pre-existing vessels, particularly in the regeneration of damaged tissues and wound healing [60]. In contrast, fetal blood vessel formation occurs through vasculogenesis, where endothelial progenitor cells join together to form dense thread-like structures that gradually develop into mature blood vessels [61]. Postnatally, existing arteries further mature and grow to form functionally mature arteries through a process called arteriogenesis [62].

In the mature body of animals and humans, vascularization of tissues insufficiently sup-

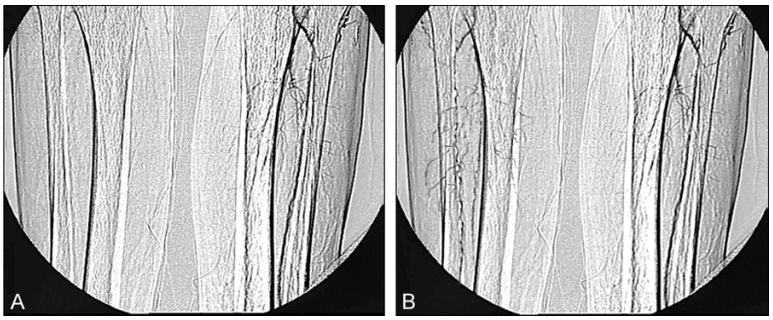

Figure 4. Clinical trial treatment of Patient T, a male with Obliterating atherosclerosis. A. Screening angiogram showing occlusion of the tibial arteries in both legs and occlusion of the femoral arteries at the right side. B. Six months after Fetal progenitor cells (FPCs) treatment, the angiogram reveals the appearance of multiple collaterals around the obliterated arteries.

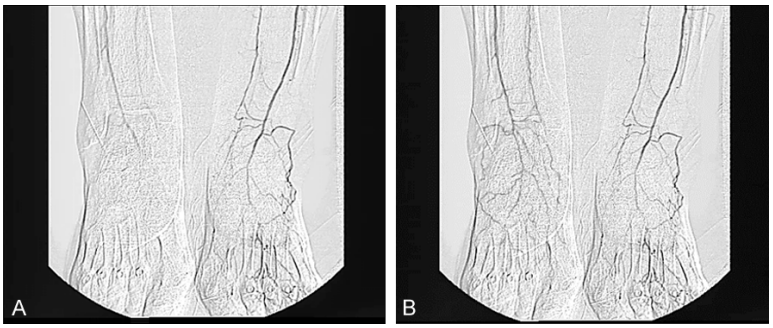

Figure 5. Clinical trial results of Patient T, a male with Obliterating atherosclerosis. A. Screening angiogram showing occlusion of the right dorsalis pedis artery. B. Six months after fetal progenitor cells (FPCs) treatment, the angiogram reveals the appearance of multiple collaterals around the obliterated arteries.

plied with blood occurs due to angiogenesis and arteriogenesis. Consequently, modulation of angio- and arteriogenesis by specific growth factors can serve as a powerful therapeutic strategy for various types of ischemic diseases [60]. However, the use of the putative main angiogenic factor, VEGF, for the treatment of chronic limb ischemia (CLI) has not provided significant therapeutic success [63].

In both preclinical studies and clinical trials, treatment of CLI using FPCs has resulted in the observation of all signs characteristic of vasculogenesis at the ultrastructural level [64-66]. Anastomosis of new capillaries and formation of the vasculature was observed up to the 3rd month after transplantation of FPCs. Capillary development occurred from proliferating marginal cells, with capillaries regenerating and anastomosing actively to form an open-loop intraorganic capillary network. Neo-capillaries formed from young endothelial cells, with erythrocytes present in their lumen.

FPCs likely produce growth factors necessary for the recapitulation of fetal vasculogenesis. Among the studied FPCs, fetal liver progenitor cells may have the most powerful potential for stimulating the growth of new vessels, as their conditioned medium contained the highest concentrations of HGF, IGF-1, ANGPT1, and VEGF-A. Notably, the concentration of VEGF was twice as high as that in the conditioned medium of fetal skin progenitor cells and ten times higher than in the conditioned medium of fetal brain progenitor cells.

Rather than one specific growth factor, a combination of growth factors produced by FPCs, participates in the stimulation of vasculogenesis and angiogenesis. We hypothesize that the combined integral action of these growth factors ensures the high effectiveness of FPCs in the treatment of CLI and wound healing. It is noteworthy that the highest concentration of FGF-7 was detected in the conditioned medium of fetal skin progenitor cells (FSPCs), along with high levels of proproliferative IGF-1 and proangiogenic ANGPT1 and VEGF-A.

The role of keratinocyte growth factor (FGF-7) in normal and wounded skin has been investigated by expression of a dominant-negative KGF receptor transgene in basal keratinocytes. Transgenic mice exhibited epidermal atrophy, abnormalities in hair follicles, and dermal hyperthickening. Upon skin injury, inhibition of KGF receptor signaling reduced the proliferation rate of epidermal keratinocytes at the wound edge, resulting in substantially delayed re-epithelialization of the wound [67]. Based on these findings, it can be hypothesized

that the specific spectrum of growth factors produced by FSPCs may contribute to the activation of wound epithelialization mechanisms observed in clinical trials utilizing these cells.

The positive clinical effects of FPCs in CLI may be associated with growth factors beyond those studied in conditioned media. It is likely that other fetal growth factors, some of which may still be unidentified, also participate in the mechanisms of activation of vasculo-, angio-, neurogenesis, epithelialization, and wound healing. We hypothesize that not only the set of specific growth factors but also the ratio of their concentrations play a crucial role in the observed therapeutic effects. Previous studies have demonstrated that higher amounts of the angiogenic factor VEGF can induce vasculogenesis, while lower concentrations can lead to angiogenesis [68]. Moreover, the use of a single growth factor in therapeutic angiogenesis may result in the development of abnormal vessels, such as the formation of dilated thin-walled vessels with increased permeability and lack of supporting pericytes [69].

FPCs provide new avenues for the development of therapeutic cell vasculo- and angiogenesis, which may be applicable in the treatment of various diseases associated with tissue ischemia. The distinctive combination and concentration of growth factors produced by FPCs likely contribute to their therapeutic efficacy in CLI and wound healing. These findings underscore the potential for further research and clinical application of FPCs in the field of regenerative medicine.

Conclusion

The clinical trials conducted in the present study and the preliminary results from ongoing clinical trials in India strongly suggest that fetal progenitor cells (FPCs) are highly effective in the treatment of chronic limb ischemia (CLI) and long-term non-healing wounds, opening up new prospects for the development of cell-based therapeutic angiogenesis in the management of peripheral arterial disease (PAD). However, further research is needed to elucidate the precise mechanisms by which FPCs stimulate vasculogenesis and angiogenesis, and to investigate whether there are any growth factors characteristic only for the period of intrauterine organogenesis during fetal development. Additionally, preclinical studies and clinical trials should be conducted to evaluate the effectiveness of FPCs in other ischemic conditions associated with diseases of the brain, lungs, heart, and intestines.

The unique properties of FPCs, derived from their developmental origins and the intrinsic mechanisms of fetal tissue repair, hold significant promise for addressing the complex pathophysiology of ischemic diseases and promoting tissue regeneration. Continued research efforts aimed at unraveling the molecular and cellular mechanisms underlying the therapeutic effects of FPCs will not only enhance our understanding of fetal development and regenerative processes but also facilitate the optimization of FPC-based therapies for a wide range of ischemic conditions, ultimately improving patient outcomes and quality of life.

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Disclosure of conflict of interest

None.

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