Chapter 22

Bone Marrow Transplantation for Research and Regenerative Therapies in the Central Nervous System

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Abstract

Bone marrow stem cells are probably the best known stem cell type and have been employed for more than 50 years, especially in pathologies related to the hematopoietic and immune systems. However, their potential for therapeutic application is much broader (because these cells can differentiate into hepatocytes, myocytes, cardiomyocytes, pneumocytes or neural cells, among others), and they can also presumably be employed to palliate neural diseases. Current research addressing the integration of bone marrow-derived cells in the neural circuits of the central nervous system together with their features and applications are *hotspots* in current Neurobiology. Nevertheless, as in other leading research lines the efficacy and possibilities of their therapeutic application depend on the technical procedures employed, which are still far from being standardized. In this chapter we shall explain one of these procedures in depth, namely the transplantation of whole bone marrow from harvested bone marrow stem cells for subsequent integration into the encephalon.

Key words Bone marrow ablation, Bone marrow harvesting, Bone marrow stem cells, Cell therapy, Cell transplantation

1 Introduction

Bone marrow stem cells (SC) constitute the best known population of the adult stem cell group. They were identified 50 years ago as the cells responsible for the formation of blood cell populations [1]. Besides their main function, it was demonstrated that bone marrow SC can differentiate in vivo into elements of a wide variety of tissues [2]. More precisely, in the late 1990s it was also reported that bone marrow-derived cells (BMDC) could differentiate into cells of the central nervous system (CNS) in vivo [3]. Three years later, two parallel works also demonstrated that BMDC could not only become glial cells but also neurons [4, 5].

Traditionally, bone marrow SC have been employed in clinical practice since 1956, when E. Donnall Thomas performed the first bone marrow transplantation in a patient with leukemia [6, 7].

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Since then, the methodologies for therapeutic treatments employing bone marrow cells have been refined. In addition, and taking into account their unexpected plasticity, bone marrow SC are currently being applied as a therapeutic tool in different models of disease (heart and coronary diseases), infarctions, stroke, graft versus host disease, bone diseases, cancers and neural diseases), and not only those related to the hematopoietic system [8].

Regarding the use of bone marrow SC in research and possible therapies in the CNS, current methodologies offer a wide range of possibilities. One of the most widely employed techniques for these purposes involves the ablation of recipient bone marrow and its subsequent replacement by BMDC so that these can reach the recipient's encephalon. In this chapter, we shall explain this methodology, dealing with the procedures for bone marrow ablation and bone marrow SC harvesting and administration.

2 Materials

Note that the suppliers are only for orientation and they may change, depending on the location of researchers.

2.1 Bone Marrow Ablation

1. Source of ^{137}Cs gamma radiation: Gammacell 1000 Elite (MDS Nordion, Ottawa, Canada).
2. Busulfan, $C_6H_{14}O_6S_7$ (Sigma-Aldrich, Steinheim, Germany).
3. Dimethylsulfoxide (DMSO).
4. Phosphate buffered Saline (PBS) pH 7.4 (25 °C): For 1 L mix 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.21 g KH_2PO_4; adjust pH with 1 M HCl.

2.2 Bone Marrow Stem Cell Extraction

1. Iscove's Modified Dulbecco's Medium (IMDM; Invitrogen; Carlsbad, USA).
2. Lysis buffer for erythrocytes; 140 mM NH_4Cl, 17 mM Tris-base; adjust pH to 7.4 with 1 M HCl ADD.
3. 70-μm pore size filter (BD Falcon; Bedford, MA, USA).
4. Falcon tubes (BD Falcon).

2.3 Cell Transplantation

1. 30G syringes (BD Falcon).
2. LE5016 clamps for mice (PanLab; Barcelona, Spain).
3. InfraCare HP3621 heating lamp (Philips Ibérica, Madrid, Spain).

3 Methods

The ablation of the recipient's bone marrow is performed before systemic injections—whether in blood vessels, intraperitoneal or intrahepatic—that are usually aimed at the replacement of such

bone marrow. It is not possible to detect the own BMDC within the encephalon of a given research animal. Therefore, regardless of whether the aim is to study the neural integration of BMDC or the therapeutic properties of such integration, it is necessary to label these cells, generally employing fluorescent proteins (the donors are usually transgenic animals that express them constitutively). Accordingly, these systemic transplantations should ensure the replacement of the recipient's bone marrow by a new one whose derivatives are easily distinguishable. Ablation of the recipients' own bone marrow is necessary to avoid problems of rejection and to achieve an empty niche for the new labeled cells [9, 10]. There are different possibilities of bone marrow ablation, and their pros and cons are summarized in Table 1.

3.1 Bone Marrow Ablation

3.1.1 Physical Ablation

Physical ablation involves the use of ionizing irradiation to destroy the bone marrow of the recipient and is the most common ablative methodology [3, 11–16].

1. Place the animal under a ^{137}Cs gamma radiation source. Different devices are useful for this purpose, ranging from special devices for mice to re-calibrated devices for human irradiation. ^{60}Co sources, although older, are also effective.
2. Irradiate the animal with a minimum dose of 7.5 Gy [13]. Higher doses can be also applied, but their side effects are stronger (*see* **Notes 1** and **2**). If doses lower than 7.5 Gy are employed, it is necessary to irradiate the animal twice or three times to achieve suitable bone marrow ablation (Table 2). Note that the irradiation time depends on the dose chosen and the progressive exhaustion of the radiation source.

Table 1
Pros and cons of ablation procedures

Methodology	Pros	Cons
Physical ablation	Widely employed Opens the blood–brain barrier Accelerates the reconstitution of the bone marrow	Severe secondary effects on cell proliferation
Chemical ablation	No major secondary effects Allows transplants in newborns (busulfan)	Does not open the blood–brain barrier Lower integration rates of BMDC into the brain?
Genetic ablation	No secondary effects Allows more physiological-like experiments Allows transplants in newborns	Needs specific mouse strains

Table 2
Doses of radiation employed for bone marrow ablation

4.5–5.5 Gy (multiple sessions)	[3, 11, 23, 26]
7.5 Gy	[12–16]
8 Gy	[21, 22]
9.5 Gy	[19, 29, 31]
10–11 Gy	[2, 25]

3.1.2 Chemical Ablation

The second possibility for ablating the bone marrow of recipients is the use of chemicals. The most widely employed compounds are also used in human chemotherapy against cancer, busulfan being the most widely employed [17, 18].

1. Weigh 25 mg of busulfan.
2. Dissolve busulfan in 500 μL DMSO.
3. Warm 24.5 mL PBS to 60 °C and maintain this temperature.
4. Add the mixture of DMSO and busulfan to the PBS and mix thoroughly. Keep it at 60 °C again.
5. Inject 20 μL/g of body weight intraperitoneally (which is equivalent to a dose of 20 g /kg). *See* **Note 3**.

Busulfan can also be injected in pregnant females in order to obtain bone marrow-ablated offspring. To achieve this, the previous procedures should be followed taking into account two variations:

1. A dose of 15.5 μL/g of the final mixture (15.5 g/kg).
2. The busulfan injections should be performed twice (on embryonic days 18.5 and 19.5) and between the caudal-most pair of nipples, to ensure a suitable effect in the embryos.

3.2 Bone Marrow Stem Cell Extraction

In order to obtain bone marrow cells, when working with small animals it is necessary to sacrifice the donors. The specific procedures to obtain such cells are as follows:

1. Sacrifice mice by cervical dislocation. When employing rats, it is also possible to decapitate them. Anesthetics are not recommended since they may interact with the mobility of bone marrow SC.
2. Remove hind legs and dissect femurs and tibias separately (*see* **Note 4**). It is important to handle the fragile bones of mice with great care in the extraction process to avoid contamination of the bone marrow. The humerus can be also dissected to obtain a higher number of cells.
3. Carefully remove muscles and other tissues surrounding the bones. Once the bones have been harvested, the following procedures should be performed under a laminar-flow hood to guarantee sterile conditions.

4. Perform injections of Iscove's Modified Dulbecco's Medium (IMDM) medium at both epiphyses of the bones to wash the bone marrow. It is recommended to perform a hole on each epiphysis prior to injecting the medium with the needle of the syringe.
5. Perform three or four injections of 1 mL of medium at each end of the bone to properly wash the bone marrow: the bone metaphysis should remain empty, becoming transparent or white instead of dark red. Other standard cell culture mediums (e.g. DMEM, Dulbecco's Modified Eagle's medium) can be also employed.
6. Collect the bone marrow wash on a 70-μm pore size filter placed on the top of a 50 mL Falcon collection tube.
7. Add more IMDM medium to the filter and gently disaggregate the bone marrow pellets with the aid of a sterile blunt object (e.g. the protective cap of the syringe needle). Stir this cell suspension gently with the blunt object to filter it into the collection tube.
8. Repeat the previous step until the collection tube contains 50 mL of medium (with the filtered cells in suspension).
9. Centrifuge the tube at 364 × *g* for 5 min at 4 °C (*see* **Note 5**).
10. Remove the supernatant.
11. Re-suspend the pellet in 5 mL of lysis buffer for 5 min to break up erythrocytes.
12. Stop the reaction by adding 45 mL of 0.1 M PBS (achieving a final volume of 50 mL).
13. Take an aliquot (e.g. 100 μL) to estimate the number of cells collected in the final volume of 50 mL. Thoma or Neubauer chambers and also automatic cell counters can be used for this purpose.
14. Centrifuge the tube at 364 × *g* for 5 min at 4 °C (*see* **Note 5**).
15. Remove the supernatant.
16. Re-suspend the pellet in PBS and keep the final suspension on ice to ensure cell survival. It is important to note that there is a maximum volume that can be transplanted in each animal.
    - For intravenous administration in adult mice, a volume of 150–200 μL should be employed; for younger mice, a slightly lower volume, about 100–150 μL, should be used.
    - For intraperitoneal injections, higher volumes can be employed.
    - Newborns are difficult to inject intravenously and hence intrahepatic injections are usually employed [18, 19]. Taking into account the tiny size of pups, no more than 50 μL should be injected.

Table 3
Number of cells for systemic transplants

≤1 million	[24, 29]
2–3 million	[3]
5–8 million	[11, 13–16, 20, 23, 25]
10 million	[18, 21, 16, 31]
10–20 million	[12]
30 million	[22]

- Finally, the desired number of cells to be transplanted in each receptor is also a factor to take into account (Table 3). Thus, the volume of PBS employed to obtain the final cell suspension depends on the desired concentration of cells (according to the volume and number of cells to be transplanted).

3.3 Cell Transplantation

There are several possibilities for administering bone marrow SC, but here we shall describe the three methods most commonly used:

- Intraperitoneal administration is the easiest method and this needs no further explanation. It is employed frequently [5, 12, 20–22]. It should be emphasized that if the procedure is not performed properly, the cells may be injected into the gut or bladder. It is therefore important to hold the animal with its head directed downwards to ensure the displacement of the organs to the thoracic cavity and enable adequate injection into the peritoneal cavity.
- Intrahepatic injections are especially suggested for newborns [18] since their skin allows the visualization of the liver (a dark red color). Injections into this structure have no additional complications (*see* **Note 6**).
- Intravenous injections require more expertise. These injections are often made in the tail vein [3, 4, 11, 13–16, 20, 21, 23–27], but they can also be performed in the retro-orbital plexus [19, 28, 29] or in the maxillo-facial vein (for pups; [17]).

There are several steps to be taken into account when performing these transplants (*see* **Note 7**):

1. The animal must be immobilized properly. For injections in the tail vein, there are some clamps for mice and rats that allow the immobilization of the animal, keeping its tail outside the device to access the blood vessels easily. For other types of injections, the animal can be anesthetized.

2. It is recommended to induce vasodilatation (especially for tail vein injections). For this purpose warm water or infrared lamps can be used. The blood vessel to be injected must be perfectly localized.
3. The needles should be thin, but their inner diameter wide enough to allow the injection of the cell suspension without massive cell lysis. 30G needles for mice and 26G needles for rats are recommended.
4. During injection, the bevel of the needle should be oriented upwards.
5. All these blood vessels are very superficial, so the needle should be inserted as parallel to the vessel as possible, and never injected deeply.
6. Once the needle is in the vein, when injecting the cells it should be possible to depress the plunger of the syringe easily, without resistance. No edema should appear and, also, it is easy to appreciate the displacement of the blood inside the blood vessel due to the liquid injected. A common signal of well performed injections (inside the blood vessel) is a small bleeding of the wound caused by the needle.

4 Notes

1. Radiation (for physical ablation) has side effects: proliferating cells are susceptible to such radiation and indeed this is the basis of radiation therapy to treat tumors or neoplastic disorders. Thus, not only bone marrow SC but also other proliferating cells are killed by irradiation, including those whose niche lies inside the encephalon. In addition radiation has a dose-dependent effect [30] and any dose necessary to ablate bone marrow –7.5 Gy or higher impairs neurogenesis [13]. Moreover, radiation also affects encephalic regions other than neurogenic zones [18].
2. Conversely, some of the side effects of radiation can be beneficial for the cells arriving at the brain (even for cell therapies); e.g. it has been reported that radiation accelerates blood reconstitution in bone marrow-transplanted animals [20], and radiation also facilitates BMDC incorporation [31], presumably due to an opening of the blood–brain barrier [32, 33].
3. Temperature of the busulfan solution should not fall below 50 °C. Therefore, it must be injected quickly to avoid excessive cooling from 60 °C.
4. To facilitate the removal of the hind legs of the donors, it is suggested that a cut be made in the fur of the lumbar region, then pulling the skin backwards to expose the underlying mus-

culature, in particular that of the legs. Then, the legs are removed and dissected by cutting them at the level of the hip, avoiding any harm to the femurs.

5. The first pellet of bone marrow cells (obtained after the first centrifugation) is red because of the presence of erythrocytes. After the lysis of these red cells, the second pellet should have a much paler color.
6. Owing to the small size of newborns, only small volumes of cell suspensions should be injected in these animals. Accordingly, the concentration of cells should be relatively high in order to transplant a suitable number. Therefore, any loss of cell suspension also involves the loss of many cells. Transplants in pups often flow back slightly because of their small size. Thus, to avoid an excessive loss of cells, care should be taken to keep the needle inside the body of the animal for a few seconds after the injection, after which it must be removed slowly.
7. The procedures explained above are aimed at achieving a bone marrow replacement for studying subsequent cell integration and its effects in the CNS. However, these procedures for bone marrow ablation and transplantation can also be employed in other research lines.

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