International Journal of ChemTech Research
CODEN (USA): IJCRGG, ISSN: 0974-4290, ISSN(Online):2455-9555
Vol.9, No.09 pp 263-269, 2016

Decreasing SDF1-CXCR4 Expression after Adipose-Derived Mesenchymal Stem Cells (ASCS) Treatment Combined with Freeze-Dried Amniotic Membrane Wrapping in Rat Sciatic Nerve Injury

Pamudji Utomo[1]*, Mochamad Aris Widodo[1], Nur Permatasari[1], Yudha Heru Fibrianto[2]

[1]Faculty of Medicine, Brawijaya University, Malang, East Java, Indonesia
[2]Faculty of Veterinary Medicine, Gadjah Mada University, Yogyakarta, Indonesia

Abstract : Nerve lesions are usually treated by end-to-end repair. Unfortunately, full recovery may never be achieved, particularly with extended lesions. Adipose-derived stem cells (ASCs) are known as one of the promising treatments in tissue regeneration.This study aimed to evaluate SDF1-CXCR4 expression after application of ASCs on sciatic nerve injury. Twenty-one Spraque-Dawley rats weighing approximately 250 g were divided into 6 control group and 6 experimental groups (n:3). Experimental groups were observed at 1st,3rd,5th,7 th 14 th and 21 st day after surgery. In the functional evaluation**,** the sciatic function index (SFI) showed the control group value was constant between -80 until -90. Whereas in the experiment group the value progressively increased until was -8 at 14 and 21 days after surgery. Treatment of ASCs decreased the expression of SDF-1 and CXCR4 on injury site at the 1st week after injury. It suggests that the presence of ASCs at injury site causes the stem cells recruitment from another site of the body (ex, bone marrow, fat tissue) does not occur accordingly and lead to the better functional outcome of nerve healing.
Keywords: adipose-derived stem cells, CXCR4, SDF1, Sciatic Nerve Injury.

Introduction

Numerous surgical procedures are performed each year for peripheral nerve repair, with a significant amount of loss function with the corresponding loss of occupation consequences[1]. Nerve lesions are usually treated by end-to-end repair. Unfortunately, full recovery may never be achieved, particularly with extended lesions. Despite good efforts on surgical techniques, functional outcome is often unsatisfactory[2].

Adipose-derived stem cells (ASCs) are known as one of the promising treatments in tissue regeneration[3]. ASCs are a large multipotent cells population in adipose tissue[4]. Application of ASCs nowadays has been suggested as a possible therapy in peripheral nerve regeneration[5]. ASCs has good potential to differentiate into neurogenic cells with capability in producing neuronal markers[6,7].

Numerous studies have reported that CXCR4 play an essential role in stromal cell derived factor-1 (SDF-1) -inducing cell chemotaxis, viability and paracrine actions of bone marrow derived MSCs. However, the function of SDF-1/CXCR4 in ASCs is not well understood[8]. This study aimed to evaluate SDF1-CXCR4 expression after application of ASCs on sciatic nerve injury.

Experimental

Study design and animals

Thirty-six Spraque-Dawley rats weighing approximately 250 g were divided into 6 control groups and 6 experimental groups (n:3). The 6 experimental group was divided into evaluation on the 1st,3rd,5th,7 th 14 th and 21 st day after surgery. Stable air humidity and a natural day/night cycle were maintained. The rats had free access to standard rodent laboratory food and tap water.

Cell isolation and culture

Adipose tissue samples were obtained from intraabdominal fat tissue of 3-month-old Spraque –Dawley rat under anesthesia. The adipose tissue was extensively washed with PBS to remove blood and fibrous material, and vessels were carefully dissected and discarded.The remaining tissue was finely minced and digested with 0.1 % of Collagenase Type I (Gibco, California, USA) for 60 min with gentle agitation. Enzyme activity was neutralized with a two-fold volume of standard medium containing Dulbecco's modified Eagle medium (DMEM, Gibco) with 20 % of fetal bovine serum (Gibco), 100 U/ml penicillin, 100 µg/ml streptomycin and centrifuged for 12 min at 400× g. The supernatant containing the lipid droplets was discarded. The stromal vascular fraction settled at the bottom was resuspended in standard medium and seeded in culture dishes. Stromal vascular fraction cultures were incubated at 37 °C in a 5 % CO2 atmosphere. After 48 h, no adherent cells were removed. When they reached 80% of confluence, adherent cells were trypsinized (0.25 % at 37 °C for 5 min, Sigma), harvested, and washed with standard medium to remove trypsin and then expanded in larger dishes. A homogenous cell population of ASCs was obtained after 3-4 weeks of culture. Cells at early passages in culture were used for the experiments[4]. Confirmation of mesenchymal stem cells was performed by immunocytochemistry with a positive marker (CD 44,CD 90,CD 105) and negative marker (CD 14, CD 19, CD 54).

Freeze-Dried Amniotic membrane

The freeze-dried amniotic membrane was produced by Tissue Bank unit of Dr. Soetomo General Hospital, Surabaya, Indonesia. We performed histology evaluation for the acellularity status of this membrane with Hematoxylin-eosin staining.

Surgical procedure

Animals were anesthetized by intramuscular administration of ketamine-xylazine (ketamine 5 %, 90 mg/kg and xylazine 2 %, 5 mg/kg). The procedure was carried out based on the guidelines of the Animal Ethics Committee of the Gadjah Mada University. The University Research Council approved all experiments. Following surgical preparation, the right sciatic nerve was exposed through a gluteal muscle incision and the sciatic nerve was injured with a knife and repaired with 7/0 monofilament non-absorbable suture. After careful homeostasis, the muscle was sutured with resorbable 4/0 sutures, and the skin was closed with 3/0 nylon. In the control group, the sciatic nerve only repaired without any specific treatment. In the experiment group, after being repaired the nerve was wrapped with freeze-dried amniotic membrane and ASCs was applied into the repaired nerve. After the expected day of evaluation has been reached, all animal were evaluated for clinical outcome with walking track analysis. Afterward, the animals were anesthetized and euthanized with cervical dislocation technique for further histopathology evaluation.

Functional assessment of nerve regeneration

Walking track analysis (WTA) was performed the 1st,3rd,5th,7th 14 th and 21 st day after surgery. The lengths of the third toe to its heel (PL), the first to the fifth toe (TS), and the second toe to the fourth toe (IT) were measured on the experimental side (E) and the contralateral normal side (N) in each rat. The sciatic function index (SFI) of each animal was calculated by the following formula[8]:

SFI : -38,3 x (EPL – NPL)/NPL + 109,5 x (ETS-NTS)/ NTS + 13,3 x (EIT –NIT)/NIT – 8,8

In general, SFI oscillates around 0 for normal nerve function, whereas around -100 SFI represents total dysfunction. The normal level was considered as 0. SFI was a negative value and a higher SFI meant the better function of the sciatic nerve.

Histological studies

The nerve in the area of repair of the control and experiment group were harvested. They were fixed in 10 % formaldehyde , dehydrated through an ethanol series and embedded in paraffin. The nerves were sectioned in 4 µm sections in the longitudinal plane then stained with Hematoxylin-eosin for general evaluation and Masson's Trichrome staining for better fibrous collagen tissue evaluation.

Immunocytochemistry

Cells were fixed with 4 % paraformaldehyde, blocked to prevent nonspecific antibody binding and incubated with primary antibodies at 4 °C overnight. Following a PBS washing, the plates were incubated with avidin/biotin blocking kit. The primary antibodies used were anti SDF1, anti CXCR4 for nerve specimens and anti CD-14, anti CD-19, anti CD-44,anti CD-54,anti CD-90, and anti CD-105 for ASCs culture (®Bioss). All the conditions were maintained in negative controls, except that the primary antibodies were eliminated. Dishes were examined under the fluorescence microscope (NIKON ECLIPSE E400).

Results

Evaluation of ASCs culture

The ASCs culture was performed until 4th passage (3-4 weeks) and reach 80 % of confluence. Afterward, the cells then ready for evaluation of Mesenchymal stem cells marker on immunocytochemistry. The result showed that ASCs were successfully confirmed with positive marker (CD 44, CD 90, CD 105) and negative marker (CD 14, CD 19, CD 54). (Figure 1).

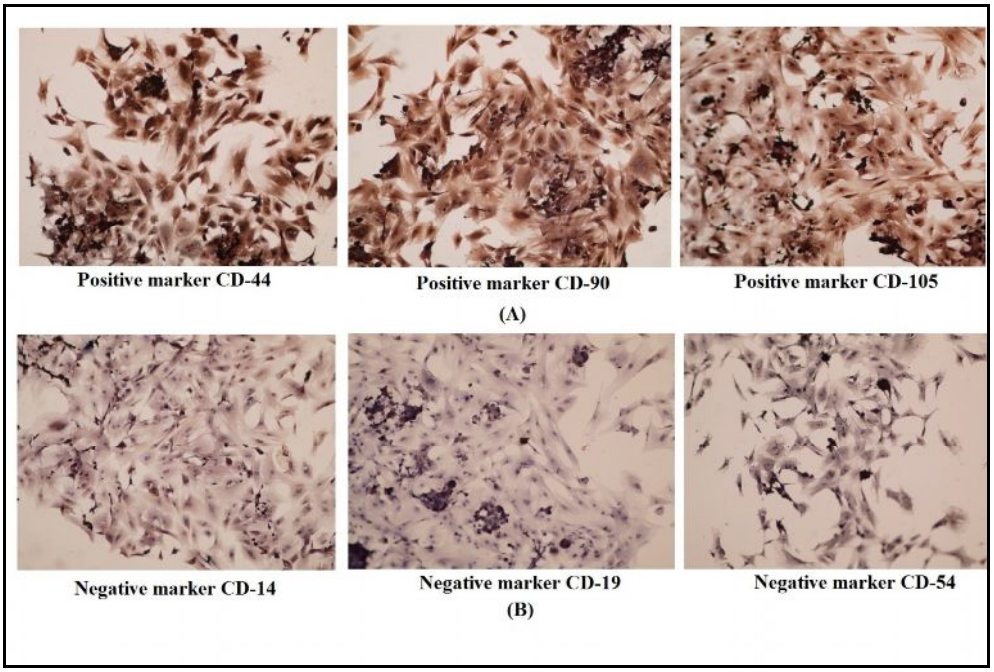

Fig 1: Staining of Mesenchymal stem cells for confirmation with positive marker (A) and negative marker (B)

After confirmed Mesenchymal stem cells, the cells then expanded until a number of cells are enough for all experiment group with dose 1 x 10^6 cells per nerve. Thus, Freeze-Dried Amniotic membrane was confirmed that has acellularity status.

Evaluation of Nerve Regeneration

In the functional evaluation**,** the sciatic function index (SFI) showed the control group value is constant between -80 until -90. Whereas in the experiment group the value progressively increases until was -8 at 14 and 21 days after surgery.

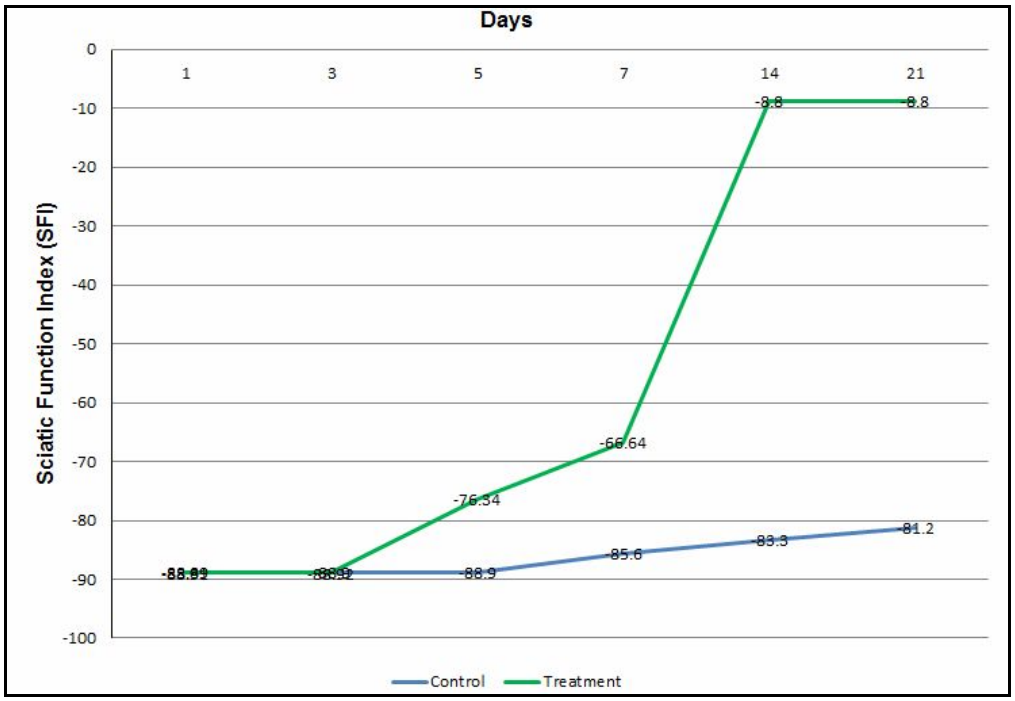

Fig 2: SFI comparison between control group and experiment group after 21 days

Histopathology Evaluation

Under microscopic evaluation, all specimens showed higher fibrosis in repair area of the control group,compared to experiment group. More obvious fibrosis showed clearly by Tricromasone staining. According to Ciu et al., nerve diameter shrunken (ND) is categorized into No (score 0), mild (1), moderate (2), severe (3)[9]. Axonal degeneration (AD) categorized into No (score 0), mild (1), moderate (2), severe (3). Nerve regeneration (NR) categorized into No (score 0), mild (1), moderate (2), good (3) (Table 1).

Table 1. Evaluation of nerve diameter (ND), Axonal degeneration (AD) and nerve regeneration (NR)

Days	ND		AD		NR	
1	3	1	3	1	1	2
3	1	3	3	2	1	1
5	1	1	3	1	1	3
7	1	0	2	1	1	3
14	1	1	2	1	1	3
21	1	1	2	1	2	3

The result of this study showed that ND Score in control group dominantly score 1 (mild), with 1 specimens scored 3 (severe). In the experiment group ND dominantly Score 0-1, with 1 specimens scored 3 (severe). The Axonal degeneration (AD) in control group range from moderate to severe, whereas in the experiment group ranges from mild to moderate. Nerve regeneration in control group dominantly mild regeneration (score 1), but in the experiment group showed dominantly good regeneration (score 3).

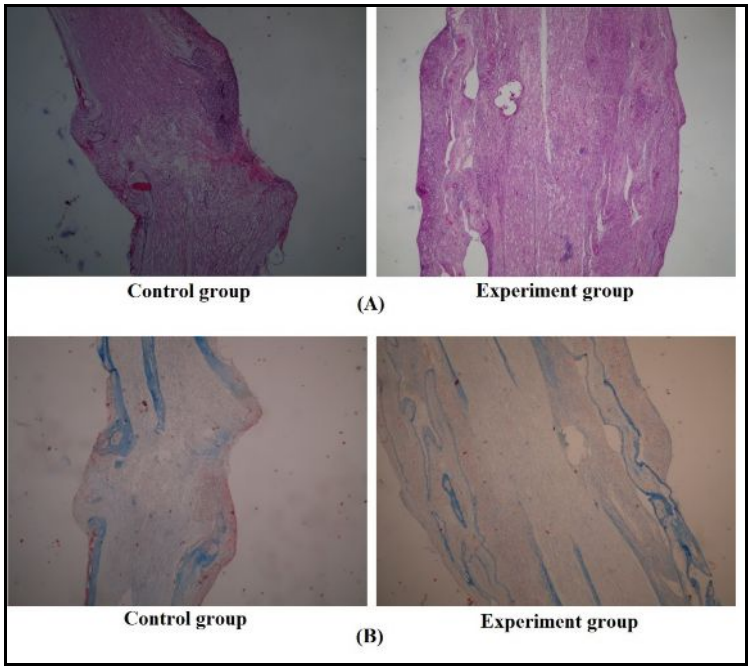

Fig 3: Histopathology at day 21st of repaired nerve with HE staining (A) and Masson's Trichrome staining (B)

Immunohistochemistry of SDF1 and CXCR4

Expression of SDF1 decreased significantly in the experiment group compared to control group. This result is consistent with all evaluation state either at the 1st,3rd,5th,7th 14th or 21 st day after surgery. Expression of SDF1-CXCR4 scored by Toyozawa et al., Score 0: absent / < 5 %, 1: 5-50 %, 2: 50-70 %, 3: 70-90 %, 4: 90-100 %[10].

Table 2. Evaluation of SDF1-CXCR4 expression on control and treatment groups

Days	Control		Treatment		p
	SDF1	CXCR4	SDF1	CXCR4	
1	4.00±0	3.33±0.57	4.00±0	1.33±0.57	0.013
3	3.67±0.57	2.33±0.57	2.00±0	1.33±0.57	0.10
5	3.67±0.57	2.33±0.57	1.67±0.57	1.33±0.57	0.10
7	3.33±0.57	3.33±0.57	0.00±0	0.33±0.57	0.03
14	2.67±0.57	2.33±0.57	2.00±0	0.33±0.57	0.013
21	1.33±0.57	2.33±0.57	3.67±0.57	1.33±0.57	0.10

The result of SDF 1 expression showed in control group range Score dominantly 3-4, whereas in experiment group dominantly score 0-2. (Figure 4A). The result showed that in experiment group SDF1 expression was decreased progressively from day 1 and significantly decreased at day 7 after injury. However, its expression was re-increase at day 14 and 21 after injury. CXCR4 expression showed in control group score range 2-3, whereas in the experiment group scores range 0-1. Expression of CXCR4 decreased significantly at day 7 and 14 after injury in the experiment group compared to control group. (Figure 4B).

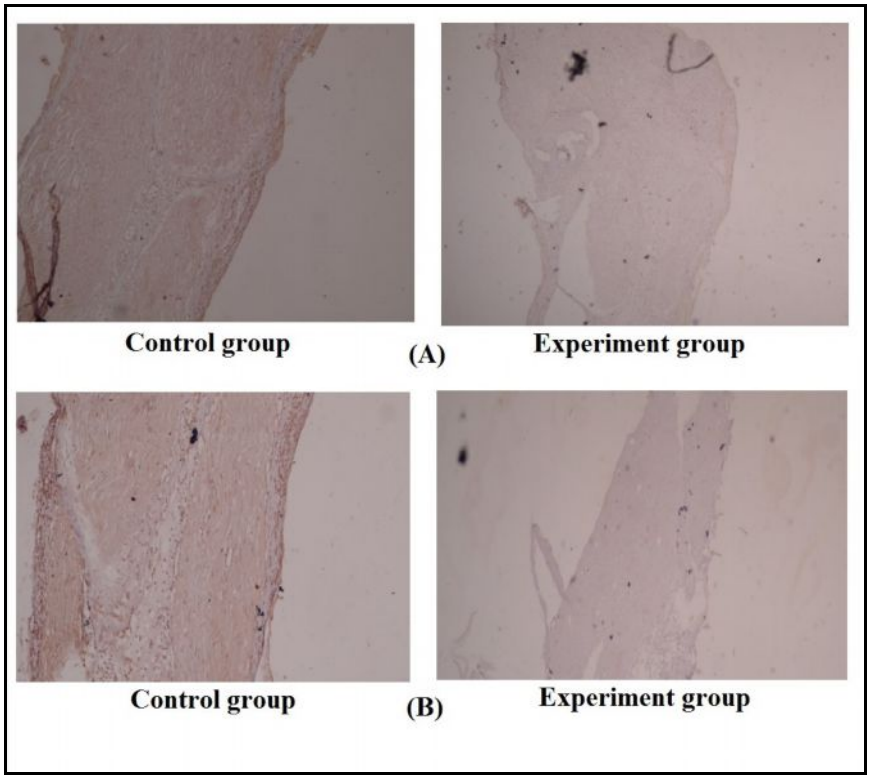

Fig 4: Analysis of SDF1 expression (A) and CXCR4 expression (B) in control and experiment group.

Discussion

SDF1 - CXCR4 axis is chemoattraction axis of Bone marrow-derived mesenchymal stem cells. The role of this axis on adipose-derived mesenchymal stem cells migration is not well understood. Li *et al* reported that angiogenesis of ASCs is, at least partly, mediated by SDF-1/CXCR4 axis. Conversely, proliferation of ASCs was induced by SDF1-CXCR7 axis[11]. The previous study by Kilpatrick and Lang[8], showed that SDF-1 expression is increased in the auditory nerve following cochlear injury.

In this study, ASCs treatment decreased expression of SDF-1 and CXCR4 on injury site especially in the first week after injury. These results indicate the presence of ASCs at injury site that causes the stem cells recruitment from another site of the body (ex,bone marrow, fat tissue) does not occur accordingly. The application of ASCs on nerve injury site may shorten the nerve healing processes by discarding the migration process. SDF-1 expression in the experiment group was re-increased at day 14 and 21 after injury, however. This condition may be due to the ASCs that have been applied are already differentiated to neural line cells. Different to the pattern of SDF1 expression, CXCR4 expression in the experimental group was not re-increase until the end of evaluation day. However, significantly decreased only occur at day 1, 7 and 14 after injury compared to control group.

Furthermore, CXCR4-SDF1 is also known as chemotactic axis in recruitment of other cells, including inflammatory cells[8]. The depression of this axis may also decrease the inflammation process. This condition generates positive impact on the functional outcome itself. In this study, better functional outcome was obtained in experimental group compared to control group significantly as shown in walking track analysis.

The result of present study is in accordance with Summa et al[4]. that uses fibrin conduit to treat sciatic nerve injury with fibrin conduit and ASC application. After 2 weeks, nerve regeneration is present on fibrin conduit. They suggest that ASCs may provide an effective cell population without the limitations of the donor-site morbidity and could be a clinically translatable route towards new methods to enhance peripheral nerve repair.

Conclusion

Underexpression of SDF1 and CXCR4 could shorten neural healing by discard the need of stem cells migration to the injury site. Better clinical outcome was obtained after application of Adipose-derived Mesenchymal Stem Cells (ASC) with freeze-dried amniotic membrane wrapping for the treatment of Sciatic nerve injury compared to control group.

References

1. Sterodimas A, de Faria J, Nicaretta B, Pitanguy I., Tissue engineering with adipose-derived stem cells (ADSCs), Current and future applications, J. Plast. Reconstr. Aesthet. Surg., 2010, 63(11): 1886-1892., DOI: 10.1016/j.bjps.2009.10.028.
2. Alborno PM, Delgado PJ, Forriol F, Maffulli N., Non-surgical therapies for peripheral nerve injury, Brit. Med. Bull., 2011, 100(1): 73-100. DOI: 10.1093/bmb/ldr005.
3. Pfister BJ, Gordon T, Loverde JR, Kochar AS, Mackinnon SE, Cullen K., Biomedical engineering strategies for peripheral nerve repair, surgical applications, state of the art, and future challenges, Crit. Rev. Biomed. Eng., 2011, 39(2): 81–124. PMID: 21488817.
4. Zuk P, Adipose-derived stem cells in tissue regeneration, a review, ISRN Stem Cells, 2013, 713959: 35 pages. DOI: 10.1155/2013/713959.
5. di Summa PG, Kinghamb PJ, Raffoul W, Wiberg M, Terenghi G, Kalbermatten DF., Adipose-derived stem cells enhance peripheral nerve regeneration, J. Plast. Reconstr. Aesthet. Surg., 2010, 63(9): 1544-1552. DOI 10.1016/j.bjps.2009.09.012.
6. Yu X, Chen D , Zhang Y , Wu X, Huang Z , Zhou H, Zhang Y, Zhang Z., Overexpression of CXCR4 in mesenchymal stem cells promotes migration,neuroprotection and angiogenesis in a rat model of stroke, J. Neurol. Sci., 2012, 316(1-2): 141-149. DOI: 10.1016/j.jns.2012.01.001.
7. Long MY, Li HH, Pen XZ, Huang MQ, Luo DY, Wang PS., Expression of chemokine receptor-4 in bonemarrow mesenchymal stem cells on experimental rat abdominal aortic aneurysms and the migration of bone marrow mesenchymal stem cells with stromal-derived factor-1, Kaohsiung J. Med. Sci., 2014, 30(5): 224-228. DOI: 10.1016/j.kjms.2013.12.005.
8. Kilpatrick L, Lang H., SDF-1 expression and stem cells in injured auditory nerve, Otolaryngolog, 2010, 143(2 suppl P104). DOI: 10.1016/j.otohns.2010.06.184.
9. Cui L, Jiang J, Wei L , Zhou X, Fraser JL, Snider BJ, Yu SP, Transplantation of embryonic stem cells improves nerve repair and functional recovery after severe sciatic nerve axotomy in rats, Stem Cells, 2008, 26(5): 1356–1365. DOI: 10.1634/stemcells.2007-0333.
10. Toyozawa S, Yamamoto Y, Ishida Y, Kondo T, Nakamura Y, Furukawa F., Immunohistochemical analysis of CXCR4 expression in fibrohistiocytic tumors, Acta Histochem. Cytochem., 2010, 43(2): 45–50. DOI: 10.1267/ahc.10003.
11. Li Q, Zhang A, Tao C, Li X, Jin P., The role of SDF-1-CXCR4/CXCR7 axis in biological behaviors of adipose tissue-derived mesenchymal stem cells in vitro, Biochem. Biophys. Res. Commun., 2013, 441(3): 675–680. DOI: 10.1016/j.bbrc.2013.10.071.

*****