COMPARATIVE CHARACTERISTICS OF THE STEM CELLS NUMBER IN THE STROMAL-VASCULAR FRACTION OF INFRAPATELLAR FAT PAD AND SUBCUTANEOUS FAT TISSUE

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Objectives. The use of infrapatellar fat pad adipose stem cells (IPFP-ASCs) show an age-independent proliferation and differentiation potential. In addition, the pronounced chondrogenic potential of IPFP-ASCs makes them promising candidates for research for use in other methods of regenerative therapy. Methods. A direct immunohistochemical study was carried out in serial paraffin sections of the stromal-vascular fraction (SVF) of the infrapatellar fat pad (IPFP) and subcutaneous tissue, using monoclonal antibodies. The minimum criteria established by the International Society for Cell Therapy to ensure the identity of MSCs use CD73, CD90 and CD105 as positive markers and CD34, CD31, CD45 as a negative. Results According to the results of histological, immunohistochemical, morphometric and statistical studies, it was found that in the SVF of IPFP and subcutaneous adipose tissue, the relative number of cells with the profile CD105+ CD73+ CD34+ CD31- CD45- in the standard field of view (x200) the SVF of IPFP was 1.58%, while the SVF of subcutaneous adipose tissue was 6.92 %, which was statistically significantly greater by 4.38 times (p < 0.05). Conclusion. The presence of a sufficient number of mesenchymal stromal cells in IPFP in combination with their topographic relationship with the structures of the joint determines the use of the stromal-vascular fraction of the IPFP for the treatment of diseases of the knee joint.

The level III of evidence.

What are the new findings:

1. The presence of mesenchymal stromal cells in infrapatellar fat pad was proven by immunohistochemical method – coexpression of stem cell markers CD105+ CD73+ CD34+ CD31- CD45-.

2. Statistically significantly more mesenchymal stromal cells in the stromal-vascular fraction of Hoff's fat pad compared to the stromal-vascular fraction of subcutaneous adipose tissue.
3. The anatomically close location and presence of the chondrogenic orientation surface markers on mesenchymal stromal cells of the Hoff’s fat pad can act as a pool of cells for the regeneration of cartilage tissue.

**Introduction.** In recent years, cell therapy, aiding in the regeneration of elements of the musculoskeletal system (such as cartilage, tendons, etc.), has begun to play an increasingly vital role in the treatment of orthopedic and traumatological patients [1-3]. Adipose stromal cells (ASC), utilized in cell therapy, are primarily isolated from subcutaneous adipose tissue by processing lipoaspirate, a technique described in the literature [4]. On the other hand, promising evidence exists for the use of infrapatellar fat pad adipose stem cells (IPFP-ASCs). Unlike other sources of mesenchymal stem cells (MSCs), IPFP-ASCs demonstrate age-independent proliferation and differentiation potential, whereas other sources tend to exhibit an inverse correlation between age and the manifestation of these properties. Moreover, the pronounced chondrogenic potential of IPFP-ASCs renders them promising candidates for further research in alternative regenerative therapy methods [5]. The infrapatellar fat pad (IPFP) is an intracapsular extrasynovial structure located in the anterior part of the knee joint, comprising approximately 20 cm³ of adipose tissue [6]. Positioned beneath the patella, the IPFP extends posteriorly to the infrapatellar plica (IPP), also known as the ligamentum mucosum. The IPP, along with the suprapatellar and mediopatellar plicas, constitutes one of the three plicas in the knee. These plicas are considered remnants of synovial plica resulting from incomplete resorption of synovial septa during embryological development of the joint [7]. It is recognized that the infrapatellar fat pad is closely associated with elements of the knee joint, functioning as a distinct organ. This association is evident not only through anatomical, physiological, and biomechanical connections but also through histological and biochemical links. However, the question regarding the regenerative potential of MSCs derived from the infrapatellar fat pad remains unresolved and necessitates further research.

The purpose of this study was to ascertain the presence and compare the relative abundance of cells exhibiting an immunohistochemical profile characteristic of adipose-
derived mesenchymal stem cells (ADMSCs) in selected samples of the stromal-vascular fraction obtained from the IPFP and subcutaneous fat tissue.

**Materials and methods.**

The research materials underwent review and approval by the bioethics committee at Zaporizhia State Medical and Pharmaceutical University (protocol No. 8, dated September 28, 2023). All patients participating in the study were provided with information about the surgical intervention plan and signed informed consent forms. For this study, 15 patients who underwent surgical or combined treatment for knee arthrosis were selected, and their data were analyzed. To mitigate the influence of potential confounding factors, we collected aspirates of subcutaneous adipose tissue from the anterior abdomen of 8 patients who showed no signs of obesity or comorbid metabolic diseases. Additionally, resected infrapatellar fat pads (IPFP) were obtained from 7 patients undergoing therapeutic and diagnostic arthroscopy, all of whom lacked signs of obesity. The average age of the patients was 44.0 ± 3.8 years, with a body mass index of 20.1 ± 1.6 kg/m². A 20 ml syringe equipped with a 3 mm diameter almond-shaped cannula was utilized to perform microliposuction under negative pressure. Prior to this, the donor site (front of the abdominal cavity) underwent treatment and anesthesia in accordance with surgical intervention principles. Following the separation of the required mesenchymal cell fraction through grinding and centrifugal separation, resuspension was conducted using autologous concentrated plasma.

The preparation of stromal-vascular fraction (SVF) from the IPFP involved similar steps, albeit with a lesser amount of final material compared to abdominal fat. Collection from the IPFP was performed using a shaver that mechanically minced the tissue.

**Morphological study.** The obtained stromal-vascular fraction from both the infrapatellar fat pad and subcutaneous tissue underwent histological processing. A standard procedure was employed for sectioning the samples, followed by staining with hematoxylin and eosin.

Microscopy was conducted using a Scope A1 microscope manufactured by "Carl Zeiss" (Germany), equipped with a Progres Gryphax Jenoptik 60N-C1"1.0x426114 camera (Germany) connected to a personal computer. Microscopic analysis was
performed utilizing the digital analysis program Progres Gryphax 1.1.4.2 developed by Jenoptik Optical System (Germany).

A direct immunohistochemical study was conducted on serial paraffin sections of the stromal-vascular fraction obtained from the infrapatellar fat pad (IPFP) (n=10) and subcutaneous tissue (n=5), utilizing monoclonal antibodies. Following deparaffinization and rehydration of the sections, high-temperature antigen unmasking was carried out by heating in a water bath in Tris-EDTA buffer (pH = 9.0). Endogenous peroxidase activity was then inhibited using a 3% hydrogen peroxide solution, followed by the application of blocking serum. Incubation with primary antibodies was conducted according to the manufacturer's instructions. The immunohistochemical reaction was visualized using the DAKO EnVision+detection system with diaminobenzidine (DAKO, USA). Finally, the sections were counterstained with Mayer's hematoxylin and embedded in Canada balsam.

As of now, there is no universally accepted specific marker for MSCs. Numerous surface markers have been identified, and there are variations in the types of markers expressed in MSCs obtained from different sources. Hence, the parallel determination of multiple markers is employed to identify MSC populations in multicellular cultures. Both positive and negative markers are identified in this process. The challenge in marker selection is further compounded by the dynamic nature of MSCs and changes in the expression of various markers during isolation and cultivation [8]. The International Society for Cell Therapy has established minimum criteria for ensuring the identity of MSCs. These criteria include using CD73, CD90, and CD105 as positive markers, while CD34, CD31, and CD45 are considered negative markers [9]. It's worth noting that adipose tissue MSCs are typically classified as CD34+, despite the well-known phenomenon of CD34 expression loss in culture [10].

After analyzing literature data to identify cells with an immunohistochemical profile characteristic of adipose-derived mesenchymal stem cells, we utilized the following markers: Monoclonal antibody Mo a-Hu CD31 Endothelial Cell Marker Ab-1, clone JC/70A (manufactured by "DAKO", Denmark). CD 105 Endoglin, clone EP274 (manufactured by "Bio SB", USA). CD73 Ecto-5'-nucleotidase (NT5E), clone RM431 (manufactured by "Bio SB", USA). CD 34, clone QBEND/10 (manufactured by "Thermo
scientific”, USA). CD 45 Leucocyte common antigen, clone PD7/26/16 + 2B11 (manufactured by "Thermo scientific", USA).

The results in each case were evaluated in 5 standardized fields of view using the Scope A1 microscope manufactured by "Carl Zeiss" (Germany), equipped with a Progres Gryphax Jenoptik 60N-C1"1.0x426114 camera (Germany) at a magnification of × 200 (eyepiece × 10, objective × 20). Digital copies of the optical image of the sections of microscopic preparations were obtained, and the relative number of cells (%) exhibiting the immunophenotype CD 105+, CD73+, CD 34+, CD 31-, CD 45- was calculated in each standard field of view at x200 magnification.

**Statistical processing of research results.** Statistical processing of the obtained results was conducted using a personal computer, employing the statistical package Statistica® for Windows 13.0 (StatSoft Inc., license № J0PZ804I382130ARCN10-J). Non-parametric criteria of statistical analysis were utilized.

To assess the hypothesis regarding the normality of the distribution of the studied indicators, the Shapiro-Wilk test was employed. The results indicated non-normally distributed data in the comparison groups. Therefore, the median and interquartile range (Me [Q1; Q3]) were reported, and the appropriate statistical test for comparison between groups - the non-parametric Mann-Whitney U-test for independent samples - was applied.

In all types of analysis, differences were considered significant at p < 0.05.

**Obtained results.**

According to the results of histological, immunohistochemical, morphometric, and statistical studies (fig.1), it was observed that in the stromal-vascular fraction of the IPFP and subcutaneous adipose tissue, the relative number of cells with the CD105+ CD73+ CD34+ CD31- CD45- profile in the standard field of view (x200) was 1.58% (1.11%; 3.10%) in the SVF of IPFP and 6.92% (5.38%; 10.71%) in the SVF of subcutaneous adipose tissue. This indicates that the relative number of these cells in the SVF of subcutaneous adipose tissue was 4.38 times higher. However, this difference was not statistically significant (p < 0.05).
Figure 1. Comparison of the relative number of cells with the CD105+ CD73+ CD34+ CD31- CD45- profile in a standard field of view (x200) in the stromal-vascular fraction of Hoff's fat pad (SVF Hoffa) and abdomen subcutaneous adipose tissue (SVF Abd).

The results of the study revealed the presence of a relative number of CD105+ cells in the SVF of IPFP 30.58 (17.84; 36.92) %, SVF of subcutaneous adipose tissue – 38.29 (25.89; 46.61) % (table 1, fig.2, fig.3), which is 1.25 times more, no statistically significant difference was found (p > 0.05).

The relative number of CD31+ cells in the SVF of IPFP was 35.90 (21.79; 49.50) %, the SVF of subcutaneous adipose tissue was 51.27 (44.44; 64.47) % (table 1, fig.2, fig.3), which is 1.43 times more, no statistically significant difference was found (p > 0.05).

The relative number of CD73+ cells in the SVF of IPFP was determined at the level of 63.39 (47.75; 75.10) %, SVF of subcutaneous adipose tissue – 38.00 (30.43; 48.11) % (table 1, fig.2, fig.3), which is 1.7 times less, a statistically significant difference (p < 0.05).

The relative number of CD34+ cells in the SVF of IPFP was 48.96 (44.87; 56.71) %, the SVF of subcutaneous adipose tissue was 89.77 (81.36; 93.88) % (table 1, fig.2,
fig.3), which is 1.83 times more, but there is no statistically significant difference (p > 0.05).

The relative number of CD45+ cells in the SVF of IPFP was determined at the level of 32.60 (25.90; 41.11) %, SVF of subcutaneous fat tissue – 19.90 (11.22; 24.44) % (table 1, fig.2, fig.3), which is 1.64 times less, the statistical difference is significant (p < 0.05).

Table 1. Relative number of cells expressing CD105+, CD31+, CD73+, CD34+, CD45+ and cells with immunohistochemical profile CD105+ CD73+ CD34+ CD31- CD45-.

<table>
<thead>
<tr>
<th>The material studied</th>
<th>CD105</th>
<th>CD31</th>
<th>CD34</th>
<th>CD73</th>
<th>CD45</th>
<th>CD105+ CD73+ CD34+ CD31- CD45-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stromal vascular fraction of infrapatellar fat pad (SVF Hoffa)</td>
<td>30.58</td>
<td>35.90</td>
<td>49.05</td>
<td>63.39</td>
<td>32.60</td>
<td>1.58 (1.11; 3.10)</td>
</tr>
<tr>
<td></td>
<td>(17.84; 36.92)</td>
<td>(21.79; 49.50)</td>
<td>(38.35; 58.03)</td>
<td>(47.75; 75.10)</td>
<td>(25.90; 41.11)</td>
<td></td>
</tr>
<tr>
<td>Stromal vascular fraction of abdomen subcutaneous adipose tissue (SVF Abd)</td>
<td>38.29</td>
<td>51.27</td>
<td>88.00</td>
<td>38.00*</td>
<td>19.90*</td>
<td>6.92* (5.38; 10.71)</td>
</tr>
<tr>
<td></td>
<td>(25.89; 46.61)</td>
<td>(44.44; 64.47)</td>
<td>(83.70; 93.42)</td>
<td>(30.43; 48.11)</td>
<td>(11.22; 24.44)</td>
<td></td>
</tr>
</tbody>
</table>

*- statistically significant difference was found when comparing the obtained results of CD+ cells in the comparison groups (p < 0.05).

The obtained data indicate the presence of adipose stem mesenchymal cells with an immunohistochemical profile of CD105+ CD73+ CD34+ CD31- CD45- in both the SVF obtained from Hoff's fat pad and subcutaneous adipose tissue. The difference between the number of these cells in our study was statistically significant, although it was 4.38 times higher in the SVF of subcutaneous adipose tissue. If we analyze the obtained results according to the expression of each marker, it is found that the relative number of positively stained CD105+ and CD31+ cells is not significantly greater in the SVF of subcutaneous adipose tissue. Similarly, CD34+ expression is not statistically significantly greater in the SVF of subcutaneous adipose tissue. However, the relative number of positively stained CD73+ and CD45+ cells is significantly greater in the SVF of the IPFP.
This can be attributed to a greater diversity of cellular composition in the IPFP and a higher number of inflammatory cells due to various traumatic and non-traumatic changes in the knee joint of donors. Of course, the limitations of our study should be taken into account. Firstly, the sample size was limited, with 10 tissue samples of the stromal-vascular fraction of the IPFP and 5 tissue samples of subcutaneous tissue. Additionally, the use of the non-parametric Mann-Whitney test has its drawbacks. When applied, it removes the requirement of normality of distribution and the requirement of equality of variance. Therefore, this criterion is less stringent than its parametric counterpart - the Student's t-test for independent samples.

Figure 2. Expression of CD31, CD105, CD73, CD45, CD34 in stromal-vascular fraction of infrapatellar fat pad in the standard field of view (Coll. X200)

Figure 3. Expression of CD31, CD105, CD73, CD45, CD34 in the stromal-vascular fraction of subcutaneous adipose tissue in the standard field of view (Coll. X200)
Discussion.

Over the past decade, numerous studies have been conducted to evaluate the immunophenotype and differentiation potential of adipose derived stromal cells (ADSCs) from the infrapatellar fat pad [11-13]. However, there have been relatively fewer studies comparing these cells with MSCs from other anatomical locations. According to Carvalho et al. (2014), freshly isolated SVF cells from subcutaneous adipose tissue exhibit significantly increased expression of the endothelial cell marker CD31 (p = 0.02) compared to IPFP-ASCs. Flow cytometry analysis of passage 2 enzyme-derived ASCs indicates that the immunophenotype does not significantly differ between the corresponding infrapatellar fat pad depot and subcutaneous donors with osteoarthritis in terms of the expression of hematopoietic (CD34, CD45) and stromal (CD29, CD44, CD73, CD90, CD105) antigens [14]. Also, in a study by SONG Sai-sai et al., 2020, no significant difference was found in stem cell expression of surface protein markers CD34 and CD31 between human subcutaneous adipose stem cells (SC-ASCs) and IPFP-ASCs, moreover, according to this study, the proliferation and chondrogenic potential of IPFPs Human-ASCs in vitro and the treatment effect of rat osteoarthritis in vivo were better than those of SC -ASCs [15]. Tangchitphisut P et al., 2016 also demonstrated in their study
that IPFP-ASC and SC-ASC have similar cell surface profiles as detected by flow
cytometry, including positive expression of CD73, CD90 and CD105 and negative
expression of CD34 and CD45. It has been demonstrated that infrapatellar fat pad-derived
adipose stem cells (IPFP-ASCs) exhibit an advantage in osteogenic and chondrogenic
differentiation over subcutaneous adipose-derived stem cells (SC-ASCs). This was
assessed by the detection of SOX-9 (a chondrogenic transcription factor) and RUNX-2 (a
transcription factor expressed in MSCs) [5, 16].

Some authors claim the presence of specific surface markers of stem cells (SOX9,
CD 44, CD49c, CD166), which indicate their potential for differentiation into the
chondrogenic side [17, 18]. Due to their strong proliferative and chondrogenic
differentiation abilities, such cells may contribute a lot to cartilage regeneration and repair
in osteoarthritis [17]. Literary sources confirm a certain uniqueness of the receptors, so it
has been confirmed to play a decisive role of SOX9 gene in the chondrogenic
differentiation stem cells, which may promote stem cells cartilage compensation in OA.

Compared with human MSCs, the mRNA level of SOX9 in such stem cells increased by
1.5 times [19]. Similar properties have been observed in other genes listed above; the only
question remains to find the most profitable source of this type of cell. Qualitatively, this
type of cells with the presented markers is also found in the infrapatellar fat pad, which,
taking into account its anatomical location, can play a positive role in the regenerative
processes of cartilage, but the question of the quantitative characteristics of these cells
and their possibilities for use in clinical practice remains open. Stem cell therapy utilizing
cartilage stem-progenitor cells, a subset of stem-like cells characterized by their enhanced
proliferation, chemotaxis capabilities, and significant potential to differentiate into
chondrocytes, holds promise as a meaningful approach for treating osteoarthritis.

Several studies investigating the potential of stem cells isolated from Hoff's
infrapatellar fat pad have concluded that the anatomical region where the cells are isolated
influences the characteristics of ASCs, and the level of chondrogenic differentiation
potential of IPFP-ASCs is higher due to the close contact of IPFPs with the synovial
membrane and fluid, suggesting that IPFP can be considered as a high-quality resource
for restorative therapy.
Conclusions:

1. Adipose stem mesenchymal cells with an immunohistochemical profile of CD105+ CD73+ CD34+ CD31- CD45- are present in SVF and subcutaneous adipose tissue. The number of these cells in SVF of subcutaneous adipose tissue is statistically significantly greater by 4.38 times than in Hoff’s SVF (p < 0.05).

2. The relative number of CD31+, CD105+, CD34+ cells in SVF of Hoff fat body and SVF of subcutaneous adipose tissue had no statistically significant differences (p > 0.05), while the relative number of CD73+ CD45+ cells was 1.7 and 1.4 times, accordingly, statistically significantly more in the SVF of Hoff's adipose body compared to the SVF of subcutaneous adipose tissue.

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: