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Original Article

Adipokine concentrations in lipoaspirates may have a role in wound healing

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ABSTRACT

Objectives: In addition to its use as a volume filler, fat grafting may have a potential role in wound healing based on the concentration of growth factors in the lipoaspirate. In this study, we compare the quantitative and qualitative concentration of the various growth factors and adipokines using the Shippert or the Coleman techniques to prepare the lipoaspirate. **Methods:** We measured leptin, adiponectin and the growth factors, i.e., acidic fibroblast growth factor (aFGF), basic FGF (bFGF), keratinocyte growth factor (KGF), bone morphogenetic protein-2 (BMP-2) and vascular endothelial growth factor (VEGF) by ELISA in solid and liquid fractions obtained with both techniques in human fat obtained with Coleman technique and Shippert technique. **Results:** All of these peptides, except BMP-2, were detected in relevant quantities in the solid fraction. The Coleman but not the Shippert technique resulted in statistically higher adiponectin concentrations in the solid tissue fraction. The other four growth factors occurred in significantly higher concentrations in the solid fractions compared to the liquid fractions, independent of the processing technique. **Conclusion:** In summary, we demonstrated that KGF, aFGF, bFGF and VEGF, as well as leptin and adiponectin, are contained in fat suspensions obtained by liposuction and in the supernatant. Only the concentration of adiponectin was in the range reported to contribute to wound healing.

KEY WORDS

Adipokines, fat, lipofilling

INTRODUCTION

Fat grafting has become a popular technique with an almost unmanageable range of applications. The regenerative capacity of fat grafts has been demonstrated in numerous experimental and some clinical studies.[1,2] Other reports suggested that the enrichment of fat grafts with stroma vascular cells (SVCs) or adipose-derived stem cells (ASCs) isolated from adipose

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tissue leads to better graft performance compared to fat grafts alone, for example, in radiation-induced ulcer healing.[2] However, focussing solely on ASC and SVC in autologous fat transplantation might underestimate the capacity of adipose tissue because adipose cells secrete various autocrine and paracrine bioactive peptides. In addition to their endocrine functions, the classical adipokines such as leptin[3] and adiponectin[4] may exert wound healing properties. Leptin, a 16-kDa protein, has been demonstrated to have positive effects in wound healing.[5,6] Local leptin synthesis and secretion are strongly upregulated in wounded skin, and leptin-deficient animals have an impaired wound healing response. Leptin exerts a mitogenic response in keratinocytes and endothelial cells *in vitro* that may be responsible for the proliferative processes induced by leptin in the skin *in vivo*. A potential role of adiponectin in the process of wound healing has recently been suggested.[7,8] Y. Fu *et al*. reported that adiponectin acts locally to maintain the adipocyte size and mass and promotes the differentiation of pre-adipocytes into adipocytes.[9] These properties may affect fat graft functioning. Typical growth factors involved in wound healing belong to the fibroblast growth factor (FGF) family.[10] The tyrosine kinase receptor activators basic FGFs (bFGFs), and acidic FGF (aFGF) are mitogenic on keratinocytes, dermal fibroblasts and vascular cells and accelerate wound healing.[11] bFGF also enhances the migration and proliferation of endothelial cells[10] and is a potent mitogenic factor for pre-adipocytes.[10] The keratinocyte growth factor (KGF), also described as FGF-7, is actively involved in the epithelialisation phase of wound healing and is considered a major growth factor in wound healing.[12] Vascular endothelial growth factor (VEGF) is a key regulator of physiological angiogenesis during embryogenesis, skeletal growth and reproductive functions and influences endothelial proliferation, migration, and viability.[13]

A variety of techniques allows harvesting adipose tissue in small and large quantities. The widely used Coleman technique[14] applies centrifugation, whereas the Shippert protocol[15] uses a filter to separate fat from tumescence solution. No data are available concerning the quantitative and qualitative differences in the transfer of growth factors using both techniques. We measured leptin, adiponectin and the growth factors, i.e., aFGF, bFGF, KGF and VEGF in harvested fat and fluid fractions of fat grafts obtained by the Coleman protocol and the Shippert technique.

MATERIALS AND METHODS

Patients

Seven healthy participants over the age of 18, without noticeable inflammation of the subcutaneous fat and undergoing elective surgery with autologous fat transfer or liposuction, were recruited for the study. Before enrolment, efforts were made to ensure that no confounding active systemic inflammatory processes were present. The analysis of fat grafts harvested with different techniques was approved by the local Institutional Review Board, and all patients provided written informed consent for the use of otherwise discarded tissue.

Lipoaspirates were harvested from the trunk according to the Coleman or Shippert protocol.[14,15] The donor sites were infiltrated with Klein solution.[16] Harvesting was carried out using a blunt-tipped 3 cc needle (Coleman number 1, Byron Medical, Mentor, Santa Barbara, CA, USA) and a 10 cc syringe or the Tissu Trans system (Shippert Medical Technology Corp, Centennial, CO, USA; Asclepios Medizintechnik, Gutach, Germany). In the Coleman protocol, solid and liquid fractions were separated by centrifugation (3 min, 920 g). The oily fraction on top was discarded. In the Shippert protocol, the solid fraction was initially separated by a filter system during the liposuction procedure and was then sedimented in a special rack for 15 min without centrifugation. Aliquots of all of the solid and liquid samples were labelled with consecutive numbers so that the person who isolated the proteins and measured the total protein content and adipokine concentrations was blinded with regard to the nature of the samples. The aliquots were transferred immediately to the laboratory for protein isolation.

Protein isolation

From the solid tissue fractions, we isolated proteins by mixing 200–250 mg of tissue with five times the amount of homogenisation buffer (phosphate-buffered saline +2% protease inhibitor mix, P8340 Sigma-Aldrich, Hamburg, Germany) and 4-6 ceramic beads with a diameter of 1.4 mm. The mixture was homogenised for 2 × 20 s at 5.500 rpm under constant cooling (Precelly Homogenisator 24, Peqlab, Erlangen, Germany). The homogenate was then centrifuged for 5 min at 6000 g, and the clear supernatant was transferred to a new tube. The liquid fractions of both lipoaspirate harvesting techniques were centrifuged as described and transferred

to new tubes. The total protein concentration in each sample was determined by the BCA method according to the protocol of the manufacturer (Micro BCA Protein Assay Kit, Thermo Fisher Scientific, Rockford, IL, USA).

Adipokine and growth factor measurements

We measured six adipokines and growth factors by ELISA according to the protocols supplied by each ELISA kit (leptin: RD191001100 BioVendor, Heidelberg, Germany; total adiponectin: DRP300; VEGF: DVE00; KGF: DKG00; bFGF: DFB50; aFGF: DFA00B Quantikine ELISA, R and D Systems, Minneapolis, MN, USA). The intra- and interassay coefficients of variation of all kits were in the range of 3.3 to 8.6%. All measurements were performed in the Infinite 200 Plate Reader (Tecan, Crailsheim, Germany). The concentrations of adipokines and growth factors in each sample were normalised to the total protein content.

Statistical analysis

Data distribution for measurements of protein content and all six adipokines and growth factors were tested by Kolmogorov–Smirnov Test (this and all other tests described were performed using the IBM SPSS Statistics 22 software package, Armonc, NY, USA). Due to the small sample size and unequal distribution of the data, median and range (minimum-maximum) are shown in Figures 1-3 and Table 1. Non-parametric tests were used to compare groups as follows: Mann-Whitney test was used for the comparison of two sample sets [Figure 1, right panel; Figure 3, all panels]; Kruskal–Wallis test was used for comparison of more than two sample sets [Figure 1, left panel; Figure 2, all sample sets]. In the case of a significant test result with Kruskal–Wallis test, Dunn's Multiple Comparison test was employed *post hoc* to identify pair-wise differences. Group differences with a $P < 0.05$ were considered statistically significant. Significant *P* values are stated as complete numbers in

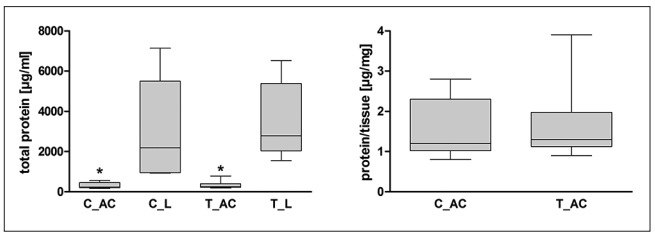

Figure 1: We compared total protein concentrations (left panel) in the liquid fractions (L) with homogenates obtained from solid fractions (AC). With both methods of lipoaspirate processing (Coleman method – C; Shippert technique – T), total protein concentration was higher in the liquid compared to the solid fractions ($P = 0.005$ with the Coleman technique, $P = 0.009$ with the Coleman technique). In comparison, however, but both techniques resulted in identical protein yield within each fraction. When normalizing total protein by the amount of solid tissue used for protein isolation (right panel), we observed no difference in protein content per mg of wet tissue with the Coleman and Shippert techniques. All data are presented as median and range (minimum-maximum). Data comparison in the left panel (total protein concentration) by Kruskal–Wallis test followed by Dunn's Multiple Comparison test to identify pair-wise differences between liquid and solid fractions with each preparation technique (*$P < 0.05$). Data comparison in the right panel (protein/tissue) by Mann–Whitney test

Table 1: The mode of cellular action and the concentrations of the wound healing-related proteins such as adiponectin, leptin, keratinocyte growth factor, acidic fibroblast growth factor, basic fibroblast growth factor and vascular endothelial growth factor in *in vivo* wound healing studies and in the fat fractions of our study

Protein	*Target cell and mode of action*	*Concentrations used in in vivo animal experiments*	*Concentrations used in human studies*	*Concentrations in the fat fraction of lipoaspirate*
Adiponectin	Keratinocyte proliferation	3.2 µg/cm²/day[7]	ND	1.01 (0.82-2.07) µg/g
	Keratinocyte migration	8.8 µg/cm²/day[8]		
Leptin	Keratinocyte proliferation	3 µg/cm²/day[6]	ND	6.12 (4.36-8.04) ng/g
	Collagen synthesis	approximately 3 µg/cm²[5]		
	Angiogenesis			
KGF	Keratinocyte proliferation	0.08 µg/cm²[17]	ND	0.44 (0.36-0.84) ng/g
		1 µg/cm²[18]		
aFGF	Fibroblast proliferation	0.1-10 µg/cm²/twice[19]	5 µg/cm²/day[22]	8.97 (6.06-21.42) ng/g
	Keratinocyte proliferation	3 µg/cm²[20]		
	Angiogenesis	6 µg/cm²/twice/week[21]		
bFGF	Keratinocyte proliferation	1 µg/cm²/day[23]	150 AU/cm²/day[11]	1.04 (0.86-1.62) ng/g
	Fibroblast proliferation	1 µg/cm²[18]	7 µg/cm²/week[27]	
	Endothelial cell proliferation	5.5 µg/cm²[24]	7.7 µg/cm²/day[28]	
		7 µg/cm²[25]	14 µg/cm²/week[27]	
		14 µg/cm²[25]		
		44 µg/cm²[26]		
VEGF	Angiogenesis	0.7 µg/cm²/day[29]	72 µg/cm²/3 × per week[31]	0.21 (0.17-0.33) ng/g
		77 µg/cm²/day[30]		

The reported effective concentrations are single applications unless otherwise stated (data in the last column are the median and interquartile range from the combined Shippert and Coleman data set). ND: None detected, VEGF: Vascular endothelial growth factor, bFGF: Basic fibroblast growth factor, KGF: Keratinocyte growth factor

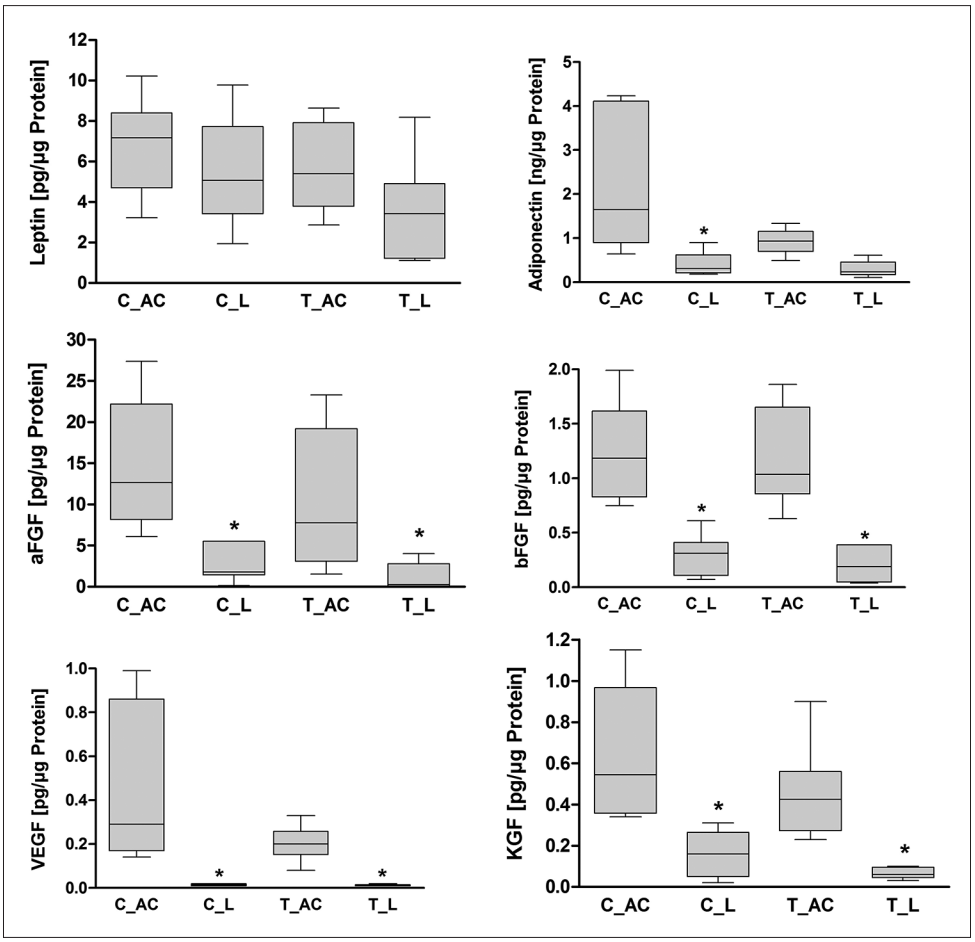

Figure 2: We measured Leptin, Adiponectin, acidic fibroblast growth factor, basic fibroblast growth factor, vascular endothelial growth factor and keratinocyte growth factor concentrations in solid (AC) and liquid (L) fractions by specific ELISAs. All results were normalised by the total protein content of the sample. Whereas we did not detect a quantitative difference between the Coleman (C) and the Shippert (T) processing procedure, at least four growth factors are much higher concentrated in the solid fractions. All data are presented as median and range (minimum-maximum). Data comparison by Kruskal–Wallis test followed by Dunn's multiple comparison test to identify pair-wise differences between liquid and solid fractions with each preparation technique (*$P < 0.05$ liquid vs. solid fraction of the same processing technique, for exact P values see text)

the text and are marked by a symbol (*) in the figures (see legends).

RESULTS

Total protein was measured in the liquid and solid fractions of the lipoaspirates [Figure 1]. The protein concentrations in the liquid fractions harvested by the Coleman or Shippert techniques were similar [Figure 1, left panel]. The protein concentrations in the solid fractions harvested by the Coleman or Shippert techniques were also similar albeit with a much larger variation due to the 10x fold larger protein content in solid fractions [Figure 1, left panel]. When normalising the total protein content of solid fractions to the amount of homogenated tissue [Figure 1, right panel], the overall yield with both techniques ranged between 0.8 mg/g minimum and 3.9 mg/g maximum (protein/wet tissue weight), with a median of 1.3 mg/g. The Coleman and Shippert techniques were identical in protein yield.

We observed a slightly larger leptin content in the solid fractions from both harvesting techniques, but the difference to the liquid fractions was not statistically significant [Figure 2]. The same trend was visible for adiponectin, and the difference between the solid and liquid fractions prepared by the Coleman technique was statistically significant ($P = 0.038$). All other growth factors measured occurred in significantly higher concentrations in the solid, cell-rich fractions compared to the liquid fractions (aFGF: $P = 0.026$ Coleman; $P = 0.031$ Shippert; bFGF: $P = 0.008$ Coleman; $P = 0.003$ Shippert; VEGF: $P = 0.001$ Coleman; $P = 0.0009$ Shippert; KGF: $P = 0.021$ Coleman; $P = 0.013$ Shippert). The more condensed nature of the Coleman preparations, which is due to the centrifugation step, resulted in slightly increased peptide

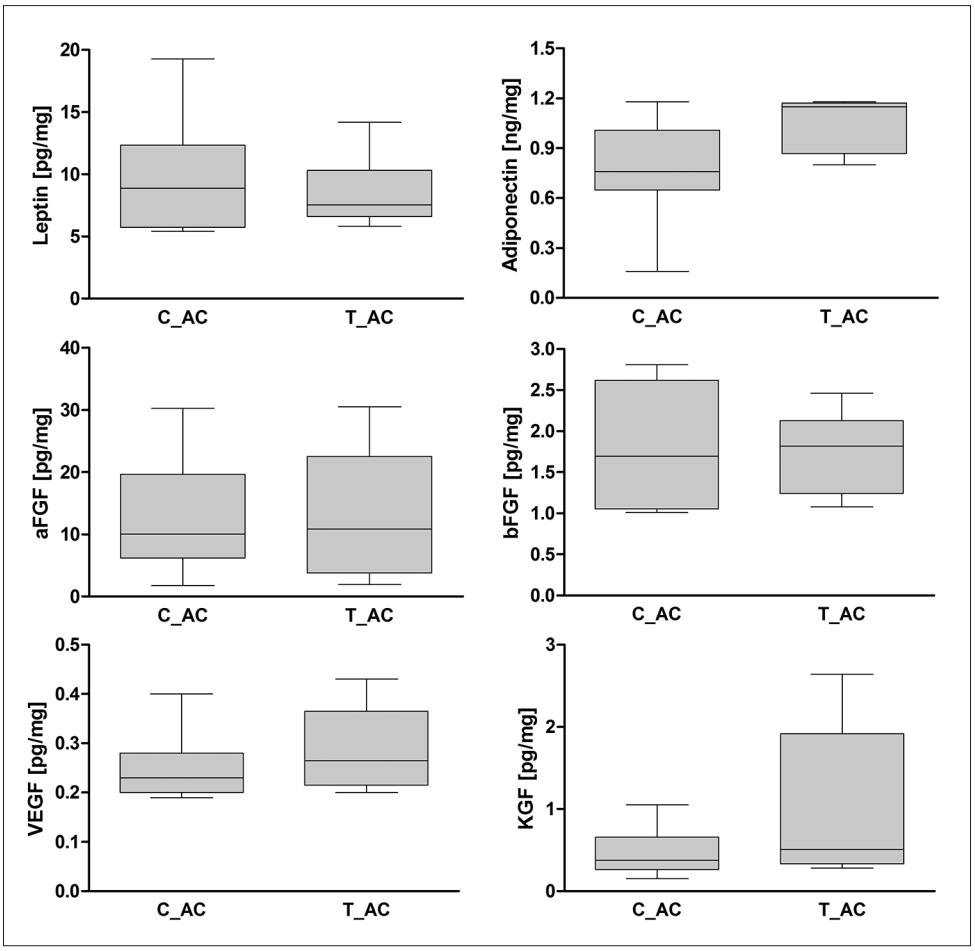

Figure 3: Normalisation of leptin, adiponectin, acidic fibroblast growth factor, basic fibroblast growth factor, vascular endothelial growth factor and keratinocyte growth factor concentrations by the amount of wet tissue used for protein isolation. The Coleman and the Shippert procedures yielded identical results in growth factor content of the solid adipose tissue fractions. All data are presented as median and range (minimum-maximum); data comparison by Mann–Whitney test

concentrations compared to the Shippert preparations without reaching statistical significance [Figure 2].

Finally, we normalised the solid, cell-rich fractions of the Coleman and the Shippert preparations by the wet tissue weight used for each protein isolation [Figure 3]. After normalisation, no differences were found for leptin, adiponectin, aFGF, bFGF, VEGF and KGF content in the solid fractions prepared by either technique. Finally, our ELISA data confirmed the well-known fact that adiponectin is one of the most abundant peptides in adipocytes and occurs in 100-1000x fold larger amounts compared to all of the other analysed growth factors and adipokines.

DISCUSSION

Various techniques of autologous fat transplantation are currently in clinical use. Most studies comparing different techniques for specific indications have focussed on volume persistence and cell viability. The paracrine activity of adipokines and growth factors secreted by adipose cells has not been specifically studied. However, paracrine activity might have an influence on the proliferation of transplanted adipocytes or skin-derived cells in wound healing indications and scar revisions. Here, we demonstrate that harvested fat cell suspensions contain significant amounts of wound healing-relevant peptides such as KGF, bFGF aFGF and VEGF. We also demonstrate that adipokines, such as leptin and adiponectin, which have recently been shown to enhance wound healing,[32] are found in significant amounts in liposuctioned solid and liquid fractions of the fat graft.

Pallua *et al*. previously presented experimental work on the secreted growth factors in harvested and cultured fat.[33] The authors analysed the growth factors bFGF, VEGF, platelet-derived growth factor-BB and insulin-like growth factor-1, which are known to improve the growth and persistence of adipose tissue. The authors concluded that

these factors may support autologous fat transplantation in a paracrine fashion. They also reported that separation by centrifugation did not reduce the quantity of growth factors in the purified fat used for transplantation.[33] These findings are in line with our findings of condensed fat grafts (the Coleman technique). We have extended these findings to fat grafts obtained by the Shippert technique and found only minimal differences in the peptide concentrations compared to the condensed fat grafts. In addition to the above-mentioned work, we focussed on wound healing-related peptides as well as adipokines and thus broadened the spectrum of analysed mediators transplanted with lipografts. We found significant concentrations of these peptides not only in the fat but also in the fluid fraction. The fluid component is normally discarded but may provide an immediate source of paracrine-acting peptides in indications such as scar remodelling and wound healing. The cell-free fluid fraction may also be a source for allogenic use. While culturing adipose cell suspension may allow for a two-stage fat transplantation approach, restrictive governmental regulations and the ease of harvesting fat favour a simultaneous harvesting and transplantation approach. In contrast to fat cells, the fluid fraction may also be cryopreserved without a significant loss of biological activity. We assume that the fluid fraction may have an even greater biological potential if the degradation by proteases could be diminished by specific protease inhibitors. Further work will have to elaborate on the biological activity of the fluid fraction, as the half-life of growth factors is relatively short.[34,35] The half-life of VEGF, for example, is only approximately 50 min.[35] This point is important when planning the use of platelet-rich plasma (PRP) in clinical settings.

The clinically observed improvements after autologous fat transplantation in wound healing[36,30] and fibrosis of the skin[2] might be explained by the release of stored growth factors and wound healing-related peptides such as KGF, aFGF, bFGF or VEGF, all of which were detected in this study. Similarly, the use of PRP with its content of growth factors, for example, EGF, VEGF and bFGF, has been described to improve fat grafting outcomes,[36,37] as well as to optimise wound healing.[38]

In vivo experiments on KGF1 utilising incisional and excisional models have demonstrated the beneficial effect on epithelialisation with concentrations as low as 80 ng KGF1/cm^2.[28] In clinical trials, bFGF was applied at doses of 7 μg per cm^2 weekly[27] or daily[28] onto cutaneous wounds. *In vivo* experiments have shown that 3 μg of aFGF per cm^2 of wound surface accelerated wound healing in diabetic mice. In patients, 5 μg of aFGF per cm^2 was sufficient to accelerate wound healing.[28] In a clinical trial, topical applied VEGF (72 μg/cm^2 VEGF165 or rhVEGF, telbermin) accelerated wound healing in diabetic neuropathic foot ulcers.[31] In the fat and fluid fractions of fat grafts, we detected only a moderate amount of VEGF (approximately 0.2 ng/g fat). Superior, but still quite low VEGF amounts, were also found in the PRP preparations. A comparison of seven commercial separation systems revealed a VEGF content at best of 47.0 ng/ml.[39]

Of note, we did not detect any stored bone morphogenetic protein-2 (BMP-2) in the fat fraction or the fluid fraction of the fat graft. BMP-2 is considered to act as an important osteogenic inducer. We hypothesised that BMP-2 is released in adipose tissue because calcifications in fat tissue have been reported in inflamed fat tissue and after fat necrosis. We cannot exclude this possibility from our findings due to the detection limit of the commercial ELSIA kit. Another possible explanation might be that BMP-2 is not readily stored in adipose cells but is rather induced *de novo* after an appropriate stimulus. Similarly, we have not addressed the intracellular origin of the additional above-mentioned growth factors.

Of great interest was the analysis of adipokines such as adiponectin and leptin. Recent studies have demonstrated the positive regulatory effect of adiponectin in wound healing.[7,8] Topical adiponectin promoted wound closure in full-thickness wounds in diabetic mice at a concentration of 2.5 μg per 6 mm punch wound per day.[8] A similar amount of adiponectin is stored in approximately two grams of fat. The administration of leptin promoted wound closure in different wound types in rodents.[6] Leptin also enhanced the gene expression of type I and III collagen, and the synthesis of collagen in wound tissue was stimulated by topically applied leptin, thus resulting in accelerated wound healing.[40] Taken together, these findings not only underline the beneficial effect of fat transplantation for skin homeostasis at the site of implantation but also suggest the therapeutic effect of fibrotic skin at the site of fat transplantation. Based on our data, the exclusive injection of the liquid fraction from lipoaspirates might also be a viable option in these conditions.

In the present study, we did not find significant differences in the content of growth factors in fat

grafts processed with the Coleman or the Shippert technique. There was a slightly higher concentration in the purified solid fat tissue after centrifugation with the Coleman technique compared to the fat tissue fraction after sedimentation for a period of minutes. The differences between the fat harvesting techniques compared in this work were not statistically significant and did not support the recommendation of performing centrifugation in general. The drawbacks associated with centrifugation have been discussed previously.[41] In a clinical setting, the significant time requirement allows the centrifugation of small volume grafts, which is greatly exceeded by the large volume grafts obtained in operations. This finding has been previously described in the literature.[33] In a previous study, we compared the cell viability of fat grafts processed with using the Coleman or the Shippert technique. We found a higher viability in the Coleman grafts, but the fat grafts were condensed due to the centrifugation step. The factor of condensation outbalanced the level of higher viability.[41]

In summary, we demonstrated that KGF, aFGF, bFGF and VEGF, as well as the adipokines such as leptin and adiponectin, are contained in fat suspensions obtained by liposuction [Table 1]. The sum of the concentrations of single growth factors and adipokines in fat suspensions may possibly affect wound healing, with adiponectin being the only protein at a concentration reported to enhance wound healing in experimental studies [Table 1].

CONCLUSION

Autologous fat grafting may not only exert a volume effect, such as that in breast reconstruction and breast augmentation but may potentially be considered a biological adjunct in wound healing, for example, for difficult to heal wounds. The biological potency of these grafts needs to be proven in randomised controlled clinical studies, as the experimental data from this study suggest a beneficial role.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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