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Nonexpanded Mesenchymal Stem Cells for Regenerative Medicine: Yield in Stromal Vascular Fraction from Adipose Tissues

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The adipose-derived stromal vascular fraction (SVF) represents a rich source of mesenchymal cells, potentially able to differentiate into adipocytes, chondrocytes, osteoblasts, myocytes, cardiomyocytes, hepatocytes, and neuronal, epithelial, and endothelial cells. These cells are ideal candidates for use in regenerative medicine, tissue engineering, including gene therapy, and cell replacement cancer therapies. In this work, we aimed to the optimization of the adipose SVF-based therapy, and the effect of the collection site, surgical procedure, and tissue processing techniques on SVF yield was evaluated in terms of cell recovery and live cells, taking into account the effect of gender, age, and body mass index. Adipose tissue samples were recovered from 125 informed subjects (37 males and 88 females; mean age: 51.31 years; range: 15–87 years), and digested in different condition with collagenase. A multivariate linear model put in evidence that in males the best collection site in terms of yield is located in the abdomen, whereas in females the biopsy region do not influence cell recovery; the collection technique, the age, and the body mass index of donor seem not to influence the cell yield. The tissue-processing procedures strongly modify the yield and the vitality of cells: a collagenase concentration of 0.2% and a digestion time of 1 h could be chosen as the best operating conditions.

Introduction

Adipose tissue has traditionally been considered as a virtually inert tissue until the mid-1990s with the discovery of leptin and adiponectin that "led to a major renaissance in this field"[1]; now, the evidence that the autologous fat transplantation can be exploited for tissue regeneration has come to the fore.[2–7] In fact, adipose tissue has been identified as a rich, easy-to-reach source of mesenchymal stem cells (MSCs), able to differentiate into adipocytes, chondrocytes, osteoblasts, myocytes, cardiomyocytes, hepatocytes, epithelial cells, and endothelial cells.[8–11] Some authors report that also neuronal/glial differentiation can occur from the morphological but not physiological point of view.[12,13]

Besides, many authors describe MSCs as cells endowed of neoangiogenic, immunomodulatory, antiinflammatory,[14–18] and putative antineoplastic[19–22] properties, and possibly employable in a plethora of degenerative pathologies. Adipose tissue is composed of two main cell populations, mature adipocytes and stromal vascular fraction (SVF): the latest is an heterogeneous fraction including preadipocytes, endothelial cells, smooth muscle cells, pericytes, macrophages, fibroblasts,[23–25] and adipose-derived stem cells (ADSCs),[9,10,24,26–33] which share several characteristics with bone marrow stem cells.[34]

Because of their stemness properties as self-renewal capacity, long-term viability, and multilineage potential, the employ of ADSCs is proposed in tissue engineering and regenerative medicine[6]; moreover, the use of SVF or ADSCs has been proposed in several chronic pathologies as Crohn disease,[35] graft-versus-host disease,[36] autoimmune pathologies (e.g., multiple sclerosis),[15] and allergic pathologies[37]: the therapeutic approach toward these pathologies can be explained by the immunoregulatory and antiinflammatory activities of ADSCs or nonexpanded SVF cells.[15] In 2010

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Caplan[38] proposed an intriguing mechanism that contribute to establish a regenerative microenvironment: after an injury, the pericytes adjacent to blood vessels activate MSCs, which in turn secrete bioactive molecules as immunomodulators, and trophic, antiapoptotic, antiscarring, angiogenic, and mitotic factors. In these perspectives, the widespread employ of MSCs in several pathologies could be explained: MSCs can be viewed as "site-regulated drug stores," and renominated "medicinal signaling cells" instead of "mesenchymal stem cells." For the clinical employ of MSCs in cell therapy, different approaches can be followed: as for a drug, administration of MSCs can be performed either by systemic intravascular perfusion or by *in situ* delivery. Moreover, either native, nonexpanded cells or *in vitro* expanded/differentiated cells can be employed. Once recovered, stem cells are separated from their natural niche; therefore, the physiological microenvironmental conditions during culture are altered. It is mandatory to explain the number of signals that regulate cells behavior when into their niche, aiming to maintain an undifferentiated stage when cultured.[39] On the other hand, Rubio *et al.*[40] have shown that human ADSCs undergo malignant transformation with prolonged passaging over >4 months; however, in this study the number of passages is not reported because of limitations in estimating the number of cell divisions.

Some authors[6,15,41] consider the use of freshly isolated nonexpanded cells as safer and more practical. For this purpose, the number of recovered cells must be sufficiently high to reach the therapeutic dosage: the most promising tissue for the recovery of MSCs is the subcutaneous white adipose tissue. Adipose tissue cell population can greatly differ depending on of harvesting site, age, gender, and body mass index (BMI); this feature leaves intriguing questions, as reported by Gimble *et al.*[7]: "Although studies are limited, adipose depot specific differences appear to exist with respect to stem cell content. […] It remains to be determined as to which human adipose tissue depot should be harvested for optimal stem cell recovery." Some research oriented toward the effect of age, gender, and anatomical region on osteogenic differentiation of ADSCs.[42,43] Recent advances support the theory that all MSCs are pericytes localized in ablumenal surface of blood vessels.[44] If MSCs are located in the adipose perivascular niche, the degree of vascularization strongly influences the number of MSCs recovered from different sites, as reported by da Silva Meirelles *et al.*[45] and by da Silva Meirelles *et al.*[46] Indeed, both immunomodulatory and trophic effects, considered as powerful therapeutic agents, seem not to be related to the differentiation properties.[38]

Moreover, the surgical procedure to obtain adipose tissue and the subsequent treatment can heavily influence the properties of SVF and ADSC vitality and yield.

In this work, we aimed to the optimization of the SVF-based therapy, and the effect of the collection site, surgical procedure, and tissue processing techniques on SVF yield was evaluated in terms of cell recovery and viability, taking into account the effect of gender, age, and BMI.

Materials and Methods

Subjects

In total, 125 informed subjects (37 males and 88 females; mean age: 51.31 years; range: 15–87 years) were enrolled. Tissue sampling and use was approved by the competent ethics committee. Age did not differ between genders (males: 51.67 ± 18.69; females: 51.15 ± 18.63; $t = 0.146$, $p = 0.88$), as well as the BMI (males: 25.33 ± 3.44; females: 26.68 ± 5.40; $t = 1.588$, $p = 0.116$). Adipose tissue was obtained as waste material after elective surgery by liposuction or manual resection. During esthetic plastic surgery, fat samples were manually harvested by liposuction of subcutaneous fat tissue, by negative pressure using a 10-mL syringe, with a blunt tip cannula and processed following Coleman's technique.[47] Resected fat samples were obtained by exeresis using a scalpel from herniated disc standard discectomy surgery, meniscectomy, or reductive mastoplasty. No cauteries were employed. By comparing the surgical techniques, male patients were submitted to resection more frequently than females (86.5% vs. 51.1%, respectively; $\chi^2 = 13.8$, $p < 0.001$). Harvesting sites were differently distributed for males and females: male donors gave samples from back (herniation, 70.3%), abdomen (abdominoplasty, 13.5%), and knee (meniscectomy, 16.2%), whereas females from back (herniation, 13.6%), abdomen (abdominoplasty, 30.7%), breast (reductive mastoplasty, 14.8%), and thigh (liposculture, 11.4%). The 2.2% of collections regarded resections from the neck zone.

Clinical sheets of patients (identity, gender, age, tissue-processing laboratories, sampling site, day of collection, and anamnesis) are filed by the structure responsible of the surgery.

From the trial were excluded patients with septicemia or extensive infections, syphilis, type B and C hepatitis, HIV, Creutzfeld-Jacobs disease, viral or unknown neurological diseases, human GH treatment, dural treatment, and malignant tumors.

Cell isolation

Procedures were conducted in a cleaning room (Class B, GMP guidelines). Each suite is supported by single-pass, positive-pressure (60 Pa), HEPA-filtered air, at a temperature of 18°C and relative humidity 50%. The flow suite is unidirectional, with entry and exit air locks. The cleaning room environment was monitored by wireless probes directly interfaced with an external computer to assure the same operating conditions during each process step, as required by the European Community Guidelines that rule for the product in advanced therapies. Low contamination levels were guaranteed by differential pressures, absolute filtration systems, and unidirectional laminar air flow. Personnel were trained in respect of the GMP guidelines. All the steps were conducted in certified ISO Class 5 (Class 100) laminar flow biosafety cabinets. Environmental cleanliness controls were performed before operation in the unmanned state (t0), and in the manned state during normal use (t1). Particulate contamination control tests were conducted on the air sample (determination of CFU/m^3 air) using sedimentation settle plates (CFU/4 h), agar contact plates (CFU/plate), and the operator's gloved hand test.

In the operating room adipose samples were suspended in phosphate-buffered saline (PBS) with penicillin/streptomycin 1%, put into a sterile box, and forwarded to a GMP laboratory at the temperature of 4°C. Adipose tissue samples were digested using collagenase in PBS without calcium/magnesium plus penicillin 100 IU/mL and streptomycin 100 μg/mL at 37°C. Two collagenase from two different

suppliers were employed (Serva or Sigma-Aldrich), and different collagenase concentration (ranging from 0.05% to 0.20%) were employed for different incubation times (ranging from 1 to 12 h). The different digestion protocols were (a) 0.05% collagenase, incubation 12 h; (b) 0.075% collagenase, incubation 12 h; (c) 0.10% collagenase, incubation 2 h; (d) 0.15% collagenase, incubation 1 h; and (e) 0.20% collagenase, incubation 1 h. Mature adipocytes and connective tissues were separated from SVF by centrifugation as reported by Zuk *et al*.[48] After centrifugation, cells were washed twice with PBS to obtain clean pellets.

Cellular yield was calculated as the number of stromal cells obtained per milliliter of bioptical tissue; counts were performed after staining with Trypan Blue in a Bürker chamber. In each sample the vitality and vital cell yield (live cells/mL treated tissue) were calculated. All samples were counted in triplicate.

Morphological investigation

Tissue digestion processes were monitored using cytospin centrifuge: slides and filters were placed into appropriate slots. Then, aliquots of 100 µL of each sample were centrifuged for 5 min with a rotation of 700 rpm. Slides were first treated with paraformaldehyde 4% for 15 min and subsequently with PBS 1% for 5 min. Then, they underwent a 10% May-Grunwald Giemsa staining for 10 min.

Statistical analyses

To evaluate the effect of subject characteristics, the collection techniques and tissue procedures on cell yield, vitality, and live cell yield, a linear multiple regression analysis was applied. As independent variables, the following subject characteristics were taken into account: gender, BMI, and age; the surgical techniques: resection or aspiration; collection site: abdomen, thigh, neck, back, breast, and knee; tissue processing conditions: (a) 0.05% collagenase, incubation 12 h; (b) 0.075% collagenase, incubation 12 h; (c) 0.10% collagenase, incubation 2 h; (d) 0.15% collagenase, incubation 1 h; and (e) 0.20% collagenase, incubation 1 h; digestion depth: total, partial, and undigested. Since vitality did not fit the model, a univariate nonparametric analysis was performed: for each independent variable, the tests of Mann–Whitney or Kruskal–Wallis were applied. Significance level was set at $\alpha = 0.05$.

Results

By employing the Zuk procedure,[48,49] samples of SVF cells were obtained. After the first 30 min of adipose tissue digestion, with collagenase concentration 0.2% (Fig. 1a), the samples appeared partially digested, whereas SVF was isolated from the extracellular matrix after 1 h (Fig. 1b).

The results of SVF yield and live cell yield are reported in Table 1 as a function of gender and surgical technique. Statistical differences between average values ($p < 0.05$) were found only for yield and live cell yield, and a significant interaction was evidenced between gender and surgical technique (data not shown): in fact, male subjects were not treated with liposuction surgical technique. The significant interaction implies that possible gender and/or surgical technique differences cannot be seen as separated factors. For these reasons the linear multivariate model was applied by stratifying by gender. The linear model results are reported in Table 2. Cell and live cell yields (in females) were not influenced by the surgical technique, whereas different yield were obtained in different collection sites in male subjects: the higher cell and live cell yield were obtained when cells were recovered from the abdomen with respect to the back ($p = 0.001$) and to the knee ($p < 0.02$). In females, no significant differences were found between collection site and cellular yield (Table 2). Digestion was performed with collagenase purchased from two suppliers: no differences were found in both cell yield and live cell yield (Table 2). Digestion conditions indeed significantly influenced both cell and live cell yield: in tissue sampled from male subjects, digestion with collagenase 0.2% per 1 h incubation gave the best results and was taken into account as reference. By reducing the collagenase concentration to 0.15%, incubation 1 h (treatment d), the cell and live cell yield decreased in a significant way ($p < 0.02$). Digestion with 0.1% collagenase (treatment c) did not increase yields after 2 h of incubation. The 12 h overnight digestion with (treatment a) 0.05% collagenase and (b) 0.075% collagenase reduced both cell yield and live cell yield ($p < 0.05$ in treatment a). In females, overnight treatment (treatment b) gave higher yield ($p < 0.001$) with respect to treatment (e) on both of the response variables. Neither in males nor in females BMI and age influenced any of the response variables. Adjusted determination coefficients for the model were higher in the male stratum than in the female one (Table 2).

Discussion

ADSCs are promising candidates in a broad range of innovative therapies, ranging from regenerative medicine to tissue engineering, in autoimmune pathologies or chronic flogosis: clearly, different cell types should be employed in different clinical situations. SVF is the starting point, for example, the raw material necessary to realize products for

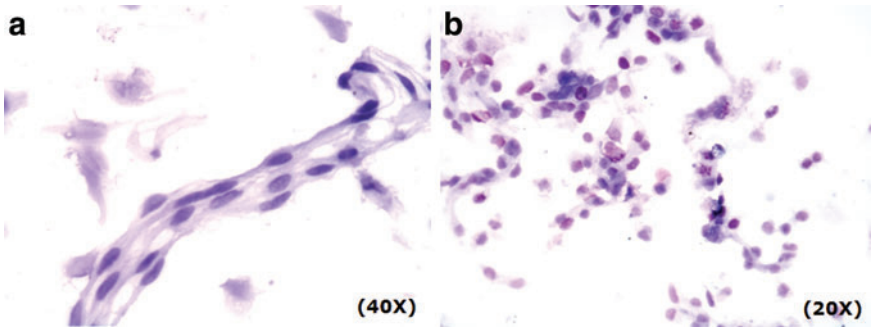

FIG. 1. Microphotograph of digested adipose tissue (collagenase 0.2%) after 30′ (**a**) and after 1 h (**b**). May-Grunwald-Giemsa staining. Color images available online at www.liebertonline.com/ten.

Table 1. Average Values and Dispersion Indices for Yield, Live Cell Yield, and Vitality Stratified for Gender and Surgical Technique

Factor	n	*Yield (log-million)*[a]	*Live cell yield (log-million)*[a]	*Vitality %*[b]
Male	36	1.44 ± 1.62	1.05 ± 0.78	77 (67–87)
Female	78	2.05 ± 1.46	1.80 ± 1.52	81 (72–88)
		$t = 2.00$, $p = 0.048$	$t = -2.33$, $p = 0.022$	MW $= -1.07$, $p = 0.283$
Resection	68	1.39 ± 1.50	1.05 ± 1.61	78.5 (70–87)
Aspiration	46	2.56 ± 1.32	2.33 ± 1.38	82 (73–88)
		$t = -4.27$, $p < 0.0001$	$t = -4.39$, $p < 0.0001$	MW $= -1.53$, $p = 0.127$

Yield and live cell yield are log-transformed.
[a]Mean ± standard deviation.
[b]Median and interquartile range.
t, *t*-test; MW, Mann–Whitney test.

Table 2. Effect of Surgical Technique, Collection Site, Type of Collagenase, Digestion Conditions, Digestion Result, Body Mass Index, and Patient's Age on Cell Yield and on Live Cell Yield

	Male					*Female*				
	MR	t-*Value*	p-*Value*	*CI 95% MR*		*MR*	t-*Value*	p-*Value*	*CI 95% MR*	
Cell yield										
Surgical technique										
Resection/liposuction	—	—	—	—	—	0.418	−1.48	0.146	0.123	1.368
Collection site										
Thigh/abdomen	—	—	—	—	—	3.657	1.75	0.086	0.872	16.169
Back/abdomen	0.057	−3.61	0.001	0.011	0.294	0.672	−0.48	0.631	0.129	3.492
Breast/abdomen	—	—	—	—	—	0.419	−1.72	0.091	0.152	1.156
Knee/abdomen	0.037	−2.54	0.018	0.002	0.541	0.812	−0.24	0.813	0.140	4.714
Type of collagenase										
Sigma/Serva	—	—	—	—	—	0.940	−0.05	0.963	0.065	13.567
Digestion conditions[a]										
a/e	0.041	−2.15	0.042	0.002	0.881	—	—	—	—	—
b/e	0.154	−1.59	0.126	0.013	1.763	8.878	4.30	<0.001	3.204	24.6
c/e	0.286	−0.82	0.422	0.012	6.791	—	—	—	—	—
d/e	0.031	−2.90	0.008	0.003	0.370	—	—	—	—	—
Digestion result										
Partial/complete	1.616	0.52	0.608	0.239	10.917	0.403	−0.95	0.348	0.059	2.756
BMI	1.131	1.34	0.192	0.936	1.366	0.975	−0.74	0.462	0.909	1.045
Age	1.014	1.02	0.319	0.986	1.042	0.982	−1.33	0.188	0.955	1.009
Adjusted determination coefficient			0.51					0.35		
Live cell yield										
Surgical technique										
Resection/liposuction	—	—	—	—	—	0.373	−1.62	0.112	0.110	1.267
Collection site										
Thigh/abdomen	—	—	—	—	—	3.915	1.79	0.080	0.846	18.121
Back/abdomen	0.024	−3.98	0.001	0.003	0.168	0.618	−0.57	0.572	0.113	3.377
Breast/abdomen	—	—	—	—	—	0.389	−1.81	0.076	0.137	1.109
Knee/abdomen	0.018	−2.63	0.015	0.001	0.422	0.850	−0.18	0.858	0.139	5.208
Type of collagenase										
Sigma/Serva	—	—	—	—	—	1.087	0.06	0.952	0.069	17.021
Digestion conditions[a]										
a/e	0.022	−2.19	0.039	0.001	0.815	—	—	—	—	—
b/e	0.089	−1.74	0.095	0.005	1.574	9.998	4.40	<0.001	3.497	28.589
c/e	0.361	−0.56	0.578	0.009	15.106	—	—	—	—	—
d/e	0.030	−2.50	0.020	0.002	0.548	—	—	—	—	—
Digestion result										
Partial/complete	1.402	0.31	0.759	0.147	13.349	0.289	−1.26	0.214	0.040	2.093
BMI	1.223	1.87	0.074	0.979	1.529	0.969	−0.87	0.388	0.902	1.041
Age	1.020	1.24	0.229	0.987	1.053	0.981	−1.36	0.181	0.953	1.009
Adjusted determination coefficient			0.43					0.36		

Results are stratified by gender. Mean ratios (MR), 95% confidence intervals of mean ratios (CI 95% MR), and statistical tests are reported.
[a](a) 0.05% collagenase, incubation 12 h; (b) 0.075% collagenase, incubation 12 h; (c) 0.10% collagenase, incubation 2 h; (d) 0.15% collagenase, incubation 1 h; and (e) 0.20% collagenase, incubation 1 h.

advanced therapy. SVF can be employed just after retrieval without cell type selection; otherwise, ADSCs can be isolated and expanded, after plating and culture in selected media. In any case, cells must be retrieved in a sufficient number to reach the therapeutic dosage and this is a constant trouble. Expansion of extracted cells could be a solution, but a high number of passages could modificate cells toward potentially tumorigenic cell lineages. After the seminal works by Zuk *et al*.[48] since 2001, a number of independent research groups retrieved MSCs from adipose tissue samples by collagenase digestion: the preliminary step was to consolidate this procedure in a relatively high sample size defining the optimal operative conditions (time and collagenase concentration) to obtain the best cell yield.

Liposuction aspirates are already employed in several clinical trials, but the articles on the optimization of isolation techniques and product characterization are scarce. In this perspective, the use of minimally manipulated fresh cells may lead to higher safety and efficacy in actual treatments.

In this article the effect of the collection site, surgical procedure, and tissue-processing techniques on SVF yield was evaluated in terms of cell recovery and viability, taking into account the effect of gender, age, and BMI. Since it was impossible to enroll male donors undergoing liposuction esthetical intervention, the interaction between gender and surgical technique was predictable: to overcome that pitfall, the linear multivariate model was applied by stratifying by gender. To better define the relations between these variables, it could be desirable to include lipoaspirated tissues from male donors. Results suggest that the effect of age and BMI are not significant, whereas the digestion conditions and the collection site have a significant effect on the output variables in both genders. In males, the best digestion conditions are set at 0.2% collagenase (1 h incubation), and in females the higher yields are reached with overnight digestion. This behavior could be due to the differences in the fibrous matrix of adipose tissue in male and female subjects. Gender-related differences between adipose tissue have been widely studied by several authors[43]: in the present work, a significant gender effect has not been fully verified, due to interaction with surgical technique, and these effects therefore cannot be considered separately.

Viability can be influenced by tissue processing (collagenase concentration and digestion timing), as reported by Bakker *et al*.[50]: in this work, the lack of significance in vitality between groups could be due to the digestion conditions that do not compromise cell vitality.

In 2004, Bakker *et al*.[50] on obese to heavily obese individuals did not find any association in BMI, age, and preadipocyte recovery from adipose tissue, whereas they observed large differences between subcutaneous and omental tissue and isolation protocol. The results presented in this article agree with those previously obtained by Bakker *et al.*, although these researchers were interested to the preadipocyte in a different context.

von Heimburg *et al*.[51] observed that if cell isolation is not performed soon after the operation, liposuction material is the better alternative for preadipocyte recovery if compared with resection. Oedayrajsingh-Varma *et al*.[52] report that adipose-tissue-collecting procedures and SVF isolation methods seem to influence the yield, viability, and replication potential of the recovered cells: the resection gave better yield results than lipoaspiration in terms of SVF yield results (almost 3% higher).

Van Harmelen *et al*.[53] demonstrated that there were no apparently significant differences between males and females involved in this study in terms of BMI, age, number of SVF cells obtained per gram of omental and subcutaneous adipose tissue starting volume, and proliferation potential of the SVF populations recovered.

In 2003, the same research group,[54] considering 189 females undergoing surgical mammary reduction, concluded that BMI correlated positively to age, mature fat cell size, and total number of adipocytes and stromal cells per body. Anyway this study also reports that BMI correlated negatively to the capacity of SVF cells to differentiate: the obese subjects showed larger stromal and fat cell numbers compared to lean subjects, but, at the same time, SVF cells were characterized by a lesser capacity to undergo adipogenic differentiation. Age did not correlate either to the number of stromal cells per gram of adipose tissue or to the differentiation potential of SVF recovered. Similar results were reported by Aust *et al*.[55]: it was estimated that, considering a 87.5% female population ($n = 18$) with a mean age ($\pm$standard deviation) of 44 ± 10 years and BMI of 24.9 ± 2.7, the mean SVF cell yield was 404.000 ± 206.000 cells/mL of lipoaspirate. Linear regression analysis of the cells derived from the female donors demonstrated a significant negative correlation between the number of cells obtained per milliliter of lipoaspirate with the BMI but not the age of the donor.

Differences in terms of SVF yield and viability can be correlated to the subcutaneous body site from which such cells are recovered. Hauner and Entermann[56] demonstrated that regional differences exist in the capacity of recovered SVF to form new fat cells when they are recovered from the abdominal rather than femoral adipose tissue depots. Such variability was noted even when the SVF was recovered from abdominal rather than hip subcutaneous depots of adipose tissue[24]: in this case, beside noticing that tissue harvested from the hip yielded 2.3-fold more CFU-F/unit volume and a 7-fold higher frequency of differentiation than that obtained from the abdomen, donor-to-donor variation in stem cell recovery was even reported.

In summary, in males the best collection site in terms of yield is located in the abdomen, whereas in females the biopsy region do not influence cell recovery; the collection technique, the age, and the BMI of donor seem not to influence the cell yield. The tissue-processing procedures strongly modify the yield and the vitality of cells: a collagenase concentration of 0.2% and a digestion time of 1 h could be chosen as the best operating conditions.

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Disclosure Statement

No competing financial interests exist.

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