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ESM-1 预处理小鼠骨髓间充质干细胞的实验研究*

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摘要 目的:探讨小鼠骨髓间充质干细胞(Bone marrow-derived mesenchymal stem cells, MSCs)经不同浓度的内皮细胞特异性分子-1(Endothelial cell-specific molecule 1, ESM-1)干预后,对其分泌的细胞因子的影响。**方法:**选择不同浓度的 ESM-1 和不同干预时间,对 MSCs 进行预处理;分析其分泌的 VEGF、ALP 等代表细胞活性的细胞因子含量改变情况;明确 ESM-1 预处理 MSCs 所需的最佳干预浓度和最佳干预时间。**结果:**通过不同 ESM-1 浓度干预 MSCs,其中 H(0.55 $\mu\text{g/mL}$ ESM-1)组条件培养液中 VEGF 和 ALP 含量同最高,分别为 (82.500 ± 3.307) 和 $(0.228 \pm 0.020)\text{pg/mL}$,差异有统计学意义, $P < 0.001$ 。选择 0.55 $\mu\text{g/mL}$ ESM-1 对 MSCs 进行不同时间的干预,结果显示 d(3 h)组条件培养液中 VEGF 含量最高, g(4.5 h)组 ALP 含量同最高,分别为 (69.112 ± 3.618) 和 $(0.352 \pm 0.030)\text{pg/mL}$,差异有统计学意义, $P < 0.001$ 。**结论:**不同干预因子预处理 MSCs 所需的最佳浓度和预处理时间不一。ESM-1 对 MSCs 的最佳干预浓度为 0.55 $\mu\text{g/mL}$ 。不同组织细胞的细胞因子的表达和所受到的影响因素也不相同。

关键词:内皮细胞特异性分子-1;骨髓间充质干细胞;血管内皮生长因子;碱性磷酸酶

中图分类号:R541.4 **文献标识码:**A **文章编号:**1673-6273(2017)18-3451-05

Experimental Study on ESM-1 Pretreatment with MSCs of Mice*

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ABSTRACT Objective: To investigate whether the secretion of Cytokines by mouse MSCs were increased after pretreated by ESM-1 with different concentration. **Methods:** The MSCs were pretreated by different concentration of ESM-1 and different intervention time, then to analyze the cytokines secretion of VEGF and ALP on behalf of cell activity changes of MSCs, in order to make clear of ESM-1 pretreatment intervention concentration and optimal intervention time needed for MSCs. **Results:** Through the different concentration of ESM-1 to pretreat of MSCs, VEG and ALP concentration in H (0.55 $\mu\text{g/mL}$ ESM-1) group of conditioned medium also reached the highest value, respectively (82.500 ± 3.307) and $(0.228 \pm 0.020)\text{pg/mL}$, and the difference of which was statistically significant, $P < 0.001$. Choosing 0.55 $\mu\text{g/mL}$ ESM-1 to intervene MSCs of different time, research shows that the concentration of VEGF is highest in the medium of d (3 h) group and the concentration of ALP is highest in the medium of g (4.5 h) group, respectively (69.112 ± 3.618) and $(0.352 \pm 0.030)\text{pg/mL}$, and the difference was statistically significant, $P < 0.001$. **Conclusion:** The optimum concentration and pretreatment time of different intervention factors required is different. The best intervention concentration of ESM-1 is 0.55 $\mu\text{g/mL}$, which pretreatment for MSCs. The expression of cytokines in different tissue cells and the influence of the process are not the same.

Key words: Endothelial cell-specific molecule 1; Bone marrow-derived mesenchymal stem cells; Vascular endothelial growth factor; Alkaline phosphatase

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前言

ESM-1 生物学活性广泛,可上调肿瘤坏死因子 α 、白介素- 1β ,下调干扰素 γ ,参与体内的细胞迁移、血管生成、炎症反应等过程^[1,2]。而单纯干细胞移植存活率低,效果不佳,是否可通过细胞因子 ESM-1 干预骨髓间充质干细胞以提高其存活率尚不明确。本课题组前期研究证实^[3,4], ESM-1 预处理 MSCs,可增加 MSCs 分泌 VEGF 和 ALP,可进一步活化干细胞。但前期研究还存在预处理干细胞所需最佳 ESM-1 浓度和预处理时间的问题。该实验拟探索预处理干细胞所需最佳 ESM-1 浓度和预

处理时间。为细胞因子干预干细胞所需干预浓度和干预时间提供理论依据。

1 材料与方法

1.1 材料、试剂和器械

健康雌性 SD 小鼠(苏州大学实验动物中心);ESM-1(Cusabio Biotech 公司);DMEM 基础培养基(Hyclone 公司);CD29、CD44 抗体(北京夏斯生物科技有限公司);VEGF ELISA 试剂盒(Assaypro 公司);ALP ELISA 试剂盒(Assaypro 公司);流式细胞仪(BD C6),倒置相差显微镜(Nikon Ts 100)。

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1.2 方法

1.2.1 MSCs 获取和鉴定 取小鼠股骨骨髓,反复吹打成单细胞悬液,以 1000 r/min 离心 5 min。用 100 mL/L FBS 稀释,以 $(1\sim5)\times 10^6$ 个/L 的密度接种于 DMEM 培养基中培养。待细胞 90 %融合时传代,第 3 代 MSCs 用于实验。应用流式细胞仪鉴定小鼠骨髓 MSCs 表面标志抗原 CD29、CD44。

1.2.2 实验分组 将 MSCs 接种至 12 孔板,用含有 10% FBS 的 DMEM 培养基培养,当细胞 90%融合时,吸去培养液,用 0.01 mol/L PBS 浸洗 2 次,加培养基 1 mL/孔。将 MSCs 随机分为两组,分别接受以下处理:

(1)空白对照组 不进行预处理组;

(2)观察组 1 用 ESM-1 预处理,根据 ESM-1 干预浓度,分为十个亚组:(A 0.2 μ g/mL、B 0.25 μ g/mL、C 0.3 μ g/mL、D 0.35 μ g/mL、E 0.4 μ g/mL、F 0.45 μ g/mL、G 0.5 μ g/mL、H 0.55 μ g/mL、I 0.6 μ g/mL、J 0.65 μ g/mL);每组设四个复孔,每孔均处理 60 min。收集每孔的培养液,检测预处理对 MSCs 分泌 VEGF 和 ALP 的影响。

(3)观察组 2 根据以上实验选择最佳 ESM-1 干预浓度进行实验。根据干预时间不同,分为十个亚组:分别干预 a 1.5

h、b 2 h、c 2.5 h、d 3 h、e 3.5 h、f 4 h、g 4.5 h、h 5 h、I 5.5 h、j 6 h。收集每孔的培养液,检测预处理时间对 MSCs 分泌 VEGF、ALP 的影响。

1.2.3 VEGF 和 ALP 检测 (1)VEGF 检测 采用美国 Assaypro 公司 ELISA 试剂盒定量各组培养基中的 VEGF 含量,检测步骤严格按照检测试剂盒的操作说明进行。(2)碱性磷酸酶(ALP)活性检测 细胞经 FBS 漂洗后胰酶消化,再次超声破碎后,吸取 0.1 mL 破碎液,加入 ALP 缓冲液及底物溶液各 0.5 mL,反应 15 min 后使用 ALP ELISA 试剂盒检测 ALP 活性。

1.3 统计学处理

计量资料采用($\bar{x}\pm s$)表示,应用 SPSS 17.0 统计学软件处理数据。3 组以上比较采用单因素方差分析,两两比较采用 q 检验,以 $P<0.05$ 为差异具有统计学意义。

2 结果

流式细胞分化抗原鉴定结果显示,超过 90 %小鼠 MSCs 性表达 CD29(细胞整合素分子)和 CD44(粘附分子),CD29 和 CD44 为干细胞特有的表面标志分子,因此间接反映干细胞含量在 90 %以上。见图 1-2。

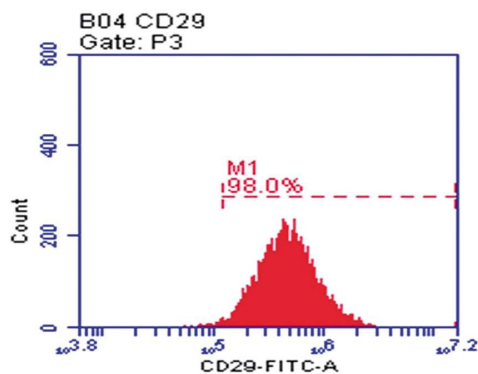


图 1 MSCs 表面标志抗原 CD29 检测结果

Fig. 1 Detection results of MSCs surface marker antigen CD29

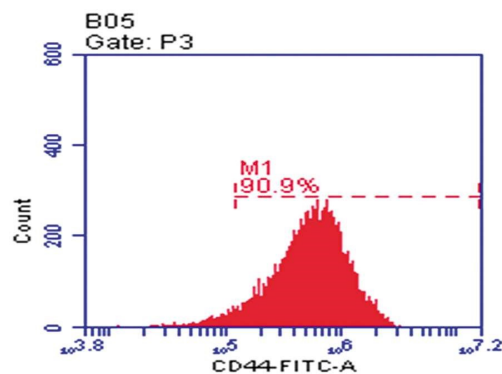


图 2 MSCs 表面标志抗原 CD44 检测结果

Fig. 2 Detection results of MSCs surface marker antigen CD44

2.1 MSCs 光镜形态

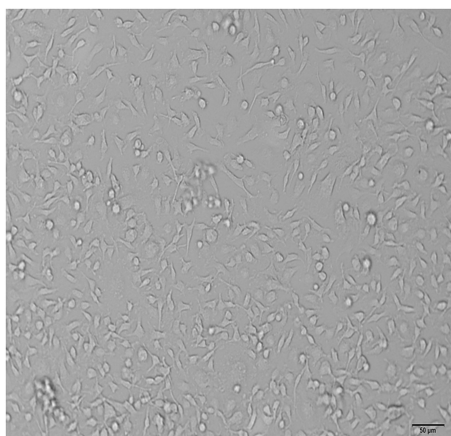


图 3 光镜下干细胞形态学观察(200 倍)

Fig.3 Morphological Observation of stem cells under light microscope (200 times)

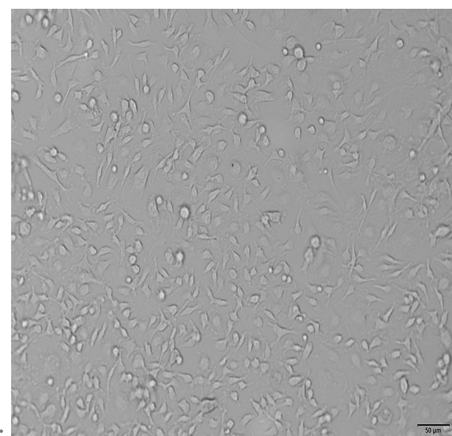


图 4 光镜下干细胞形态学观察(200 倍)

Note: Fig. 3-4 showed that MSCs cells were spindle shaped and arranged regularly, the growth rate stable.

2.2 最佳干预浓度

不同 ESM-1 干预浓度组条件培养液中, H (0.55 $\mu\text{g/mL}$ ESM-1) 组条件培养液中 VEGF 和 ALP 含量同时达到最高值,

分别为 (82.500 ± 3.307) 和 (0.228 ± 0.020) pg/mL , 差异有统计学意义, $P < 0.001$ (见表 1 和图 5-6)。

表 1 不同 ESM-1 浓度组条件培养液中 VEG 和 ALP 浓度($\bar{x} \pm s$)

Table 1 Concentration of VEG and ALP in culture medium of different ESM-1 concentration groups

Groups		VEGF concentration (pg/mL)	ALP concentration (pg/mL)
Blank Control Group (n=4)		23.000 ± 1.109	0.023 ± 0.002
Observation Group 1 (n=4)	A	30.188 ± 2.726^a	0.034 ± 0.002
	B	35.438 ± 2.173^{ab}	0.050 ± 0.004^{ag}
	C	44.438 ± 3.418^{aaa}	0.069 ± 0.005^{aah}
	D	48.953 ± 1.711^{aaac}	0.083 ± 0.002^{aaai}
	E	55.313 ± 3.125^{aaaad}	0.095 ± 0.003^{aaag}
	F	65.313 ± 2.135^{aaaaa}	0.118 ± 0.016^{aaaaaa}
	G	$71.688 \pm 2.357^{aaaaad}$	0.189 ± 0.005^{aaaaaa}
	H	$82.500 \pm 3.307^{aaaaaaa}$	$0.228 \pm 0.020^{aaaaaaa}$
	I	$78.438 \pm 1.197^{aaaaaaac}$	$0.186 \pm 0.005^{aaaaaaa}$
	J	$73.313 \pm 2.561^{aaaaaaaf}$	$0.167 \pm 0.006^{aaaaaaah}$
F-value		276.313	272.273
P-value		<0.001	<0.001

Note: Compared with Blank Control Group $^aP < 0.001$; Compared with A Group $^aP < 0.001$, $^bP = 0.05$, $^cP = 0.011$; Compared with B Group $^aP < 0.001$, $^bP = 0.003$; Compared with C Group $^aP < 0.001$, $^cP = 0.014$, $^dP = 0.031$; Compared with D Group $^aP < 0.001$, $^dP = 0.001$, $^eP = 0.049$; Compared with E Group $^aP < 0.001$; Compared with F Group $^aP < 0.001$, $^dP = 0.001$; Compared with G Group $^aP < 0.001$; Compared with H Group $^aP < 0.001$, $^fP = 0.026$; Compared with I Group $^fP = 0.006$, $^hP = 0.003$, $^iP = 0.001$.

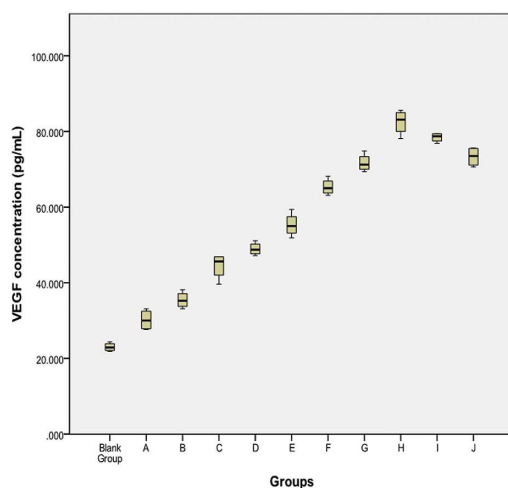


Fig.5 VEGF concentration trend chart

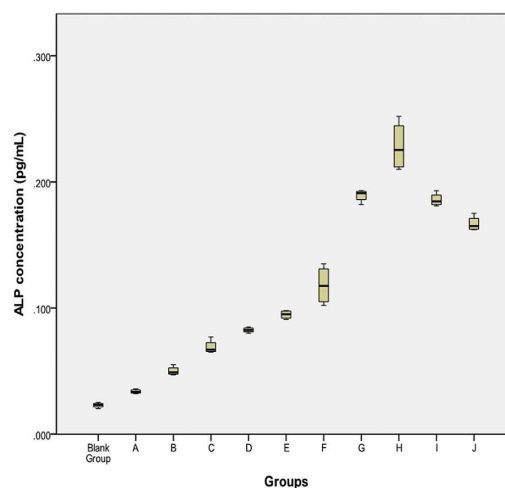


Fig.6 ALP concentration trend chart

图 5-6 VEGF 和 ALP 含量随 ESM-1 不同干预浓度变化箱图

Fig.5-6 VEGF and ALP concentration box diagram with the change of different intervention concentration by ESM-1

2.3 最佳干预时间

不同 ESM-1 干预时间组条件培养液中, 干预 3 小时后的 d 组条件培养液中 VEGF 含量最高, 干预 4.5 小时后的 g 组 ALP 含量同最高, 分别为 (69.112 ± 3.618) 和 (0.352 ± 0.030) pg/mL , 差异有统计学意义, $P < 0.001$ (见表 2 和图 7-8)。

3 讨论

骨髓间充质干细胞(MSCs)是多能细胞, 自体或异体干细胞的使用被认为是治疗疾病的一种很有前途的替代治疗方法^[5]。但是单纯干细胞移植存在迁移率低、存活率低的问题, 预处理 MSCs 可能是提高治疗性血管生成的一种可行方法^[6]。本实验通过不同浓度的 ESM-1、不同干预时间, 对 MSCs 进行预处理以提高干细胞潜能; 同时进一步明确 ESM-1 干预 MSCs 所需的最佳干预浓度和预处理时间。

表 2 不同 ESM-1 干预时间组条件培养液中 VEG 及 ALP 浓度($\bar{x} \pm s$)

Table 2 Concentration of VEG and ALP in the conditioned medium of different ESM-1 intervention time groups

Groups		VEGF concentration (pg/mL)	ALP concentration (pg/mL)
Blank Control Group (n=4)		13.515 ± 1.197	0.046 ± 0.022
Observation Group 2(n=4)	a	18.179 ± 2.127 ^a	0.060 ± 0.015
	b	29.602 ± 3.416 ^{bb}	0.116 ± 0.018 ^{cc}
	c	37.817 ± 1.184 ^{bbb}	0.130 ± 0.029 ^{dc}
	d	69.112 ± 3.618 ^{bbbb}	0.220 ± 0.022 ^{bbb}
	e	64.959 ± 2.434 ^{bbbb}	0.249 ± 0.012 ^{bbb}
	f	60.725 ± 3.416 ^{bbbbb}	0.327 ± 0.061 ^{bbbgf}
	g	49.143 ± 5.002 ^{bbbbb}	0.352 ± 0.030 ^{bbbbb}
	h	46.321 ± 2.228 ^{bbbbb}	0.274 ± 0.041 ^{bbgd}
	i	36.541 ± 3.618 ^{bbbbb}	0.255 ± 0.034 ^{bbcb}
	j	34.281 ± 2.26 ^{bbbbb}	0.242 ± 0.026 ^{bbbbb}
F-value		149.290	43.860
P-value		<0.001	<0.001

Note: Compared with Blank Control Group ^aP=0.034, ^bP<0.001, ^cP=0.003, ^dP=0.001; Compared with a Group ^bP<0.001, ^cP=0.016, ^dP=0.003; Compared with b Group ^bP<0.001, ^cP=0.002, ^dP=0.034; Compared with c Group ^bP<0.001; Compared with d Group ^bP<0.001, ^cP=0.02; Compared with e Group ^bP<0.001, ^cP=0.01; Compared with f Group ^bP<0.001, ^cP=0.022, ^dP=0.003; Compared with g Group ^bP<0.001, ^cP=0.001; Compared with h Group ^bP<0.001.

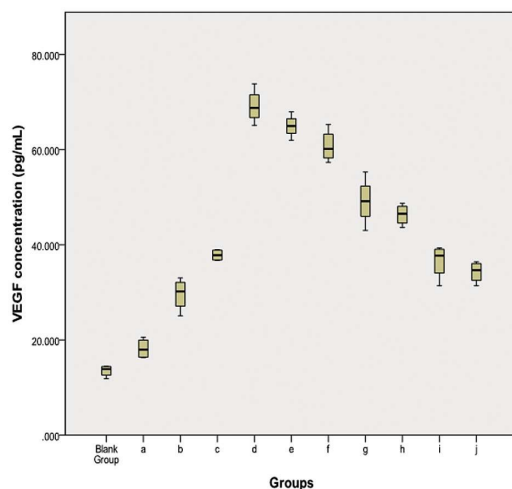


Fig.7 VEG concentration trend chart

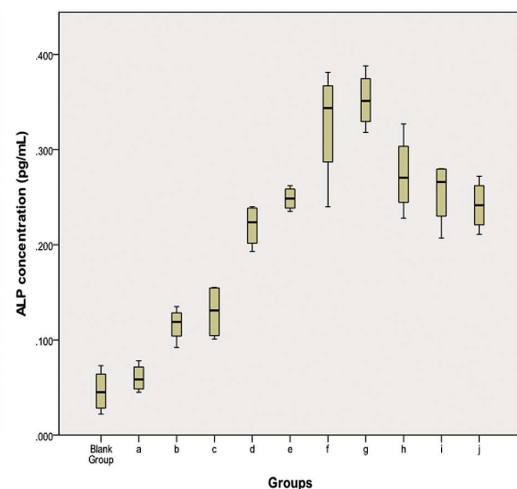


Fig.8 ALP concentration trend chart

图 7-8 VEGF 和 ALP 含量随 ESM-1 不同干预时间变化箱图

Fig.7-8 VEG and ALP concentration box diagram with the change of different intervention time by ESM-1

不同干预因子所需的最佳干预浓度不一。目前研究对于不同的干预因子干预浓度范围变化较大,从 0.1 nmol/L~100.0 mmol/L^[7,8]。国外一项研究使用 7~10 mol/L Ang II 刺激人足细胞 24 h 后,VEGF 的表达明显增加^[9]。一项关于人内皮细胞和胎儿股骨干细胞共培养的研究显示^[10],加入 100 ng/mL 的血管内皮生长因子培养 7 天后,ALP 基因表达明显增加,活性增强。本实验通过不同 ESM-1 浓度干预 MSCs,以刺激 MSCs 增加 VEGF 和 ALP 等代表细胞活性的细胞因子的分泌。结果显示,H(0.55 μg/mL ESM-1)组条件培养液中 VEGF 和 ALP 含量同时达到最高值,分别为 (82.500 ± 3.307) 和 (0.228 ± 0.020) pg/mL,差异有统计学意义,P<0.001。因此,0.55 μg/mL ESM-1

为 MSCs 所需的最佳干预浓度,可同时促进 VEGF 和 ALP 的分泌。国内刘宏博等^[11]使用 1.0 μg/mL 脂多糖 10 μL 处理 MSCs 后 48 h,可促进 MSCs 的增殖及细胞移植后新生血管的生成。另一项研究使用 0.1 ng/mL 的 TGF-β1 预处理 MSCs,可增强细胞外基质成分的表达,细胞外基质可能有助 MSCs 的存活和扩增,从而提高细胞生存和潜在的治疗效益^[12]。基质细胞衍生因子 1(SDF-1),主要是促进细胞外基质的合成,SDF-1 预处理脂肪干细胞,通过 Caspase 信号通路的激活,可防止干细胞在缺氧和无血清条件下的细胞凋亡^[13]。也说明细胞外基质在干细胞存活和扩增方面有着重要作用。ALP 是代表细胞活性的细胞因子,标志着细胞的成熟程度和功能,随着细胞的成熟,酶

的活性也逐渐增强。VEGF 可在体内诱导血管生成,增加血管通透性,提高干细胞的存活,以达到治疗心梗的目的^[14]。

目前关于干细胞预处理时间方面的研究,波动范围从数小时到数天。国内一项关于预处理时间方面的研究证实,阻断冠脉 5~10 min 的短暂缺血,30 min 内导致 VEGF 的最高表达^[15]。国外有研究采用氯化钴预处理小鼠 MSCs 以模拟缺氧条件,预处理后 6、12、24 和 48 h,VEGF mRNA 的表达明显增加。其次,还可以上调 ALP 的分泌并影响干细胞的多向分化^[16]。最近报道的类似研究使用去铁胺、氯化钴化学剂预处理 MSCs,同样去模拟缺氧条件,预处理 24 h 后可促进乙酰化组蛋白 H3 的启动子表达增加,特别是在 16 h 和 24 h 之间的差别最大^[17]。国内近期研究,使用 10 μ m 的人参皂苷预处理大鼠 MSCs 24 h,结果显示其对氧化应激诱导的 MSCs 凋亡具有明显的保护作用^[18]。还有学者使用抗氧化剂依达拉奉干预 MSCs 24 h,证实骨髓间充质干细胞的细胞因子分泌明显增加^[19]。以上研究对于不同因子预处理的时间大多集中在 24 h 以内。本实验选择最佳干预浓度的 ESM-1(0.55 μ g/mL)对 MSCs 进行不同时间的干预实验,结果显示 d(3 h)组条件培养液中 VEGF 含量最高,g(4.5 h)组 ALP 含量最高,分别为(69.112 $\pm$ 3.618)和(0.352 $\pm$ 0.030)pg/mL,差异有统计学意义,P<0.001。结果证实最佳干预时间并不统一。其原因,一方面考虑干预时间差别不明显,相隔 0.5 h;其次考虑 VEGF 和 ALP 分泌时间不一。不同组织细胞 VEGF 和 ALP 的表达不一,而且也受到多种因素的影响。其中缺血、缺氧以及细胞因子如肿瘤坏死因子 α (TNF α)、转化生长因子(TGF)等对 VEGF 的表达也有调控作用^[20]。因此,不同组织细胞因子的表达和所受到的影响因素并不统一,预处理时间方面也存在明显的差别。

综上所述,不同干预因子预处理骨髓间充质干细胞所需的最佳浓度和预处理时间不一;不同组织细胞的细胞因子的表达和所受到的影响因素不一。选择最佳干预浓度和干预时间有助于进一步提高干细胞的潜能。

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