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·论著·

应用颅骨-破骨细胞联合培养体系研究先天性成骨不全破骨细胞骨吸收活性

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摘要：**目的** 先天性成骨不全(OI)的主要临床表现为骨矿化过程不良，骨量丢失，骨骼畸形和骨折。但是其发病机理，尤其在其骨再建过程中成骨细胞(OB)及破骨细胞(OC)的功能改变尚不清楚。本实验以先天性成骨不全小鼠模型，oim/oim 为基础，应用破骨细胞-颅骨联合培养体系研究 OB 和 OC 两种细胞在骨再建过程中的功能改变和相互作用。**方法** 本实验采用小鼠颅骨(CAL)组织培养模型。本模型采用颅骨组织培养，利用颅骨中成骨细胞可以从颅骨片游离出到培养皿及颅骨表面，从而支持培养皿及颅骨表面前体破骨细胞分化成为成熟破骨细胞，并吸收颅骨产生吸收陷窝。本实验中，共 2 组颅骨-破骨细胞联合培养体系：(1) 对照组(WT)颅骨与对照破骨细胞(WTCAL-WTOC)；(2) OI 颅骨与 OI 破骨细胞(OICAL-OIOC)。联合培养颅骨及骨髓组织 14 日后，以 TRAP 免疫组化染色方法识别破骨细胞，ALP 免疫组化染色方法识别成骨细胞，计算 OC/OB。破骨细胞骨吸收活性以颅骨表面骨吸收陷窝占颅骨表面百分比并除以培养系统中的破骨细胞数表达。**结果** 第 14 日，OICAL-OIOC 组的破骨细胞数低于 WTCAL-WTOC 组(92. 50 + 23. 18/mm^2 对比 379. 00 + 136. 53/mm^2，$P < 0.01$)；OICAL-OIOC 组的 OC/OB 明显低于 WTCAL-WTOC 组(0. 68 + 0. 57 对比 1. 65 + 0. 67，$P < 0.01$)；OICAL-OIOC 组 OI 破骨细胞的吸收能力高于 WTCAL-WTOC 组(27. 76 + 22. 81 对比 7. 32 + 5. 09，$P < 0.001$)。**结论** oim/oim 小鼠破骨细胞-颅骨培养体系中破骨细胞的数目明显减少，成骨细胞支持破骨细胞分化能力减低；但其破骨细胞骨吸收活性明显增强，以代偿成骨细胞功能，维持骨再建过程中成骨过程及骨吸收过程的平衡。

关键词：破骨细胞；先天性成骨不良；破骨细胞-颅骨联合培养模型

In vitro study of absorption activity in osteoclasts of congenial osteogenesis imperfecta through calvaria-osteoclast co-culture system

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Abstract: **Objective** The major clinical manifestations of osteogenesis imperfecta (OI) includes abnormal bone mineralization, bone mass loss, skeletal deformities, and fractures. However, the pathogenesis of OI, especially the abnormalities in osteoblast (OB) and osteoclast (OC) activity in bone remodeling is not well defined. This paper aimed to explore the functional variation and interaction of OB and OC during the bone remodeling by in vitro OC-Calvaria (CAL) co-culture system in a mouse model of OI, *oim/oim*. **Methods** Calvaria culture model was used in this paper. The OBs originated from the calvaria and spread into the culture plate, providing support for differentiation of OC precursors into osteoclasts. Two co-culture systems were used in experiment, including WTCAL-WTOC as control group and OICAL-OIOC as experimental group. OCs was identified using tartrate-resistant acid phosphatase (TRAP) staining and OBs was identified using histochemical assessment of alkaline phosphatase (ALP) staining after 14 days. OC/OB ratio was calculated. The bone absorption activity of OCs was defined using the percentage of absorption pits in the whole calvarial surface. **Results** The number of OC in the OICAL-OIOC group (92. 50 ± 23. 18/mm^2)

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were less that that in the WTCAL-WTOC (379.00 ± 136.53/mm^2) group after 14 days ($P < 0.01$). OC/OB ratio in OICAL-OIOC group (0.68 ± 0.57) was significantly lower than that in the WTCAL-WTOC group (1.65 ± 0.67, $P < 0.01$). The bone absorption ability of OCs in the OICAl-OIOC group (27.76 ± 22.81) was significantly higher than that in the WTCAl-WTOC group (7.32 ± 5.09, $P < 0.001$). **Conclusion** The number of OCs in OICAL-OIOC group significantly and osteoclastogenesis ability supported by OBs decrease significantly. The absorption ability of OCs in the OICAL-OIOC group increases to compensate the OBs function and to maintain the balance of bone absorption and bone formation during bone remodeling.

Key words: Osteoclast; Congenial osteogenesis imperfect; Osteocalst-calvaria

先天性成骨不全是一种骨 I 型胶原蛋白基因缺陷的先天性疾病[1,2]。基因突变最多的位置在 COL1A1 或 COL1A2 甘氨酸残基的位点[3]。先天性成骨不全临床表现虽有一定变异,但主要特点有骨矿化过程不良、骨量丢失、骨骼畸形和多发骨折[4]。

现阶段先天性成骨不良的治疗目标是增加患者的骨量、增加皮质骨的厚度并减少松质骨的成分[5,6]。组织形态学研究表明 OI 患者的骨再建速度加快,可能缘于其破骨细胞的活动性能增强,导致骨组织总量减少[7]。有关破骨细胞的形态剂量学研究表明先天性成骨不全患者的骨吸收表面百分比增加,但是单位破骨细胞吸收功能减低[8]。作者前期研究表明研究证实 oim/oim 小鼠模型的破骨细胞 (Osteoclast, OC) 虽然具有正常破骨细胞表型 (CD61 及 CD51),但胞体较大,内含较多的细胞核及吞噬颗粒,并有较强的骨吸收能力[9]。但至今尚缺乏有关 OI 体内骨再建过程破骨细胞骨吸收活性的实验研究。

oim/oim 小鼠的染色体突变位置在于第 6 染色体,靠近 COLA-2 基因位点。小鼠临床表现类似于 OI 第一和第三型,如显示出骨皮质变薄、长骨畸形、多发骨折。由于该小鼠在基因突变位点,生物化学特性和临床表型均类似于 OI 患者[10]。该小鼠为研究 OI 发病机理中的骨再建过程提供了良好的模型[11]。

研究骨再建的试验模型有多种,其中包括颅骨或长骨骨组织培养模型[12,13]。不同种模型可提供不同的研究骨再建途径[14]。本实验采用了破骨细胞-颅骨联合培养体系,在本模型中,成骨细胞和破骨细胞维持了活体组织的离体结构,这为研究骨再建及破骨细胞活性提供了良好的试验手段。

1 材料与方法

1.1 实验分组

破骨细胞-颅骨模型的原理是利用颅骨组织中成骨细胞游离并分布到颅骨表面及培养皿表面,从而支持骨髓原始细胞中前体破骨细胞分化成成熟的破骨细胞,并吸收颅骨表面骨组织。选用 5 ~ 8 周大小的 10 只 B6C3Fe 野生型(WT)和 oim/oim(OI)小鼠(Jackson Laboratory, Harbor, ME, USA)。手术取出其颅骨,去除软组织,培养颅骨组织,以支持破骨细胞分化。共分成两组:(1) WTCAL-WTOC;(2) OICAL-OIOC。各组共重复实验 6 次。

1.2 颅骨组织培养

颅骨组织培养参照前研究[15]。以无菌手术取出 5 ~ 8 周 WT 及 oim/oim 小鼠颅骨组织。在 PBS 溶液中洗涤颅骨。以孔钻取 3mm 大小的颅骨组织片,见图 1A。颅骨组织在原始培养基 (alpha modified essential medium [α-MEM] containing L-glutamine, nucleoside (GIBCO, Invitrogen Corp., USA), supplemented with 10% fetal bovine serum

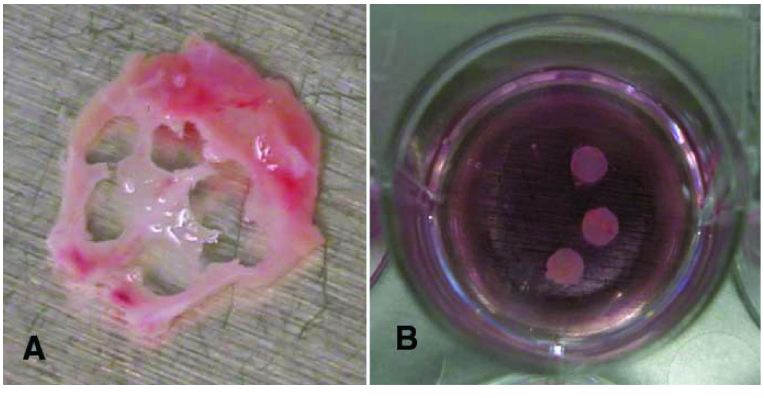

图 1 A:颅骨片取材后的 oim/oim 小鼠颅骨;B: 24 孔培养板中颅骨片

(GIBCO), and 1% antibiotics [penicillin and streptomycin] (GIBCO)) 中培养 24 小时待用。

1.3 破骨细胞-颅骨培养体系

骨髓基质细胞取材参照前实验[16]。利用已被取颅骨的小鼠,无菌取出其胫骨及股骨,清除软组织。切除两侧骨端,以原始培养基冲洗出骨髓。以 PBS 溶解红细胞。原始骨髓组织在有 5 ng/ml recombinant murine M-CSF (Pepro Tech, Rocky Hill, NJ, USA)中培养过夜。随后取悬浮细胞,种植在已培养过夜的颅骨于 24 孔培养板中,浓度为 0.5 (10^7/每孔,见图 1B。于原始培养基添加 10^{-8} mol/L 1,25-$(OH)_2D_3$ and 10^{-6} mol/L prostaglandine E2 以支持成骨细胞再生。于第 14 日取出颅骨片及培养板做组化染色及骨吸收研究。

1.4 组织化学染色研究

本实验以酒石酸酶(TRAP)及碱性磷酸酶(ALP)双染色方法同时鉴别体系中 OB 及 OC;TRAP 辨别破骨细胞,即多核细胞(3 个核以上),染色为红色。ALP 染色识别 OB,为蓝色,见图 2。以 10 倍显微镜扫描培养孔中的 OB 及 OC 数量。二者相除得出破骨细胞/成骨细胞比例。这一比例可作为成骨细胞支持破骨细胞能力的参照。

图 2 对照组培养体系中 ALP 及 TRAP 双染色
A:第 14 日仅 ALP 染色显示模型中成骨细胞
B:ALP-TRAP 染色显示红色多核破骨细胞,及浅蓝色成骨细胞

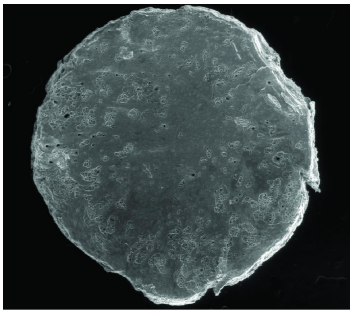

图 3 第 14 日 OIOC 组,扫描电镜下颅骨片表面骨吸收陷窝(×80)

1.5 扫描电镜研究骨吸收活性

以漂白液清除颅骨表面上的细胞,并迅速于 PBS 内洗涤 3 ~ 5 次。颅骨被放大 80 倍,以 Scanning Electronic Microscope FEI Model XL30 取其图像,见图 3。以 Image J 软件计算骨吸收陷窝占颅骨总面积的百分比。以该百分比除以培养皿中的总破骨细胞数,从而得出单位破骨细胞骨吸收能力。

1.6 统计学处理

两组数据以 Independent-Samples T Test 统计,小于 0.05 为显著性差异。

2 结果

2.1 OB 和 OC 数

第 14 日,两组间 OB 数目无区别。与 WTCAL-WTOC 组相比,OICAL-OIOC 组的 OC 数明显减少[(92.50 ± 23.18)/mm^2 对比 (379.00 ± 136.53)/mm^2, $P < 0.01$],见图 4;OICAL-OIOC 组的 OC/OB 比例明显减低(0.68 ± 0.57 对比 1.65 ± 0.67, $P < 0.01$),见图 5。该结果显示 OICAL-OIOC 组成骨细胞支持破骨细胞骨再生能力明显减低。

2.2 骨吸收功能研究

第 14 日,两组破骨细胞骨吸收活性显示,OICAl-OIOC 组较 WTCAL-WTOC 组明显增强(27.76 ± 22.81 对比 7.32 ± 5.09, $P < 0.001$),见图 6。这表明,OI 破骨细胞的骨吸收能力较对照组明显增强。

3 讨论

3.1 本实验首次建立了破骨细胞-颅骨联合培养模型,揭示了骨再建过程中,成骨细胞与破骨细胞功能及对于骨吸收过程的调节作用。以往颅骨骨组织培养模型多用于研究成骨细胞分化[17]、骨缺损修复[18]和骨再生过程[19]。ANDERSSON[20]曾应用颅骨组织培养研究骨再建过程,但作者并未观察到颅骨表面有骨吸收陷窝。本实验成功利用颅骨与其自身骨髓细胞培养,在无生长因子环境下,分化成成熟破骨细胞,并在颅骨片表面形成骨陷窝,扫描电镜直接显示了破骨细胞吸收活性。目前其他破骨细胞研究多应用破骨细胞分化因子,如 RANKL,在玻璃[10]、牙片或塑料培养板[21]中刺激前体破骨细胞分化。与这些模型相比,本实验颅骨及骨髓组织取自同一小鼠,最大限度的模仿了体内骨再建中骨吸

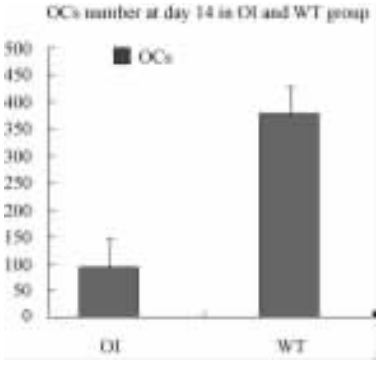

图 4 破骨细胞-颅骨联合培养体系中破骨细胞数的统计结果

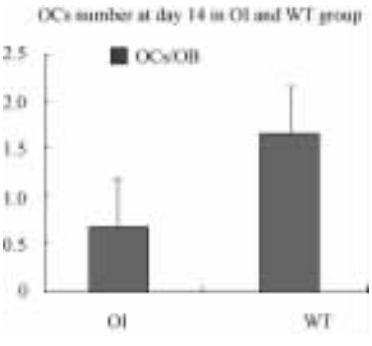

图 5 第 14 天破骨细胞-颅骨联合培养体系中 OC/OB 比例

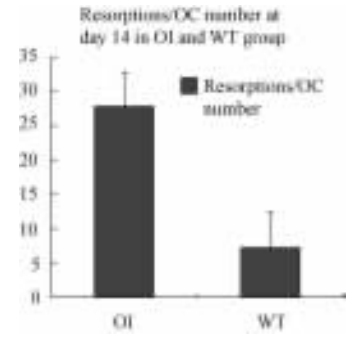

图 6 破骨细胞-颅骨联合培养体系中骨吸收活性实验结果

收过程及其调节机制。其次,颅骨组织培养最大限度的保存了活体组织内细胞与细胞间、细胞与骨基质间的立体空间结构及体内微环境,模仿了 OI 或其他骨疾病病理机制。最后,其他模型中破骨细胞吸收的底物为玻璃、塑料或牙板,而非自体骨组织;而本模型为克服了这一缺点,这样会去除其他影响因素,例如免疫排斥等。本颅骨-破骨细胞联合培养模型体系应用于骨再建研究过程尚属国内外首创。

3.2 由于 OI 患者发病年龄、临床类型等因素变异,有关 OI 骨再建缺陷的机理及解释并不统一。Baron[22]等发现 OI 患者骨量减低但其骨转化率加快,作者认为 OI 患者的成骨细胞功能出现障碍,但其骨量并未减低,而非体内骨吸收过程加快。Jones[23]等发现,OI 五型患者骨再建障碍主要由于患者体内矿化不良。Cepollaro[24]发现 OI 患者的 BMD 较正常人明显减少。McCarthy[25]则发现 OI 患者的类骨质表面减少,骨转化率明显减低。有关 OI 患者 OB 和 OC 细胞数量的研究也存在较大的分歧。Iwamoto[8]认为 OI 患者的骨吸收表面增加,OC 数量增加,患者骨量减低,其原因主要为破骨细胞活性增加而成骨细胞功能减低。Munns[26]等发现 OI 患者的 OB 和 OC 数均较正常增加。Rauch[27]等发现 OI 患者 OC 数量在二磷酸钠治疗前后均明显减少。Ste-Marie[28]则发现 OI 患者 OC 明显减少,而 OB 却较正常增加。所有以上研究结果表明 OI 骨再建中成骨细胞、破骨细胞及其相互作用的研究还有着较大分歧,且尚无实验依据。

3.3 本实验显示 oim/oim 小鼠的成骨细胞数量与对照组无区别,但破骨细胞数量减少,因模型内无破骨细胞生长因子,表明 OI 组成骨细胞促破骨细胞分化能力降低。该结果支持有关 OI 破骨分化障碍的临床发现。本实验发现 OI 组破骨细胞的吸收功能较对照组增强。作者认为系因为 OI 小鼠破骨细胞为代偿其细胞数减少的一种现象,反映了骨再建中成骨及破骨过程平衡的一种机制。这一点也支持作者前期体外破骨细胞培养实验所证实的 oim/oim 小鼠破骨细胞活性增强的发现[9]。所不同的是,前期实验引用了 RANKL 作为生长因子,促进破骨细胞分化,而本实验应用了自身活体成骨细胞诱导其破骨细胞分化。

现阶段研究证实成骨细胞可支持破骨细胞分化并与破骨细胞间存在密切的相互作用[29]。目前研究证实 OI 患者和 oim/oim 小鼠的成骨细胞存在缺陷[30,31]。本实验证实虽然 oim/oim 小鼠颅骨细胞可支持其破骨细胞分化,但其支持破骨细胞分化的功能明显弱于对照组。目前治疗 OI 患者主要应用二磷酸钠等抗破骨细胞类药物,但 OI 患者破骨细胞活性增强是其骨再建过程中代偿机制。如果该机制被二磷酸钠等药物抑制,将导致 OI 患者骨再建失衡,并导致其他如骨折、骨脆化、骨硬化等副作用[32]。因此,需近一步探讨如何保持 OI 患者正常的骨代谢及骨量平衡。

3.4 综上所述,本试验首次采用了破骨细胞-颅骨联合培养模型研究 oim/oim 小鼠骨再建缺陷的病理机制,证实 oim/oim 小鼠成骨细胞支持破骨细胞分化过程存在缺陷,但其破骨细胞吸收能力却增强,以代偿破骨细胞减少及维持 oim/oim 小鼠体内的骨量平衡。本研究提出不同的 OI 骨再建理论,为今后有关 OI 基础及临床应用研究提供了实验模型和理论基础。

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应用颅骨-破骨细胞联合培养体系研究先天性成骨不全破骨细胞骨吸收活性

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