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· 基础研究 ·

miR-34a骨粉复合胶原基水凝胶促进辐照区骨缺损修复

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【摘要】 目的　探讨负载miR-34a的Bio-Oss®骨粉与转谷氨酰胺酶交联明胶(transglutaminase crosslinked gelatin，Col-Tgel)的联合使用对辐照损伤大鼠骨髓间充质干细胞(bone marrow mesenchymal stem cells，BMSCs)的成骨分化作用及对辐照区骨缺损修复的作用。方法　本实验已获得单位实验动物伦理委员会批准。取2周龄SD大鼠长骨骨髓，培养BMSCs并进行鉴定。当BMSCs生长至贴满瓶底80%时，进行2 Gy剂量X线照射，制备BMSCs辐照损伤模型备用。将2.5、5 μL Col-Tgel分别加入10 mg Bio-Oss®骨粉(P)中，制备复合骨替代材料PG-2.5和PG-5，通过体外和体内实验筛选骨粉与水凝胶合适比例。将lipofectamine 2000分别与Cy3-agomiR-34a、agomiR-34a或agomiR NC混合，然后将各组混合液分别加入10 mg Bio-Oss®骨粉(P)并进行冷冻干燥。将上述负载各组miR的10 mg Bio-Oss®骨粉和未负载miR的Bio-Oss®骨粉分别与2.5 μL Col-Tgel混合，制备PG-Cy3-miR-34a、PG-miR-34a、PG-miR NC、PG组复合骨替代材料。将辐照后的BMSCs与PG-Cy3-miR-34a组复合骨替代材料共培养，使用共聚焦显微镜观察转染效果。将辐照后的BMSCs与PG-miR-34a组、PG-miR NC组、PG组复合骨替代材料共培养，使用RT-qPCR检测miR-34a表达、CCK-8检测细胞增殖，并在成骨诱导14 d后利用RT-qPCR检测成骨相关基因Runt相关转录因子2(Runt related transcription factor 2，Runx2)、碱性磷酸酶(alkaline phosphatase，ALP)、骨钙素(osteocalcin，OCN)的表达。取8周龄SD大鼠进行双侧胫骨15 Gy剂量X线照射，3周后在胫骨干骺端骨骺线下方2~3 mm处制备直径3 mm、深度2 mm的胫骨缺损，缺损区分别置入PG-miR-34a组、PG-miR NC组、PG组复合骨替代材料，植入8周后取材行micro-CT和HE切片观察体内骨缺损修复效果。结果　2 Gy辐照影响BMSCs成骨分化能力，辐照组ALP染色浅于非辐照组，辐照组茜素红染色矿化结节少于非辐照组。10 mg Bio-Oss®骨粉与2.5 μL Col-Tgel构建的复合材料具有较好的操作性能和成骨性能，并用于后续实验。PG-Cy3-miR-34a可将负载的Cy3-agomiR-34a转染入辐照损伤BMSCs中。PG-miR-34a可提高辐照损伤BMSCs中miR-34a的表达水平，对细胞增殖无抑制作用，并能显著促进成骨相关基因Runx2、ALP、OCN的表达。在辐照区骨缺损修复实验中，micro-CT显示PG-miR-34a组骨缺损区新生骨体积高于其他组，HE切片染色结果也验证了PG-miR-34a可促进骨缺损修复。结论　miR-34a骨粉复合胶原基水凝胶可促进辐照损伤BMSCs体外成骨分化，促进辐照区骨缺损修复。

【关键词】 骨粉；　骨髓间充质干细胞；　成骨分化；　骨修复；　miR-34a；
转谷氨酰胺酶交联明胶；　辐照损伤；　放疗

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Combinational use of miR-34a functionalized bone powder with Col-Tgel enhances bone regeneration in irradiated bone defects

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【**Abstract**】 **Objective** To study the effect of the combinational use of miR-34a-functionalized Bio-Oss® bone powder with transglutaminase crosslinked gelatin (Col-Tgel) on the osteoblastic differentiation of bone marrow mesenchymal stem cells (BMSCs) and bone defect healing after irradiation. **Methods** The experiment was approved by the Animal Ethics Committee. BMSCs were isolated from the bone marrow of 2-week-old Sprague-Dawley (SD) rats and identified. After reaching 80% confluence, BMSCs were irradiated with 2 Gy of X-ray radiation to establish a radiation-damaged BMSC model for further experimentation. 2.5 μL or 5 μL of Col-Tgel was mixed with 10 mg of Bio-Oss® (P) to prepare PG-2.5 and PG-5. The optimal proportion of Bio-Oss® (P) and Col-Tgel was determined through *in vitro* and *in vivo* experiments. Cy3-labeled agomiR-34a, agomiR-34a, or agomiR NC was mixed with lipofectamine 2000 and added to 10 mg of Bio-Oss® (P). The mixtures were lyophilized, and 2.5 μL Col-Tgel was added to each group of lyophilized Bio-Oss®/lipofectamine/miRNA complexes or to 10 mg of Bio-Oss® to obtain PG-Cy3-miR-34a, PG-miR-34a, PG-miR NC, and PG. Irradiated BMSCs were cocultured with PG-Cy3-miR-34a to evaluate cellular uptake of Cy3-agomiR-34a using confocal microscopy. Then, irradiated BMSCs were cocultured with PG-miR-34a, PG-miR NC, and PG. The expression of miR-34a was tested by RT-qPCR and cell proliferation was tested by CCK-8 assay. After 14 days of osteogenic induction, the mRNA expression of Runt-related transcription factor 2 (Runx2), alkaline phosphatase (ALP), and osteocalcin (OCN) was tested by RT-qPCR. The bilateral tibias of 8-week-old SD rats were irradiated with a single dose of 15 Gy of X-ray radiation. Three weeks later, tibial defects with a diameter of 3 mm and a depth of 2 mm were created 2-3 mm below the epiphyseal line in the tibial metaphysis. The composite bone substitute materials of PG-miR-34a, PG-miR NC, and PG were implanted into the defect area. Eight weeks after implantation, the tibias were harvested and evaluated for bone regeneration using micro-CT analysis and HE staining. **Results** The results demonstrated that 2 Gy irradiation adversely affected the osteogenic differentiation capacity of BMSCs, evidenced by the decreased ALP staining and number of mineralized nodules stained with Alizarin red in the irradiated group compared to the non-irradiated group. The composite material consisting of 10 mg Bio-Oss® and 2.5 μL Col-Tgel exhibited good osteogenic induction capability and handling properties and was used for subsequent experiments. The PG-Cy3-miR-34a could deliver the loaded Cy3-agomiR-34a into irradiated BMSCs. PG-miR-34a enhanced the expression of miR-34a in irradiated BMSCs without affecting cell proliferation. PG-miR-34a significantly upregulated the expression of osteogenic-related genes, including Runx2, ALP, and OCN. In the experiment of bone defect healing in irradiated tibias, micro-CT analysis showed that PG-miR-34a group had a higher bone volume in the bone defect area compared to other groups. The HE staining results also confirmed that implantation of PG-miR-34a can promote the healing of bone defects in irradiated tibias. **Conclusion** The combinational use of miR-34a-functionalized Bio-Oss® bone powder with Col-Tgel could promote the osteogenic differentiation of irradiated BMSCs and enhance bone regeneration in irradiated bone defects.

【**Key words**】 bone powder; bone marrow mesenchymal stem cells; osteogenic differentiation; bone repair; miR-34a; transglutaminase crosslinked gelatin; radiation damage; radiotherapy

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手术结合放疗是恶性肿瘤的重要治疗方式，但放疗区骨缺损修复是治疗中的重要挑战。血管化自体骨移植是放疗区骨缺损修复的首选方法，但需开辟第二术区，且感染风险较高[1]。利用高压氧作为辅助治疗，可改善修复效果，但其疗效仍具有争议[2]。采用骨替代材料进行修复也是常用的治疗方式，但辐照区骨再生能力受损，修复效果不理想[3]。干细胞[4-5]和细胞因子可用于促进辐照区骨再生，与骨替代材料结合使用可改善放疗区骨缺损愈合[6-7]，但有促进肿瘤复发的风险[8]。因此，

制备适合放疗区骨缺损修复的新型骨替代材料具有重要意义。

miR-34a是一种具有抑瘤作用的miRNA[9]，miR-34a也参与成骨调控。前期研究发现miR-34a可促进辐照损伤后骨髓间充质干细胞（bone marrow mesenchymal stem cells，BMSCs）的成骨分化和辐照区骨缺损修复[10]。在辐照区骨缺损修复中利用骨替代材料携带并释放miR-34a，可在促进局部成骨功能的同时，利用其抑瘤作用降低肿瘤的复发风险，从而具有良好的安全性和临床应用前景。

放疗区骨组织抗感染能力差[11]。颗粒型骨替代材料缺乏内聚性，在临床应用中由于松散而延长临床操作时间，增加感染风险。使用赋形剂可提高颗粒型骨替代材料的内聚性和可塑性，便于临床操作[12]。水凝胶类材料是常见的赋形剂，包括透明质酸[13]、明胶[14]、壳聚糖[15]等。转谷氨酰胺酶交联明胶（transglutaminase crosslinked gelatin，Col-Tgel）是一种酶交联水凝胶，是明胶片段在转谷氨酰胺酶的作用下相互交联形成，具有良好的生物相容性、低免疫原性和可控成胶的特点[16]，可作为赋形剂提高骨替代材料的可操作性。

本研究将采用冷冻干燥法将miR-34a负载于Bio-Oss®骨粉颗粒表面，然后将Bio-Oss®骨粉与胶原基质水凝胶复合，通过体内外实验验证其对放疗区骨缺损的修复作用。

1　材料和方法

1.1　主要试剂和仪器

主要试剂：α-MEM培养基（11900073，Gibco，美国），胎牛血清（11011，四季青，中国），PBS（G4202，赛维尔，中国），抗坏血酸（49752，Sigma，美国），地塞米松（HY-14648，MCE，美国），β-甘油磷酸钠（G5422，Sigma，美国），Col-Tgel（P1720H，Bioruo，中国），Bio-Oss®骨粉（82100724，Geistlish，瑞士），Lipofectamine 2000（11668019，Invitrigon，美国），U6反转录引物及RT-qPCR引物（Q1124、锐博，中国），miR-34a反转录引物及RT-qPCR引物（U1018、U0712、V0525，锐博，中国），agomiR-34a（miR-4000085，锐博，中国），agomiR control（miR4N0000002-4-5，锐博，中国）。

主要仪器：冷冻干燥机（LyoQuest，Teslstar，西班牙），荧光定量PCR仪器（CFX，BIO-RAD，美国），酶标仪（Multiscan，Thermo，美国），倒置荧光显微镜（IX83，OLYMPUS，日本），正置荧光显微镜（BX63，OLYMPUS，日本），激光共聚焦显微镜（880，Carl Zeiss，德国），X线辐照仪（X-Rad 320，PRECISION，美国），micro-CT（AX-2000，Always Imaging，中国）。

1.2　BMSCs的培养、鉴定与辐照

本实验经中国人民解放军陆军军医大学实验动物福利伦理审查委员会审批通过（AMU-WEC2020042）。取2周SD大鼠长骨骨髓，使用全骨髓培养法培养BMSCs，BMSCs行三系诱导分化鉴定，成脂诱导14 d后行油红O染色，成骨诱导21 d后行茜素红染色，成软骨诱导28 d后行阿利新蓝染色。BMSCs生长至贴满瓶底80%时，进行剂量为2 Gy的单次X线照射，剂量率为1.1 Gy/min[10]。BMSCs辐照6 h后进行消化并制备细胞悬液，接种于各组骨替代材料。

1.3　复合骨替代材料的制备

Col-Tgel与Bio-Oss®骨粉混合材料的制备：将2.5 μL或5 μL Col-Tgel加入10 mg Bio-Oss®骨粉（P）中，制备复合骨替代材料PG-2.5和PG-5。

复合骨替代材料制备：200 μL无酶去离子水中加入5 μL lipofectamine 2000，轻柔吹打混匀，室温静置5 min；200μL无酶去离子水加入Cy3-agomiR-34a、agomiR-34a或agomiR NC（500pmol）轻柔吹打混匀，室温静置5 min；将各组miRNA与转染试剂lipofectamine 2000稀释液吹打混匀，室温静置20 min；上述各组混合液与10mg Bio-Oss®骨粉混合，快速放置于冰上静置15 min后移入−80 ℃冰箱内放置2 h；将上述样品转移到已预冷至−80 ℃的真空冷冻干燥机内，保持温度−80℃冻干24 h，将2.5μL Col-Tgel分别加入负载了Cy3-agomiR-34a、agomiR-34a、agomiR NC、未负载miR的10 mg Bio-Oss®骨粉中，制备PG-Cy3-miR-34a、PG-miR-34a、PG-miR NC、PG复合骨替代材料。

1.4　Col-Tgel与Bio-Oss®骨粉混合材料对辐照后BMSCs成骨分化作用检测

将P、PG-2.5和PG-5组骨替代材料置于12孔板中，加入2 Gy辐照的BMSCs，48 h后更换为成骨诱导液。细胞成骨诱导14 d后利用RT-qPCR检测Runx2、ALP、OCN基因表达，引物序列见表1。

1.5　Col-Tgel与Bio-Oss®骨粉混合材料对辐照区骨缺损修复的作用检测

8周SD大鼠进行双侧胫骨15 Gy射线照射，剂量率为1.1 Gy/min，辐照3周后进行骨缺损模型的

建立，在胫骨干骺端骨骺线下方2 ~ 3mm处制备直径为3mm、深度2mm的胫骨缺损[10]。缺损区置入P组、PG-2.5组、PG-5组复合骨替代材料，植入8周后取材行HE染色，采用Image J分析骨缺损区域内骨面积/总面积。

1.6　复合骨替代材料的转染效果检测

将PG-Cy3-miR-34a组、PG-miR NC组、PG组复合骨替代材料置于12孔板中，加入2 Gy辐照的BMSCs，48 h后hoechst染细胞核，使用共聚焦显微镜观察并拍照；将PG-miR-34a组、PG-miR NC组、PG组复合骨替代材料置于12孔板中，加入2 Gy辐照的BMSCs，48 h后提取RNA，RT-qPCR检测miR-34a表达。

1.7　细胞增殖检测

将PG-miR-34a组、PG-miR NC组、PG组复合骨替代材料置于12孔板中，加入2Gy辐照的BMSCs，于培养2、4、7 d采用CCK-8试剂盒检测细胞增殖，孵育2 h后，每孔吸出200 μL溶液于96孔板，酶标仪检测450 nm波长的吸光度。

1.8　复合骨替代材料对辐照后BMSCs成骨分化作用检测

将PG-miR-34a组、PG-miR NC组、PG组复合骨替代材料置于12孔板中，加入2 Gy辐照的BMSCs，48 h后更换为成骨诱导液。细胞成骨诱导14 d后利用RT-qPCR检测ALP、Runx2、OCN基因表达，引物序列见表1。

表1　RT-qPCR引物

Table 1　Primers used for RT-qPCR

Gene	Forward primer sequence (5′-3′)	Reverse primer sequence (5′-3′)
Runx2	5′ AGA CCA GCA GCA CTC CAT AT 3′	5′ CTC ATC CAT TCT GCC GCT AGA 3′
ALP	5′ ATG GCT CAC CTG CTT CAC G 3′	5′ TCA GAA CAG GGT GCG TAG G 3′
OCN	5′ AGG GCA GTA AGG TGG TGA AT 3′	5′ GCA TTA ACC AAC ACG GGG TA 3′
GAPDH	5′ GGCACAGTCAAGGCTGAGAATG 3′	5′ ATGGTGGTGAAGACGCCAGTA 3′

Runx2: runt related transcription factor 2；ALP: alkaline phosphatase；OCN: osteocalcin

1.9　复合骨替代材料对辐照区骨缺损修复的作用检测

8周SD大鼠进行双侧胫骨单次15 Gy剂量照射，剂量率为1.1 Gy/min，辐照3周后进行骨缺损模型的建立，在胫骨干骺端骨骺线下方2 ~ 3 mm处制备直径为3 mm、深度2 mm的胫骨缺损[10]。缺损区置入PG-miR-34a组、PG-miR NC组、PG组复合骨替代材料，植入8周后取材行显微CT（micro computed tomography，micro-CT）检测（Always Imaging，中国），扫描分辨率为9 μm，采用VG StudioMAX（Volume Graphics，德国）进行三维重建和分析，感兴趣区域（ROI）为原骨缺损部位（L，2 mm；φ，3 mm），阈值28 500为骨与材料，阈值33 550为材料，阈值内体积相减为新生骨体积，比较骨体积分数（bone volume fraction，BV/TV）。检测完成后进行脱钙处理，用于HE染色。

1.10　统计学分析

统计学分析软件为Graphpad prism 8，计量资料以均数±标准差表示，采用单因素方差分析，组间使用Tukey post-hoc检验，$P < 0.05$时认为差异有统计学意义。

2　结　果

2.1　辐照对BMSCs成骨分化的影响

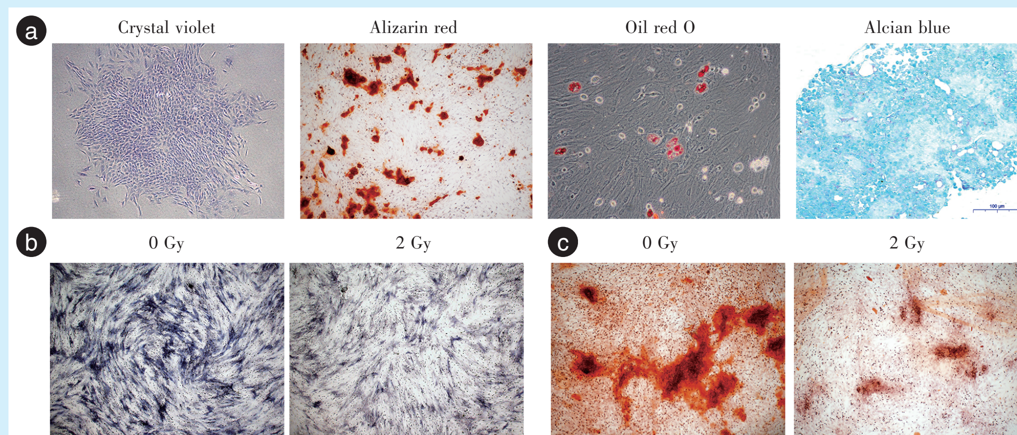
培养的细胞符合BMSCs特点，原代细胞成集落生长，经成骨诱导后可成矿化结节，成脂诱导后可观测到脂滴形成，成软骨诱导后可形成软骨组织（图1a）。2 Gy辐照影响BMSCs成骨分化能力。成骨诱导1周，辐照组ALP染色比非辐照组浅（图1b）；成骨诱导3周，辐照组茜素红染色矿化结节少于非辐照组（图1c）。

2.2　不同比例Col-Tgel与Bio-Oss®骨粉混合后对Bio-Oss®骨粉操作性能的影响

Bio-Oss®骨粉（P）可塑形性差，不易转移，接触水后易被冲散。2.5μL Col-Tgel与Bio-Oss®骨粉混合后（PG-2.5）可赋予Bio-Oss®骨粉黏性，Bio-Oss®骨粉不易分散，但可定型性不佳，用镊子转移时易被夹散。5 μL Col-Tgel与Bio-Oss®骨粉混合后（PG-5）可赋予Bio-Oss®骨粉可定形性，不易分散（图2）。

2.3　不同比例Col-Tgel与Bio-Oss®骨粉混合后对成骨功能的影响

接种2 Gy辐照的BMSCs并进行体外成骨诱导14 d，P、PG-2.5、PG-5组之间Runx2、ALP、OCN表达



a: characterization of BMSCs. The colony of primary BMSCs stained with crystal violet (×100), mineral node stained with Alizarin red (×40), fat droplets stained with oil red O (×200), proteoglycans stained with Alcian blue (scale bar: 100 μm). b: ALP staining of non-irradiated and 2 Gy-irradiated BMSCs after 7 days of osteogenic induction (×40). c: Alizarin red staining of non-irradiated and 2 Gy-irradiated BMSCs after 21 days of osteogenic induction (×40). BMSCs: bone marrow mesenchymal stem cells, ALP: alkaline phosphatase

Figure 1 Osteoblastic differentiation of non-irradiated and 2 Gy-irradiated BMSCs

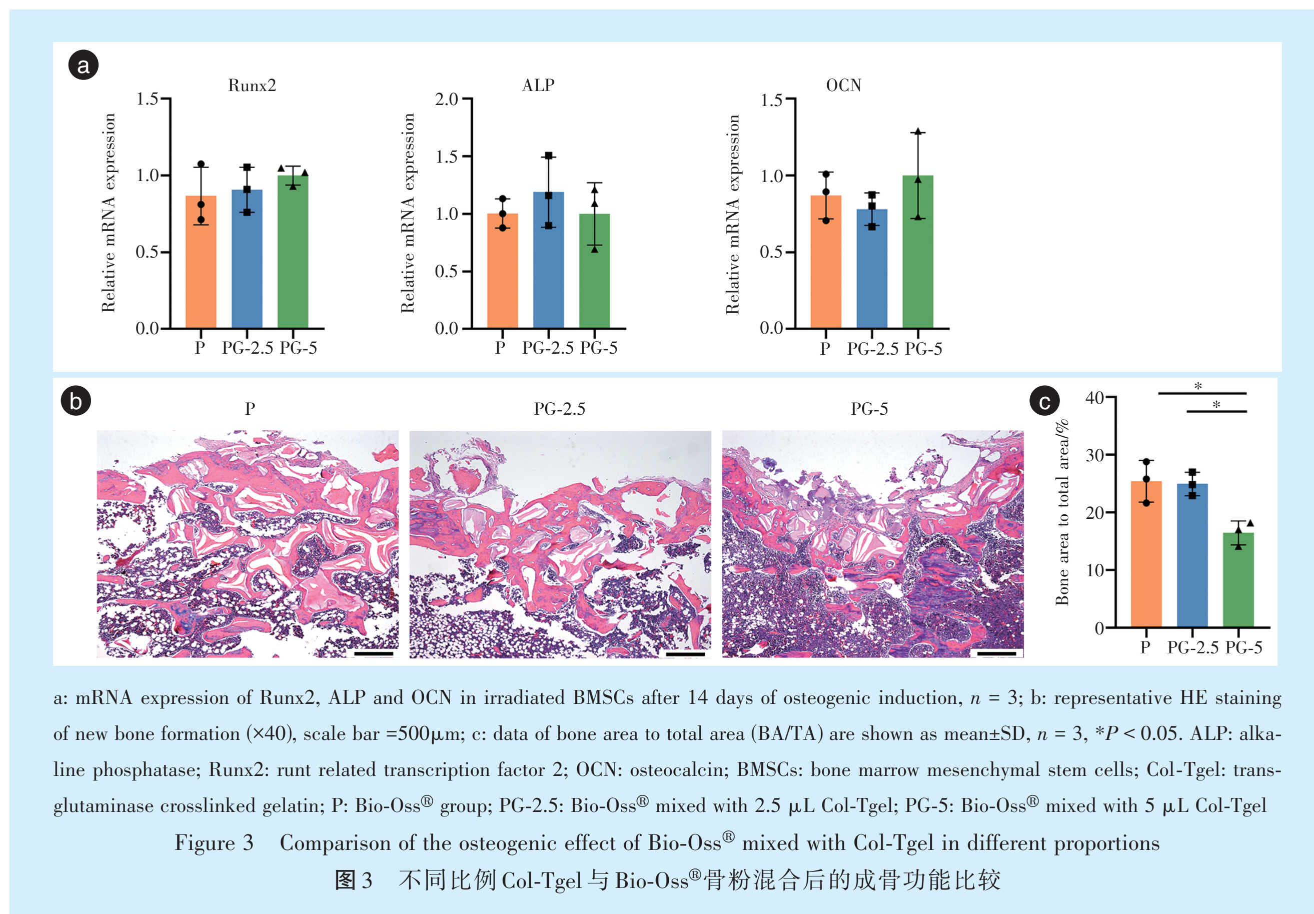
图1 2 Gy剂量辐照对BMSCs成骨分化的影响

a: morphology of P, PG-2.5 or PG-5. b: mobility of P, PG-2.5 or PG-5. c: stabilization of P, P-2.5 or P-5 was tested by immersion in water. d: filling rat tibial bone defects with P, PG-2.5 or PG-5. e: rat tibial bone defects filled with P, PG-2.5 or PG-5. Col-Tgel: transglutaminase crosslinked gelatin; P: Bio-Oss® group; PG-2.5: Bio-Oss® mixed with 2.5 μL Col-Tgel; PG-5: Bio-Oss® mixed with 5 μL Col-Tgel

Figure 2 Handling properties of Bio-Oss® mixed with Col-Tgel

图2 Col-Tgel对Bio-Oss®骨粉操作性能的影响

无统计学差异($P > 0.05$)(图3a)。3组材料修复大鼠胫骨缺损实验发现,各组均可见骨粉颗粒及新骨形成,P组和PG-2.5组骨轮廓恢复较好,PG-5组骨轮廓恢复不佳(图3b)。3组间骨缺损区骨面积/总面积(BA/TA)有统计学差异($F = 10.6$,$P = 0.011$),P组和PG-2.5组BA/TA大于PG-5组($P = 0.015$,$P = 0.019$)(图3c)。基于以上结果,选择PG-2.5比例进行后续实验。



a: mRNA expression of Runx2, ALP and OCN in irradiated BMSCs after 14 days of osteogenic induction, $n = 3$; b: representative HE staining of new bone formation (×40), scale bar =500μm; c: data of bone area to total area (BA/TA) are shown as mean±SD, $n = 3$, *$P < 0.05$. ALP: alkaline phosphatase; Runx2: runt related transcription factor 2; OCN: osteocalcin; BMSCs: bone marrow mesenchymal stem cells; Col-Tgel: transglutaminase crosslinked gelatin; P: Bio-Oss® group; PG-2.5: Bio-Oss® mixed with 2.5 μL Col-Tgel; PG-5: Bio-Oss® mixed with 5 μL Col-Tgel

Figure 3 Comparison of the osteogenic effect of Bio-Oss® mixed with Col-Tgel in different proportions

图3 不同比例Col-Tgel与Bio-Oss®骨粉混合后的成骨功能比较

2.4 miR-34a骨粉-Col-Tgel复合材料可提高辐照损伤BMSCs中miR-34a的表达水平

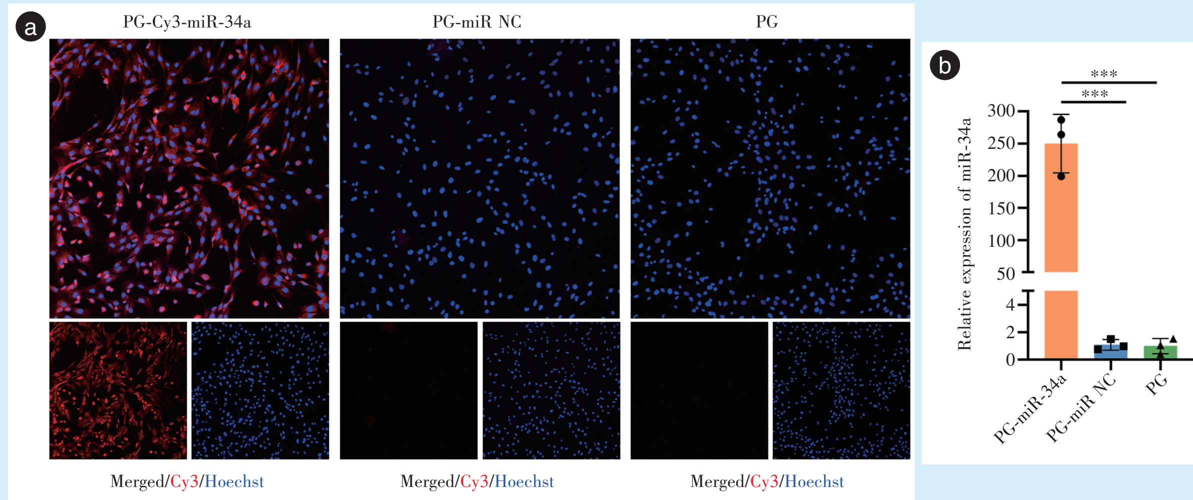
共聚焦显微镜观察发现,2Gy辐照的BMSCs与PG-Cy3-miR-34a共培养后,Cy3标记的agomiR-34a可进入细胞,BMSCs显示红色荧光(图4a)。RT-qPCR检测结果显示,PG-miR-34a可显著提高miR-34a表达水平($F = 89.69$,$P < 0.001$)(图4b)。

2.5 miR-34a骨粉-Col-Tgel复合材料促进辐照损伤BMSCs体外成骨分化

2 Gy辐照后的BMSCs接种于PG-miR-34a组、PG-miR NC组和PG组复合骨替代材料,培养2、4、7 d,CCK8检测结果显示3组材料对细胞增殖无明显差异($P > 0.05$)(图5)。接种于3组复合材料并成骨诱导2周后,检测成骨相关基因表达。RT-qPCR结果显示,PG-miR-34a组成骨相关基因表达高于PG-miR NC组(Runx2:$P = 0.050$,ALP:$P < 0.001$,OCN:$P = 0.003$)和PG组(Runx2:$P = 0.033$,ALP:$P < 0.001$,OCN:$P = 0.002$),PG-miR NC组和PG组间表达水平无差异($P > 0.05$)(图6)。

2.6 miR-34a骨粉-Col-Tgel复合材料促进辐照区骨缺损修复

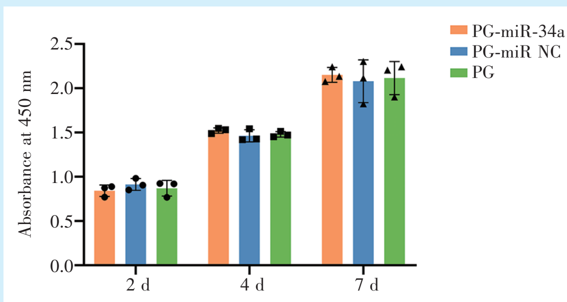
Micro-CT检测骨缺损修复术后8周各组新骨形成情况,各组均可见骨缺损区新骨形成,仍能看到骨粉颗粒存在,新生骨组织与骨粉颗粒交织、融合。PG-miR-34a组骨皮质连续性恢复较好,PG-miR NC组和PG组骨皮质区存在空隙(图7a)。三组间骨体积/总体积(BV/TV)存在统计学差异($F = 61.20$,$P < 0.001$),PG-miR-34a组新生高于其他两组($P < 0.001$)(图7b)。HE染色结果显示,PG-miR-34a组骨粉颗粒周围有大量新骨沉积,骨与骨粉颗粒间融合,PG-miR NC组和PG组骨粉颗粒周围也形成新骨沉积,但部分骨粉颗粒间存在结缔组织(图7c)。



a: images of 2 Gy-irradiated BMSCs cultured with PG-Cy3-miR-34a, PG-miR NC or PG (×100). b: miR-34a expression determined by RT-qPCR in BMSCs 48 hours after transfection. n = 3; $^{***}P < 0.001$. BMSCs: bone marrow mesenchymal stem cells; Col-Tgel: transglutaminase crosslinked gelatin; PG-Cy3-miR-34a: Cy3-agomiR-34a loaded Bio-Oss® mixed with Col-Tgel; PG -miR-34a: agomiR-34a loaded Bio-Oss® mixed with Col-Tgel; PG-miR NC: agomiR control loaded Bio-Oss® mixed with Col-Tgel; PG: Bio-Oss® mixed with Col-Tgel

Figure 4 Effect of PG-miR-34a on the expression level of miR-34a in irradiated BMSCs

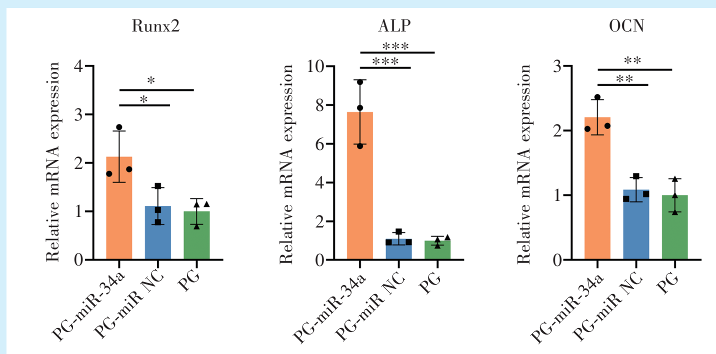
图4 miR-34a骨粉-Col-Tgel复合材料对辐照损伤BMSCs中miR-34a表达水平的影响



Two Gy-irradiated BMSCs were cultured with PG-miR-34a, PG-miR NC or PG. Cell proliferation was measured by CCK-8 2, 4 and 7 days after culture (n = 3). BMSCs: bone marrow mesenchymal stem cells; Col-Tgel: transglutaminase crosslinked gelatin; PG-miR-34a: agomiR-34a loaded Bio-Oss® mixed with Col-Tgel; PG-miR NC: agomiR control loaded Bio-Oss® mixed with Col-Tgel; PG: Bio-Oss® mixed with Col-Tgel

Figure 5 Effect of PG-miR-34a on the proliferation of irradiated BMSCs

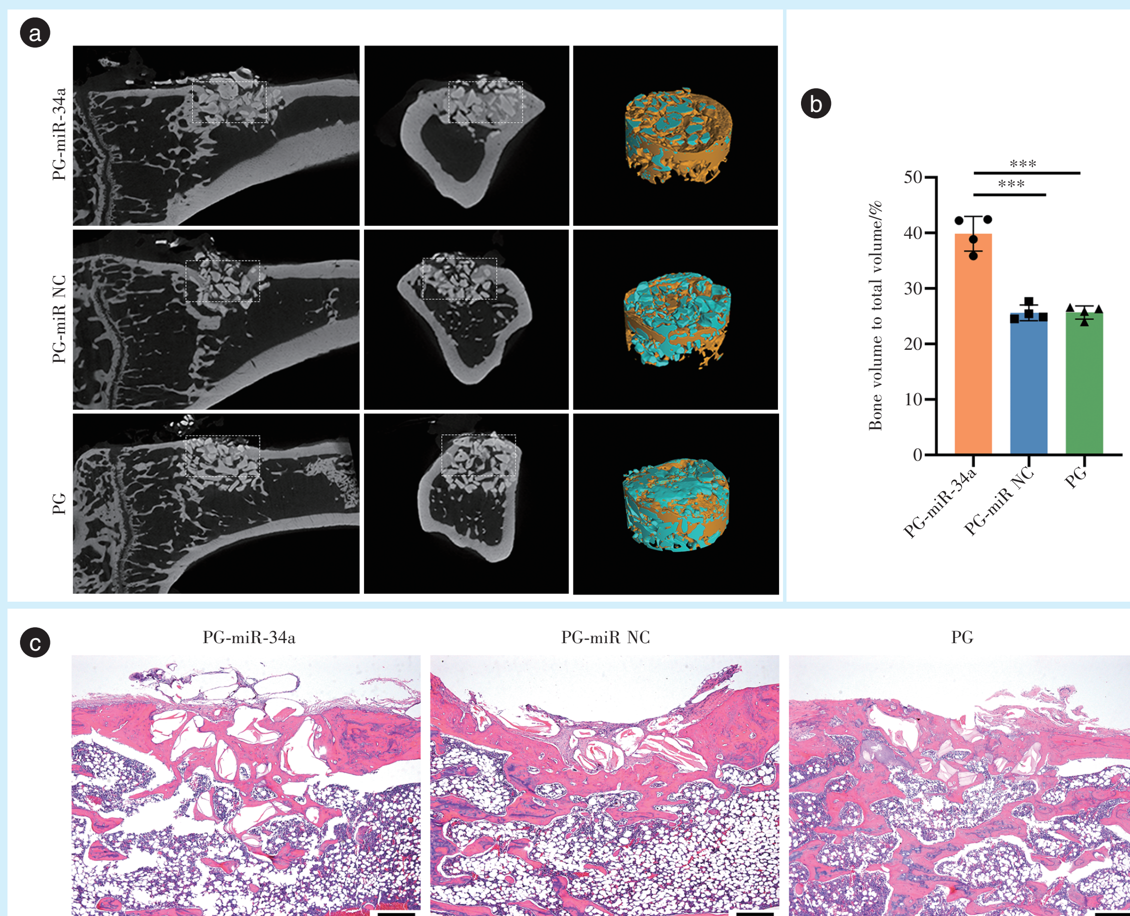
图5 miR-34a骨粉-Col-Tgel复合材料对辐照损伤BMSCs增殖的作用



Two Gy-irradiated BMSCs were cultured with PG-miR-34a, PG-miR NC or PG. Gene expression of Runx2, ALP and OCN were tested by RT-qPCR after 14 days of osteogenic induction. Data are shown as mean±SD, n = 3; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. BMSCs: bone marrow mesenchymal stem cells; Col-Tgel: transglutaminase crosslinked gelatin; PG-miR-34a: agomiR-34a loaded Bio-Oss® mixed with Col-Tgel; PG-miR NC: agomiR control loaded Bio-Oss® mixed with Col-Tgel; PG: Bio-Oss® mixed with Col-Tgel; Runx2: runt related transcription factor 2; ALP: alkaline phosphatase; OCN: osteocalcin

Figure 6 Inducing effect of PG-miR-34a on the *in vitro* osteogenesis of irradiated BMSCs

图6 miR-34a骨粉-Col-Tgel复合材料对辐照损伤BMSCs体外成骨的诱导作用



a: two- and three-dimensional (2D and 3D) micro-CT images of bone formation in the defect area of irradiated tibias 8 weeks after transplantation. The dotted boxes indicate the original bone defects. In the 3D images, the yellow parts represent the newly formed bone, and the blue parts represent bone substitudes. b: the morphometric analysis of BV/TV for micro-CT. Data are shown as the mean ± SD, n = 4; $^{***}P < 0.001$. c: representative HE staining of new bone formation (×40), scale bar =500μm. Col-Tgel: transglutaminase crosslinked gelatin; PG-miR-34a: agomiR-34a loaded Bio-Oss® mixed with Col-Tgel; PG-miR NC: agomiR control loaded Bio-Oss® mixed with Col-Tgel; PG: Bio-Oss® mixed with Col-Tgel

Figure 7 Effect of PG-miR-34a on bone formation in irradiated bone defects

图7 miR-34a骨粉-Col-Tgel复合材料对辐照区骨缺损修复的作用

3 讨 论

多项研究将胶原基水凝胶与骨替代材料联合使用改进骨替代材料性能[17]。胶原基水凝胶可作为赋形剂改善颗粒状骨替代材料的塑形性，形成凝胶后的三维网状结构可改善骨替代材料的力学性能[14, 18]。同时，胶原基水凝胶还可作为载体传递药物、生长因子和干细胞。本实验所使用的Col-Tgel是一种酶交联胶原基水凝胶，其凝胶强度可控，高强度Col-Tgel可用于促进骨修复[19]。在联合使用两种材料时，二者的特性及含量对复合材料的性能有较大影响。10 mg Bio-Oss®颗粒型骨粉与5 μL Col-Tgel混合后，Bio-Oss®骨粉颗粒被水凝胶包裹，在水凝胶凝固前，复合材料具有较好的可塑性。10 mg Bio-Oss®骨粉与2.5 μL Col-Tgel混合后，骨粉颗粒互相黏附，但由于凝胶含量少，部分颗粒容易分散。单纯Bio-Oss®骨粉缺乏黏附性呈松散状，不易塑形。置于培养板内，两组复合材料组胶原基水凝胶可作为黏性介质提高Bio-Oss®骨粉颗粒内聚性，滴加液体时不易冲散，单纯Bio-Oss®骨粉易被冲散。BMSCs在体外成骨诱导中，3种材料对成骨相关基因表达无明显影响。在骨缺损修复实验中，与单纯Bio-Oss®骨粉组相比，两组复合材料易充填于骨缺损处。8周后PG-2.5组与Bio-Oss®骨粉组均得到了较好的修复，PG-5组骨皮质轮廓形态恢复不良。这可能与凝胶体积增加，影响成骨相关细胞进入凝胶内部有关，影响颗粒型骨替

代材料发挥骨引导作用，成骨相关细胞以复合材料表面为引导形成新骨。本实验选择10 mg与2.5 μL的比例以提高骨替代材料的操作性，且不影响体内骨修复效果。本研究采用大鼠胫骨缺损模型进行筛选，骨缺损范围较小，与临床应用存在一定差异，后续研究需利用其他动物模型制备较大范围骨缺损，进一步筛选合适的比例。

miRNA可用于构建生物活性支架材料促进骨再生[20]。有机材料如壳聚糖[21-22]、聚乙二醇[21, 23]、聚乙烯亚胺[24]等可用作miRNA载体或支架材料用于调控局部成骨环境促进骨再生。无机材料如金属材料[25-26]和磷酸钙类材料[27]也可通过物理吸附或化学连接与miRNA复合。材料表面介导的转染具有较高的转染效率，细胞既可与材料表面的转染复合物直接接触，又可吸收从材料表面进入培养基的转染复合物[28]。笔者在前期研究中发现，miR-34a通过冷冻干燥法与羟基磷灰石纳米颗粒结合后仍具有转染能力[29]。但纳米磷灰石颗粒不直接用作充填类骨替代材料。在本研究中选择临床常用的Bio-Oss®骨粉作为加载miR-34a的支架材料，构建更符合临床治疗中使用的功能性骨替代材料。加载miR-34a的骨粉与Col-Tgel结合后接种辐照后的BMSCs，可提高细胞内miR-34a水平。

miR-34a参与骨代谢调控，可促进BMSCs[30]、牙根尖乳头干细胞、人脂肪间充质干细胞的成骨分化。已有研究将miR-34a加载于植入材料促进骨损伤修复，miR-34a可通过羟基磷灰石/介孔有机二氧化硅纳米颗粒加载于金属植入物促进骨折愈合[31]。在前期研究中，笔者发现miR-34a可促进辐照后骨再生[10]。本实验中所构建的miR-34a复合骨替代材料也可通过促进辐照损伤BMSCs的成骨相关基因表达促进体外成骨分化，用作辐照区骨缺损处的骨充填材料可促进骨缺损修复。恶性肿瘤手术后的骨缺损修复会因放疗和化疗而影响骨再生能力，使用生物活性因子可促进局部骨再生，但又会带来肿瘤复发的风险。miR-34a可通过调控细胞凋亡、细胞周期、肿瘤干细胞干性等抑制肿瘤发生、生长和转移[32]，用于肿瘤手术后的骨缺损修复具有生物安全性，但仍需进一步验证。

综上，本研究将miR-34a模拟物通过冷冻干燥法加载于Bio-Oss®骨粉表面并以Col-Tgel为黏性载体构建复合骨替代材料，该材料可促进辐照损伤条件下BMSCs的体外成骨分化，用作骨缺损充填材料可促进辐照区骨缺损修复，为促进放疗后骨缺损修复重建提供了新方法。

【Author contributions】 Liu H and Wu X performed the experiments and wrote the article. Liu H designed the study and revised the article. All authors read and approved the final manuscript as submitted.

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