

LIPUS调控FOXO1磷酸化促进氧化损伤的人 牙周膜干细胞成骨分化的作用研究

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摘要 该研究探讨低强度脉冲超声(low-intensity pulsed ultrasound, LIPUS)对氧化损伤的人牙周膜干细胞(human periodontal ligament stem cells, hPDLSCs)成骨分化的保护作用及机制。采用浓度为300 μmol/L的H₂O₂构建细胞氧化损伤模型, 将其分为对照组、H₂O₂组、LIPUS组和LIPUS+H₂O₂组。DCFH-DA探针和TBA比色法分别检测各组细胞活性氧(reactive oxygen species, ROS)和丙二醛(malondialdehyde, MDA)水平; 碱性磷酸酶(alkaline phosphatase, ALP)和茜素红染色分别评估各组细胞早期及晚期成骨分化能力; WB(Western blot)评价抗氧化酶[过氧化氢酶(catalase, CAT)和超氧化物歧化酶(superoxide dismutase 2, SOD-2)]、成骨相关蛋白[矮小相关转录因子2(runt-related transcription factor-2, RUNX2)、ALP和骨桥蛋白(osteopontin, OPN)]、叉头框蛋白O1(forkhead box protein O1, FOXO1)和(phospho-FOXO1, p-FOXO1)表达水平。随后采用FOXO1抑制剂AS1842856(100 nmol/L)处理细胞, 实验分为对照组、H₂O₂+二甲基亚砜(dimethyl sulfoxide, DMSO)组、LIPUS+H₂O₂+DMSO组和LIPUS+H₂O₂+AS1842856组。CCK-8(cell counting kit-8)法检测细胞活力; DCFH-DA探针检测各组细胞ROS水平, WB检测各组RUNX2、ALP和OPN的蛋白表达情况。结果发现与空白对照组相比, H₂O₂组ROS与MDA水平均升高, 抗氧化酶蛋白表达下调($P<0.0001$); ALP和茜素红阳性染色以及成骨相关蛋白表达水平均显著降低($P<0.0001$); p-FOXO1/FOXO1蛋白值上升($P<0.0001$)。与H₂O₂组比较, LIPUS+H₂O₂组的ROS与MDA水平均下降, 抗氧化酶蛋白表达上调($P<0.0001$); 成骨染色及相关蛋白表达量显著增加($P<0.0001$); p-FOXO1/FOXO1蛋白值降低($P<0.0001$)。相较于LIPUS+H₂O₂+DMSO组, LIPUS+H₂O₂+AS1842856组细胞活力下降, p-FOXO1/FOXO1蛋白表达上调, ROS水平升高且成骨分化相关蛋白表达量显著降低($P<0.0001$)。研究结果显示LIPUS通过调节FOXO1的磷酸化水平, 发挥抗氧化防御作用并提高氧化损伤的hPDLSCs成骨分化能力。

关键词 低强度脉冲超声; 人牙周膜干细胞; 氧化损伤; 叉头框蛋白O1; 成骨分化

LIPUS Regulates FOXO1 Phosphorylation to Promote Osteogenic Differentiation of Oxidatively Damaged Human Periodontal Stem Cells

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Abstract This study was to investigate the protective effect and mechanism of LIPUS (low-intensity pulsed ultrasound) on osteogenic differentiation of oxidatively damaged hPDLSCs (human periodontal ligament stem cells). The cellular oxidative damage model was constructed by using H₂O₂ at an action concentration of 300 μmol/L, which was divided into the control, H₂O₂, LIPUS and LIPUS+H₂O₂ groups. Cellular ROS (reactive oxygen species) and MDA (malondialdehyde) levels were detected by DCFH-DA probe and TBA colorimetric assay in each group, respectively. ALP (alkaline phosphatase) and alizarin red staining were used to assess the early and late osteogenic differentiation capacities of each group, respectively. WB (Western blot) evaluation of antioxidant enzymes [CAT (catalase) and SOD-2 (superoxide dismutase 2)], osteogenesis-related protein [Runx2 (runt-related transcription factor-2), ALP and OPN (osteopontin)], FOXO1 (forkhead box protein O1) and p-FOXO1 (phospho-FOXO1). Subsequently, the cells were treated with FOXO1 inhibitor AS1842856 (100 nmol/L), and the experiments were divided into control, H₂O₂+DMSO, LIPUS+H₂O₂+DMSO, and LIPUS+H₂O₂+AS1842856 groups. CCK-8 (cell counting kit-8) assay detected cell viability, DCFH-DA probe detected the level of cellular ROS in each group, and WB detected the protein expression of RUNX2, ALP, and OPN in each group. Compared with the control group, both ROS and MDA levels were elevated and antioxidant enzyme protein expression was down-regulated in the H₂O₂ group ($P<0.0001$); ALP and alizarin red positive staining as well as expression of osteogenesis-related protein were significantly reduced ($P<0.0001$); and the p-FOXO1/FOXO1 protein ratio rose ($P<0.0001$). Compared with the H₂O₂ group, the LIPUS+H₂O₂ group showed decreased ROS and MDA levels and upregulated expression of antioxidant enzyme proteins ($P<0.0001$); ALP and alizarin red positive staining as well as protein expression of osteogenesis-related were significantly increased ($P<0.0001$); and p-FOXO1/FOXO1 protein ratio decreased ($P<0.0001$). Compared with the LIPUS+H₂O₂+DMSO group, the LIPUS+H₂O₂+AS1842856 group showed decreased cell viability, up-regulation of p-FOXO1/FOXO1 protein expression, elevated ROS levels and significantly decreased protein expression of osteogenic differentiation ($P<0.0001$). The results revealed that LIPUS exerted antioxidant defense and improved osteogenic differentiation of oxidatively damaged hPDLSCs by regulating the phosphorylation level of FOXO1.

Keywords low-intensity pulsed ultrasound; human periodontal stem cells; oxidative damage; forkhead box protein O1; osteogenic differentiation

牙周炎是一种由牙菌斑细菌引起的慢性病理性炎症,引起牙周组织不可逆的渐进性破坏^[1]。临床上牙周炎的治疗方式主要包括机械清创、抗生素治疗与外科干预等。上述疗法虽然可以延缓牙周炎进展,但无法恢复已经受损的牙周组织结构及其功能^[2]。

多项研究提示,活性氧(reactive oxygen species, ROS)累积所形成的氧化应激微环境是导致牙周炎发生发展的核心因素^[3-5]。氧化应激能够降低牙周膜干细胞(periodontal ligament stem cells, PDLSCs)的成骨分化能力,PDLSCs的成骨能力降低则被认为是限制牙周炎牙槽骨再生的关键环节^[6-7]。

低强度脉冲超声(low-intensity pulsed ultrasound, LIPUS)作为一种安全无创的机械刺激,已被报道能够有效抵抗氧化应激、促进骨再生与控制炎症进程等^[8]。YING等^[7]认为在牙周炎中,LIPUS可以通过降低细胞内ROS水平进而部分恢复PDLSCs成骨活性并减轻牙槽骨破坏,提示LIPUS可作为有潜力的新兴物理疗法用于促进牙周炎骨再生,但其具体作用机制仍有待被阐明。研究报道叉头框蛋白O1(forkhead box protein O1, FOXO1)能够响应机械应力,并且可能作为关键调节因子参与成骨细胞抗氧化防御、细胞增殖与骨形成等过程^[9-11]。因此,本研究

拟采用H₂O₂构建人牙周膜干细胞氧化损伤模型,探讨LIPUS的抗氧化和成骨分化保护作用,以及FOXO1在这个过程中可能发挥的调控作用,为LIPUS在临床上治疗牙周炎提供理论基础与实验依据。

1 材料与方法

1.1 主要试剂和仪器

低强度脉冲超声(LIPUS)仪由重庆融海超声医学工程研究中心研制;人牙周膜干细胞购自上海赛百慷生物技术股份有限公司;质量分数3%的H₂O₂水溶液、DMSO购自美国Sigma-Aldrich公司; α -MEM培养基购自大连美仑生物技术有限公司;胎牛血清购自美国Gibco公司;人相关干成骨诱导分化试剂盒购自广州赛业生物科技有限公司;1%茜素红染色液购自北京Solarbio科技有限公司;ROS检测试剂盒、MDA检测试剂盒、ALP染色试剂盒均购自上海碧云天生物技术有限公司;AS1842856购自美国MCE公司;RUNX2、ALP、OPN、SOD-2、CAT和 β -actin抗体均购自武汉三鹰生物技术有限公司;4-HNE、3-NT、FOXO1和p-FOXO1抗体均购自美国CST公司。

1.2 方法

1.2.1 细胞培养 将hPDLSCs培养于含10%胎牛血清、1%双抗的 α -MEM培养基中,置于37℃、5%CO₂细胞培养箱中培养,每2天换液1次,细胞密度达到80%即可进行传代。

1.2.2 CCK-8 将细胞以 5×10^3 /孔、每孔100 μ L接种于96孔板,培养24 h后进行处理,处理结束后,弃去孔内液体,每孔加入100 μ L培养基及10 μ L CCK-8工作液,置于37℃孵育2 h,用酶标仪在波长为450 nm处测定各孔吸光度(D)值。

1.2.3 成骨诱导及设计分组 将hPDLSCs以50%的密度接种于24孔板,相应处理如下:普通培养基培养1天后更换为成骨培养基(1 mL/孔),每隔3天换液1次。实验分为四组:对照组(Control)、H₂O₂组(300 μ mol/L H₂O₂诱导6 h)、LIPUS组(照射剂量100 mW/cm²,照射时间10 min/次)、LIPUS+H₂O₂组。

1.2.4 ROS检测 将细胞接种于6孔板,培养24 h后按分组处理,然后将6孔板中的细胞与1 mL在 α -MEM中稀释的0.1% DCFH-DA在37℃下避光孵育30 min,用PBS冲洗2次后在荧光显微镜下观察荧光强度。

1.2.5 MDA检测 将细胞接种于6孔板,培养24 h后按分组处理,收集细胞并提取蛋白,使用BCA蛋

白测定试剂盒测定蛋白浓度,根据上海碧云天生物技术有限公司MDA检测试剂盒说明书步骤,检测MDA活性。MDA浓度以mol/g蛋白表示。

1.2.6 碱性磷酸酶(ALP)染色 将细胞接种于24孔板,培养24 h后进行分组处理,成骨诱导7天后,根据试剂盒说明书按比例配制染色液。取出细胞后用PBS洗涤2次,4%多聚甲醛室温固定1 min, PBS再洗2次,加入染色液工作液,在37℃孵箱中避光孵育30 min,镜下呈现蓝紫色染色时弃染色液,加少量PBS,显微镜下拍照。

1.2.7 茜素红染色 将细胞接种于24孔板,培养24 h后进行分组处理,成骨诱导21天后,取出细胞用PBS清洗2次,4%多聚甲醛室温固定1 min, PBS再洗2次,加1%茜素红染液,在37℃孵箱中避光孵育30 min,镜下观察到红色钙结节,即可吸出染色液,加少量PBS,显微镜下拍照。

1.2.8 Western blot 分组处理后,取出细胞,先用PBS清洗细胞2次,然后每孔加入上样缓冲液与电泳液SDS-PAGE体积比为1:4的溶液,静置2 min,待细胞完全脱落后吸入EP管中,100℃放置10 min,使蛋白完全变性。每组取10 μ L蛋白上样,电泳后根据蛋白分子量选择合适的切胶范围以及转膜时间,切下的胶条进行半干转膜。转膜完毕后TBST清洗5 min \times 3次(下面用于清洗的溶液均为TBST),快速封闭液室温封闭25 min,洗5 min \times 3次。加入相应的一抗(RUNX2、ALP、OPN、SOD-2、CAT及 β -actin, 1:1 000稀释;4-HNE、3-NT、FOXO1及p-FOXO1, 1:500稀释),置于4℃孵育12~14 h。洗5 min \times 3次,再加入相应HRG标记的IgG二抗(1:5 000稀释),室温摇床孵育1 h。洗5 min \times 3次,用化学发光法进行曝光显影。

1.2.9 AS1842856处理 将hPDLSCs用AS1842856(100 nmol/L)或DMSO(体积分数为0.02%)处理。实验分为对照组、H₂O₂+DMSO组、LIPUS+H₂O₂+DMSO组和LIPUS+H₂O₂+AS1842856组。

1.2.10 统计学处理 以上各项实验均进行3次重复独立实验,Graphpad Prism 9.0用于统计分析,用均数 \pm 标准差($\bar{x} \pm s$)来表示计量资料。各组间差异比较则采用单因素方差分析(One-Way ANOVA)这种统计学方法。 $P < 0.05$ 为差异有统计学意义。

2 结果

2.1 H₂O₂构建hPDLSCs氧化损伤模型

采用不同浓度的H₂O₂处理细胞6 h,与对照组相

比, 300 $\mu\text{mol/L}$ H_2O_2 作用后细胞活力降低($P<0.01$), 400、500 $\mu\text{mol/L}$ H_2O_2 处理后细胞活力显著下降($P<0.0001$), 呈浓度依赖性。因此, 选取300 $\mu\text{mol/L}$ 的 H_2O_2 作用浓度作为后续实验条件(图1)。

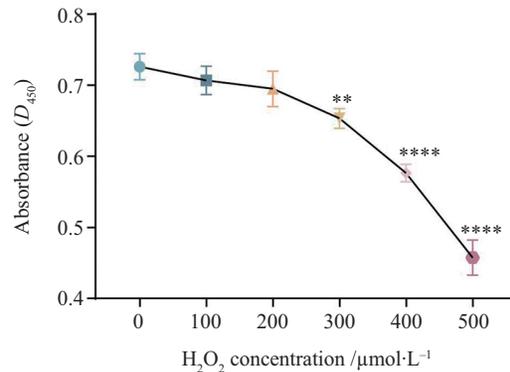
2.2 在 H_2O_2 诱导的氧化应激环境下LIPUS能够发挥抗氧化防御作用

与对照组相比, H_2O_2 组细胞的ROS和MDA水平明显升高($P<0.0001$), 同时, 抗氧化酶CAT和SOD-2的蛋白表达量明显降低($P<0.0001$)。与 H_2O_2 组比

较, LIPUS+ H_2O_2 组胞内ROS与MDA水平显著下降($P<0.0001$), CAT和SOD-2的蛋白表达量明显增多($P<0.0001$)(图2和图3)。结果表明, LIPUS能够降低 H_2O_2 诱导的胞内ROS与MDA水平, 上调 H_2O_2 抑制的抗氧化酶表达, 发挥抗氧化防御作用。

2.3 LIPUS提高氧化损伤的hPDLSCs成骨分化能力

与对照组相比, H_2O_2 组的阳性蓝紫色和红色钙结节区域明显减少, 成骨分化相关蛋白RUNX2、ALP和OPN的表达量显著降低($P<0.0001$)。与 H_2O_2

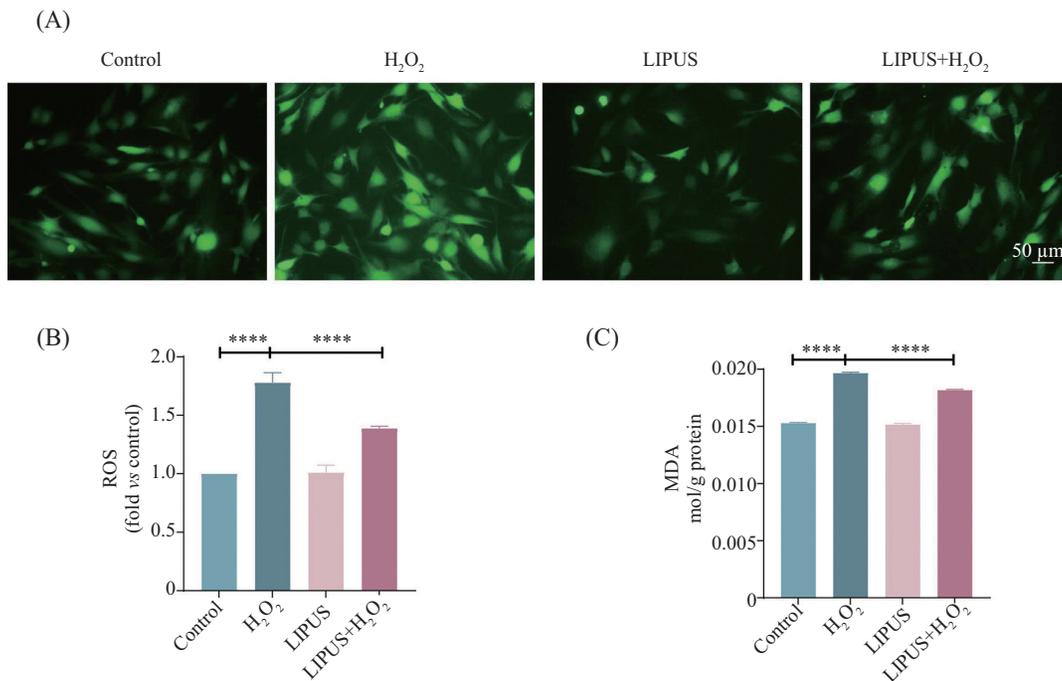


CCK-8法筛选适宜的 H_2O_2 作用浓度, ** $P<0.01$, **** $P<0.0001$, 与0 $\mu\text{mol/L}$ 组比较。

Screening of suitable H_2O_2 action concentration by CCK-8 method, ** $P<0.01$, **** $P<0.0001$ compared with 0 $\mu\text{mol/L}$ group.

图1 CCK-8法筛选适宜 H_2O_2 作用浓度构建hPDLSCs氧化损伤模型

Fig.1 Screening of suitable H_2O_2 action concentration by CCK-8 method for constructing oxidative damage model of hPDLSCs



A: DCFH-DA染色; B: 图A DCFH-DA染色相关定量分析; C: MDA检测试剂盒测定各组MDA水平。**** $P<0.0001$ 。

A: DCFH-DA staining; B: figure A DCFH-DA staining correlation quantitative analysis; C: determination of MDA level of each group by MDA detection kit. **** $P<0.0001$.

图2 各组细胞内ROS和MDA水平比较

Fig.2 Comparison of intracellular ROS and MDA levels in each group

组比较, LIPUS+H₂O₂组的阳性染色区域明显增加, RUNX2、ALP和OPN的蛋白表达量显著增加($P<0.0001$)(图4和图5)。结果表明LIPUS可以提高H₂O₂作用下受损的成骨分化能力。

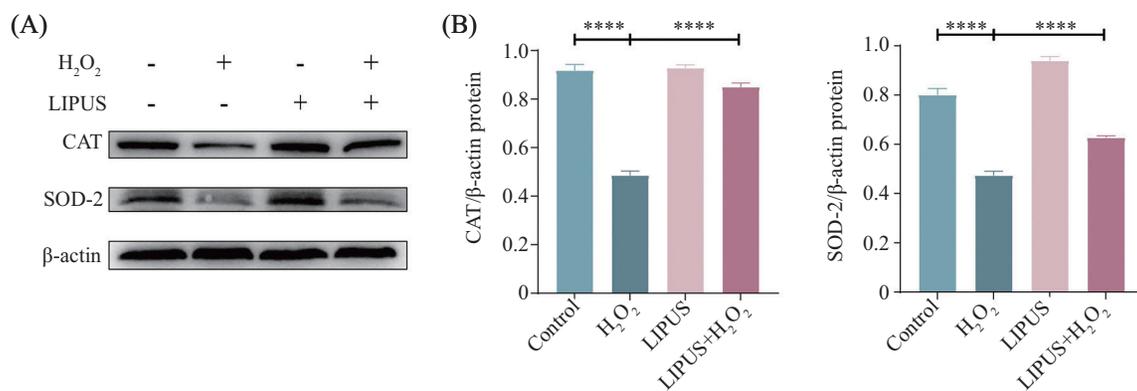
2.4 LIPUS抑制氧化应激诱导的FOXO1磷酸化

与对照组比较, H₂O₂处理后 p-FOXO1/FOXO1蛋白表达水平明显增加($P<0.0001$)。与H₂O₂组相比, LIPUS+H₂O₂组 p-FOXO1/FOXO1蛋白表达水平显著下降($P<0.0001$)(图6)。结果提示在氧化应激环境下

FOXO1磷酸化水平升高, 然而, LIPUS可以抑制氧化诱导的FOXO1磷酸化表达。

2.5 FOXO1可能是LIPUS发挥氧化损伤保护作用的关键调控靶点

与H₂O₂+DMSO组比较, LIPUS+H₂O₂+DMSO组细胞活力增加($P<0.0001$), p-FOXO1/FOXO1蛋白值显著降低($P<0.0001$), 胞内ROS水平明显下降($P<0.0001$)且成骨分化相关蛋白RUNX2、ALP和OPN表达量显著上调($P<0.0001$)。采用FOXO1抑制剂AS1842856处理后, 与LIPUS+H₂O₂+DMSO组相比,

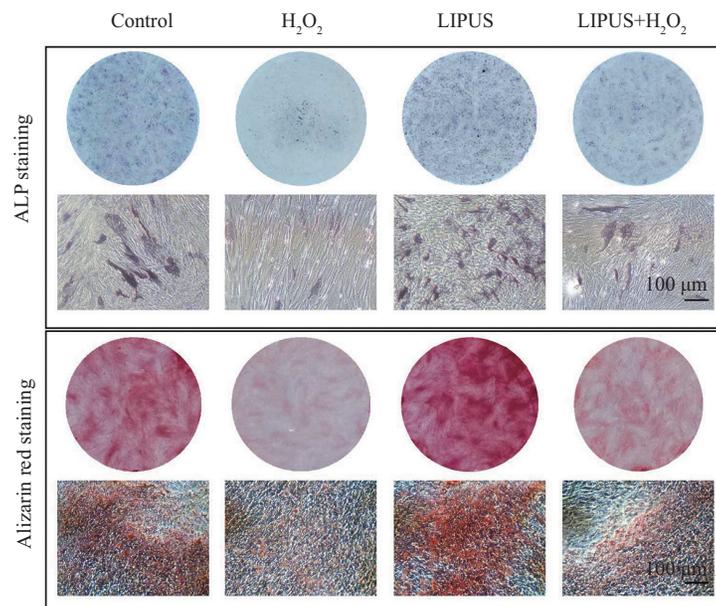


A: Western blot检测各组抗氧化酶相关蛋白CAT与SOD-2表达情况; B: 相应灰度值分析。**** $P<0.0001$ 。

A: Western blot to detect the expression of antioxidant enzyme-related protein CAT and SOD-2 in each group; B: corresponding gray value analysis. **** $P<0.0001$.

图3 各组细胞抗氧化酶的蛋白表达水平

Fig.3 Protein expression levels of antioxidant enzymes in each group

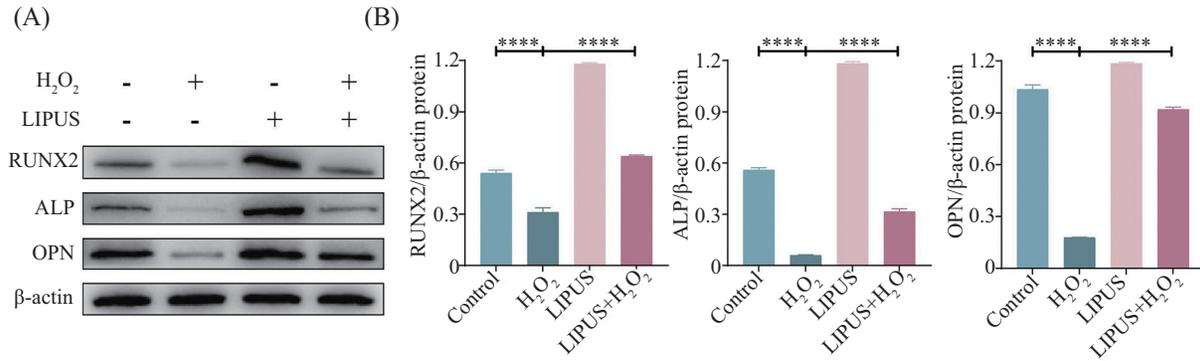


ALP和茜素红染色观察各组细胞早期及晚期成骨分化能力。

ALP and alizarin red staining to observe the early and late osteogenic differentiation ability of each groups.

图4 LIPUS作用对H₂O₂诱导下的hPDLSCs成骨分化的影响

Fig.4 Effect of LIPUS action on H₂O₂-induced osteogenic differentiation of hPDLSCs

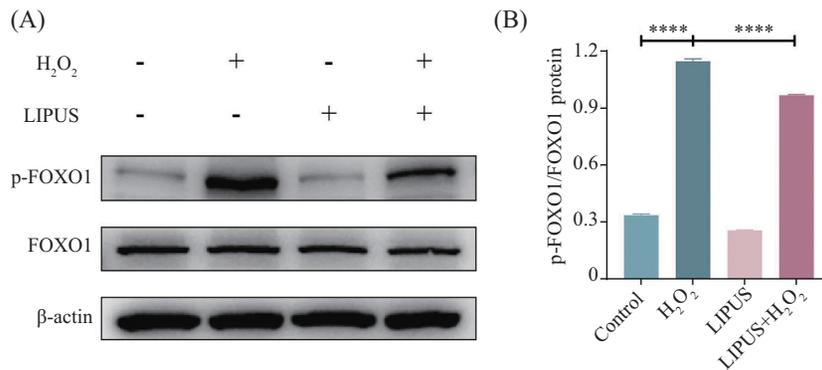


A: Western blot检测各组成骨分化相关蛋白RUNX2、ALP和OPN表达情况; B: 相应灰度值分析。**** $P<0.0001$ 。

A: Western blot to detect the expression of each group of osteogenic differentiation-related protein RUNX2, ALP, and OPN; B: corresponding grayscale value analysis. **** $P<0.0001$.

图5 LIPUS作用对H₂O₂诱导下的hPDLSCs成骨相关蛋白的影响

Fig.5 Effect of LIPUS action on H₂O₂-induced osteogenic proteins of hPDLSCs



A: Western blot检测各组p-FOXO1/FOXO1蛋白表达情况; B: 相应灰度值分析。**** $P<0.0001$ 。

A: Western blot for p-FOXO1/FOXO1 protein expression in each group; B: corresponding gray value analysis. **** $P<0.0001$ 。

图6 LIPUS处理对H₂O₂作用下p-FOXO1/FOXO1蛋白表达的影响

Fig.6 Effect of LIPUS treatment on p-FOXO1/FOXO1 protein expression in the presence of H₂O₂

LIPUS+H₂O₂+AS1842856组细胞活力下降($P<0.0001$), p-FOXO1/FOXO1蛋白表达明显上调($P<0.0001$), 胞内ROS水平显著升高($P<0.0001$), 并伴随着成骨分化标志蛋白表达水平降低($P<0.0001$)(图7~图9)。结果提示, 上调FOXO1磷酸化水平后, LIPUS对氧化损伤的hPDLSCs的保护作用明显减弱, 表明FOXO1可能是LIPUS发挥氧化损伤保护作用的关键调控靶点。

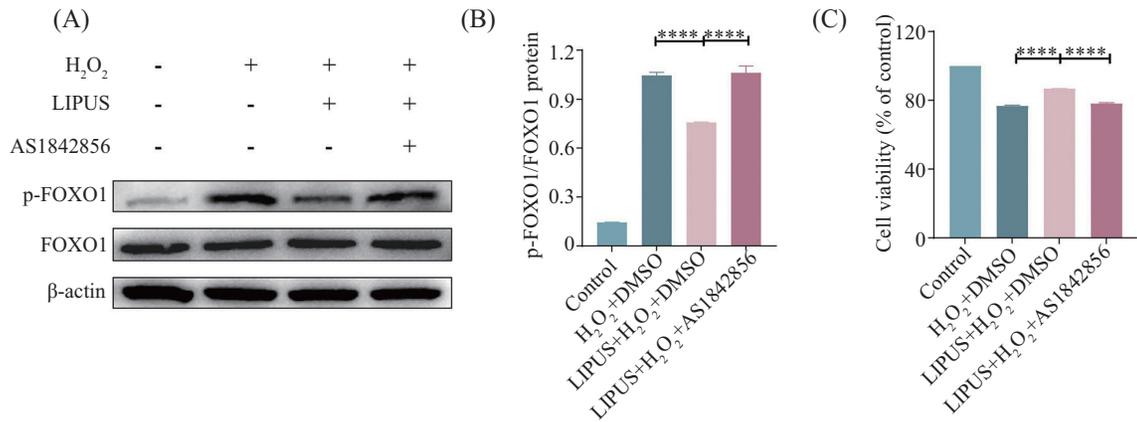
3 讨论

作为一种慢性炎症性疾病, 牙周炎会逐渐引起牙周支持组织甚至牙槽骨的丢失。提高PDLSCs的成骨分化能力是牙周炎组织再生的重要策略之一。LIPUS在炎症调节和组织再生等方面取得的积极效应显示出其在牙周组织再生方面的治疗潜力, 然而, 其具体作用机制尚不清楚。

牙周炎炎症微环境导致的氧化应激失衡, 能

抑制成骨细胞分化和刺激破骨细胞发生, 加速骨丢失^[12]。PDLSCs表现出良好的自我更新、多向分化能力和免疫调节作用, 是维持牙周组织稳态的重要细胞之一, 也是牙周骨组织重建过程中重要的种子细胞^[13]。氧化应激的失衡会抑制PDLSCs的成骨分化, 从而导致其骨再生效率降低^[14-16]。本研究主要探讨了LIPUS对氧化损伤的hPDLSCs的保护作用。结果发现, 在H₂O₂诱导的氧化应激环境下, LIPUS能够提高hPDLSCs抗氧化能力并恢复其受损的成骨分化水平, 提示hPDLSCs是LIPUS在牙周炎的治疗中重要的效应细胞之一, LIPUS对牙周其余细胞, 如破骨细胞的作用有待进一步探讨。

FOXO1是骨稳态的关键介质, 在成骨细胞中高表达, 参与调控细胞分化、ROS清除和免疫稳态等。FOXO1的转录活性通过一系列翻译后修饰(包括磷酸化、乙酰化、泛素化等)来调节, 其中磷

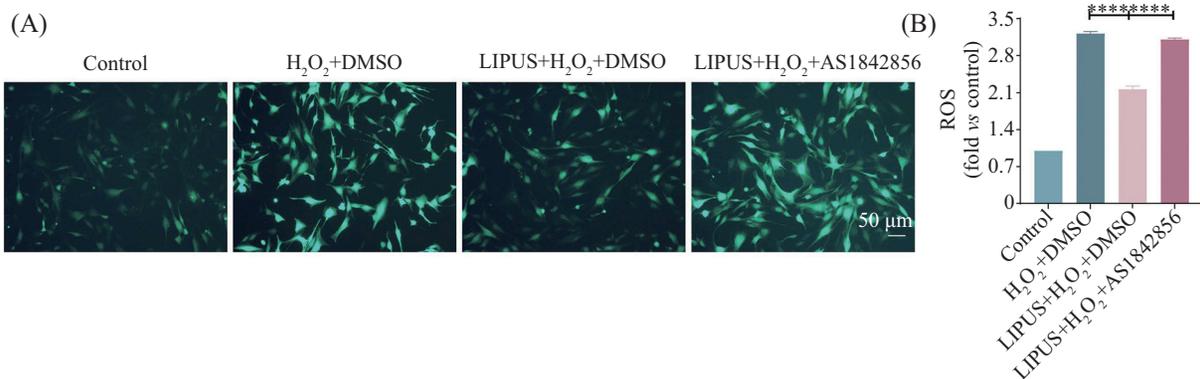


A: Western blot检测各组p-FOXO1/FOXO1蛋白表达情况; B: 相应灰度值分析; C: CCK-8检测细胞力。**** $P < 0.0001$ 。

A: Western blot detection of p-FOXO1/FOXO1 protein expression in each group; B: corresponding gray value analysis; C: CCK-8 assay for cell viability. **** $P < 0.0001$.

图7 FOXO1抑制剂对p-FOXO1/FOXO1蛋白表达和细胞活力的影响

Fig.7 Effects of FOXO1 inhibitor on p-FOXO1/FOXO1 protein expression and cell viability

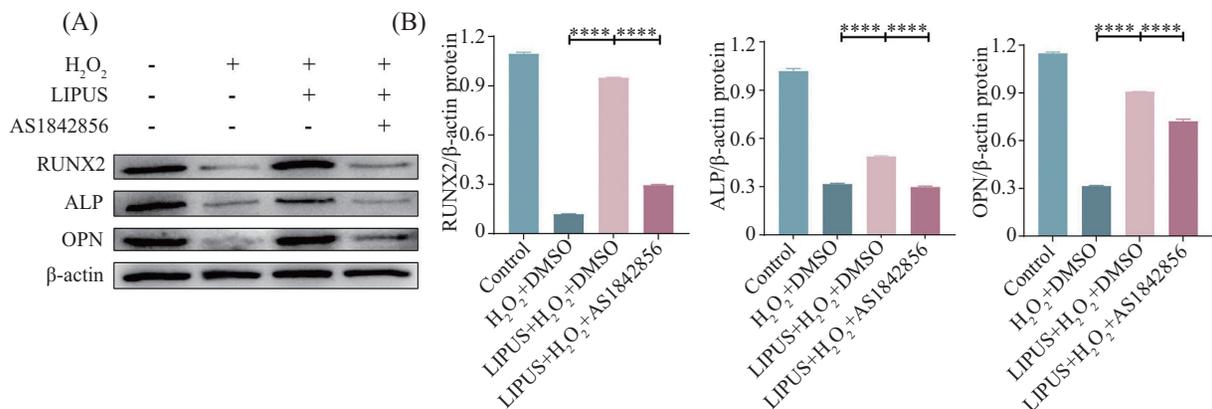


A: DCFH-DA染色; B: DCFH-DA染色相关定量分析。**** $P < 0.0001$ 。

A: DCFH-DA staining; B: quantitative analysis related to DCFH-DA staining. **** $P < 0.0001$ 。

图8 抑制FOXO1对LIPUS介导hPDLSCs抗氧化防御能力的影响

Fig.8 Effect of FOXO1 inhibition on LIPUS-mediated antioxidant defense in hPDLSCs



A: Western blot检测各组成骨分化相关蛋白RUNX2、ALP和OPN的表达情况; B: 相应灰度值分析。**** $P < 0.0001$ 。

A: Western blot detection of the expression of osteogenic differentiation-related protein RUNX2, ALP and OPN, in each group; B: corresponding gray value analysis. **** $P < 0.0001$ 。

图9 抑制FOXO1对LIPUS保护hPDLSCs成骨分化能力的影响

Fig.9 Effect of FOXO1 inhibition on LIPUS-protected osteogenic differentiation of hPDLSCs

酸化作为FOXO1重要的转录后修饰之一,可从根本上改变FOXO1活性及其亚细胞定位^[17-18]。位于细胞核内的FOXO1以去磷酸化形式存在且具有转录活性,能够调节多种靶点,如参与抗氧化的基因、成骨分化相关基因、凋亡和自噬相关基因等^[17]。研究发现FOXO1在Ser256位点的磷酸化可促进FOXO1与14-3-3蛋白结合并将p-FOXO1易位至胞质,导致FOXO1转录活性降低^[19-20]。以往的研究报道,氧化应激促进FOXO1磷酸化出核,引起胞质p-FOXO1增多和胞核FOXO1减少^[21]。我们的研究也发现氧化应激环境下FOXO1磷酸化水平明显上调,可能导致FOXO1转录活性下降,抑制抗氧化与成骨分化相关基因的表达。

FOXO1是一个机械应答因子,一些机械应力如层流诱导的剪切应力能够促进FOXO1核表达,发挥细胞保护作用^[22]。CHEN等^[23]报道LIPUS可以提高hPDLSCs的FOXO1核积累,促进RUNX2表达和基质矿化。本研究发现LIPUS作为一种机械应力,能够降低ROS蓄积微环境中上调的FOXO1磷酸化水平。AS1842856是一种特异性的FOXO1抑制剂,可以直接结合活化的FOXO1并抑制其介导的反式激活^[24]。LI等^[25]的研究发现用AS1842856处理后,胆固醇诱导的肌腱源性干细胞中磷酸化FOXO1减少这一过程受到抑制,说明AS1842856也可以增加磷酸化FOXO1的水平。本研究结果也发现使用AS1842856处理使得细胞内FOXO1磷酸化水平升高,导致LIPUS对氧化损伤的hPDLSCs的保护作用明显减弱。提示LIPUS的机械刺激可能通过降低FOXO1的磷酸化水平来提高hPDLSCs抗氧化防御能力,进而恢复氧化损伤的成骨分化能力。

综上所述,本研究发现了LIPUS能改善氧化应激,促进hPDLSCs成骨分化,FOXO1在这一过程中可能发挥关键调控作用,为深入理解LIPUS应用于牙周炎疗效的相关分子作用机制提供了理论支持与参考指导。

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