

缺氧缺血性脑损伤后乳酸通过GPR81抑制细胞凋亡

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摘要 **目的** 观察缺氧缺血性脑损伤后乳酸(Lac)受体G蛋白偶联受体81(GPR81)与细胞凋亡间的动态变化,明确Lac干预通过GPR81对细胞凋亡的作用。**方法** 选取30头新生大约克夏猪。将21头新生猪随机分为Sham组和缺血缺氧(HI)模型组,HI模型组细分为0~2 h、2~6 h、6~12 h、12~24 h、24~48 h、48~72 h HI组,每组3头,采用免疫组织化学和TUNEL染色观察基底节区GPR81和细胞凋亡变化。将9头新生猪随机分为Sham+生理盐水(NS)组、HI+NS组和HI+Lac组,每组3头。观察基底节区GPR81表达水平、凋亡细胞比例以及PI3K、AKT、Bax和Bcl-2表达水平。**结果** HI脑损伤后细胞凋亡率逐渐上升,24~48 h达到峰值($P < 0.05$)。GPR81表达水平先升后降,2~6 h达到峰值($P < 0.05$)。与Sham+NS组相比,HI+NS组细胞凋亡率、Bax表达水平升高,p-AKT/AKT比值以及PI3K、Bcl-2表达水平降低(均 $P < 0.05$);与HI+NS组相比,HI+Lac组细胞凋亡率、Bax表达水平降低,p-AKT/AKT比值以及GPR81、PI3K、Bcl-2表达水平升高(均 $P < 0.05$)。**结论** Lac干预能够通过GPR81介导PI3K/AKT信号通路活化,发挥抑制细胞凋亡的作用。

关键词 缺氧缺血; G蛋白偶联受体81; 凋亡; 乳酸

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Inhibitory effect of lactate on the cell apoptosis via GPR81 after hypoxic-ischemic brain damage

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Abstract **Objective** To observe changes in the lactate (Lac) receptor, G protein-coupled receptor 81 (GPR81), and apoptosis after hypoxic ischemic brain injury in newborn piglets, and to clarify the mechanism of Lac/GPR81 signal pathway on the cell apoptosis through the intervention of Lac. **Methods** Thirty newborn piglets were selected. A total of 21 newborn piglets were randomly divided into Sham and hypoxia-ischemia (HI) model groups, and the HI model group was subdivided into 0–2 h, 2–6 h, 6–12 h, 12–24 h, 24–48 h, and 48–72 h HI groups, with $n = 3$ each group. Changes in GPR81 expression and apoptosis in the basal ganglia after HI injury were observed using immunohistochemical and TUNEL staining. Nine newborn piglets were randomly divided into Sham+normal saline (NS), HI+NS, and HI+Lac groups, with $n = 3$ each group. The effects of Lac on the expression of GPR81, apoptotic cell percentage, and the expressions of PI3K, AKT, Bcl-2, and Bax in the basal ganglia after HI injury were observed. **Results** The cell apoptosis rate increased gradually after HI injury and peaked at 24–48 h ($P < 0.05$). After HI injury, GPR81 expression initially increased and then decreased, peaking at 2–6 h ($P < 0.05$). The cell apoptosis rate and the expressions of Bax were higher and p-AKT/AKT ratio and the expressions of PI3K and Bcl-2 were lower in the HI+NS group compared with the Sham+NS group (all $P < 0.05$). The cell apoptosis rate and the expressions of Bax were lower and p-AKT/AKT ratio and the expressions of GPR81, PI3K, and Bcl-2 were higher in the HI+Lac group compared with the HI+NS group (all $P < 0.05$). **Conclusion** After HI injury, exogenous Lac can inhibit cell apoptosis through activation of the PI3K/AKT signaling pathway mediated by GPR81.

Keywords hypoxia-ischemia; G protein-coupled receptor 81; apoptosis; lactate

新生儿缺氧缺血性脑损伤是由于围产期脑内缺氧缺血(hypoxia-ischemia, HI)造成线粒体氧化代

谢障碍、酸碱离子和神经递质紊乱,进而造成细胞坏死和凋亡,缺氧和脑血流减少会抑制细胞有氧代谢,导致无氧代谢产物的积累^[1]。在HI后早期,乳酸(lactate, Lac)为糖酵解的产物,能作为能量底物和信号分子发挥神经保护作用。Lac作为配体,与膜特异性G蛋白偶联受体81(G protein-coupled receptor 81, GPR81)结合,发挥其信号分子作用^[2]。GPR81激活

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具有调节突触活动以及减轻神经炎症等功能^[3]。既往研究^[4]发现,HI后磷脂酰肌醇-3-激酶(phosphatidylinositol-3-kinase, PI3K)/蛋白激酶B (protein kinase B, AKT) 信号通路是调节细胞凋亡的关键途径,通过调控凋亡相关蛋白表达,发挥神经保护作用。然而,GPR81对细胞凋亡的具体调节作用尚不明确,需要进行体内实验证实HI后Lac对GPR81及细胞凋亡的影响。本研究应用新生猪HI模型,通过外源性给予Lac,研究Lac对GPR81表达、PI3K/AKT信号通路以及Bcl-2、Bax等凋亡蛋白表达的影响,探究HI后Lac通过GPR81对细胞凋亡的抑制作用及其分子机制。

1 材料与方法

1.1 实验动物和分组

本研究共选用30头3~5日龄新生大约克夏猪,体重1.0~1.5 kg,雌雄不限。将21头新生猪按是否进行HI处理,随机分为假手术组(Sham组, $n = 3$)和HI模型组($n = 18$),并将根据HI后时间,将HI模型组细分为0~2 h HI组、2~6 h HI组、6~12 h HI组、12~24 h HI组、24~48 h HI组和48~72 h HI组,每组3头。将其余9头新生猪按HI后是否进行Lac干预,随机分为Sham+生理盐水(normal Saline, NS)组、HI+NS组、HI+Lac组,每组3头。

Sham组和Sham+NS组新生猪仅游离双侧颈总动脉,不进行HI处理。造模结束后,HI+Lac组立即腹腔注射L-乳酸钠,总剂量为1 g/kg,体积为3 mL/kg^[5],HI+NS组和Sham+NS组注射等体积NS(3 mL/kg)。Sham+NS、HI+NS和HI+Lac组新生猪均在HI后24~48 h内处死,并进行组织学检测。本研究获得我院医学伦理委员会审核批准。

1.2 HI模型的制备

将新生猪肌内注射速眠新II(0.6 mL/kg)麻醉。麻醉后行气管插管,连接TKR-200C小动物呼吸机(江西特力麻醉呼吸设备有限公司)。颈部皮肤消毒后行正中切开,暴露双侧颈总动脉,用动脉钳夹闭血流,通入6%氧气浓度的氮氧混合气体。40 min后恢复血供,通入100%氧气,缝合切口。待动物恢复自主呼吸后,停止通气并拔管。全程密切监控动物的血氧饱和度和心率。

1.3 免疫组织化学染色

将新生猪处死并取出全脑,置于4%多聚甲醛中4℃固定72 h。将脑组织标本沿冠状位切成4 mm的组织片,脱水、透明、石蜡包埋后切片(4 μm)。选取含有基底节层面的切片,进行脱蜡、水化、抗原修复、封闭、一抗和二抗孵育以及封片。普通光学显微镜(E800,日本Nikon公司)下拍照,在400倍光镜下每张切片随机选择3个等大区域,细胞染色呈棕黄色为阳性表达。应用ImageJ软件测量平均光密度值。

1.4 TUNEL染色

切片的制备和选取与免疫组织化学染色相同,进行脱蜡、水化、蛋白酶K修复、滴加反应液、DAPI细胞核复染以及封片。荧光显微镜(Y-TV55,日本Nikon公司)下拍照,在400倍光镜下每张切片随机选择3个等大区域,计数阳性细胞,取平均值。细胞呈绿色荧光染色为阳性表达,计算神经细胞凋亡率,神经细胞凋亡率(%)=TUNEL阳性细胞数/DAPI阳性细胞总数×100。

1.5 Western blotting

每组取100 mg新生猪脑基底节组织,研磨裂解。离心后收集上清液,测定蛋白浓度。对已提取的蛋白进行电泳、转膜、封闭以及一抗和二抗孵育。按比例配制适量增强型化学发光液,使用Tanon成像系统(Tanon-4600,上海天能科技有限公司),自动曝光。应用ImageJ软件进行图像定量分析。

1.6 统计学分析

采用SPSS 26.0软件进行统计学分析。所有数据经Kolmogorov-Smirnov检验符合正态分布,用 $\bar{x} \pm s$ 表示。采用Levene检验分析数据是否符合方差齐性,若方差齐,采用单因素方差分析进行比较,进一步两两比较采用LSD-*t*检验;若方差不齐,采用Brown-Forsythe检验进行比较,进一步两两比较采用Dunnett T3检验。 $P < 0.05$ 为差异有统计学意义。

2 结果

2.1 HI后新生猪基底节区细胞凋亡的动态变化

计算HI后细胞凋亡率,分析基底节区神经细胞凋亡的变化。结果显示,与Sham组相比,0~2 h HI组细胞凋亡率显著升高($P = 0.005$),24~48 h HI组细胞凋亡率达到峰值($P < 0.001$)。结果表明,HI后基底节区细胞凋亡不断加重,24~48 h最显著。见图1。

2.2 HI后基底节区GPR81表达的变化

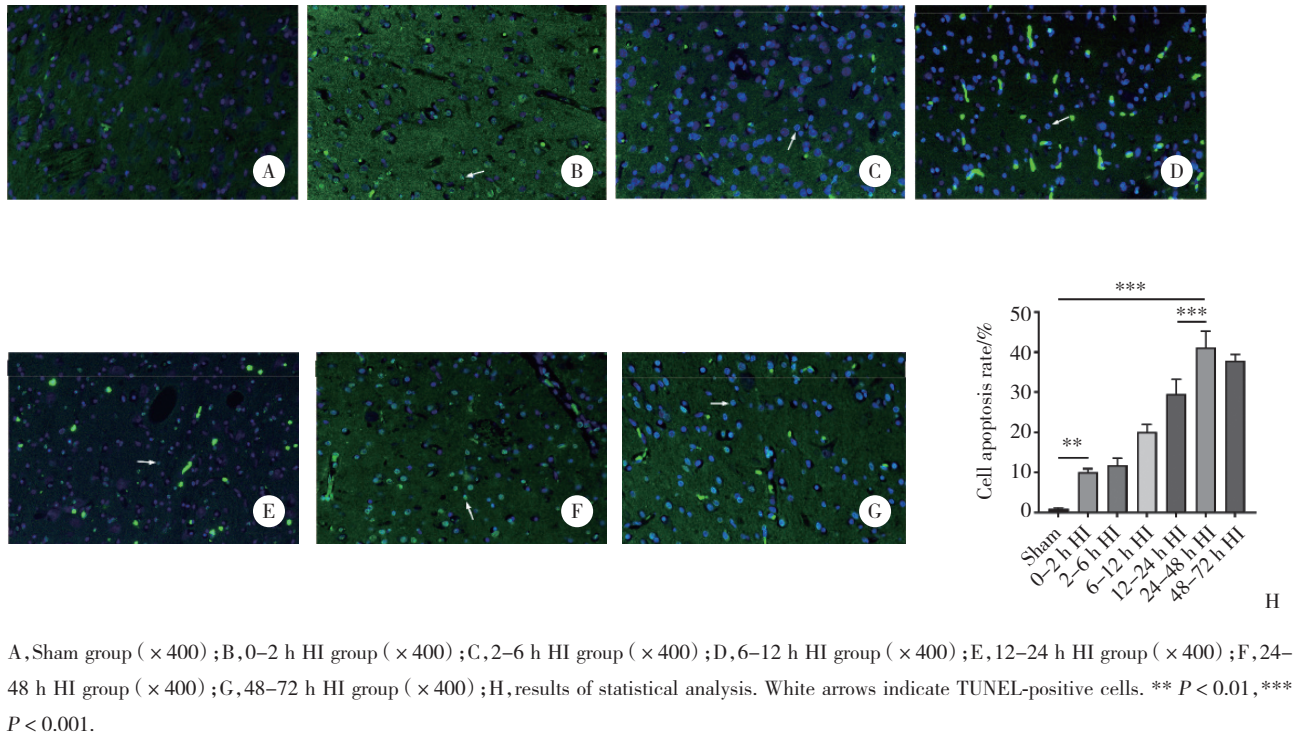


图1 HI后基底节区细胞凋亡率的变化

Fig.1 Changes in the cell apoptosis rate in the basal ganglia after HI injury

免疫组织化学染色结果显示,HI损伤后GPR81表达水平逐渐升高,2~6 h达到峰值,然后降低。与Sham组相比,0~2 h HI组GPR81表达水平显著升高($P <$

0.001)。与2~6 h HI组相比,0~2 h HI组、6~12 h HI组GPR81表达水平显著降低($P < 0.001$)。见图2。

2.3 HI后外源性Lac增加基底节区GPR81的表达

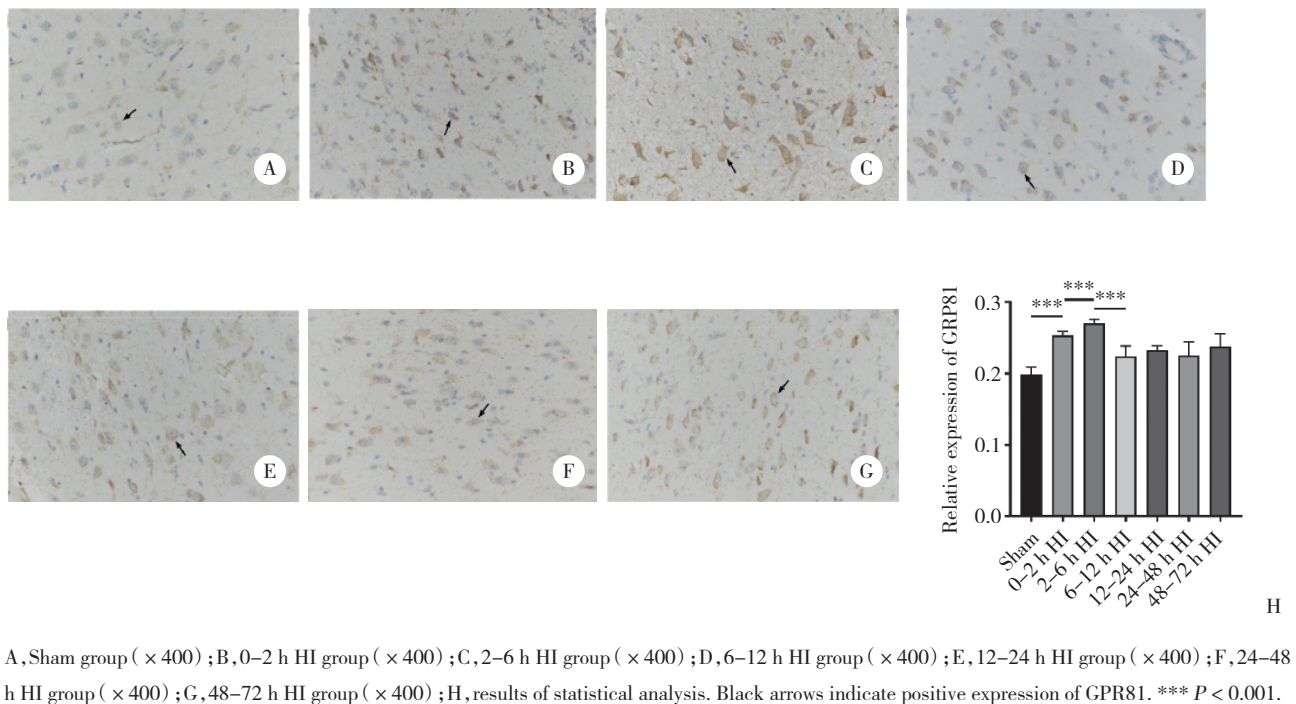


图2 HI后基底节区GPR81表达的变化

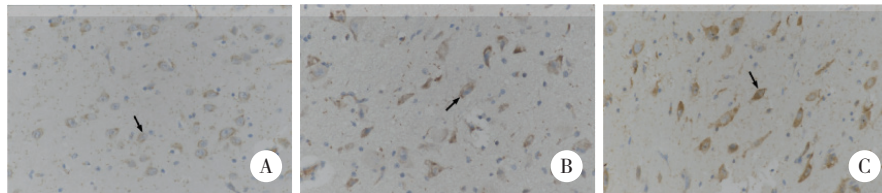
Fig.2 Changes in GPR81 expression in the basal ganglia after HI injury

免疫组织化学染色结果显示,与HI+NS组相比,HI+Lac组GPR81表达水平显著升高($P < 0.001$)。见图3、表1。

2.4 HI后外源性Lac激活PI3K/AKT信号通路、调控凋亡相关因子表达并减轻细胞凋亡

Western blotting和TUNEL染色结果显示,与Sham+

NS组相比,HI+NS组PI3K和Bcl-2蛋白表达水平、p-AKT/AKT比值显著降低(均 $P < 0.05$),Bax蛋白表达水平和细胞凋亡率显著升高(均 $P < 0.05$);与HI+NS组相比,HI+Lac组PI3K和Bcl-2蛋白表达水平、p-AKT/AKT比值显著升高(均 $P < 0.05$),Bax蛋白表达水平和细胞凋亡率显著降低(均 $P < 0.05$)。见图4、图5、表1。



A, Sham+NS group; B, HI+NS group; C, HI+Lac group. Black arrows indicate positive expression of GPR81.

图3 外源性Lac干预后基底节区GPR81表达的变化 ×400

Fig.3 Changes in GPR81 expression in the basal ganglia after exogenous Lac intervention ×400

表1 3组GPR81、PI3K、Bax、Bcl-2表达以及p-AKT/AKT比值、细胞凋亡率的比较

Tab.1 Comparison of the expression of GPR81, PI3K, Bax, and Bcl-2, p-AKT/AKT ratio, and the cell apoptosis rate among three groups

Item	Sham+NS group (n = 3)	HI+NS group (n = 3)	HI+Lac group (n = 3)
GPR81	0.259 ± 0.016	0.290 ± 0.018	0.435 ± 0.030 ^{1),2)}
PI3K	0.847 ± 0.282	0.581 ± 0.323 ¹⁾	0.978 ± 0.283 ²⁾
Bax	0.665 ± 0.148	0.885 ± 0.069 ¹⁾	0.624 ± 0.153 ²⁾
Bcl-2	0.718 ± 0.140	0.246 ± 0.100 ¹⁾	0.564 ± 0.097 ^{1),2)}
p-AKT/AKT ratio	0.909 ± 0.118	0.253 ± 0.066 ¹⁾	0.438 ± 0.112 ^{1),2)}
Cell apoptosis rate (%)	1.0 ± 0.8	36.1 ± 4.5 ¹⁾	10.7 ± 5.4 ^{1),2)}

1) $P < 0.05$ vs. Sham+NS group; 2) $P < 0.05$ vs. HI+NS group.

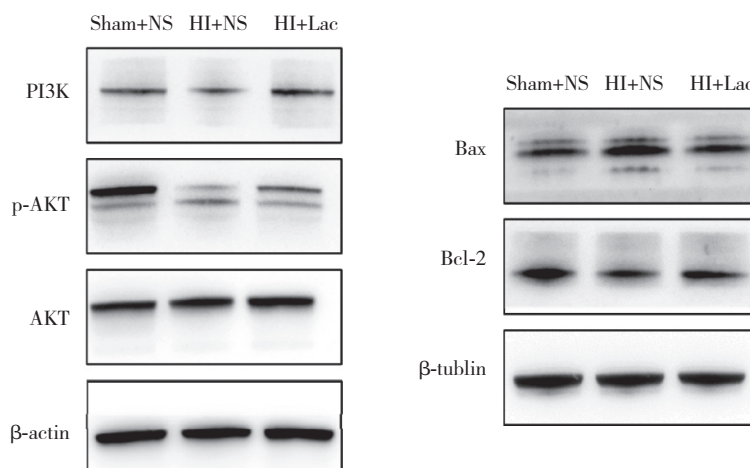
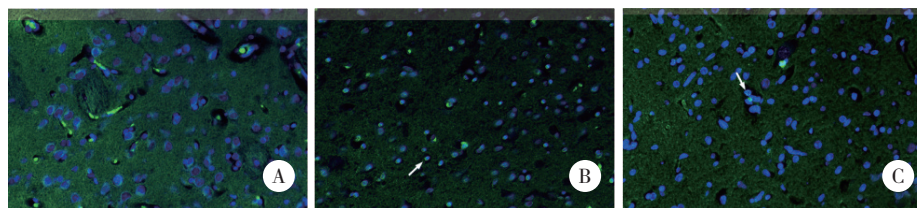


图4 Western blotting检测PI3K、p-AKT、AKT、Bax、Bcl-2蛋白表达

Fig.4 Expression of PI3K, p-AKT, AKT, Bax, and Bcl-2 proteins detected by Western blotting



A, Sham+NS group; B, HI+NS group; C, HI+Lac group.

图5 外源性Lac干预后细胞凋亡的变化 ×400

Fig.5 Changes in the cell apoptosis after exogenous Lac intervention ×400

3 讨论

既往研究^[6]中,Lac常被视为HI后早期脑内有氧代谢障碍的生物标志物。随着星形胶质细胞-神经元乳酸穿梭假说的提出^[7],Lac在中枢能量代谢中的重要作用逐渐引起人们的重视。近年来,陆续有研究^[8-9]将外源性Lac作为治疗创伤性脑损伤和新生儿脑缺血的手段。在GPR81作为Lac受体在大脑中的表达和分布得到证实后,Lac通过结合GPR81发挥信号分子的作用更加明确^[2]。但Lac作为信号分子在新生儿HI脑损伤中的作用及下游调节机制,仍有待深入研究。

本课题组前期应用1H-MRS检测了新生猪HI脑损伤后脑内Lac含量的动态变化。研究^[10]结果显示,HI后0~6 h Lac含量一过性升高,随后在6~12 h含量下降,而且证实了HI后Lac通过单羧酸转运体和葡萄糖转运体协同调节星形胶质细胞和神经元的能量代谢,这为深入探讨Lac在HI后的作用机制奠定了基础。在此基础上,本研究进一步分析了Lac作为信号分子的调控机制。结果显示,HI后早期GPR81表达水平同样出现一过性升高,在2~6 h达到峰值,6~12 h表达水平下降,这与前期研究结果中Lac含量变化趋势一致,提示在HI后早期Lac与GPR81发挥协同作用。既往研究^[9]发现,Lac通过激活GPR81可增加创伤性脑损伤后突触可塑性相关蛋白的表达,从而减缓神经功能损伤。此外,缺血性脑损伤后GPR81激活具有调控细胞凋亡的功能^[11]。本研究结果显示,HI后6~12 h Lac浓度以及GPR81表达水平下降,而脑组织细胞凋亡率逐渐升高,24~48 h达到峰值,提示HI后细胞凋亡的增加与GPR81表达水平降低有关。

本研究外源性注入与GPR81亲和力较高的L-乳酸钠^[12],进一步观察Lac对GPR81及下游凋亡相

关分子通路的影响。PI3K/AKT通路是HI损伤后抑制细胞凋亡的关键通路^[13]。PI3K能够接收GPR81传递的信号,通过磷酸化AKT,上调抗凋亡蛋白,发挥其抑制细胞凋亡的作用^[14]。本研究结果证实,HI后24~48 h,PI3K表达水平、p-AKT/AKT比值和抗凋亡因子Bcl-2表达水平降低,凋亡因子Bax表达水平和细胞凋亡率升高,提示PI3K/AKT通路活性受到抑制,细胞凋亡增加。外源性注射Lac后,GPR81表达水平升高,并且PI3K表达水平、p-AKT/AKT比值、抗凋亡因子Bcl-2表达水平升高,凋亡因子Bax表达水平和细胞凋亡率降低,提示外源性Lac能够活化GPR81,通过介导激活PI3K-AKT信号通路,抑制细胞凋亡。

本研究存在一定局限性:第一,本研究注射的L-乳酸钠剂量为1 g/kg,是参考既往研究^[5]的剂量进行了等效转化。大鼠腹腔注射2 g/kg L-乳酸钠 30 min后,血浆Lac浓度增加约4倍,下丘脑Lac含量上升至GPR81激活范围^[12]。本研究在新生猪腹腔注射1 g/kg L-乳酸钠后,也观察到GPR81表达水平升高,但仍需设置多个L-乳酸钠浓度,以确定效果最佳、不良反应最小的药物浓度。第二,本研究中,因为HI后24~48 h时细胞凋亡最显著,故选择此时点研究外源性Lac对GPR81表达水平的影响,可能存在一定的偏倚。

综上所述,本研究利用新生猪HI模型研究Lac/GPR81信号通路变化,尝试研究外源性Lac对Lac/GPR81信号通路的调节,证实了外源性注射Lac后GPR81表达水平升高,抗凋亡信号通路PI3K/AKT活化,脑组织凋亡细胞减少,说明Lac通过GPR81介导的PI3K/AKT信号通路活化发挥抑制细胞凋亡的作用。

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