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Effect of Artesunate on the Expression of ICAM-1 and MMP-9 in Vascular Endothelial Cells under High Glucose Condition

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Abstract

Objective: To investigate the effect of ART on the expression of ICAM-1 and MMP-9 in vascular endothelial cells under high glucose condition. Methods: Human Umbilical Vein Endothelial Cells (HUVEC) were divided into glucose (G) group, 40 mmol/L G + ART (G40 + ART) group, mannitol (M) control group, dimethyl sulfoxide (DMSO) control group. The concentration gradient of G group is 5.5 mmol/L G (G5.5), 25 mmol/L G (G25), 40 mmol/L G (G40); the concentration gradient of M control group is 5.5 mmol/L G + 19.5 mmol/L M (M25), 5.5 mmol/L G + 34.5 mmol/L M (M40); the concentration gradient of ART of G40 + ART group is G40 + 10 ug /ml ART (10A), G40 + 20 ug/ml ART (20A), G40 + 40 ug/ml ART (40A); the volume of DMSO in the DMSO control group is the same as it is in the 40A group. Western blot and cell immunofluorescence technique were used to detect the protein expression of Intercellular adhesion molecule-1 (ICAM-1) and Matrix metalloproteinase-9 (MMP-9) in each group. Results: The protein expression of ICAM-1 and MMP-9 in G25 group was higher than that in G5.5 group (P < 0.01), and it increased in G40 group compared with G25 group (P < 0.01); the protein expression of ICAM-1 and MMP-9 in G25 group was higher than that of M25 group (P < 0.01), and it increased in G40 group compared with M40 (P < 0.01); the protein expression of ICAM-1 and MMP-9 of G40 + ART group was lower than that of G40 group, in which it was lower in 20A group than that of 10A group (P < 0.01), and it was lower in 40A group compared with 20A group (P < 0.01). The DMSO control group showed that the protein expression of ICAM-1 and MMP-9 in G40 + ART was lower than that of G40 + DMSO group (P < 0.01). Conclusion: The expression of ICAM-1 and MMP-9 protein was increased under high glucose condition in a concentration-dependent manner. ART inhibited the expression of ICAM-1 and MMP-9 protein in vascular endothelial cells under high glucose condition in a concentration-dependent manner. This experiment lays the foundation for further study of the changes of ICAM-1 and MMP-9 expression in the mechanism of ART inhibiting retinal neovascularization and leakage.

Keywords

Artesunate, Intercellular Adhesion Molecule-1, Matrix Metalloproteinase-9, Vascular Endothelial

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Cells, Diabetic Retinopathy

青蒿琥酯对高糖条件下血管内皮细胞ICAM-1和MMP-9表达的影响

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摘　要

目的：探讨ART对高糖条件下血管内皮细胞ICAM-1和MMP-9表达的影响。方法：将人脐静脉内皮细胞(Human Umbilical Vein Endothelial Cells, HUVEC)作为实验对象，分为葡萄糖(Glucose, G)组、40 mmol/L G + ART (G40 + ART)组，甘露醇(Mannitol, M)对照组、G40 + DMSO (DMSO)对照组，其中G组浓度梯度分为5.5 mmol/L G(G5.5)、25 mmol/L G(G25)、40 mmol/L G(G40)；M对照组浓度梯度为5.5 mmol/L G + 19.5 mmol/L M(M25)，5.5 mmol/L G + 34.5 mmol/L M(M40)，G40 + ART组中依据ART浓度梯度分为G40 + 10 ug/ml ART(10A)、G40 + 20 ug/ml ART(20A)、G40 + 40 ug/ml ART(40A)；DMSO对照组中DMSO用量与40A组中溶解ART所用DMSO体积相同。采用Western blot、细胞免疫荧光技术分别检测各组细胞间黏附分子-1(ICAM-1)、基质金属蛋白酶-9(MMP-9)蛋白表达情况。结果：1) ICAM-1、MMP-9蛋白在G25组较G5.5组表达升高(P < 0.01)，且G40组较G25组表达升高(P < 0.01)；ICAM-1、MMP-9蛋白在G40 + ART组较G40组表达下降，其中，10A组低于G40组(P < 0.01)，20A组低于10A组(P < 0.01)，40A组低于20A组(P < 0.01)。2) ICAM-1、MMP-9蛋白在G25组较M25组表达升高(P < 0.01)；G40组较M40组表达升高(P < 0.01)。3) DMSO对照组显示G40 + ART组ICAM-1、MMP-9蛋白表达低于DMSO组(P < 0.01)。结论：ICAM-1和MMP-9蛋白在高糖条件下表达升高，且具有浓度依赖性，ART可抑制高糖条件下血管内皮细胞ICAM-1和MMP-9蛋白的表达，且具有浓度依赖性，为进一步研究ART抑制视网膜新生血管形成和渗漏的机制中ICAM-1和MMP-9表达变化奠定基础。

关键词

青蒿琥酯，细胞间粘附分子-1，基质金属蛋白酶-9，血管内皮细胞，糖尿病性视网膜病变

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1. 引言

在糖尿病导致的微血管并发症中，DR 是最常见的[1]，并成为全球工作年龄失明的主要原因[2]。

DR 的临床特征是血管通透性增加，微血管瘤形成，血管增生和黄斑水肿[3]。ART 是一类新型抗疟药，近年来已经报道了其抗肿瘤作用，而且在肿瘤中拮抗血管生成的作用也引起了广泛的关注[4]。ART 具有抑制血管内皮细胞增殖、迁移和管腔形成的作用，可以剂量依赖性地有效抑制肿瘤血管的形成和生长[5]。最近研究表明 ART 能抑制视网膜新生血管(NV)的生长和渗漏[6]。新生血管形成早期阶段细胞间粘附分子-1(ICAM1)及基质金属蛋白酶-9(MMP-9)至关重要。首先，MMP-9 可以降解细胞外基质，此为新生血管形成的前提条件[7]，DR 患者荧光素血管造影的临床证据表明，血–视网膜屏障是血管渗漏导致黄斑水肿的主要部位，MMP-9 可促进血管渗透性增加[8]。其次，ICAM-1 在白细胞与血管内皮细胞粘附[9] [10]，诱导毛细血管阻塞[11]，导致内皮细胞损伤和死亡[12]，进而导致视网膜毛细血管无灌注和新生血管形成[13] [14]。现阶段针对 DR 中新生血管形成和渗漏的主要治疗手段为抗 VEGF 治疗，但是，单纯抗 VEGF 治疗具有一定局限性，如抗 VEGF 药物靶点单一、半衰期短。因此，我们需要根据新生血管形成的分子机制阐明更多治疗靶点，应用一种能够多靶点、更加长久发挥治疗作用的药物，以预防和治疗这种疾病。之前研究表明，青蒿琥酯(Artesunate, ART)可通过多靶点抑制视网膜新生血管形成和渗漏，本实验应用 ART 处理高糖条件下 HUVEC，并用 Western blot、细胞免疫荧光检测 ICAM-1、MMP-9 蛋白表达情况，探讨 ART 对高糖条件下血管内皮细胞 ICAM-1 和 MMP-9 表达的影响，为进一步研究 ART 抑制视网膜新生血管形成和渗漏的机制中 ICAM-1 和 MMP-9 表达变化奠定基础。

2. 材料与方法

2.1. 材料

2.1.1. 细胞型号及来源

HUVEC-C(型号：ZQ0446，规格：5×10^5 cells/vial)来源于上海中乔新舟生物科技有限公司。

2.1.2. 主要试剂

DMEM 低糖培养基(中国 Solarbio)，DMEM 高糖培养基(中国 Solarbio)，胎牛血清(美国 HyClone)，青链霉素混合液(100X) (中国 Solarbio)，0.25%胰蛋白酶消化液(中国 Solarbio)，二甲基亚砜(美国 Sigma)，兔抗人 MMP-9 抗体(英国 Abcam)，兔抗人 ICAM-1 抗体(英国 Abcam)，兔抗人 GAPDH 抗体(中国 Elabscience)，羊抗兔 Western blot 二抗(中国 Elabscience)，羊抗兔荧光二抗(中国 Elabscience)，WB 专用一抗二抗稀释液(美国 博士得)，荧光抗体稀释液(中国 Solarbio)。

2.1.3. 主要仪器设备

洁净工作台(中国 苏州安泰空气技术有限公司)，CO_2 恒温培养箱(德国 Heraeus)，倒置显微镜(日本 OLYMPUS)，倒置荧光显微镜(日本 OLYMPUS)，多功能成像系统(法国 Vilber)，低速台式离心机(中国 北京时代北利离心机有限公司)，台式低温高速离心机(美国 Sigma)，电子天平(美国 METTLER TOLEDO)。

2.2. 方法

2.2.1. 细胞培养

将冻存管内的 HUVEC 复苏，加入含 10% FBS 的低糖(5.5 mmol/L 葡萄糖) DMEM 培养液，调整细胞密度为 1×10^5/mL，以每瓶 4 ml 接种于 25 cm^2 培养瓶，放于 37℃、5% CO_2 恒温培养箱恒温培养。

2.2.2. Western Blotting 检测 ICAM-1 和 MMP-9 蛋白表达

采用 SDS-PAGE 进行蛋白质印迹分析，将蛋白质样品与 5XSDS-PAGE 蛋白上样缓冲液按比例(4:1)

混匀，依据目的蛋白分子量，配制 10%分离胶，5%浓缩胶进行电泳。将蛋白转移至 PVDF 膜上，用配制好的奶粉封闭液阻断膜上非特异性结合位点。用兔抗人 MMP-9 抗体(英国 Abcam)，兔抗人 ICAM-1 抗体(英国 Abcam)进行一抗孵育，和兔抗人 GAPDH 抗体(中国 Elabscience)作为内参对照。然后以羊抗兔 Western blot 二抗(中国 Elabscience)进行二抗孵育。使用 ECL 发光液用凝胶成像分析仪显影；使用 Image J 软件定量分析条带灰度值，将各目的条带灰度值与内参 GAPDH 条带灰度值之比作为该蛋白的相对表达含量，重复三次实验的平均值作为最后统计值。

2.2.3. 细胞免疫荧光检测 ICAM-1 和 MMP-9 蛋白表达

根据实验需要将无菌盖玻片置于 24 孔板的 12 孔或 10 孔中。将 HUVEC 以 1×10^5/ml 的密度接种于盖玻片上 37℃培养 24 h。然后去除处理液，用 PBS 洗涤，4%多聚甲醛固定 10 分钟，并在室温下用 0.1% Triton X 100 通透 1 分钟。将细胞在含有 10%封闭用正常山羊血清 PBS 中封闭 1 小时。用兔抗人 ICAM-1 荧光抗体(英国 Abcam)在 4℃孵育过夜，同时对应一孔不加一抗作为阴性对照。用羊抗兔荧光二抗(中国 Elabscience)在室温下孵育 1 小时。使用 DAPI 复染核 10 min。使用抗荧光衰减封片剂封片，通过放大倍数为 200 倍的荧光显微镜观察并拍摄结果。

2.3. 统计学方法

运用 SPSS 17.0 对数据进行统计学分析，各组数据经 *Shapiro-Wilk* 检验均呈正态分布，数据以均数±标准差表示，两组数据之间比较采用 *t* 检验，以 $P < 0.01$ 或 $P < 0.05$ 为差异具有统计学意义。

3. 结果

3.1. 高糖对 HUVEC ICAM-1 和 MMP-9 蛋白表达的影响及 ART 对高糖条件下 HUVEC ICAM-1 和 MMP-9 蛋白表达的影响

应用 G5.5，G25，G40 的培养液处理细胞，运用 Western blot、细胞免疫荧光检测 ICAM-1、MMP-9 蛋白表达。结果表明 ICAM-1、MMP-9 蛋白在 G25 组较 G5.5 组表达升高，且 G40 组较 G25 组表达升高，差异有统计学意义($t = 4.796$、17.31、3.430、2.987，均为 $P < 0.01$)。应用 10 A，20 A，40 A 三种药物浓度对细胞进行处理，运用 Western blot、细胞免疫荧光检测 ICAM-1、MMP-9 蛋白表达，结果表明 ICAM-1、MMP-9 蛋白在 G40 + ART 组较 G40 组表达下降，其中，10 A 组低于 G40 组，差异有统计学意义($t = 3.846$、8.887，均为 $P < 0.01$)，20A 组低 10A 组，差异有统计学意义($t = 6.536$、5.329，$P < 0.01$)，40 A 组低于 20 A 组，差异有统计学意义($t = 6.169$、3.947，$P < 0.01$) (如图 1A~图 1D)。

3.2. 高渗透压对 HUVEC ICAM-1、MMP-9 蛋白表达的影响

对于 M 对照组，应用 Western blot、细胞免疫荧光检测 HUVEC ICAM-1、MMP-9 蛋白表达情况，结果显示 ICAM-1、MMP-9 蛋白表达在 G25 组较 M25 组表达升高，G40 组较 M40 组表达升高，差异具有统计学意义($t = 11.84$、3.845、8.803、15.30，均为 $P < 0.01$) (如图 2A~图 2D)。

3.3. 为进一步证明 ART 对高糖条件下 HUVEC ICAM-1、MMP-9 蛋白表达的影响，设置 DMSO 对照组

DMSO 对照组中 DMSO 用量与 40A 组中溶解 ART 所用 DMSO 体积相同，采用 Western blot、细胞免疫荧光检测 ICAM-1、MMP-9 蛋白表达，结果显示 G40 + ART 组 ICAM-1、MMP-9 蛋白表达低于 DMSO 组($t = 5.997$、10.31、21.43、3.378、8.229、18.19，均为 $P < 0.01$) (如图 3A~图 3D)。

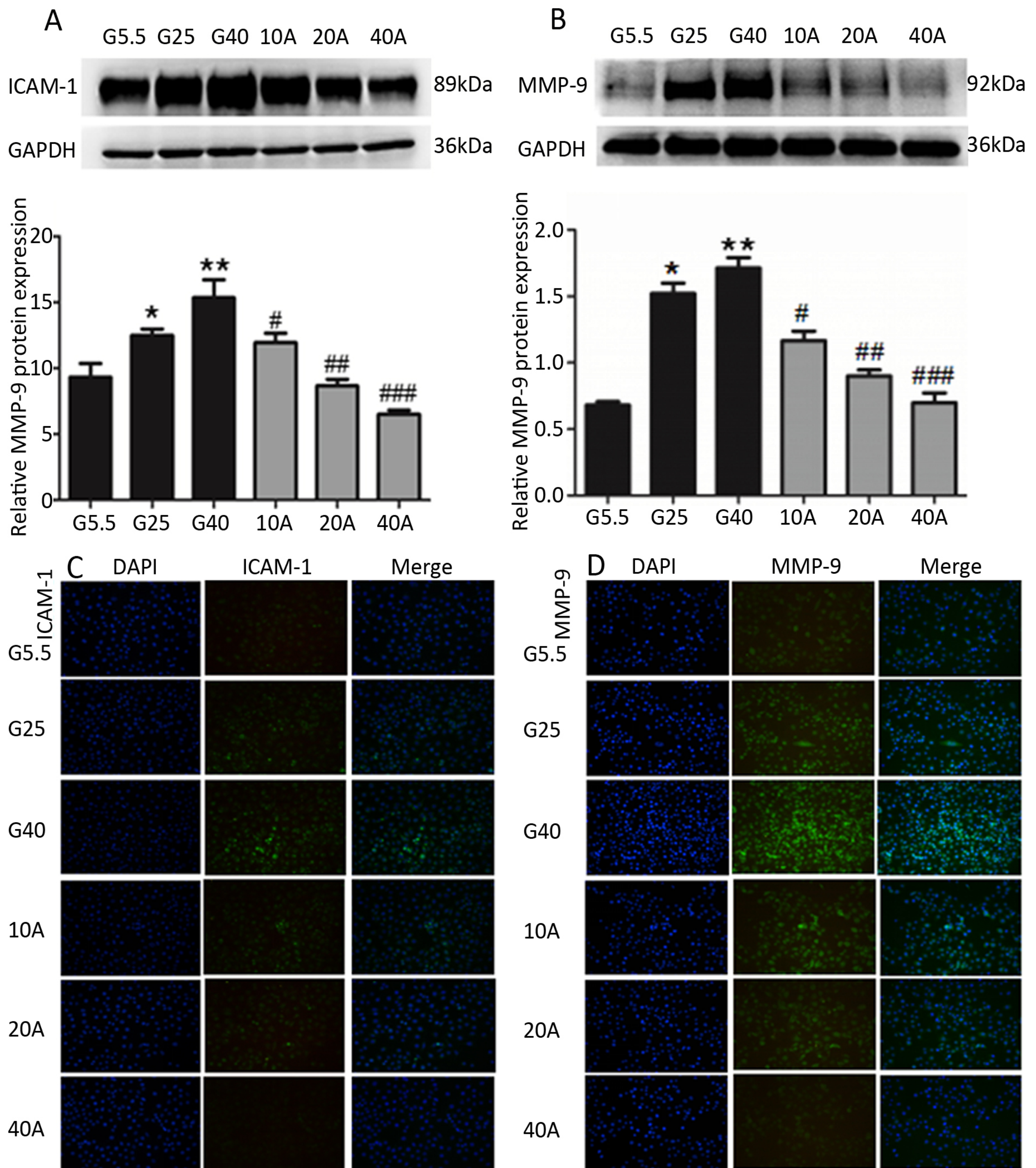

图 C、D 放大倍数(200×)

Figure 1. A, B, C, D The results of Western blot and cell Immunofluorescence showed that the expression of HUVEC ICAM-1 and MMP-9 increased under high glucose condition in a concentration-dependent manner. ART inhibits the expression of HUCAMC ICAM1 and MMP-9 proteins under high glucose conditions in a concentration-dependent manner. (*indicates $P < 0.01$ compared with G5.5 group; **indicates $P < 0.01$ compared with G25 group, # indicates $P < 0.01$ compared with G40 group, ##indicates $P < 0.01$ compared with 10A group, ####indicates $P < 0.01$ compared with 20A group)

图 1. A、B、C、D Western blot、细胞免疫荧光结果显示 HUVEC ICAM-1、MMP-9 在高糖条件下表达升高，且具有浓度依赖性；ART 可抑制高糖条件下 HUVEC ICAM1、MMP-9 蛋白表达，且具有浓度依赖性(*表示与 G5.5 组相比 $P < 0.01$；**表示与 G25 组相比 $P < 0.01$，#表示与 G40 相比 $P < 0.01$，##表示与 10A 组相比 $P < 0.01$，####表示与 20A 组相比 $P < 0.01$)

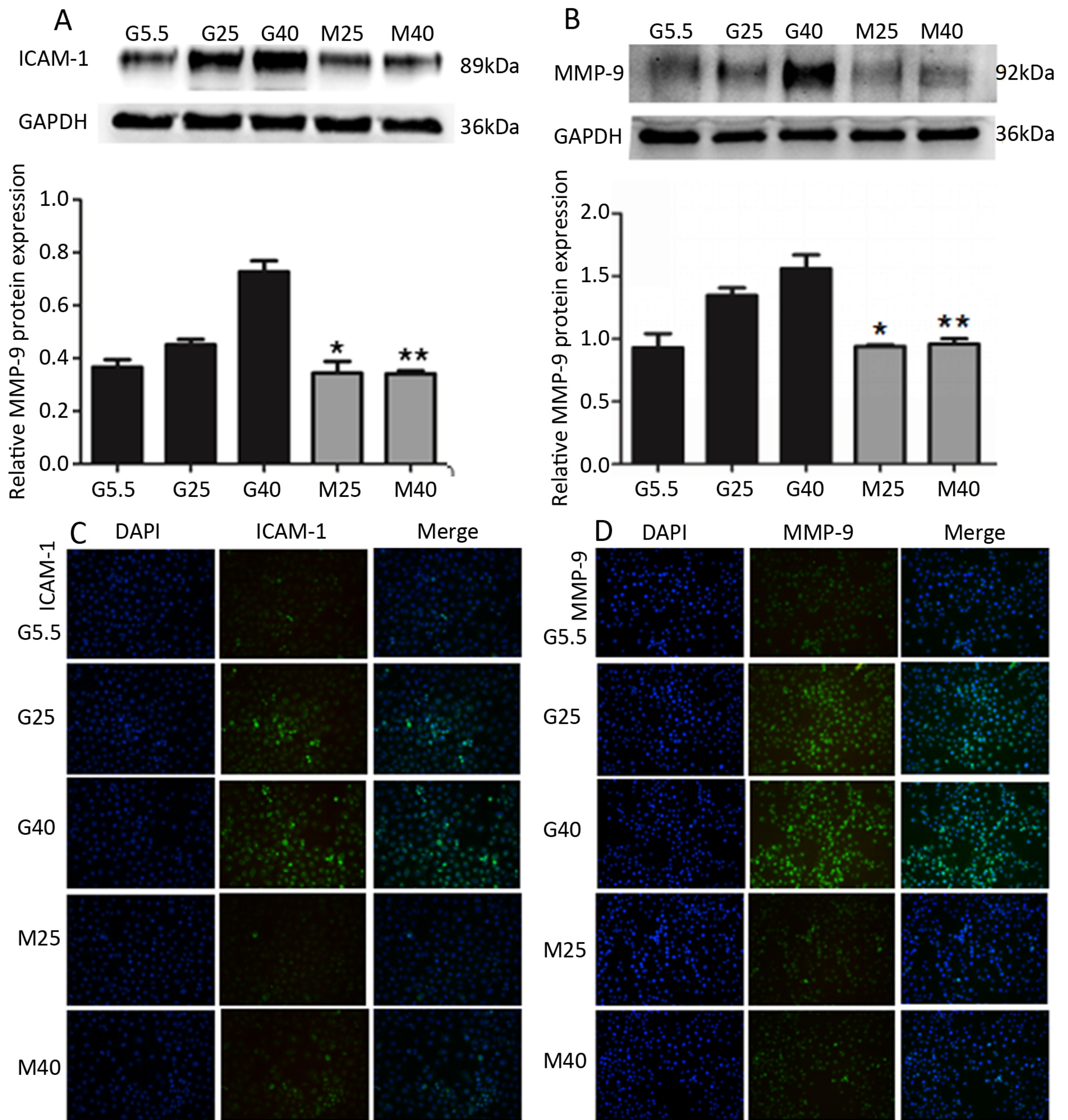

图 C、D 放大倍数(200×)

Figure 2. A, B, C, D Western blot and cell Immunofluorescence showed that high osmotic pressure had no significant effect on ICAM-1 and MMP-9 protein expression. (*indicates $P < 0.01$ compared with G25, **indicates $P < 0.01$ compared with G40)

图 2. A、B、C、D Western blot、细胞免疫荧光结果显示高渗透压对 ICAM-1、MMP-9 蛋白表达无显著影响。(*表示与 G25 相比 $P < 0.01$，**表示与 G40 相比 $P < 0.01$)

4. 讨论

本研究在细胞水平证实了 ICAM-1 和 MMP-9 蛋白在高糖条件下表达升高，且具有浓度依赖性，ART 可抑制高糖条件下血管内皮细胞 ICAM-1 和 MMP-9 蛋白的表达，且具有浓度依赖性。

有越来越多的证据表明炎症在 DR 的发病机制中起着重要作用，DR 始于低度慢性炎症性疾病[15]，

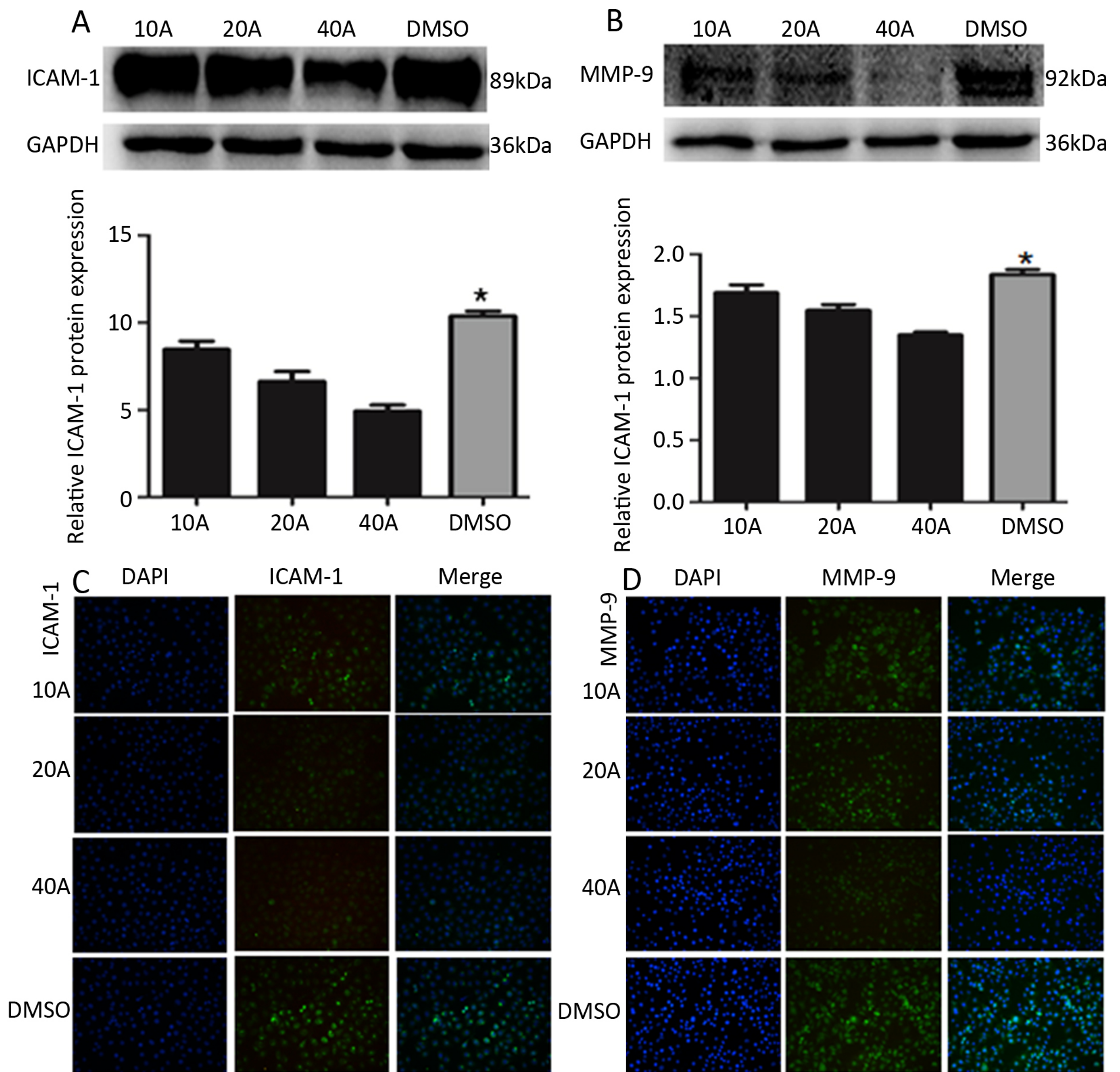

Figure 3. A, B, C, D Western blot and cell Immunofluorescence showed that the expression of ICAM-1 and MMP-9 in ART group was significantly lower than that in DMSO group. (*indicates P < 0.01 compared with the 10 A, 20 A, and 40 A groups)

图 3. A、B、C、D Western blot、细胞免疫荧光结果显示 ART 组 ICAM-1、MMP-9 蛋白表达显著低于 DMSO 组(*表示与 10 A、20 A、40 A 组相比均为 P < 0.01)

在 DR 发病过程中促炎因子、趋化因子、白细胞粘附增加[16]，白细胞瘀滞是炎症过程的主要组成部分[17]，内皮细胞表达的 ICAM-1 不仅可以调节白细胞与内皮细胞的粘附，而且在血视网膜屏障破坏、血管通透性的调节中也起着重要作用[18] [19] [20]，可诱导视网膜毛细血管无灌注和新生血管形成[13] [14]。MMP-9 可促进白细胞瘀[21]，其机制与基底膜降解，白细胞聚集于受损组织处有关[22]。

此外，DR 新生血管生成的阶段包括：发生基底膜降解，内皮细胞迁移和增殖，随后毛细血管形成。这种组织的迁移和重塑受基质金属蛋白酶的调节[17]。MMP-9 是基质金属蛋白酶家族中最大的成员[23]，其可促进血管渗透性增加[24]，降解毛细血管基底膜，毛细血管基底膜是细胞外基质的一部分[7]，这是内皮细胞在内皮下基质中渗透和形成新腔的必要条件[8]。而且，MMP-9 可降解 BRB 内皮细胞紧密连接

蛋白组分，增加血管渗漏[24]。

研究表明高糖条件下血管内皮细胞及 DR 患者中 ICAM-1、MMP-9 表达升高[8] [25] [26] [27]，且血管内皮细胞 ICAM-1 的升高呈葡萄糖浓度依赖性[28]，本实验结果与之前研究结果一致，此外，本实验还进一步证明随着葡萄糖浓度升高，血管内皮细胞 MMP-9 表达会随之升高，提示更高的血糖浓度可能加重糖尿病性视网膜病严重程度。本研究结果显示 ART 可抑制高糖条件下血管内皮细胞 ICAM-1，MMP-9 表达，并且呈现浓度依赖性。ART 通过下调 VEGFR2，PKC*α* 和 PDGFR 的表达来抑制兔的虹膜和视网膜新生血管，并缓解猴子的黄斑水肿[6]。本实验为 ART 抑制视网膜新生血管的形成和渗漏提供了新的作用靶点和理论支持。糖尿病患者不仅 ICAM-1 水平高，而且其配体 CD11a/CD18 和 CD11b/CD18 的水平也增加[29]。阻断 ICAM-1 或 CD18 的表达减弱了糖尿病动物的视网膜血管中的白细胞瘀滞、内皮细胞死亡和血管渗漏[30]。在敲除 ICAM-1 和 CD18 基因的 DR 小鼠模型中视网膜血管粘附的白细胞数量减少，血管内皮细胞损伤数目减少，血－视网膜屏障破坏降低，视网膜血管组织病理学改变减轻[16]。通过抑制 MMP-9 的活性可抑制角膜新生血管形成并保护 BRB 功能的完整性，减少视网膜血管渗漏[31] [32]。因此，结合本实验在细胞水平研究结果，ICAM-1、MMP-9 可能成为 ART 治疗 DR 的新作用位点，并发挥治疗作用。

DR 发病过程中，血管内皮细胞生长因子(VEGF)为促使新生血管和黄斑水肿形成的主要因子[33]，但是，其它一些因子也具有同样作用，如 ICAM-1、MMP-9，HMGB-1 [34] [35] [36]，TNF*α*[37] [38]等，因此，VEGF 并非新生血管形成和渗漏的唯一因素。目前针对新生血管及黄斑水肿的治疗主要为抗 VEGF 药物的应用。抗 VEGF 药物可抑制新生血管形成和渗漏，并提升黄斑水肿患者的视力[33] [39]。但是，抗 VEGF 药物存在一定局限性，如半衰期短[40]，药物作用持续时间短，例如，目前广泛应用的雷珠单抗需每月玻璃体腔注射以维持药物在眼内组织的治疗水平[41]；靶点单一，仅能以 VEGF 某些亚型为治疗靶点，仅 Aflibercept 和 Conbercept 能额外以胎盘生长因子为治疗靶点[40]；最重要的是，只有少于 50%的患者接受抗 VEGF 治疗后获得视力的提升[42] [43] [44] [45]。研究认为严格控制血糖可降低 DR 致盲率[46]，但临床上维持正常血糖水平是困难的，有时甚至是不可能的，需要根据其发展的分子机制阐明更多治疗靶点以预防和治疗这种疾病。由于 ART 可以通过作用于 VEGFR2，PKC*α*，PDGFR 三个靶点抑制视网膜新生血管的发生、发展和渗漏，药物作用时间长达 6 个月[6]。而且本实验在细胞水平证明 ICAM-1、MMP-9 可作为 ART 两个新作用位点。此外，ART 耐受性较好[47]，青蒿素及其衍生物抗新生血管的作用浓度仅为临床用于疟疾治疗剂量千分之一[48]，在非大剂量(10 mg/L)全身长期应用条件下没有额外副作用[46] [49] [50] [51] [52] [53]，因此，ART 应用于 DR 的早期和晚期治疗成为可能。

总之，本实验在细胞模型中证明 ICAM-1 和 MMP-9 的升高具有葡萄糖浓度依赖性，根据之前研究在一定程度上提示 DR 患者应严格控制血糖水平；此外，ART 可抑制高糖条件下血管内皮细胞 ICAM-1 和 MMP-9 蛋白的表达，且具有浓度依赖性，ICAM-1 和 MMP-9 可能作为 ART 治疗 DR 新作用位点，为其成为治疗 DR 的新型药物及 ART 抑制视网膜新生血管形成和渗漏的机制中 ICAM-1 和 MMP-9 表达变化、发现其更多潜在治疗价值提供帮助。但是，本实验仅局限于细胞水平 ART 对 ICAM-1 和 MMP-9 的影响，对于 ART 抑制视网膜新生血管形成和渗漏的机制中 ICAM-1 和 MMP-9 表达变化有待进一步实验研究。

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