基于海分枝杆菌感染斑马鱼模型的抗结核药物评价方法研究

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摘要: 结核病 (tuberculosis, TB) 是由结核分枝杆菌导致的一种严重感染性疾病。近年来随着耐药结核菌不断出现, 发展新型抗结核药物迫在眉睫。本研究利用与结核分枝杆菌高度类似的海分枝杆菌为模式生物, 建立了海分枝杆菌-斑马鱼幼鱼感染模型和细菌菌数定量 PCR (quantitative PCR, qPCR) 分析方法。研究表明, 卵黄囊注射海分枝杆菌是一种高效、便捷的感染斑马鱼胚胎方式。通过测定感染的斑马鱼存活率、斑马鱼体内细菌数目和 Ziehl-Neelsen 抗酸性染色指标, 对抗结核药物异烟肼和利福平的药效、药物之间的协同作用等多个参数进行了分析和比较, 结果表明 3 种评价方法具有较好的一致性。本研究证实了海分枝杆菌-斑马鱼感染模型结合细菌菌数 qPCR 分析方法是一种简单、高效的抗结核药物体内筛选和评价系统。本研究依据中国医学科学院医药生物技术研究所《实验动物管理条例》中相关伦理规定, 开展动物实验。

关键词: 海分枝杆菌; 抗结核药物; 斑马鱼模型; 药物评价; 定量 PCR
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Application of *Mycobacterium marinum* infected zebrafish for evaluation of anti-tuberculosis drugs

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Abstract: Tuberculosis (TB) is a serious infectious disease caused by *Mycobacterium. tuberculosis.* In recent years, with the emergence of drug-resistant forms, the development of new anti-tuberculosis drugs is urgently needed. In this study, we used *Mycobacterium marinum* (*M. marinum*), which is highly similar to *M. tuberculosis*, to establish a *M. marinum* infected-zebrafish model and quantitative PCR (qPCR) method for bacterial count analysis. The results showed that injecting *M. marinum* into the yolk sac is an efficient and convenient way to infect zebrafish embryos. By counting the survival rate of infected zebrafish and the number of bacteria in zebrafish by Ziehl-Neelsen staining, we analyzed the efficacy of isoniazid and rifampicin as anti-tuberculosis drugs and the synergistic effect of drugs. The results suggested that three evaluation methods exhibit good consistency. This study demonstrated that zebrafish-*M. marinum* infection model combined with qPCR analysis is a simple and efficient method for *in vivo* screening and evaluation of anti-tuberculosis drugs. Animal experiments were carried out in accordance with the provisions for animal ethics in the Regulations on Laboratory Animals of Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences.

Key words: *Mycobacterium marinum*; anti-tuberculosis drug; zebrafish model; drug evaluation; qPCR

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结核病是由结核杆菌导致的一种死亡率较高的感染性疾病, 全球现有结核患者约2 000万, 每年新增加约900万患者, 死亡人数高达170万, 严重威胁着全球的公共卫生健康[1-3]。虽然异烟肼 (isoniazid)、利福平 (rifampicin)、吡嗪酰胺 (pyrazinamide)、乙胺丁醇 (ethambutol) 和链霉素 (streptomycin) 等抗结核药物可以有效治疗由药物敏感菌感染引起的结核病, 然而近年来随着耐多药结核菌 (multidrug-resistant-tuberculosis, MDR-TB) 以及广泛耐药结核菌 (extensively drug-resistant-TB, XDR-TB) 的不断出现, 结核的发病率日益上升, 因此, 发展新型抗结核药物以及更有效的疫苗迫在眉睫[4-9]。

理想的体内动物模型是药物研发的重要保证。由于结核分枝杆菌生长缓慢、技术操作要求高、风险大, 所以结核病的体内动物模型一直是抗结核药物研发的瓶颈问题。尽管一些灵长类动物可以模拟人类结核病的很多病理特征, 但是由于伦理以及成本等原因使其不适合作为结核病药物筛选及理论研究的动物模型[10], 而小鼠、豚鼠等动物模型虽然已经应用于抗结核活性确证及治疗方案评价, 但是均无法完全模拟出结核病损伤在人体中的关键过程, 例如肉芽肿的形成和成熟[7,11]。此外, 小鼠体内筛选模型成本较高, 因此建立新的体内抗结核药物筛选平台对于新药研发十分重要。

由于便于遗传学操作和饲养, 斑马鱼作为新型的模式生物日益成为体内试验理想的动物模型[12-15]。斑马鱼拥有与人类相似的复杂的免疫系统[16,17], 已经被用作研究许多疾病致病机制的工具[18,19], 而以斑马鱼作为天然宿主的海分枝杆菌, 是研究结核分枝杆菌的优良模式病原, 其生长快、毒力小、传染性低, 只需在二级生物实验室内进行研究, 并且其与结核分枝杆菌的基因相似性高达85%以上[20]。海分枝杆菌可感染斑马鱼, 并表现为感染早期细菌数目的急剧增多以及干酪样肉芽肿的形成, 与结核杆菌感染人的病程和病理表现非常相似[21-24], 并且可以对细菌的感染以及损伤进行实时观察[22]。基于上述特点, 斑马鱼海分枝杆菌模型已广泛应用于结核病的致病机制和发病病程等基础研究, 如肉芽肿的形成和成熟等[5,21,22,25,26]。虽然海分枝杆菌感染斑马鱼模型在基础研究中已被广泛应用, 但其在抗结核药物的筛选与评价中的应用仍然十分有限。主要的研究方法包括利用携带表达红色荧光蛋白质粒的海分枝杆菌感染斑马鱼, 进而通过荧光分析的技术手段评价给药效果[27,28], 或者根据海分枝杆菌感染斑马鱼的死亡率来衡量[29-31]。上述方法均存在着易受干扰、难以准确和绝对定量的缺陷。

目前, 国际上常用的海分枝杆菌感染斑马鱼的办法是通过显微注射的办法将海分枝杆菌注射到受精后一天的斑马鱼幼鱼的臀脉中[22,32]。但是该办法步骤繁琐, 不适合用于大规模的药物筛选[32]。菌液浸泡感染斑马鱼幼鱼的方法虽然简单, 但是效果很不稳定。本研究通过卵黄囊注射方式, 建立了海分枝杆菌感染斑马鱼模型, 应用qPCR细菌计数法测定细菌在斑马鱼体内的数量, 结果表明qPCR细菌计数法可以对药物效果定量评价。

材料与方法

菌 株 *Mycobacterium marinum* (*M. marinum*) ATCC 927 strain和*M. marinum* strains M (ATCC BAA-535) 接种于7H11固体培养基, 30 ℃倒置培养5～7天。挑取海分枝杆菌单菌落, 转入7H9液体培养基的试管中, 30 ℃静置培养5～7天。以1∶100体积比例将上述海分枝杆菌接种于7H9液体培养基中, 30 ℃、180 r·min^{-1}振摇培养至对数生长期。

实验动物 斑马鱼 (*Danio rerio*) AB系, 本院张靖溥教授惠赠。斑马鱼养殖于28 ℃的人工海水 (海水盐浓度28%), 采用人工光源对斑马鱼的生活周期进行调控, 照明14 h/黑暗10 h交替。饵料选用孵化24 h的丰年虾, 每日中午饲喂1次。依据中国医学科学院医药生物技术研究所《实验动物管理条例》中相关实验动物伦理规定, 开展本研究动物实验。

显微注射 将菌液吸入由毛细管制成的玻璃注射针 (3～5 μmol·L^{-1}), 注射入1～4细胞期受精卵的卵黄囊中, 注射的溶液体积一般约10 pL。

斑马鱼切片染色 斑马鱼幼鱼用福尔马林液固定过夜后, 依次脱水、透明、包埋和切片。石蜡切片脱蜡后, 进行HE染色和Ziehl-Neelsen染色。组织采集照片放大倍数为200。

qPCR测定海分枝杆菌DNA 扩增16S～23S ITS引物为: F:5'-CACCACGAGAAACACTCCAA-3'; R: 5'-ACATCCCGAAACCAACAGAG-3'。采用SYBR Premix Ex Taq II Tli (RNaseH Plus) 试剂盒 (TAKARA)。反应体系为20 μL, Mix 12.5 μL; 10 mmol·L^{-1}引物各1 μL; DNA 3 μL, 用ddH_2O补至20 μL。循环条件为95 ℃ 10 s, 65 ℃ 34 s, 40个循环, 实时荧光定量PCR进行扩增 (Agilent Stratagene MX3000P)。每个样品设置3个重复, 统计各个样品的CT值。

细菌数的计算 将梯度稀释 (1×10^2、1×10^3、1×10^4、1×10^5、1×10^6、1×10^7) 并经涂布平板计数的海分枝杆菌927与20个斑马鱼幼鱼混合后, 用Qiagen试剂盒提取基因组获取标准样品。根据标准样品的CT值用软件GraphPad Prism 5.01绘制标准曲线, 然后根据待测样

品的CT值和标准样品的回归方程，得到20条幼鱼体内的细菌数，单条幼鱼体内的细菌数=20条幼鱼体内的细菌总数/20。

半数抑制浓度 (half maximal inhibitory concentration, IC_{50}) 的计算 根据对照组和实验组斑马鱼体内的细菌数，算出各浓度组对应的抑制率，然后用GraphPad Prism 5.01计算每个药物的IC_{50}。

统计学方法 所有数据以$\bar{x} \pm s$表示，采用GraphPad Prism 5.01软件对数据进行分析，两组间均数比较采用t检验，多组间比较采用单因素方差分析，以$P<0.05$为具有统计学显著性差异。

结果

1 海分枝杆菌927株-斑马鱼幼鱼感染模型的建立

本实验采用人工注射的方式，注射的位置为斑马鱼的卵黄囊，注射的时间为斑马鱼鱼卵受精后的2 h左右，即胚胎发育的64细胞时期。在之前的很多研究中，海分枝杆菌感染斑马鱼鱼卵用的菌株都是使用毒力较强的海分枝杆菌M株，关于毒力较弱海分枝杆菌927株进行斑马鱼鱼卵感染的研究目前还没有报道。本实验分别以海分枝杆菌927株和M株采用卵黄囊注射的方式对斑马鱼胚胎进行注射感染实验，以灭活的菌株作为对照。结果表明，当海分枝杆菌927株的注射剂量大于90个细菌时，第8天斑马鱼的死亡率可以达到90%，而海分枝杆菌M株的注射剂量达到35个细菌时，斑马鱼鱼卵的死亡率就可以达到90%。当海分枝杆菌M的注射剂量为大于141个细菌时，斑马鱼鱼卵的发育会受到严重的影响，而927株在达到379个细菌的数目时斑马鱼鱼卵发育正常 (图1)。研究结果初步说明，海分枝杆菌927株可以用于斑马鱼鱼卵感染模型的构建，且卵黄囊注射的方式是一种可行的感染方式。

为了进一步确定卵黄囊注射方式的可行性，本研究用Ziehl-Neelsen抗酸性染色法对注射感染后5天的斑马鱼幼鱼染色，对其体内细菌的分布情况进行观察。如图2所示，海分枝杆菌感染斑马鱼5天后，斑马鱼的头部、尾部以及腹部都有海分枝杆菌的存在，表明细菌已经从注射的卵黄囊位置扩散到了全身，说明卵黄囊注射方式是一种可行的注射方式。鉴于本研究拟采用qPCR方法分析细菌数量，细菌的数目越高，其准确性越高，因此选用海分枝杆菌927株作为实验菌株。

2 应用qPCR的方法在幼鱼中评价药物的疗效

为了初步验证感染模型是否可以用于药效评价，本研究首先选择异烟肼和利福平处理感染海分枝杆菌927的斑马鱼鱼卵，通过分析斑马鱼鱼卵存活率、体内细菌数计数和抗酸性染色3种方法，对药物的给药效果进行评价。结果如图3所示，2 mmol·L^{-1}异烟肼和400 μmol·L^{-1}利福平对感染了海分枝杆菌927株的斑

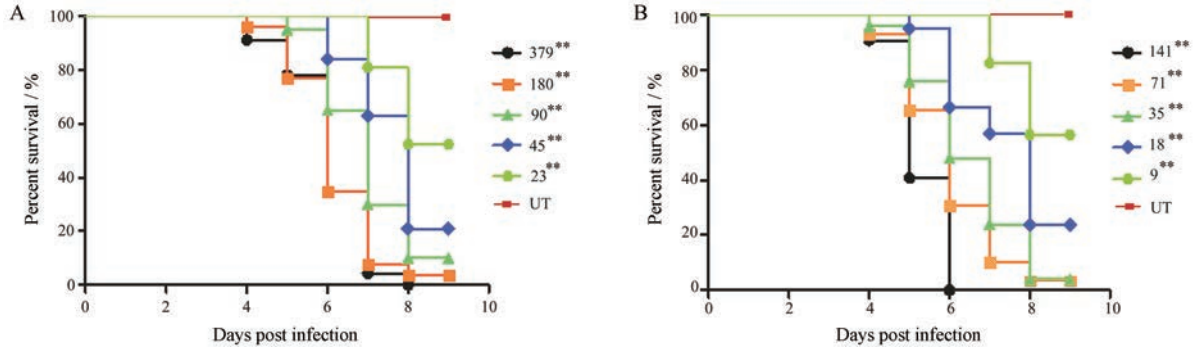

Figure 1 Lethal effects of zebrafish embryos infected with different concentrations of *Mycobacterium marinum* (*M. marinum*) strain 927 and *M. marinum* strain M. Zebrafish embryos were infected with *M. marinum* strains 927 (A) and strains M (B) at the indicated number of bacterial, then the survival ratio in the first ten days was recorded. n = 20, $\bar{x} \pm s$. $^{**}P<0.01$ *vs* the uninfected control group (UT)

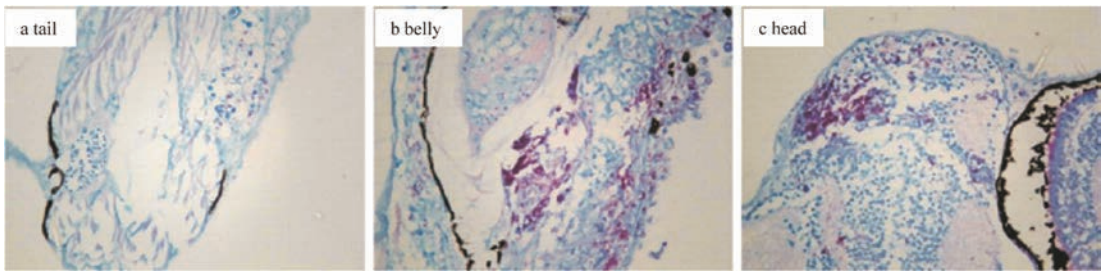

Figure 2 The distribution of bacteria in tail (a), belly (b) and head (c) of zebrafish was assessed using Ziehl-Neelsen staining at 5 days post infection. Zebrafish embryos were infected with *M. marinum* strains 927 (156 ± 23 cfu). Magnification of 200 ×

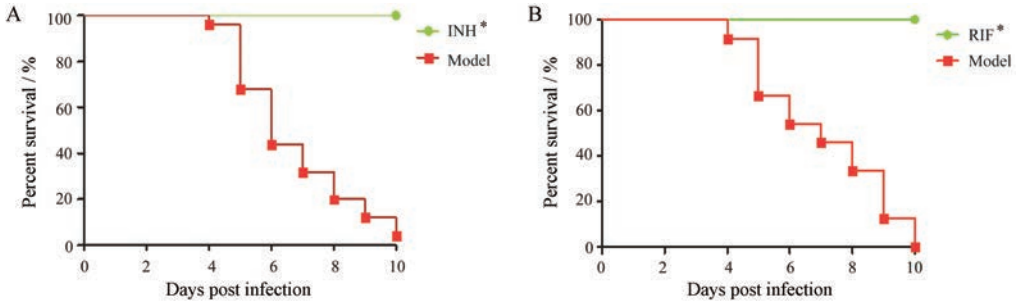

Figure 3 Isoniazid (INH, A) and rifampicin (RIF, B) can rescue infected embryos. Zebrafish embryos were infected with *M. marinum* strains 927 (186 ± 31 cfu), then randomly split into two equal groups. One group was exposed to 2 mmol·L^{-1} INH or 400 μmol·L^{-1} RIF for 10 days (exposure to the drugs achieved by adding compounds to egg water; antibiotics refreshed once daily) and the model group was followed without treatment (water refreshed once daily). n = 20, $\bar{x} \pm s$. $^{*}P<0.05$ *vs* model group

马鱼具有明显的治疗效果, 可以显著地降低死亡率。

同时, 为了精确测定斑马鱼幼鱼体内的细菌数目, 本研究建立了一套基于 qPCR 的细菌计数方法, 通过检测海分枝杆菌 16S-23S rRNA 的拷贝数来对斑马鱼体内的细菌数目进行定量, 16S-23S ITS 通常在一套海分枝杆菌的基因组里只有一个拷贝[33,34], 因此, 16S-23S ITS 基因 PCR 产物的水平可以用于反映细菌的数目。为了验证 qPCR 的办法可以用于抗结核药物的筛选, 本研究分别用 2 mmol·L^{-1} 异烟肼和 400 μmol·L^{-1} 利福平对感染了海分枝杆菌的斑马鱼进行治疗, 通过 qPCR 计数法对斑马鱼体内的细菌数进行计数, 对药物的给药效果做出评价。结果如图 4 所示, 在斑马鱼胚胎发育的过程中, 模型组鱼卵内的细菌数目逐渐增加, 其增长速度接近于不受抑制的呈指数式增长, 异烟肼和利福平给药组的斑马鱼幼鱼体内海分枝杆菌受到药物的抑制, 不再生长。

为了验证 qPCR 计数法的可靠性, 本研究将感染了海分枝杆菌的斑马鱼鱼卵分别进行药物处理, 并以

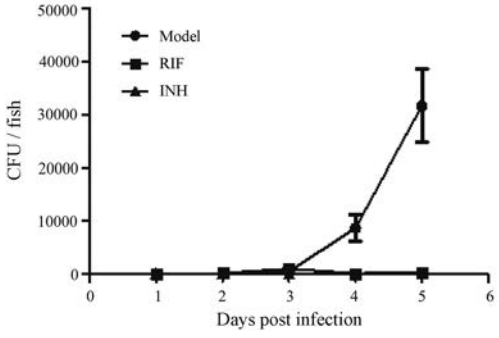

Figure 4 The effect of INH and RIF on bacterial growth in zebrafish. Zebrafish embryos were infected with *M. marinum* strains 927 (176 ± 39 cfu), then randomly split into equal groups that were exposed to 2 mmol·L^{-1} INH, 400 μmol·L^{-1} RIF or buffer, respectively. The bacteria number in zebrafish was calculated by qPCR daily until five days of post-infection. n = 20, $\bar{x} \pm s$

模型组作对照, 于第 5 天对斑马鱼行 Ziehl-Neelsen 抗酸性染色, 结果如图 5 所示, 结果表明, 与模型组相比, 给药组斑马鱼体内海分枝杆菌的分布很少, 仅在异烟肼给药组斑马鱼的腹部有少许细菌的存在, 而模型组的斑马鱼的腹部、头部以及尾部都有大量海分枝杆菌的存在, 说明抗结核一线药物异烟肼和利福平可以明显减少鱼卵内的细菌含量, 与 qPCR 计数法的曲线相一致。

3 应用 qPCR 计数法计算抗结核药物异烟肼和利福平的 IC_{50}

如图 6 所示, 应用斑马鱼死亡率 (图 6A 和 B) 计算利福平和异烟肼的 IC_{50} 分别是 166 和 840 μmol·L^{-1}, 应用 qPCR 计数法计算利福平和异烟肼的 IC_{50} 分别为 42.3 和 358 μmol·L^{-1}, 两种方法计算得到的 IC_{50} 基本一致。上述结果表明, 应用 qPCR 计数法计算抗结核药物异烟肼和利福平的 IC_{50} 非常可信。

4 利福平与异烟肼的协同作用

本研究通过观察斑马鱼的死亡率以及通过应用 qPCR 计数法来计算幼鱼体内的细菌数来检测异烟肼和利福平之间的协同作用。研究结果发现, 单独给药异烟肼组和利福平组斑马鱼的成活率要明显高于模型组, 但却又明显低于异烟肼与利福平联合给药组。由于低剂量给药只能延缓斑马鱼的死亡, 超过第 10 天, 各个给药组的斑马鱼大部分都会死亡。与生存率结果相吻合, 异烟肼与利福平联合给药组鱼卵内 (第 5 天) 的细菌数目要明显低于异烟肼以及利福平的单独给药组 (图 7)。上述结果表明, qPCR 计数法也可用于抗结核药物协同作用分析。

讨论

目前国际上关于斑马鱼鱼卵的感染实验用的菌株主要是海分枝杆菌 M 株, 采用的注射方式为尾臀注

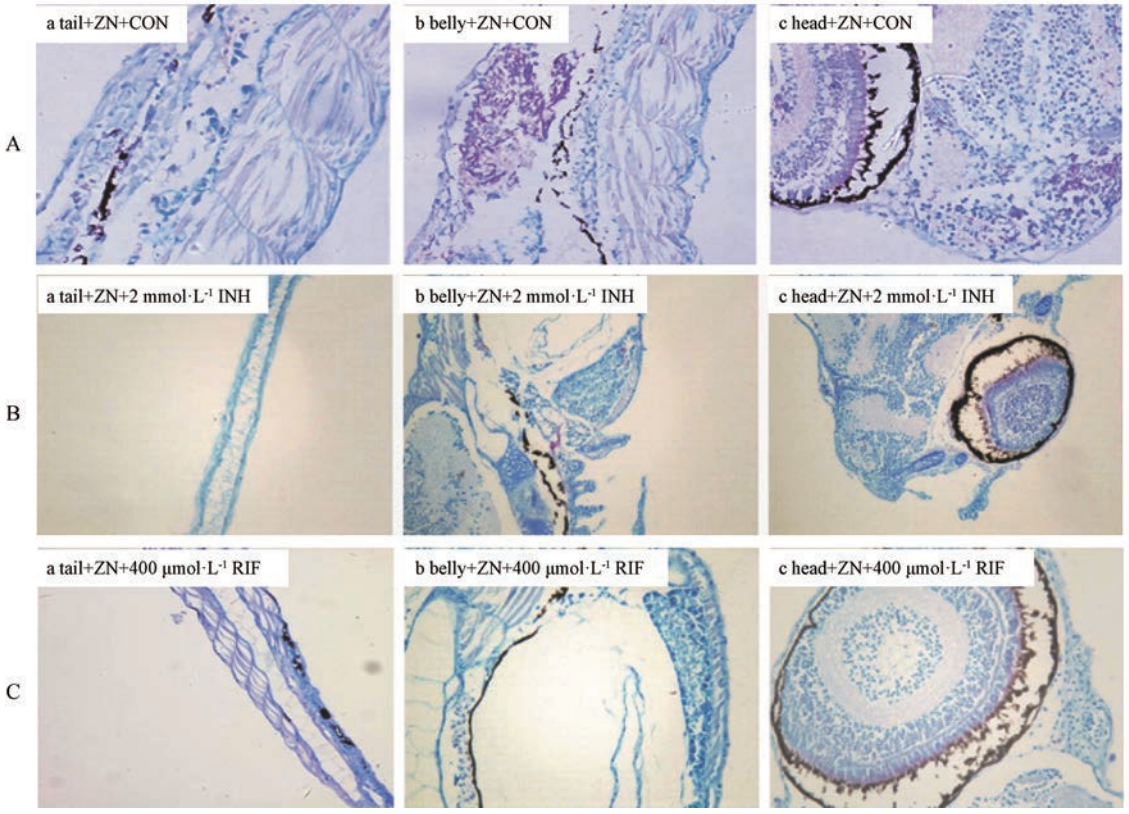

Figure 5 The effect of INH or RIF on the distribution of *M. marinum* in infected zebrafish. Zebrafish embryos were infected with *M. marinum* strains 927 (138 ± 27 cfu), then randomly split into equal groups that were exposed to buffer (A), 2 mmol·L^{-1} INH (B) or 400 μmol·L^{-1} RIF (C), respectively. At 5 days post infection, the distribution of bacteria in tail (a), belly (b) and head (c) of zebrafish was assessed using Ziehl-Neelsen staining

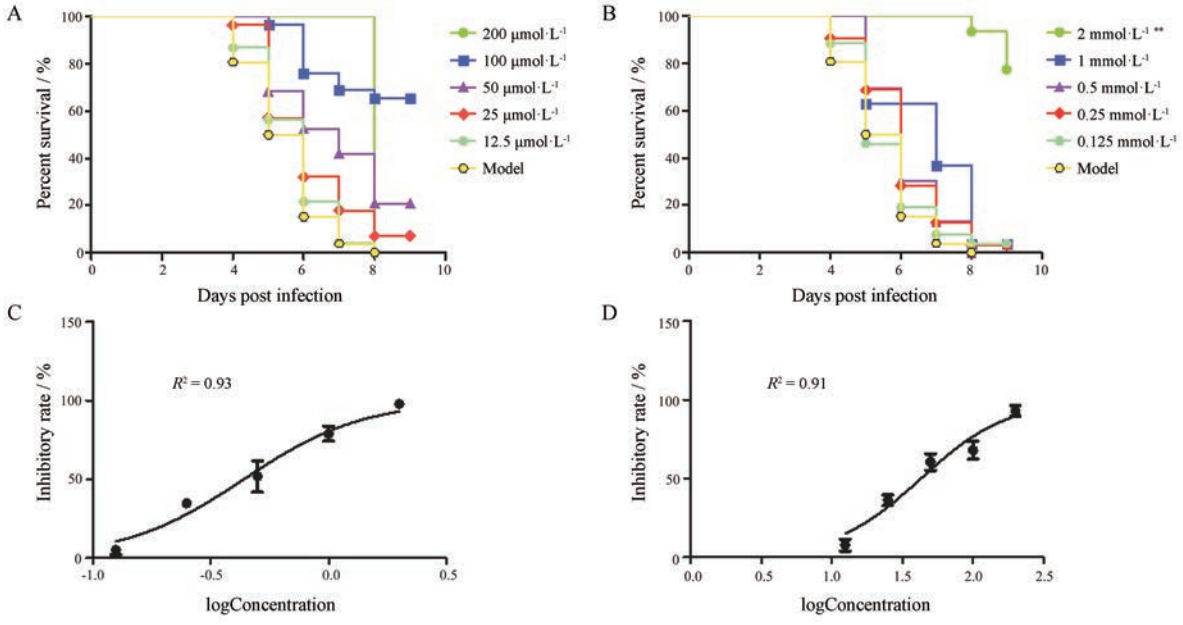

Figure 6 The dose response analysis of RIF and INH in a *M. marinum* infected zebrafish-model. Zebrafish embryos were infected with *M. marinum* strains 927 (199 ± 33 cfu), then randomly split into 6 equal groups, followed by the treatment with different concentration of RIF (200, 100, 50, 25, 12.5 μmol·L^{-1}) (A, C) or INH (2, 1, 0.5, 0.25, 0.125 mmol·L^{-1}) (B, D). At 5th day of post-infection, the bacteria numbers of 20 larvae were determined by qPCR, which is used to calculate IC_{50} by GraphPad Prism 5.01. Concentration inhibition rate of RIF (R^2 = 0.91) or INH (R^2 = 0.93) was detected, respectively. n = 20, $\bar{x} \pm s$. $^{**}P$<0.01 *vs* model group

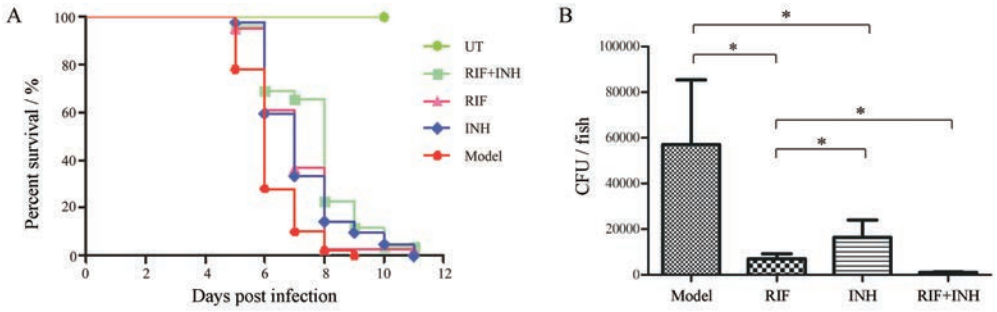

Figure 7 Synergistic drug interactions in the zebrafish larval infection model. Zebrafish embryos were infected with *M. marinum* strains 927 (158 ± 19 cfu), then randomly split into 4 equal groups, followed by the treatment with 100 μmol · L^{-1} RIF and 0.5 mmol · L^{-1} INH or both, respectively. At 5th day of post-infection, the bacteria numbers of 20 larvae were determined by qPCR. A: The survival rate of zebrafish in various treatments; B: Numbers of bacteria of zebrafish in various treatments. n = 20, $\bar{x} \pm s$. *P<0.05

射[13,16,21,28,35-37]。本课题组通过实验证明, 应用海分枝杆菌927株在斑马鱼受精卵受精后约2 h采用卵黄囊注射的方式也可以达到致死的效果, 并且用尼森染色证实细菌在斑马鱼幼鱼体内是以聚集状态存在, 表明注射的效果与臀脉注射的效果相同, 但是注射的效率大为提高。经过初步估计, 采用卵黄囊注射的方式, 每小时的注射量能有2 000个, 感染率接近100%。

本研究建立了一套依赖qPCR计数法的抗结核药物药效评价系统, 首先将该系统在海分枝杆菌感染幼鱼模型上进行验证, 通过结合斑马鱼鱼卵内的细菌数以及斑马鱼的成活率对药物的治疗效果进行综合评价, 评价的周期仅仅为5天, 与其他动物模型相比, 时间大为缩短, 明显提高了药物评价效率。同时, qPCR计数法可以对斑马鱼体内的细菌数目进行准确定量, 进而更为量化地对药物的给药效果进行评价。

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