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二甲双胍通过GSK3β/Wnt通路抑制涡虫再生---再生与长寿的新思考

Metformin Suppresses Planaria Regeneration through the GSK3β/Wnt Pathway ---New Insights on the Association between Regeneration and Longevity

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二甲双胍通过 GSK3β/Wnt 通路抑制涡虫再生

---再生与长寿的新思考

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论文摘要：

现代医学中，二甲双胍在延长寿命和预防癌症上展现出了出乎意料的潜力。除二甲双胍之外，另一个在长寿领域的大热话题是再生的过程。再生能力与长寿到底有什么具体关联？如果二甲双胍在许多生物模型中表现出可以延长寿命的作用，那么它对再生的进程又有什么影响呢？二甲双胍在生物体中对再生的作用途径又具体是什么？为了试图回答这三个问题, 我们在常见的再生模型涡虫身上试验和观察了二甲双胍在不同浓度下对涡虫再生的影响。然后，我们还通过免疫组化来探索二甲双胍在神经系统再生进程有什么作用。进一步，我们通过 qPCR(荧光定量 PCR)来探寻在二甲双胍作用下涡虫体内与再生相关的基因的表达变化。在表型的观察中，结果出乎我们的推测：二甲双胍实际上却减缓了涡虫再生修复的过程。在 qPCR 的结果中，我们发现二甲双胍可以显著增加涡虫体内 GSK-3β的表达，暗示二甲双胍可能通过 GSK-3β/Wnt 通路调控涡虫的再生。为了验证我们的假设，我们随后通过小分子抑制剂抑制了实验组涡虫的 GSK-3β的活性。结果表明，在抑制 GSK-3β之后，涡虫被二甲双胍减缓的再生过程得到了恢复。同时，神经系统再生也得到了恢复。通过这些实验结果表明 GSK-3β/Wnt 通路在二甲双胍生抑制涡虫再生过程中发挥重要作用。再生与长寿的关系远远比简单的促进要复杂，再生只是长寿中一个相关的因素。二甲双胍在增加长寿中有着巨大作用，而在我们的实验中发现的二甲双胍抑制再生的现象看似是与我们的推测相悖，事实上却是在解释再生与长寿有比我们的猜测更复杂的关系。

关键词：二甲双胍；涡虫；再生

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论文正文

前言：

要说永生领域中的一个新星那一定就是二甲双胍。二甲双胍本来是一种通过降低血糖来治疗二型糖尿病的口服处方药。二甲双胍在人体中会减少肝脏的葡萄糖的产生，同时让人体的细胞对胰岛素更加敏感。最近的多项研究却发现了二甲双胍除了治疗二型糖尿病以外多项潜在用途，其中就包括增加寿命。

二甲双胍到底是什么东西，应用范围是什么?在人工胰岛素技术还未出现之前，二型糖尿病病人只能求助于猪的胰岛素去代替自身不足的胰岛素。但是猪胰岛素生产价格昂贵，所以在二甲双胍的历史中它一直是一种十分受欢迎的药物。二甲双胍有非常长的历史。最早在一本被鉴定来自于 17 世纪的药草指南里，二甲双胍就已经被记录为一种药物了(Tomas et al, 2017)。当时的居民已经学会了从法国丁香花中提取二甲双胍这种物质。虽然他们还没有发明“二型糖尿病的名字”，却也已经像现代人一样用其治疗糖尿病的症状。在近代的历史中，科学家们首次在 1992 年人工合成了二甲双胍的核心有效物质 dimethyl biguanide (Tomas et al, 2016). 但是在二甲双胍历史的大部分时间里，它都仅仅被人们当做一种简单的糖尿病药物。

最近，很多科学家提出了一些二甲双胍的潜在用途。首先在 2005，来自英 Stirling 大学的教授 J.M.M Evans 就猜测二甲双胍可以减少二性糖尿病病人的癌症发生率（Evans, 2005）。二甲双胍通过腺苷酸活化蛋白激酶来调控血糖的高低，腺苷酸活化蛋白激酶可以引导人体细胞从血糖中吸收更多的葡萄糖。而腺苷酸活化蛋白激酶的一个上游控制器是 LKB1. LKB1 可以通过编 serine threonine 来活化腺苷酸活化蛋白激酶(Shackelford et al,2009)。LBK1 同时也是一个广为人知得到肿瘤抑制物质。这个作用通道让二甲双胍将细胞新陈代谢与有丝分裂调控联系起来(Evans et al,2005)。许多其他的研究却表明，二甲双胍是一个潜在的能够治疗和高龄疾病基本起因的方法。在 2019 年 1 月发表的最新研究中，研究者们发现二甲双胍以影响能量使用的分布的方式延长了蚕的寿命。这个发现似乎反映了二甲双胍在影响腺苷一磷酸中的作用：触发了激酶 p53 基因叉头蛋白质的盒类 O 信号通

路，并因此增加了这些蚕的抗压能力和抗氧化能力，还降低了蚕生产蚕丝所需的能量。这三个效果综合在一起，延长了蚕的寿命（Song et al,2019）。在另外一个用雄性老鼠的实验当中，其结果表现了从中年开始的长期的二甲双胍摄入增加了这些受试目标的健康状况和寿命。在细节层面上，这里二甲双胍表现出的效益与卡路里限制极其相似，例如胰岛素敏感性增加(Montalvo et al,2013)。

在二甲双胍方面最新的相关研究中披露，在美国加利福尼亚州展开的小型临床研究首次表明（Fahy et al, 2019），人体中用于指示生理年龄的表观遗传时钟可以被逆转。表观遗传时钟依赖于身体的表观基因组，其包含标记 DNA 的化学修饰。这些标签的模式在生命过程中发生变化，并跟踪一个人的生物年龄，这可能落后于或超过实际年龄。研究者令 9 名健康志愿者服用了三种常用药物 - 生长激素和两种糖尿病药物（其中包括脱氢表雄酮（DHEA）和二甲双胍）并且通过分析一个人基因组上的标记来测量其平均生命年龄，结果表明每人平均年龄减少约 2.5 岁。参与者的免疫系统也显示出恢复活力的迹象。然而，这只是一个初步的实验结果并且还存在许多缺点，如实验规模很小，没有设置对照组等。

虽然二甲双胍发现早历史久远，但是其具体的反应机制与完全的作用途径仍然不清楚。十年之前药理学家们认为二甲双胍的唯一作用靶点就是腺苷酸活化蛋白激酶(AMPK adenosine monophosphate-activated protein kinase), 但到现在我们发现二甲双胍有很多与腺苷酸活化蛋白激酶相关与不相关的作用途径。近几年来科学家们也甚至发现了其他更多的二甲双胍的新兴用途，治疗肥胖，预防癌症。在其中各种新发现中，减缓衰老是当今二甲双胍研究中的重点。虽然二甲双胍的作用可以被简简单单地描述为“增加寿命”，但众多信号通路在药物作用下如何产生了这个总效应的原理仍然不明确。

而长寿与再生由于什么关系呢?

长寿最大的敌人就是衰老。在本质来看，人类总体的衰老过程其实就是人体中的各个器官组织老化的体现。当器官老化是，这些器官的作用也慢慢恶化衰弱。衰老这个过程本身并不致命，真正致命的是伴随着衰老的各种慢性病。一个

这样的慢性病的例子就是通过二甲双胍治疗的二型糖尿病, 2016 年由二型糖尿病直接造成的死亡在全球就有 150,0000 例(WHO)。为了促进长寿增加寿命，最为必要的一步就是保持器官的活性。器官老化只要是因为器官中的细胞老化凋亡速度超过了新细胞产生的速度（端粒变短，使得基因信息无法被完全复制）。没有新的细胞来代替，老化的细胞就需要继续依靠自身维持其作为体细胞的功能。但是老化的细胞并不能如同新细胞一样有效地工作。当一个器官中老化细胞的占比渐渐上升，这个器官的功能表现也会慢慢变差。这个过程并不会在年轻人的身体里面发生，因为年轻人的细胞再生能力都还比较强。他们的干细胞仍然有能力去产生新细胞去代替器官中受损与老化的细胞。但老年人的细胞再生无法像年轻人这样快速有效地进行。没有代替的新细胞，他们的器官就会慢慢老化。由此来说，细胞再生是衰老的根本解决方法。对再生能力的增强从某些角度来说就是对寿命的增强。细胞再生过慢是衰老的主要原因，是在长寿中一个绕不开的话题。

如果当今很多研究都表明二甲双胍可以增加寿命，那么二甲双胍有可能会加强细胞再生的能力吗？有可能是二甲双胍通过加强再生能力来增加了这些研究中受试目标的寿命吗？那么，如果二甲双胍可以延长寿命，那么它对再生又会有什么影响呢？为了回答这两个问题，我们在涡虫的再生模型上探索了二甲双胍的作用。涡虫是分子生物学实验中常见的再生模型。来自涡虫纲的涡虫以他们强大的再生能力而著称.一些学者也认为涡虫强大的再生能力可以为人类的永生带来新的解题思路。 其广为人知的强大再生能力源自于涡虫体类广泛存在的新胚叶(neoblast). 这些由高度集中的干细胞组成的新胚叶使得涡虫能够从其身体干重的 273 分之 1 再生出一整只新涡虫(Morgan et al,1898)。普通的成年涡虫身长约为 10mm，但与其小巧的身形不同的是涡虫具有与高等动物相似的神经结构。

为了研究二甲双胍对再生的影响，具有的强大的再生能力的涡虫是我们实验的理想的研究模型。所以，实验中我们用不同的二甲双胍浓度处理涡虫，观察他们再生过程的变化。然后通过 qPCR, 通路干扰的方式探究二甲双胍对涡虫作用的机制（实验流程见图 1）：

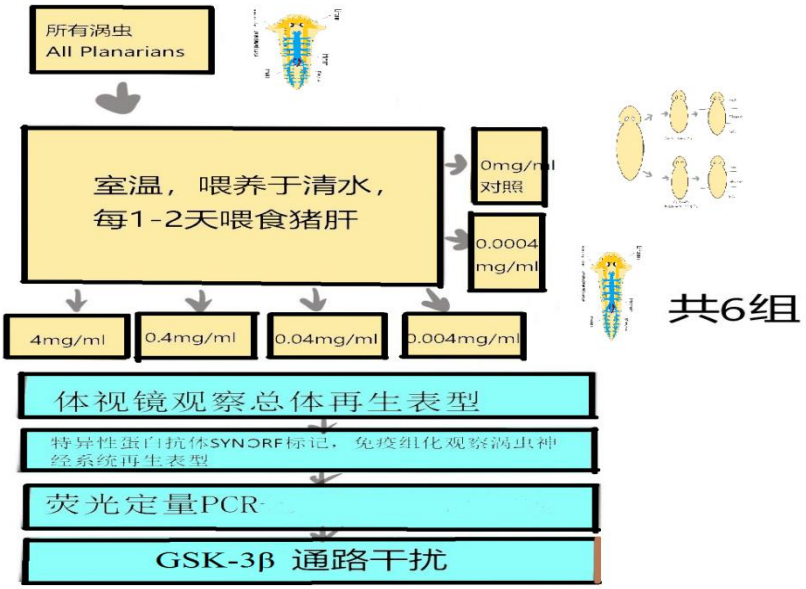

图 1. 实验流程图

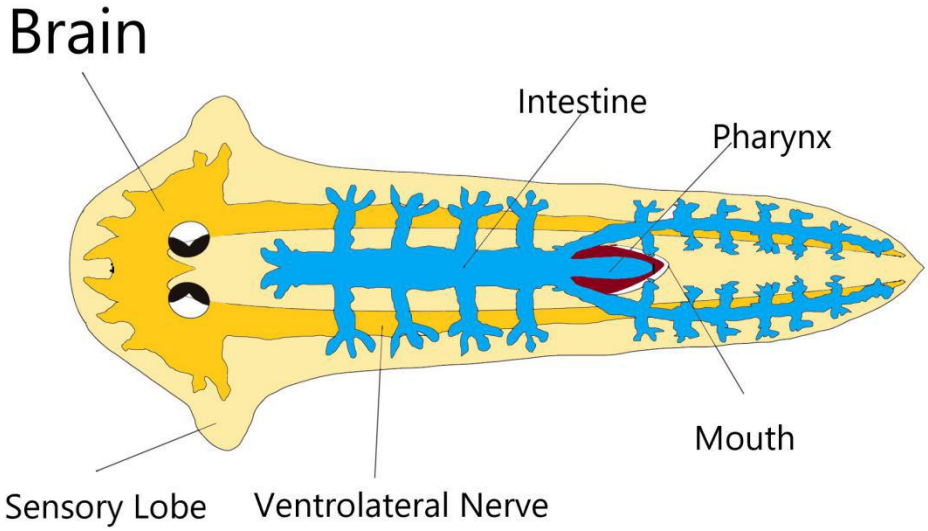

图 2：涡虫结构示意图

方法:

(1) 涡虫处理与切割:

本实验中采取的涡虫是东亚三角涡虫(*Dugesia japonica*)（见图 2）。将其在室温下喂养于盛清水的玻璃碗中，每 1～2 天进行喂食猪肝。在进行实验前，先将涡虫进行切割（见图 3）。培养皿中，将涡虫切成 3 段：头，腹，尾。对照组五只涡虫同样将涡虫分为三段，放置在装有清水得细胞培养板中。在用二甲双胍处理涡虫时，每一组涡虫数量为五只（实验组一共有两批涡虫，第一批受二甲双胍处理后进行体视镜拍照与免疫组化，另一组受相同的二甲双胍处理后进行 qPCR）。实验组将每一只涡虫的三段分别放置在装有不同浓度等体积的二甲双胍溶液的 24 孔细胞培养板中，分别为： 4 mg/ml，$4*10^{-1}$ mg/ml，$4*10^{-2}$ mg/ml，$4*10^{-3}$ mg/m，$4*10^{-4}$ mg/ml。

其中的中间浓度 $4*10^{-2}$ mg/ml 是我们按照二甲双胍的成年人药用指南根据药物量/单位重量得来的。然后我们再依照这个浓度像两边稀释得到了其他的浓度值。对照组同样也在切割后浸入清水环境中。

体视镜下观察再生的表型:

在持续处理 6h，1d，3d，7d 后，首先通过体视镜观察不同组涡虫的再生情况。为避免涡虫蠕动，将实验组和对照组涡虫放在置于碎冰表面的载玻片上。待其完全伸展，通过体视镜进行拍照。

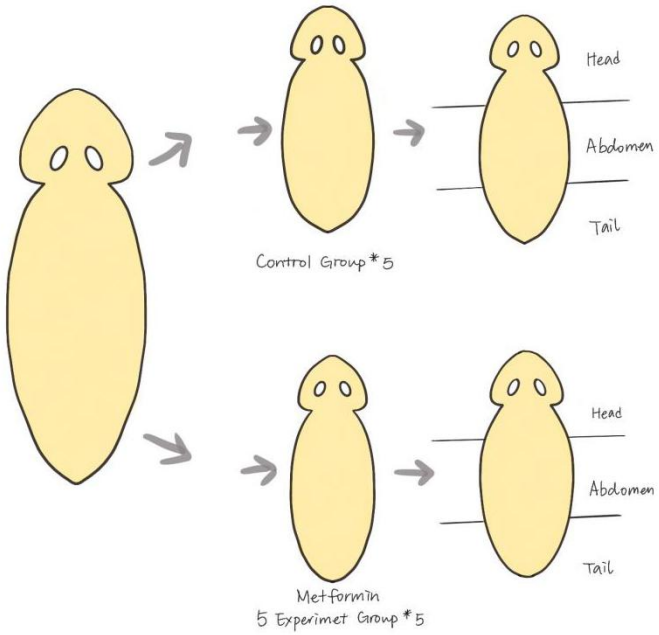

图 3：涡虫切割方法

(2) 涡虫神经系统的免疫组化分析

涡虫切后 0h，2 天，7 天时，我们通过免疫组化对对照组和实验组的涡虫用神经系统特异性标记的蛋白抗体 SYNORF1（购置于 DSHB 公司）进行再生情况的分析。整个实验包含 11 个步骤，依次为：先用 2%的 HCl 溶液处理处理涡虫 10 分钟 （处理涡虫），再将涡虫放置在 4%的多聚甲醛溶液中 2h（固定涡虫），然后在零下 20℃的环境下放置在 100%的甲醇溶液让涡虫脱水 1h（脱水），再在 1%的 SDS 溶液下通透涡虫 25min（通透），通透完成后用不同浓度的甲醇溶液（75%，50%，25%）各处理涡虫十分钟（水化），将涡虫放置在牛奶中封闭 1h（封闭）后，在 4 的条件下，将涡虫放在 1:30（牛奶：抗体）的溶液中放置过夜（一抗孵育），再用 PBST 溶液清洗涡虫五次，每次 5-10 分钟（洗涤），最后涡虫在 1:200（牛奶：抗体）的溶液中，4℃的环境下过夜（二抗孵育），抗体孵育完成后在显微镜下过检。其中，封闭、一二抗孵育，洗涤均在摇床上完成。

(3) RNA 的提取与反转录

总体来说，先使用 Trizol(TaKaRa)从涡虫中提取总 RNA，然后用 RNA 合成 cDNA。先取五只涡虫放在灭过菌的 1.5ml 离心管里，把多余的水分吸掉，加入 500ml RNA 提取试剂 Trizol，用枪头戳涡虫，吹打使样品充分裂解。再加入三氯甲烷，猛烈晃动 20s，室温放置 3min。放入离心机在 1200g，4℃的条件下离心。由此，我们可以得到水相和 RNA，再加入等体积的异丙醇，室温静置 10min，离心。然后，在离心管中加入乙醇清洗，得到相对纯净的 RNA。最后，将提取出的涡虫总 RNA，保存于-80℃的冰箱中备用。

反转录时，先取五只涡虫，加入五成的 gDNA buffer 和 1 微升的 gDNA eraser 去除原涡虫体内的 DNA，在 42℃ 2min，4℃forever 的条件下离心，随后上述体系再加入 mix，primer，RNase Free H20，将 RNA 反转为 DNA，在 37℃ 15min，85℃ 5s，4℃forever 的条件下离心，并在-20℃的冰箱里保存。

(4) 荧光定量 PCR

实验和对照组选取凋亡和增殖基因作为观察对象，对应和 PCNA，以 actin 为对照组进行 qPCR。实时荧光定量 PCR 引物通过 Primer Premier 软件设计获得，序列见表 1。

每个反应体系 25 微升需加二成的 mix，正反引物，水，目标 cDNA。PCR 反应程序参数为：每个循环，在 95℃的条件下 DNA 变性 5min，随后在 60℃下退火 15sec，最后在 75℃的条件下延伸 30sec。PCR 反应结束后，取 5 微升进行琼脂糖凝胶电泳检测。

表 1：PCR 引物序列表

	Sequence (5’-3’)
Bcl2L qPCR-F	GCATTGTGGCTTTCTTCTCCTTCG
Bcl2L qPCR-R	CATCGTTCCCATAGAGGTCCACAAA
DjMCM2 qPCR-F	GATTTGTGGTGATCCTGGCACT
DjMCM2 qPCR-R	ACTAACGCTCCTGCTTCCA
DjPiwiA qPCR- F	TCTGATAGCGAAAGAAGCAA
DjPiwiA qPCR- R	ACGACCACGAATAGTAATAGG
DjPiwiB qPCR- F	GACCTTACGATTAGTTGCGACCAT
DjPiwiB qPCR- R	TTGCGAGGAACTTCGTCTTT
GSK3β qPCR-F	GGAATCCAGACCCTT
GSK3β qPCR-R	CAATAACTAAACTGGCTGA
MCL-1 qPCR-F	CCTCCTGTGCGTTCTTAT
MCL-1 qPCR-R	TCTGTTCCCAAACCAATAA
p53 qPCR-F	CCCCACTTTCTTGACCAT
p53 qPCR-R	CGTAAACGCTTCGAGATGTTCC
PCNA qPCR-F	AAGAAAGTGATGCCGTAA
PCNA qPCR-R	ATAACTGCTGGAACA
WNT qPCR-F	TGTCATCACATCCTTGCCATAT
WNT qPCR-R	ATGCCATGCTCAGTGCTAG

(5) 抑制剂处理：

为了验证我们的猜想，即 GSK-3β/Wnt 通路是二甲双胍抑制涡虫再生过程的主要方式之一，我们用相同的方式喂养新的一组共三只的涡虫，作为三个重复样本。在处理涡虫时，将每只涡虫的头部与尾部切除，留下中间部分好以同时观察每个样本的头部与尾部的再生进度。

在给涡虫处理二甲双胍时，我们选取了之前实验组中二甲双胍抑制涡虫再生现象最为明显浓度，即 4 mg/ml。GSK-3β 的抑制剂采用的是购置于陶素

TOPSCIENCE 的小分子 CHIR-99021，具体靶点为 GSK-3α和 GSK-3β，浓度为 0.1μm/ml。为让涡虫在其现有环境之下尽可能充分地再生，因普通未处理的涡虫绝大部分会在 7 天之内完全再生，我们也让抑制剂组的涡虫在体系中用 7 天的时间修复。

7 天后，我们再次用同样的步骤对这三个重复样本进行体视镜观察与神经系统免疫组化分析。以此，我们利用再生表型判断二甲双胍对涡虫的抑制作用有没有被抑制剂阻断或减轻，进而验证或推翻我们的猜想。

结果:

(1) 涡虫再生情况观察

涡虫在被切为头，腹，尾三段后，6 小时时用显微镜进行观察，头部，腹部，尾部均有明显的被切除后的伤口痕迹，尚未出现愈合的标志。在涡虫被处理完成的 1 天后，其头部，腹部，尾部均出现少量胚基。3 天后，胚基增大，尾部再生部分出现眼点。在被处理完成 7 天后，涡虫再生完整。其中，头，腹，尾的再生快慢并没有非常明显的差别，但是总体上来说，头部完成再生的速率在相较之下更快(图 4 与图 5)。

(2) 二甲双胍抑制涡虫再生

实验组涡虫被不同浓度的二甲双胍溶液处理完成后，将涡虫分为三段。不同时间观察实验组涡虫头部，腹部和尾部再生情况。并拍照记录。

本实验二甲双胍溶液浓度梯度依次为：4 mg/ml，$4*10^{-1}$ mg/ml，$4*10^{-2}$ mg/ml，$4*10^{-3}$ mg/ml，$4*10^{-4}$ mg/ml。观察发现，二甲双胍在不同浓度都有对涡虫再生的抑制作用，但抑制作用的程度也表现出了浓度依赖性。在高低端浓度条件下（4 mg/ml，$4*10^{-4}$ mg/ml），涡虫的伤口才出现愈合趋势，出现极少量的胚基。而在中间浓度梯度（$4*10^{-1}$ mg/ml，$4*10^{-2}$ mg/ml，$4*10^{-3}$ mg/ml）涡虫再生相对较快速，但相较于对照组仍然有明显的减缓。如图 4 及图 5，在切割 1 天后，

对照组和实验组涡虫基本都没有开始再生。在切割 4 天后，对照组涡虫已再生出眼点，胚基部分有少量的色素开始沉着，而实验组中间和极端浓度梯度的涡虫眼点部分再生均不明显，胚基未出现少量的色素沉淀。在切割后七天，对照组涡虫已经完成了再生，但实验组中中间浓度梯度的涡虫伤口仍然没有完全愈合，意味着没有再生完全。只有其中用 $4*10^{-2}$ mg/ml 二甲双胍浓度处理过的涡虫再生情况相对良好，7 天后基本上趋于再生完整。在相同的二甲双胍浓度中，头，腹，尾之间的再生速率差异并不明显（图 4 与图 5）。神经系统的再生也有类似的结果（图 6）。

图 4：不同浓度二甲双胍对涡虫再生的影响（背部）

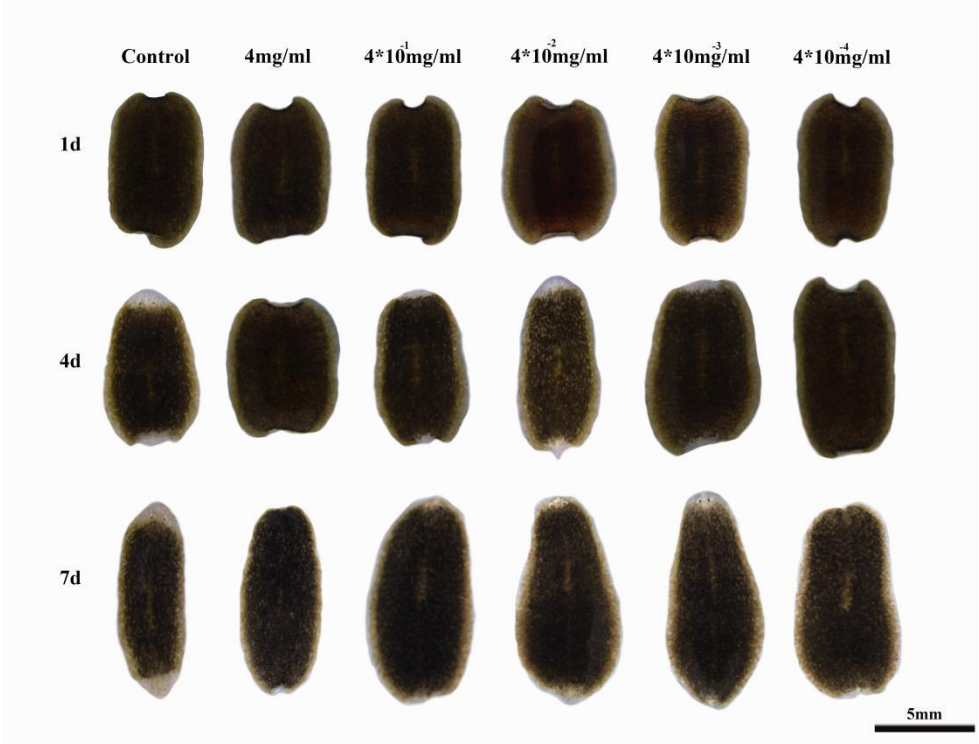

图 5：不同浓度二甲双胍对涡虫再生的影响（腹部）

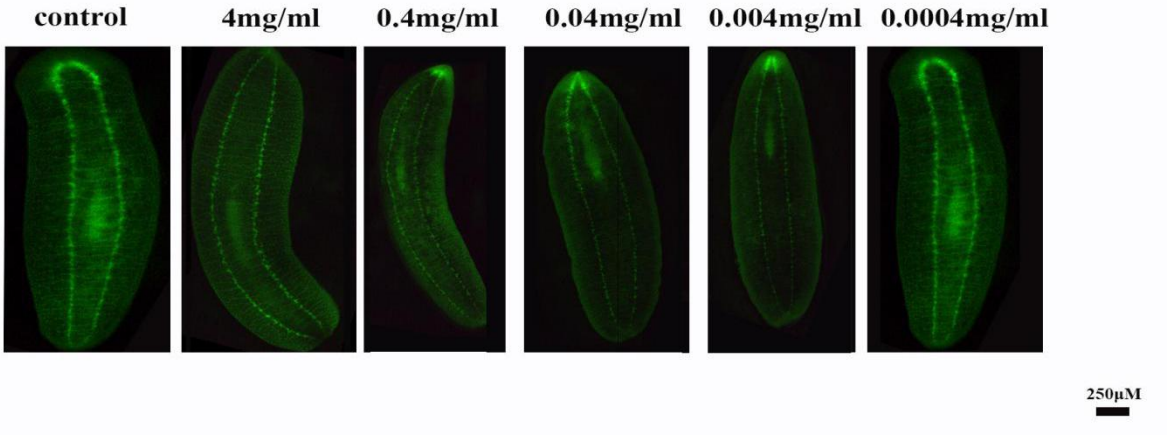

图 6：不同浓度二甲双胍对涡虫神经系统再生的影响

(3) 二甲双胍在涡虫再生过程中的作用机制探索

为了探索二甲双胍对再生的影响，我们利用 qPCR 检测了几个与再生相关的基因的表达，包括与干细胞相关的 DiPiWiA, DjPiWIB; 与增殖相关的 PCNA; 与凋亡相关的 P53 基因等等。

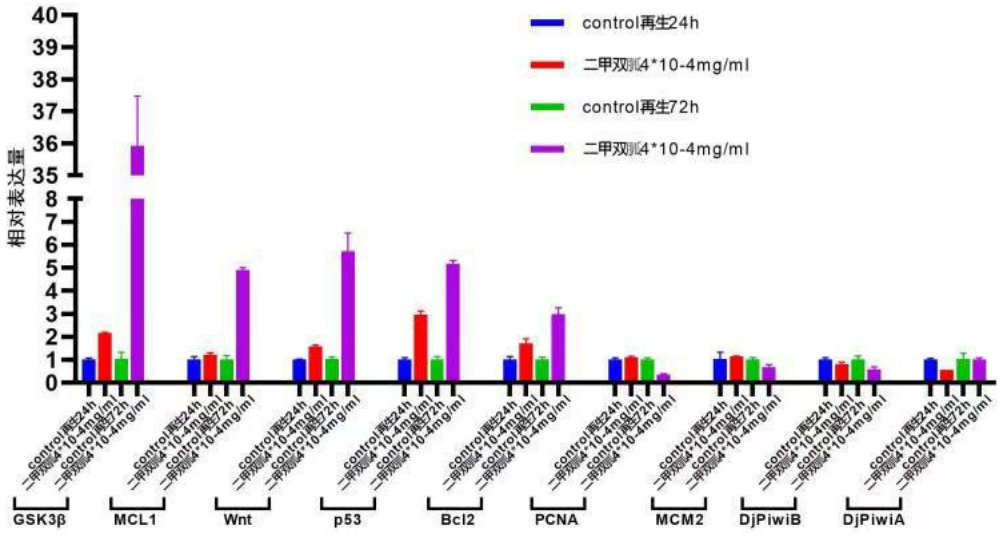

图 7：二甲双胍处理涡虫后，相关基因的表达变化

图 7 所示，与对照组相比实验组的 DjPiWiA 基因与 DjPiWiB 的表达有统计学上明显的减少。PCNA 的基因表达变化与 MCM2 的基因表达变化趋势相似。二甲双胍处理后，两者都在 72 小时有明显的下降。与之相反的是，与对照组相比，实验组的 P53、BCL2、MCL-1 的表达在二甲双胍处理 72 小时后有显著的上调。有意思的是，Wnt 信号通路中关键基因 GSK-3β，在二甲双胍处理再生 72 小时后，基因的表达量有非常显著的增加（图 7）。

(4) 抑制 GSK-3β能显著的缓解由二甲双胍所引起的涡虫再生抑制

qPCR 结果显示二甲双胍处理涡虫后 GSK-3β的表达显著上升。GSK-3β是 Wnt 信号通路中的关键基因，而 Wnt 信号通路在涡虫再生过程中发挥着重要作用，因此我们猜测二甲双胍可能通过 GSK-3β/Wnt 信号通路调控涡虫再生。为了

验证该猜想，我们选用了 GSK-3β的小分子抑制剂 CHIR-99021 对涡虫进行处理，观察是否会影响二甲双胍所引起的涡虫再生抑制。

如图 8 所示，在只加入了 $4*10^{-4}$ mg/ml 的二甲双胍处理的涡虫中，我们可以看到再生依然如同实验组中同浓度涡虫组一样减缓。7 天后伤口仍然没有完全闭合，只出现了极少量的胚基。眼点完全没有出现，也没有出现眼点和明显的头部尾部。而在加入了 0.1 μm GSK-3β小分子抑制剂 CHIR-99021 的涡虫中，再生相较于只加入了同浓度二甲双胍的涡虫有大幅度改善。虽然 7 天后也没有同未受任何处理处理的涡虫一样完成再生，但是已经出现了大量的胚基和一点在再生过程中的眼点。伤口出也已经愈合，有较明显的头部和尾部。而在加入了 0.01 μm CHIR-99021 的涡虫中，再生相较于只加入了同浓度二甲双胍的涡虫也有一定的改善。虽然也没有出现大量的胚基，但是伤口已经愈合。同时也可以看出在头部再生出现一点正在再生过程中的眼点。

进一步的免疫组化结果也表明在加入了 0.1 μm CHIR-99021 的涡虫中，神经系统再生相较于加入了同浓度的二甲双胍的涡虫进步明显。神经链已经完全闭合，神经系统已经完全再生完成。其 7 天的再生恢复水平与对照组内未做任何处理的涡虫相当。而在加入了 0.01μm CHIR-99021 的涡虫中神经系统再生也有一定幅度的加快，神经链趋于闭合，相较于只加入了同浓度的二甲双胍的涡虫有一定的提升（图 9）。

以上结果说明 GSK-3β/Wnt 信号通路在二甲双胍抑制涡虫再生中有重要作用。

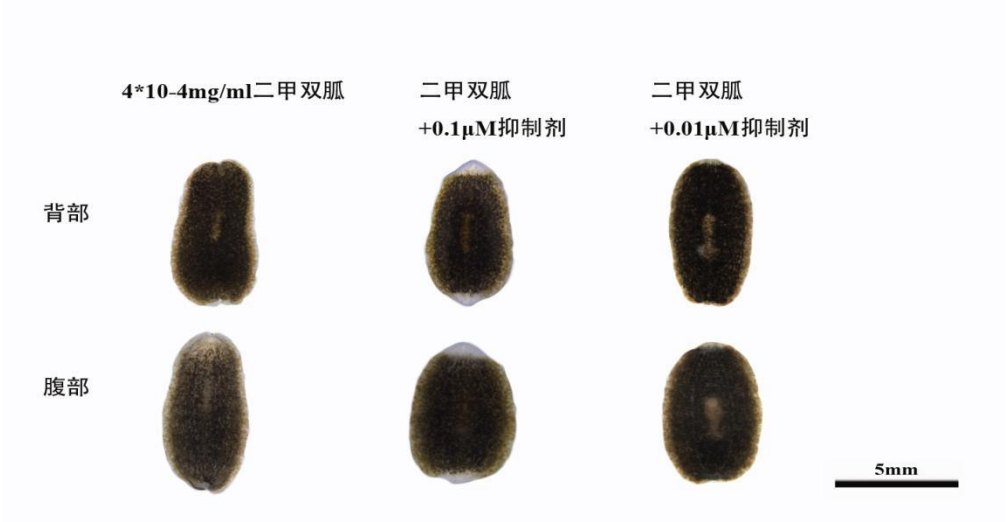

图 8：GSK3β抑制剂 CHIR-99021 缓解二甲双胍对涡虫再生的抑制

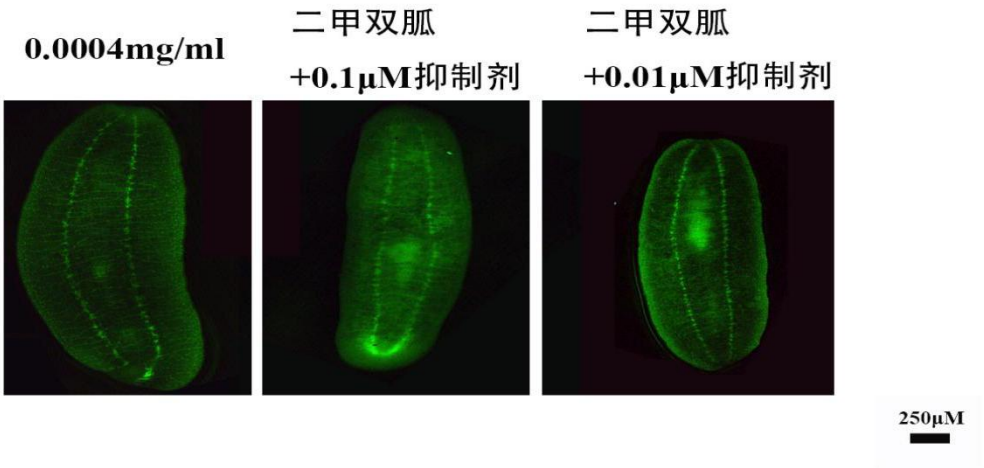

图 9：GSK3β抑制剂 CHIR-99021 缓解二甲双胍对涡虫神经系统再生的抑制

讨论：

(1)二甲双胍抑制涡虫再生

在没有二甲双胍处理的涡虫中，被切为头，腹，尾三段的涡虫部分都在 7 天后再生完整，由此可以看出涡虫具有强大的再生能力。而在被二甲双胍溶液处理完成七天后，涡虫再生结果随浓度的变化而变化。总体来说，与未经过二甲双胍

溶液处理过的对照组涡虫比较，在每个时间段的再生情况均有所下降。由此得出，二甲双胍溶液对涡虫再生有抑制作用，且极端浓度抑制效果更佳明显。

因为再生情况的不同，我们分析了涡虫神经系统的表达方式。涡虫在被处理完成七天后，被极端浓度（4 mg/ml，$4*10^{-4}$ mg/ml）所处理的涡虫神经系统表达如下图。相比起中间浓度梯度，极端浓度处理的涡虫神经系统头部的脑神经节表达相对较弱，而在腹部，在腹神经索通向体后，横神经相连的腹部区域，神经系统表达较明显。由此可见，二甲双胍抑制涡虫再生对涡虫神经系统有一定的影响。

在荧光定量 PCR 分析的几个基因中，DjPiwiB 和 DjPiwiA 表达量的减少明显，且随着二甲双胍的浓度变化波动很大，暗示可能是受到了二甲双胍作用的影响。DjPiwiA 和 DjPiwiB 的表达是干细胞再生时不可缺少的。为了保证新胚叶中干细胞再生的正常进行，必须让大部分转座子沉默，不干扰其他基因的复制。涡虫的体细胞自身无法表达 PIWI 蛋白，但 PIWI 类蛋白对转座子沉默的过程十分重要。通过将 DjPiwiA 和 DjPiwiB 从新胚叶干细胞遗传到其再生出来的体细胞，这些不能表达 PIWI 蛋白的体细胞才得以使得他们自己的转座子沉默(Shibata et al，2016)。二甲双胍的作用下，涡虫的 DjPiwiA 和 DjPiwiB 表达量大幅度减少。这一变化可能阻碍了新胚叶中干细胞的再生。而干细胞产生新细胞的过程是涡虫再生修复的主要过程和不可或缺的充要条件，没有新的体细胞涡虫就没有办法补全自身缺少的部分。所以宏观的角度来说，干细胞的复制再生受阻使得涡虫整体的再生修复变缓，与我们在体视镜与免疫组化中观察到的情况一致。

PCNA 是一种 DNA 聚合酶的辅助蛋白，存在于正在增殖的细胞中，是一个细胞增殖情况的良好指标。在荧光相对定量 PCR 中实验组的 PCNA 蛋白表达相较于对照组有大幅度的增加，但是实验组涡虫的再生对比对照组却有减缓。这是因为虽然细胞增殖受刺激而增加，但增殖的充要条件干细胞再生却减缓。在这些因素的共同作用下，涡虫的总体再生才会减缓。

P53，作为一种肿瘤抑制基因，会在细胞的分裂再生中起到减缓并监视的作用。如果在 DNA 复制中基因的变异较少，那么 P53 会帮助这个细胞恢复自己的变异基因。继而反之，如果一个正在复制的细胞的基因已经有大量的变异，P53 无法修复，那么 P53 就会引导这个变异的细胞凋亡。二甲双胍的作用使得涡虫中 P53 的表达增加。更多的 P53 意味着再生之中的细胞受到更多来自于 P53 的检查。受于更高表达的 P53 基因牵制，干细胞再生总体减缓，使得涡虫再生修复也减缓。这一结果也与体视镜与免疫组化中观察到的情况相互解释。

BCL-2 和 MCL-1 是重要的细胞凋亡调控基因，具有抑制凋亡作用。在二甲双胍的作用下，实验组的涡虫相较于对照组的涡虫表达量增加，实验组中涡虫的细胞凋亡受到抑制。细胞中的受编程凋亡通常发生在生物体受伤伤口的位置。最新的多项以果蝇，水螅，与非洲爪蟾蜍为模型研究表明细胞凋亡的过程同时能够引发促进有丝分裂的信号，使得干细胞与祖细胞增殖再生修复伤口(Bergmann et al, 2010)。荧光相对定量 PCR 中表明实验组的 BCL-2 和 MCL-1 的表达量增加，说明凋亡可能受到抑制。再生过程中需要凋亡发生，凋亡抑制就会影响再生。由此，涡虫的再生在二甲双胍的作用下变缓。

除了上面所述的五个基因的表达的变化之外，还有一个在二甲双胍作用下的实验组中表现出统计学上显著性差异的就是 GSK-3β。GSK-3β广泛存在与包括人类与涡虫的动物中。GSK-3 在人体中有多种作用，包括参与肝糖的代谢与细胞的增殖，分化与凋亡。在 2019 年 5 月一篇发布在 Cancer Cell 上的论文中，研究人员们发现在禁食降低血糖与二甲双胍的共同作用之下抑制了肿瘤的生长(Elgendy et al, 2019)。具体来说，他们发现二甲双胍与禁食引起 GSK-3β在细胞中的过度活化。在后来进一步实验中将 GSK-3β抑制后，二甲双胍与禁食对肿瘤的抑制效果作用降低。作为结论，这篇文章表示 PP2A-GSK-3β-MCL-1 通路是肿瘤治疗潜在的新靶点。在我们的涡虫再生模型实验中，荧光相对定量 PCR 的结果显示二甲双胍也对 GSK-3β的表达产生了影响。在实验组中，受二甲双胍作用的涡虫 GSK-3β表达明显增加，且在不同的浓度中波动很大。

(2)二甲双胍通过 GSK-3β/Wnt 通路调控涡虫再生

已有研究证实 Wnt 信号通路在涡虫再生中有重要作用，而 GSK-3β是 Wnt 信号通路上游的关键基因。此外，在 2019 年十月发表在 Science 的一篇文章中，作者表示 GSK-3β/Wnt 信号通路在大部分哺乳类生物组织连续再生，替代老细胞的过程中起了关键的作用。如果没有 GSK-3β/Wnt 信号通路的多种细胞外发育信号蛋白，那组织的修复再生就无法进行(Clevers et al，2019)。同时 GSK-3β/Wnt 通路在生物进化历史中起源久远，最先来自于最简单早期多细胞生命体。因其在进化树上出现早，GSK-3β/Wnt 通路在高等浦乳类与涡虫中都起到了相似的作用。因此二甲双胍很有可能通过 GSK-3β/Wnt 调控涡虫再生。为了回答这个问题，我们通过小分子抑制剂 CHIR-99021 抑制 GSK-3β，进一步分析 GSK-3β/Wnt 通路在二甲双胍所引起的涡虫再生抑制中的作用。

通过体视镜观察与涡虫神经系统免疫组化分析，我们发现在 CHIR-99021 与二甲双胍共同处理后涡虫总体再生的情况比单独用同样浓度二甲双胍处理的涡虫更快速。同时，涡虫神经系统的再生也有相似的结果。由此可见，二甲双胍处理后，GSK-3β表达量增加，Wnt 通路被阻断，涡虫再生受到抑制。而在 GSK-3β被抑制后，Wnt 通路可能会被激活，从而二甲双胍对涡虫再生的抑制作用有明显的减弱。这个结果表明，GSK-3β/Wnt 通路在二甲双胍作用于涡虫再生过程中有重要作用。

(3) 再生与长寿的关系：

长寿和再生之间的关系并没有我们之前认为的那么简单。科学界中，有很多科学家已经能够证明二甲双胍能够在一定程度上延年益寿，但是这似乎和我们的结果冲突。在我们的推论中，再生能力应该和长寿的能力呈正比关系，因而再生速度越快，一个人的寿命就越长。这是由于我们认为为了促进长寿增加寿命，最为必要的一步就是保持器官的活性。因而快速再生能够快速补上衰老的细胞，从而维持器官的活性而让一个生命体活得更久。但是我们的实验似乎结果和这个推论完全相反。在和涡虫的实验中，二甲双胍不但没有推进再生速度，反倒是严重

减缓了再生的速度。通过对 GSK-3β/Wnt 通路的影响，二甲双胍对于涡虫的再生产生了阻碍作用。这让我们对再生与长寿的关系提出了疑问。倘若再生速度真的和长寿应当为正比的话，那这代表着除了对再生速度的影响，二甲双胍对生物体还有其他我们没有研究的作用，再生也不会是决定长寿的最高决定性因素。这将会带来许多新的与长寿相关的研究课题。再则可能是我们最开始的“长寿和再生速度之间应当是正关系”这个推论是一个不严谨，或者是一个会被我们实验结果给证伪的一个推论。在这种情况下，再生和长寿究竟有什么关联，到底是呈正关系还是副关系，将会是一个重点探讨话题。

改进:

我们的实验仍有去多不足，其中，我们只研究了 GSK-3β一种蛋白酶在二甲双胍作用机制里的作用，所以我们无法知道是否还有其他蛋白酶和蛋白通路影响着二甲双胍的作用机制和细胞的再生。另外，我们只研究了其中的一个功效——抑制细胞的再生，实验表明，二甲双胍会抑制涡虫的再生，但是二甲双胍被称为“神药”，其有很多健康功效，二甲双胍让人长寿这一说法被一些科学家所印证，这与我们的实验结果构成了一个矛盾的说法,可能再生与长寿的关系远远没有简单的促进这么简单。

意义:

我国糖尿病的发病率已由低于 3%的低发病率上升为中发病率（3%-10%）国家，在人口基数大的因素影响下，我国已成为世界上糖尿病患者数量最多的国家，二甲双胍作为治疗糖尿病的药物，需求量大，且在医学和药学上都有很大的意义和应用价值。生活中，二甲双胍又被人们称作“神药”，并具有许多功效，如减肥，降低胆固醇，长寿等。因此，以二甲双胍为研究对象，旨在研究其作用原理，以及是否应证二甲双胍能给人们带来的益处。二甲双胍价格亲民，容易获得，二甲双胍若是对长寿健康有极大的改善，那么各个人群都可以收益。我们的研究初步揭示了一些再生和长寿之间的联系，为进一步的抗衰老相关研究提供了一些新的思路。

Metformin Suppresses Planaria Regeneration through the GSK-3β/Wnt pathway ---New Insights on the Association between Regeneration and Longevity

Abstract:

Metformin has shown the potential to prolong life expectancy and prevent cancer as modern medicine. Apart from metformin, another one of the inevitable topics in the field of immortality is regeneration. How is regeneration ability related to long lifespan? If metformin promotes longevity, then, how is it going to impact regeneration? How does metformin exert its effect on regeneration? Here, we sought to look into the answer of these three questions by performing the metformin effect experiment on planarian, the common regeneration model. Groups of planarian are treated under different concentration of metformin. We observe how their overall regeneration process is affected by the microscope. Then, we see how their nervous system regeneration is affected by immunohistochemistry. We further explored its influence on their internal gene expression with qPCR. The results came out very unexpected. Although metformin has shown the potential to promote longevity, it stagnated the regeneration process in planarians. To understand such a phenomenon, we went to further investigate how this observation should be explained. From the result of the qPCR, we find a potential pathway that metformin exerts its effect through, the GSK-3β/Wnt pathway. To confirm our hypothesis, we furthered our experiment to block the GSK-3β/Wnt pathway while applying metformin on planarian. Then we repeated the same process to see if the previously observed stagnation of regeneration is still present. As the results(from a microscope, immunohistochemistry, qPCR) turned out, the stagnation of regeneration is significantly alleviated after blocking the GSK-3β/Wnt pathway. This suggests that the GSK-3β/Wnt pathway is the primary pathway through which metformin exerts its effect to slow down regeneration. The relationship between regeneration and longevity is much more complicated than mere promoting. Regeneration is rather one among the multiple

factors in longevity, necessary but not decisive. Metformin has great importance in the promotion of longevity, but we found a counter-intuitive result from our experiment: that metformin deters regeneration. This suggests that regeneration has a much more complicated relationship.

Introduction:

A great recent hit in the field of immortality is a medicine called Metformin. It was originally presented as an oral diabetes medicine(for type 2 diabetes) for its ability in the reduction of blood sugar level. The substance can reduce glucose production in the liver and boost the human system's sensibility to insulin. Recent researches, on the other hand, discovers many potentials uses of the medicine besides just treating type2 diabetes, including the promotion of longevity.

What is metformin exactly? What is its range of use? During the long history of metformin, it was especially popular when diabetes patients still had to resort to expensive pig-produced insulin for substitute. The medicine comes with a long history which dates back to the 17th century. In a herbal guide that dates back to that time, the substance was recorded as a natural remedy(Tomas et al, 2017). At that time the local inhabitants already learned the method to derive the substance from French lilac. They used it, like just modern people, to treat the disease we now know as diabetes. In recent history, scientists first succeeded in synthesizing its core compound (dimethyl biguanide) artificially in the year 1922(Tomas et al, 2017). Through most of its history, metformin is known as nothing more than a diabetes treatment.

In the modern age, however, researchers proposed many potential uses of metformin. First, in the year 2005, Evans et al. Hypothesize that the use of metformin in patients with type 2 diabetes could reduce their cancer risks. Metformin regulates glucose by targeting AMPK(AMP-activated protein kinase), which induces cells to take more glucose from circulating blood. AMPK has an upstream regulator LKB1, which is also a well-known tumor suppressor,(Shackelford et al,2009). This discovery links

metformin to both cell metabolism and mitosis control. Many other studies, on the other hand, find metformin a potential geroprotector(a therapeutic than aims to affect the root cause of aging and aging-related diseases). In a more recent study published in January 2019, researchers found that metformin prolonged lifespan in silkworms by remodeling their energy distribution. They suggest metformin's action in the adenosine monophosphate-activated protein kinase-p53-forkhead box class O signaling pathway increased stress resistance and antioxidative ability in these silkworms while reducing the energy for silk production. The combination of these three effects leads to the extension of lifespan in those tested silkworms(Song et al,2019). In another study conducted on male mouse demonstrated that long-term metformin intake starting from middle ages improved the healthspan and lifespan of the subjects. In particular, the benefits resembles that of calorie restriction, such as increased insulin sensitivity(Montalvo et al,2013).

Recently, according to a recent study published in the September issue of Nature, a small clinical study conducted in California, USA, shows for the first time that the epigenetic clock used to indicate physiological age in humans can be reversed. The epigenetic clock is dependent on the body's epigenetic genome, which contains chemical modifications of the labeled DNA. The patterns of these tags change throughout life and track a person's biological age, which may lag or exceed the actual age. The researchers asked nine healthy volunteers to take three commonly used drugs - growth hormone and two diabetes medications (including DHEA and metformin) and measure their average age by analyzing the markers on a human genome. The result showing that their biological ages shed about 2.5 years. Participants' immune systems also showed signs of rejuvenation. However, this is only a preliminary experimental result and there are still many shortcomings, such as the small scale of the experiment, the absence of a control group, etc.

Despite its early discovery and long history, metformin's exact mechanism remains unclear. AMPK is no longer the only known way of how metformin exerts its influence. Through the years, scientists have also proposed even many other new applications of this old medicine: obesity treatment, cancer prevention, and so on. Among the discoveries, anti-aging is has been the topic with the most focus. Although the effect of

metformin can be simply described as "lifespan extension", how the substance affects numerous pathways to come to produce this combined effect is complicated.

The biggest rival of longevity is aging. The gradual process of aging in human is primarily a manifestation of the aging process of internal tissues. With the aging of the organs, their function gradually deteriorates and weaken. Aging is not fatal in itself. It is rather the various chronic diseases that come with aging that kill the person. One very common example of such “chronically fatal” disease is type two diabetes, to which metformin is a treatment. According to the WHO, the World Health Organization, type 2 diabetes caused an estimated death of 1.5 million in the year 2016(WHO). To promote longevity and increase life span, one inevitable step would be to preserve organ liveliness. Organs waken because their cells age and die at a faster rate than new cells are generated（shortening of telomeres that deters the full copying of genetic material）. Without replacement by new cells, the old cells keep on serving their role. The old cells, however, does not work as efficiently as new cells. With an increasing proportion of old cells within a certain organ, the overall performance of the organ worsens gradually. This wouldn’t have happened for the organs of young people because their cell regeneration ability is still strong. Their stem cells are still capable of replacing all the compromised cells within the organ. For old people, however, their cell regeneration doesn’t work as effectively. Without substitute cells, their organs deteriorate gradually. Thus, the regeneration of new cells is the root solution to deal with aging. From this point of view, slow cell regeneration is the main cause of aging, and it is a topic that cannot be avoided in the promotion of longevity. For being the root cause of aging, the promotions of longevity would be itself a promotion of better regeneration ability.

If studies have indicated metformin has the function to increase life span, could metformin be boosting regeneration ability? Is it possible that metformin increases lifespan in the way that it restores the regeneration ability in those test subjects? And how does metformin affect regeneration if it promoted longevity? Planarian are common models in molecular biology studies. To answer such question, we make use of the planaria model, planarian(class Turbellaria) have been known primarily for its incredible capability in regeneration. Many also believe that their incredible regeneration ability could shed light on human being’s quest to immortality. This salient trait is made possible by their pervasive neoblasts throughout the body. These abundant clusters of

highly concentrated stem cells make it possible for them to regenerate the whole body from a fragment 1/273 of their original weight(Morgan, 1898). Often 10mm in length, what is opposing to its small size is that the species shares neurological traits with high-level animals, a fairly uncommon phenomenon in its class.

To study how metformin influences regeneration process, planarian's incredible regeneration ability makes them an ideal model for our experiment. For this reason, we treated different groups of planarian to different concentrations of metformin solution and observe how their regeneration process is affected. Then, through qPCR and pathway interference, the internal mechanism of metformin from the perspective of molecular biology can be known. Refer to Diagram1 for experiment procedure.

Experiment Sequence:

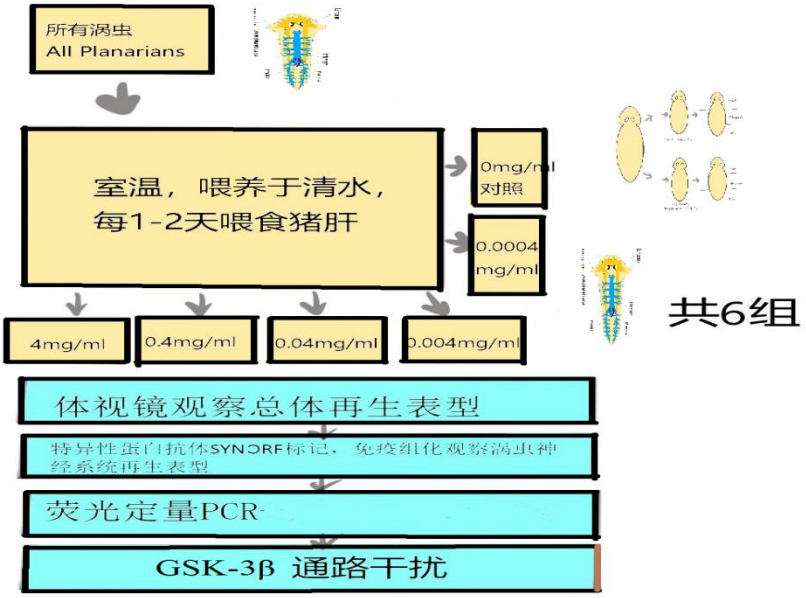

Diagram 1. procedure of the experiment

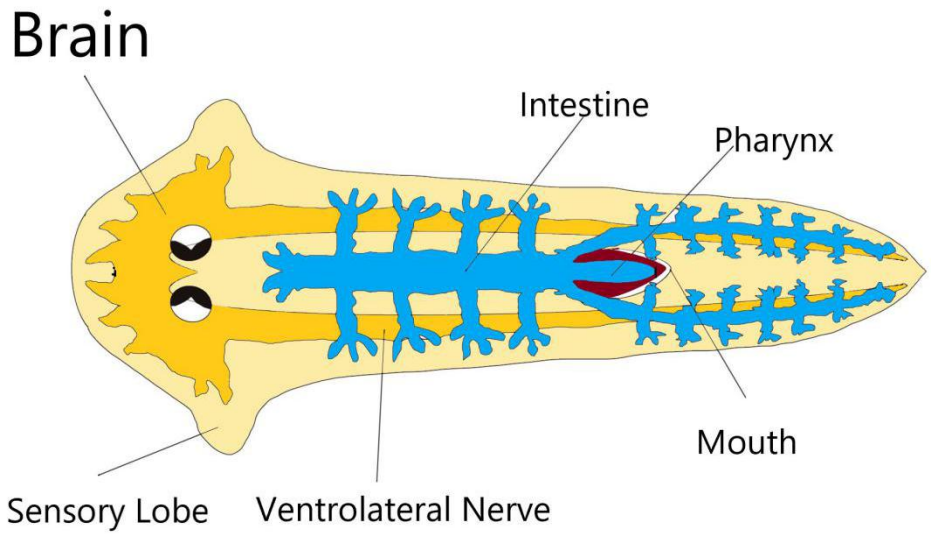

Diagram 2. structure of planarian

Methods:

(1)Cutting Planarian

Planarian (*Dugesia japonica*) was used for the experiment(Refer to Diagram2) Planarian used for the study is all bred in the lab. It was bred in a glass bowl with clear water at room temperature and pig liver was fed every 1 to 2 days. The planarian is cut before metformin treatment(Refer to Diagram 3). When treating planarian with metformin, five planarians are classified as one group, and within each group, each planarian would be chopped into three parts, classified as the ”head”, the “body” and the “tail”.(There are two sub0groups of planarian of equal number for the experiment group, the first sub-group was used for microscope and immunohistochemistry after metformin treatment, the other group was used in qPCR after the exactly same metformin treatment) For the experiment group, the 5 groups of planarian are put in solutions of metformin of descending concentration: 4 mg/ml , $4*10^{-1}$ mg/ml , $4*10^{-2}$ mg/ml , $4*10^{-3}$ mg/m , $4*10^{-4}$ mg/ml, in labeled Petri dishes. The middle concentration $4*10^{-2}$ mg/ml was derived from the prescription guide of metformin, from which we calculated the medicine weight/body weight proportion. Then we dilated or concentrated by a factor of 10 to derive other concentration of metformin. A comparison group is also cut and

placed with a similar procedure but allocated in pure H_2O.

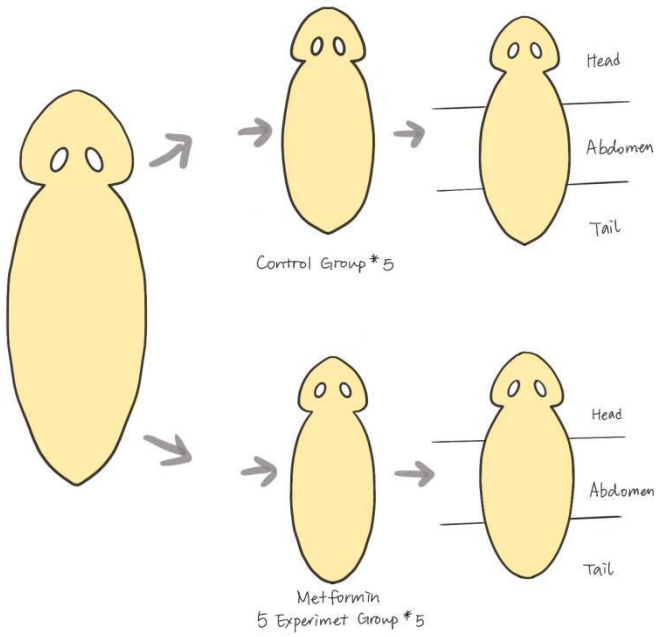

Diagram 3. Cutting planarians

(2)Observation of Regeneration Under Electronic Microscope

After 6 hours, 1 day, 3 days and 7 days, the planarian is placed on the slide, and a high-resolution picture is taken of their current regeneration progress. To minimize the interference of the footage, the slide is placed on ice to minimize planarian's movement.

(3)Immunohistochemical Analysis

After the planarian have been cut for 0 days, 2 days and 7 days respectively, the comparison group and the experiment group are marked using SYNORF1 protein antibody - purchased at DSHB company for the immunohistochemical analysis. This step of the experiment has 11 steps, in sequence:

First, submerge the planarian in 2% HCL solution for 10 minutes. Then, saturate the planarian in 4% Paraformaldehyde solution for 2 hours. Third, place planarian in 100% methanol solutions at temperature minus 20 ℃ for one hour. This step is for dehydration.

Fourth, place planarian under 1% SDS solution. After 25min, use different concentrations of methanol solutions (75%, 50%, 25%) and place one planarian in each of them for 10 minutes. Fifth, Place the planarian in milk for 1h, separated from light and air. Then, under 4 ℃, place the planarian under a solution of 1 to 30 Milk to Antibodies solution overnight. After more than 12hours, we then wash the planarian with PBST solution, each time for 5 to 10 minutes. Finally, under 4 ℃, place the planarian under a solution of 1 to 200 Milk to Antibodies solution overnight. After finishing all the process above, we can then place them under microscopes to collect data.

(4)Rna Extraction and Reverse transcription

In brief, total RNA was extracted from each pool of planarian using Trizol(TaKaRa), and cDNA was synthesized from RNA. Three samples were run in parallel for each condition. Elongation factor 2 (EF-2) was used as an internal control. Primers used for quantitative real-time PCR were designed using the software Primer Premier.

First, we put five planarians into a sterilized 1.5 ml centrifuge tube, suck out all the excess water, and add in 500 ml of RNA extractor, Trizol. Then, use the tip of the dropper to poke the planarian and make sure that they are completely turned into small pieces. Third, we add in trichloromethane and shake the container of these materials to mix up the substances. Then remain the tube stationary for 3 minutes. After that, put the containers into the centrifuge under conditions of 1200Gs and 4 ℃. We now have a rather see-through water layer and the RNA. Add in isopropanol of the same amount, but it stationary for 10 minutes, and then centrifuge it again. After that, add in Ethanol to wash out the liquid, and then we would have acquired relatively highly concentrated RNAs. Extract those RNAs, and store them in a fridge at minus 80 Celsius.

During the Reverse Transcription process, take five planarians, and a 50% gDNA buffer and 1 micrometer of gDNA eraser to purge all DNA in the original Planarian's body. Place it under 42 ℃, and then centrifuge it under 4 ℃. When done with the previous steps, repeat it, but add mix, primer, and RNase Free H20 instead to turn RNA

back to DNA. Centrifuge under conditions 37 ℃ for 15 min, 85 ℃ for 5 seconds, and then 4 ℃ for as long as possible. When the centrifuge's done, put it in the fridge at minus 20 ℃ to preserve it.

Table 1. PCR Primer Sequence Index

	Sequence (5'-3')
Bcl2L qPCR-F	GCATTGTGGCTTTCTTCTCCTTCG
Bcl2L qPCR-R	CATCGTTCCCATAGAGGTCCACAAA
DjMCM2 qPCR-F	GATTTGTGGTGATCCTGGCACT
DjMCM2 qPCR-R	ACTAACGCTCCTGCTTCCA
DjPiwiA qPCR- F	TCTGATAGCGAAAGAAGCAA
DjPiwiA qPCR- R	ACGACCACGAATAGTAATAGG
DjPiwiB qPCR- F	GACCTTACGATTAGTTGCGACCAT
DjPiwiB qPCR- R	TTGCGAGGAACTTCGTCTTT
GSK3β qPCR-F	GGAATCCAGACCCTT
GSK3β qPCR-R	CAATAACTAAACTGGCTGA
MCL-1 qPCR-F	CCTCCTGTGCGTTCTTAT
MCL-1 qPCR-R	TCTGTTCCCAAACCAATAA
p53 qPCR-F	CCCCACTTTCTTGACCAT
p53 qPCR-R	CGTAAACGCTTCGAGATGTTCC
PCNA qPCR-F	AAGAAAGTGATGCCGTAA
PCNA qPCR-R	ATAACTGCTGGAACA
WNT qPCR-F	TGTCATCACATCCTTGCCATAT
WNT qPCR-R	ATGCCATGCTCAGTGCTAG

(5) Fluoreszenzquant qPCR:

Using the special primer F and R designed by TsingKe, choosing the dead or expanded genes in the experiment and comparison group, corresponding to Bax and PCNA, putting actin as the control group to perform PCR.

For every individual, 20% of the mix, both positive/negative primers, pure H2O, and the

target cDNA. The PCR parameter is: DNA was denaturated at 95℃ for 5min, then annealed at 60℃ for 15sec, and finally extended at 75℃ for 30sec. When the PCR is over, extra 5 ml to perform agarose gel electrophoresis detection.

(6)Inhibitor Processing:

To prove our hypothesis, which is that GSK-3β/Wnt pathway is the main ways the Metformin inhibits planarian regeneration, we fed 3 new planarians in the same way as three new repeating samples. When processing the planarians, we cut the head and tail off of all planarians, and only leave the middle part so we can simultaneously observe the head and tail regeneration of each sample.

In the treatment of planarians with metformin, we selected the most significant concentration of metformin in the previous experimental group to inhibit the regeneration of the planarian, ie 4 mg/ml. Inhibitors used for GSK-3β/Wnt pathway blockade were the CHIR-99021 inhibitor of TOSCCIENCE, IC50 was only 10/6.7 nM, and the specific targets were GSK-3α and GSK-3β at a concentration of 0.1 μm/ml. To allow the planarian to regenerate as fully as possible under its existing environment as most of the untreated planarians will be completely regenerated within 7 days, we also let the planarians of the inhibitor group to use 7 days.

After 7 days, we again used the same procedure to perform stereoscopic observation and neuroimmunohistochemical analysis of these three replicates. In this way, we used the regenerative phenotype to judge whether the inhibitory effect of metformin on the planarian was blocked or alleviated by the inhibitor, thereby verifying or overturning our conjecture.

Results:

(1)Observation of regeneration of planarians

The control group was transversely cut into three segments: head, abdomen, and

tail. The regeneration of the worm was observed after different periods. The results of regeneration observation are shown in the figure.

After being cut into three segments of head, abdomen, and tail, the worm was observed under a microscope at 6 hours. The head, abdomen, and tail all had obvious wound marks after being removed, and no sign of healing was found. One day after the treatment, a few embryonic bases appeared in the head, abdomen, and tail. After 3 days, the embryo base enlarged and the eyespot appeared in the regeneration part of the tail. After 7 days of treatment, the regeneration of the planarian was complete.

There was no significant difference in the rate of regeneration among head, tail, and abdomen. However, the planaria head complete full repair faster comparing to the two other segments.

(2)Metformin Deters the Regeneration Process in Planarian

After being treated with different concentrations of metformin solution, the experimental group was divided into three segments. The regeneration of the head, abdomen, and tail was observed at different time. Photographs were taken.

The concentration gradients of metformin solution were 4 mg/ml, $4*10^{-1}$ mg/ml, $4*10^{-2}$ mg/ml, $4*10^{-3}$ mg/ml, $4*10^{-4}$ mg/ml, respectively.

Through observation, we noticed that metformin suppressed planarian regeneration at all concentration. Referring to Diagram 4 and 5, the extent to which the regeneration process is suppressed, however, exhibits concentration dependence. It was observed that the wound healed only slightly at the extreme concentration (4mg/ml, $4*10^{-4}$ mg/ml) and no embryo base appeared. However, the regeneration rate of planarian was fast at the intermediate concentration gradient ($4*10^{-1}$ mg/ml, $4*10^{-2}$ mg/ml, $4*10^{-3}$ mg/ml), but only comparing to the two extreme concentrations. Planarian in the control group has already fully repaired themselves 7 days after the injury. The planarian from the middle-

range concentrations, however, still haven't fully healed their wound at 7 days. Only the regeneration of planarian treated with $4*10^{-2}$ mg/ml metformin was good and tended to be complete after 7 days. At the same concentration, the regeneration rate doesn't vary significantly among head, abdomen, and tail segments (Diagram 4 and 5). Meformin's effect for the nerve system demonstrates similar results (Diagram 6).

Diagram 4. planarian under different concentration of metformin (back)

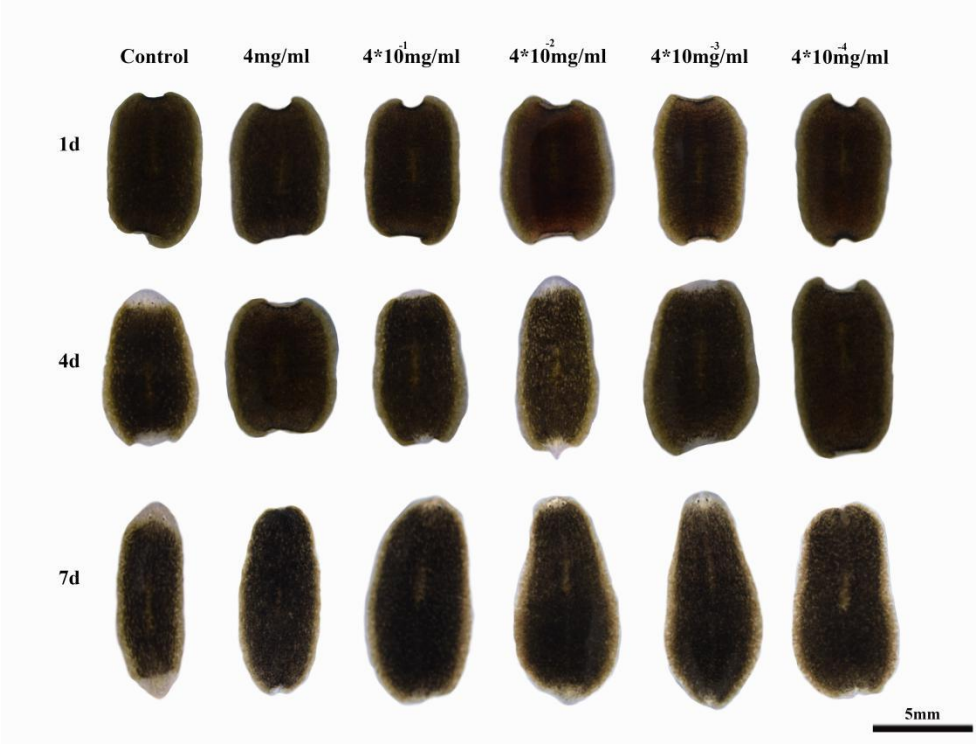

Diagram 5. planarian under different concentration of metformin (abdomen)

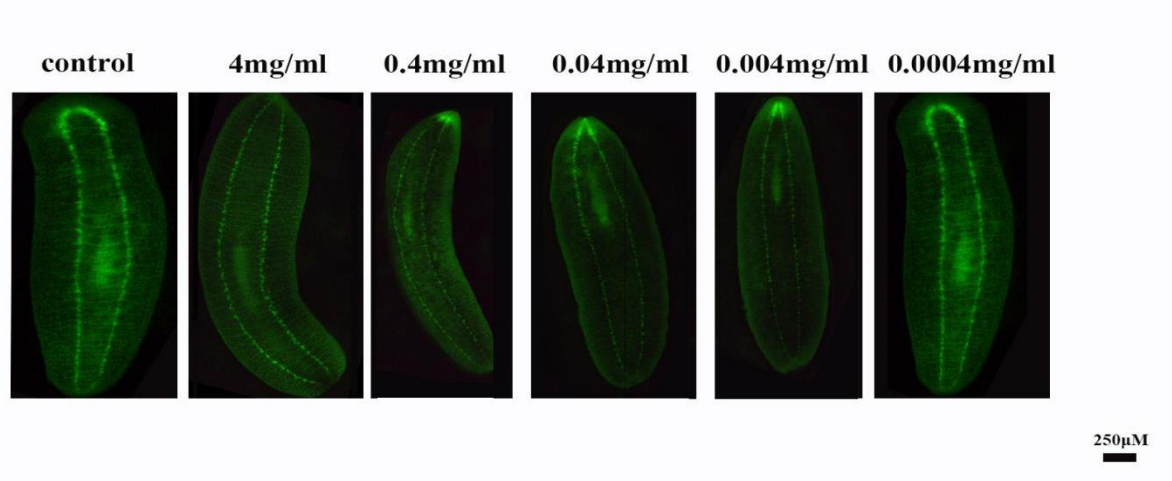

Diagram 6. planarian nerve system under different concentration of metformin

qPCR result histogram

(3)Exploration on the Mechanism of Metformin's Effect on Planaria Regeneration

In the relative quantitative fluorescent PCR using Actin as an internal reference

gene, metformin had a significant effect on the expression of several tested genes. We chose some of the gene expressions related to regeneration to be tested in PCR, DiPiWiA and DiPiWiB relating to stem cells; PCNA relating to multiplication; P53 relating to apotosis, etc.

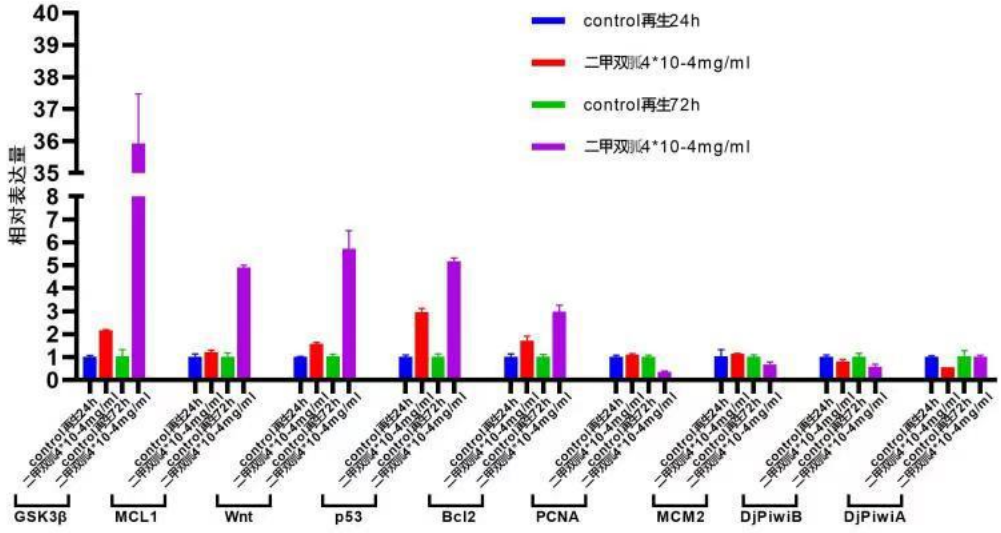

Diagram 7. gene expression under the effect of metformin

As demonstrated by Diagram 7, among them, when compared with the control group, the expression of DjPiWiA and DjPiWiB gene in the experimental group was statistically significantly reduced. The gene expression of DiPiWiA fell to 55% of the control group in 24 hours but later rise to approximately the same level as the control group at 72 hours. DjPiWiB's gene expression, on the other hand, keeps falling. It fell to 81% of the control group in 24 hours and 58% in 72 hours.

The fluctuation pattern is similar between that of PCNA and MCM2. They both increased slightly in 24 hours(9% and 15% respectively). Then they both fell far below the control group(35% and 67% of the control group respectively).

Compared with the control group, there was a statistically significant increase in the expression of P53 in the experimental group. It increased to 3 times of that of the control group and 5 times of the control group in 24 and 72 hours respectively.

Similarly, the expression of the BCL2, MCL-1 gene in the experimental group was statistically significantly higher than that in the control group. They reached to 3 and 5

times of the control group respectively in 72 hours.

Compared with the control group, the expression of GSK-3β gene and Wnt gene in the experimental group showed a statistically significant increase. In 24 hours the gene expression of GSK-3β and Wnt rose to 2 and 1.5 times of the control group respectively. In 72 hours, they rose to approximately 36 times and 7 times of the control group respectively.

(4) The Regeneration of the planarians in the inhibitor group

Observed under the stereoscopic microscope:

In the planarians treated with only 4*10-4 mg/ml of metformin concentration, we can see that the regeneration is still as slow as the planarian group treated with the same density of planarians in the experimental group. After 7 days, the wound was still not completely closed, and only a very small amount of embryonic base appeared. The eye point did not appear at all, and there was no obvious tail or head forming as well.

In the planarians treated with an addition of 0.1 um inhibitor, the regeneration speed was significantly improved compared to the planarians only treated in the same concentration of metformin. Although regeneration was still not completed after 7 days as a planarian without any treatment would, a large number of embryonic bases and a little eye point were present during the regenration. The wound has also healed, with a distinct head and tail.

In the planarian treated with an addition of 0.01 um inhibitor, the regeneration phase was somewhat improved compared to the planarian of the same concentration of metformin. Although there is not a large number of embryo bases, the wound has healed. At the same time, there is a little bit of eye point that can be seen within the head part, and the regeneration process has a generally small increase in the whole body.

Immunohistochemical analysis:

Referring to Figure 8, in the metformin treated with only 4*10-4 mg/ml metformin, the nerve chain remained unintact after 7 days of regeneration, and the nerve

regeneration was incomplete.

Within the planarian treated with an addition of 0.1 um inhibitor, the regeneration of the nervous system was significantly improved compared to those treated only with the same concentration of metformin. The nerve chain has been completely closed and the nervous system has been completely regenerated. The 7-day recovery level was comparable to that of the control group without any treatment.

In the planarian treated with an addition of 0.01um inhibitor, the regeneration of the nervous system was accelerated to a certain extent, and the nerve chain is showing tendency to close. Compared with the planarians with only the same concentration of metformin, there is a comparable increase (Figure 9).

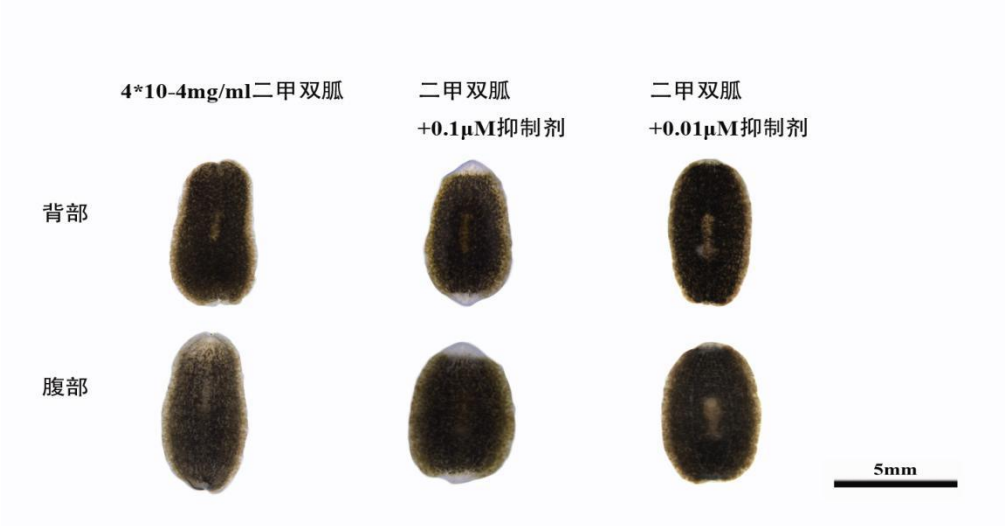

Figure 8. Inhibitor CHIR-99021 alleviates the negative effect metformin has on planarian regeneration

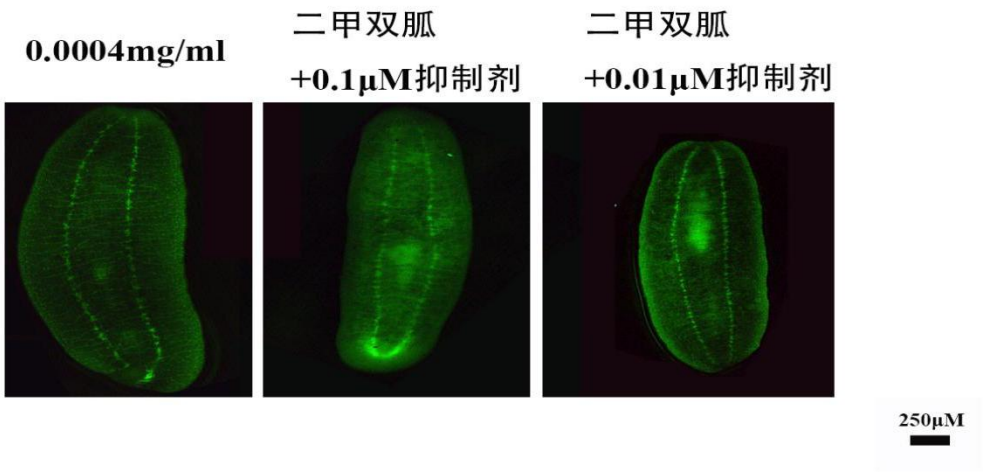

Figure 9. Inhibitor CHIR-99021 alleviates the negative effect metformin has on planarian nerve system regeneration

Discussion:

(1)Metformin Suppresses Planaria Regeneration

Planarian has been cut into head, abdomen, and tail, and regenerated completely after 7 days. It can be seen that the planarian has strong regeneration ability.

After being treated with metformin solution for seven days, the regeneration results of Vorticella varied with the concentration. Generally speaking, the regeneration rate of planarian in each period decreased compared with that in the control group which had not been treated with metformin solution. It was concluded that metformin solution could inhibit the regeneration of planarian, and the inhibition effect of extreme concentration was better.

Because of the different regeneration conditions, we analyzed the expression patterns of the nervous system of the planarian. Seven days after the treatment, the expression of the nervous system of planarian treated with extreme concentration (4

mg/ml， $4*10^{-4}$ mg/ml) is shown below. Compared with the intermediate concentration gradient, the expression of the brain ganglion in the head of the nervous system of the extreme concentration treatment was relatively weak, while in the abdomen, the expression of the nervous system was more obvious in the abdominal region where the transverse nerve was connected after the abdominal nerve cord reached the body. It can be concluded that metformin inhibits the regeneration of planarian and has certain effects on the nervous system of them.

Among the several genes tested, the decrease in DjPiwiB, DjPiWiA expression was significant and fluctuated greatly with the concentration of metformin, suggesting that it may have been the effect of metformin. Expression of DjPiwiB is an indispensable process in stem cell regeneration. To ensure the normal regeneration of stem cells in the new embryo leaves, most of the transposons must be silenced without interfering with the replication of other genes. The somatic cells of the planarian cannot express the PIWI protein by themselves, but the PIWI-like protein is important for the process of silencing the transposon. By inheriting DjPiwiB from new embryonic stem cells to their regenerated somatic cells, these somatic cells that do not express PIWI proteins silence their transposons (Shibata et al, 2016). Under the effect of metformin, the expression of DjPiwiB in the planarian was greatly reduced. This change microscopically hinders the regeneration of stem cells in the new embryo. The process by which stem cells produce new cells is the main process and necessary for the regeneration of the planarian. Without the new somatic cells, there is no way to supplement the missing parts for the planarians. Therefore, from a macroscopic point of view, the regenerative reproduction of stem cells is hindered, and the regeneration of the whole planarian is slowed down, which is consistent with what we observed in light microscope and immunohistochemistry.

PCNA is an accessory protein of DNA polymerase, present in cells that are proliferating and is a good indicator of cell proliferation. In the relative quantitative fluorescence PCR, the expression of PCNA protein in the experimental group was significantly higher compared with the control group, but the regeneration of the experimental group was slowed compared with the control group. This is because

although cell proliferation is stimulated and increased, the stem cell regeneration, a necessary condition for regeneration, is slowed down. Under the combined effect of these factors, the overall regeneration of the planarian will be slowed down.

P53, as a tumor suppressor gene, plays a role in slowing down and monitoring in the cell's division and regeneration. If there is less mutation within the gene in DNA replication, then P53 will help the cell recover its variant gene. On the contrary, if the gene of a cell that is being replicated has a large number of mutations and P53 cannot be repaired it, then P53 will induce apoptosis of this variant cell. The effect of metformin increased the expression of P53 protein in the planarian. More P53 protein means that the cells in regeneration are more examined from the P53 protein. Constrained by the higher expression of the P53 gene, the stem cell regeneration is generally slowed down, and the regeneration of the planarian is also slowed down. This result is also explained by the observations observed in the light microscope and immunohistochemistry.

The expression of BCL-2 is an important indicator of apoptosis and a protein that inhibits apoptosis. Under the effect of metformin, the expression of BCL-2 in planarians in the experimental group was increased compared with that of the control group, and the apoptosis of the planarian was inhibited in the experimental group. The programmed apoptosis in the cell usually occurs at the site of the wound. The latest studies using Drosophila, leeches, and Xenopus laevis have shown that the process of apoptosis can simultaneously trigger mitogenic signaling, allowing stem and progenitor cells to proliferate and regenerate wounds (Bergmann et al, 2010). Fluorescence relative quantitative PCR showed an increase in the expression level of BCL-2 protein in the experimental group. Therefore, the apoptosis of the experimental group was inhibited in the wound cells, and the regeneration signal from the aphid was reduced, and the overall regeneration was slowed down. The results of BCL-2 in qPCR were also consistent with those observed in light microscopy and immunohistochemistry.

The gene expression of MCL-1 is indicated to have increased by our qPCR. This is a sign that the apoptosis process is oppressed in the system. For the same reasoning as BCL-2, when cell apoptosis is oppressed, the regeneration signal triggered by apoptosis is also oppressed. Apoptosis is a necessary process in regeneration, the influence in

apoptosis will also affect regeneration. So to speak, the planarian regeneration process is slowed down under the effect of metformin, corresponding to the qPCR result of MCL-1.

In addition to the changes in the expression of the five genes reported above, there is also a statistically significant difference in another thing under the effect of metformin, which is GSK-3β kinase. GSK-3β kinase is widely present in animals including humans and planarians. The expression of GSK-3β kinase has many functions in the human body, including participating in hepatic glucose metabolism and cell proliferation, differentiation, and apoptosis. In a paper published in Cancer Cell in May 2019, the researchers found that the combination of fasting and lowering blood sugar combined with metformin inhibited tumor growth (Elgendy et al, 2019). Specifically, they found that metformin and fasting caused excessive activation of GSK-3β in cells. After further inhibition of GSK-3β enzyme in subsequent experiments, the inhibitory effect of both metformin and fasting on tumors was reduced. As a conclusion, this article indicates that the PP2A-GSK-3β-MCL-1 pathway is a potential new target for tumor therapy.

In our planarian regeneration model experiment, the fluorescence relative quantitative PCR showed results that proves metformin's effect on GSK-3βexpression. In the experimental group, the expression of GSK-3β was significantly increased by metformin and fluctuated greatly in different concentrations.

(2)GSK-3β/Wnt Pathway Plays an Important Role in Metformin's Effect on Planarian Regeneration

Studies have confirmed that Wnt signaling pathway plays an important role in the regeneration of planarians, and GSK-3β is a key gene of Wnt upstring signaling pathway. In addition, in an article published in Science in October 2019, the authors stated that the GSK-3β/Wnt signaling pathway plays a key role in the continuous regeneration of most mammalian tissues and the process of replacement of old cells. Without the multiple extracellular developmental signaling proteins of the GSK-3β/Wnt signaling pathway, tissue repair and regeneration would not be possible (Clevers et al, 2019). At the same time, the GSK-3β/Wnt pathway has a long history in biological evolution, first emerging the simplest early multicellular life. Because of its early appearance in the phylogenetic tree, the GSK-3β/Wnt pathway plays a similar role in both the high-level mammals and

the planarian. Therefore, metformin is likely to regulate the regeneration of planarians by GSK-3β/Wnt. To prove this claim, we inhibited GSK-3β by the small molecule inhibitor CHIR-99021, and further analyzed the role of the GSK-3β/Wnt pathway in the inhibition of cyclone regeneration induced by metformin.

By stereoscopic observation and immunohistochemical analysis of the planarian nervous system, we found that the overall regeneration of the planarian after both CHIR-99021 and metformin treatment was faster than the planarians treated with the same concentration of metformin alone. At the same time, the regeneration of the planarian nervous system has similar results. It can be seen that after treatment with metformin, the expression of GSK-3β is increased, the Wnt pathway is blocked, and the regeneration of planarian is inhibited. However, after GSK-3β is inhibited, the Wnt pathway may be activated, and the inhibitory effect of metformin on the regeneration of planarian is significantly reduced. This result indicates that the GSK-3β/Wnt pathway plays an important role in the action of metformin on the regeneration of planarian.

(3)Relationship between Regeneration and Longevity

The relationship between longevity and regeneration is not as simple as we thought. In the scientific community, many scientists have been able to prove that metformin can prolong life to some extent, but this seems to conflict with our results. In our hypothesis, the ability to regenerate should be directly proportional to the ability to live longer, so the faster the regeneration, the longer the life a person leads. This is because we believe that in order to promote longevity and increase life expectancy, the most necessary step is to maintain the activity of the organs. Therefore, rapid regeneration can quickly make up the aging cells, thereby maintaining the activity of the organs and allowing an organism to live longer. But our experiments seem to be the opposite of this hypothesis. In the experiment with the planarian, metformin not only did not advance the rate of regeneration, but actually slowed down the rate of regeneration. Through the influence of the GSK-3β/Wnt pathway, metformin has an inhibitory effect on the regeneration of planarians. This raises questions about the relationship between regeneration and longevity. If the rate of regeneration is really proportional to longevity, it means that in addition to the effect on the rate of regeneration, metformin has other effects on the organism that was not part of this study, and regeneration is not the most decisive factor

in determining longevity after all. This will bring many new research topics related to longevity and its determining factors. Then it may be that our initial claim of "relationship between longevity and regenerative speed should be positive" is a corollary that will be falsified by our experimental results. In this case, "what is the relationship between regeneration and longevity", and whether it is a positive or a negative relationship, will be a key topic of discussion.

Improvements:

Our experiments still have many problems. For instance, we only studied the role of GSK3b, a protease, in the mechanism and effects of metformin, so we have no way to know whether there are other proteases and protein pathways that affect the mechanism of metformin's impact on cell regeneration. In addition, we have only studied one of these effects – metformin inhibiting cell regeneration. Experiments have shown that metformin inhibits the regeneration of planarians, but metformin is called "the god medicine", which has many health effects. The claim that metformin makes people live longer is also confirmed by some scientists. That however, constitutes a contradiction with our experiment results. Maybe the relationship between regeneration and longevity is far from that simple.

Impact:

The incidence of occurrences of diabetes in China has risen from a low incidence rate of less than 3% to a medium incidence rate (3%-10%). Due to the large population base, China has become the country with the largest number of diabetic patients in the world. As a drug use to treat diabetes, metformin has a large demand and has great significance and application value in medicine and pharmacy. In other ordinary situations, metformin is also known as "the god medicine" and has many effects, such as weight loss, lowering cholesterol, and increasing longevity. Therefore, we studied metformin as our research subject, aiming to study its how it works to see if it proves the benefits that metformin can bring to people. The price of metformin is low and therefore easy to obtain, and if it is really greatly improves longevity, then all individual groups can benefit from the discovery. Our experiment had touched on the relationship between regeneration of longevity, thus laying the ground work for future studies on longevity.

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请如实说明:

1.每一个队员在研究报告撰写中承担的工作以及贡献:

整个项目由王珂、邓雅心、汤晟宇三人共同讨论、实验、结果分析、论文撰写完成。其中，王珂和邓雅心负责了主要的实验研究。汤晟宇负责了资料收集，涡虫表型观察与分析。实验工作主要在2019年6-8月份完成。论文撰写第一稿于2019年8月底完成。

2. 指导老师与学生的关系，在论文写作过程中所起的作用，及指导是否有偿:

指导老师彭锐教授是四川大学生命科学学院老师，与我们的升学指导老师林恩恩系朋友关系，通过林老师介绍，我们认识了彭老师，并请求彭老师对我们的项目进行必要的指导。在论文写作过程中，彭老师对我们论文初稿的格式、语言表达、逻辑关系进行无偿指导。

3. 他人协助完成的研究成果:

无

如有必要，请附上团队成员和指导老师的简历。

致谢:

首先感谢来自川大的彭锐教授，彭教授不仅为我们提供了实验中必备的硬件条件，同时也启发我们发现了我们感兴趣的

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还要感谢我们的升学指导林老师，林老师引导我们探索了我们的兴趣所在，也给我们三个认识了彼此。林老师是我们三个之间友谊的链接。

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这个暑假能够来到川大进行实验探索很是幸运，不仅丰富了我们的暑假生活，同时还让我们与同学之间有更多的相处。在实验与编撰写论文的过程中，我们三位同学都经历了许多挫折，但是我们都能够彼此鼓励并克服困难，培养了团队合作精神，更体会了实验成功的欣喜。

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2019 年 9 月 15 日