

· 论 著 ·

桃红四物汤对泼尼松诱导斑马鱼骨质疏松的治疗作用

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摘要: **目的** 利用泼尼松诱导的斑马鱼骨质疏松模型评价中药复方桃红四物汤的抗骨质疏松的作用。**方法** 选择发育正常的 3 dpf 斑马鱼胚胎, 用 10 $\mu\text{g/mL}$ 泼尼松处理建立斑马鱼骨质疏松症模型, 加入不同浓度 (50、150 和 450 $\mu\text{g/mL}$) 的中药复方桃红四物汤。同时设置正常对照组、模型对照组和阳性对照药依替膦酸二钠组。4 d 后, 用 0.2 % 钙黄绿素进行染色, 在体视荧光显微镜下观察, 拍照并计算斑马鱼脊椎骨的荧光强度。**结果** 与正常对照组相比, 泼尼松处理的模型对照组的斑马鱼脊椎骨荧光强度显著减少; 与模型对照组比较, 依替膦酸二钠能增加斑马鱼脊椎骨矿化量并有效防止泼尼松诱导斑马鱼产生的骨质疏松; 与模型对照组比较, 450 $\mu\text{g/mL}$ 浓度的桃红四物汤能显著提高斑马鱼脊椎骨骨骼的荧光强度, 而 50 $\mu\text{g/mL}$ 和 150 $\mu\text{g/mL}$ 浓度的桃红四物汤溶液组效果均不明显。**结论** 桃红四物汤对斑马鱼的最大耐药浓度 MTC 为 450 $\mu\text{g/mL}$, 并对泼尼松诱导的斑马鱼骨质疏松模型有明显的改善治疗作用。

关键词: 斑马鱼; 骨质疏松; 泼尼松; 桃红四物汤; 依替膦酸二钠

Therapeutic effect of Taohong Siwu Decoction on prednisone induced osteoporosis on zebrafish

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Abstract: Objective To evaluate the anti-osteoporosis effect of traditional Chinese medicine Taohong Siwu Decoction by the use of prednisone-induced zebrafish osteoporosis model. **Methods** We selected 3 dpf normal growth zebrafish embryos, established zebrafish osteoporosis models using prednisone treatment, which were then treated with different concentrations (50, 150 and 450 $\mu\text{g/mL}$) of lyophilized powder of traditional Chinese medicine compound Taohong Siwu Decoction. At the same time, the normal control group, the model control group and the positive control group were established. After 4 days, these zebrafish were dyed for 2 h by 0.2 % calcein, and then the fluorescence intensity of vertebrae of zebrafish were observed and calculated using stereo fluorescence microscope. **Results** Compared with the normal control group, prednisone-treated model group had significantly lower vertebral fluorescent density; compared with the model group, Etidronate Disodium of 300 $\mu\text{g/mL}$ can increase the amount of vertebrae mineralization and effectively prevent osteoporosis induced by prednisone; compared with the model group, 450 $\mu\text{g/mL}$ concentration of Taohong Siwu Decoction can significantly improve the zebrafish vertebral fluorescence intensity, while the effects of the two groups of Taohong Siwu Decoction solution at 50 $\mu\text{g/mL}$ and 150 $\mu\text{g/mL}$ concentration were not obvious. **Conclusion** The Compound Chinese medicine Taohong Siwu Decoction, with a maximum concentration of drug resistance MTC of 450 $\mu\text{g/mL}$ on zebrafish, had obvious therapeutic effects on zebrafish osteoporosis model induced by prednisone.

Key words: Zebrafish; Osteoporosis; Prednisone; Taohong Siwu Decoction; Etidronate Disodium

桃红四物汤源自于清代吴谦的《医宗金鉴》由桃仁、红花、当归、白芍、熟地黄、川芎等 6 味中药组成,

是遵循“活血祛瘀”的原则所组成的基本复方, 在各种血栓性疾病的治疗, 如血管性头痛、冠心病、血栓性静脉炎等方面, 都取得了满意的疗效^[1]; 又有基础研究^[2]表明, 桃红四物汤在骨伤科方面有一定促进骨折愈合、改善颈椎病、腰腿痛等症状的作用, 但其基础机理和临床应用有待于进一步的开发和研究。

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随着老龄化社会的到来,越来越多的老年问题也随之出现。骨质疏松症作为影响老年人日常生活的普遍问题之一,已得到普遍的关注和重视。在骨骼研究领域人们已经成功建立了许多动物骨骼模型,而新型模式生物斑马鱼则越来越受到人们的关注。斑马鱼个体小、易于饲养、胚胎透明易于观察和操作,易于观察骨骼的发育^[3-5],并且它的重要器官即使出现畸形也能存活很长时间^[6],同时在研究领域还有些实验样品用量少、实验周期短和可实现高通量筛选等优点。现代研究表明,其骨骼形成机制与哺乳动物一样,且其参与调控的关键基因与哺乳动物的相关基因具有高度的同源性^[7],因此斑马鱼已被广泛用于骨骼发育及相关疾病机制的研究与治疗^[8-11]。

因此,本研究利用斑马鱼模型,研究中药复方桃红四物汤对泼尼松诱导的斑马鱼骨质疏松的治疗作用,为探讨该复方对骨质疏松有无疗效提供了科学依据。

1 材料和方法

1.1 材料

1.1.1 药品与材料:10 $\mu\text{g/mL}$ 泼尼松(上海阿拉丁生化科技股份有限公司,批号 28778),依替膦酸二钠(TargetMol,批号为 T1210),0.2% 钙黄绿素(美国 sigma 公司,批号为 C0875-5G),桃红四物汤(桃仁 9 g,红花 6 g,当归 12 g,川芎 6 g,白芍 9 g,熟地黄 12 g),每个复方大约 54 g,加 10 倍体积的水煎煮 60 min,离心取上清,经低温冷冻干燥成粉末状, $-80\text{ }^{\circ}\text{C}$ 保存备用。解剖显微镜(SZX7, OLYMPUS, Japan);电动聚焦连续变倍荧光显微镜(AZ100, Nikon 公司);6 孔板(Nest Biotech);甲基纤维素(Aladdin, Shanghai, China)。

1.1.2 实验动物:野生型 AB 系斑马鱼,由浙江杭州环特生物公司提供,以自然成对交配繁殖方式进行。年龄为受精后 3 天(3 dpf),共 180 尾,用于评价桃红四物汤对斑马鱼骨质疏松治疗作用实验。饲养于 $28\text{ }^{\circ}\text{C}$ 的养鱼用水中(水质:每 1 L 反渗透水中加入 200 mg 速溶海盐,电导率为 $480\sim 510\text{ }\mu\text{S/cm}$; pH 为 $6.9\sim 7.2$;硬度为 $53.7\sim 71.6\text{ mg/L CaCO}_3$),实验动物使用许可证号为:SYXK(浙)2012-0171。饲养管理符合国际 AAALAC 认证的要求。

1.2 方法

1.2.1 桃红四物汤的最大耐受浓度(MTC)测定:随机选取 3 dpf 正常野生型 AB 系斑马鱼于六孔板中,每孔(即每浓度组)30 尾,用泼尼松处理正常斑马鱼建立斑马鱼骨质疏松症模型,分别水溶给予桃

红四物汤 350、400、450 和 $500\text{ }\mu\text{g/mL}$ 浓度,每孔容量为 3 mL,同时设置正常对照组和模型对照组。 $28\text{ }^{\circ}\text{C}$ 培养箱孵育一段时间后,观察并统计斑马鱼死亡数量与毒性情况,确定桃红四物汤对斑马鱼的最大耐受浓度(MTC)。

1.2.2 桃红四物汤对泼尼松诱导的骨质疏松的作用:随机选取 3 dpf 正常野生型 AB 系斑马鱼于六孔板中,每孔(即每浓度组)30 尾,用泼尼松处理正常斑马鱼建立斑马鱼骨质疏松症模型,分别水溶给予桃红四物汤 50、150 和 $450\text{ }\mu\text{g/mL}$ 浓度,阳性对照药依替膦酸二钠 $300\text{ }\mu\text{g/mL}$ 浓度,每孔 3 mL 容量,同时设置正常对照组和模型对照组。 $28\text{ }^{\circ}\text{C}$ 培养箱孵育 4 d 后,用 0.2% 钙黄绿素进行染色,染色后进行拍照。采集数据,分析统计斑马鱼脊椎骨前三节的荧光强度(S),桃红四物汤对斑马鱼骨质疏松治疗作用计算公式如下:

骨质疏松治疗作用(%) =

$$\frac{S(\text{供试品组}) - S(\text{模型对照组})}{S(\text{正常对照组}) - S(\text{模型对照组})} \times 100\%$$

1.3 统计学处理

全部统计学处理结果用 $\bar{x} \pm s$ 表示,利用方差分析和 Dunnett's-T 检验两种统计学方法, $P < 0.05$ 认为差异具有统计学意义。

2 结果

2.1 桃红四物汤对斑马鱼的最大耐药浓度

通过对各浓度组桃红四物汤引起斑马鱼幼鱼死亡率的统计分析,结果显示,桃红四物汤在 350、400 和 $450\text{ }\mu\text{g/mL}$ 浓度时,斑马鱼反应正常,均未引起斑马鱼死亡。当浓度为 $500\text{ }\mu\text{g/mL}$ 时,3.3% 斑马鱼死亡,表明本实验中,桃红四物汤对斑马鱼的最大耐药浓度 MTC 为 $450\text{ }\mu\text{g/mL}$ 。因此,后续的骨质疏松治疗作用的实验浓度设置为 50、150 和 $450\text{ }\mu\text{g/mL}$ 。详见表 1。

表 1 桃红四物汤对斑马鱼的最大耐药浓度($n=30$)

Table 1 The maximum concentration of drug resistance of Taohong Siwu Decoction on zebrafish ($n=30$)

Group	Concentration ($\mu\text{g/mL}$)	Zebrafish response	Death number	Mortality (%)
Normal control group	—	normal	0	0
Prednisone-treated model group	—	normal	0	0
Taohong Siwu decoction group	350	normal	0	0
	400	normal	0	0
	450	normal	0	0
	500	normal	1	3.3

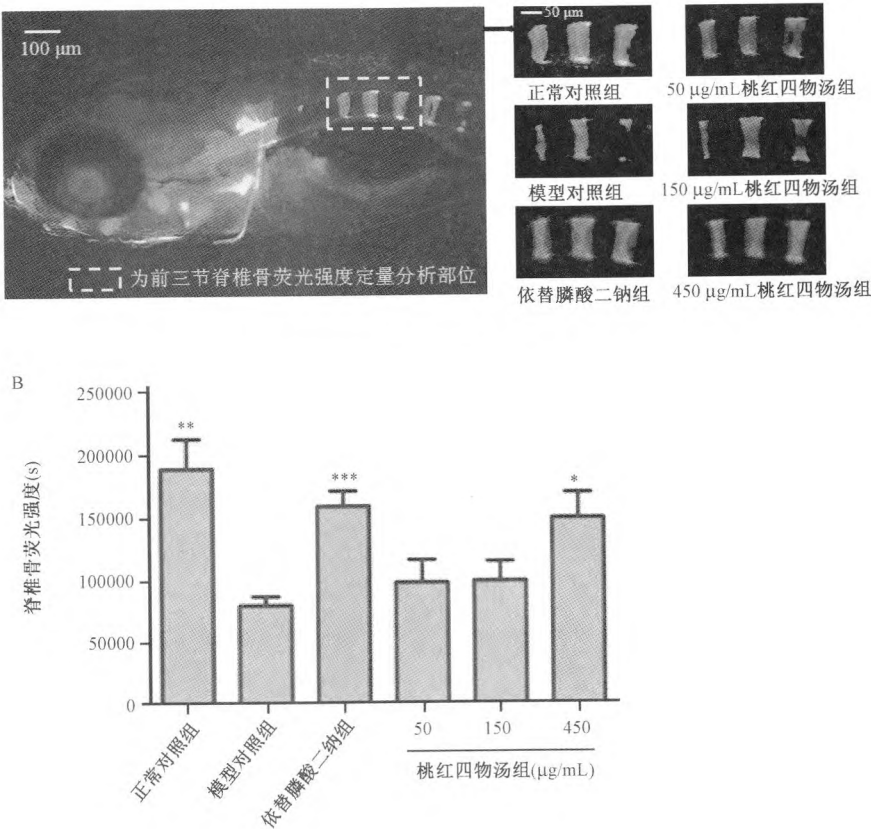


图 1 桃红四物汤对斑马鱼骨质疏松的作用 (n = 30)

A 泼尼松处理斑马鱼建立骨质疏松动物模型,设正常对照组和模型对照组,桃红四物汤三个浓度组,依替膦酸二钠组六个处理组,分别处理 4 d;B 定量分析斑马鱼脊椎骨前三节的荧光强度。30 次独立重复实验的结果用 $\bar{x} \pm s$ 表示。与模型对照组比较, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Fig. 1 Effect of different concentrations of Taohong Siwu Decoction on vertebral osteoporosis in zebrafish (n = 30)

A: Zebrafish osteoporosis models were established using prednisone and 6 study groups including Normal control group, Model group, Taohong Siwu Decoction groups of three different concentrations, and Etidronate disodium group received respective treatment for 4 days; B The fluorescence intensity of the first three vertebrae of zebrafish in different groups were measured using quantitative analysis. All data represent as the means $\pm$ SD of thirty independent experiments. Compared with the model group, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

2.2 桃红四物汤对泼尼松诱导的骨质疏松的作用

实验共有 6 个组,为正常对照组,模型对照组,依替膦酸二钠组和 3 个浓度的桃红四物汤组,作用斑马鱼模型,结果见图 1~2,表 2。

模型对照组的斑马鱼脊椎骨荧光强度小于正常对照组, $P < 0.01$; 阳性药物依替膦酸二钠组的斑马鱼脊椎骨荧光强度大于模型对照组, $P < 0.001$,提示斑马鱼骨质疏松模型构建成功,也表示依替膦酸二钠对斑马鱼骨质疏松具有明显的治疗作用。

桃红四物汤在浓度为 50 $\mu\text{g/mL}$ 和 150 $\mu\text{g/mL}$ 时,对斑马鱼骨质疏松治疗作用分别为 16.4 % 和 17.8 %,斑马鱼脊椎骨荧光强度与模型对照组比较 $P > 0.05$,提示桃红四物汤在 50 $\mu\text{g/mL}$ 和 150 $\mu\text{g/mL}$

表 2 桃红四物汤对斑马鱼骨质疏松的治疗作用 (n = 30)

Table 2 Therapeutic effect of Taohong Siwu Decoction on zebrafish osteoporosis (n = 30)

Group	Concentration (µg/mL)	Vertebrae fluorescence intensity (Mean ± SE)	Therapeutic effect of osteoporosis (%)
Normal control group	-	188135 ± 23590 **	-
Prednisone-treated model group	-	78843 ± 7225	-
Etidronate disodium group	300	158086 ± 11963 ***	72.5 ***
Taohong Siwu Decoction group	50	96783 ± 18241	16.4
	150	98247 ± 15419	17.8
	450	148217 ± 20167 *	63.5 *

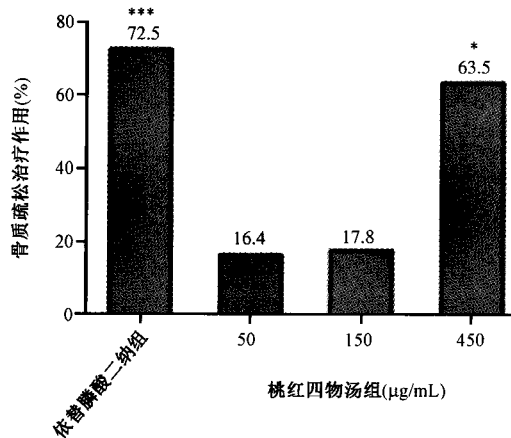


图2 桃红四物汤对斑马鱼骨质疏松的治疗作用 (%) 按公式(2.2)计算桃红四物汤各组对骨质疏松的治疗作用。与模型对照组比较(图中未列出), * $P < 0.05$, *** $P < 0.001$

Fig.2 Therapeutic effects of Taohong Siwu Decoction on zebrafish osteoporosis (%). The therapeutic effects of Taohong Siwu Decoction on zebrafish osteoporosis were calculated according to the formula (2.2); Compared with the model group (not in the figure), * $P < 0.05$, *** $P < 0.001$.

浓度时,对斑马鱼骨质疏松的治疗作用不明显;但桃红四物汤在 450 $\mu\text{g/mL}$ 浓度时,其斑马鱼骨质疏松治疗作用为 63.5%,斑马鱼脊椎骨荧光强度与模型对照组比较 $P < 0.05$;提示桃红四物汤在 450 $\mu\text{g/mL}$ 浓度时,对斑马鱼骨质疏松具有明显的治疗作用。

3 讨论

桃红四物汤作为活血化瘀类的经典中药复方,方中熟地、白芍是血中的血药,当归、川芎是血中的气药,阴阳动静相配,故能补血,又能和血,加入活血祛瘀的桃仁、红花为主药,突出了活血化瘀的作用,由于桃仁、红花的活血作用比较缓和,再配合四物汤养血扶正,故本方是一首比较平和和有效的活血祛瘀方剂^[1]。中医认为,脉者,血之府也,凡血液流通之处,皆可称为血府,因此桃红四物汤也被广泛用于骨科各疾病的治疗,如骨折、腰腿痛等^[2]。

由于桃红四物汤多在骨伤科方面用做壮骨强肾药的辅助用药,单独探讨其对骨质疏松症作用的研究较为少见,本研究探讨在泼尼松诱导的斑马鱼骨质疏松模型中,桃红四物汤对其的最大耐药浓度和治疗作用,结果发现,在经泼尼松处理的斑马鱼骨质

疏松模型组的基础上,加入的桃红四物汤在 350、400 和 450 $\mu\text{g/mL}$ 浓度时,斑马鱼反应正常,未有死亡。而在 500 $\mu\text{g/mL}$ 浓度时引起 3.3% 斑马鱼死亡,即本方对斑马鱼的 MTC 为 450 $\mu\text{g/mL}$,在此基础上,进一步评价了阳性对照药和桃红四物汤的抗骨质疏松活性,结果发现,依替膦酸二钠的抗骨质疏松的作用略强于桃红四物汤,而桃红四物汤的疗效存在着一定的量效关系,另一方面,提示斑马鱼骨质疏松模型也可用于中药复方抗骨质疏松活性的疗效关系研究。

由于斑马鱼骨质疏松模型是一种介于单细胞和整体哺乳动物之间的在体模式动物模型,可作为细胞实验和常规动物实验评价之间的桥梁,体积小而有完整骨骼,可实现在体、无损伤地快速观察药物的作用。这对开发我国丰富的中药资源,尤其是能够快速发现和甄别具有抗骨质疏松作用或骨骼毒副作用的某些经典复方^[12],有着不可否认的促进作用。

【参考文献】

- [1] 卫穗转,张自强,祁文兵,等. 桃红四物汤在骨科临床中的应用. 中医临床研究, 2011, 3(16): 89-90.
Wei SZ, Zhang ZQ, Qi WB, et al. Application of taohong siwu decoction on treating orthopedics in clinical. Clinical Journal of Chinese, 2011, 03(16): 89-90. (in Chinese)
- [2] 熊辉,李前,谈立明,等. 桃红四物汤有效成分的交互作用及其对骨髓微血管形态学的影响. 中医正骨, 2010, 22(11): 11-14.
Xiong H, Li Q, Tan LM, et al. The interactions of Taohongsiwu Decoction's effective constituents and the influences on the histomorphology and the microangiogenesis of callus. The Journal of Traditional Chinese Orthopedics and Traumatology, 2010, 22(11): 11-14. (in Chinese)
- [3] Kari G., Rodeck U, Dicker AP. Zebrafish: An emerging model system for human disease and drug discovery. Clinical Pharmacology & Therapeutics, 2007, 82(1): 70-80.
- [4] Penberthy WT, Shafizadeh E, Lin S. The zebrafish as a model for human disease. Front Biosci, 2002, 7(1-3): 1439-1453.
- [5] 李森. 地塞米松磷酸钠导致斑马鱼颅面部发育异常的研究. 华中科技大学, 2009.
Li M. Craniofacial dysplasia in zebrafish induced by dexamethasone sodium phosphate. Huazhong University of Science and Technology, 2009. (in Chinese)
- [6] Jurković N, Kolb N, Colić I. Nutritive value of marine algae Laminaria japonica and Undaria pinnatifida. Molecular Nutrition & Food Research, 1995, 39(1): 63-66.

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- [5] Katsimpardi L, Litterman NK, Schein PA, et al. Vascular and neurogenic rejuvenation of the ageing mouse brain by young systemic factors. *Science*, 2014, 344(6184):630-634.
- [6] Sinha M, Jang YC, Oh J, et al. Restoring systemic GDF11 levels reverses age-related dysfunction in mouse skeletal muscle. *Science*, 2014, 344(6184): 649-652.
- [7] Jin M, Song S, Guo L, et al. Increased serum GDF11 concentration is associated with a high prevalence of osteoporosis in elderly native Chinese women. *Clin Exp Pharmacol Physiol*, 2016, 43(11):1145-1147.
- [8] Egerman MA, Cadena SM, Gilbert JA. GDF11 increases with age and inhibits skeletal muscle regeneration. *Cell Metab*, 2015, 22(1):164-174.
- [9] Wu XY, Zhang H, Xie H, et al. Reference intervals of bone turnover markers determined by using their curve-fitting valley for adult females in China. *Osteoporosis International*, 2014, 25: 943-952.
- [10] Kanis JA, Melton LJ, Christiansen C, et al. The diagnosis of osteoporosis. *J Bone Miner Res*, 1994, 9(8):1137-1141.
- [11] Brincat SD, Borg M, Camilleri G, et al. The role of cytokines in postmenopausal osteoporosis. *Minerva Ginecol*, 2014, 66:391-407.
- [12] Molnar I, Bohaty I, Somogyi-Vari E. IL - 17A-mediated sRANK ligand elevation involved in postmenopausal osteoporosis. *Osteoporos Int*, 2014, 25:783-786.
- [13] Zhao R. Immune regulation of bone loss by Th17 cells in oestrogen-deficient osteoporosis. *Eur J Clin Invest*, 2013, 43: 1195-1202.
- [14] Sridharan M, Cheung J, Moore AE, et al. Circulating fibroblast growth factor 23 increases following intermittent parathyroid hormone (1-34) in postmenopausal osteoporosis: association with biomarker of bone formation. *Calcif Tissue Int*, 2010, 87(5): 398-415.
- [15] Sun J, Zhang C, Xu L, et al. The transforming growth factor- β 1 (TGF- β 1) gene polymorphisms (TGF- β 1 T869C and TGF- β 1 T29C) and susceptibility to postmenopausal osteoporosis: a meta-analysis. *Medicine (Baltimore)*, 2015, 94(4):e461.
- [16] 赵燕, 王艳, 张燕, 等. GLP-1 对 2 型糖尿病大鼠血清 OPG、RANKL 及骨密度的影响研究. *中国骨质疏松杂志*, 2016, 22(6):700-705.
- Zhao Y, Wang Y, Zhang Y, et al. The effect of GLP-1 on serum concentrations of OPG and RANKL and bone mineral density in type 2 diabetic rats. *Chin J Osteoporos*, 2016, 22(6):700-705.
- [17] Scudellari M. Ageing research: Blood to blood. *Nature*, 2015, 517(7535):426-429.
- [18] Bitto A, Kaeberlein M. Rejuvenation: it's in our blood. *Cell Metab*, 2014, 20(1):2-4.
- [19] Zhang Y, Shao J, Wang Z. Growth differentiation factor 11 is a protective factor for osteoblastogenesis by targeting PPAR γ . *Gene*, 2015, 557(2):209-214.
- [20] Li Z, Zeng F, Mitchell AD, et al. Transgenic overexpression of bone morphogenetic protein 11 propeptide in skeleton enhances bone formation. *Biochem Biophys Res Commun*, 2011, 416(3-4):289-292.
- [21] Lu Q, Tu ML, Li CJ, et al. GDF11 Inhibits Bone Formation by Activating Smad2/3 in Bone Marrow Mesenchymal Stem Cells. *Calcif Tissue Int*, 2016, 99(5):500-509.

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- [7] Fleming A, Sato M, Goldsmith P. High-throughput in vivo screening for bone anabolic compounds with zebrafish. *Journal of Biomolecular Screening*, 2006, 10(8): 823-831.
- [8] 鞠黎. 胚胎期多氯联苯暴露对斑马鱼骨骼发育的影响. 南京医科大学, 2012.
- Ju L. Effects of exposure to PCBs during embryonic period on skeletal development of zebrafish. Nanjing Medical University, 2012. (in Chinese)
- [9] 刘晨, 陈斌, 徐又佳. 斑马鱼疾病模型. *中华骨质疏松和骨矿盐疾病杂志*, 2014, 7(3): 277-280.
- Liu CH, Chen B, Xu YJ. Zebrafish as an animal model of human disease. *Chinese Journal of Osteoporosis and Bone Mineral Disease*, 2014, 7(3): 277-280. (in Chinese)
- [10] Siccaldi AJ III, Padgett-Vasquez S, Garris HW, et al. Dietary strontium increases bone mineral density in intact zebrafish (*Danio rerio*): a potential model system for bone research. *Zebrafish*, 2010, 7(3): 267-273.
- [11] Jobgen WS, Jobgen SC, Li H, et al. Analysis of nitrite and nitrate biological samples using high-performance liquid chromatography. *Journal of Chromatography B*, 2007, 851(1-2): 71-82.
- [12] 彭维兵, 韩建, 陈维云, 等. 昆布多糖对氯化镉诱导斑马鱼骨骼发育毒性的改善作用. *山东科学*, 2015, 28(5): 22-26.
- Han WB, Han J, Chen WY, et al. Recovery effect of laminarin on cadmium chloride induced developmental toxicity of zebrafish skeleton[J]. *Shan dong Science*, 2015, 28(5): 22-26. (in Chinese)

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