Zebrafish as a research tool for human diseases pathogenesis and drug development

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(Received July 2, 2021: Revised May 23, 2022: Accepted May 24, 2022)

요 약 : 다양한 동물 모델이 인간 질병, 의약품의 효능 및 작용 메커니즘을 연구하는 데 사용되고 있다. Zebrafish(*Danio rerio*)는 여러 가지 장점이 있어 인간 질병에 대한 중개 연구의 모델로 점점 더 폭넓게 활용되고 있다. 본 논문은 Pubmed, Google Scholar, Scopus에서 2020년 12월까지 최근 10년간 zebrafish 모델, 천연물(한약), in vivo 스크리닝의 키워드를 사용하여 저널에 게재된 논문을 검토하여 필요한 정보를 얻었다. 이 리뷰에서 우리는 천연물(한약) 연구에 대한 다양한 제브라피쉬 질병 모델의 최근 경향에 대해 논의하였다. 특히, 암, 안질환, 혈관 질환, 당뇨병 및 합병증, 피부질환에 중점을 두었고, zebrafish 배아를 사용하여 이들 질병에 대한 의약품의 분자 작용 메커니즘에 관해 언급하였다. Zebrafish는 실험실에서 임상 연구까지의 격차를 줄이는 데 중추적 역할을 할 수 있는 중요한 동물 모델이다. Zebrafish는 의약품이나 화장품 개발, 질병의 병인론을 이해하기 위해 사용되고, 이로 인해 생의학 연구에서 설치류의 사용을 줄이는 데 크게 기여하고 있다.

Abstract : Various animal models have been used to study the efficacy and action mechanisms of human diseases and medicines. Zebrafish (*Danio rerio*) is increasingly and successfully used as a model in translational research on human diseases. We obtained necessary information from original peer reviewed articles published in scientific 54 journals, such as Pubmed, Google Scholar, Scopus scince their inception until Dec, 2020 using the following terms: zebrafish animal models, herbal medicine, *in vivo* screening. In this review, we discuss the recent contributions of the various zebrafish disease models to study of herbal medicines. We focused on cancer, eye diseases, vascular diseases, diabetes and its complications, and cosmetic dermatology. We also highlight the molecular action mechanisms of medicines against these disease, demonstrated using zebrafish embryo. Zebrafish can be pivotal in bridging the gap from lab to clinical bedside. It is used as a model to understand human diseases pathogenies with further scope for drug development. Furthermore, zebrafish can reduce rat and mouse animals in biomedical research.

Keywords : zebrafish (Danio rerio); disease models; drug development; in vivo screening; translational research

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1. Introduction

Herbal medicines such as medicinal plants, natural products have been used to treat various diseases and enhance the general health and wellbeing. According to the World Health Organization (WHO), herbal medicines are the mainstay treatment for approximately 80% of the global population, especially in developing countries. In Korea, herbal medicines with acupuncture have been used as a clinical treatment for 500 years. In recent years, herbal medicines are considered as dietary supplements for disease prevention and as alternative/complementary medicines.

Pharmaceutical companies have focused on discovering drugs for various disease using medicinal plants, natural products, and active compounds. The application of modern technologies and methodologies in herbal medicine development is required to examine their action mechanisms and to prove their novel effects using *in vitro* and *in vivo* experimental models. New scientific techniques have been applied in herbal drug discovery or mechanism studies. For example, high throughput screening is used in drug discovery, and it involves automated testing with thousands of chemical and/or biological experiments per day [1]. Systems biology is used to understand the interaction of chemical and metabolic processes interaction in the body and to measure the responses of body to the mixtures of compounds in herbal extracts [2]. Metabolomics is an advanced technique used in drug discovery to systematically study a complex mixture, such as plant extracts [3]. For *in vivo* assay, worms (nematodes) have been used to examine the efficacy of herbal extracts. *Caenorhabditis elegans* (C. elegans) is a free-living transparent nematode, enabling to observe them by microscopy. It is beneficial in aging research owing to it relatively short lifespan (average 17 days at 20 ℃) [4].

Zebrafish embryos and adults have been used as a vertebrate model in the toxicity assessment of herbal medicines, analysis of anti-angiogenesis effects, and disease-related research for drug development [5] [6]. Mice and rabbits are the most widely used animal models (Fig. 1). These animal models have great advantages, but they also have from several limitations such as and animal welfare. All research involving in vivo models should comply with standard ethics committee guidelines, where the 3Rs (replacement, refinement and reduction) principle should be implemented. In accordance with these principles, zebrafish (*Danio rerio*) is being increasingly used as an animal model in academic research in the field of genetics, development, diseases, toxicology safety, and drug discovery since the early 1980s [6]. The number of studies using zebrafish as a model in biomedical research has significantly increased (Fig. 2). According to the zebrafish model organism database, The Zebrafish Information Network (ZFIN, http://zfin.org), at least 71.4% of human genes have at least one ortholog, and 82% of the morbid genes have a related in zebrafish [6, 7]. Major universities, and research centres worldwide, as well as pharmaceutical companies such as AstraZeneca, Novartis, and Pfizer, use larval zebrafish in seizure study; absorption, distribution, metabolism and excretion; and safety pharmacology. In develop the drug discovery, natural product (herb) extracts are an attractive starting material because many possess structural complexity that cannot be achieved using chemical synthesis [4]. As shown in Fig. 3, zebrafish can be used at multiple points in the drug discovery process. In this review, we focus on the use of zebrafish disease model in the study of herbal medicines and their novel and synergistic effects and identification active compounds in herbs.
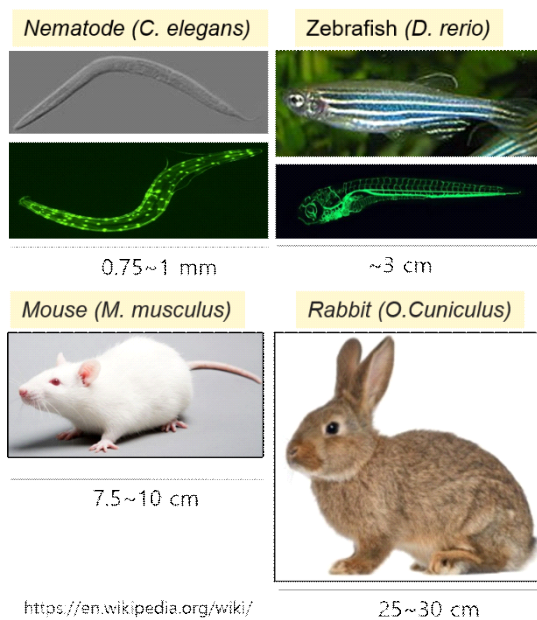
Fig. 1. *In vivo* assay models for studying the efficacy of herbal medicines. *Caenorhadbitis. elegans* is a good model for studying aging and zebrafish for studying toxicity, efficacy, and for drug screenings.
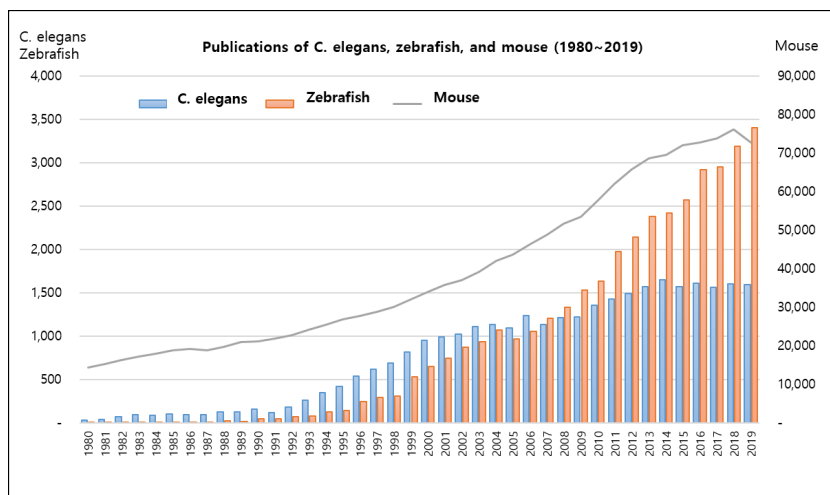

Fig. 2. Studies on *Caenorhadbitis. elegans*, zebrafish, and mouse published on PubMed per year. The number of zebrafish publications was determined using the following keywords in PubMed advanced search: '*C. elegans*', 'zebrafish', or 'mouse'. *Caenorhadbitis. elegans* and zebrafish are indicated on the left axis and mouse on the right axis. PubMed searches were conducted on April 30, 2020.
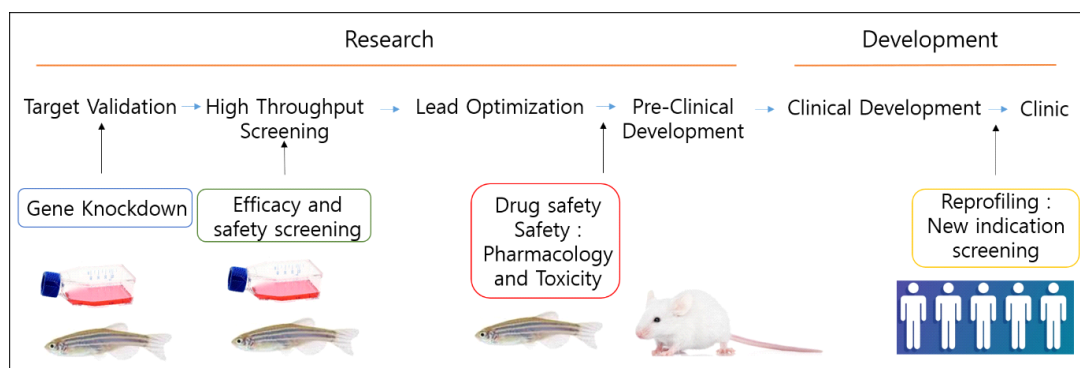
Fig. 3. Schematic diagram illustrating where assays with zebrafish can be used in drug discovery and development. Assays with larval zebrafish can be used at multiple points in the drug discovery process, namely, target validation, efficacy, and safety liabilities. Assays with zebrafish can also be used for reprofiling/re-purposing/re-positioning to identify new applications of approved or generic drugs and compounds that have failed in Phase 2 trials for reasons other than safety.

2. Methods

In this review, we obtained the necessary information for this review from original peer reviewed articles from database, such as PubMed, Google Scholar, and Scopus using the terms from their inception until Dec, 2020 using the following terms: zebrafish animal model, herbal medicine, traditional Chinese medicine, and *in vivo* screening.

3. Zebrafish as disease models

Zebrafish is an important animal model for drug screening owing to the following: rapid development and limited space requirement for maintenance and experimentation. Studies have focused on developing specific diseases model through genome editing tools using zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated (Cas) system [8, 9]. Information about zebrafish can be obtained from several zebrafish databases (Table 1). The

ZFIN is a database of genetic and genomic data of zebrafish. ZFIN provides gene marker clones and extensive information on zebrafish, including genomics data, developmental stages, publications and molecular tools.

3.1. Cancer

Zebrafish has several attributes that cancer researchers find attractive compared to mice. Zebrafish handling requires minimal care, and its maintenance in the laboratory is inexpensive. Zebrafish as a human cancer model are used as mutant lines, transgenic lines, and xenotransplants (2~5 day-old or 30 days-old) [10]. Chemical carcinogenesis is the first approach used to induce tumor formation in zebrafish. Water-soluble carcinogens such as dimethylbenzanthracene (hepatic and intestinal tumours), ethylnitrosourea (hyperplasia and papillomas), and diethylnitrosamine (hepatoma) induce various tumors in zebrafish, especially tumors of the liver, skin (papillomas), muscle (rhabdomyosarcomas and leiomyosarcomas), vasculature (hemangiosarcomas), testis (seminomas) and pancreas (pancreatic carcinomas) [11-14]. The knockout of p53, a

Table 1. Zebrafish databases and the URLs

	Database	URLWeb site
ZFIN	Zebrafish model organism database	http://zfin.org/
ZDMS	Zebrafish disease model society	http://zdmsociety.org/
VEGA	Zebrafish gemone annotation	http://vega.sanger.ac.uk/Danio_rerio/Info/Index

Table 2. The technology and the types of tumor induced in zebrafish

Models	Treatment	Types of induced tumor	Ref.
Chemical-induced model	Demethylbenzanthracene (DMBA)	Hepatic and interstinal tumors	[13]
	Ethylnitrosourea (ENU)	Hyperplasia and paillomas	[11]
	Diethylnitrosamine (DEN)	Hepatoma, cholangiocarcinoma and pancreatic carcinoma	[14]
Gene knockout model	*p53*	Malignant peripheral nerve sheath tumors	[15]
	Adenomatous polyposis coli (*APC*)	Interstinal, hepatic and pancreatic neoplasias	[16]
	BRCA2	Testicular cancer	[18]
	GSTT1	Lymphoma	[17]
Gene overexpression model	*Myc*	T-cell leukemia and hepatoma	[19, 20]
	MYCN and *fgf8*	Neuroblastoma	[21]
	KRAS(G12D)	Rhabdomyosarcoma	[22]
	Akt1	Lipoma	[23]

tumor suppressor gene, in zebrafish has result in elucidation of a novel mechanism of p53 regulation and the importance of these mechanism *in vivo* [15]. The knockdown of the cancer-related genes such as *APC, BRCA2* and *GSTT1* induces cancer in specific organs [16-18]. Overexpression of genes such as Myc, MYCN and fgf8, KARS, and Akt1 are related cancer and can be used for the mechanism and animal models (Table 2).

3.2. Eye disease

Zebrafish are visually responsive at 72-hour post fertilization (hpf) and the retinal morphology of adult zebrafish is anatomically and functionally similar to that of humans [24]. Zebrafish, which has a cone-rich retina, is a suitable disease model of ophthalmology, where there is a lack of drug discovery effect and drug development efforts. Rodents have evolved to nocturnal existence and have a rod-dominant retina. The rod cells in the retina are reactive at low levels of light, and they don't mediate colour vision. High glucose induces changes in hyaloid-retinal vessels during early ocular development in zebrafish. This system could be used for rapid screening of drugs for diabetic retinopathy in zebrafish larvae [25].

Table 3. Comparison of human rhodopsin with that in other species [26]

Species	Nucleoside similarity
Rat	85.6%
Zebrafish	80.8%
Mouse	80.5%
C. elegans	44.0%

The homology of protein is nearly 100% for key domains.

3.3. Vascular biology

Angiogenesis is the growth of new microvessels from the existing vasculature, and it regulates endothelial cell proliferation and migration [27]. It is associated with wound healing and tissue function. Moreover, pathological angiogenesis has been observed in cancer, proliferative diabetic retinopathy, and age-related macular degeneration. Transgenic flk1:EGFP (the endothelial-specific flk1/VEGFR2 promoter directing enhanced green fluorescent protein) zebrafish allows for the vascular imaging [28]. Fli:EGFP zebrafish is a similar transgenic line, in which the fli1 promoter is expressed by enhanced GFP in all blood vessels throughout embryogenesis [29]. Models for vascular development and angiogenesis have been established. The knockdown of growth arrest-specific 6 (Gas6) inhibits angiogenesis in the developing vessels of zebrafish, and Gas6 signaling regulates angiogenesis *via* ERK1/2 kinase [28].

Herbal medicines traditionally used in Asia, such as *Centipeda minima*, *Croton crassifolius*, *Wedelia chinensis*, erxian decoction, and chlorogenic acid from extract of *Ilex kudingcha* is have been tested for their anti-angiogenic activity using transgenic (fli1a:EGFP or flk1:EGFP) zebrafish models [30-34]. The results reveals that the herbal medicines can block both inter-segmental blood vessels and sub-intestinal vessels. The results also suggested that compounds isolated from herbal extracts are promising candidates as anti-angiogenic agents.

3.4. Diabetes and its complications

Diabetes mellitus (types 1 and 2) is a chronic disease that results in diabetic complications such as retinopathy, nephropathy, foot ulceration, and neuropathy. Genetically modified- or chemical-induced rodent models have been used to study the mechanism and pathogenesis of the disease and to test the effects of drugs. Several diabetic models of zebrafish have been established and used for high- throughput screening of anti-diabetic drugs [4, 35, 36]. Transgenic(Tg) zebrafish expressing GFP in the beta cells (ins:H2BGFP) and Tg zebrafish (ins:CFP- NTR) expressing nitroreductase specifically in their beta cells are used for screening of anti-diabetic drugs and diet-induced obesity zebrafish is also used a model for type 2 diabetic mellitus (T2DM) [36, 37]. After overfeeding for 8 weeks, this obesity model shows high fasting blood glucose levels, glucose intolerance and insulin resistance [38]. Culturing under high glucose induces the elevated blood and tissue glucose in zebrafish and reduces the inner plexiform layer and inner nuclear layer in zebrafish retinas and suggest as an animal model to study diabetic retinopathy [39]. High glucose induced dilation of hyaloid-retinal vessels in larval zebrafish and polyphenols from *Euporbia pekinensis* and *Osteomeles schwerinae*, flavonoids from *Litsea japonica*, lignans from the *Brandisia hancei*, proanthocyanidins from *Spenceria ramalana* [40-43].

3.5. Cosmetic dermatology

Zebrafish, as a depigmentation animal model, has gained attention in the medicinal and cosmetic industries [44]. Depigmentation studies using zebrafish models are important for investigation the anti- melanogenic activity of bioactive compounds from herbal extracts in vivo. Phenotype-based screening, and analysis of melanin content, tyrosinase inhibitory activity, proteins activities, and transcription genes have been performed to depigematation effect of herbal extracts, using zebrafish [45]. Human skin pigmentation and zebrafish pigmentation are related in terms molecular genetics, genetic development, and molecular biology [46-48]. As shown in Fig. 4, bioxchanin A from *Trifolium pratense* inhibits melanogenesis in zebrafish [49]. Kazinol U from *Broussonetia kazinoki* Sieb (Moraceae) also inhibits melanogensis by inhibiting of tyrosinase-related proteins via AMP kinase activation [50].
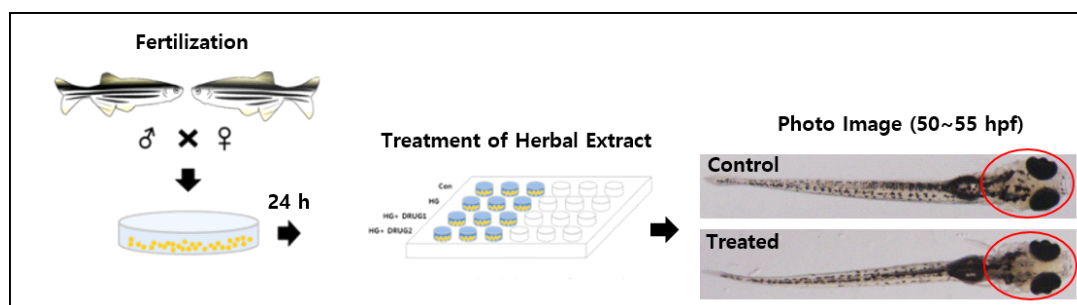
Fig. 4. Overall strategy for a rapid screening system to identify herbs with depigmentation effect *in vivo* using zebrafish larvae. Zebrafish pairs are bred to produce hundreds of fertilized embryos for development *ex vivo*. The embryos are arrayed in 24-well plates and maintained in embryonic water containing candidate herbs. Larvae deposited in 24-well plates were photographed using a stereomicroscope at 50~55 hour post fertilization (hpf).

Skin wound healing and regeneration are complex process of functions of injured tissues. The skin is consisted the epidermis, dermis, and skin appendages, including the hair follicle. Various types of skin wounds appear mechanical wounds such as cuts, stab wounds, and abrasion, chemical wounds, and radiation-based wounds. In adult zebrafish, regeneration of dermal scales that share some similarities to mammalian hair follicles occurs after partial complete ablation during skin wounding [51]. A requirement for cells residing in hair follicle in mouse and scales in zebrafish has been described to allow the regeneration [52]. *Piper sarmentosum* extract induce fin tissue regeneration [53]. However, researchers have observed a significant decrease in the regenerative capacity of the caudal fin amputated zebrafish exposed to cigarette smoke extract [54].

4. Conclusions and future research directions

Understanding the physiology of zebrafish will help demonstrate the efficacy and the action mechanisms of herbal medicines and the pathogenesis of diseases. In conclusion, zebrafish represent a superior vertebrate model for drug screening, and they can provide deeper understanding of the action mechanisms of herbal medicines in the future. Also, it is a useful *in vivo* model to evaluate the synergistic effect of small amounts of natural products.

Conflicts of interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Ethical statement
Not applicable.

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