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Mini Review

Organoid models for Chinese herbal medicine studies

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ABSTRACT

Organoids are three-dimensional cell accusations generated from pluripotent stem cells or adult stem cells *in vitro*. With many advantages over cell and animal models, organoids have been increasingly used in drug and clinical medical research in recent years. Chinese herbal medicine (CHM) is characterized by multi-target and multi-pathway treatment methods; however, there is no commonly accepted study method regarding efficacy and underlying mechanisms. In this review we summarized the important applications of organoid models in pharmacodynamic mechanism studies, efficacy and safety evaluations, and CHM personalized medicine, thus providing the theoretical basis for its development and innovation.

Keywords: organoids, Chinese herbal medicine, three-dimensional model, *in vitro* experiments

1. INTRODUCTION

Organoids are tissue analogs with specific spatial organization created via three-dimensional stem cell cultivation *in vitro* [1]. An organoid is a miniature *in vitro* organ model that closely resembles the properties of actual organs *in vivo*. Organoids are derived from adult stem cells (ASCs) or pluripotent stem cells (PSCs). During organoid culture, stem cells proliferate and differentiate into organ-representative cell types in an environment with multiple signaling factors, mimicking the natural environment *in vivo*. Moreover, organoids can be applied to various species and tissues.

Advances in stem cell technology have driven the development of organoids. Research involving organoids has made remarkable progress in the past decade. Intestinal organoids were first constructed in the laboratory of Hans Clevers, the originator of organoids, in 2009 [2]. In 2013, brain organoids developed from human PSCs were successfully cultivated [3]. Subsequently, various organoids originating from different germ layers were gradually developed [4]. Compared to conventional two-dimensional cell culture models, organoids have more complex and diverse cell types with a more stable genome, mimicking and reflecting the interaction between cells and the extracellular environment. At present, various kinds of organoids have been established for numerous potential applications, such as drug screening [5].

Compared with traditional animal models, organoids avoid the differences between species and thereby simulate human traits more accurately [6]. Of all animal models, non-human primates are most closely related to humans, making experimental monkeys suitable models for evaluating drugs; however, monkey experiments are often lengthy at a high cost. By way of comparison, drug screening and organoid evaluation requires less time at a lower cost. Aside from the time and costs, guidelines have been issued to reduce animal testing. Specifically, the European Union issued a directive in 2010 to reduce

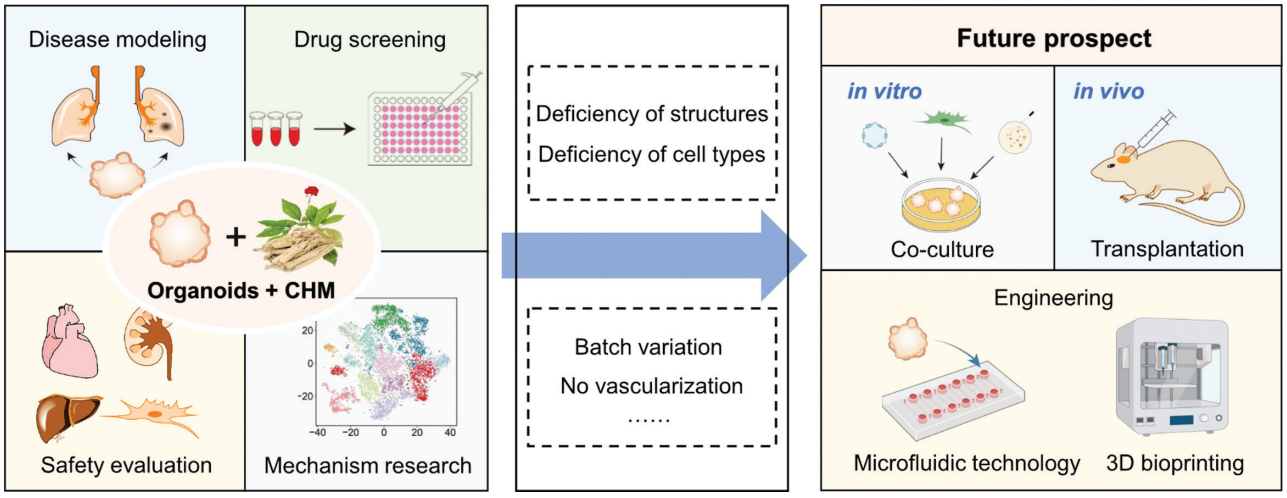

Figure 1 | Application and prospect of organoids in the research of CHM.

animal testing [7]. Recently, the U.S. Senate passed the FDA Modernization Act 2.0 to reduce the testing of new drugs on dogs, primates, and other animals. Thus, the most likely alternative to animal testing technology involves organoids, which simulate three-dimensional organs *in vitro* [8].

ASC-derived organoid cultures are typically established by embedding isolated ASCs or single-cell suspensions into extracellular matrix hydrogels. PSCs, including induced stem and embryonic stem cells, can also take advantage of self-renewal and differentiation capabilities to generate organoids [5]. Tumor cells isolated from tumor tissues of patients, which contain cancer stem cells, can be cultured in a microenvironment simulating *in vivo* conditions after adding growth factors and small molecules to generate tumor organoids, which can be stably expanded in an *in vitro* culture system. Growth factors are important substances that need to be added to organoid cultures. Growth factors direct stem cell differentiation by regulating signaling pathways. For most organoid types, growth factors required for most organoid types include Wnt and R-spondin-1 [2, 3]. The type of organoids needed for specific experiments should be determined based on the sample source, experimental content, and conditions.

Chinese herbal medicine (CHM) is regarded as one of the world's greatest treasures. Due to impressive curative effects and negligible side effects, CHM has become increasingly popular for treating specific disorders, such as cardiovascular disease [9], coronavirus disease 2019 (COVID-19) [10], and cancer [11]. Unlike chemical drugs of a single chemical compound that in most cases act on a specific target, CHMs are usually prescribed in the form of 'formulae,' which are a combination of herbs. Because the herbs target different organ, the incidence of drug resistance in CHM is relatively lows.

CHM is characterized as multi-targets and -pathways, inducing many limitations in studies involving conventional animal and cell models. Of note, it is effective to use organoids to avoid deviations in experimental results caused by differences between species in animal models. More importantly, organoids make up for the lack of integrity and heterogeneity in the cell culture model. Owing to the substantial work in past decades, organoids have emerged to have an important role in regulating disease occurrence and development, hunting for drug targets, and conducting drug screening and safety evaluations [12]. In view of the above, we have summarized the application of organoids in CHM research regarding efficacy, drug screening, safety, and underlying mechanisms, and advanced solutions and prospects for existing problems, such as the deficiency of structures and cell types, and batch variation (**Figure 1**).

2. EFFICACY EVALUATION

Organoids have a significant role in the evaluation of drug efficacy, thus providing references for medications used clinically. There have been numerous reports focusing on organoids in Western medicine [13]. Although there are relatively few reports on CHM (**Table 1**), organoids have enormous development potential.

Organoids are rich in cell types and contain tissue structures that can be utilized to create disease models by modifying culture conditions, gene editing, and obtaining cell sources from patients to evaluate the efficacy of CHM treatment.

Fan et al. [14] assessed the mechanism and pharmacodynamic impact of the CHM extraction, Guanxinning injection (GXNI), using an organoid model of cardiac hypertrophy. The model was comprised of cardiac fibroblasts, cardiomyocytes, and endothelial cells. Cells in the model were cardiac-like in shape with extracellular matrix components and exhibited spontaneous, rhythmic contractile, and diastolic activities. With respect to human organoids, Du et al. [15] created a blood-brain

Table 1 | Application of organoids in efficacy evaluation and drug screening of CHM.

Type of organoids	Species	Origin	Models of disease	CHM	Dose	Reference
Cardiac organoids	Rats	Cardiac fibroblasts (CFs), cardiomyocytes (CMs), and endothelial cells (ECs)	Myocardial hypertrophy	Guanxining Injection (GXNI)		[14]
BBB organoids	Human	Human brain microvascular endothelial cells (HBMEC), human brain astrocytes (HA) and human brain vascular pericytes (HBVP)	Oxygen-glucose deprivation/ reoxygenation (OGD/R)	Guanxining Injection (GXNI)	10 μL/mL	[15]
Small intestinal organoids	Mice	Intestinal crypt stem cells	Normal	Glycyrrhetinic acid	10, 20 μM	[16]
Small intestinal organoids	Mice	Intestinal crypt stem cells	Normal	Sijunzi decoction		[17]
Small intestinal organoids	Mice	Intestinal crypt stem cells	Normal	St. John's wort extract and hyperforin	SW ethanol extract (0.8, 8.0 μg/ml); hyperforin (0.2, 1.0 μM)	[18]
Hepatobiliary organoids	Human	Human induced pluripotent stem cell (hiPSC)	Normal	Cholesterol + MIX (Chinese pending patent number: ZL 201810211144.X)		[19]
Patient-derived organoids	Human	Tissues from patients with colorectal cancer	Colorectal cancer	Triptolide, cantharidin, and bufalin	Triptolide (0.0008, 0.004, 0.02, 0.1, 0.5 μM); cantharidin (0.3, 1.5, 7.5, 37.5, 187.5 μM), and bufalin (0.0096, 0.048, 0.24, 1.2, 6 μM)	[20]
Patient-derived organoids	Human	Tissues from patients with colorectal cancer	Colorectal cancer	Celastrol	0.0562, 0.2808, 1.404, 7.02, 35.1 μmol/L	[21]
Patient-derived organoids	Human	Tissues from patients with non-small cell lung cancer	Non-small cell lung cancer	Chelerythrine chloride, cantharidin, harmine, berberine and betaine	1.56–2.88 μM	[22]

barrier (BBB) organoid oxygen-glucose deprivation model to investigate the protective effect of GXNI on BBB dysfunction induced by ischemic encephalopathy, which is useful for the development of central nervous system-targeted drugs. These organoids are spheroids formed by various types of cells, and the cells are recombined through the secreted cytoplasmic matrix. They can simulate tissue characteristics *in vivo*, but may lack other cell types.

In addition, organoids derived from stem cells can also be established for drug intervention to study the protective effect of CHM on specific organs. Chen et al. [16] established intestinal organoids and showed that glycyrrhetinic acid increases the level of human antigen R and downstream proliferation associated nuclear antigen Ki67 to promote the development of intestinal organoids and maintain intestinal homeostasis. Wang et al. [17] determined the impact of polysaccharide extracts of the CHM formula, Sijunzi decoction, on the growth of intestinal organoids. Wang et al. [17] used the dry crypt cell mass of mouse small intestine to cultivate into intestinal organoids *in vitro*, and observed and characterized the morphologic traits and protein expression of intestinal organoids. ASCs are isolated from mouse tissues and cultured to obtain organoids, which can produce a large number of organoids in a short time using a simple procedure. Of note, there are species differences between mouse-derived organoids and human organs.

In a study involving liver protection, Wu et al. [19] added the CHM product, cholesterol+ MIX (Chinese pending patent number, ZL 201810211144.X), in the process of forming hepatobiliary organoids. Wu et al. [19] reported that the organoids have better secretion and drug metabolism abilities, and a longer survival time after transplantation *in vivo*, which provides a new direction for further study. In this study, human-induced pluripotent stem cells (hiPSCs) were shown to differentiate into different types of organoids. HiPSCs are readily accessible cell types, and organoids derived from hiPSCs have greater potential for translation into clinical applications; however, this type of organoid requires a relatively long culture time.

3. DRUG SCREENING

Organoids can be cultured in a relatively short period of time in large quantities with high genomic stability. Therefore, organoids have been gradually applied to drug screening [23]. Colorectal cancer organoids were established to evaluate the effects of several toxic CHM monomer components on their activity, including triptolide, cantharidin, and bufalin. At the same time, colorectal normal organoids were established to evaluate toxicity and verify the feasibility of anti-cancer methods [20]. Other studies involved patient-derived colorectal cancer organoids and showed that celastrol inhibits organoid growth, and the inhibitory effect of celastrol was stronger than the positive drug, L-OHP [21]. These studies provide a new methodologic reference for screening the anti-cancer activity of CHM. Human tissues were isolated, stem cells were extracted, and organoids were cultured in these studies. Organoids with donor characteristics can be grown in a relatively short period of time; however, donor samples are scarce, and some types of organoids cannot be generated from human tissues.

In addition, the establishment of organoid biobanks has an important role in CHM screening, especially for cancer [24]. Li et al. [22] established living non-small cell lung cancer organoids biobanks for drug research; these tumor organoids have similar pathologic features to primary tumors. Li et al. [22] screened some CHM monomers, including chelerythrine chloride, cantharidin, harmine, berberine, and betaine, and reported that the latter three monomers had anti-cancer activity against lung cancer organoids.

Patient-derived cancer cells (PDCs) and patient-derived xenotransplantation mice (PDXs) are often used as tumor models in drug screening; however, PDCs lack diversity in cell types and environments and PDXs have a low transplant success rate and require a long culture time [25, 26]. When compared, tumor organoids maintain the genetic characteristics and individual heterogeneity of patient tumors more efficiently, serve as a good model for drug screening, and are helpful for individualized drug use.

CHM has had a significant impact on cancer treatment as a complementary and alternative therapy [27]. Numerous studies have shown that the combination of CHM and chemotherapy drugs enhance the anti-tumor effect of chemotherapy drugs via a variety of molecular mechanisms and overcomes the resistance of molecularly-targeted drugs [28]. Although chemotherapy is widely used, chemotherapy has potent toxicity and side effects on normal tissues. The use of CHM to mobilize the internal resistance to disease reduces the toxic side effects of Western medicine and achieves the effect of overall regulation [29]. In addition, CHM therapy attaches importance to the individualized medical care that Chinese medicine practitioners (CMPs) embrace by adjusting the combination of herbs and dosage of medications based on the specific body constitution of the patient. Organoids originating from human cells or tissues preserve donor heterogeneity, especially tumor organoids. The notion of using an organoid model to evaluate the impact of drug therapy and screen drugs is consistent with the treatment concept of CHM.

4. SAFETY EVALUATION

In recent years CHM has been widely applied and safety has received additional attention. It is challenging to study the safety of CHM due to the complexity of CHM components and the limitations of conventional models. Because cell models lack intercellular connections and animal models require a long drug administration process, organoids are promising models for the safety evaluation of CHM.

Organoids are used to access hepatotoxicity. The liver is the main organ of metabolism and is prone to drug-induced injury. It has been reported that many CHMs have potential hepatotoxicity, leading to serious clinical adverse events, such as liver fibrosis [30]. Previous studies used PSC-induced hepatocytes to evaluate the hepatotoxicity of CHM and found that the toxicity pattern of such hepatocytes is similar to human primary cultured hepatocytes [31, 32]. Compared with liver cells cultured *in vitro*, liver organoids induced by PSCs more closely resemble the human tissue microenvironment and are thus more suitable for hepatotoxicity assessment. Li et al. [33] used a droplet overlapping method to construct liver organoids. Combined with high-intensity imaging, 2,3,5,4′-tetrahydroxy-trans-stilbene-2-O-β-glucoside (trans-SG; ingredients of *Polygonum multiflorum* [He Shou Wu, *Fallopia multiflora*]) toxicity was shown to be significantly lower than its cis-isomer (cis-SG), which is consistent with previous animal results. Zhu et al. [34] combined 3D printing and microfluidic chip technology to obtain liver organoids derived from human cells, and evaluated the hepatotoxicity of commercially-available CHM injections. Indeed, the evaluation results were more accurate than the 2D cell model.

The kidney is the main excretory organ of the human body and is vulnerable to drugs. Renal toxicity of CHM is currently evaluated using animal models based on biomarkers and histopathologic results. Kidney organoids have overcome the shortcomings of animal models, such as the time-consuming requirements. Gu et al. [35] used human-induced PSCs to obtain renal organoids and applied this organoids to evaluate the nephrotoxicity of *Phytolacca* saponins. It was found that application of organoids reduce the time and amount of drug required.

Cardiotoxicity is an important indicator that must be considered before implementing clinical trials of newly-developed drugs [36]. Recently, various CHMs, such as aconite (Wu Tou, *Aconitum carmichaeli*) [37], *Tripterygium wilfordii* (Lei Gong Teng, *Tripterygium wilfordii Hook. f.*) [38], and oleander (Jia Zhu Tao, *Nerium*

oleander L.) [39], have been reported with the corresponding cardiotoxicities. Liu et al. [40] summarized the application and advantages of cardiac stereoscopic cell models and cardiac organoids in the evaluation of CHM cardiotoxicity. Liu et al. [40] proposed that the combination of cardiac organoids with automatic patch clamp technology and high-connotation cell image analysis technology is more effective than traditional models for CHM cardiac safety research.

Neurotoxicity is also one of the possible toxicities caused by CHM. In evaluating neurotoxicity, previous *in vitro* models mainly consisted of neurons and glial cells derived from human neural stem cells [41], but these models lack 3D structures and may not accurately summarize the key events of neural development. When compared, brain organoids mimic more structure and neural functions of the human brain [42], which can be used to evaluate neurotoxicity and solve the clinical challenge of obtaining human brain samples. Using brain organoids, Huang et al. [43] reported that cadmium exposure induces neuronal apoptosis, inhibits the proliferation of neural progenitor cells, and impairs ciliogenesis. With respect to CHM, our team is using brain organoids to evaluate the neurotoxicity of some CHM treatments for neurologic diseases.

5. MECHANISM STUDY

Organoids, as three-dimensional culture models *in vitro*, have more complicated structures and biological functions. Organoids can be used to better characterize CHM efficacy or toxicity mechanisms using a real-time quantitative polymerase chain reaction (qPCR), Western blot analysis, and imaging technologies [44]. Xu et al. [45] established human patient-derived colorectal cancer organoids, utilized flow cytometry analysis, and showed that atractylenolide I enhanced T cell cytotoxicity and improved tumor response to immunotherapy. Yan et al. [18] established intestinal organoids via qPCR and found that St. John's wort (*Hypericum perforatum*) extract and the active component, hyperforin, activate progesterone X receptor and inhibit NFκB activation for the prevention and treatment of inflammatory bowel disease. Using a combination of fluorescent probes and high-intensity imaging technique, the mechanism underlying cis-SG hepatotoxicity was shown to primarily involve mitochondrial damage [46]. When combined with high-intensity imaging, organoids predict the toxicity of CHM and explain the mechanism in a low-cost and high-throughput manner.

CHM is known for multi-component, multi-target, and diverse modes of action. It is difficult to interpret the molecular mechanism of CHM action and identify the differences in gene expression of different cells caused by CHM intervention. Organoid models facilitate the analysis of molecular mechanisms and action of CHM targets on disease treatment from a systematic perspective involving genomics, transcriptomics, proteomics, and metabolomics.

Single-cell RNA sequencing (scRNA-seq) not only maps the single-cell profiles of developing and mature organoids, but also reveals the potential mechanisms of disease by identifying the cell types expressing disease-related genes. Moreover, scRNA-seq provides higher resolution experimental data for studying the action of CHM targets, and further builds a 'component-target-pathway' spatial regulatory network [47]. There are currently Western medicine studies applying the combination of scRNA-seq and organoid technology [48], but no studies pertaining to the application of CHM have been published. Our team has recently successfully constructed brain organoids and applied scRNA-seq to study the efficacy and mechanism underlying the Chinese herbal compound, GY-1. In addition, our team has elucidated the dynamic regulation of immune cells on the repair process of myocardial infarction using scRNA-seq, and further elucidated the specific mechanism of Tanshinone IIA in the treatment of myocardial infarction [49].

In addition, other omics techniques have an important role in organoid research; proteomics and metabonomics are two such examples. Proteomics study specific proteins in organisms from an overall perspective that can quantitatively demonstrate the changes in functional pathways and biological processes with high precision and sensitivity [50]. Metabonomics provide more comprehensive phenotypic characteristics and physiologic interactions of diseases. Compared with two-dimensional cell culture, three-dimensional cultures more efficiently simulate the physiologic environment *in vivo*, thus are more suitable for combined research with metabonomics [51, 52].

6. CHALLENGES AND OPPORTUNITIES

6.1 Existing problems

Even though organoids are superior to cell and animal models, organoids also have limitations. First, unlike the internal environment, organoids have no vascular structures and no connections between tissues, and are thus not compatible with CM holistic dialectic theory. Second, there are large differences between batches when using organoids to detect the efficacy and toxicity of CHM, and it is difficult to replicate experimental results. Moreover, there are no reference standards for clinical drug use, and it is challenging to match the dose of organoid pharmaceuticals to the human body dose. Nevertheless, more and more studies have combined organoid models with other technologies to address these issues, as will be discussed below.

6.2 Future prospect

Compared with Western medicine studies, there are relatively few reports involving organoids in CHM studies, but the limited studies have great potential. First, dialectic treatment is one of the main CHM concepts

that fully reflects the characteristics of individualized treatment. CHM treatment is an ancient form of precision medicine, while organoids are the embodiment of modern precision medicine. In terms of individualized precise treatment, organoid studies are consistent with CHM concepts. In addition, CHM treatment has a wealth of clinical cases, and the combination of CHM and organoid studies is conducive to treatment of more difficult and miscellaneous diseases. Moreover, there is a large number of CHMs, including compounds, single drugs, and active ingredients that provide an abundance of choices for organoid studies.

CHM has the characteristics of holistic body regulation, therefore it is not sufficient to describe the mechanism of action from a local perspective, such as a single organ. Although there are many cell types and tissue structures in organoids, the interaction between organoids and the surrounding environment cannot be fully simulated, which differs the situation *in vivo*, thus resulting in an inability to determine the holistic mechanism underlying CHM. To better mimic the cellular microenvironment, organoids are occasionally co-cultured with different cell types or microbes. For example, Hans Clevers [53] at Nature Protocols described the microinjection approach for co-culture of intestinal organoids and microbes. These intestinal organoids were derived from ASCs and provide fully differentiated and ancestral epithelial cell types, thus co-culture with microorganisms facilitates studies involving host-microbe interactions [53]. Cattaneo et al. [54] co-cultured tumor organoids and peripheral blood lymphocytes to expand tumor-reactive T cells from peripheral blood, which was conducive to evaluating reactivity and lethality to tumor cells. In addition, some studies have shown that co-culturing specific cells in the tumor microenvironment (TME) with tumor organoids partially simulates some characteristics of the TME [55].

The lack of organoid vascularization has always been an issue. Indeed, organoid vascularization has been attempted with organoid transplantation to mice and rats [56, 57]. Another emerging method is to co-culture organoids with human umbilical vein endothelial cells, but there is a large gap between the time and space of early development *in vivo*. Recently, Salmon et al. [58] obtained a neurovascular organoid by controlling the physical interaction of endothelial cells, pericytes, and organoids spatially using microfluidic chips via three-dimensional printing and microfluidic technology. This study achieved a highly synchronized vascularization process of organoids in time and space, and provided a new approach for the study of tissue vascularization *in vitro*.

Another challenge of organoid construction is the large batch variation. Existing studies have shown that the three-dimensional bioprinting of organoids has greatly improved with a very low coefficient of size difference (1%–4%) [59]. Three-dimensional bioprinting technology enables a high-throughput organoid construction in terms of biomimetic quality and production speed. Lawlor et al. [60] demonstrated that automatic construction of renal organoids can be achieved using three-dimensional bioprinting, with morphologic component cell types and gene expression levels consistent with those of manual construction.

7. CONCLUSION

CHM has long lacked an appropriate research model due to the intricacy of CHM. Organoids are useful for investigating CHM because of the special benefits. Future studies, however, will need to address the shortcomings of current organoids in CHM research, such as the accuracy of CMH with respect to clinical dosage and the large variation of organoid batches. With improvement of organoid technologies, organoids will be better applied in studies of CHM efficacy and safety, and may gradually replace other conventional research models.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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