REVIEW

3D organoids derived from the small intestine: An emerging tool for drug transport research

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Abstract
Small intestine \textit{in vitro} models play a crucial role in drug transport research. Although conventional 2D cell culture models, such as Caco-2 monolayer, possess many advantages, they should be interpreted with caution because they have relatively poor physiologically reproducible phenotypes and functions. With the development of 3D culture technology, pluripotent stem cells (PSCs) and adult somatic stem cells (ASCs) show remarkable self-organization characteristics, which leads to the development of intestinal organoids. Based on previous studies, this paper reviews the application of intestinal 3D organoids in drug transport mediated by P-glycoprotein (P-gp), breast cancer resistance protein (BCRP) and multidrug resistance protein 2 (MRP2). The advantages and limitations of this model are also discussed. Although there are still many challenges, intestinal 3D organoid model has the potential to be an excellent tool for drug transport research.

Abbreviations: ASCs, adult somatic stem cells; BCRP, breast cancer resistance protein; BMP, bone morphogenetic protein; CDF, 5(6)-carboxy-2',7'-dichlorofluorescein; cMOAT, canalicular multispecific organic anion transporter; DDI, drug–drug interactions; EGF, epidermal growth factor; ER, efflux ratio; ESCs, embryonic stem cells; FGF, fibroblast growth factor; iPSCs, induced pluripotent stem cells; Lgr5\textsuperscript{\textdagger}, leucine-rich-repeat-containing G-protein-coupled receptor 5 positive; MCT, monocarboxylate transporter protein; MRP2, multidrug resistance protein 2; NBD, nucleotide-binding domain; OATP, organic anion transporting polypeptide; OCT, organic cation transporter; OCTN, carnitine/organic cation transporter; \( P_{\text{app}} \), apparent permeability coefficient; PEPT, peptide transporter protein; P-gp, P-glycoprotein; PMAT, plasma membrane monoamine transporter; PSCs, pluripotent stem cells; Rh123, rhodamine 123; SLC, solute carrier; TEER, transepithelial electrical resistance; TMDs, transmembrane domains.

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1. Introduction

Drug absorption, distribution and elimination are closely related to drug transport, which plays an important role in drug discovery and development\(^1\)\(^-\)\(^3\). It has been generally accepted that small intestine is the main site of drug absorption because of its more permeable epithelium and larger surface area. Therefore, the establishment of an in vitro model that can highly simulate the physiological structure and function of the small intestine will provide a reliable tool for the evaluation of drug absorption and excretion.

Up to now, various cell models have been used to imitate the intestinal epithelium for drug transport study in vitro. In particular, the Caco-2 cell monolayer model has been widely used as a standard tool for high-throughput screening of drug permeability and identification of substrates and/or inhibitors of drug transporters due to its advantageous differentiation similar to the intestinal enterocytes\(^3\)\(^-\)\(^4\). However, it should be noted that the immortalized cell monolayer could not recapitulate the tissue architecture of the intestinal mucus layer realistically. In addition, the interaction between different types of cells of intestine (including the enterocytes, Paneth cell, enteroendocrine cell, and goblet cell, etc.) does not exist. Thus, Caco-2 monolayer model has its own limitations in drug transport research.

With the development of 3D cell culture technology, 3D organoids are emerging as a burgeoning in vitro culture model which can be used in disease model construction\(^4\)\(^-\)\(^7\), human organ development\(^8\), drug screening and toxicity testing\(^8\)\(^,\)\(^10\), and personalized medicine research\(^11\). Organoids are 3D self-organizing structure derived from pluripotent stem cells (PSCs) or adult somatic stem cells (ASCs), which can recapitulate the structure and function of organs or tissues in vivo\(^5\)\(^,\)\(^11\). Different from traditional 2D cell models, organoid is composed of various cell types with regenerative ability, which perform their own functions and are arranged into three-dimensional tissues. Therefore, the main characteristics of organoid are self-organized, multicellular and functional\(^13\)

Since the first intestinal organoid model was cultured from ASCs of mouse small intestine in 2009\(^14\), more and more organoid culturing systems have been adapted to generate different kinds of mouse and human organoids. As the first generation of organoid, small intestinal 3D organoid may be the most representative example of organoid characteristics. The origins of intestinal organoids are the purified intestinal crypts or single leucine-rich-repeat-containing G-protein coupled receptor 5 positive (Lgr5\(^+\)) stem cells, which have the capability of long-term growth and expansion of intestinal epithelia cells in Matrigel with a series of essential components of intestinal stem cell niche, including R-spondin-1, epidermal growth factor (EGF) and bone morphogenetic protein (BMP) inhibitor Noggin. Intestinal organoids develop crypt-villus structures with stratified epithelium, which consist of all the major different types of cell lineages in the gut, including columnar epithelial enterocytes with a brush border of apical microvilli (Fig. 1). These organoids show the biological functions of the gut, including absorption and secretion activities\(^14\)\(^,\)\(^15\).

As one of the determinants of pharmacokinetics, drug transporters, more specifically, membrane transporters have a great impact on the safety and efficacy of drugs\(^16\). After oral administration, the drug must pass through the intestinal mucosa to the portal vein. And the membrane transporters expressed in the small intestine play a decisive role in drug absorption. Until now, there are few reports on drug transport mediated by membrane transporters with usage of 3D organoids. In recent years, we have studied the expression and function of ATP-binding-cassette transporters, containing P-glycoprotein (P-gp), breast cancer resistance protein (BCRP) and multidiuregulation associated protein 2 (MRP2) in 3D organoids for drug efflux transport research.

In this paper, we reviewed the methods of cultivating mouse and human small intestinal 3D organoids in ASCs and PSCs, and summarized the application of intestinal 3D organoids in transporter-mediated drug transport based on previous research.

2. Intestinal 3D organoids culture

Intestinal organoid is stem cell-derived and self-organizing 3D culture model, which simulates the phenotypic structure, cell composition and partial function of the small intestine\(^7\). A special media composition can recapitulate the stem cell niche signaling pathway in vivo, which is essential for the growth and development of intestinal organoid. The media composition has the ability to maintain the function of stem cells and drive their expansion, and eventually their differentiation\(^17\)\(^,\)\(^18\). Organoids can be initiated from two main types of stem cells: (1) PSCs, including embryonic stem cells (ESCs) and the synthetic induced pluripotent stem cells (iPSCs), which are responsible for organ embryonic development; and (2) ASCs, organ-restricted stem cells, which are crucial for mature organ regeneration and homeostasis\(^12\)\(^,\)\(^17\). For ESCs and iPSCs, the infinite expansion potential is a necessary prerequisite for their discovery and development. On the contrary, once cultured in the proper matrix and the right growth factors, ASCs have shown the ability to grow into organoids, even though they were not thought to proliferate in vitro.

2.1. Intestinal organoids derived from pluripotent stem cells

Since the establishment of PSC cell lines, biologists have established several types of differentiated cells with the support of developmental biology\(^2\)\(^,\)\(^19\)\(^,\)\(^20\). So far, it has been reported that human intestinal organoids come from ESC and iPSC, while mouse intestinal organoids are generated from ESC\(^1\). WNT and fibroblast growth factor (FGF) signals are known to promote the transformation of endoderm into the middle/hind gut. By adding WNT3A and FGF4, human PSCs treated with activin were cultured into 3D middle/hind gut spheroids from the 2D monolayer epithelium\(^21\)\(^-\)\(^25\). These 3D spheroids were further cultured in Matrigel along with R-spondin-1, EGF and noggin, and eventually differentiated into intestinal organoids. After 1–3 months of
expansion, the organoids form a polarized intestinal epithelium with villus-like structures and crypt-like regions, containing all known intestinal cell types\textsuperscript{24}.

2.2. Intestinal organoids derived from adult stem cells

Different from the organoids derived from PSCs, ASCs can be induced to form 3D organoids by providing suitable conditions to simulate the niche environment of intestinal stem cells in the process of tissue self-renewal. In 2009, a culture system was established, which allowed the long-term growth and expansion of villus-like epithelial domain from a single sorted Lgr5\textsuperscript{+} stem cell or purified intestinal crypts\textsuperscript{14,24,25}. The single Lgr5\textsuperscript{+} stem cell or whole crypts were embedded in laminin-rich Matrigel and cultured in serum-free medium, including R-spondin-1, EGF and BMP inhibitor Noggin\textsuperscript{14,27}. Within a few days, cystic single cell epithelial structures and central lumen were formed, known as “crypt-like budding”. Then the single crypt-like structure changed into multiple discrete budding crypts, in which all types of cells were distributed at normal numbers\textsuperscript{14,27}. The 3D organoids showed simple high polarization, the enterocyte brush border of intestinal epithelial cells formed the luminal surface, and the basolateral side faced outward\textsuperscript{28}. The organoids are remarkably stable, and can grow every 5–7 days continuously for years, without genetic and phenotypic changes\textsuperscript{29}.

The growth and differentiation of crypt stem cells are mainly regulated by four signaling pathways\textsuperscript{12,30}. First of all, WNT is the key pathway to promote the proliferation and maintain the survival of stem cells. Secondly, the NOTCH pathway helps to maintain the undifferentiated state of proliferating cells. Thirdly, the EGF signals play an important role in promoting the mitosis of stem cells. Finally, the BMP signaling pathway takes active part in the process of 3D organoids development. Therefore, inhibiting BMP signal is a key measure to create a suitable environment for crypt differentiation.

3. Application of intestinal 3D organoids in drug transport research

In recent years, the cultivation technology of 3D organoids is becoming more and more mature and has been widely used in various fields. However, although 3D organoids are a good model, there are few studies on their applications in drug metabolism and pharmacokinetics. In the past few years, we have studied and developed this model, especially in the field of drug delivery. In the next section, we will summarize the applications of 3D organoids in transporter-mediated drug transport research in combination with the published studies and knowledge of this model (Fig. 2).

3.1. P-gp mediated drug transport in intestinal 3D organoids

P-gp, an ATP-dependent membrane transport protein, is a 170-kDa single polypeptide with 1280 residues. It consists of two homologous half parts, each containing one nucleotide-binding domain (NBD) and six transmembrane domains (TMDs)\textsuperscript{31}. P-gp is present in various tissues and organs, extremely in the small intestine, colon, liver, kidney and brain–blood barrier, which is involved in the pharmacokinetics of drugs\textsuperscript{32}. It acts as an efflux pump to affect the absorption of endogenous and exogenous substances, and to protect the body from toxins and xenobiotics\textsuperscript{33}. However, the efflux effect can also inhibit drug absorption, promote drug clearance and reduce the bioavailability of oral drugs. In addition, overexpression of P-gp is associated with many diseases, such as Alzheimers disease\textsuperscript{34}, HIV\textsuperscript{35}, inflammatory bowel disease\textsuperscript{36} and various cancers.

P-gp expressed in intestinal 3D organoids has been detected at both mRNA and protein levels. At the mRNA level, the expression of P-gp in mouse and human intestinal organoids was similar to that in normal intestinal tissues and isolated intestinal crypts\textsuperscript{21,28}. At the protein level, immunohistochemical results showed that P-gp maintained physiological expression and located in the apical...
side of mouse and human 3D intestinal organoids, which was consistent with that in small intestine tissue.

The intestinal 3D organoids have been used in the study of drug efflux transport mediated by P-gp. Rhodamine 123 (Rh123), as a typical autofluorescence substrate of P-gp, has been widely used in the detection of drug efflux transport mediated by P-gp. Rh123 can be conducted active trans-epithelial transport by 3D organoids in the basolateral to apical direction after supplement in the medium\(^3\). In addition, P-gp inhibitors, including verapamil, quinidine and mitotane, were utilized to assess the inhibition of the Rh123 transporting across 3D organoids\(^3\),\(^7\). Apart from this, the intestinal 3D organoids were also used as a model to evaluate the inhibitory effect of cucurbitacin E on P-gp, which was consistent with the results of Caco-2 monolayer model\(^4\).

### 3.2. BCRP mediated drug transport in intestinal 3D organoids

BCRP is a 72-kDa membrane transport protein with 655 amino acids, belonging to ABCG gene family. As an efflux transporter, BCRP is highly expressed in apical epithelium of small intestine and colon, which can restrict the absorption of its substrates\(^5\). Recent studies have shown the importance of BCRP in diabetes\(^6\), rheumatoid arthritis\(^7\), and various cancers\(^8\).

Both mRNA and protein expression levels of BCRP in intestinal 3D organoids have been investigated\(^9\). Results in our previous study showed that the mRNA levels of BCRP in crypts and villus were different among proximal, middle, and distal small intestine, while no significant difference was found in the organoids cultured from three parts of small intestinal crypts. Therefore, in the study of BCRP-mediated drug transport via culturing organoids, it is advisable to select the part from the proximal to the middle of the small intestine. In addition, the mRNA expression of BCRP was not affected by the culture time. Hoechst 33342, the fluorescence probe substrate of BCRP, has been used in the study of drug transport in intestinal 3D organs. BCRP inhibitors Ko143 and YHO-13177 significantly reduced the fluorescence intensity of Hoechst 33342 in the 3D organoids\(^10\),\(^47\), which indicated that 3D organoids were a sensitive tool for screening BCRP specific inhibitors.

### 3.3. MRP2-mediated drug transport in intestinal 3D organoids

As an important efflux transporter involved in drug–drug interactions (DDI), MRP2 has attracted more and more attention. The transmembrane protein MRP2 contains 1545 amino acids, also known as canalicular multispecific organic anion transporter (cMOAT). MRP2 is expressed in the apical membrane of multiple normal tissues, such as small intestine, liver, kidney, brain and placenta. A large number of studies have shown the relationship between MRP2 and gastric cancer\(^10\), colon cancer\(^9\), breast cancer\(^10\), lung cancer\(^1\) and other tumors\(^5\).

MRP2 was also expressed in mouse intestinal 3D organoids\(^5\), which was located on the inner surface of organoids, the same as P-gp and BCRP. In addition, there were no significant differences in mRNA levels between the proximal, middle and distal parts of the small intestine. MK-571 and probenecid were selected as inhibitors, and 5(6)-carboxy-2',7'-dichlorofluorescein (CDF) as the substrate of MRP2 for drug efflux transport study. MK-571 and probenecid significantly decreased the accumulation of CDF in 3D organoids. Results showed that 3D intestinal organoid model was a feasible model to evaluate MRP2-mediated drug transport\(^5\).

### 4. Discussion

Recently, advanced 3D culture technology has made PSCs and ASCs exhibit their remarkable self-organizing properties\(^12\). Cultured in specific developmental medium, the brain, inner ear, intestine, kidney, liver, lung, pancreas, retina, stomach, thyroid organoids have been derived from the PSCs isolated from mouse and human tissues. PSCs have been cultured in vitro for a long time (murine in 1981 and human in 1998), while ASCs are initially thought to have limited proliferation in vitro\(^5\),\(^5\). Since the initial 3D organoids are generated from mouse intestinal isolated ASCs\(^4\), there has been increasing organoid culturing systems from a broad range of mouse and human organs, including breast, colon, esophagus, intestine, lingual epithelium, liver, lung, ovary, pancreas, prostate, salivary gland and stomach. And most of these organoids have been used in the construction of disease and tumor models, human organ regeneration, host–pathogen
### Table 1  The murine and human organoids derived from PSCs (including ESCs and iPSCs) and ASCs, and their application in different research and clinical aspects.

| Tissue          | Species source | Stem cell type | Application                              | Ref.       |
|-----------------|----------------|----------------|------------------------------------------|------------|
| Brain           | Human          | ESCs/iPSCs     | Development model                        | 7,56–61    |
|                 | Mouse          | ESCs           | Disease model                            |            |
|                 |                |                | Tumor model                              |            |
|                 |                |                | Host-pathogen interaction                |            |
| Breast          | Human          | ASCs           | Tumor model                              | 62         |
|                 | Human/mouse    | ASCs           | Disease model                            | 63–69      |
|                 |                |                | Tumor model                              |            |
|                 |                |                | Host-pathogen interaction                |            |
| Colon           | Human/mouse    | ASCs           | Tumor model                              | 66,70      |
|                 | Human/mouse    | ASCs           | Development model                        |            |
| Esophagus       | Human/mouse    | ASCs           | Disease model                            | 6,9,14,37–41,47, |
|                 | Human/mouse    | ESCs/iPSCs     | Tumor model                              | 53,71–77   |
|                 | Human          | ESCs/iPSCs     | Gene correction                          |            |
|                 |                |                | Drug transporters model                   |            |
|                 |                |                | Host-pathogen interaction                |            |
| Intestine       | Human          | ESCs/iPSCs     | Disease model                            | 78,79      |
|                 |                |                | Tumor model                              |            |
|                 |                |                | Kidney regeneration                      |            |
| Kidney          | Mouse          | ASCs           | Tumor model                              | 80–82      |
| Lingual         | Human/Mouse    | ASCs/iPSC      | Disease model                            | 8,83–90    |
|                 | Human          | ASCs/iPSC      | Tumor model                              |            |
|                 |                |                | Liver regeneration                       |            |
|                 |                |                | Drug metabolism model                    |            |
| Liver           | Human          | ASCs           | Development model                        | 88,91–95   |
|                 | Human          | ESCs/iPSCs     | Disease model                            |            |
|                 |                |                | Tumor model                              |            |
|                 |                |                | Host-pathogen interaction                |            |
| Lung            | Human          | ASCs           | Tumor model                              | 96         |
| Ovary           | Human          | ASCs           | Tumor model                              |            |

*(continued on next page)*
interaction, gene correction and drug testing. Various murine and human organoids and their application in different research and clinical aspects are summarized in Table 1.

Drug transport is important in the process of drug discovery and development for assessing the DDI. The most relevant organ responsible for drug transport is the small intestine. Therefore, the establishment of a reliable and physiological model of intestinal mucosa in vitro is the key to evaluate drug absorption and excretion. Intestinal 3D organoids have been used in gene research and therapy, disease modeling, intestinal organ transplantation, and intestinal tissue development. Although a relatively perfect culture system has been established, until now, there are few studies on the evaluation of drug transport and metabolism by using 3D organoids. Based on previous studies, this paper reviews the application of intestinal 3D organoids in drug efflux transport mediated by P-gp, BCRP and MRP2.

As we all know, Caco-2 monolayer model has been widely recommended by regulatory authorities and pharmaceutical companies as a standard screening method for drug transport evaluation, mainly because it is similar to the innate differentiation of intestinal epithelial cells. Caco-2 cells (from human colon cancer) were cultured in DMEM medium for 21 days to form a well polarized monolayer connected by tight junctions (Fig. 1C). However, it should be noted that the Caco-2 monolayer model is composed of a single cell type, which cannot really recapitulate the tissue architecture of the intestinal mucosa. Under normal physiological conditions, there are five main cell types in the intestinal epithelial layer, including enterocytes, goblet cells, enteroendocrine cells, Paneth cells and stem cells. Each of these cell lines plays an important role in cytoprotection of the intestinal mucosa. Enterocytes, also known as absorptive cells, are responsible for most of the absorption in the small intestine. Goblet cells can release trefoil peptides and mucins, which are the key components of the protective barrier to protect mucosal surface, and also participate in the absorption. Mature enteroendocrine cells secrete chromaffin granules and cholecystokinin, which are thought to be involved in the regulation of absorption and metabolism. Paneth cells can produce lysozymes and crypt defensins, involving in mucosal barrier. In addition, Paneth cells secrete growth factors and provide a niche for stem cells. Stem cells are responsible for producing these four cell types described above. The 3D organoids are derived from crypts or single stem cells, and have all five different cell types. Therefore, compared with Caco-2 monolayer model, the 3D organoid model is closer to intestinal epithelium in physiology. In addition, it has been reported that the tight junction of Caco-2 cells is more stringent than that of human intestinal epithelial cells, resulting in a decrease of paracellular permeability. The same time, the expression of drug transporters, such as P-gp, in Caco-2 cells is higher than that in primary intestinal epithelial cells, which will enhance the transport activity during drug absorption. Therefore, Caco-2 monolayer model has its own limitations in drug transport research. The key features, advantages and limitations between Caco-2 cell monolayer model and 3D organoid model are summarized in Table 2.

### Table 1 (continued)

| Tissue    | Species source | Stem cell type | Application                  | Ref.     |
|-----------|----------------|----------------|------------------------------|----------|
| Pancreas  | Human/mouse    | ASCs iPSCs     | Disease model                | 97–101   |
| Prostate  | Mouse          | ESCs           | Retinal regeneration         | 105–107  |
| Retina    | Human/mouse    | ASCs iPSCs     | Disease model                | 22,108–111 |
| Stomach   | Mouse          | ESCs           | Host-pathogen interaction    | 107–109  |
| Thyroid   | Human          | ASCs           | Thyroid regeneration         | 112      |

ESCs, embryonic stem cells; iPSCs, induced pluripotent stem cells; ASCs, adult stem cells.

Yuanjin Zhang et al.
Table 2: The comparison between Caco-2 cell monolayer model and 3D organoid model.

| Comparison item | Caco-2 cell monolayer model | 3D organoid model |
|-----------------|-----------------------------|-------------------|
| Key features    |                             |                   |
| Origin          | Derived from human colon cancer | Derived from PSCs or ASCs |
| Stemness and multipotency | No | Yes |
| In vivo-like complexity | No | Yes |
| Advantages      |                             |                   |
|                  | > Mature evaluation system | > Various cell types with different functions |
|                  | > Recommended as a standard screening method for permeability evaluation | > Closer to intestinal epithelium in physiology |
|                  | > Low cost | > Multiple applications |
|                  | > Higher expression of drug transporters | > Unsound evaluation system for drug transport study |
|                  | > Time consuming | > High cost |
| Limitations     |                             |                   |
|                  | > Single cell type | > Multiple applications |
|                  | > More stringent tight junction than small intestine epithelial | > Multiple applications |
|                  | > Higher expression of drug transporters | > High cost |

Of course, 3D organoids are derived from normal small intestine cells, and their physiological structure and function are closer to the real situation. However, there are still many aspects of 3D organoids that need to be improved before being accepted by regulatory agencies and pharmaceutical companies for transporter-mediated drug transport evaluation. On the one hand, there is no standard evaluation system. For Caco-2 monolayer model, the robustness criteria of monolayer integrity and drug permeability have been widely recognized. In general, the trans-epithelial electrical resistance (TEER) values > 300 Ω/cm² is usually used as a standard to evaluate the integrity of the monolayers. Apart from this, the apparent permeability coefficient ($P_{app}$) value is used as a reference for permeability evaluation, and the efflux ratio (ER) value can be used as an indicator for assessing P-gp substrate or inhibitor. On the other hand, the choice of transporter probe substrates is limited. According to current reports, only specific fluorescent substrates have been used in drug transport studies. In the future, the HPLC–MS instrument is expected to be applied for the detection of other common substrates. Therefore, further research is needed to support the improvement of the model.

In intestinal epithelium, the solute carrier (SLC) is another important classification of drug transporter, which is involved in drug absorption and transport study. The SLC transporter family mainly includes organic anion transporting polypeptide (OATP), organic cation transporter (OCT), carnitine/organic cation transporter (OCTN), peptide transporter protein (PEPT), monocarboxylate transporter protein (MCT), and plasma membrane monoamine transporter (PMAT). Although the expression of different SLCs in human iPSCs derived organoids is explored, the research on drug transport mediated by SLCs still faces great challenges. Due to the valgus structure, SLCs are expressed in the lumen side of intestinal organoids, which makes it hard to directly monitor the transport process. Besides the intestinal tract, transporters expressed in other organs, especially in the liver, are also of great significance. Liver is the main organ of drug metabolism, and intrahepatic transporter-mediated drug evaluation is a hot spot in pharmaceutical research. The 3D organoids derived from the liver have been used in the development of organoid-based drug metabolism model and drug-induced liver fibrosis test. Further studies are needed to identify the expression and function of ABC and SLC transporters in liver.

5. Conclusions

The intestinal organoids, however, do not come without shortcomings, that is, they lack the natural microenvironment composed of innervation, blood vessels and immune cells. Despite of these limitations, the successful development of intestinal organoid culture provides useful knowledge for the study of intestinal epithelial cells and has become a physiological related drug transport model in vitro. In this review, we have summarized the application of 3D organoids derived from small intestine in the study of drug efflux transport mediated by P-gp, BCRP and MRP2. The advantages and limitations of the model are also discussed. Although there are still many challenges, intestinal 3D organoid model is becoming a powerful tool for drug transport evaluation. The application of this organ derived model system is expected to narrow the gap between traditional “cell” and “animal” in drug transport evaluation.

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Author contributions

Xin Wang was responsible for the conception and design of the review. Yuanjin Zhang, Shengbo Huang, Wenxia Chen and Bingyi Yao collected literatures. Yuanjin Zhang, Shengbo Huang, Weiguo Zhong and Xin Wang analyzed literatures and summarized results. Yuanjin Zhang and Shengbo Huang drafted the manuscript. Xin Wang and Weiguo Zhong revised the manuscript.
Conflicts of interest

The authors declare no conflicts of interest.

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