The Toxicity Evaluation of Novel Carbon-Based Nanoparticle Medicine and How to Choose the Suitable Animal Toxicity Test Models for It

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ABSTRACT
Carbon nanoparticles have massive potential in different biology and medical applications areas. In order to use the novel carbon nanomaterials medicine for human treatment, safety issues are necessary to be considered, and it is essential to determine the best animal test models to evaluate the toxicity of carbon-based nanomaterials. In order to find out the most suitable method to evaluate the potential toxicity of therapeutic carbon-based nanoparticles, this author conducted several different research papers and concluded that even for the same carbon-based nanomaterials, in different animal testing models, different animal growth stages or different test technique, there might show different effect result and toxicity. Therefore, researchers who work on carbon nanoparticles should scientifically consider the effects of those novel materials' toxicity. Moreover, it is necessary to choose the best models to investigate the potential toxicity of the new therapeutic carbon nanomaterials. The profiles should be carefully considered when developing carbon nanoparticle medicine for human use and conducting comparative studies.

Keywords: *Carbon nanomaterials, Nanotoxicology evaluation, Animal toxicity test models, Medicine development, Animal science*

1. INTRODUCTION

The applications of carbon-based nanoparticles have become an essential research field recently. Carbon nanoparticles have been widely used in different medical areas, such as disease diagnosis, vaccine technology, and biomedical imaging, especially pharmaceutical design. Since their unique optical properties, high biocompatibility, and easily derivatized features, carbon nanoparticle medicines have enormous potential for reducing toxicity, precision delivery improvement, and pharmacodynamics compared with traditional small-molecule drugs [1]. For instance, in the current fight against coronavirus disease 2019 (COVID-19) research, carbon nanoparticles have accelerated the research of the COVID-19 newest vaccines and home test kits [2]. However, for a carbon nanoparticle with clinical medicine potential, before its large-scale application, toxicity tests are virtual experiments to be executed to ensure patient safety [3]. The chemical manufacturing-controlled definition and pharmacodynamic assessment of carbon nanoparticle drugs are limited in toxicity testing for small molecule medicines. Another controversial concept is that many carbon nanoparticle medicines are food grade and are synthesized in a way that resembles food design processing [4]. In addition to these, many previous studies only used inconsistent safety assessment methods, which is hard to compare the toxicities of various carbon nanoparticle medicine [1]. In order to overcome those shortcomings in previous studies, it is essential to choose the most suitable toxicity assessments for specific novel carbon nanoparticle medicines by understanding the toxicity in various animal models, different animals growth stages, and different tests technique. Therefore, different recent papers were reviewed from these three directions. Zebrafish were used as a model to evaluate the toxicity in animals' different growth stages investigation, and different animals' models, for instance, zebrafish, rodents, and chickens were also used to compare the toxicity differences. I will focus on three different kinds of tests for the test technique: inhalation toxicity, irritating skin test, and microbiota analyses. Then their findings were combined to conclude and clarify the potential biological hazards of carbon nanoparticle medicines, which are

helpful for future safety evaluation criteria for such materials.

2. ANIMAL TOXICITY TEST MODELS IN CARBON NANOPARTICLE MEDICINES POTENTIAL TOXICITY EVALUATION

2.1. Different Stage

Recently, many studies have found that the same carbon nanoparticle medicines might lead to various toxic effects on different experimental animals' growth stages. According to the article, zebrafish models have been conducted to investigate the toxicity of carbon nanoparticles in different growth stages. Researchers have begun to use zebrafish as animal toxicity test models since they have three different growth stages: embryos, eleutheroembryos (a free-floating embryo of a fish), and adults. Use Lin's research as an example; they adopted zebrafish in the different growth stages as models to test the potential toxicity of the newest carbon nanoparticles, lysine-carbonized nanogels (Lys-CNGs), which can be used as a basis for drug design as its antiviral capability and broad-spectrum antibacterial effect. They conducted acute toxicity tests on zebrafish embryos, eleutheroembryos, and adults to find out the toxicity of Lys-CNGs in different growth stages. For the embryos and eleutheroembryos, 20 samples of each stage were randomly assigned wells, in which different concentrations of Lys-CNGs were added. Ten adult zebrafish were divided into two groups according to the food provided: the commercial food and food with Lys-CNGs. During the experiments, the death samples were selected to calculate the mortality. Based on the result, they found out that when the Lys-CNGs concentration is higher than 100 ppm, the mortality of the zebrafish embryos will significantly increase. However, in the zebrafish eleutheroembryo stage, if Lys-CNGs concentration exceeded ten ppm, the mortality of the zebrafish embryos will significantly increase, in which the concentration was lower compared with the embryos stage. There was no death of any adult zebrafish samples, but these carbon nanoparticles significant change the weight of the fish. For instance, the weight of those fish fed with commercial food only increased by 12.1%, while those fed with Lys-CNGs feeder increased by 38.6%. In other research that Chung conducted, they also used zebrafish with their different growth stage to investigate novel carbon nanoparticles, carbon quantum dots (CQDs), which also have a wide variety of applications and high biocompatibility [5]. Through acute toxicity test, long-term weight monitoring and evaluation of the fertility and egg hatch rate, they also found out that zebrafish eleutheroembryo were more sensitive to these carbon nanoparticles than the embryo, which is similar to the first example. In the acute toxicity test, the LD50 of eleutheroembryo was significantly lower than the embryo. What was different from the first experiments, they investigated the zebrafish offspring's hatchability and deformity. They found out that there was no significant difference regardless of the food fed type.

In conclusion, those results indicated that even for the same materials and animal models, they still could cause different effects for different growth stages. Therefore, when we apply the novel materials to a new medicine design, we need to consider patients' age distribution and avoid drug toxicity. Meanwhile, it is also essential to consider the effects of drugs on pregnant women and their offspring.

2.2. Different Animal Models

Many different animal models were also applied in carbon nanoparticle medicines toxicity tests to meet different requirements. As the 2.1 Different stage section showed, zebrafish have been widely applied in the biological and pharmacological research area, for instance, in gene recombination studies and assessing the oxidative toxic effects of nanoparticles. The Economic Cooperation and Development (OECD) suggested using zebrafish for acute toxicity testing since this kind of fish shares more than 10,000 genes with humans, representing approximately 70% of the human genome. Meanwhile, human drugs always elicit similar physiological responses in zebrafish, making them an ideal model for toxicity assessments [5]. Zebrafish can reproduce large numbers of offspring, and their embryo could affect development very fast with a short generation time; therefore, low price is another advantage compared with other animal models. Apart from acute toxicity testing, since zebrafish have a readily observable digestive system, bioaccumulation of carbon quantum dots (CQDs) in the zebrafish body could also be tracked easily. For instance, Kang observed the distribution, metabolism, absorption and excretion of carbon nanoparticle medicines in zebrafish embryos by using fluorescence techniques and found that carbon quantum dots do not accumulate in zebrafish embryos and do not interfere with their development [6]. However, for some biological research experiments, some of the specific characteristics of the zebrafish were problematic. The most distinct disadvantage of the zebrafish model, especially for the human-related problem, is that zebrafish are not mammal species. This fact indicated that some carbon nanoparticles medicines might be metabolized differently from mammals, or at least at a different rate when compared with mammal species, which may alter their function [7]. The gender of the zebrafish is also determined differently from mammals since it does not appear to be genetically controlled [8].

Three rodent species are usually conducted in toxicity test models: rat, mouse, and hamster, in which the mouse and the rat have been widely applied in biology or pharmacology experiments. Although more expensive than zebrafish, the choice of rodent species is still based

on economics and practicality. They have a small body size, short life span, short gestation period, and similar metabolism processes to human beings, making them an ideal laboratory animal [9]. For instance, in Strojny's research, they use Wistar rats as an animal model to test the toxicity of graphite nanoparticles and graphene oxide, novel carbon nanoparticles have been found to have the potential for biomedical applications [10]. They measured rats' blood morphology and their inflammatory state of the liver and observed that aggregates of carbon nanoparticles could be found around the injection site. However, none of the carbon nanoparticles influenced the rats' health. When compared with mice, although the size of rats is bigger than mice, they are easier to handle and show less stress than mice. In another experiment, to find out carbon nanoparticles' oxidative stress and DNA damage response, Wessels used rats and mice simultaneously. Through mRNA expression analyses and bronchoalveolar lavage, they found no toxicity nor pulmonary inflammation in both mice and rats [11]. They combined two different rodent test models; for example, mice's whole lungs were used to measure the oxidative DNA damage by fpg-modified comet assay, while they chose to examine the lung epithelial cells extracted from exposed rats. However, the rat is not usually the model of choice for diseases related to tumours, blood and immune function, so more giant mammals were also used as animal toxicity test models. Still use Lin's research as an example; other than zebrafish and rats, they used rabbits to conduct the skin irritation test and the skin sensitivity test of Lys-CNGs by using guinea pigs [1]. Their result indicated that the rabbit had no obvious discomfort during the experiment, with nobody changing weights. No irritating physiological reaction could be found in rabbit models after being treated with Lys-CNGs. Like the rabbit, the skin sensitization test in guinea pigs also had no adverse skin reactions with no weight change, indicating that this kind of novel nanoparticles did not harm the mammals' skin. More giant mammals have more complex systems when compared with rodent species, but it is necessary to consider the budget and the ethical problems.

It is interesting to note that some scientists are now using chickens as animal toxicity test models. The chicken embryo is particular in animal models when used for toxicity experiments. It has also made significant contributions to cancer research, gene recombination, virology and immunology. When testing the carbon nanoparticles, Chicken embryos have several advantages as animal toxicity test models. They have excellent characteristics such as rapid growth, easy accessibility, and are suitable for experimental observation. The early stages of chicken embryonic development provide a good model for investigating the potentially toxic effects, oxidative stress, and body weight. For instance, Sawosz used the chicken-embryo model to test the toxicity of six different types of carbon nanoparticles [12]. This study treated fertilized chicken eggs with CNPs, such as placebo, pristine graphene and graphene oxide. Their results indicated that CNPs could maintain blood circulation and did not have any significant side effects, which suggested that CNPs have the potential applicability as vehicles for drug delivery. However, chickens are also not a mammal species, so there are still many problems that need to be overcome when using the chicken models, which are similar to zebrafish models.

2.3. Different Tests

With the more and more different kinds of animals being used in toxicity assessment, different tests models must be considered when designing the experiments. Through a summary of the other literature, this author found several basic tests which usually be conducted in animal toxicity test models, which are inhalation toxicity test, skin-irritating test and microbiota test. Acute inhalation toxicity is the sum of the adverse effects caused by the experimental materials after a short period of uninterrupted exposure to them by inhalation. The skin irritation test is an in vitro animal test to identify chemicals or mixtures that may cause skin responses. Furthermore, the microbiota tests measure the microorganisms in animals' gastrointestinal tract and can give the animal model an idea of the types and amounts of microorganisms in their gut after using carbon-based nanomaterials medicine.

Yokoyama used pulmonary reducing ability mice as animal models for the inhalation toxicity and exposed them to NiO or C60 nanoparticles for two weeks [13]. An electron paramagnetic resonance (EPR)study was conducted to determine how those carbon nanoparticles will influence the biological systems. The result indicated that NiO nanoparticles could significantly reduce pulmonary after two weeks while C60 nanoparticles cannot. Compared with the methods commonly used in the previous research, intratracheal instillation exposure, inhalation toxicity has its advantage; for instance, inhalation toxicity could result in a more even distribution throughout the lung lobes. However, there are still various problems that need to be overcome while using this technique to investigate potential toxicity. The shortcomings of this method related to inhalation, such as volume of material administered, dose, vehicles, and anaesthetic agent [14].

Different methods could be conducted to process the skin-irritating; Lin's research is an example, and they use two different skin-irritating to investigate the toxicity of Lys-CNGs [1]. First is the skin irritation test on rabbits; they had three white rabbits' backs' fur shaved, from the middle back to the shoulder, to expose the skin. They soaked the sterile gauze in the Lys-CNGs solution and then put it on the rabbits' backs for four hours. The skin reactions score was recorded for 1-, 24-, 28-, and 72-hours timepoint to evaluate the symptoms; for instance,

zero indicated there was no oedema, one indicated very slight oedema, two indicated well-defined oedema, until score four, which indicated the beef redness eschar formation, with the number increases, the more the skin reacts. For another skin sensitivity test, they experimented with the guinea pig. Fifteen female samples had their back shaved and divided into two groups. They were injected with the Lys-CNGs solution, or PBS solution was mixed with FCA separately. After seven and fourteen days, a gauze patch containing the Lys-CNGs solution was placed on the injection site for 48 hours. The skin response to this test was recorded after one day and two days after the gauze patches were removed. A score was also applied to evaluate the skin response, similar to the skin irritation in rabbits, by combining two different skin tests. Those authors found that their skin had no physiological reaction when exposed to Lys-CNGs or saline solution. Moreover, similar to the skin sensitization test in guinea pigs, there was no skin adverse reactions and weight change, indicating that high doses of Lys-CNGs did not damage the skin of mammal species. The essential issue that needs to be overcome by the researcher when conducting skin irritating or sensitive tests is the ethical problem, for instance, how to alleviate the suffering of experimental animals.

Microbiota analyses were usually used to determine the composition and function of a community of microorganisms in a particular location. Still, use Lin's study as an example; they euthanized the sample zebrafish, fed by Lys-CNGs and took their intestinal tissues through a sterile instrument [1]. All the tissue samples were stored at -80 degrees centigrade, and DNA was extracted for intestinal microbiota analysis, then analyzed by the Biotechnology Company. The results showed that long-term feeding of low concentrations of this kind of novel carbon nanoparticle did not influence the dominant species in the intestinal flora of adult zebrafish. Like skin irritation or sensitive test, it is also necessary to solve ethical problems.

3. CONCLUSION

According to this review, the toxicity assessment of different types of carbon nanoparticles exists as an objective comparative benchmark to comprehensively understand the safety of different novel carbon nanoparticle medicines.

Carbon nanoparticles have great potential for pharmacy applications, particularly in infectious diseases and tumours treatments; however, only a few studies on carbon nanoparticles' safety and toxicity. In order to investigate the safety in human beings' therapeutic applications, safety investigation and the establishment of appropriate evaluation criteria are necessary to be conducted before being widely used. In conclusion, the same carbon nanoparticle might show different toxicity effects for various animal models, animals' growth stages, or different investigation techniques. For instance, Lys-CNGs exhibit substantial toxicity in zebrafish's embryonic and eleutheroembryo stages, while no adverse effects were observed in adult mammalian models when Lys-CNGs were administered externally or orally. On the other hand, the zebrafish model provides a low-cost and rapid analysis model for testing the potential toxicity of carbon nanoparticles. However, as their different structure from mammal animals, for example, the highly toxic effect could be found in zebrafish embryos. However, there were no adverse reactions in larger mammals, indicating that further studies are needed to be conducted to investigate the reason caused those embryos died and if it will happen in mammals or human beings.

Furthermore, to meet the experiment's requirements and ethical considerations, the kind of animal model and the test animal's age we choose to test certain materials are essential. Sometimes, combining different methods is also necessary when we design experiments. Last but not least, whether those phenomena would be applied to all the other carbon nanoparticle medicines requires further investigation. Therefore, this author encourages the strategies mentioned above to be used in the studies to test the biocompatibility and toxicity of new carbon nanoparticles medicine, particularly following international standards for assessment methods.

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