Experimental Nanobiotechnology

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Lecture 21: In vivo Toxicity Studies Using Zebrafish Embryo

Hello everyone, today we are going to learn in vivo toxicity studies using zebrafish embryo. In today's lecture we are going to learn various experimental models and advantages of zebrafish as an animal model. In today's lecture we are also going to learn how we can use zebrafish for understanding the toxicity of nanomaterials both theoretically as well as practically through demonstration. Let us see some of the current in vivo and in vitro models in human research.

The in vitro model system we already learnt in the some of the previous lectures. So we can make organoids, we can have spheroids and tumoroids for understanding the toxicity of materials. But the major problem is low clinical relevance. And the advantages are like it has a high availability for skinning and reduced cost and high throughput. So these are the various advantages of this in vitro model.

But the drawback is failure to replicate the native conditions. Whereas this in vivo models, it provides a various degree of physiological similarities to the humans and also there are various genetic tools are available for genetically modifying these animals. But major challenge is very expensive to maintain and limited availability and also failure to predict the human response and also we have the ethical and

Societal challenges arise when we use this kind of animal model. Let us see some of the common in vivo laboratory models. We have the fruit fly, zebrafish, and mouse models. Most labs use these kinds of in vivo models for their research work. Initially, we start with in vitro models to understand the toxicity of particular materials.

Because cell culture—this in vitro model—is very easy to work with, low-cost to maintain, and has a short experimental cycle. So, these are the advantages of the in vitro model system. But as I mentioned earlier, these are highly controlled and simplified systems. So, there is poor correlation with the in vivo mechanism.

That is why we have to move from in vitro to in vivo models. But in the in vivo model, as I mentioned earlier, there are many challenges. We have to select the right in vivo model to understand our material's toxicity. We can use the fruit fly. It is very easy to work with because it has a short generation time and is very low-cost to maintain.

But it has the limitation like it is genetically distant from the humans and it is relatively simple anatomy and it has a no adaptive immune system. We can use the mouse. The mouse has a lot of advantages like it has a complex behavior and organ homologues to humans. and genetic similarity to humans but the limitations we told earlier like these are very expensive to maintain and experimental cycle is long and ethical constraint whenever we use animal like mouse model

we have to take the ethical committee approval animal ethics committee approval and it's a it takes some time to get the approval as well as to maintain these animals also expensive So that is why like the zebrafish is an alternate to an animal model and it is a very simple system. Advantages like it has a high reproductive rate and the development is external and again it also has the genetic similarity to the humans. And the limitations like again it has the moderate flexibility and moderate predictivity and also moderate translational value.

Let us see why zebrafish is a human model and what are the advantages of this zebrafish. Zebrafish is a common animal model for scientific research because of their many similarities to humans and easy to maintain as well as easy to genetically modify. And if you see that humans and zebrafish share almost 70% of their genes and this zebrafish embryos are transparent. So, it will be very useful for the researchers to observe their growth under microscope

And track the effects of drugs or genetic modifications. We can add drugs, we can add nanoparticles, and we can monitor them under the microscope. And also, these zebrafish are very small. So, they can be easily kept in large groupings. Also, these zebrafish may breed all year round and produce hundreds of embryos per week.

And they also grow very fast. So these are the advantages of using zebrafish as a human model. Let us see why zebrafish are used for toxicology studies. Because, as I mentioned earlier, it is easy to propagate and monitor their developmental stages. Because zebrafish embryos are transparent by nature.

And here, DPF means days post fertilization. And HPF means hours post fertilization. Additionally, zebrafish also have 70% genetic homology with humans, as I mentioned earlier. So, zebrafish can be a very good model for understanding various human diseases. From this slide, we can clearly understand the conserved organ systems between zebrafish and humans.

So, that is why the zebrafish can be a very good alternative animal model for understanding various human diseases. Let us see what the various experimental studies we can perform using the zebrafish model are. The first one is behavioral studies. For example, we can use the zebrafish model to understand anxiety. In pharmacology, we can use the zebrafish model to understand diet or drug-induced obesity.

We can also use the zebrafish as a model system to understand Type 2 diabetes, as well as non-alcoholic fatty liver diseases and other intestinal diseases. In toxicology, we can conduct various toxicity studies, including neurotoxicity, genotoxicity, and also nephrotoxicity. That is why the zebrafish is a very good model system for understanding the toxicity of nanomaterials.

Let us see how we can use the zebrafish model in aquatic ecotoxicology. We can use the zebrafish model to understand heavy metal pollutant toxicity, and if there is heavy metal contamination in the water, it can block neural pathways, increase cell death apoptosis, and lead to myocardial hypertrophy. Additionally, a high amount of organic pollutants in the water will decrease the activity of zebrafish.

And also, there will be a skeletal abnormality, and we can also use zebrafish for understanding the nanoparticle toxicity in water. For example, if there is nanoparticle toxicity that leads to embryonic malformation or death of the zebrafish. Or it leads to impaired immune function as well as various inflammatory reactions. Let us see how we can study the toxicity of silver nanoparticles using zebrafish embryos.

So, the zebrafish embryos will be placed in a 96-well plate. So, we have to keep the zebrafish embryo in the 96-well plate, then add various concentrations of silver nanoparticles. So, when you add various concentrations of nanoparticles and after incubation, you can see the zebrafish embryo under the microscope. At low concentration, you may observe this kind of normal embryo, meaning that particular concentration is safe for use. So, there is no change in the morphology, and the embryo is normal.

At different concentrations, you can observe the embryo is malformed. If you observe this kind of malformed embryo, that means that concentration is not safe to use for further studies. And if you use a high concentration of silver nanoparticles, you may observe this kind of dead embryo. The toxicity of silver nanoparticles can also be studied by observing the heart rate of zebrafish embryos. With respect to the increasing concentration of silver nanoparticles,

The heart rate is decreasing, and the mortality rate is increasing. Let us see another example for understanding the toxicity of nanomaterials using zebrafish embryos. Here, we are going to learn about nanoceria. This nanoceria nanomaterial has antioxidant activity. It mimics the enzyme SOD, which is the superoxide dismutase enzyme.

And it removes the reactive oxygen species. Here, we are going to use these nanoceria, which are encapsulated in an albumin nanoparticle, and we are going to understand its protective efficiency—how it protects the zebrafish embryo in the presence of hydrogen peroxide. So, for that, we added these nanoceria to the zebrafish embryo, and here we did not add any nanoceria.

These are the different concentrations of nanoceria added to the zebrafish embryo. Then, we added hydrogen peroxide. We can see here that when there is no nanoceria, the reactive oxygen species is higher. In cases where different concentrations of nanoceria are used, you can see here the reactive oxygen species decreasing because this nanoceria removes these ROSs.

By its antioxidant enzyme-like activity. For this assay, we use the DCFHDA assay, the principle of which you already know. Most of you know that in the DCFHDA assay, if you have more ROS, the fluorescence will be high. You can see that when using nanoceria alone, there is no fluorescence. Also, in the control where we did not add any hydrogen peroxide or nanoceria, there is no fluorescence.

Nanoceria was not added, and there is no fluorescence. But when treating only with hydrogen peroxide, you can see high fluorescence. This is due to the high amount of ROS production. However, when incubating the zebrafish embryo with nanoceria, you can see that the ROS is suppressed. That is why you can see there is no fluorescence here.

This means the ROS is suppressed by nanoceria, which has enzyme-like activity and removes all the ROS. We can also assess the toxicity of this nanoceria. When using

nanoceria at different concentrations, you can see there is no toxicity. It is almost 100 percent. The survival rate is almost 100 percent.

And when you are using this nanoceria in presence of hydrogen peroxide, you can also see here when you are not using the nanoceria, So, the survival rate is going down. But when you are using this nanoceria, with respect to concentration, the survival rate is also increasing. That means, so the nanoceria is protecting the zebrafish embryo from the reactive oxygen species.

So, from this slide, we understood how the nanoceria can increase the survival rate of zebrafish embryo and also the protective effect of nanoceria against the hydrogen peroxide induced embryotoxicity in zebrafish. Let us learn how to identify the male and female zebrafish. You can see here this is the male zebrafish and male zebrafish can be identified by the small and slim body size as well as we can see the bright color when compared to the female and also it has the long fin.

So these are the identification marks for the male fish and in case of female fish you can see here it has a rounder body when compared to the the male and also it has a large size and it has a dull color when compared to the male and it has a small fin when compared to the male so based on this we can identify which is male and which is female fish. To get the embryo for bioimaging experiment we have to keep the fish for breeding before we keep it for breeding as per the OECD guidelines test number 236 that is the fish embryo acute toxicity test we have to keep two male

And one female fish in the spawning unit. And this is the spawning unit. And this is the recommended male to female ratio for zebrafish breeding. Because this ratio increases the chances of successful fertilization. And here in the spawning unit, the male and female fish are separated by a transparent barrier for overnight.

Then the next day, remove the transplant barrier, allow the fish to breed. After breeding, the fish lays the eggs which can be collected and counted for the imaging studies. And we have to select the healthy embryo for our studies. So, how to select the healthy embryos? If the egg is transparent and shiny, it is a healthy developing embryo.

Instead, if you observe white opaque egg, then it is a unfertilized egg or it contains a dead embryo. once you select the correct embryo count it then add the carbon dots and incubate it. after incubation you can observe the carbon dots labeled fluorescent embryo using fluorescence microscope. From this picture we can understand that after incubating

zebrafish embryos with carbon dots you can observe the fluorescence and this is a bright field image and this is the fluorescent image

And this is the merged image. From this image, you can clearly understand that there is no toxicity from carbon dioxide compared to the control. We can also understand the bioimaging applications of carbon dioxide from this image. Let us see the maintenance, breeding, and typical conditions for zebrafish embryo acute toxicity tests as per the OECD guidelines. We have to feed the zebrafish 3 to 5 times daily with dry flake food.

Additionally, we also have to give brine shrimp, which is Artemia, because feeding live food provides a source of environmental enrichment. To guarantee optimal water quality, the excess food and feces should be removed approximately 1 hour after feeding. Other parameters, such as water temperature and water quality, have to be maintained properly. Also, we have to maintain proper illumination and follow the photoperiod, which is 12 hours of light and 12 hours off. As I mentioned earlier, we have to follow the recommended male-to-female ratio for breeding.

Based on the egg structure and appearance, we can select healthy developing embryos. As I mentioned earlier, if the egg is transparent and shiny, it is a healthy developing embryo. However, if you observe a white opaque egg, it is an unfertilized egg or contains a dead embryo. The spawning rate mainly depends on the strain. Usually, a single mature female spawns at least 50 to 80 eggs per day.

I hope you got the overall idea about how we can use the zebrafish embryo for understanding the toxicity of nanomaterials. Let us go to lab and learn this more in detail. In this experiment, we will learn how to do bioimaging of carbon dots by using zebrafish embryos. So to begin the experiment, we require zebrafish embryos.

First, we need to separate the male and female fishes in a 2 is to 1 ratio using transparent glass barrier. Once this is done, switch off the light and leave them overnight. Now we have switched on the light. The next step is to place the chamber at a slanted angle and remove the transparent glass barrier placed between the male and female fish. Leave the setup undisturbed for 20 to 30 minutes.

The fish may seem to be fighting or chasing each other. These are the signs of spawning. The female fish releases eggs into the water, which are then fertilized by sperm from the male fish. Here we can observe the eggs in the bottom of the chamber. Now we will remove the fish and place them in another chamber to collect and clean the embryo.

The separated fish can be further divided into females and males. They will be fed and ready for breeding in the next few days. Next, we will observe the embryos under the microscope to identify and separate the fertilized ones. To clean them, we will use E3 media. It is important to handle the embryos carefully to avoid damage.

Now, we will view the embryos under the stereo zoom microscope. We will see the embryos on this large screen. First, we will open the software on the computer. Then, we will place the plate under the microscope. Switch on the background light.

And change the light mode to bright field. Once the embryo is clearly visible in the eyepiece, adjust the knob from bright field to dark mode so that we can see the embryo on the monitor. Switch to live mode. Adjust the settings.

and capture the image for reference. We have separated the fertilized embryos. Now we require the test material that is carbon dots. We will prepare the dilution of carbon dots to achieve the desired concentration. Once we have prepared the desired dilution, we will treat the embryos.

First we will place the exact count of embryos in the 12-well plate. Remove the media and for the control we will add the E3 media. For the treatment we will add carbon dots. Incubate it for at least 2 hours.

Now we will place the control embryos on a slide. For the carbon dots treated embryos, we will wash them using E3 media and then place them on the slide. We will now view these slides under stereo zoom microscope for bioimaging. First, we will observe the control embryo. For bright field, we will follow the same procedure as I told earlier.

To observe under the GFP filter, press the GFP button and switch it on. Then, turn off the background light. As you can see, there is no fluorescence in the control embryo. And it is not clearly visible compared to the bright field. Now, we will place the carbon dot-treated embryo.

First, we will observe it under the bright field. You can see the embryo containing carbon dots. Next, we will switch on the GFP filter, then turn off the background light. Now, we can observe the green fluorescence in the embryo, confirming that the carbon dots have entered the embryo, demonstrating their bioimaging property.

Now, let's compare the carbon dot-treated embryo with the control embryo in the bright field. Finally, we will save all the images. In this lab session, we have learned how to use

zebrafish embryos to study the bioimaging properties of carbon dots. As a summary, in today's lecture, we learned about various experimental models used in human research, and we also learned how we can use zebrafish as an alternative toxicological model.

Through practical demonstration, we also learned about the bioimaging experiment using zebrafish embryos. Thank you for your kind attention. All the very best for your exams.