Medical Biomaterials
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Lecture - 20
Biological Responses/Animal Studies

Hello every one welcome to the course on Medical Biomaterials. In this class we will again talk about biological responses and slowly move towards in vivo and animal studies. Any biomaterial before it goes into clinical trials has to be tested on animal, so we will start talking about animal also soon. Large number of animals are used like rabbits, mouse, rats, going right up to dog, sheep and so on. So, let us continue some more on the biological response. So, as soon as the biomaterial and its placed inside the body.

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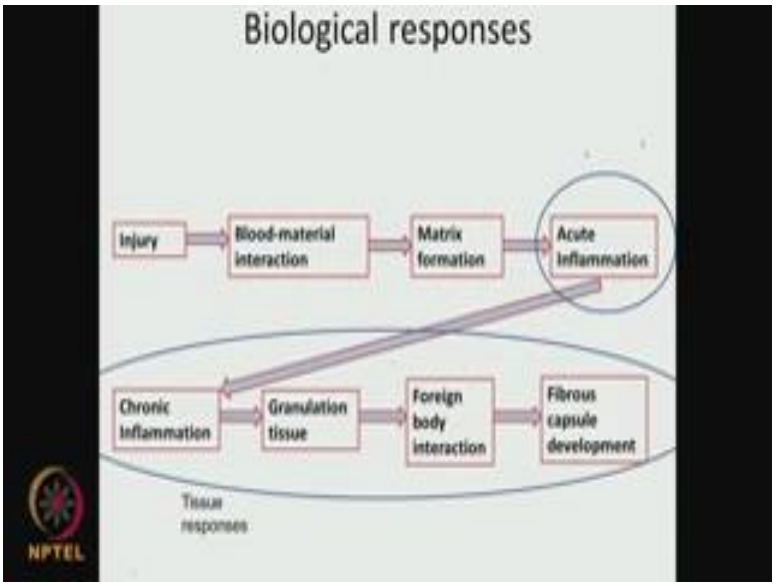

I said in the previous class there is going to be a blood material interaction leading to activation of the coagulation, pathway and then the activation of the complement path way both of them are part of the immune respond system, then what happens is things start happening we have the inflammation parts of the tissue response. Acute inflammation, the chronic inflammation then the tissue formation foreign body interactions and fibrous encapsulation of the material and so on actually. So, lot of tissue response starts happening here. So, we will look at it slightly in more detail.

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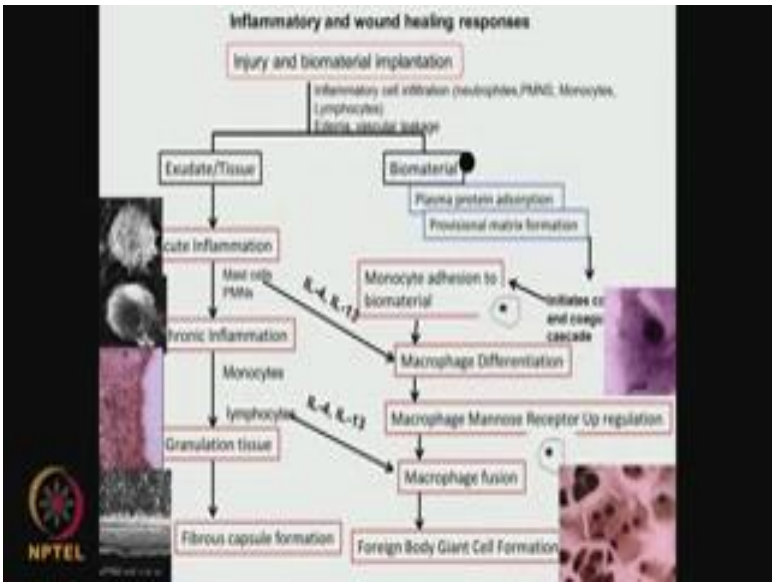

So, when there is an inflammation there are lot of wound healing response because when the surgeon tries to open the host body and then place a biomaterial; obviously, it is like an wound. So, the wound healing process start taking place because the surgeon has created an injury because of the biomaterial implantation, because there is going to be inflammatory cell infiltration. So, lots of cell like neutrophils, pmns, monocytes, lymphocytes start going towards at then there is going to be edema vascular leakage, so all this start happening actually. So, the biomaterial is placed inside.

So, there is plasma protein adsorption, then you have provisional matrix formation monocyte adhesion to the biomaterial which here you have the complement and coagulation which we talk in detail, then we have the macrophage differentiation and macrophage place a very important role in forming lot of foreign body giant cell and try to engulf or encapsulate the biomaterial. So, there are all interrelated. As you can see here we have the acute inflammation chronic inflammation and then the tissues are formed here and your biomaterial gets completely fibrous capsule formation.

So, both the things happen simultaneously when you have the inflammation healing process. Now these are some of the inflammatory cells as you can we see this picture here and these are granulation tissues as you can see this tissues are formed around the biomaterial and they try to completely encapsulate your fibers and as you can see these are some of those monocytes which are formed near the site of inflammation or other site

of where the biomaterial is placed. So, these are part of the inflammation and wound healing process. So, lot of different types of cells are formed here and then finally, in the biomaterial is inert it can completely get encapsulated. Of course, as I said if the biomaterial is toxic or the excudent are toxic then you could cell death happening there and these are some again pictures of foreign body giant cell formation as you can see here this is called a foreign body giant formation.

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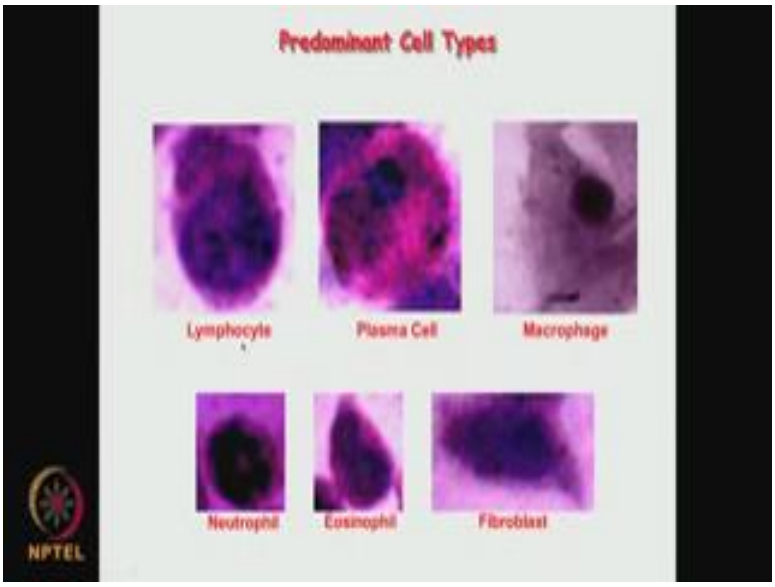

So, different types of cells are formed I should this picture long time back, lymphocytes, plasma cell, macrophages, fibroblast, neutrophil, eosinophil. So, all this are formed here in this particular step here.

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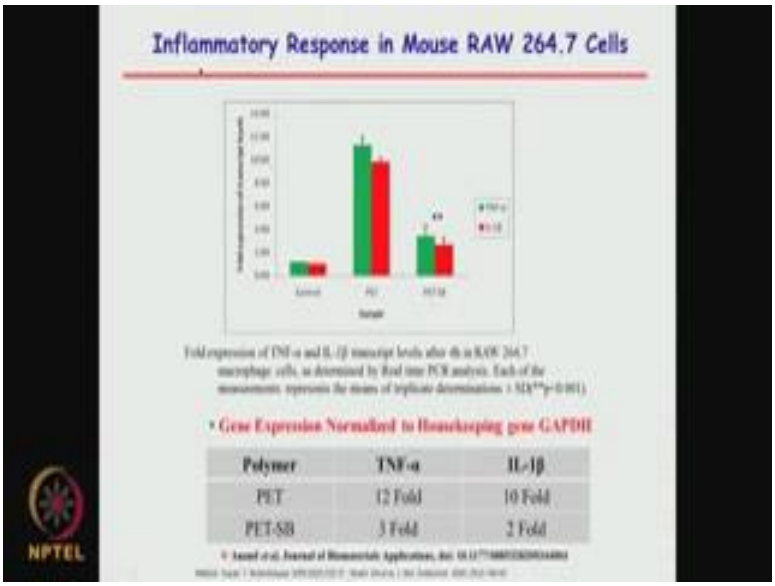

So, look at this there is an experiment. So, when you use the mouse raw 264.7 cells what happens when it is in contact with the biomaterial like polyester or then this is a polyester, this is a modified polyester. So, what are the inflammatory response when the mouse cells are in contact with this polyesters. This figure tells you the expressions of two important transcriptions which are involved inflammation - one is called TNF alpha the other one is called IL 1 beta. These two are markers, which tells you if there is a inflammation. As you can see in the control they are very very low amount of the level of TNF alpha as well as IL 1 beta. But as soon as come in contact with the biomaterial made up of polyester here, you can see both of them have gone tremendously by almost 12 fold where as when they are in the control; that means, there is no contact with biomaterial it is almost like one fold. So, it is shot up.

So, what we do? We do some modifications to the polymer polyester. So, you are able to bring it down from almost 12 fold to 4 fold. So, three fold reduction in the inflammatory response, which you have achieved by modifying the surface; because surface modification helps you to reduce the inflammatory response of the biomaterial to this particular cell line. So, these types of experiments have to be done in your lab before you to take this biomaterial further for animal studies. So, these are called in vitro cell line based studies. So, we are using this particular cell line you can test other muscle cell lines and so on and as you can see as soon as they come in contact with the polyester there is a big increase in the inflammatory markers. So, most of the in vitro studies with

cells lines focus towards these two particular inflammatory markers - one is called the TNF alpha the other one is IL 1 beta. So, they look at the transcription levels of these two markers and then we will be able to tell whether the biomaterial is creating a inflammatory response.

So, we do some surface modification to this biomaterial and as you can see they come down dramatically, but still it is higher than the control, but it is comes down dramatically. So, how do you do these experiments? There is something called real time PCR polymerized chain reaction this is an instrument which can tell you what happens to various jeans. So, we can focus on a particular jean and then tell whether jean levels are up and when they are in contact with a material and so on and so it tells you the mechanism this is called mechanistic study. So, we are able to tell there is a inflammatory responds because this particular two jeans mainly TNF alpha and IL 1 beta have gone up by almost from 1 fold to 12 times increasing, 12 times increasing and this 1 is 10 time increasing, but when you do a surface modification you are able to bring it down only 3 times increase in TNF and 2 times increase in IL 1 beta. So, do you understand?

So, this type of experimental studies are very essential to understand the inflammatory responses these materials create and then you can modify the surface of the materials. So, that the inflammatory response is dramatically reduced. So, we may be able to reduce it still further by another type of modification we will not go too much into that of as now. On surface modification, but surface modification of biomaterial is a very important topic you can do it through different approaches plasma bombarding, UV, radiation, immobilizing or coating, anti bacterial material, yesterday I talked about coating aphern, when aphern is coated it prevents the complement activation or even platelet activation immobilizing enzymes and so on. So, there are so many different ways of modifying the surface. So, that we can reduce the blood coagulation we can prevent the activation of platelets, we can reduce the inflammatory responses and so on actually this type of in vitro studies very very important before actually go into in vivo animal studies.

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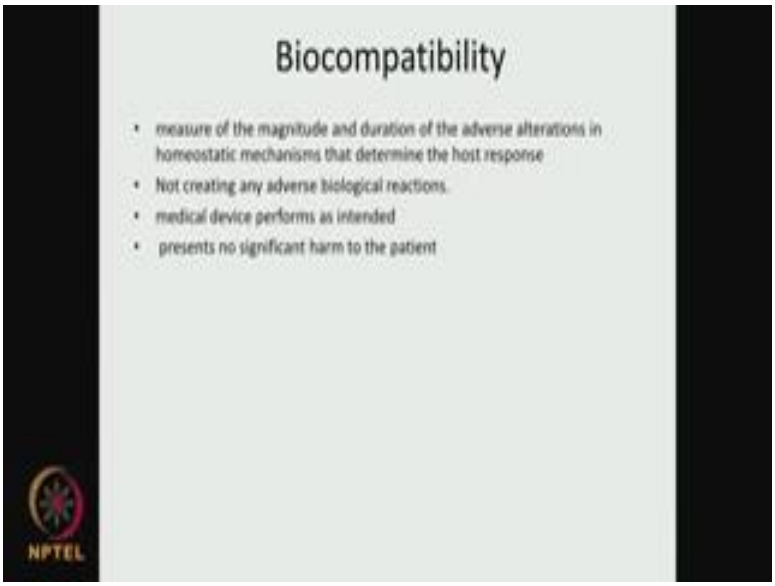

So, biocompatibility basically the measure of magnitude and duration of the adverse alteration, it is not only how bad the responses it, but how long it last in the homeostatic system in the host. We should not create any adverse biological reactions at the same time the medical device should perform as intended; it should not present any significant harm to the patient. So, these are all necessary when you want to say the material is biocompatible.

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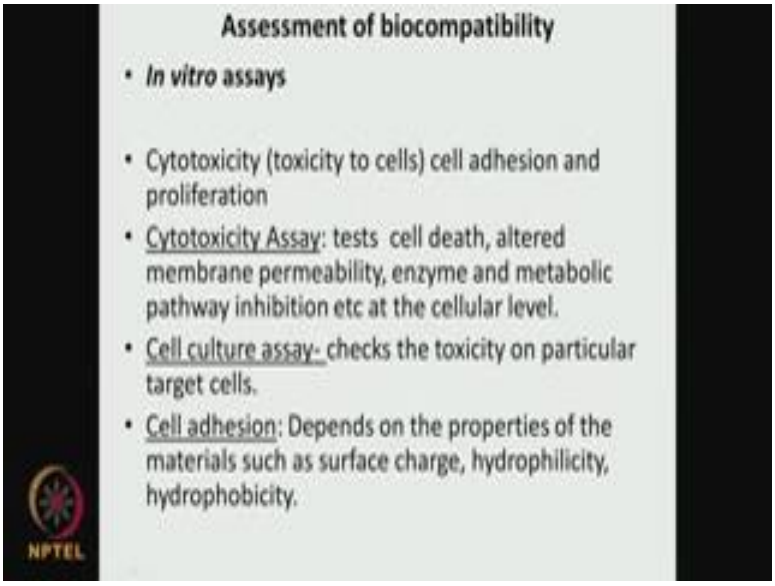

So, so many assays are there for assessing the biocompatibility I have been talking about it previous classes also. So, sort of we will summarize them there are in vitro assays that we can do it in lab, there are in vivo assays where you do it in animals. So, in vitro assays I mentioned it long time back cyto toxicity, toxicity to cells, cell adhesion that means, are the cells adhering properly and then are they proliferating properly. Is there a problem in adhering and is there a reduction in the proliferation; that means, cells are dying cells are not growing that is called cytotoxicity.

So, we are looking at cell death altered membrane permeability. So, as the membrane become very porous. So, some of the enzyme inside which are important come out at the cellular level. So, we can monitor that also we can look at whether membrane as become permeable using a different types of dyes and looking under a microscope. So, we can fluoresce dyes and if the membrane is permeable then those dyes may go inside and then we can look at it using a microscope the fluorescent microscope. We can also look at specific sizes, there are so many different types of cells we can look at like I showed in the previous case mouse, cells, we can look at human health cell, muscle cells, we can look at bone narrow cells so on. So, we can look at the cell culture on specific cells. So, we then look at whether cells are adhering properly because that is also very important.

Adhesion is very important. So, the cells can start proliferating. So, the adhesion depends upon properties of the biomaterial like surface charge, hydrophilicity, hydro phobicity, and what is the surface roughness and so on. So, we can do that sort of cells adhesion studies experimentally in the lab.

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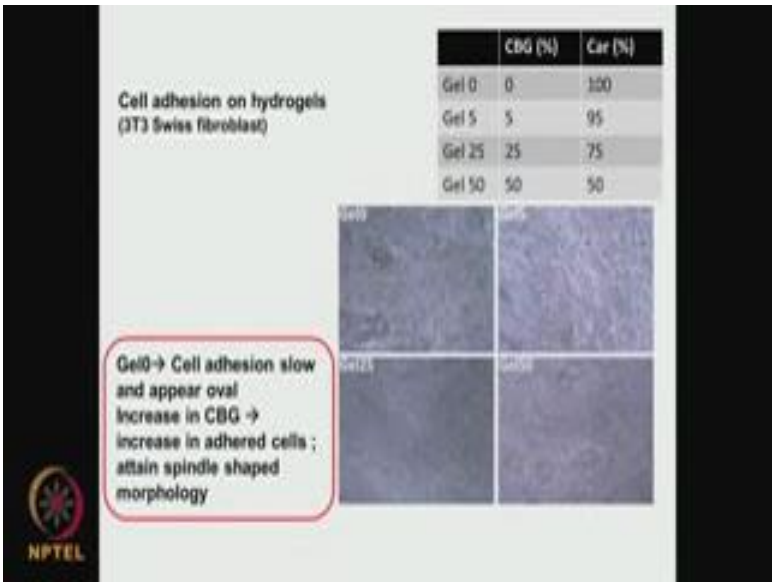

In vitro based for example, I just want an example of how to do. What we did was we have a biomaterial made where we use different types of beta glucan; glucans are produced by bacteria these all are bacterial glucans. So, they are generally some of them are water soluble in this particular cases water soluble they are very biocompatible carrageenan.

So, we prepared four types of gels - one with the no glucan only carrageenan beta glucan, it is called beta glucans are supposed to be immuno modulating properties and this gel is prepared only with 100 percent carrageenan, this gel is prepared with 5 percent beta glucan and 95 carrageenan, this gel 25 is prepared by 25 percent weight percent and 75 percent carrageenan, this gel prepared 50, 50, so 4 different gels with the increasing beta glucan. As I said beta glucan are produced by bacteria they are compatible they are water soluble some of them are water soluble, in this particular case its water soluble molecular weight about 1500 taltons and then we grow cells on them, we grow this particular cell 3T3 Swiss Fibroblast. So, look at this, on gel zero that means, there is no beta glucan only carrageenan cells are not adhering properly and cells have to look spindle shape where as they all look spherical shape; obviously, this particular material surface is not very conducive. So, its toxic to the cells 3T3 cells.

Now look here, as we keep adding beta glucan 5 percent, 25 percent, 50 percent we can see cells are becoming spindle shape this is the correct morphology of the cells they are

becoming spindle shaped here very nice, very nice. So, by adding more beta glucan which is biocompatible you are allowing the cells to adive as well as grow in a proper shape which is the spindle shape where as if you look here there is no beta glucan there is spherical; obviously, this is not the correct.

So, addition of beta glucan is improving the biocompatibility of the material. So, you can do this type of studies in vitro in the lab. So, the amount of adhered cells also increases as you increase the beta glucan and they also achieve they are spindle shape of morphology you can see this right. So, these are called adherent tests which is carried out in vitro we can look at any type of cells and then we grow the cells and then we look at them under a microscope. So, it is quite easy actually.

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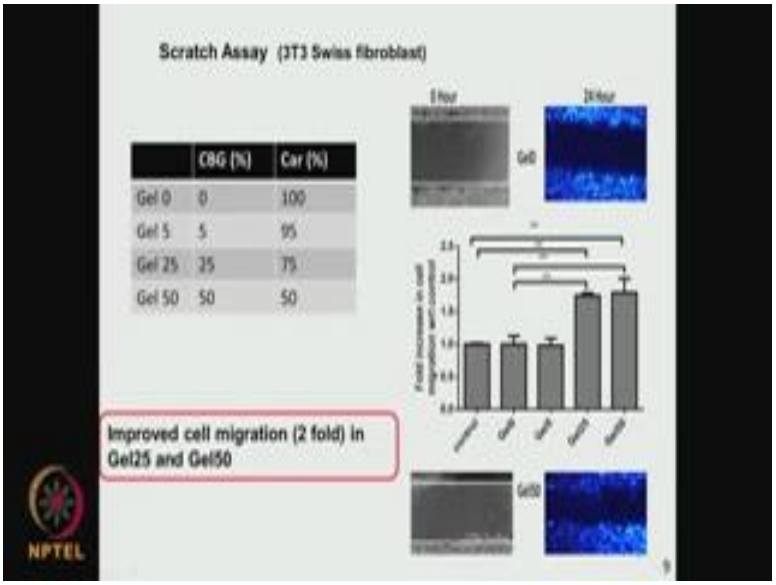

You can do another study that is called scratch assay, this again in vitro in vitro means you can do it in the lab and the scratch assay tells you whether the cells are migrating; that means, they grow and start migrating. If the surface is very biocompatible cells will nicely migrate, the surface is not biocompatible its toxic then the cell migration will be slow or completely retarded. Same material we took four different surfaces and this has got 100 percent carrageenan, this gel 5 has got 5 percent beta glucan 95 percent carrageenan, this gel has 25 percent beta glucan and 75 percent carrageenan, this gel has 50 50 beta glucan and carrageenan.

So, what you do is we grow the cells for 24 hours and then create artificially a scratch, scratch means we make a mark with the very fine needle and then we start seeing whether the cells starts migrating. So, slowly slowly the scratch should completely disappear. So, this is zero time. So, this is zero time on gel 0, gel 5, gel 25, gel into 50 we created a scratch and then after 24 hours we are monitoring the scratch this is a color image we put it in a dye, we can see only carrageenan is there the gap is very large whereas, the gel 50 which is 50 50 carrageenan and beta glucan gap is very small; that means, cell has proliferated, migrated and started reducing the scratch.

That means, the material is very conducive for the cell growth, it is a very interesting experiment which can do in vitro and this experiment also tells you we can use this type of material because they help the cells to proliferate, migrate and which are very very important and if you want to have a very high biocompatible material. Whereas, this particular gel 0 which is 100 percent carrageenan look at this even after 24 hours the migration is very poor; that means, it is slightly toxic. As you keep increasing the beta glucan we can see the migration proliferation is very very good. This is shown numerically here, so gel 0 migration if you take it as 1 and the 25 and 50 the migration goes to almost two times. So, double the rate at which cells are migrating.

So, this assays in vitro assays I talked about in the previous slide the adhesion in the morphology development and the scratch assay all these assays are very useful to determine whether a biomaterial is conducive to cells and these are experiments done in vitro; that means, in the lab. So, when we use different types of cells as I said you know here I am showing three t three we can use a raw cells mouse and so on actually.

So, I showed you lots of different in vitro based experiments, monitoring the inflammatory marker likes TNF alpha or IL 1 beta, looking at cell adhesion proliferation, looking at a cell migration using the scratch assay all these are experiments which we carry out in the lab to prove or disprove whether the material is biocompatible or non cyto toxic. Then we can modify the surface and again see whether the cyto toxicity is reduced, finally, once you are satisfied we can go to animal studies.

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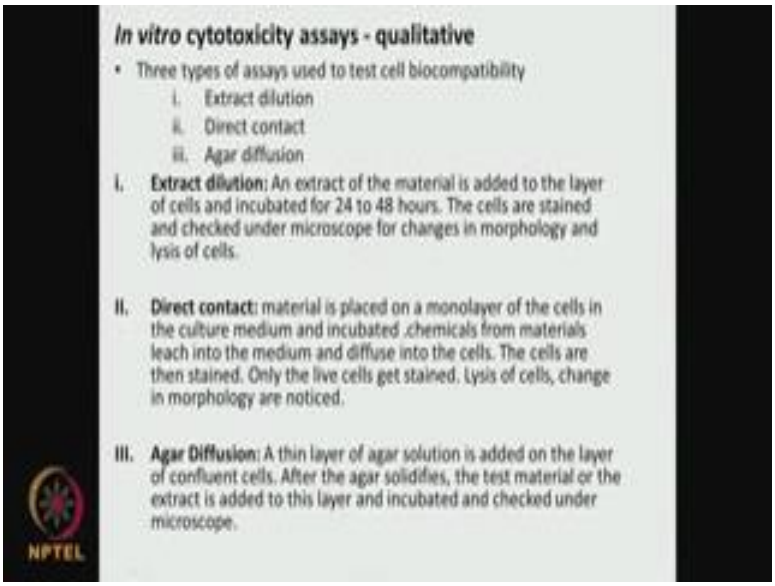

So, the in vitro cytotoxicity assays is we take the material polymer for example, we see whether the extract from the polymer are going to create toxicity. This is very useful especially in dental in plants for example, dental pmma poly methyl methacrelate is used widely how do they do they take a methacrylic acid and its polymerized using UV. So, most of the methacrylic acid is becoming polymer the most metal methacrylic acids become polymeric metal methacrelate little bit of monomeries left behind little bit which may slowly leach of out a period of very very long time. So, is this leachants toxic? So, this type of assay is very useful. So, what we do is extract from the polymer and use that extracted solution and see whether the cells grow in there, whether the cells die in there, and so on actually.

For example you are using amalgam, lot of amalgam based materials are used in dental fillings. So, they can slowly leach out over a period of years. So, are they toxic? So, we this particular assay is very useful. So, you take out a samples and extract its liquid from the material and then test whether that liquid is toxic to the cells.

Direct contact, what you do you? You incubate the material biomaterial with the cell line and it is called direct contact. So, that approach is called agar diffusion. So, what you do is we have cell nicely growing. So, we add a thin layer of agar solution on top, agar is used for growth of cells bacteria and so on then once the agar solidifies your biomaterial is added to this and then you check. So, if the biomaterial is toxic the cells will not grow

and closure to the biomaterial, but if the biomaterial is not toxic cells will completely grow even near the biomaterial and start angle filling practically. So, that is called the agar diffusion. So, and this method is almost like your cell attachment and proliferation and migration whereas, these two methods are like self proliferation or cell death. So, these are in vitro methods these are more qualitative because we cannot get a quantitative number, but there are quantitative methods are also there I talked about it long time back if you remember we use to do MTT assay.

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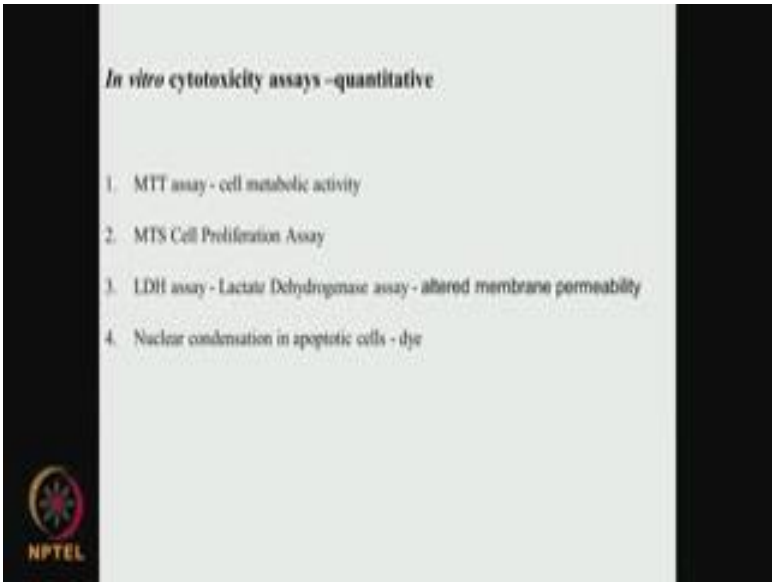

Do you remember in our old class we did otherwise you can go back and check it tells you the cell metabolic activity. So, we can calculate in percentage with respect to control how many cells are viable cells are there, we can say 90 percent viable cells, 80 percent viable cell. Something called MTS assay again looking at the cell proliferation percentage of cells alive when it is with the biomaterial with respect to the control where we do not have a biomaterial. Then we have the LDH assay because there is an this particular enzyme called lactate dehydrogenase which is present inside the active cells or cells which have a good metabolic activity if the cells die because of the necrosis then the membrane becomes damaged. So, the membrane because of the reduction in membrane permeability this particular enzyme comes out into the solution and the measure of the amode of this is the measure of the cell that is died because of necrosis or membrane damage.

Another is nuclear condensation in apoptotic cells. So, if the cells die naturally which is called apoptotic and we add some dye we can see the nuclear condensation under a fluorescent microscope and we can tell whether it has died because of apoptotic. If it has died because of unnatural causes like necrosis there will not be condensation of the nucleus the nucleus will be spread all over the place and so we can tell the necrosis. These are called quantitative assays I talked about it some time back and these are the qualitative assay. So, all these assays are very useful in vitro to understand the cyto toxicity.

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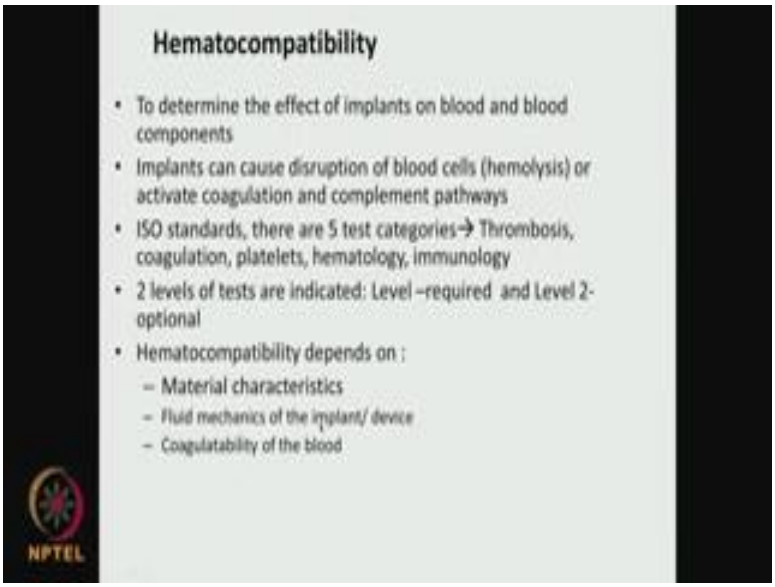

Then hematocompatibility that is the blood related compatibility you want to know whether the effect of implant on blood and blood components again I talked about it long time back because the implants can cause disruptions of blood cells that is called hemolysis or they can activate the coagulation pathway leading to blood clots if you remember we talked about fibrin which and then complement pathway, so the implants can activate the complement pathway which leads to several proteins getting activated.

So, the ISO standards are there which talks about 5 different test categories thrombosis, coagulation, platelet, hematology, immunology. So, one can do all these test to understand whether the material is biocompatible and there are two levels of test - the basic level and the advance level.

So, again the hematocompatibility depends on the material characteristics, fluid mechanics of the implant device coagula ability of the blood. So, if somebody is having cardio vascular problem and he or she is given a blood tinning like rephern or even aspirin, so the coagulation does not happen so easily whereas a normal person the coagulation may happen easily. So, hemotocompatibility also depends on that. So, I also talked about how to reduce the activation of the coagulation as well as the complement pathways there are coatings, coatings which can reduce that we talked about it some time back and we also said there are certain synthetic polymers which are very hemotocompatible and which does not activate these coagulation and complement pathways. So, one can think about using those biomaterials.

Or think about the coating the biomaterial which will reduce the activation of these particular two pathways that is called hemotocompatibility.

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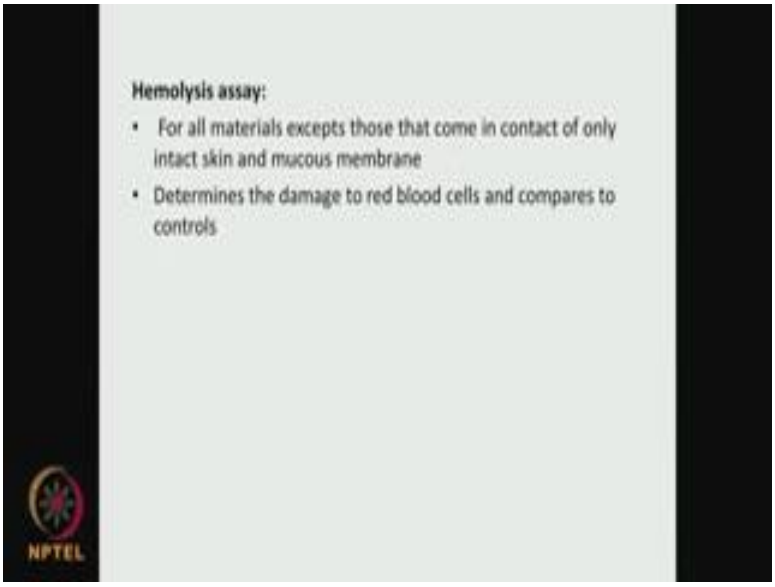

So, there is a Hemolysis assay, for all materials except those that come in contact of only intact skin and mucous membrane. So, this assay tells you how much of the red blood cells are damaged with respect to the control.

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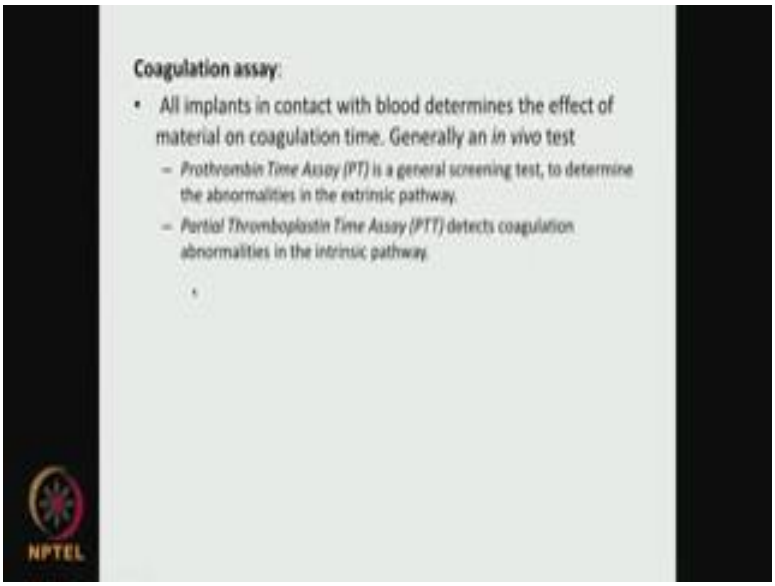

So, you also have the coagulation assay. So, it tells you the effect of the material on the coagulation time - one is called the prothrombin time assay, other is called the partial thromboplastin time assay, both these assays are quantitative. So, it tells you the effect of the biomaterial on the coagulation with respect to the control, control means without the bio material.

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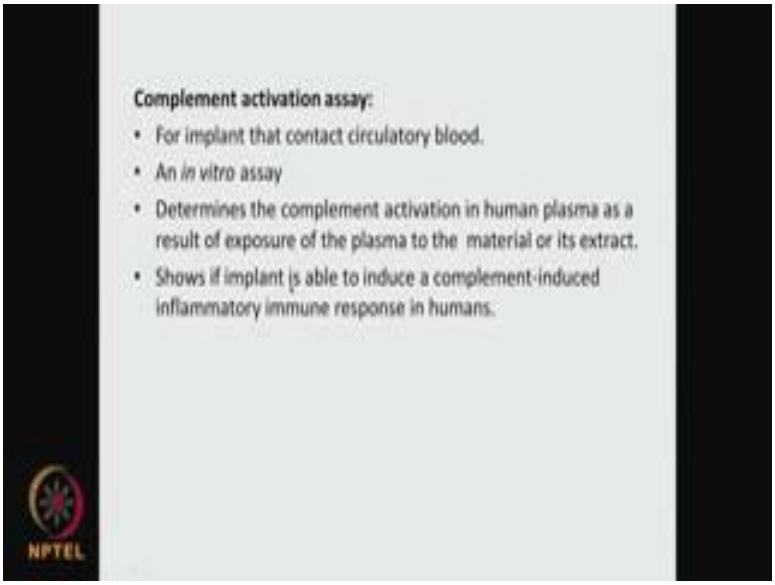

Then there is algae in assays for complement activation. So, again as I said the complement activation with respect to the circulatory blood this also in vitro assay. So, it

determines the complement activation in human plasma as a result of exposure of the plasma to the material. So, this assay is very useful as I tells you whether the complements will get activated. So, we have previous coagulation based assay or hemolysis based assay, hemo which tells you whether the blood the plasma getting going to get damaged whether the biomaterial is going to activate your thromboses and the coagulation pathway and then comes whether the biomaterial is going to activate your complement system. So, all these are assays which tells you sometimes qualitative sometimes the quantitative effect of the biomaterial on especially when they are coming in contact.

So, these are in vitro assays. So, I can do it with cell lines, I can look at the jean expressions, I can take plasma blood plasma and then I can do a experiments with a blood plasma and so on actually. So, these are all now comes the next step where you are taking the biomaterial to animals that is called in vivo.

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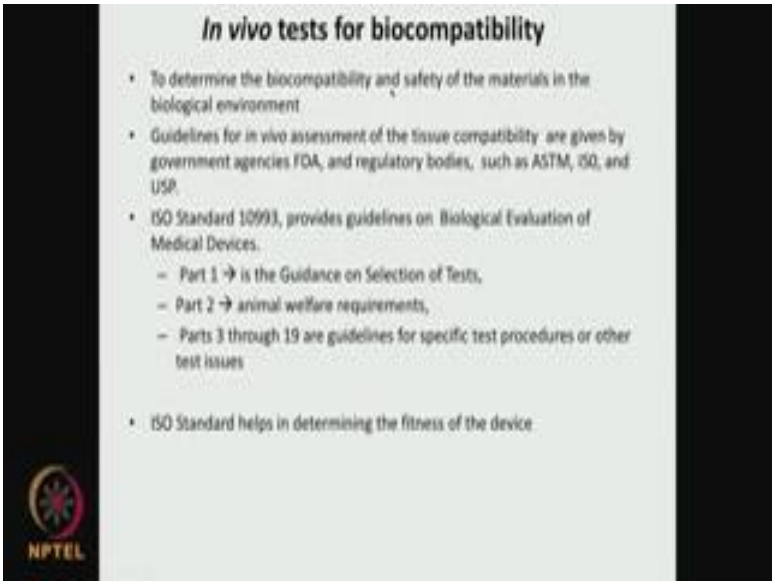

To determine the biocompatibility and safety of the material in the biological environment, so whatever we do in the lab actually it is not really biological I can use different types of cell lines, I can take the blood plasma, red blood cells but still it is not really in vivo, but then when we take it to an animal then it becomes in vivo. So, there are many guidelines for in vivo assessment of tissue compatibility, FDA has some guidelines there are regulatory bodies such as ASTM, ISO, USP they have many

guidelines which tells you what are the types of test you need to do when you are working with animals, biological evaluation of medical devices, guidance on selection of tests animal welfare requirements because when we carry over experiments with animals we have to follow lot of animal welfare guideline, we have to abide by them. There are 19 guidelines and specific test procedures. So, all these guidelines we have to undertake especially when we go into the animal studies.

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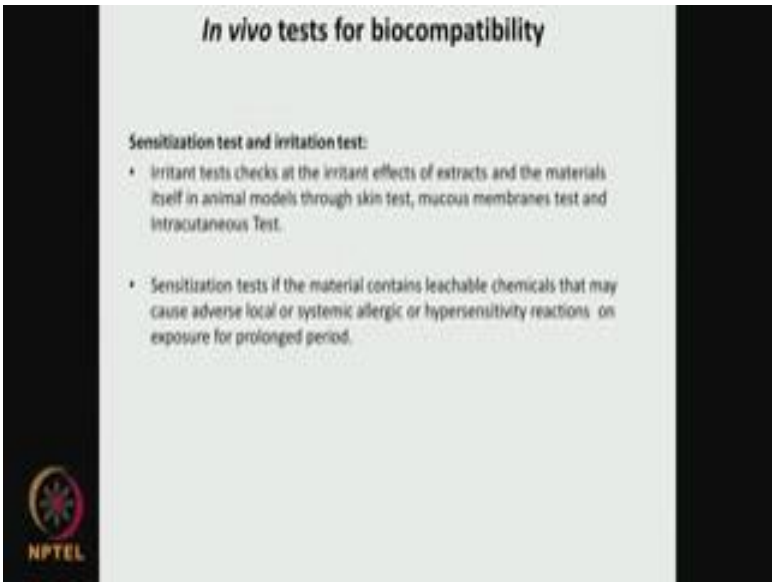

So, we will look at these more in detail in the next class.

Thank you very much for your time.