Introduction to Biomaterials

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Module No. # 01

Lecture No. # 09

So, we will continue our discussion, on the, in vitro toxicity and apoptosis of bone cells. Particularly, we have, you, I will show you that, you know, how human ((…)) cells can undergo apoptosis, due to the treatment by nanoparticles.

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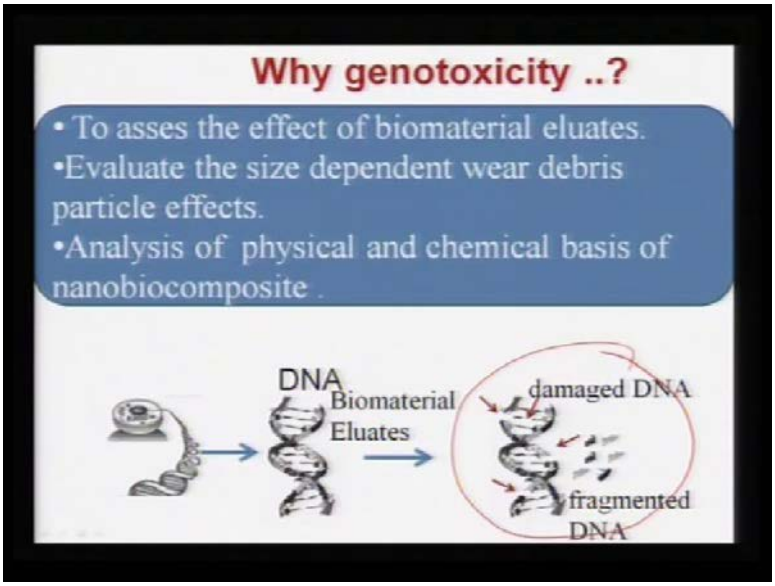

So, the first question that I will address is the - what is Genotoxicity? And, if you know, that in the human genome, like in DNA, so DNA has a typically, double helix structure and this DNA, when they are treated, when the particular cell is, treated with nanoparticles. Then, what will happen? If then, if the particles are very fine, then they can be internalized. Internalized means, they can be taken up by the cell nucleus. And, once they are taken up by the cell nucleus, then they can preferentially, damage the double helix pattern of the DNA.

Now, what will happen? If the double helix patterns are the individual strands, are broken by the Nano particle treatment, now, if you go back to that. To that, firstly let me answer this one.

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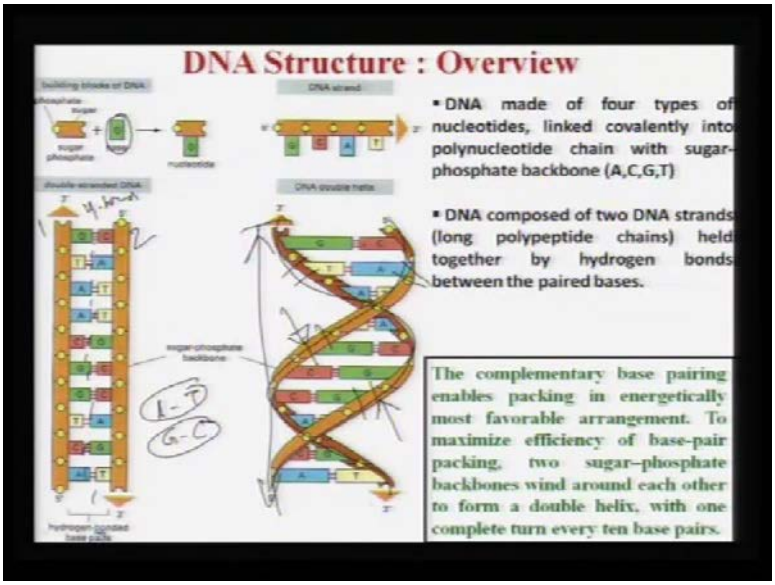

If you remember, the DNA structure, which is not very complicated, if you understand it in a logical sequence. Now, let me, remind you, so this is the strand 1 and this is your stand 2. And, how these strands are made? Strands are made by Phosphate sugar ((mol...)), Phosphate sugar molecule and 1 base.

Now, this base can be either A that stands for Adenine; T stands for Thymine; G stands for Guanine and C stands for Cytosine. So, any of these four things can be there, and the, where this base pairing is typically take place is A, T and G, C. So, this is the typical base pairing like these two bases can come, and they form a hydrogen bonds in between. So, you can see this red ones, these are like, you know hydrogen bond or weak bond.

Now, A, T, G, C and this is the double strand. Now, in order to minimize the total energy of the system, typically in the natural state, this DNA forms a double helix. And, this double helix pattern also, it is not in arbitrary manner, the ten base pairings, each one complete turn of the double helix that requires ten bases pairing at least. So, that means between A and T and G, C at least, there will be ten base pairings. So, if you continue, if you count here 1, 2, 3, 4, 5, 6 7, 8,9,10. So, that means this is a total, one complete turn

of the double helix from this one to this one. Now, as I said that if you treat cells with the very fine Particles and if they are internalized by cells and suppose, they would, they are breaking this double helix pattern. Breaking means there are certain breaks in the continuity of these different strands. Now, these strands can be broken, at any arbitrary manner either at this place, this place or any other place. Now, what will happen if the strands are broken?

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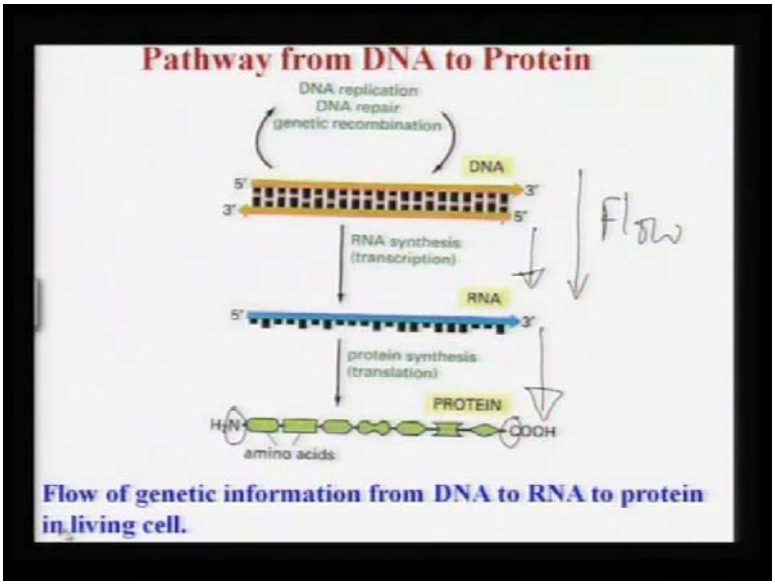

You know, the DNA structure typically (( )), typically produces protein by a process known as a transcription, translation processes. What is transcription processes? The double helix pattern of the DNA can give rise to RNA, inside the nucleus in a eukaryotic cell. RNA further, undergoes translation process to produce the protein. And, this is the protein structure, where this is C terminal on the right and this is N terminal on the left of the protein structure. And, that is the way that flow takes place, genetic information from DNA to RNA to protein in living cells.

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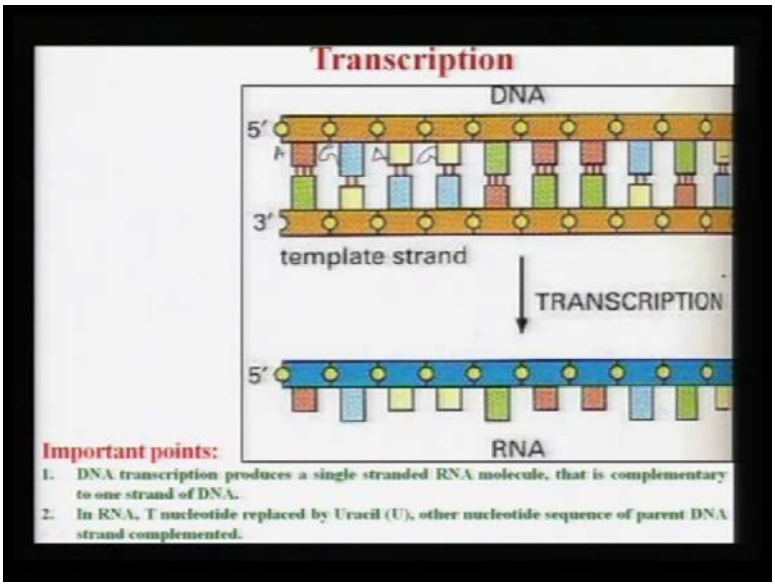

So, this is that in a more, few of this DNA transcription process, like you know, you have a single strand here. This is single strand here, this can be A, this can be G or whatever sequence it can be. Now, this sequence, if you see, in the DNA you have A, T, G, C.

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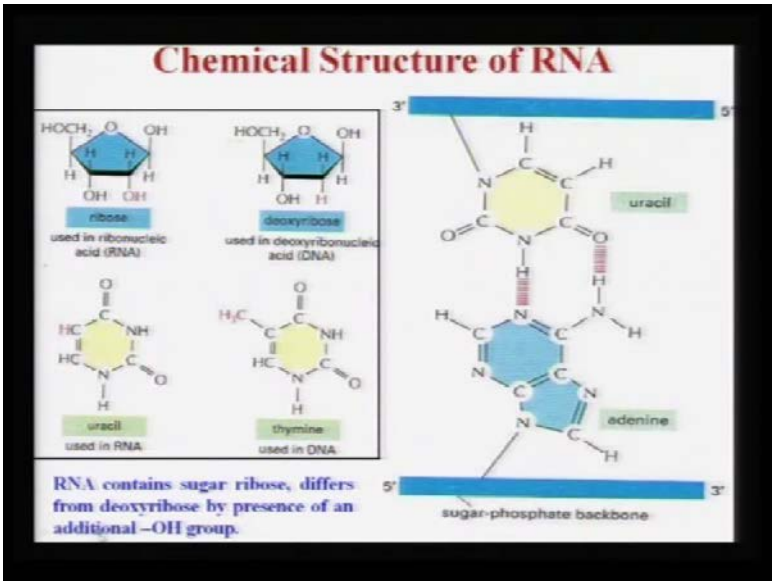

But, in case of RNA, you have so in DNA, you have that A, T, G, C.

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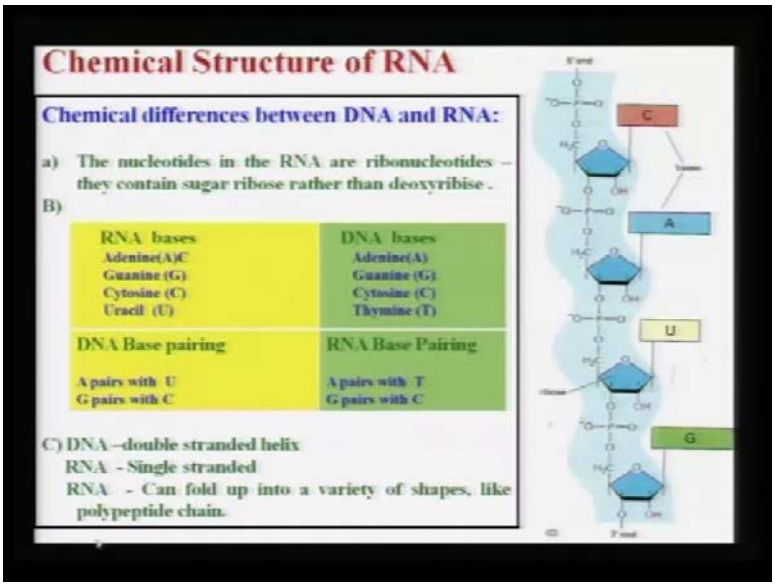

In case of the RNA, that thymine group T is replaced by Uracil U. And, typically, RNA structure is made of the single strand. As, you can see that RNA structure typically made of a single strand; not like a double strand, as you have seen for DNA. And, here you have the Cytosine, you have the Adenine, you have the uracil and you have the Guanine. These are the four bases.

And, this basis also can be in parti cular in a particular order. So, therefore, if you look at the chemical differences between DNA and RNA, the nucleotides in the RNA are essentially, ribonucleotide because they contain sugar ribose; rather than deoxyribose. And, the second one is that in terms of bases like, Uracil replacing the Thymine. And, third one is that DNA has a double helix strand and whereas, RNA has a single strand. So these are the three important differences between DNA and RNA.

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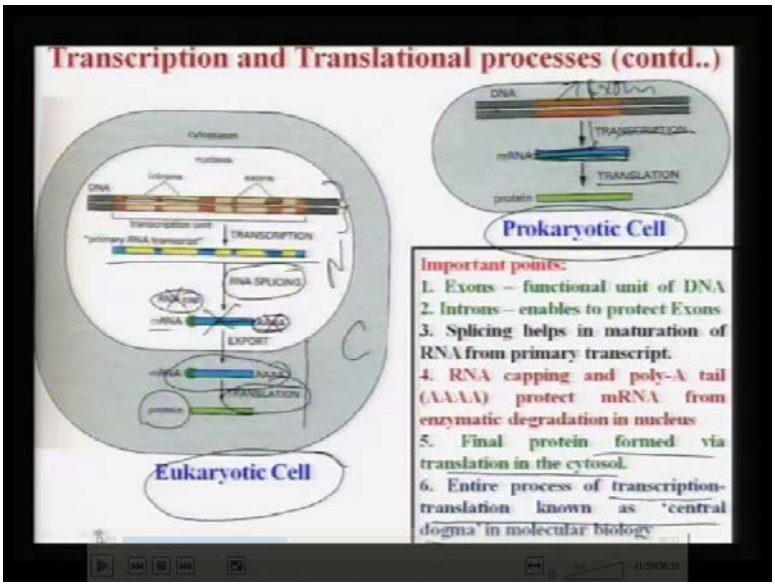

Now, if the DNA double strand is... And this is a particular thing that you know. This is a more, few of the translational processes. Now, this is your double strand DNA, which is there inside the nucleus.

Now, it undergoes transcription. Now, how it undergoes transcription? Like ((you know there is a)) single strand here, there it is just being dissociated and it forms only one single strand, that is what, is RNA molecule. Now, there is two couple of things that requires explanation here. You have the Exons and Introns. What are the Exons? Exons is actually functional unit. Now, which is red in color, you see that becomes blue in the case of the RNA. But, exactly their expression also is same. So, Exons here, what I mean by this? Is the red one and this is here the blue one. So, that total expression of these Exons in the DNA will remain, almost similar in case of, the RNA also. And Introns, Introns enables to protect Exons. So Introns is this non-red color thing. And, these Introns are typically required in DNA, to protect the RNA part of the…, to protect the Exons.

Now, once this transcription makes this primary RNA transcribe, then what will happen? These RNA will undergo further Biochemical processing which is known as the splicing. So, splicing is nothing but, that is a biochemical process, which makes primary RNA to mRNA. mRNA stands for messenger RNA. So, there are three types of RNA. There is one is mRNA, that is messenger RNA. And, this is the coat for proteins. That means that

whatever proteins, will have amino acids sequence, that coat is there inside the messenger RNA or in other, in other way messenger RNA carries the coat for the amino acids, which will be finally, formed outside the nucleus.

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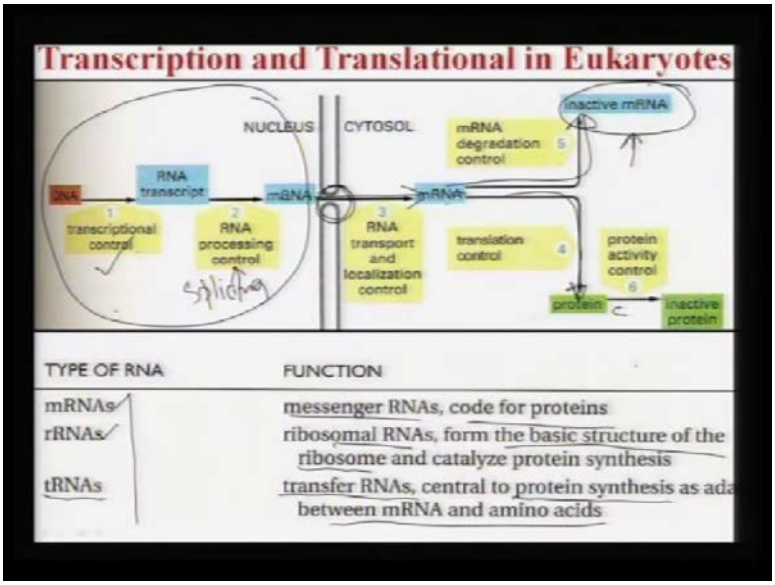

There is another RNA called rRNAs. That is called ribosomal RNAs. Now, ribosomal RNAs, this forms a basic structure of the ribosome. What is ribosome? Ribosome is the protein synthesis unit, which is there in the cytoplasm. Right. Outside the nucleus. And there is called tRNAs. tRNAs means, this is called transfer RNAs, that is central to protein synthesis and this is that, between mRNA and amino acids. So, there are three primary RNAs or three important RNAs: one is an mRNAs, one is rRNAs and one is the tRNAs.

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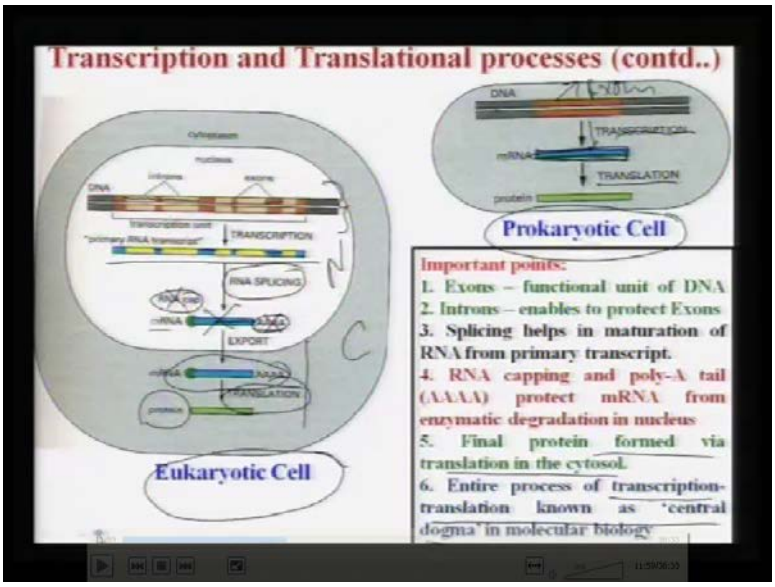

Now, what you see in this particular? This is the eukaryotic cells, in the case of, eukaryotic cell, your transcription process; everything is computed within the nucleus itself. So this is your A and this is your C. N stands for Nucleus C stands for Cytoplasm. Now, translational process, that occurs, outside the nucleus in the cytoplasm. And, they are the ((protein...)), ultimately synthesized.

Now, what you see here? In case of that mRNA; the mRNAs are two things. One is the mRNA cap and one is that AAAA. That is that poly- A tail. Now why these two things are required in case of the mRNA? RNA cap actually protects RNA capping and then, AAAA poly- A tail, this actually, protects inter RNA molecule from degradation.

Now, if there is no capping and there is no poly-A tail, why it is known as poly-A tail? Because there is multiple A and that is why, it is called poly-A tail. Now, if there is no capping and no tail here then, what will happen? This RNA, mRNA cannot come out of the nucleus. And, they itself will be degraded by certain enzymes inside the nucleus. In other words, this mRNA functions will be lost. They will not be able to carry the coats for the proteins, for synthesis outside the nucleus.

Now, what is the requirement for this RNA cap and poly-A tail? RNA cap and poly-A tail is essential to protect the mRNA molecules. If there is no cap and no tail then, what will happen? This mRNA will be degraded by the attack from the enzymes inside the

nucleus. So this mRNA cannot be synthesize by, mRNA cannot be protected. Now, what will happen? The final protein, that will be synthesized from mRNA, via the process called translation that takes place outside the nucleus. So, that takes place in the cytoplasm itself. And entire process of transcription and translation it is known as the central Dogma. As, I have already mentioned in the last lecture also. Now, in case of, prokaryotic cell what will happen ?You have the DNA and you have that Exons here.This red one is Exons and when you see that, it is the transcription process, this red one Exons actually transformed to completely blue one and then, in the translation process it goes to the protein synthesis in the Eukaryotic, prokaryotic cell for example, in bacterial cells the transcription and translation process both, occurs in the cytoplasm because there is no distinct nucleus in the bacterial cells. So, all the DNAs, they are like loosely spread out in the cytoplasm. Therefore, transcription, translation processes actually take place in the cytoplasm itself. Yeah. This is what, I have already mentioned to you that, up to the DNA, through the mRNA process, transcriptional control and RNA processing control; RNA processing control, which is known as the Splicing.

So, RNA processing control everything takes place inside the nucleus. Now, this mRNA is transported from nucleus to cytoplasm by the nuclear pore. So, this is the nuclear pore and this, was this mRNA is coming out of the nuclear pore then, if it goes to the translational control; then it goes to the protein and protein activity control. If it goes to the mRNA degradation control, then it becomes inactive mRNA. So, outside the cytoplasm also, mRNA can become active or can become inactive, depending on, whether, it is attacked by some hormones or some enzymes and so on. So, if it is not active, then it goes to the inactive mRNA and eventually it will be degraded. And, if it is active and it goes to translational control, then it makes the protein, with the C terminal at the right and N terminal at the left.

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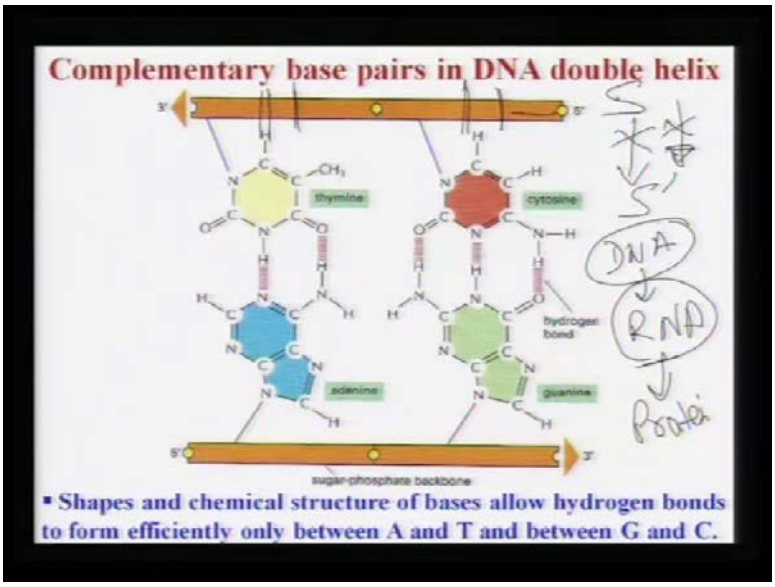

So, and also, another thing that you know that in the DNA structure, if it is broken at these different junctions, then what will happen? The individual strands, suppose, this is S, it cannot make S prime because the strand is now broken. Therefore, DNA replication process are from one DNA to multiple DNA that, replication process also cannot takes place. Also, it cannot take place is another thing is that DNA to mRNA or DNA to RNA transcription process because your basic structure is now faulted or this basic structure is now broken. Therefore, this Genotoxicity means that is the DNA damage. And, DNA damage means this (( )), with this DNA can neither form complimentary this backbone chains and therefore, the DNA cannot be replicated; nor this DNA can produce RNA and can produce further proteins. So, entire transcription, translations will control also, will not be activated because of faulted structure, also, that replication process, will not be also, not activated because of the DNA becomes damaged now.

So, this is known as the Genotoxicity process. Now, you know, what is the consequence of the damage DNA? And, that once this one of the strand or both the stands are broken up in various places, then both the DNA replication, transcription and translation process cannot takes place in the nucleus or in the Eukaryotic cells.

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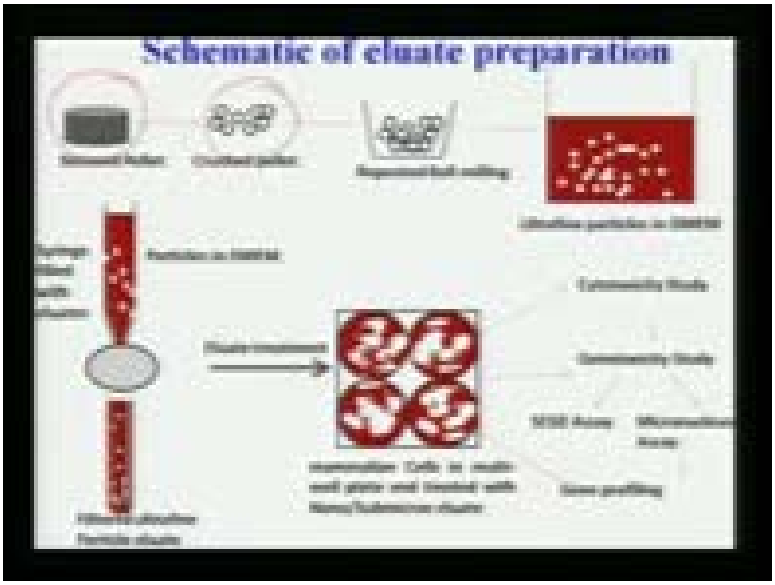

Now, the question is that, how we can prepare this Nanoparticle? Although, I have mentioned it, in very first, in the last lecture. So, let me, just refresh your mind, you have the Sintered Pellet. This sintered pellet means, it is a Compact, of the some powder based material and then, if you crush it, to make coarser particles, then, you can do it the repeated ball milling process here. After, Repeated Ball Milling, it is ultra-fine particles in the DMEM, that is the dublacos modified eggers medium that typical calcium medium for the, of ((prokaryotic and)) eukaryotic cell. Then, after you do that medium then you can filter it, with certain filters, lets a point to micron filter and so on. So, that very fine particle can be dispersed in the DNA. Now, after this, you treat this Eluate. So, this is the four well plates, for example. So, 4 well plate means, like there are four different wells and each well, you would, you put the cells and then, you treat them with the Nanoparticles carried by the DNA.

Now, once you do that, then you can do the Cytotoxicity, as a…, that is called MTT Assay, then you can do the Genotoxicity as a, like a Single Cell Gel Electrophoresis Assay and Micronucleus Assay and finally, you can do Gene profiling also.

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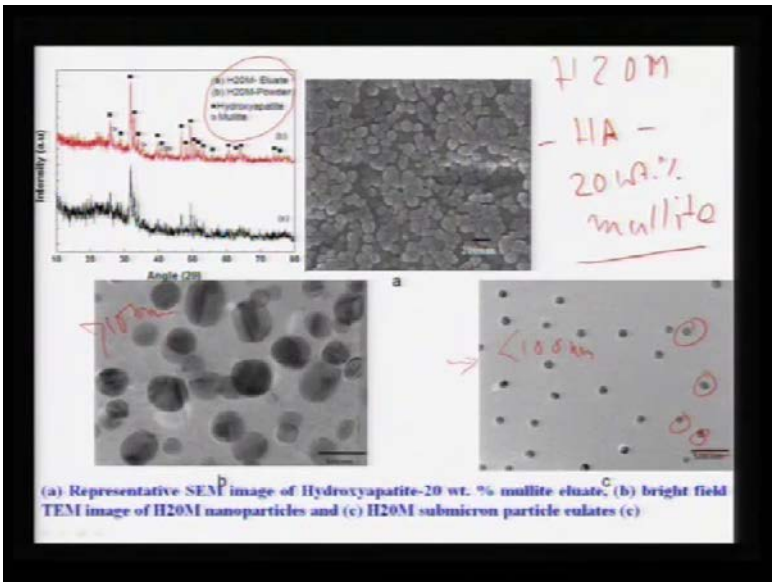

All those things, I will show you, that how to carry out in the laboratory scale. Now, the other things that, I have that, I would mention here, that the, as a case study, what I will show that Hydroxyapatite, which is a bioactive material and which is also inorganic composition of the human tissue or bone. Now, Hydroxyapatite as a monolithic material, it is very difficult to be used because it is very brittle in nature. So, therefore, what we have developed? We have developed H20M stands for Hydroxyapatite HA 20 weight percentage Mullite. Why Mullite? Mullite is essentially, used to increase the physical properties, like strength, like fracture toughness of this ceramic material. So, that they can be more suited for a long term applications and load bearing applications.

Now, once we make this; so, therefore, if you start with this one, so, you have to start with that HA 20 Mullite. So, this is the Sintered Pellet. So, from this Sintered Pellet, you crush it, you put it ball milling and then, you filter it and then you make that DMEM. So, this ultra-fine particles loaded DMEM solution and this loaded solution, then it is strutting the human fit elastoplasts cells and this is what two types of particles, you can see. This is, less than 100 Nanometer and this is greater than 100 Nanometer. You can clearly see this is your five hundred nanometer bar. So, therefore, individually these particles are like less than hundred nanometers.

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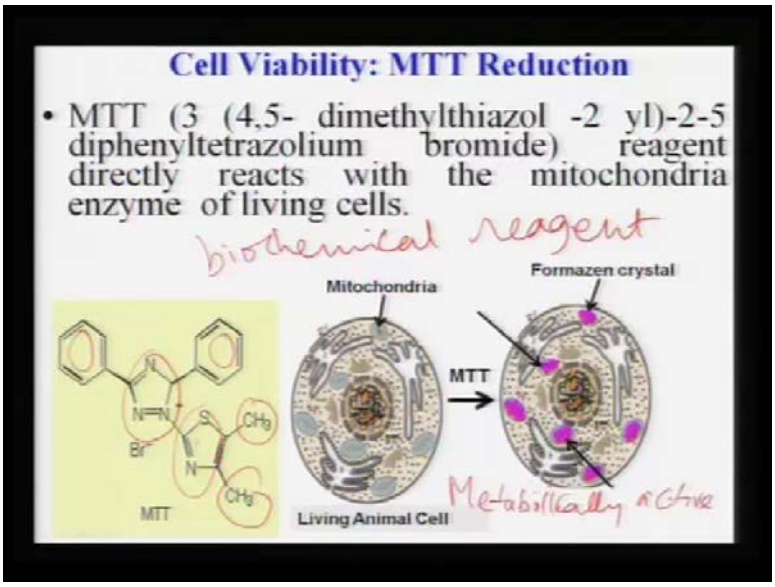

So, then we do this MTT Assay, So MTT Assay is essentially, what is MTT Assay? MTT Assay is a particularly, biochemical reagent and this is the typical structure of the MTT. What you see in the MTT? You have several Benzene rings, this is one ring and this is another ring and these rings also, there is, different other bonding here, in this case and there are methyl group are also loaded here. So, this MTT, when they are reacting with a live cell then, what will happen? This MTT will react preferential with the mitochondria of the metabolically active cells. And then, what it will make? It will make the Formazan crystal. And, this Formazan crystal, they will be violet in color. Therefore, the number of ((...)) cells are there in the solution; the more intensity of this violet color, the solution will give, when it will be analyzed by the ((…)) micro plate of the Optical Density.

You understand, what I am saying? So, if there is less number of ((…)) cells, this violet color, intensity also, optical density also will be less. So, from that optical density, you can directly say that higher optical density means more number of viable cells is there less optical density means less number of viable cells there. And, this viable means, here, as I have mentioned, this is called metabolically active cells. Metabolically active cells means that is, mitochondrial active cells that means mitochondria are still active and they are, like in the typically, this mitochondria is still. Mitochondria are known as the power house of the cell like, all this ADP, ATP transformation, everything is occurring in the

mitochondria. So, that means is the mitochondria is active means, cells are getting enough energy for its survival.

So that is the basis for the MTT deduction process.

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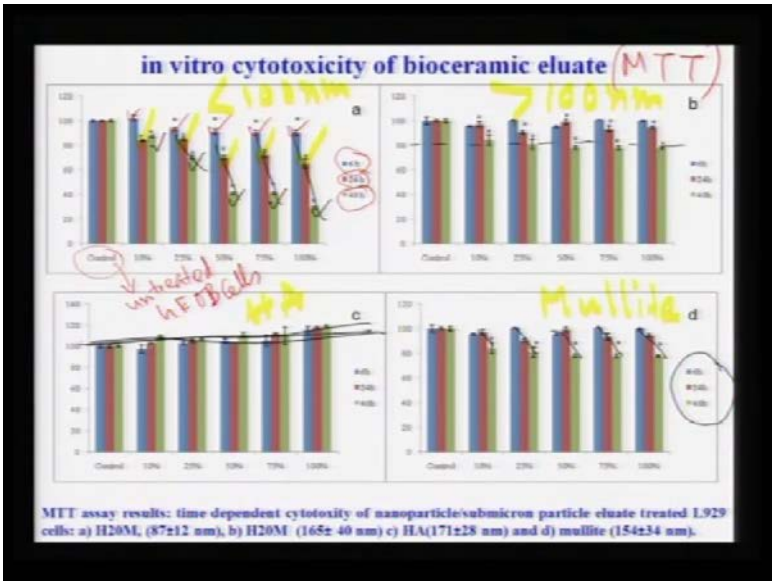

Now this is the MTT results. Now what you see here? MTT results here, that you know that there are ten percentages to hundred percentage Eluate concentration here. And this is control. Control means untreated cells.

So, control means untreated human ((…)) osteoblast cells and this is ten percentage concentration means total in this solution that DMEM loaded solution here; it has only ten percentages of the particles. And so, ten percentages of the particles are loaded here. Now, these are three different timescale, this MTT deduction process take place. One is six hour; one is twenty four hours and one is forty eight hours. That six hour, twenty four hours, forty eight hours, from which point it is counted? Like, the moment in a culture medium, you treat or you inject the particles the Eluate particles. Then, from after those six hours, you take your MTT values, optical density values. Now, what you see in the six hours? Typically optical density values are relatively higher, that means most of the cells are now, survival.

Now, after twenty four hours, what you see? That, this number of cells is, decreased for all concentration. Do, you agree with that? And, this is the case for, the less than hundred

nanometer particle cells. This is the case, for the greater than hundred nanometer particle cells. This is, for pure Hydroxyapatite and this is for pure Mullite.

Now, what you see here? That this is, the less than hundred and this is the greater than, hundred nanometer. So, if you do notice that, depending on, the treatment time six hour or twenty four hours; there is a general tendency of decreasing the MTT values, with increasing the time duration.

Now, if you look at the forty eight hour values that means this is the green one. And, green one also, in all the cases, the MTT values are less than that of the twenty four hour case.

So, what it means? It means that, the toxicity potential, increases with more treatment time or increase in treatment time. It is because from six hours to twenty four hours to forty eight hours your MTT optical density values are progressively decreasing independent of all the concentrations.

Now, in case of, greater than hundred nanometer, what you see here? This kind of progressive decrease, you see this decrease, this decrease, this decrease is significantly lesser because in all the cases your MTT values is eighty percentage of higher. You understand, what I am saying? So, that means when you are treating the same solid culture solution with the same cells, with the Nanoparticles less than hundred nanometer.

Then, the toxicity values are much more, compare to, that when you are treating with the more than hundred Nanometer particle cells. Is it clear? In case of the Hydroxyapatite, you do not see any toxicity at all. Because your MTT values are at hundred percentage or little bit above hundred percentage. In case of Mullite, again your MTT values is decreasing and this decreases is also, more or less independent of the concentration of six or twenty four and forty eight hours.

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So, this is the typical cell morphology, after treatment with different concentration of that H 20 M Hydroxyapatite twenty percentage Mullite, of less than, the hundred Nanometer. And, you can see that morphology also, it changes depending on the treatment concentration or treatment time.

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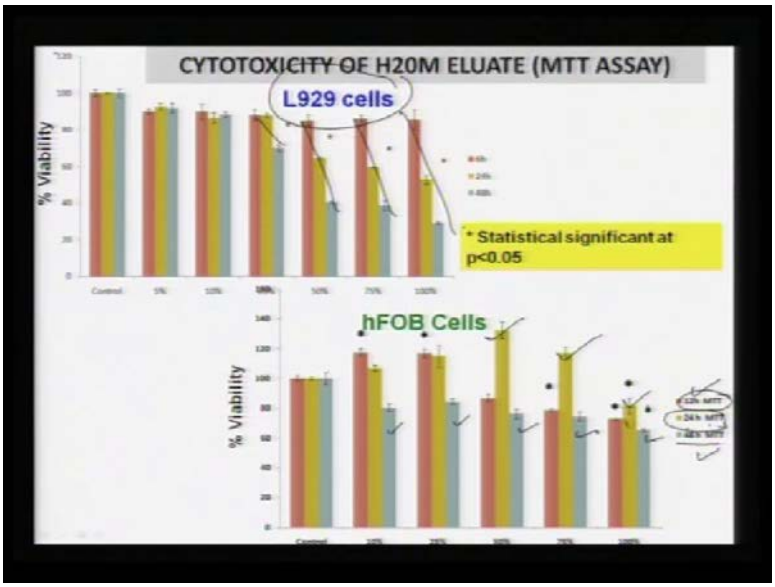

Now, in case of the L929 cells, if you see this decrease is also significant. But, here, in case of, the hFOB cells, there is some change, which could not be established because

after twenty four hour treatment. That MTT values is increasing whereas, forty eight hours this MTT values are decreasing in dependent of the concentrations.

So, we do see, there are some trains, like if you consider the twelve hour to forty eight hour, from twelve hour there are two forty eight hour, the MTT values decreases. But, we cannot confident to say, based on this results, that it decreases with increase in time because in the twenty four hour case, we have seen independent of the concentration that MTT values decreases from twelve hour.

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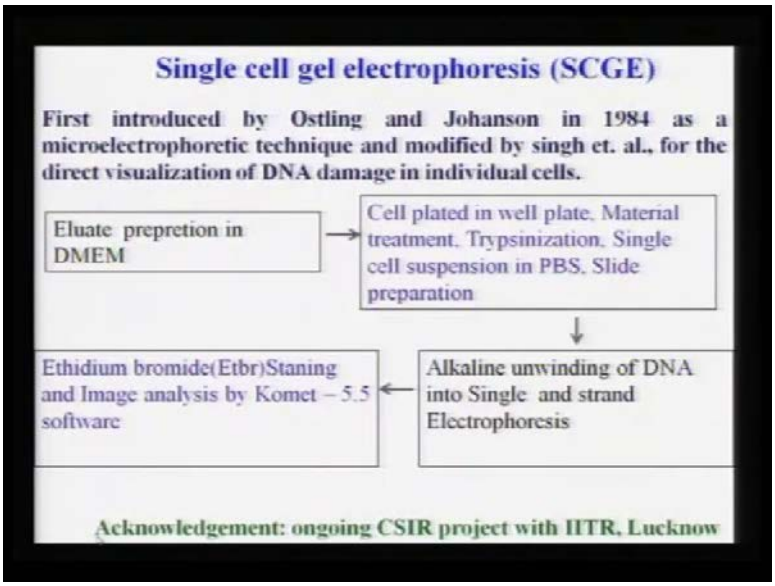

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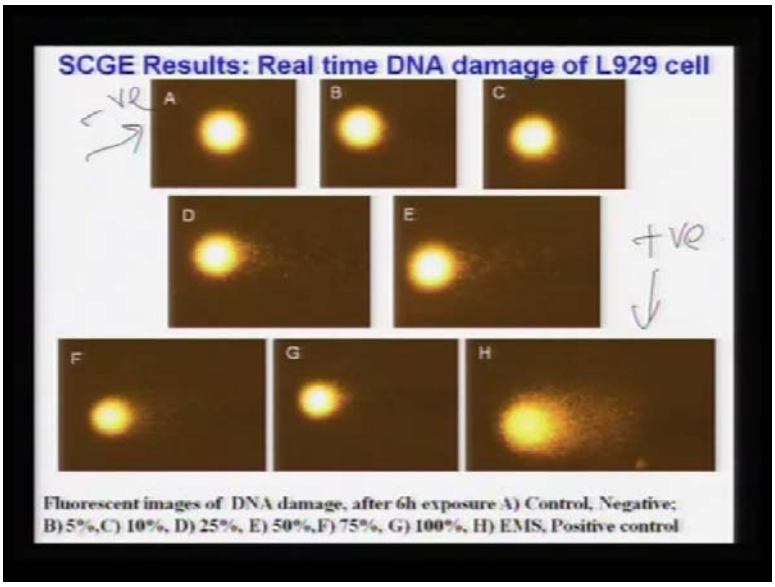

This is Single Cell Gel Electrophoresis, which I have already, mention to you in the last lecture. It is essentially, it shows that real time DNA damage.

So, this is the negative control and this is called positive control. Positive control means that we use some solution, when you treat cells with that particular solution then it will show Extensive Genotoxicity. Extensive Genotoxicity means if you see the DNA fragmentation, so, all these dots essentially reflect the DNA fragmentation around the nucleus of the cells.

In case of the negative control, you do not see any DNA fragmentation. And, that is what is mentioned. When the, that is what you also shown here negative and positive control.

Now, if you treat them with five percentages or ten percentages, twenty percentages and fifty percentages, hundred percentages then, what you notice? With increase in concentration that your DNA damage capability is also, to some extent increases.

However, this increase, if you compare with this positive control, it is much less. That means that although, this Hydroxyapatite twenty percentage Mullite particles, they can contribute to the Genotoxicity property, but at a much less significant manner, compared to the positive control material.

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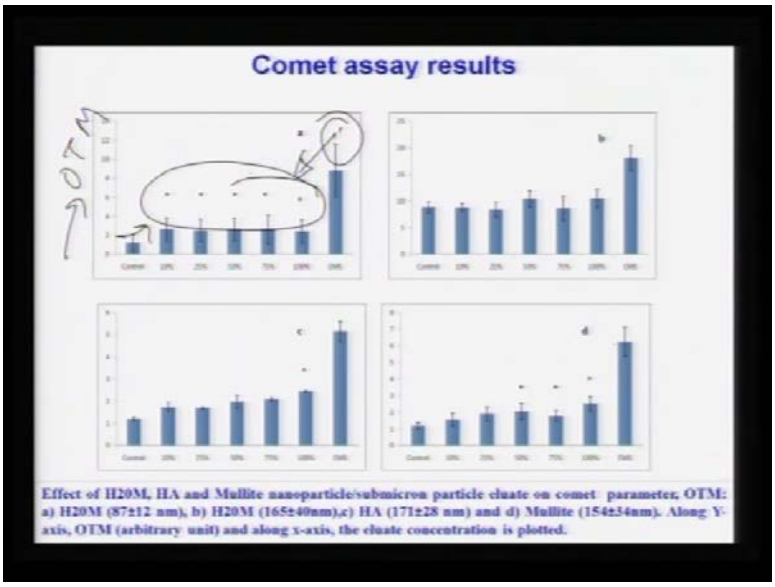

So, this is the quantification of the Comet assay results. Now, how it is quantified? Normally, within with some software, one can use that Olive Tail Movement. Olive Tail movement means that quantify that how much comet shape is distorted? And, how much DNA is been damaged? Here.

And, this Olive Tail Movement, when you plot it with concentration, what you see? That they show the statistically significant values, compared to this negative control as well as, the positive control.

But, olive tail movement values here, it is much less than that of the positive control, in all the cases.

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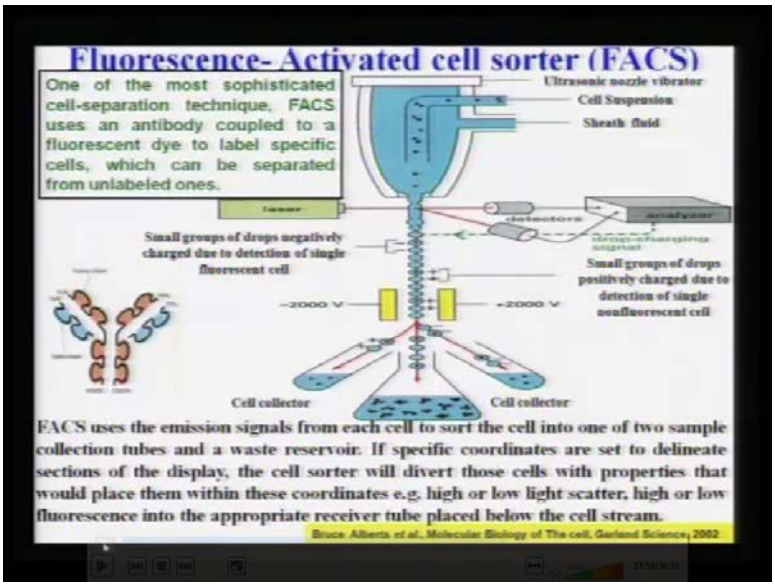

Coming to the fluorescent activated cell sorter, till now, you know that what is the toxicity at the cellular level? Now, we would like to know that after you treat the cells with the Hydroxyapatite, Mullite solution that what is the fraction of the treated cells? They are in the Apoptotic stage or they are in the Necrotic stage or they are in the late Apoptotic stage or they are live cells.

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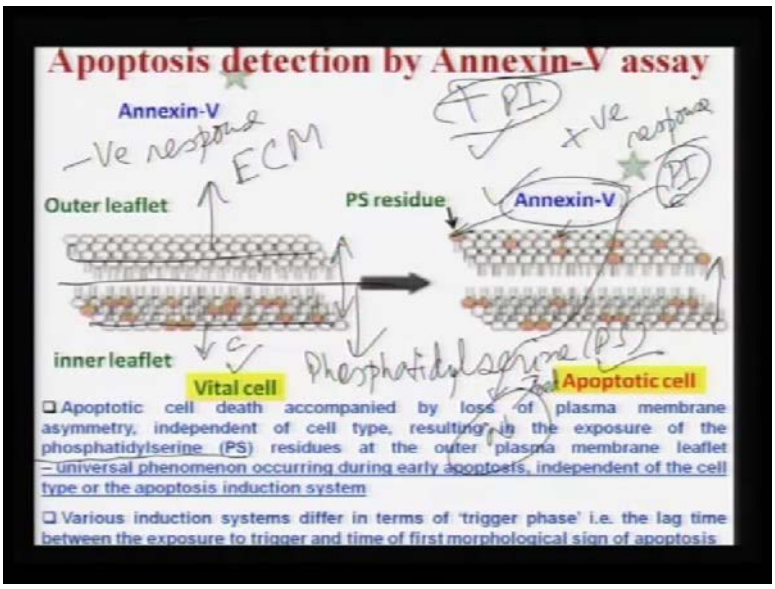

What is the difference between apoptotic and vital cells? Vital means viable cells.

So, these are cells alive, these are cells are apoptotic. Now, this is the typical structure of the plasma membrane; which is a double layer kind of a structure. So, this is the top layer, which is exposed to ECM, here Extracellular Matrix.

This is the bottom layer, which is exposed to cytoplasm C. Now in this double layer structure, there are certain molecules. Which is known as Phosphatidylserine? Now, these molecules Phosphatidylserine, so, this is called known as the PS molecule. Now these PS molecules, they are actually appearing here, is a colored molecule, here in this slide.

Now, when it is a live cell or vital cells; this Phosphatidylserine molecules, they are exposed to the cytoplasm or bottom side or inner leaflet. When the cell is apoptotic, then what will happen? There is asymmetry of the plasma membrane structure. Asymmetry means some of the Phosphatidylserine molecule, will now be exposed to the Extracellular matrix in the outer leaflet.

But, in the live cells, all the Phosphatidylserine molecules, they are exposed to the cytoplasm only. Now, once they are exposed to the Extracellular matrix. Now, there is a dye called Annexin-V, it is like another dye, like you know MTT type of dye.

Now, this Annexin-V, they can be club together with this Phosphatidylserine residue. And, as a result, what will happen? That, the more the.., Annexin-V molecules are being reactive, with these Phosphatidylserine molecules. What it will indicate? It will indicate that, more number of cells is in the apoptotic stage. Is it clear?

So, if the Annexin-V dye molecule shows the positive response, I repeat, if the Annexin-V dye molecule shows the positive response, what it means? The cells are in the apoptosis stage this is the called positive response. If the Annexin-V molecules, shows a negative response, what it means? The cells are now; live cells or cells are vital cells.

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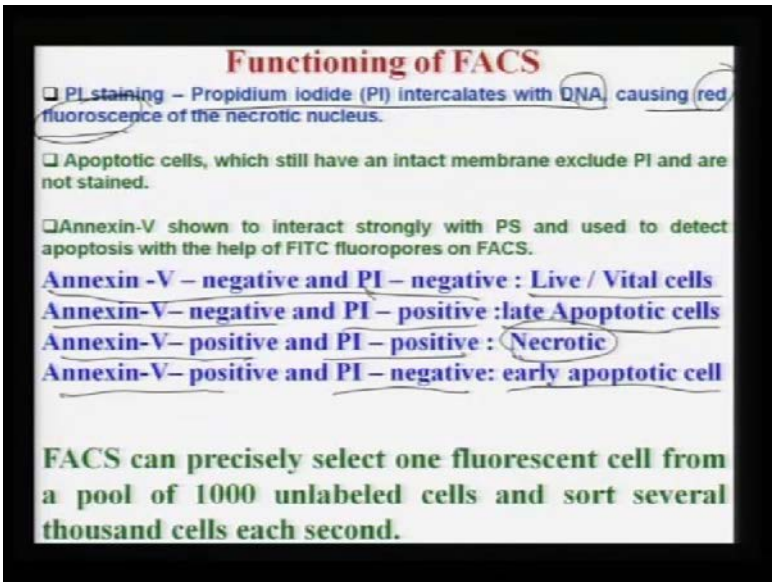

Now, there are other molecules, which are known as the PI that Propidium Iodide. Now, Propidium Iodide typically intercalates with DNA and causing red fluorescence, of the Necrotic cells. Now, this is, you have seen about that this Annexin-V treatment, it directly reacts with the plasma membrane; outer leaflet. Now, there is another molecule, which is known as Propidium Iodide. What it does? It goes through the cytoplasm, then it goes to the nucleus, it reacts with the DNA of the nucleus and then, if the cells are Necrotic, then Propidium Iodide will make red color nucleus.

Now, if the nucleus is becomes red, after this Propidium Iodide treatment, then, what will happen? Then, you can say that these many cells are in the Necrosis stage. So, there are two ways we are detecting. One is that Annexin-V and another one is the PI.

Now, if that Annexin-V is also positive, PI is also positive; then, certainly the cells are not viable at all; cells are all dead. If the PI is negative, Annexin-V is negative; cells are live cells; they are vital cells. Now, you can follow this slide very easily. First one, I have written Annexin-V is negative, PI is negative; cells are live cells or vital cells.

Now, I said that Annexin-V is negative and PI is positive; means cells are going towards the Necrosis stage. But, not yet, all the cells are Necrosis. We, call it as, late Apoptotic cells.

If Annexin-V is positive that means cells are Apoptotic, PI is positive; that means cells are already dead. Then, we call them Necrotic cells. If Annexin-V is positive and PI is negative, that means, cells are early Apoptotic cells. If we go back to this one, then it is what I said? I said if both the Annexin-V and PI are negative then, cells are live cells. No confusion at all.

If Annexin-V is negative, PI is positive; that means cells are going through the early Apoptotic stage. If Annexin-V is positive, PI is negative then, cells are going to the late Apoptotic stage. If Annexin-V is negative, PI is positive then, it is called late Apoptotic stage and if both are positive both are positive means like Annexin-V is positive and PI is also positive then, cells are Necrotic cells.

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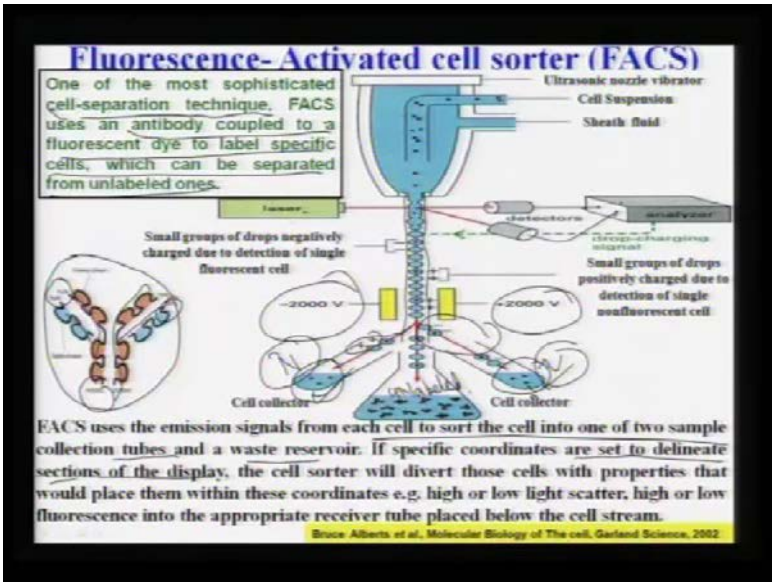

Now, how you can determine these using that fact? You send through that cell suspension, which are treated with Hydroxyapatite Mullite and then, what will happen? You allow them to go through a particular stream, jet like a pattern here, where you also apply certain voltage, externally like 2 kilogram voltage. Voltage difference is 2000 voltage, 2 kilogram voltage.

Now, here you are putting this laser light and their detectors and the analyzers. So, this detectors and analyzer and this laser system, they are, you are actually allowing the quantification to take place. And, depending on whether the cells will finally, carry

positive charge or negative charge and how they are stained with this Annexin-V and the PI, from that it can be sorted.

So, Fluorescence-Activated means this Annexin-V is a fluromolecule, PI Propidium Iodide is also fluoromolecule. So, when you treat the cells with Annexin-V and Propidium Iodide, they will respond differently, depending on whether the cells are in the live cells, Apoptotic cells or Necrotic cells and from the color difference, through this laser light you can essentially, detect how much color is red ?how much color is different color ?With the Annexin-V staining and from the stain cells, each of the cells can be monitored by this analyzer and then, you can quantify that, how much cells are live cells? How much cells are early Apoptotic, late Apoptotic or necrotic cells?

So, that is, what has been mentioned here, that facts, actually, uses this emission signals, from each cell to sort the cell into one or two sample collection tubes and waste reservoir. If specific coordinated are set to delineate sections of the display. Specific coordinates means you tell the system that the test tube, which is placed at this forty five degree angle, plus forty five degree or negative minus forty five degree angle that will be vital cells, which is now, which is in the minus forty five degree, they are necrotic cells. And similarly, all other type of cells, you can essentially, mention. And, then system will tell, will guide in such a way, that you know, exactly vital cells will be going to the plus forty five degree test tube minus, Necrotic cells will go to minus forty five degree test tube and so on. And therefore, you can actually collect the cells, which is vital, which is Necrotic, which is late Apoptotic.

So, Cell Sorter means depending on whether, the cells are live or Necrotic, this machine will be able to sort the cells or will be, able to distribute, distinguish the cells depending on whether it is vital or Apoptotic cells and this is one of the most powerful cell separation technique. Cell separation means a distinguish cell depending on whether there is antibody, coupled to a fluorescent dye to label specific cells, which are to desperate from the unlabeled ones.

So, this is your unlabeled cells and this is your labeled cells. And, what is Antibody? Antibody is typically another protein and then, it has a typically NN here and CN here, as you can see, this is that one type of protein molecule; this is another type of protein molecule. So, it has a typically complicated structure.