Introduction to Biomaterials

Prof. Bikramjit Basu

Prof. Kantesh Balani

Department of Materials and Metallurgical Engineering

Indian Institute of Technology, Kanpur

Module No. # 01

Lecture No. # 10

Cell Apoptosis-II

So, we will continue this FACS analysis. So, here it is, another set of figure, which is little bit more details, as you can see. So, you have a flow chamber here. So, from flow chamber, individual cells are being charged particles here, and in these charged particles, they will go in the positive side and negative side.

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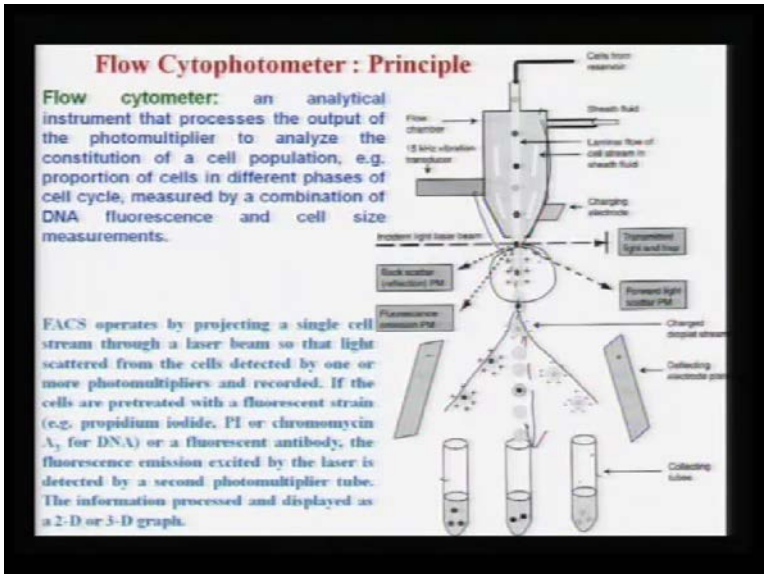

So, negatively charged particles will be attracted towards the positive deflecting electrode, and negatively and positively charged cells, will be deflected towards the negative electrode, right? So, this is based on a very simple technique, and then, these uncharged particles, they will be going to this, straight.

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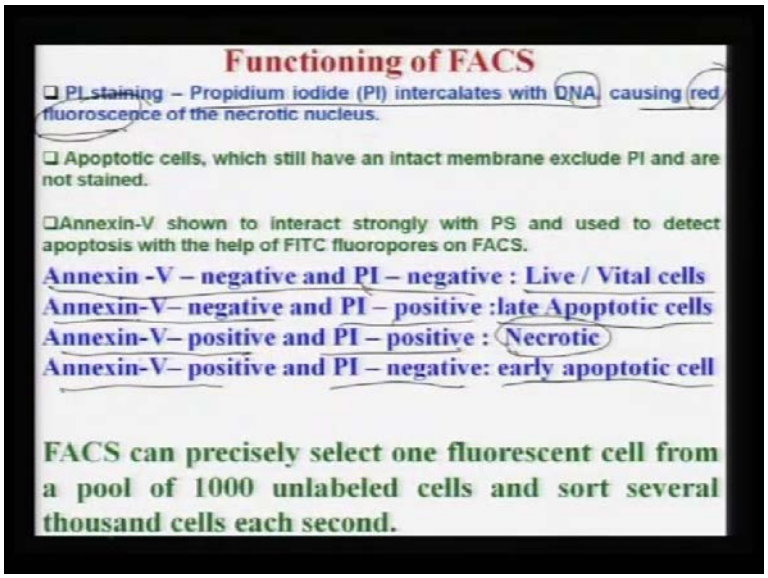

So, these charged cells, they will be collected at two different test tubes, different test tubes. And now, let us see that how these results will look like.

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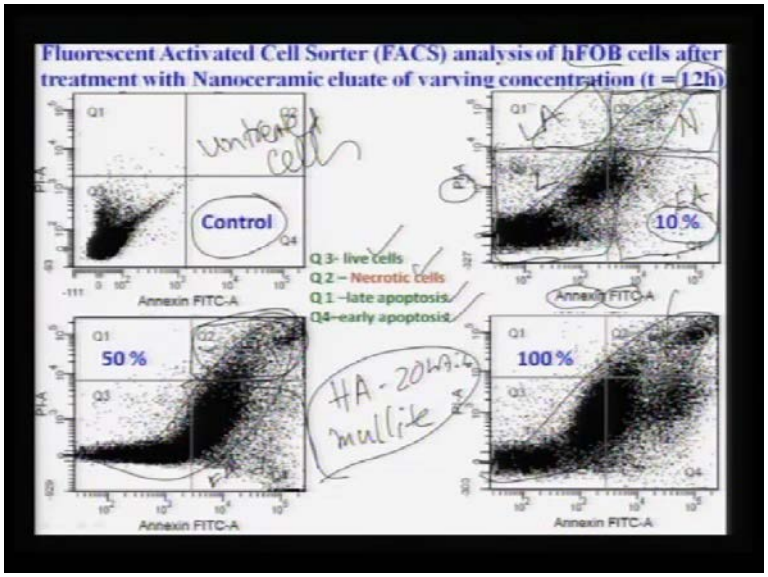

This is that hydroxyapatite 20 percent mullite particles. The eluate preparation and the particle preparation are the same, as I have discussed earlier, in case of the cytotoxicity experiments and single cell (( )) experiment.

So, control means, essentially this is like untreated cells. So that hFOB cells, hFOB stands for Human Fetal Osteoblast Cells. HFOB cells, when they are treated with h 20 m eluate, then after 6 to 12 hours, what are the fractions of the live cells. Fraction of the necrotic cells, fraction of the late apoptosis cells, and fraction of the early apoptosis cells, how you can distinguish using the FACS machine and here is the results.

Here, you see that PI stands for propidium iodide. It interacts with DNA, causing the rate T nucleus in case of the necrotic cells. Annexin, you know that annexin 5 is another a dime, and then, FITC is the steropore. So, annexin, FITC - they in combination, they will stay indifferently if it is apoptosis cells. So, what are the different coordinate cells? Q3, that means, this one is for the live cells. Q2, that means, this one diagonally up, that is for necrotic cells. Q4, that is for the early apoptosis cells EA. Q1, that is, for the late apoptosis cells.

So, what is the pattern, evolution pattern you see? It is more like a sigmoidal, e sigmoidal curve type of pattern. You see that after this treatment, cells are following this kind of pattern. Now, let me first tell you each data point here, you see that each data point. So, each data point corresponds to the information of one single cell. Now, after 10 percent eluate treatment, that means after 10 percent of the hydroxyapatite 20 percent mullite eluate, what you see your life cell coordinate, this is your life cell coordinate. Here, you have most number of the cells that means your most of the cells are live. However, you too see noticeable cells in the necrotic fetch. Also, noticeable numbers of cells are in the early apoptosis with the few cells, they are entering in the late apoptosis stage.

When you put this treatment as 50 percent, what you notice here, that more number of cells are now going to the early apoptosis phase, and also similarly, higher number of cells compare to 10 percent. If you see that concentration of the dots, right, concentration of the dots in the necrotic stage is much lower after treatment with 10 percent compare to the concentration of the necrotic phase here, after 50 percent treatment. If you now see when they are treated with 100 percent eluate, you see more number of dots are here, and more number of dots means, more number of cells are in the necrotic stage.

Another important thing, if you notice the concentration of the dots in the early apoptosis coordinates, look at the slide here, and you yourself will be able to realise that if you increase the concentration of the eluated particles from 10 percent to 50 percent to 100

percent. If you see the number of dots in the early apoptosis coordinates, increases progressively from 10 percent to 50 percent, from 50 percent to 100 percent. Clear?

So, therefore, that more cell fractions of the total cell populations are increasingly entering into the early apoptosis stage, when you are treating them with the progressively higher and higher concentration of the hydroxyapatite 20 percent eluate concentration. Is it clear? Now, you know how to read the FACS data right. So, you have 4 different coordinates and in this 4 different coordinates, you have different dots. Each dot corresponds to one single cell information ok.

So, you can quantify using the standards of pair that what is the total fraction of the cells that are now in the apoptosis stage, early apoptosis, late apoptosis necrotic and all this stage.

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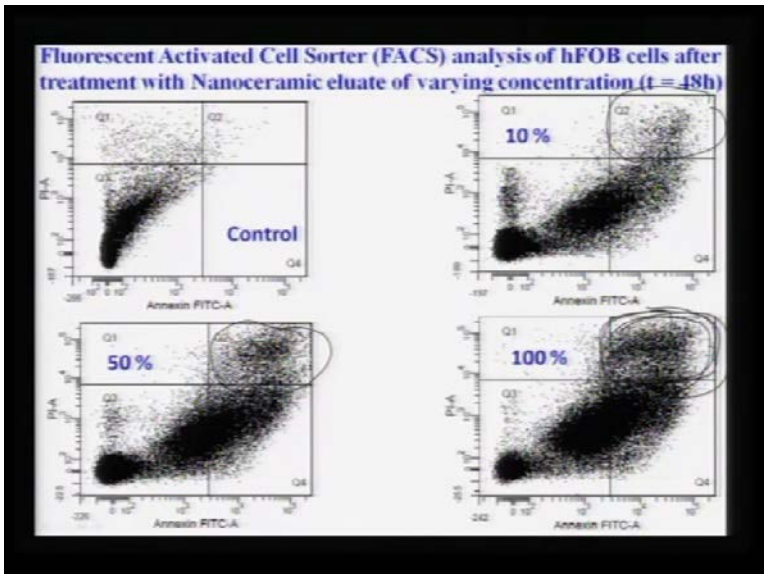

Now, what happens when you treat them for 24 hours? This is control means again is the untreated cells. There is no treatment of the hydroxyapatite 20 percent mullite particles. In case of the untreated cells, what you notice here, that in the 10 percent concentration, your some cells fractions are going to the early apoptosis.

50 percent more number of dots means, more number of cells are in the early apoptosis. 100 percent clearly more number of dots here, that means more number of cells in early apoptosis. So, there are 2 things. One is that your concentration of eluate and one is your

time treatment time. So, with 24 hours, you see that with the higher and higher concentration, more number of cells is going to the early apoptosis stage and necrotic stage.

What happens in the 48 hours treatment? 48 hours you notice that really large numbers of cells are going to the necrosis stage you see and these numbers, if you qualitatively compare, these numbers are more than what you have seen at the corresponding concentration after 12 hour treatment. Do you understand what I am saying? I am saying that let us now compare what happens after 10 percent eluate treatment, after 12 hour and after 48 hours. So, after 48 hour, here you see that 10 percent, 100 percent concentration, more number of cells is going to the necrotic stage. That means the severity of the cell death, increases with increasing concentration or with increasing treatment time.

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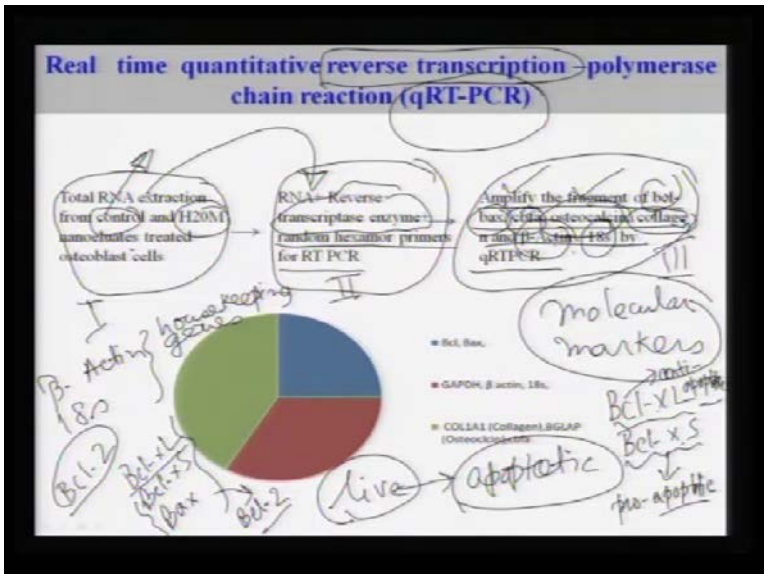

Now, in a next few slides, I will be showing you that how different molecular markers, molecular markers means different genes which are involved in the bone cell differentiation process. Bone cell differentiation means when it is going to live cells to apoptosis cells right. Bone cells, live cells your DNA will have certain arrangement, your plasma membrane will have certain function. In the apoptosis cells, your plasma membrane also will have different functions and DNA will not be able to replicate and make the protein or synthesize the protein like in the live cells. Agree on this point?

So, therefore, the ability to DNA to transcript and translate to make the proteins or the ability of DNA to go through the replication process, that will be different depending on the cells are live or the cells are apoptosis or necrotic. Now, this is known as that qRT-PCR technique which is known as q stands for quantitative. RT stands for reverse transcription, PCR stands for polymers chain reaction.

Now, you can now follow because you you know that how from DNA, how this RNA is produced and then, these proteins are produced. Now, total RNA is initially extracted from the control. What is control here? Control means untreated cells that mean, cells which has not received any treatment of the particles, like 100 to 20 percent mullite particles. So, total RNA extraction, if your total RNA is extracted from the control untreated cell as well as H20M nano eluates. H20M stands for hydroxyapatite 20 percent mullite nano particle eluates.

Now, this RNA which is extracted plus reverse transcriptase enzyme, so reverse transcriptase enzyme means, if you want this reverse transcription process. Reverse transcription means what? DNA to RNA is transcription. Reverse transcription means RNA to goes back to DNA. So, that is why I often say that all these biological terms are very easy, if you follow it in a logical manner. So, you require certain enzyme to be added to RNA in order for the RNA to go back to the DNA DNA double helix pattern and random hexamer primers for the RT PCR. So, they are primers, I will come back to that. Now, you have to amplify the fragment of the Bcl, Bax, CBFA, osteocalcin, collagen, beta-actin, 18s, by qRT-PCR. Now, what is what it what it is means? What is the meaning of this statement?

So, up to the step on it is clear that you can extract RNA from the untreated cells as well as the treated cells. Step 2 is also clear. Now, reverse transcriptions means mRNA goes back to DNA, but you require certain enzyme to be added and some primer to be added. Now, these primers are essentially combination of the nucleotide. What is the combination of the nucleotide?

You you know for DNA, what are the nucleotides that you require, ATGC. So, certain sequence of the nucleotide you have to act to the RNA to make RNA go back to the DNA. Why? Because RNA has different nucleotides because RNA has AUGC, Adenine Uracil Guanine and Cytosine, whereas, DNA has adenine thymine guanine and cytosine.

Now, what is stage 3? Now, RT PCR. What RT PCR does? After you do this treatment this RNA plus reverse transcriptase enzyme, then what will happen that it will produce certain fragment of Bcl, Bax. These are genes, CBFA, they are genes, Osteocalcin is another genes and collagen that is another genes.

These last 2 beta-actin and 18s, they are known as the house keeping gene. What is known as the house keeping gene? What is the name suggests? Name suggests is that housekeeping means, if you want total nucleus to be in the order. In order means, this nucleus should be able to replicate during the cell division process and nucleus you want then all this chromosome and DNA to carry exactly the genetic information. Then, you require certain genes to be very active in the cells.

So, beta-actin and 18s are the 2 genes independent on the cell type. They are always active in case of the live cells. Do you understand what I mean? I repeat independent of the cell type means, it does not matter whether it is a fibroblast cells, osteoblast cells, endothelial cells, you will always find beta-actin and 18s. These are 2 different types of genes which are present in the nucleus, so that the normal functionality of the nucleus can be maintained. So, in other words it is typically known as UBQS genes. That means it is present in all cell type. Now, why do we require to amplify the fragment? What happens, after this RNA transcription, RNA reverse transcription process, the DNA that will be produced and this Bcl, CBFA, osteocalcin, collagen, these genes that are expressed, expressed means the amount that will be formed of all these genes that will be very smaller in number or smaller in quantity, you have to amplify it. Otherwise, you cannot distinguish between the amounts of Bcl gene, amount of CBFA gene, amount of osteocalcin gene. Are you getting my point?

You have to amplify their expression. You have to amplify, so what the way they are expressed, you have to amplify to 10 times, so that you can distinguish the difference between these 2 ok. So for example, suppose if I say that the strength of the certain 2 materials, they are like 1.2 gigapascal and 1.1 gigapascal. So, you may see that 1.2 gigapascal, 1.1 gigapascal, it is 0.1 gigapascal difference. The moment I say, the strength of 2 materials is 1200 mpa and 1100 mpa, then you can immediately say that this there is a hundred megapascal difference, that means hundred megapascal is large.

So, there you were going down in the unit, so to amplify the difference. So, in in here, you have 2 expressions that Bcl, Bax, these are like different genes and these genes Bcl that is a class of genes. This is the Bcl 2 is a class of genes which are activated in osteoblast cells. Now, in Bcl family you have the different genes, that is, Bcl-XL gene, then you have called Bcl-XS gene. Now, Bcl-XS is the genes which are called pro-apoptotic genes. Pro means what? It will help in the apoptosis process. Bcl-XL is called anti-apoptotic genes. Anti-apoptotic means, this particular gene, if it is expressed more that means cells are more live or more vital, that means are not going to the apoptosis process. Is it clear?

So, I repeat so, Bcl-XL, Bcl-XS and Bax. So, these are 3 genes Bcl-XL, Bcl-XS and Bax. It is 3 genes. They all belong to the family of the Bcl 2 family of the genes. Now, this Bcl 2 family of the genes, they are important for this toxicity process, apoptotic process. However, in this family of gene, the first gene, this is anti-apoptotic. The 2 other genes, these are like pro-apoptotic. Pro-apoptotic means, if these genes are expressed more, that means cells will undergo definitely to the apoptosis process. If the first gene is expressed more, then cells will be, then cells will be more live cells ok. Therefore, in this first slide, what you what you what should be clear to you, that qRT-PCR process is helpful to identify the molecular markers. Molecular markers means, the genes here, genes of in today's which are helpful or which are responsible for the apoptosis process.

FACS machine is useful to quantify the fraction of the cells which are live, which are apoptotic, which are late necrotic, which are late apoptosis process but, FACS machine cannot identify which are the genes are expressed more or which are the genes they are responsible for this apoptotic process. Do you understand what I am saying? So, therefore, qRT-PCR is an indispensable process in understanding the molecular level of understanding of the apoptosis process.

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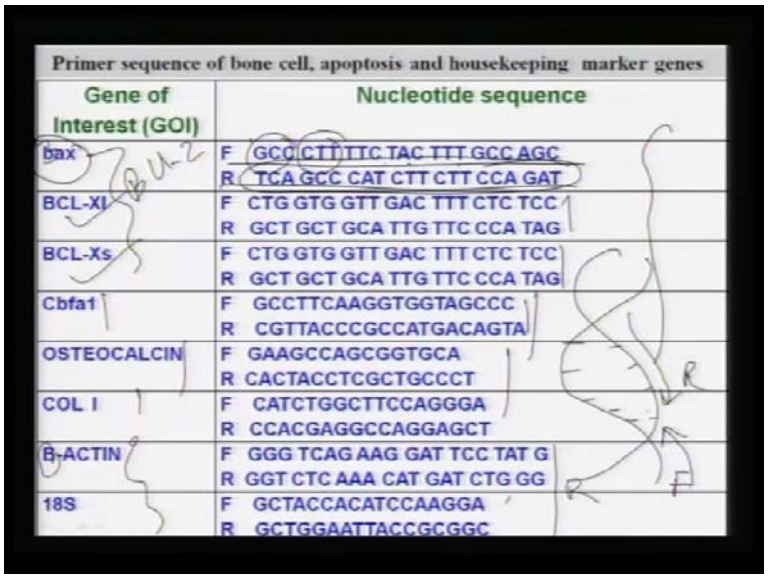

Now, in the molecular biology literature, it is called gene of interest or GOI. GOI here is not Government of India but, GOI is here genes of interest. Now, genes of interest as I said that the first 1 is the bax, BCL-Xl, BCL-Xs, these are like BCL 2 family of genes as I said earlier. Now, I told you right that what is called primer. These primers, they are forward transcription and reverse transcription. Why forward and reverse is required? Because RNA is your single strand, DNA is your double strand right.

So, 1 is forward, means 1 is, it will go this way. 1 is reverse means, 1 it will go this way. So, what is the prime? Primer as I said primer sequence means, GCC that means 1 guanine that will be followed by 2 cytosines. Then, comes CTT. That means, after 2 cytosine's, there will be 1 cytosine and 2 thymine's, TTC, TAC, TTT all those things. So, these are very specific to the Bax gene forward primer. Forward primer means, here this 1 would be simply added. You understand what I am saying? So, 1 is forward. Let us say this is forward and this is reverse. This is reverse means, this is reverse yeah ok.

So, this is the forward primer sequence that means nucleotides sequence. This is the reverse primer sequence, that is, the reverse nucleotide sequence. Now, this sequence is necessary to to make the transcription, reverse transcription process from RNA to DNA in order to enable that process. Now, depending on the genes of interest BCL-Xl, BCL-Xs, all those things you see, these are the primer sequence. FA1 you have the primer

sequence, osteocalcin called 1; last 2 are beta-actin. No, this is not beta-actin and 18s, they are like housekeeping genes ok.

In this house keeping genes, you see that here also, that primer sequence is different. If you notice, if you carefully notice, that all these genes of interest that Bax 2 up to 18s, they are nuclear type sequence can never be identical. There must be some difference in certain nucleotides sequence. If you carefully go through it, you do not find any identical nucleotide sequence.

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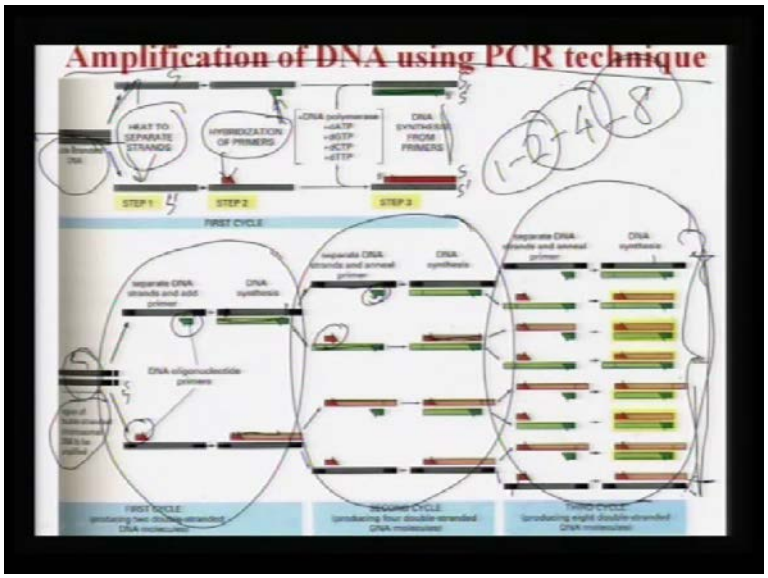

So, this is that one. So, in the first cycle what will happen if you have double-sided DNA? Then, heat to separate the strands. So, if you heat it to little bit higher temperature like 30 degree, 50 degree or 70 degree, see these 2 double strands DNA, they will be separated. Then, step 2 is what, hybridization of the primers. Hybridization of the primers means those primers that we have discussed earlier slide. These primers can be added here.

Now, stage 3 means that is the DNA synthesis from primers. So, here what will happen, this will replicate. So, s becomes, s will produce another complimentary strand s prime. If it is s prime, then it will make the another complimentary strand s. So, here it is s, and it is s prime in the step 1. Step 3, once these primers are added, then they themselves will produce another complimentary strand. In case of s prime, it is s in case of s, it is s prime, so that DNA synthesis is completed.

Now, the other things that we can use that amplification of DNA using the PCR technique polymers chain reaction, suppose you have a region of double standard chromosomal DNA to be amplified, so this is your s and this is your s prime. Now, I will now I am showing you how to amplify only a small part of s and s prime to larger quantity, so that you can measure by polymers chain reaction. Now, what you can do is, you can separate these DNA strands and add primer to that. So, separate means, this is your small strand here and then, you add green primer here and this is the red primer to other things. So, this primer would be certainly of a different nucleotide sequence ok.

Then, what will happen? It will produce another strand here, complimentary strand. Now, this process from here, if you heat it to little bit higher temperature as I have said here, so again this prime, this DNA strands will be separated. So, the black one will be separated from the green one. Again, you can anneal the primer and then, you can add this green and the red one. So, again it will form another strand here, complimentary strand and these process can be repeated, and so that from 2 1 double helix pattern of DNA, you can end up having so many number of DNA, replicated DNA with that particular genes of interest.

So, if this single DNA helix here, whatever the expression, the expression of this final quantity, final number of this DNA would be much more and that would be detectable right, but in the process, what you see that there are couples of cycles here. This is the cycle number 1, this is the cycle number 2 producing 4 double standard DNA molecules and cycle number 3, this is the 8 double standard molecules. So, 2 to 4, 4 to 8 and this is 1 to 2. 1 to 2 is the first cycle, 2 to 4 is the second cycle and 4 to 8 is the third cycle ok. So, similarly, you can have capital N number of cycles and large number of cycles for the expression of the DNA here.

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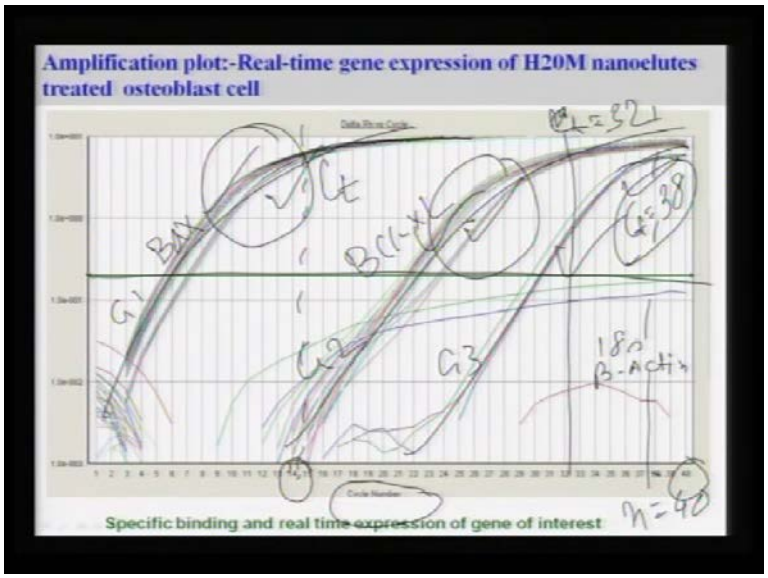

This is the other one. Now, you can see what is the typical amplification plot? That is the real time gene expression of this osteoblast cells Now, these are like you know different genes of interest, that how they are amplified. This is your cycle number. You now know that what is the cycle that consists of? So, you can see these cycles can go up to 40 here. So, that is huge number of cycles. So, N is equal to 40.

Now, these cycles what you see, this is the reference values. With increasing cycle number, your genes of interest are actually amplified; all the genes of interest are amplified ok. Now, these genes of interest can be either, osteocalcin or Cbfa 1 or different types of genes are there. In each group of genes if you see that with increase in number of cycles, they are being more amplified. That means, there concentration or their expression, that is getting much and much more.

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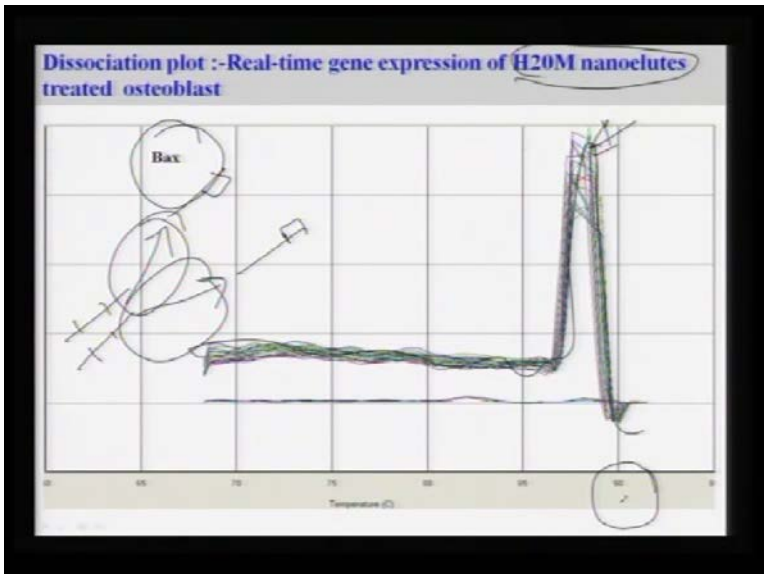

Then, coming to the Bax gene. Bax is the pro-apoptotic gene right. Pro-apoptotic gene means these are like responsible for the apoptosis process. Now, this is with the temperature. Why temperature is required? You remember that you have the double strand DNA you want to amplify. You have to heat them, so that to separate the strand. Then, you have to add some primer here. So, what is the temperature of this heat treatment with which it can see that in case of the Bax gene, it goes through a single peak and this essentially tells you that this Bax gene is expressed in a natural manner, the way it should be expressed after the treatment with the hydroxyapatite 20 percent mullite elates. Now, it is the quantitative values that I am giving that CT is the cycle threshold value.

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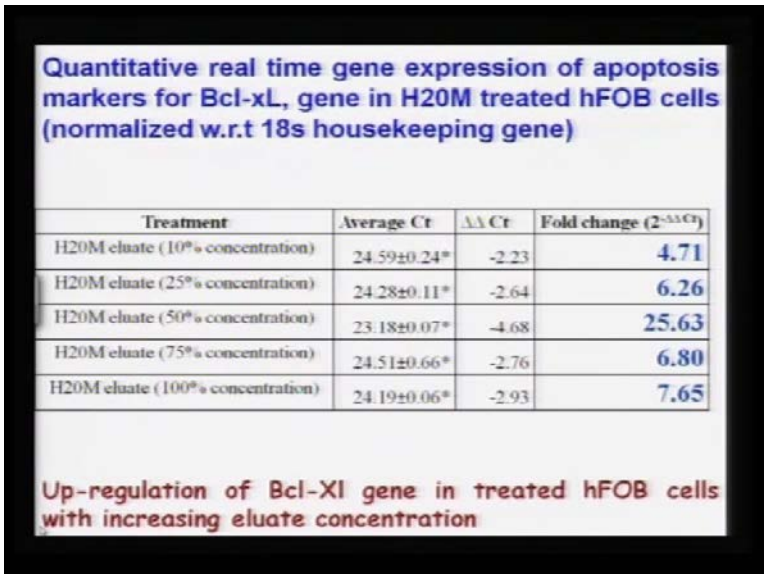

Now, what is cycle threshold value? If you go back to this one, cycle threshold value for a particular gene of interest, for example, if it is the Bax or it can be the BCL-Xl ok. Cycle threshold value means, this is for example your CT values. That means, total number of cycles at which these particular genes of interest are expressed to the highest expression level. You understand what I am saying?

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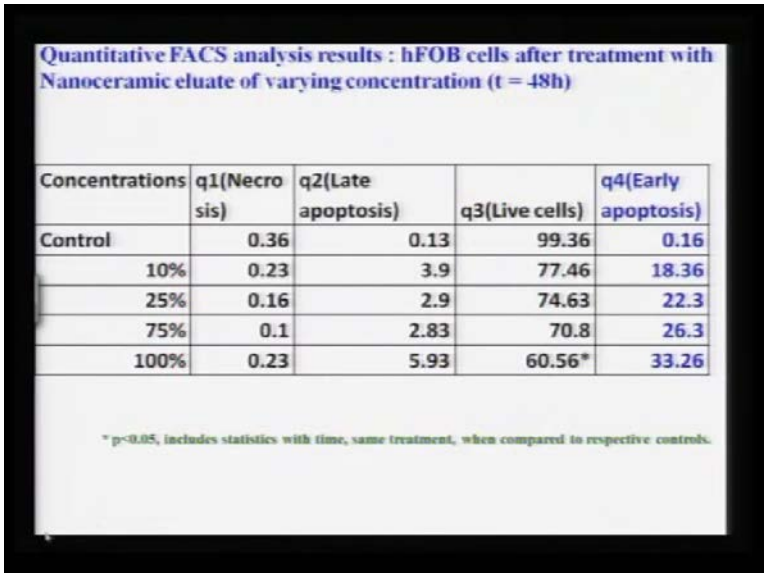

What I am saying here, if you look at that this is the Bax gene, this is they say gene number 1, this is gene number 2, this is gene number 3. For the gene number 1, the cycle

threshold value means, suppose this is the 14 cycles. Beyond 14 cycles, they are expressed to the highest possible level after the amplification process. So, their CT value is 14 here. If you go to gene number 2 here, here CT values are much higher and it can be CT value is 32. That means after 32 numbers of cycles, gene 2 will be expressed to its maximum amplified value. If you go through the gene 3, then here the cycle threshold value is 38 for example. So, that means here after 38 numbers of cycles, that gene 3 is expressed to its highest possible value.

So, accordingly, for each of the genes, you can find out that what the typical CT value is. So, for example, here it is the Bax gene, that is the pro-apoptotic gene. So, what you see that cycle threshold value, after 10 percent eluate concentration, it is 23.46 and delta delta CT value means, this is your CT values 23.46. I have mentioned here very critically, this is normalized with respect to beta-actin housekeeping gene.

So, what it means? This means if you go back to this amplification plot, you have to follow it little bit carefully. So, this g1, g2, g3 these are the genes of interest, but at the same time, you have that 18s gene and you have the beta-actin gene. These are like housekeeping gene. So, these 2 genes, they can also be amplified with respect to certain cycles, PCR cycles. So, you have to continuously measure that what is the cycle threshold for the genes of interest and what the cycle threshold is for the 18s or beta-actin housekeeping gene, so that you can find out that what is the normalized values. This delta delta CT values means that this is the cycle threshold difference of the particular genes of interest that Bax gene with respect to beta-actin gene. So, it is minus 1.74 and typically the fold change, fold change is typically 2 to the power minus delta delta CT and if you do little bit calculation, it is coming out 3 to 3.35.

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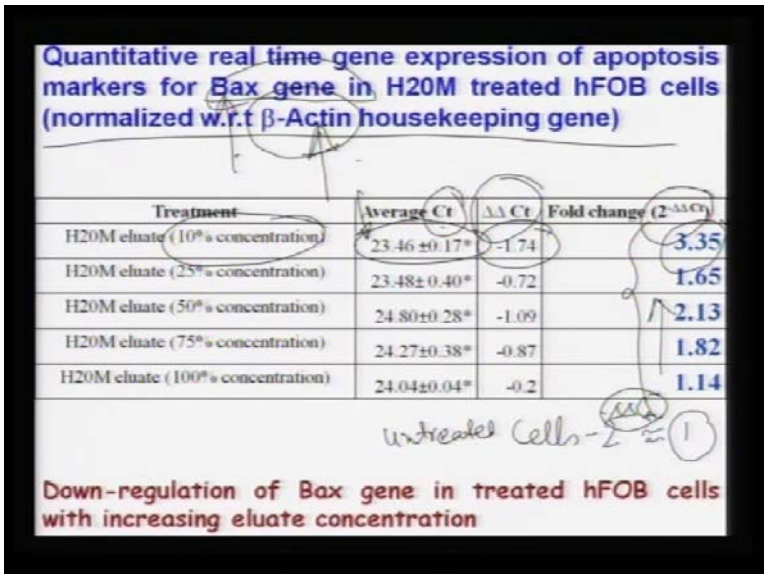

Now, the question that should come to your mind what is the significance of these values? Now, typically for your control or untreated cells, this 2 to the power minus delta delta CT is typically taken as 1. That means delta delta CT values are 0. Why it is 0? Because whenever you are considering in that untreated cells, there beta-actin expression will be the same as the normal cells. So, there will be no difference. So, there will be no difference between the beta-actin and the Bax gene. It will be 0 and therefore, 2 to the power 0 is nothing but 1. Now, anything higher than 1 here, it is actually showing that more up-regulations. So, this is not that much, this is not that much higher than 1 if you go back to this 1, if you see that another BCL-Xl gene and then, it is compared or it is normalized with respect to 18s housekeeping gene.

Now, here is that average CT value if you see and delta delta CT values. So, average CT value means these are the number of cycles at which, BCL-Xl gene is amplified to the highest possible expression level ok. Now, when you are doing it with respect to the 18s housekeeping gene, it is coming out to be negative and the fold change, therefore it is going to be positive. This is that 4.71, but what you notice here that there is something going on at the 50 percent concentration level. Why something going on? It is going from 4 to 6 to 25, but again above 50 percent concentration, then your fold change values are going down. There is up-regulation. Up-regulation means that fold change values is typically greater than 1. 1 stands for control cells or control sample like an untreated sample and with respect to 1; it is severely up-regulated, even it goes to 25.

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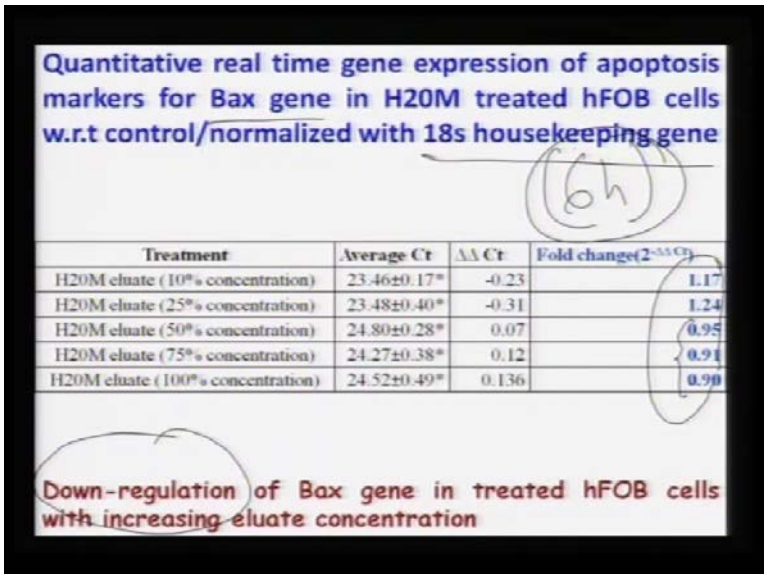

Now, if you go to that another 1 Bax gene with respect to the 18s housekeeping gene, similar analysis, artificial analysis, what it shows? That it is very close to 1 and sometimes, it is less than 1. So, that means Bax gene is down-regulated. Down-regulation and up-regulation means, how these genes are expressed in the treated cells with respect to the untreated cells.

So, if it is down-regulated, that means Bax genes are not that much active if it has to undergo very much highly apoptosis process and typically, these analysis are done after 6 hours treatment ok. So, that means the cells are not really down-regulated. Cells are not really undergoing severe apoptosis process.

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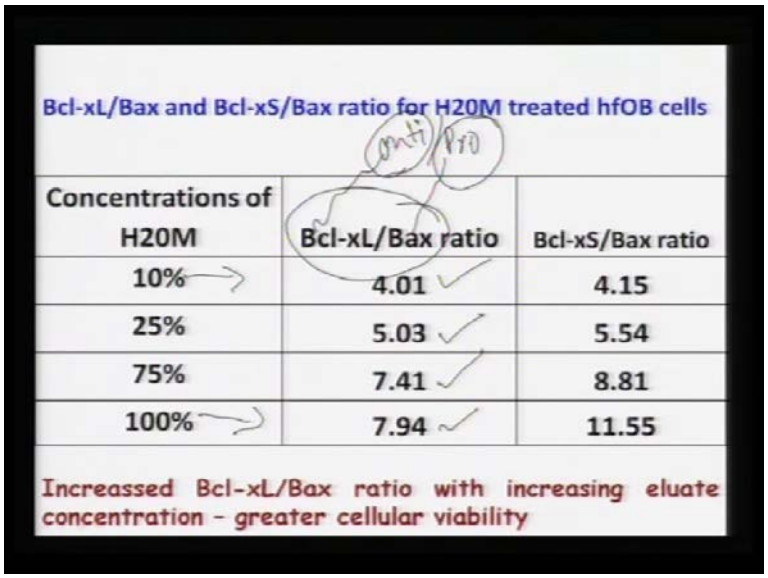

Now, BCL-Xl to Bax ratio actually determines what? It determines the ratio of the anti-apoptotic gene to the pro-apoptotic gene. So, this is the anti by pro-apoptotic gene because BCL-Xl values after 10 percent concentration is 4.01, 25 percent concentration is 5.03, 75 percent concentration is 7.41 and 100 percent concentration is 7.94.

So, what you notice, if you go from 10 percent to 100 percent, your BCL-Xl to Bax ratio is just double, almost double 4.01 to 7.94. So, what is the meaning of that? The meaning of that is that this hydroxyapatite 20 percent mullite concentration, if it is increased from 10 percent to 100 percent, the BCL-Xl anti-apoptotic to pro-apoptotic gene ratio, it also increases twice. That means, there is increased BCL-Xl to Bax ratio. What it means? It means there is more expression of anti-apoptotic gene with respect to the pro-apoptotic gene. That means the cells will not undergo normal apoptotic process, if you increase the concentration from 10 to 100 percent, simply because of the abundance of the anti-apoptotic gene, which will restrict the cells to undergo the normal apoptosis process. Is it clear?

So, for any kind of this artificial analysis with the cells, one has to always determine the final values of the BCL-Xl by Bax ratio because that is the anti-apoptotic to the pro-apoptotic gene expression ratio.

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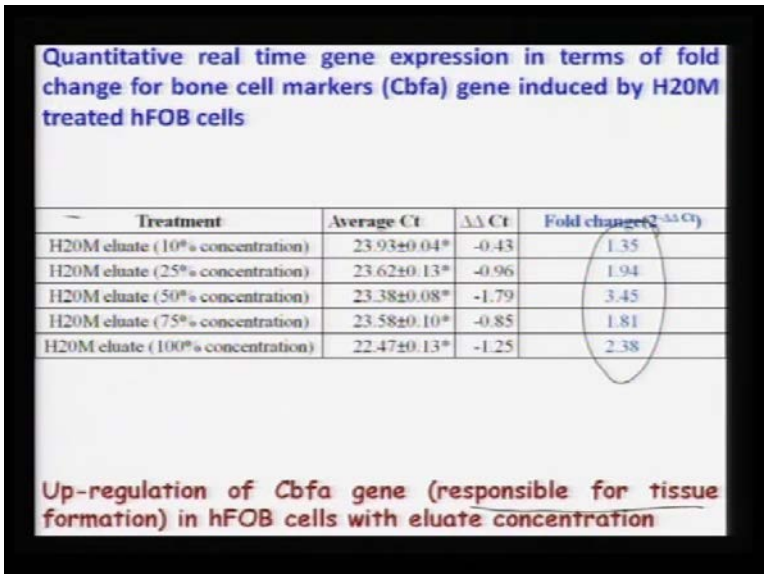

Now, there is some other gene which is also known as the Cbfa genes. Now, Cbfa genes are essentially responsible for the tissue formation. Now, if it is Cbfa genes are more than 1, as the way it is here, you can say that the Cbfa genes are up-regulated and therefore, this hydroxyapatite 20 percent mullite eluates, they will essentially induce the more tissue formation.

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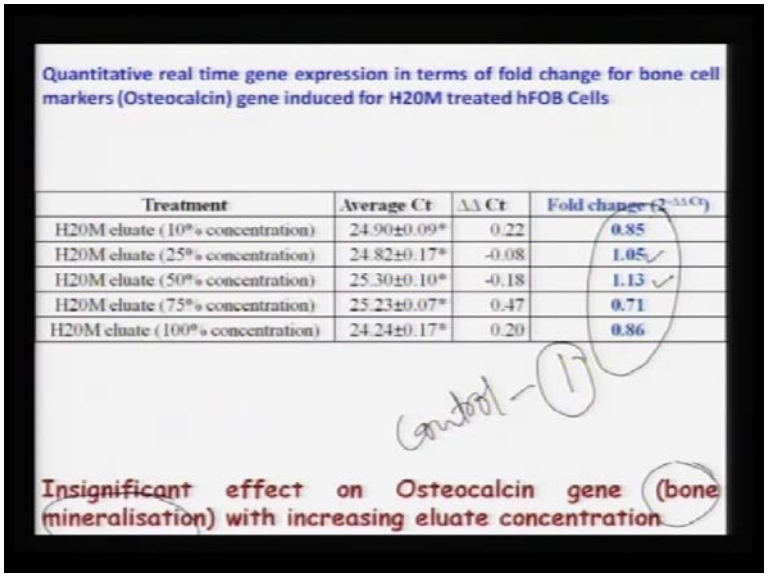

There are other genes also known as osteocalcin genes. Now, osteocalcin genes are mostly responsible for the bone mineralization process, but osteocalcin what you notice

here, this is down-regulated or this is insignificant effect because for the control cells like untreated cells as I said, your fold change is 1. So, with respect to 1, it is hardly 1.0 to 1.13. It is there is not much difference. So, the control cells, osteocalcin expression is same as the osteocalcin expression in this different eluate concentration ok.

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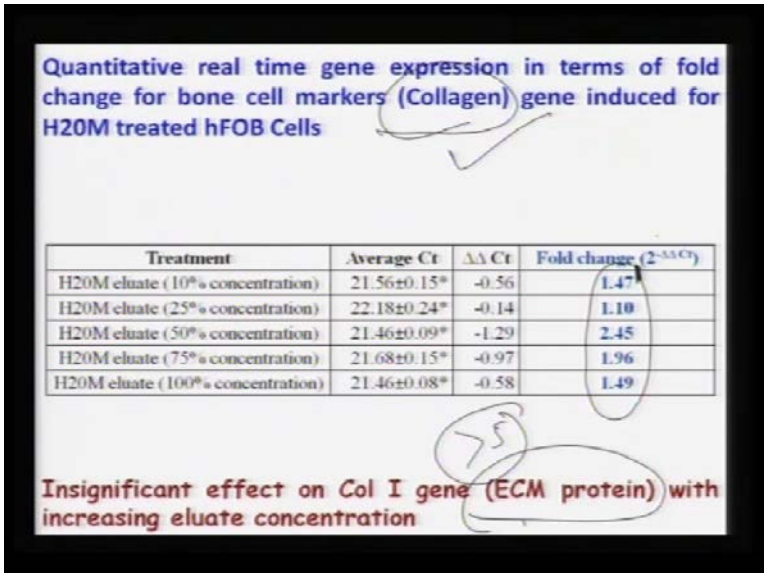

Now, this is another gene is known as the collagen gene expression. Now, in collagen gene what you notice here, the fold change is little bit higher than 1, but it is certainly this fold change is not greater than 5 or something like that. So, there is insignificant effect on the fold change values in the case of the collagen genes. Therefore, this is the ECM protein collagen is nothing but, the extracellular matrix protein. So, this particular eluate will not have much effect in terms of the collagen synthesis also.

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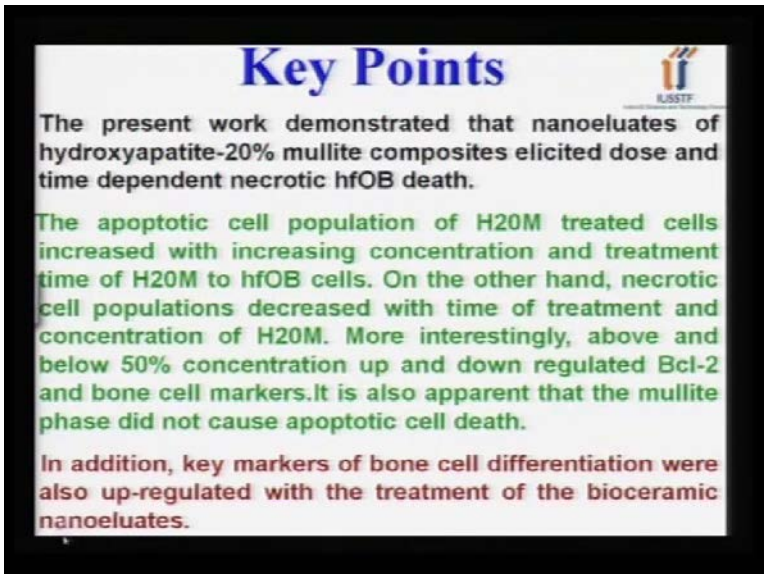

Now, you can see that what the different mechanisms are. So, ultimately this is the last slide that possible mechanism, it is that hydroxyapatite mullite, hydroxyapatite CA10, PO4 whole 6 OH whole 2 and mullite is alumina and silica, so 3 aluminum 2 silica.

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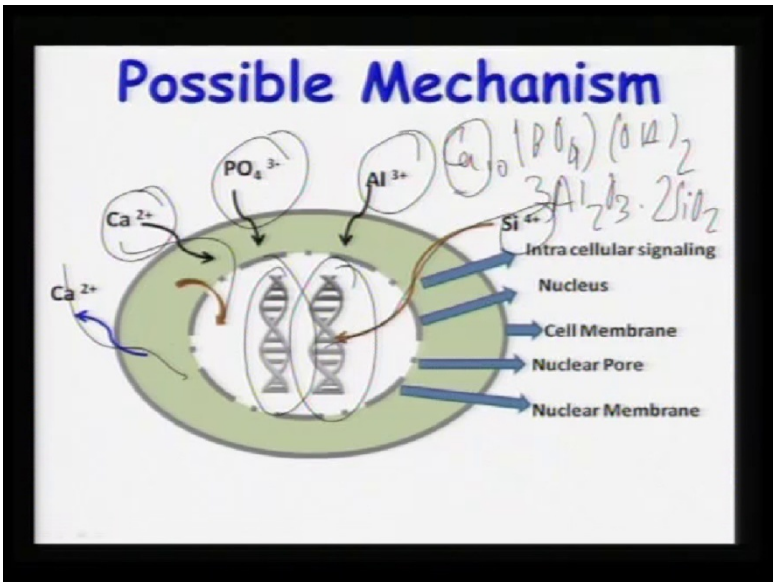

So, what has happen in case of these eluate treatments, your calcium plus 2 ions are there, your phosphate ions are there and silicon ions are there and Al plus 3 ions are there. So, these calcium ions can go inside the cytoplasm. Some side calcium ions can go outside the cytoplasm also, but what we proposed that silicon ions are getting in and they

are causing the damage to the double helix pattern of the nucleus in the DNA. That is causing the more toxicity or more cell death, when you are treating the hydroxyapatite mullite matrix. Ok.