Biomaterials for Bone Tissue Engineering Applications
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Module 7
Lecture No 35

So, in this module I will discuss that the rational for the development of the Hydroxyapatite-Zinc Oxide composites. So, particularly this Zinc Oxide.

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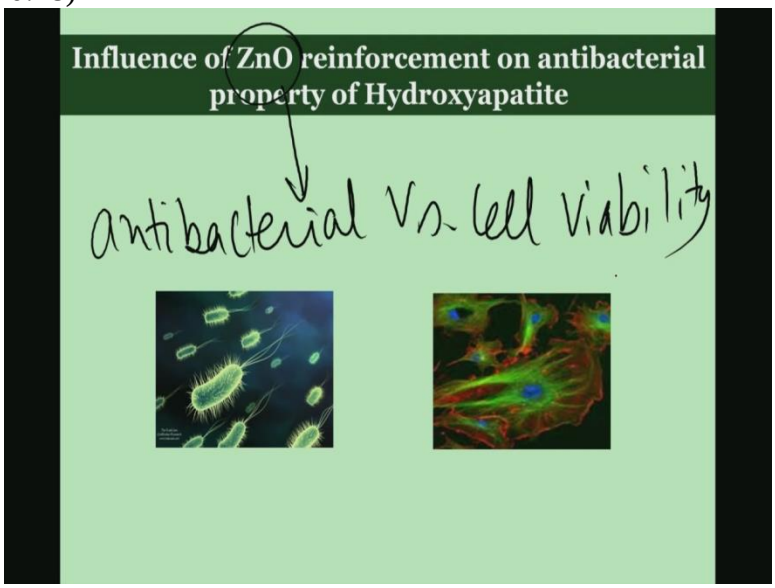

So, this Zinc Oxide is known for this anti-microbial anti-bacterial properties. So, this anti-bacterial properties are has been has been utilized here to develop Hydroxyapatite Zinc Oxide composite. So, one of the things that I must mention at the beginning of this module is that there is something called anti-bacterial v/s cell viability. I would not use the term anti-microbial simply because microorganism means that you know that bacteria, protozoa and all other microorganisms.

So but here we are discussing only on the anti-bacterial property of Hydroxyapatite Zinc Oxide composite. So the idea is that one can add the Zinc Oxide to an optimal amount so that it will cause anti-bacterial or it will induce anti-bacterial property or bactericidal property. Now bactericidal property and bacteriostatic property this has been mentioned in one of the earlier

modules. So, here we will see that what is the bactericidal property because of the addition of Zinc Oxide.

But at the same time the Zinc Oxide addition should not invade the cell growth on the substrate or should not invade the cell function on the substrate. So that is very typical because the moment you start adding the Silver or Zinc Oxide it may potentially cause toxicity to the eukaryotic cells. So, it may kill the prokaryotic cells but it may cause toxicity to the eukaryotic cells and therefore eukaryotic cell viability will be disturbed.

So, we show that how Zinc Oxide content is to be tailored while developing Hydroxyl Zinc Oxide composite so that it will induce anti-bacterial property, at the same time the cell viability property is not compromised.

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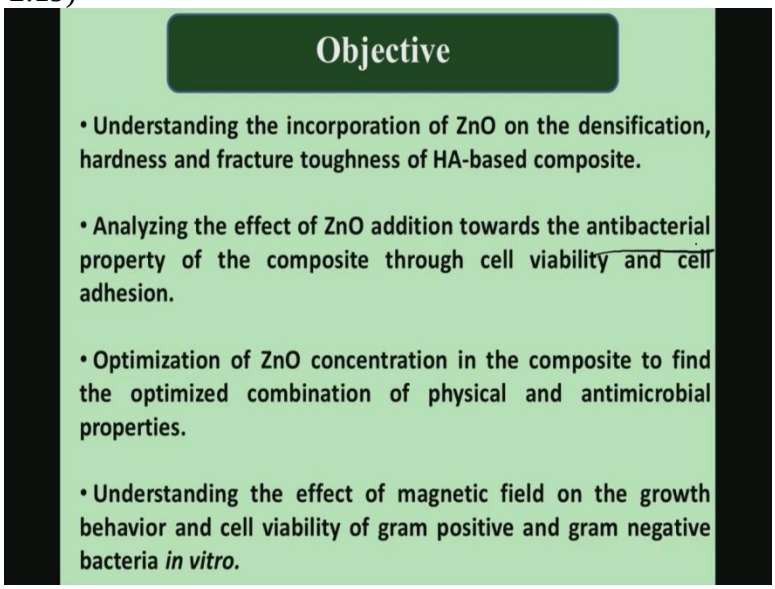

So, having said this that this the objective of this present module to discuss that whether this incorporation Zinc Oxide has any influence on densification hardness fracture toughness how it will influence the anti-bacterial property on this materials and then in terms of both physical anti-bacterial properties and so on and cell viability properties.

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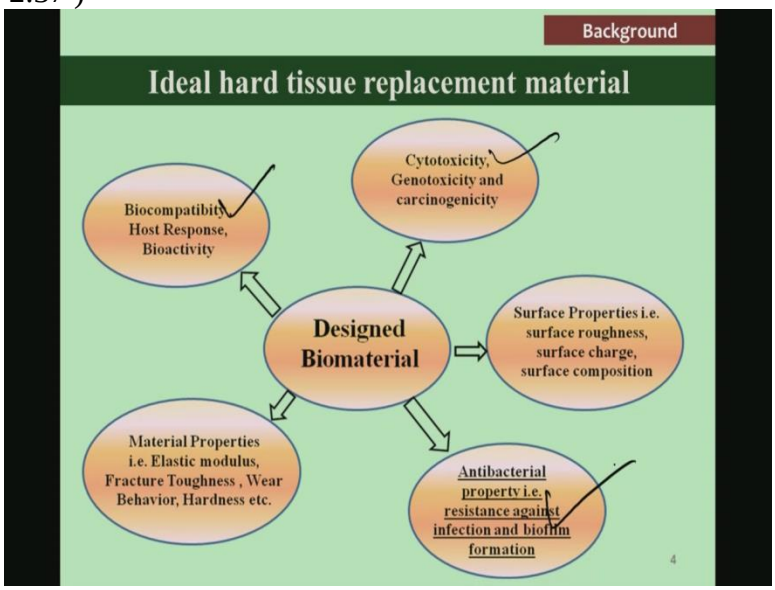

So, just to review some of that earlier discussion that is that we develop several biomaterials or if for a matter of fact that world-wide that a large nber of biomaterials are investigated for orthopedic applications, for dental applications, for other general, for cardio vascular applications and so on. But all these development the development of all these materials are inspired to address certain issues and these issues include for example Cytotoxicity or biocompatibility host response or anti-bacterial property resistance against infe infection and biofilm information.

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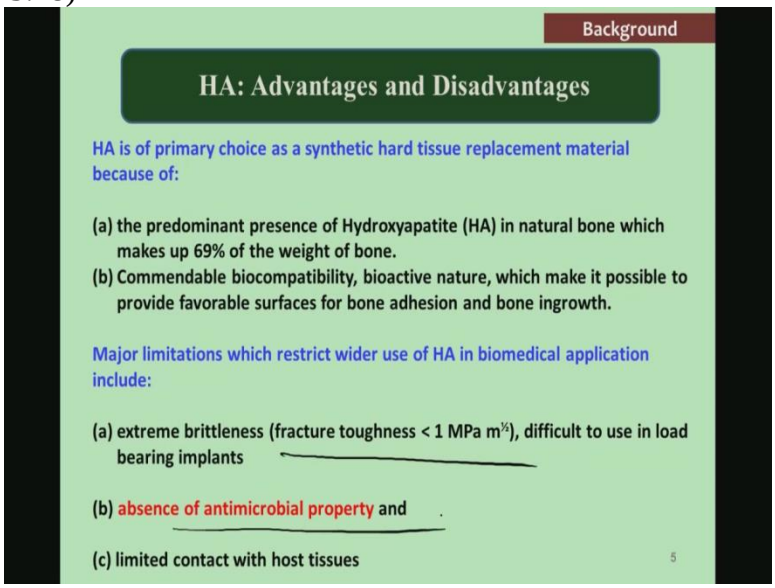

So hydroxyapatite is a primary choice as you know that it has been mentioned in this particular NPTEL course that Hydroxyapatite has the inorganic composition of the natural bone. So therefore Hydroxyapatite has been investigated quite widely. But some other major limitations Hydroxyapatite is that it has very low fracture toughness, it has absence of anti-microbial property and limited contact with host issues.

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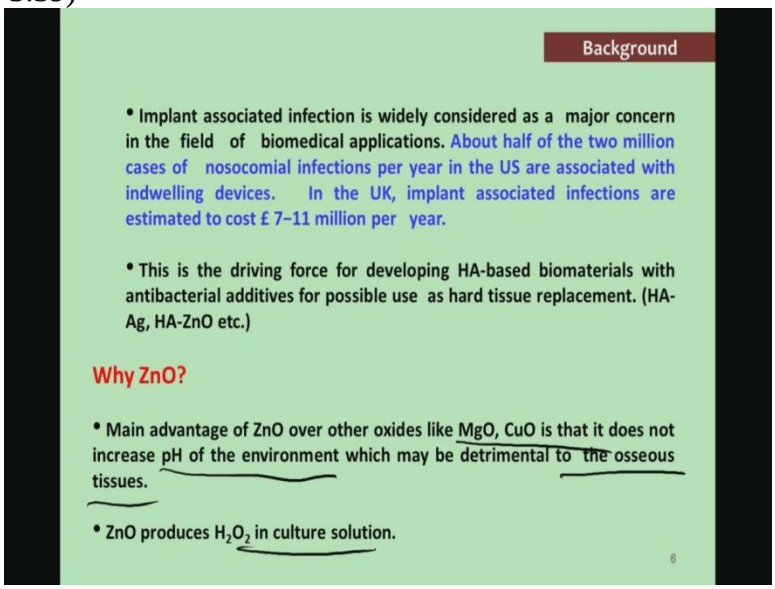

.And why Zinc oxide? Because Zinc Oxide like MgO, CuO it is increases the pH of the environment which may be detrimental for the osseous tissues and it can produce H2 O2 in the cultured conditions.

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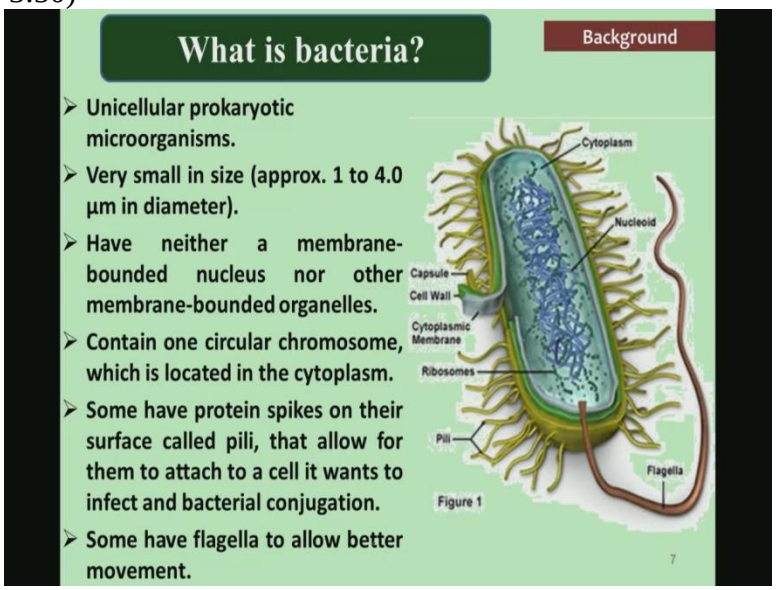

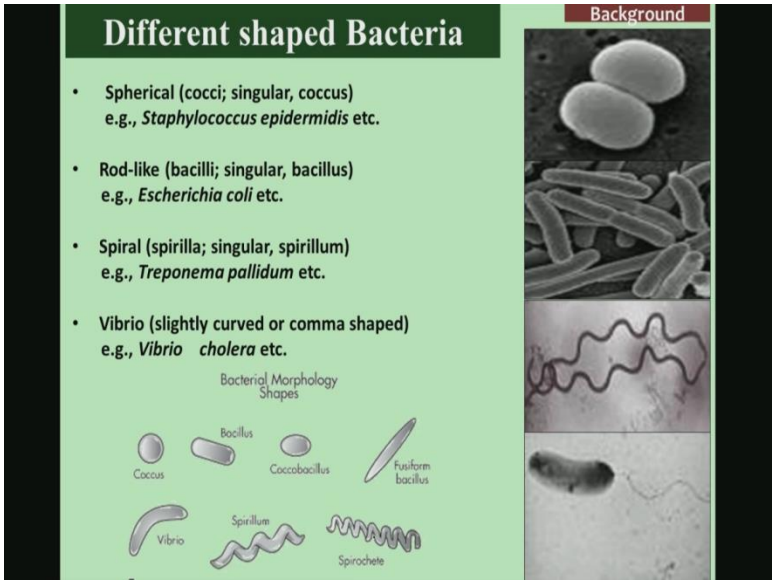

So, just to recall what is bacteria, bacteria is a unicellular prokaryotic microorganisms it is very small in size like 1 to 4 micron in diameter. And there is different shaped bacteria but out of that most pathogenic bacteria which can kill which can cause pathetic infection is Staphylococcus or Staphylococci, Staphylococcus epidermidis. Most widely investigated bacteria is Escherichia coli for most in the context of the biomaterials applications.

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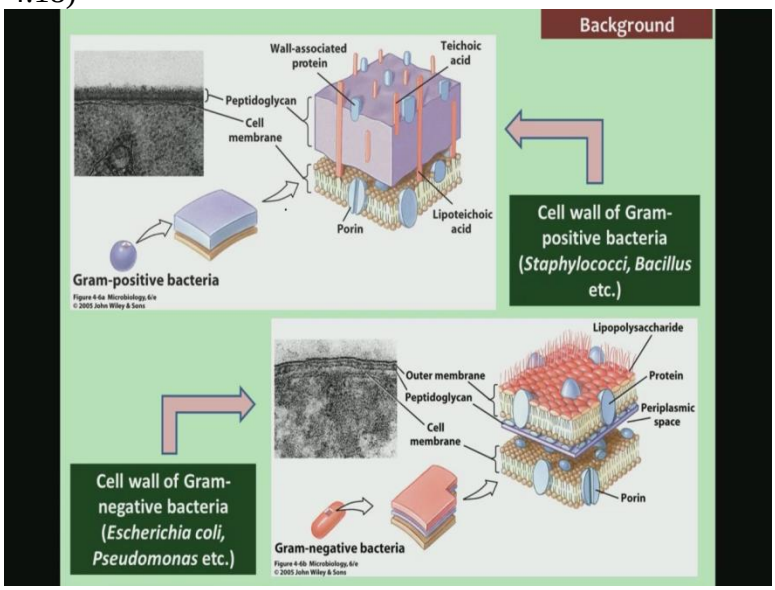

Now these things has been mentioned very critically and sufficiently in earlier lectures on the bacteria that structure characteristics wise it is the cell membrane structure which is distinctively different in between that gram positive and gram negative bacteria. And in case of the gram negative bacteria it is a triple layer structure you have an additional intermediate layer of the peptidoglycan layer which is between the two outer membrane and inner membrane. Whereas this cell one of the gram positive bacteria you have the peptidoglycan layer is very thick and you have that additional cell membrane as usual.

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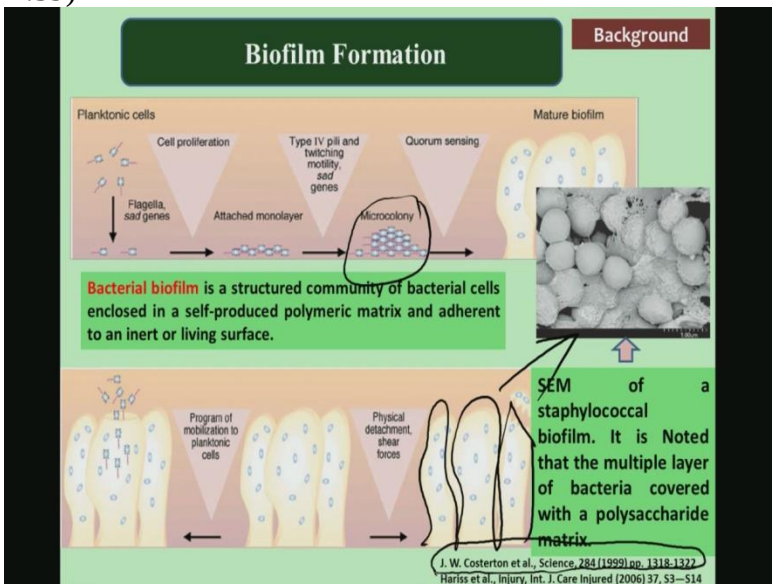

Now, coming to biofilm formation which starts with this colony formation of the ability of colony formation of a single bacteria, now once this colony grows and then it makes that micro colony and once it grows then it is essentially it can potentially form this large, stable biofilm. And this biofilm you can also investigate using scanning electron microscopy just you see that it is a like a spherical bacteria which is spherical she was staphylococci bacteria which is pathogenic bacteria and once it forms its biofilm then it spreads all over the place and then it forms an (())(5:37) matrix like features and which will help adherence of the in nerous bacteria on a material substance.

These the depending since it is very important this biofilm formation this has also been published in some of the high impact journal like Science in 16 to 17 years ago by Costerton and his group.

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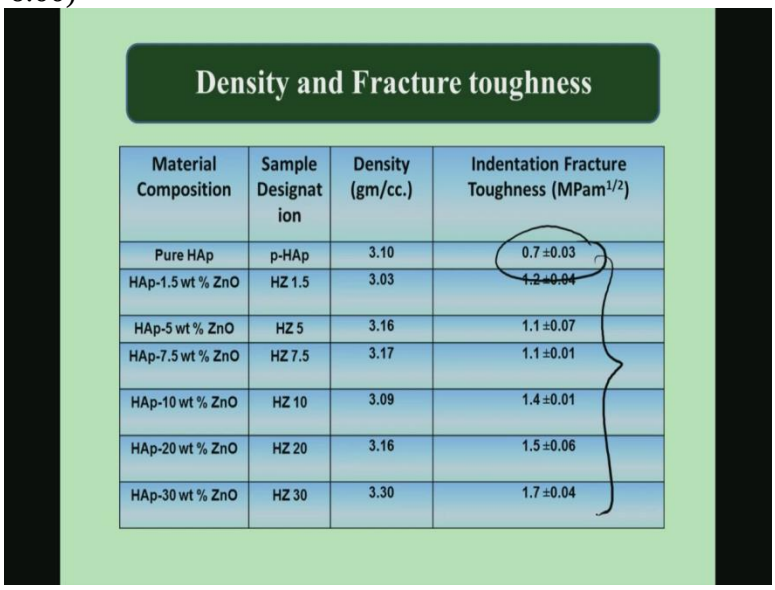

Now Hydroxyl Zinc Oxide it has reasonably good density because this is all conventionally stinted materials it is not even spark plasma sintered. In normal 1200 degree Celsius, this material could be sinted in air . As far as the fracture toughness is concerned this fracture toughness of pure hydroxy is 0.7 and then as you see that if you keep on increasing the Zinc Oxide content fracture toughness does not increase significantly to a greater extent but to a reasonable extent $ 0 7 it goes to up to 1.5 0r 1.7 mps square root metre.

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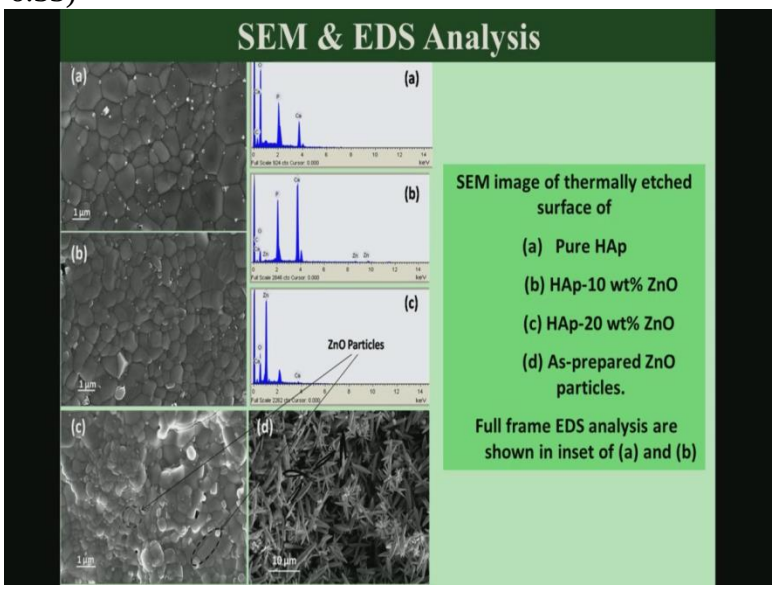

Now, when you see the distribution of Zinc Oxide Zinc Oxide in the original unsintered conditions as a particulate it has a more like needle like shaped morphology. Now when you do this needle like separate morphology if you add to hydroxyapatite then during sintering it grows as a more like a (())(6:51). And this has been confirmed from the EDS Analysis that indeed indeed that equate separate morphology is that Zinc Oxide particles.

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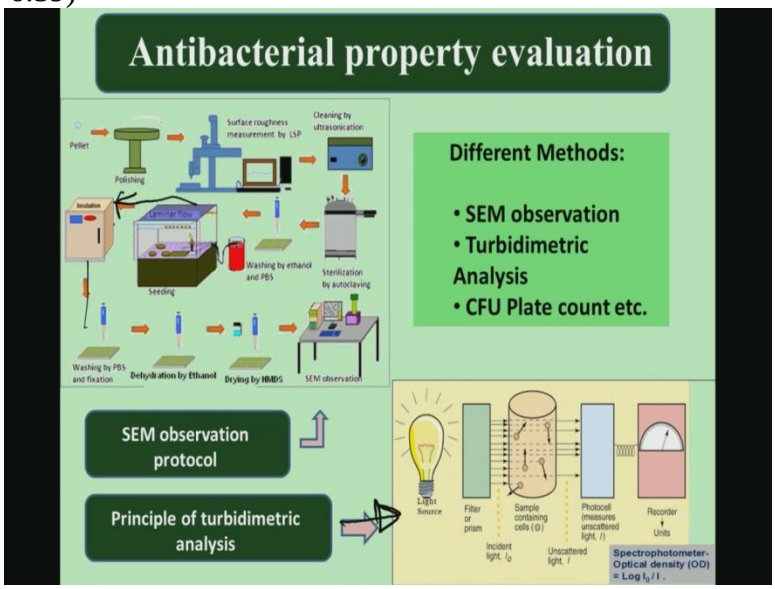

Now, anti-bacterial property of this Zinc Oxide composites so, you we have one can use this standard methodology like you start with that polishing of the sample then you do surface (())(7:12) measurements, cleaning, sterilization . Then washing by PBS and then finally incubation and then incubation it goes like in 37 degree Celsius in a normal ambient atmosphere and then subsequently you do Terbidimetric analysis just to quantify that what is the bacterial viability in growth medi.

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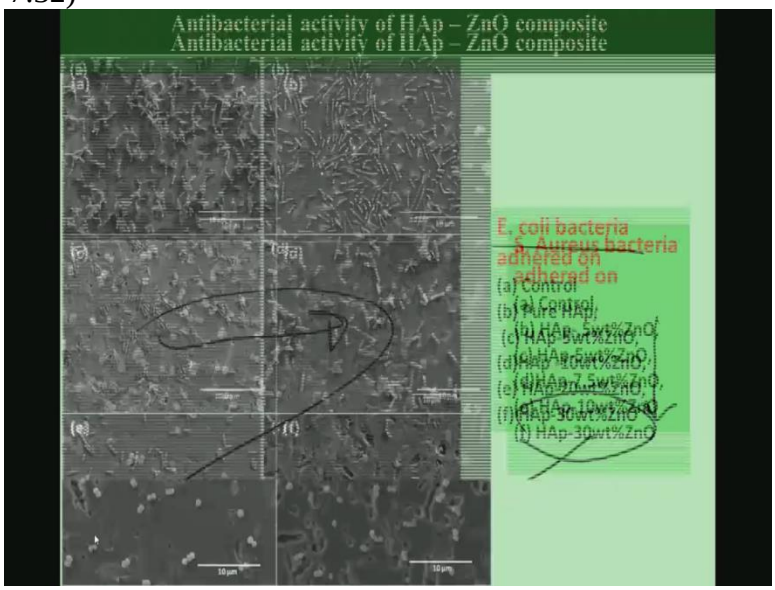

Now, in terms of the anti-bacterial viability on the Hydroxyapatite Zinc Oxide composite when you grow that E.coli that as I said that is widely used bacteria. It has a very rod shaped morphology and as you increase the Zinc Oxide content and this way it is increased. Ok? So, as you increase the Zinc Oxide content you see at the highest 38% Zinc Oxide hardly you see any bacteria. But at the same time you see certain features on the material surfaces which indicate that Zinc Oxide essentially is leached out.

So, Zinc Oxide is leached out and causing the bacterial death and suddenly once it increases the Zinc Oxide you see the colony formation ability is also suddenly reduced and then bacteria is more like a more like isolated conditions not like in a in a in a in a colonized form.

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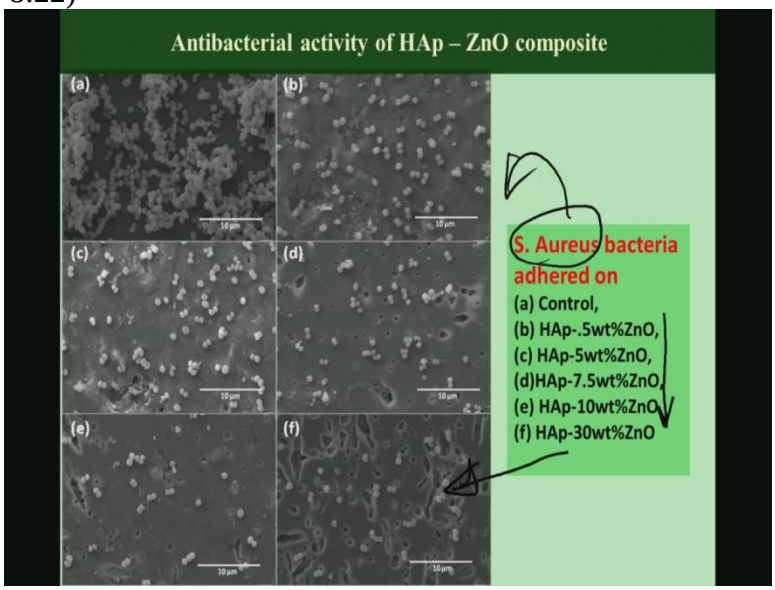

Now as I said in one of the earlier module that any type of cell related study or bacterial study must be conducted using not only one strain but with multiple strain. So, that cell strain type dependent behavior can be assessed more appropriately while assessing that biocompatibility or anti-bacterial property or cyto-compatibility property of this material. In the context of bacteria, what is recommended is to use some one or two strains of the gram negative bacteria, one or two strains of the gram positive bacteria.

So, that relatively moderate spectr of the bacteria strains are investigated to see they are to assess their viability, to assess their biofilm formation ability on any biomaterial substance. In this context, we have also done since this is from our published results and essentially that Mtech work of Nore Saha, IIT Kanpur, winners at IIT Kanpur, so we have used staphylococcus aureus bacteriain addition to E.coli bacteria and staphylococcus as expected from the morphology it is spherical shaped morphology, spherical morphology and as you see that as you increase that Zinc Oxide content certainly less and less nber of bacteria is seen on the SEM image and this also it is not many times in a very clustered conditions but may be one or two bacteria together.

So, colony forming ability and then nber of bacteriawhich is still adhering on thematerial substrate suddenly is reduced by the addition of Zinc Oxide and then we have assessed up to 38% Zinc Oxide conditions.

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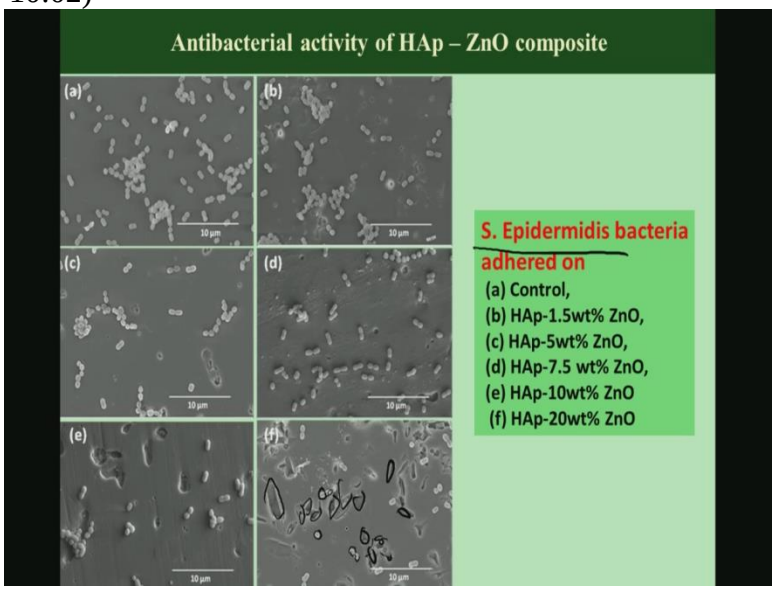

So, not only staphylococcus aureus that is the pathogenic bacteria another bacteria strain which is gram positive that is staphylococcus epidermidis and using the staphylococcus epidermidis also we have established we have seen similar observations but here again I would like to show your attention draw your attention that some of the places you see that Zinc Oxide is leached out ok?

And this Zinc Oxide is leached out and then it leaves certain impression behind that shows that Zinc Oxide is leached out and causing leads bacteria to adhere and survive on a bacterial substrate. And you hardly see one or two bacterias or this bacteria is often one bacteria and it is in the dividing stage. So if you grow closely and see that higher magnification image of this this bacteria, you will see that bacteria want to two that binary fusion is taking place and in the process it causes some furrow at that or cleavage in the bacterial morphology.

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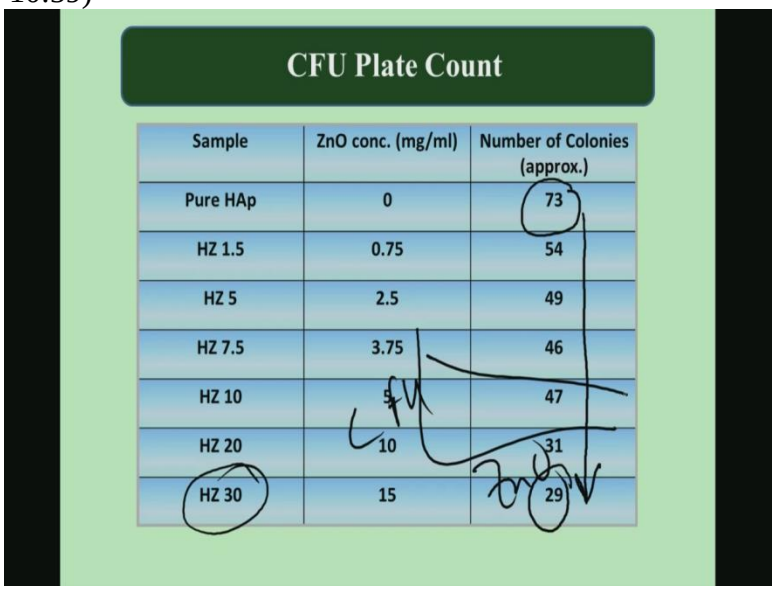

So, this is that CFU Plate Count what you see that pure Hydroxyapatite colony nbers is 73 and it sub significantly it is reduced to less than the half in the 29 or 30 in case of going 30% Zinc Oxide add is added. So, if you now plot this 73 with to 29 it certainly shows that if you plot CFU v/s Zinc Oxide content, so certainly it is reduced like this almost like systematically may not be in a linear manner but certainly it will show systematic reduction in the CFU count with the addition of the Zinc Oxide to Hydroxyapatite.

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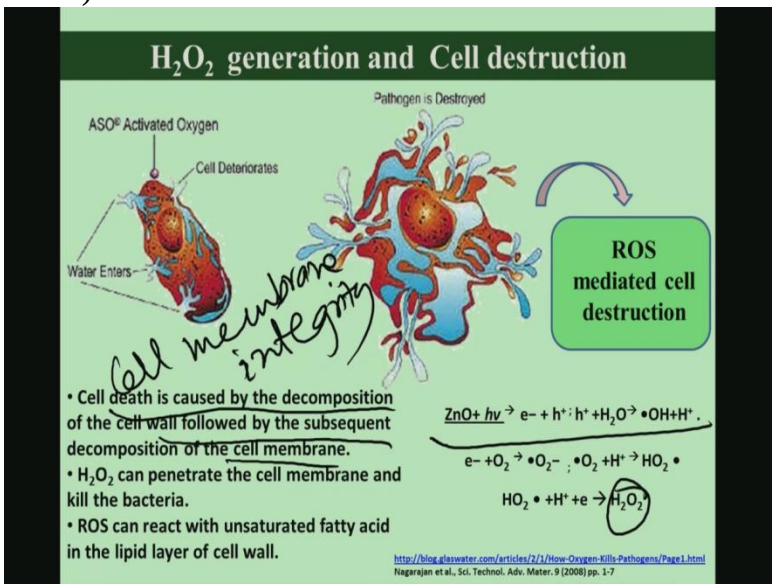

Now, what is the role that Zinc Oxide plays in causing this anti-bacterial property? That is one of the role that Zinc Oxide plays is H2O2 formation. So, essentially what is expected is that cell death is caused by the decomposition of the cell wall followed by subsequent decomposition of the cell membrane. So, essentially cell membrane integrity which I have mentioned while discussing some of the anti-bacterial assay.

So cell membrane integrity is disturbed by this Zinc Oxide addition and some of the potential reaction that I have seen that if you see that it essentially forms that H2O2 and this H2O2 is one of the aureus species which can penetrate to the cell membrane and kill the bacteria. In fact, H2O2 treated cells are often used as a control which will definitely show some cell death.

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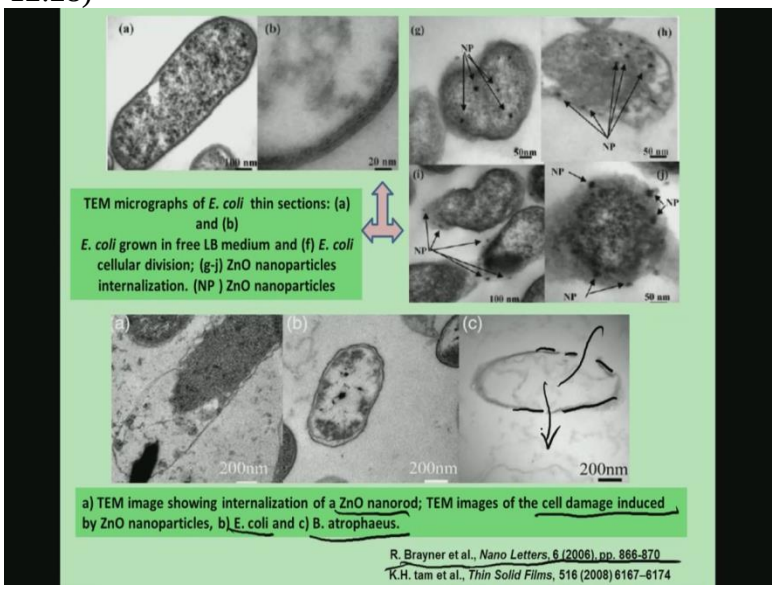

Ok. Now, this statement can be substantiated by using some of the published literature not from our group but from elsewhere. For example Briner and Briner and team published a paper in nano letters in 2006 where they have reported that how E.coli bacteria can be killed in the thin section they their morphology can be disrupted quite significantly by addition of Zinc Oxide nano particles.

Similarly another team has also shown that TEM image of the Zinc Oxide nano rod and then how TEM and then very thin slice of the bacteria particularly E.coli and some of the other bacteria B. atrophaeus and these bacteria also shows that there is show signs of the membrane

disintegrity as you can see that there is more coercion in the membrane. So, once this membrane disintegrity is lost I.. sorry membrane disintegrity is taking place then bacteria cannot survive essentially all the cyto cytoplasmic material organals or cytoplasmic material can come out and causing the bacterial death.

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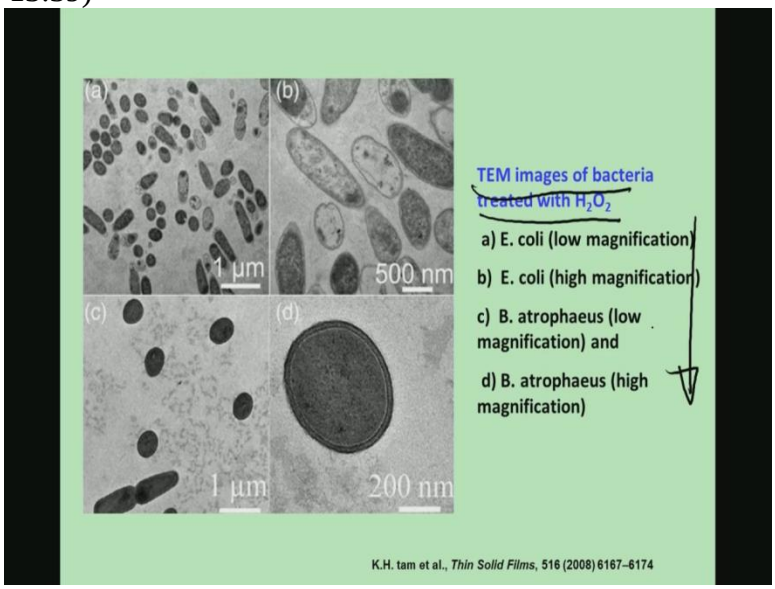

This is the another one by this E.coli uh.. b.. just to show that TEM image of the bacteria treated with the H2O2 and here we can one can confirm that how this bacterial morphology also is disturb is disturbed or disrupted because of the H2O2 treatment.

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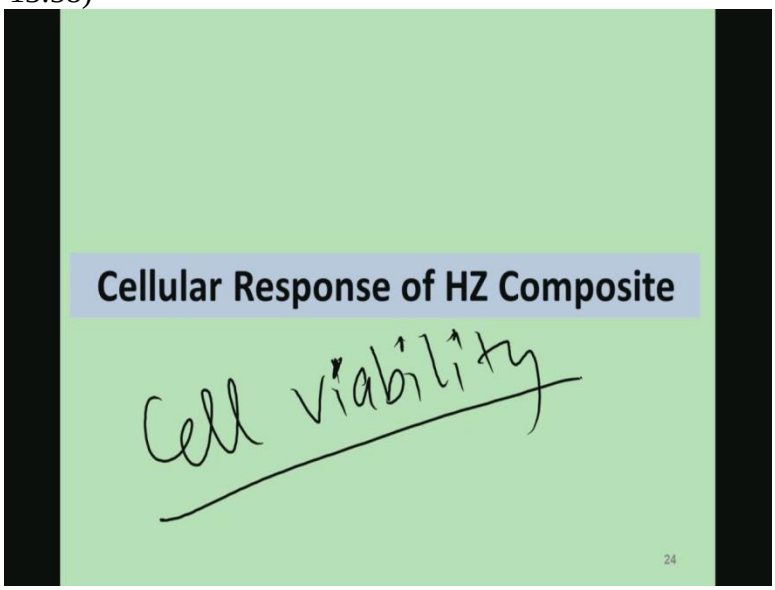

Now, as I said that anti-bacterial property all this is fine but at the same time one has to function one has to find out that what is the cellular viability or cell viability in this Hydroxyapatite Zinc Oxide composite. So, not only Zinc Oxide addition should cause anti-bacterial property but it should not distract the cell viability properties because of the Zinc Oxide addition.

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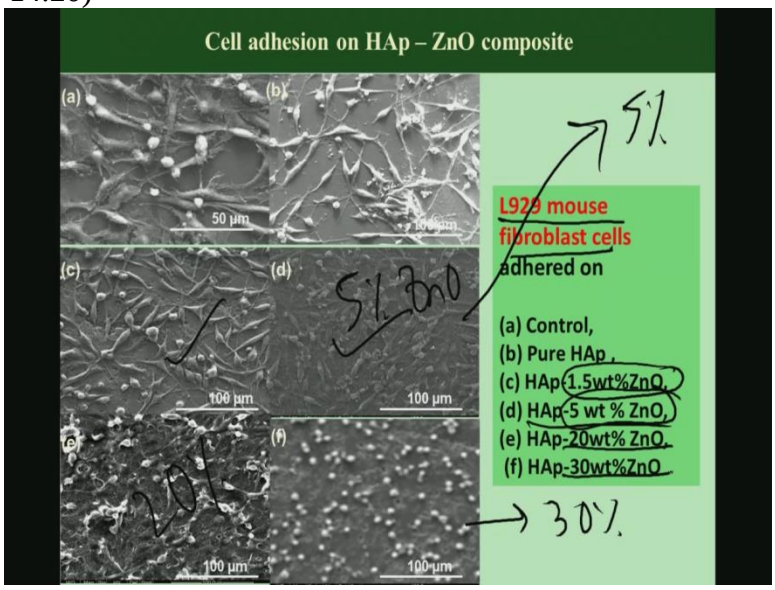

Here one has to show very carefully now let me write down this `D' essentially means that 5% Zinc Oxide, 5% Zinc Oxide. So, this is essentially 5% Zinc Oxide and you see that there is certain morphological changes that is started taking place from 5% onwards and this is the 30%.

This 30% cells are I cells are spherical in nature which certainly shows cell does not cell does not undergo any morphological alterations or in other word cell morphology remains spherical while they are being attached to the substrate.

So, that shows that kind of cells are not adhering and expanding and that is the kind of indication that cells are perhaps are not viable. Now, one has to quantify all these viability things using that entity as a which I will show you later. But before that I will also discuss with that how this L99 mouse fibroblast cells they adhere and proliferate to some extent on lower Hydroxy Zinc Oxide content material that is 1.5 and 5 weight percent.

Once you add 20% Zinc Oxide, here again it does not show very regular spindle like morphology like a fibroblast cell should exhibit, like in this case and this case. So certainly you can say that 20%, 30% would not favour the cellular response the way lower the Zinc Oxide content materials they would essentially show.

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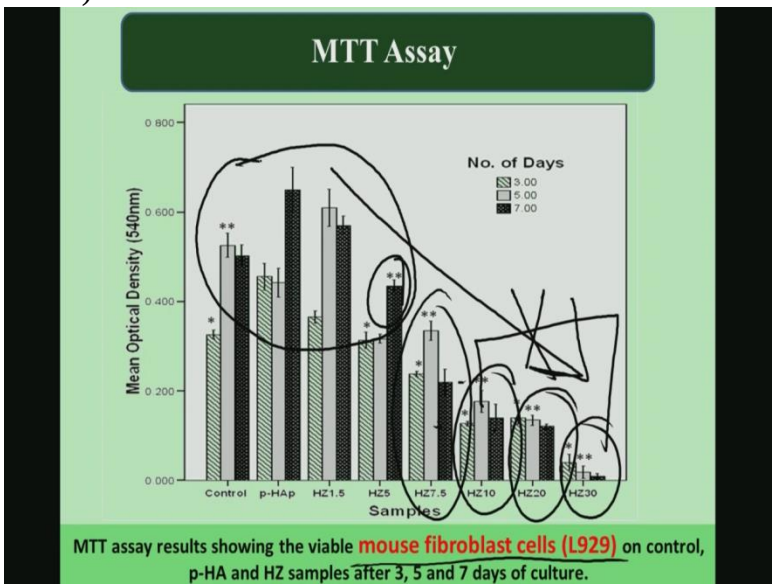

Now same thing we have seen also when you use that sarcomastersarcoma cells that is han osteoblast cells and you see that 1.5,5 and 7.5% materials that shows this kind of typical cellular morphology. 20% still ok not bad but 30% certainly is not good. So, 30% Zinc Oxide does not show any good cell response properties and this is kind of reflected here in the MTT Assay.

So, the first one is that mouse fibroblast cell that is L929 cells which is a connective tissue cells which is widely used by biomaterials research. You see that there is a progressive, systematic decrease in the cell viability as you increase that Zinc Oxide content in the material. And perhaps that more significant, statistically significant results are obtained from 5% onwards and 7uh5% , 10% is certainly worse 20 and 30. So, this 10,20,30 one should not use, people should restrict that Hydroxyapatite Zinc Oxide composite at up to 5% Zinc Oxide.

So, beyond 5%count Zinc Oxide composite there is a seriously systematic, significant, statistically significant difference in terms of cell viability with L929 mouse uh..fibroblast cells.

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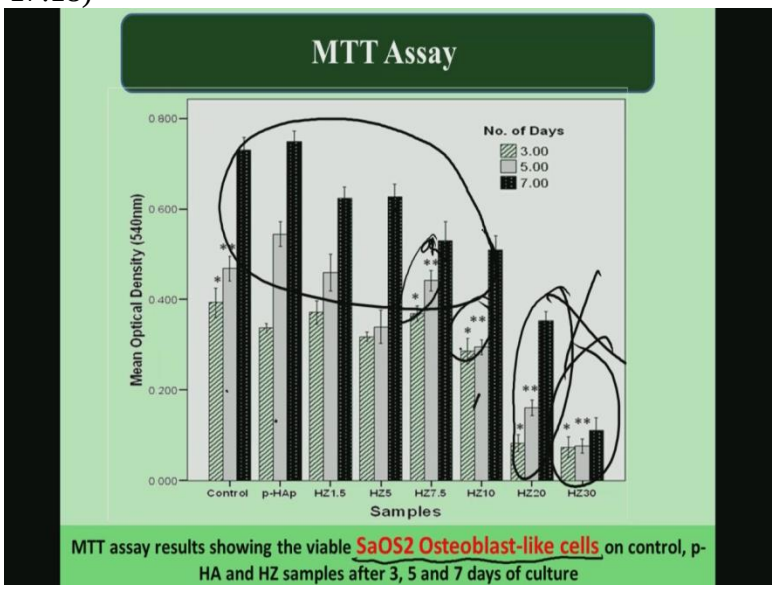

The similar observation to some extent are largely valid when we have quantified the cell viability using that osteoblast like cells sarcomaster sarcoma cells. Here you see that very clearly 20%, 30% is is worse in terms of that supporting the cell viability. 10% is fine but the there are certain stars are there so stars means they are statistically significant results but then when you see that with respect to control parent Hydroxyapatite up to 10% this is still ok. Up to 10% one can consider but in case of fibroblast cells you say that from 5% onwards it is not that good.

So, osteoblast cells show up to some tolerance level up to 10% Zinc Oxide but overall one would recommend that cell compatibility wise that hydroxyapatite 5% Zinc Oxide is more safe than 5 then 10% or 20%.

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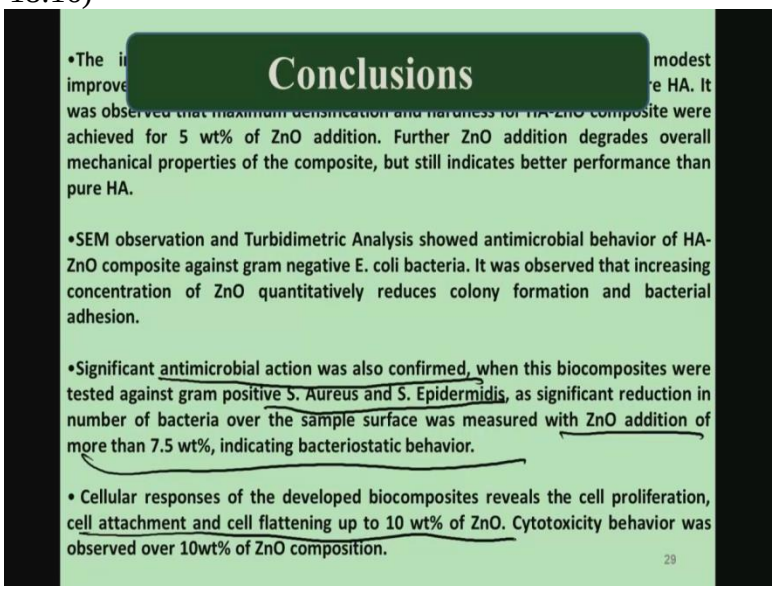

So this conclude this so significant micro anti-microbial action has been confirmed using that Hydroxyapatite Zinc Oxide using S. Aureus and S. Epidermidis strain which is more pathogenic strain and it was measured that with Zinc Oxide addition more than 7.5% indicating that static behavior. Now, cellular responses of this materials cell attachment flattening is observed only up to 10%.

So, somewhere between 5% and 10% is the optim Zinc Oxide content which can be useful for further biomedical applications. And however this thing needs to be tested in that pre-clinical study in the animal model and but for that one of the useful composition can be Hydroxyapatite, perhaps in the middle range is the 7.5 or 8% Zinc Oxide additions which shows reasonable cell viability in terms of sarcomaster sarcoma cells as well as fibroblast cells and at the same way it has reasonable anti-bacterial property. So, Thank you.