COMPARISON BETWEEN A NEW NANOTECHNOLOGY-BASED AGENT AND CONVENTIONAL TALC IN THE CONTROL OF MALIGNANT PLEURAL EFFUSIONS

B. Romero Romero[1], A. Montes Worboys[2], E. Arellano Orden[2], V. Sánchez López[2], L. Padrón Fraysse[1], J. T. Martín Juan[1], F. Rodríguez Panadero[2].

[1]Surgical Medical Unit for Respiratory Diseases. Virgen del Rocío Hospital in Seville. [2] Institute of Biomedicine of Seville (IBIS)

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Abstract

For this study, we used titanium dioxide (TiO_2), produced using nanotechnology. To show its superiority with respect to talc, we completed an in vitro study comparing the pro-inflammatory response of both agents towards malignant and benign mesothelial cells; researching the possible apoptosis induction and possible inhibition of angiogenesis for both agents. We took a culture of cell lines derived from human mesothelioma, originating from human biphasic mesothelioma and human bronchial adenocarcinoma. The cells were cocultured with different doses of talc and TiO_2 nanoparticles. The levels of different inflammatory mediators were analyzed for each culture supernatant sample. The apoptosis rate was analyzed using caspase-3 expression. The endostatin levels were determined for the angiostasis study using the ELISA technique. We observed that the viability of the benign mesothelial cells is much lower after using TiO_2. In the case of malignant mesothelial cells, the same effect was observed with a high dose of TiO_2. In adenocarcinoma of the lung, the viability of these cells exposed to talc was distinctly lower than that which was observed in the benign cell line. IL-8 production was much higher in neoplastic mesothelial cells than in benign cells and increased following a dose-dependent pattern with talc, while it decreased with TiO_2. According to these results, we can see that talc is superior to TiO_2 in its ability to produce mediators which favor pleurodesis for the control of malignant pleural effusions.

Key words: talc, nanoparticles, pleurodesis.

COMPARACION DE UN NUEVO AGENTE BASADO EN NANOTECNOLOGÍA CON EL TALCO CONVENCIONAL PARA EL CONTROL DE LOS DERRAMES PLEURALES MALIGNOS

Resumen

Resumen: En este trabajo usamos dióxido de titanio (TiO_2), fabricado mediante nanotecnología. Para demostrar su superioridad respecto al talco, realizamos un estudio *in vitro* comparando la respuesta pro-inflamatoria de ambos agentes sobre células malignas y mesoteliales benignas; investigando la posible inducción de apoptosis y la posible inhibición de angiogénesis también por ambos agentes. Realizamos cultivo de líneas celulares derivadas de mesotelio humano, procedente de mesotelioma bifásico humano y adenocarcinoma bronquial humano. Las células se co-cultivaron con diferentes dosis de talco y de nanopartículas de TiO_2. En todas las muestras de sobrenadantes de los cultivos se analizaron los niveles de diferentes mediadores inflamatorios. La tasa de apoptosis se analizó por la expresión de Caspasa-3. Para el estudio de angiostasis se determinaron los niveles de endostatina mediante técnica ELISA. Observamos que la viabilidad de las células mesoteliales benignas es mucho menor al emplear TiO_2. En el caso de las células mesoteliales malignas, se observó el mismo efecto con dosis alta de TiO_2. En el adenocarcinoma de pulmón, la viabilidad de estas células expuestas al talco fue netamente inferior a la que se observó en la línea celular benigna. La producción de IL–8 fue mucho mayor por parte de las células mesoteliales neoplásicas que por las benignas y aumentó siguiendo un patrón dosis dependiente frente al talco, mientras que cayó con el TiO_2. Según estos resultados, se demuestra que el talco es superior al TiO_2 en su capacidad de producir mediadores que favorecerían la pleurodesis para el control del derrame pleural maligno.

Palabras clave: Talco, nanopartículas, pleurodesis.

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Beatriz Romero Romero
bearomererromero3@gmail.com

INTRODUCTION

The presence of a malignant pleural effusion indicates the existence of an advanced neoplastic disease in which curative treatment is no longer possible. Within the palliative framework for this treatment, pleurodesis is especially important, which has the key role of adhering the two pleura in order for the lung to remain re-expanded, thus avoiding the accumulation of liquid in the pleural space.

Throughout history, different sclerosing agents have been used to adequately control malignant pleural effusions, with talc proving to be the most effective. While the success rate for pleurodesis with talc is indeed high and, generally, its intrapleural application is well tolerated[1], important side effects have occasionally been described, both when it is applied as a slurry mixed with saline and as a powder[2, 3].

Based on different published work in the literature, a close relationship is thought to exist between the particle size and shape of the talc used and its absorption through the pores of the pleura and, as a result, its systemic dissemination and the appearance of associated complications[4-9].

Jannsen et al. *(Talc Safety Study Group)*[10] studied 558 patients with malignant pleural effusion who underwent pleurodesis with talc, always using the same type of talc (with calibrated particle size, Steritalc®, Novatech, France), reaching the conclusion that its use was sufficiently safe, without observing any cases of acute respiratory distress. On average, the particles of this talc were large in size (24.5 μm) and there was a low concentration (11%) of small particles (<5 μm).

The mechanisms involved in pleurodesis are not completely explained. It is clear that there is a primary inflammatory response initiated by the mesothelium, which is the first target of any stimulant applied in the pleural space, as well as a secondary response, which is caused by the different cells trapped in the pleural space after the first stimulation of the mesothelium and whose magnitude and complexity are not yet completely clear[11]. It has been shown that the addition of talc to a human mesothelium culture provokes the release of a large amount of interleukin 8 (IL-8, known chemotactic factor for polymorphonuclear neutrophils) and also MCP-1 (monocyte chemoattractant protein), which are the two types of cells that, after mesothelial stimulation, are most directly involved in the pleural inflammation process that leads to pleurodesis[12-14]. In recent years, two additional beneficial effects have been considered for the use of talc in malignant pleural effusions which are based on apoptosis induction after intrapleural application[15] and also on angiogenesis inhibition related to tumor progression (angiostasis) which, among other factors, would be measured by endostatin production[16].

APPROACH TO PROBLEM

Until now, the role that the specific surface of the particles used in pleurodesis could play has not been researched. It is likely that smaller particles (which would have a greater contact surface area with mesothelial cells per unit of weight) are much more efficient in inducing a strong inflammatory and fibrotic response. However, these more efficient talc particles (= smaller) are also more dangerous due to the risk of systemic dissemination. If we were able to obtain big enough particles to impede systemic dissemination, but which also had a large specific surface area, we would achieve a very efficient sclerosant with few associated complications.

For our research, we used a new material (consisting of titanium dioxide, TiO_2) which is produced with a controlled size and shape using nanotechnology, provided by the PERC (Particle Engineering Research Center) at the University of Florida. It is free of impurities and the size of the particles is larger and more uniform than the majority of talc used in clinical practice (Steritalc®, Novatech, France), while its width is adjusted to 50 nanometers, allowing it to maintain extensive contact with the cellular membrane for more effective activation. Additionally, these new particles can be given an antitumoral effect by coupling them with different chemotherapy agents. They can also be used in fluorescence studies, which allows quantifying the "cellular load" with nanoparticles and locating/quantifying the particles adhered to the cellular membrane, but which have not penetrated it, and those that have been internalized[18], as well as also completing *in vivo* translocation studies[19]. In this way, *in vitro* tests can not only be done on the efficiency of inducing an inflammatory and fibrotic response, but also on the ability to slow the growth of neoplastic cells, either through apoptosis induction or angiogenesis inhibition (angiostasis).

OBJECTIVES OF STUDY

In order to prove the superiority of a new agent for pleurodesis that was designed with nanotechnology, an *in vitro* comparative study was done with the following specific objectives:

1. Compare the pro-inflammatory/fibrotic and pro-angiogenic response of new TiO2 nanoparticles compared to calibrated talc (Steritalc®, Novatech, France) in malignant cells (mesothelioma and adenocarcinoma) and benign mesothelial cells
2. Research possible apoptosis induction via TiO2 nanoparticles and calibrated talc particles
3. Research possible angiogenesis inhibition (angiostasis) via TiO2 nanoparticles and calibrated talc particles

MATERIAL AND METHODS

1. Study on cellular viability and pro-inflammatory/fibrotic response.

1.1. Benign and malignant (mesothelioma and adenocarcinoma) cell culture, and particle stimulation.

1.1.1 Culture of the cell lines CRL-9444 (Met5-A), derived from human mesothelium, and CRL-2081(MSTO-211H), originating from human biphasic mesothelioma, obtained from the ATCC (American Type Culture Collection, Rockville, MD, USA). Additionally, the cell line CRL-5911 (NCI-H2009) (human bronchial adenocarcinoma) was used, also provided by the ATCC. The cells were spread on polystyrene plates treated for cell cultures (Nunc) and were stored under constant conditions in an incubator at 37º C, 5% CO_2 and saturation humidity. The culture medium used for the Met5A line was M-199 (Gibco Laboratories, Grand Island, NY). This medium contains the essential nutrients and was additionally supplemented with 10% fetal bovine serum (FBS) inactivated by heat for 1 hour at 56º C, penicillin (100 U/mL), streptomycin (100 mg/mL), human insulin (5.75 mg/L) (Sigma-Aldrich) and epidermal growth factor (EGF) (5.75 mg/L) (Sigma-Aldrich). The MSTO-211H cells were cultured with RPMI-1640 supplemented with 10% heat inactivated FBS, penicillin (100 U/mL) and streptomycin (100 mg/mL).

1.1.2. *In vitro* stimulation of cell lines with different types of particles. The *in vitro* stimulation experiments were done between procedures 5 and 10, once the cultures reached confluence with the medium at 0% FBS. The cells were cocultured at different times (6, 24, 48 and 72 hours) at different doses (dose-response study) of talc (Steritalc®, Novatech, France) and nanoparticles (TiO_2) produced by the PERC (Particle Engineering Research Center) at the University of Florida. The doses consisted of 0, 3, 6, 12 and 24 μg/cm², according to Nasreen et al.[15]. Wells were prepared with benign and malignant mesothelial cells without particles as a control, and wells without cells as a blank. This procedure was repeated 10 times (replicas) for each cell type. Experiment monitoring was done at 6, 24, 48 and 72 hours after the start of the *in vitro* treatment. All of the samples taken from the culture mediums were centrifuged at 1,000 rpm for 5 minutes to eliminate dead cells and the supernatant was frozen in aliquots at -80º C until later determinations. For the cellular viability and proliferation study, we analyzed the behavior of the cell lines incubated with talc and nanoparticles using the Trypan-Blue exclusion test and MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. The cells for each tested dose were also photographed at each point in the experiment.

1.2. Pro-inflammatory and fibrotic response study.
Determining cytokine levels in supernatants: levels of the following cytokines were analyzed in all of the culture supernatant samples: IL-8, TNF-a, VEGF, FGFb, TGFβ-1, MCP-1. Determinations were made using ELISA (Quantikine® R&D Systems, Minneapolis, MN, USA) following the manufacturer's recommendations.

2. Apoptosis induction study.

The cells were cocultured with different doses of talc (0, 100, 200, 400, 500 and 800 μg/cm², Steritalc®, Novatech, France) (dose-response study) and with 0, 3, 6, 12 and 24 μg/cm² of TiO_2 nanoparticles produced by the PERC (Particle Engineering Research Center) at the University of Florida at different times (6, 24, 48 and 72 hours), according to talc indications published by Nasreen et al.[15]. All of the samples taken from the culture mediums were centrifuged at 1,000 rpm for 5 minutes to eliminate dead cells and the supernatant was frozen in aliquots at -80º C

until later determinations. RNA was extracted from the cell pellet with a specific kit (Qiagen Diagnostics, GmbH) following the manufacturer's recommendations. RNA quantity and quality was measured with a Nanodrop, ND-1000 spectrophotometer (Wilmington, DE, USA), and the RNA was retrotranscribed to cDNA with the SuperScript II kit (Invitrogen) in a conventional thermal cycler. The apoptosis rate was analyzed using levels of caspase-3 expression by the cells in the CRL-2081 and CRL-9444 lines after incubation with talc or TiO_2.

3. Angiogenesis inhibition (angiostasis) study.

According to the methodology followed by Nasreen et al. [16], HUVECs (Human umbilical vein endothelial cells) (Cell Applications, Inc., San Diego, CA, USA) were cultured using the medium recommended by the cell line supply company. When they reached 60-80% confluence, they were stimulated with the supernatants obtained from the cultures described in section 1.1.2 for 24 hours, applying the following doses of conditioned medium: 10, 25, 50, 100 and 200 μg/cm^2. After stimulation with supernatants for 24 hours, the HUVEC culture supernatants were extracted, aliquoted and kept frozen at -80º C until endostatin determination was made. Cell viability was analyzed using the Trypan-Blue exclusion test and proliferation was analyzed using the MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. The cells for each tested dose were also photographed at each point in the experiment. The experiments were repeated 10 times (replicas) in order to obtain enough precision in the supernatant analysis results (ELISA). Later, the same analyses described in section 2 were applied and endostatin levels were determined in HUVEC culture supernatants using the ELISA technique (Chemicon International, Inc., Temecula, CA, USA).

4. Data analysis

4.1. Cytokine levels in cell culture supernatants.

- ANOVA of factor to compare levels of each of the cytokines between the cultures stimulated with TiO_2 nanoparticles and talc particles, with application of the Bonferroni test (post hoc).
- T test to compare independent averages (intergroup, different cultures), with the application of the Mann-Whitney U non-parametric test if the equality of variances requirement was not met.
- T test to compare related averages (intergroup), with the application of the Wilcoxon non-parametric test if the equality of variances requirement was not met.

4.2. Comparative analysis of cell viability, apoptosis and necrosis.

- K test for independent samples (variance of a Kruskal-Wallis factor).
- Additionally, the systematic taking of a series of photographs of the samples at all of the points when the medium was changed and supernatants were extracted allowed for a qualitative analysis of the experiments.

RESULTS

1. Cell viability (MTT).

1.1. CRL-9444 line (benign mesothelial cells): at 24 hours, cell proliferation after coculturing with talc and TiO_2 was very similar, with a moderate reduction in the highest does of talc and TiO_2. However, viability was much lower when using TiO_2 heated to 450º C (which changes its crystalline structure with regard to the other form of TiO_2 used in our experiments). At 48 hours, we observed a clear drop in viability of the cells cultured with high doses of the three agents (talc, TiO_2 and TiO_2 heated to 450º), always noticing a more pronounced drop with TiO_2 in either of its two forms (without statistically significant differences). These findings in benign mesothelial cells would put the potential use of TiO_2 as a sclerosing agent at a disadvantage (Figure 1).

1.2. CRL-2081 line (malignant mesothelial cells): the viability of these cells was reduced very slightly when cocultured with talc (in relation to the controls), however there was a marked drop when using a >16 μg/cm2 dose of TiO_2, without reaching a statistically significant difference. This could explain the scarce mesothelial response to pleurodesis in clinical practice.

1.3. CRL-5911 line (adenocarcinoma of the lung): the cell proliferation of this line of tumor cells was clearly lower in the cells stimulated with talc than with TiO_2, in either of its two forms (TiO_2 vs TiO_2 heated to 450º). Additionally, the viability of the malignant cells exposed to talc was clearly inferior to that observed in the line of benign cells (without reaching statistically significant difference) which could have interesting implications to reinforce the use of talc in pleurodesis in malignant pleural effusions due to pleural metastasis of adenocarcinoma (which is the most common).

1.4. HUVECs exposed to supernatants (related to the response to angiogenic stimulants).

1.4.1. CRL-9444: at 24 hours, the proliferation of HUVECs stimulated with talc and TiO_2 supernatants was very similar, with a slight drop at the highest doses.

1.4.2. CRL-2081**:** after 24 hours, the proliferation of HUVECs stimulated with talc, TiO2 and TiO2 heated to 450º supernatants was very similar, without statistically significant differences (Figure 1).

2. Cytokine production (related to the response to the sclerosant) by cells stimulated with different types of particles.

2.1. IL-8: its production was distinctly lower in benign mesothelial cells (CRL-9444) than in HUVECs. It increased following a dose-dependent pattern for talc, primarily in benign mesothelial cells, although without statistically significant differences with regard to the other cell lines. IL-8 production was greater in HUVECs exposed to supernatant from benign mesothelial cells incubated with talc, also following a dose-dependent pattern, although the difference was also not statistically significant with respect to the other cell lines studied. In contrast, IL-8 production by HUVECs exposed to supernatant from benign mesothelial cells incubated with TiO_2 followed an inverse pattern in comparison to that observed with talc (a higher dose of TiO_2 resulted in lower IL-8 production). The same results were observed for TiO_2 heated to 450º, without finding statistically significant differences in any case (Figure 2).

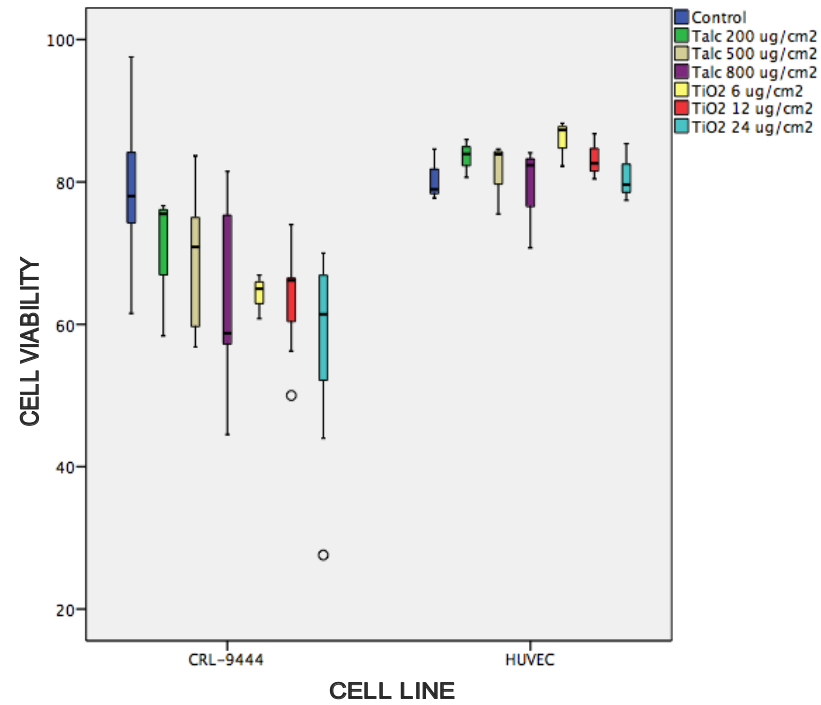

Figure 1. Cell viability for benign mesothelial cells (CRL- 9444) and HUVECs stimulated with benign mesothelial cell supernatant with different agents at different doses (talc, TiO2 and TiO2 heated to 450ºC).

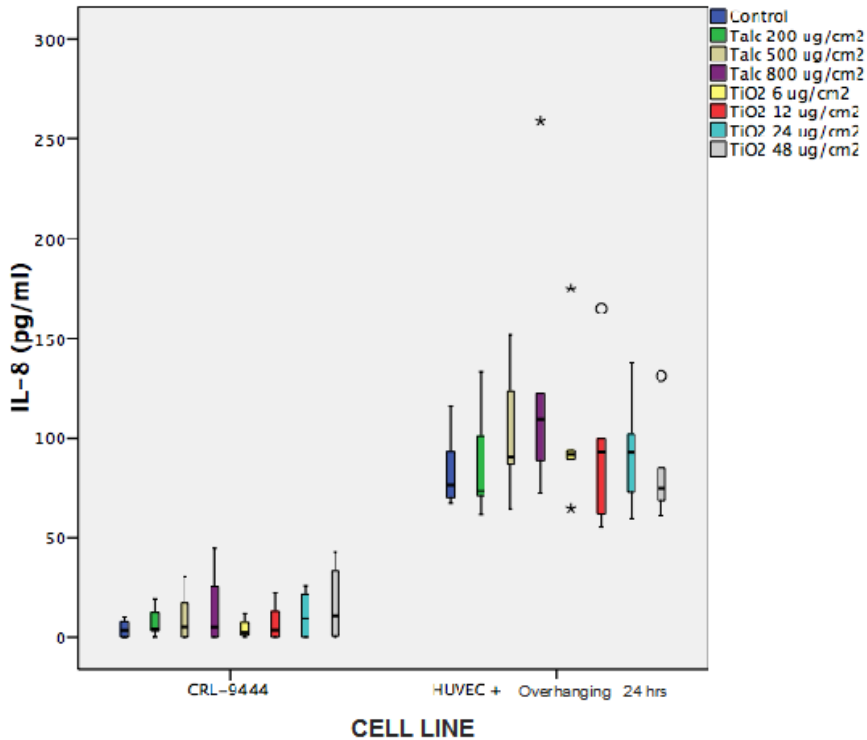

Figure 2. IL-8 production by benign mesothelial cells (CRL-9444) and HUVECs stimulated with benign mesothelial cell (CRL-9444) supernatant with different agents at different doses (talc, TiO2 and TiO2 heated to 450ºC).

2.2. FGFb and VEGF: higher levels were observed in the neoplastic mesothelial cells stimulated with talc, and a drop was seen when cocultured with TiO_2, following a pattern inversely proportional to the dose applied. A similar pattern was observed in the case of the VEGF.

3. Endostatin production (related to angiostasis) by the benign and malignant cell lines, stimulated with different types of particles.

3.1. Benign mesothelial cells (CRL-9444): endostatin levels were always lower than controls (cells not stimulated with any type of particle) both for stimulation with talc and TiO_2 or TiO_2 heated to 450º, although levels in the case of talc were higher than for TiO_2.

3.2. Malignant mesothelial cells (CRL-2081): endostatin production by these cells was also lower than the controls, although talc was again shown to be superior to TiO_2 (without significant differences). Globally, the malignant cell line produced less endostatin than the benign line for talc and TiO_2 (without significant differences). This finding may explain the poor mesothelial response to pleurodesis with talc and also suggest the modulating role the benign pleural mesothelium may play with regard to malignant lesions in the case of pleural mesothelioma.

4. Endostatin production by HUVECs after stimulation with supernatants from cocultures of benign and malignant cell lines exposed to different types of particles.

4.1. In the case of HUVECs stimulated with benign mesothelial cell supernatant, the same pattern was again observed with regard to endostatin production, both in the higher levels produced by the controls and the superiority of the effect of supernatants from cocultures with talc compared to TiO_2 (Figure 3).

4.2. A similar pattern was observed when the HUVECs were incubated with supernatants from malignant mesothelial cells (CRL-2081). However, levels were lower than in cases where the HUVECs were stimulated with benign mesothelial cell supernatant, which suggests that neoplastic cells are more resistant to angiogenesis inhibition from any of the tested agents.

5. Morphological findings with regard to different types of particles.

Photographs have been taken of all of the experiments. The most striking findings are the following:

- In general, scarce signs of cell deterioration have been observed in the benign mesothelial cells co-incubated with talc (Figure 4A, B), in agreement with the acceptable levels of cell viability and proliferation outlined previously. In the case of TiO_2, signs suggesting a higher degree of cell

damage were observed, with likely necrosis (Figure 4 A, B, C, D).

- We observed that many of the TiO_2 particles, which in theory were of ideal size to stimulate the cells by having a large specific surface area, broke apart until reaching sizes of 1 µm (or even smaller), and were internalized in many of the cells studied (Figure 5).

6. Apoptosis in different cell lines.

We observed that at 48 hours after exposure to talc and TiO_2 particles, the benign mesothelial cells expressed twice as much caspase-3 as the controls, which indicates that both talc and TiO_2 induce apoptosis in these cells (Figure 6).

- Greater apoptosis is induced in malignant mesothelial cells when they are treated with talc in comparison with TiO_2 particles.
- In the case of human umbilical vein endothelial cells (HUVECs), talc induces a higher rate of apoptosis than TiO_2 particles.

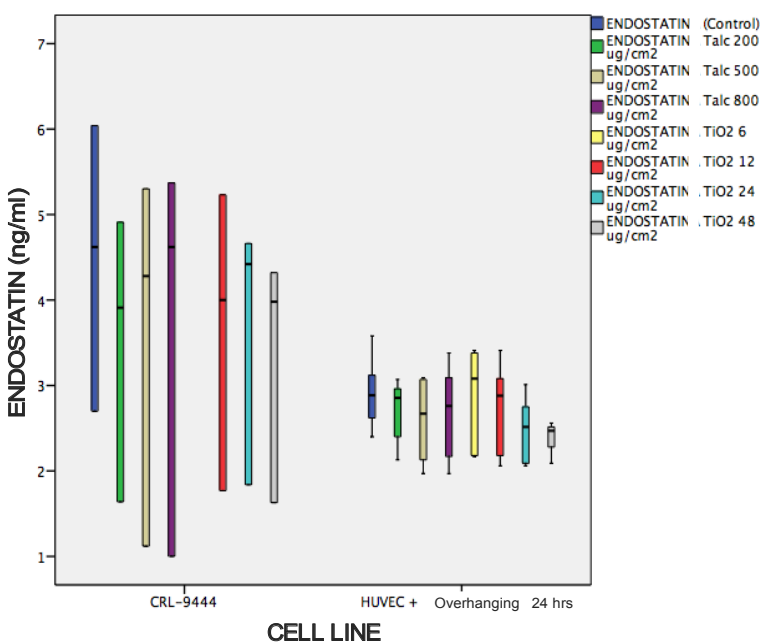

Figure 3. Endostatin production by benign mesothelial cells (CRL-9444) and HUVECs stimulated with benign mesothelial cell (CRL-9444) supernatant with different agents at different doses (talc, TiO_2 and TiO_2 heated to 450ºC).

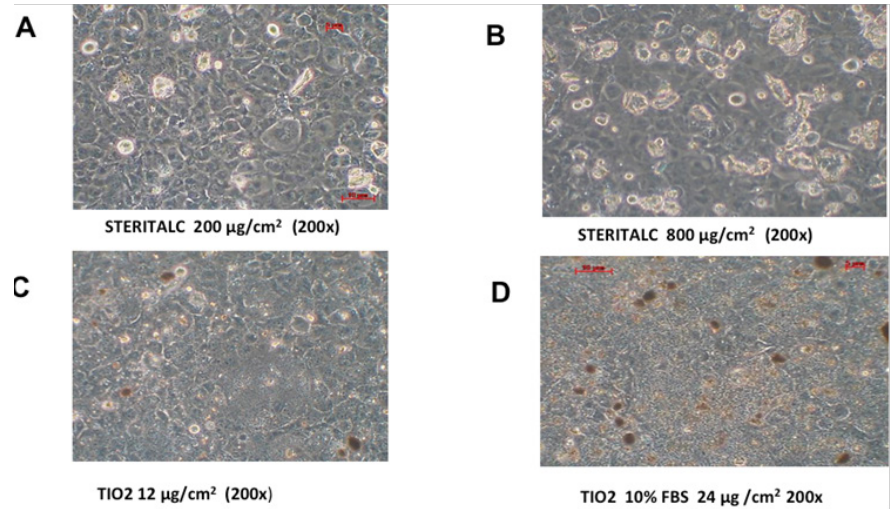

Figure 4: Benign mesothelial cells (CRL-9444) cocultured with talc (panels A and B) and with TiO_2 (C, D). The larger size of talc particles can be observed in comparison with those of TiO_2, and there are also signs of greater deterioration when mesothelial cells are cocultured with TiO_2.

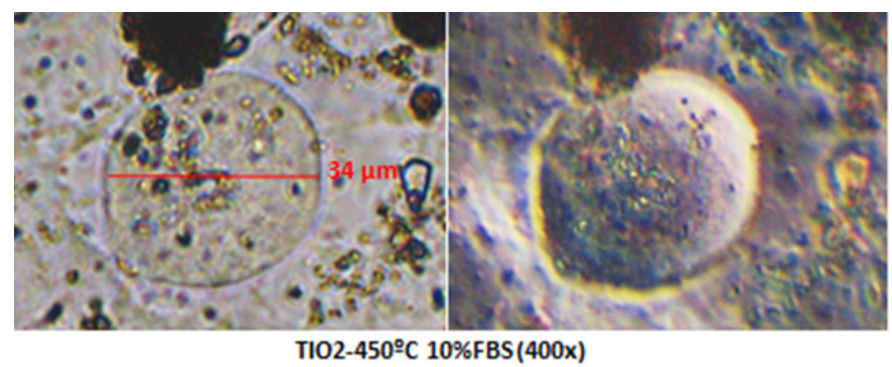

Figure 5. Internalization of small TiO_2 subparticles after coculturing the benign mesothelial cells (CRL -9444) with this type of particle.

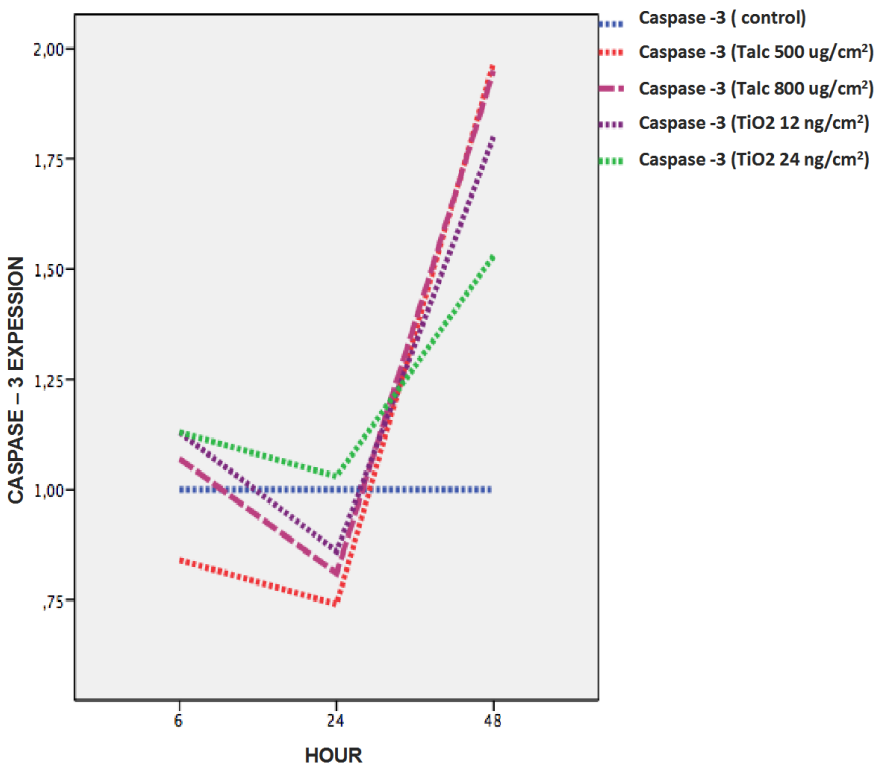

Figure 6. Caspase-3 expression in benign mesothelial cells (CRL–9444) without exposure to any agent (control), and exposed to talc and TiO_2.

DISCUSSION

At present, talc is the safest and most efficient sclerosant in the treatment of relapsing malignant pleural effusions. The majority of the complications described when used in this type of effusion are related to morphology (length, shape, width of different talc particles). Keeping in mind that talc is a naturally occurring mineral, its characteristics cannot be precisely controlled, thus the particles are subject to source extraction and processing conditions. With the major developments achieved by nanotechnology, it is possible to design particles with characteristics which are in theory ideal to induce successful pleurodesis.

TiO_2 is produced with a controlled size and shape using nanotechnology. It is free of impurities and the original size of the particles is larger than the majority of talc used in clinical practice (Steritalc®, Novatech, France), while its width is adjusted to 50 nanometers, allowing it to maintain extensive contact with the cellular membrane for more effective activation. On top of that, the crystalline state and micro/nanoporosity of this new material can be adjusted (it is well-accepted as a biocompatible material and has been used in human implants for decades) which, from the theoretical perspective, gives it great behavior versatility according to its on-demand design.

The ideal agent for pleurodesis in malignant effusions should, at least, meet the following characteristics:

a) induce a good fibrotic response in the pleural space, with the objective of eliminating the pleural space though the adhesion of the visceral and parietal layers of the pleura, eliminating the possibility of the reaccumulation of pleural liquid.
b) inhibit the proliferation of neoplastic cells, respecting the proliferation of benign mesothelial cells to the degree possible.
c) inhibit the angiogenesis that commonly accompanies invasive tumors.
d) have the physical properties to impede their passage from the pleural space to systemic circulation.

In the viability studies completed in our work, we observed that the viability of benign mesothelial cells is much lower after applying TiO_2, which would detract from its use as a sclerosing agent from the outset. The mechanisms implied in pleurodesis are not completely explained and, although it is known that practically all sclerosing agents provoke acute inflammation in the pleural space, there are few studies that have researched the specific mechanisms involved in this process[21]. The role of the mesothelium seems essential, because it clearly initiates a primary response, which is the first target of any stimulant applied in the pleural space. There is also a secondary response, which is caused by the different cells trapped in the pleural space after the first stimulation

of the mesothelium and whose magnitude and complexity are not yet completely clear.

In the case of malignant mesothelial cells, the same effect was observed, primarily with the high dose of TiO_2 (greater than 16 micrograms/cm^2). The scarce decrease in the viability of these malignant mesothelial cells treated with talc could explain the limited mesothelial response to pleurodesis with talc in clinical practice. On the other hand, malignant mesothelioma usually extensively and diffusely affects the majority of the pleural cavity, thus the modulating response a non-tumorous mesothelium could have is voided or greatly reduced, which again contributes to explaining the limited or non-response that a malignant pleural mesothelioma usually has to pleurodesis or any other of the treatments commonly attempted with this type of neoplasm.

In adenocarcinoma of the lung, the viability of the cells exposed to talc was clearly inferior to that observed in the line of benign cells, which could have interesting implications to reinforce the use of talc in pleurodesis in malignant pleural effusions due to pleural metastasis of adenocarcinoma (which is the most common).

IL-8 (known chemotactic factor for polymorphonuclear neutrophils) and MCP-1 (monocyte chemoattractant protein) are known to be the two types of cells that, after mesothelial stimulus, are most directly involved in the pleural inflammation process that leads to pleurodesis[13,14]. In our study, IL-8 production was much higher in neoplastic mesothelial cells than in benign cells and increased following a dose-dependent pattern with talc, while it decreased with TiO_2, with a pattern inversely proportional to the applied dose of TiO_2. Similar results were found for VEGF production. According to these results, we can see that talc is superior to TiO_2 in its ability to produce mediators which favor pleurodesis for the control of malignant pleural effusions.

The line of malignant mesothelioma cells proved particularly resistant to the angiostatic action of talc in previous studies[16] as well as in our *in vitro* experiments, showing a clear correspondence with the clinical results previously published by our Group which show that this tumor has the worst results in pleurodesis attempts. On the contrary, adenocarcinoma has proven to be more sensitive to talc than to the other two agents tested with regard to the inhibition of angiogenesis measured by endostatin[16].

The results obtained show that talc (sterile with calibrated particles) is currently the most efficient sclerosant used in the control of relapsing malignant pleural effusions and the *in vitro* results obtained for the use of TiO_2 are clearly inferior. In our study, we have seen that the TiO_2 particles had broken apart, reaching sizes under 1 mm, having been internalized in many of the cells studied (Figure 4 A, B, C, D and Figure 5). This can explain some of the negative effects observed in our TiO_2 experiments and invalidate its potential as a sclerosing agent in humans (at least in its current form) due to the risk of systemic dissemination. We still do not have an explanation for this unexpected finding which supposes a clear limitation for the use of this agent, which seems to be stronger for TiO_2 heated to 450º C, although this is likely related to certain instability in its crystalline structure, which will need to be studied in the future.

This is an experimental "*in vitro*" study performed with cultures of cell lines, thus these preliminary results with TiO_2 cannot be extrapolated to "*in vivo*" studies. However, we continue to think that nanotechnology offers great future design possibilities for an agent which obtains better results than talc. We believe it is necessary to continue to perform additional studies to obtain a sclerosing agent with the ideal characteristics.

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