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Original Research

Lowered Cyclin E levels increase the efficiency and the specificity of capsaicin against cancerous cells of mesothelium

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Abstract: Capsaicin is one of the most extensively studied phytochemicals and its cytotoxicity on a various types of cancer has been demonstrated both *in vitro* and *in vivo*. The evaluation of its effect on mesothelioma, however, has remained quite limited. In this study, we investigated the anti-mesothelioma potential of capsaicin by observing its cytotoxicity on healthy, immortalized and cancerous cells of mesothelium *in vitro* and how this potential be affected by lowered Cyclin E levels, a key regulator of G1/S transition of cell cycle. For this purpose, we determined and compared the IC_{50} values of capsaicin in both FBS (Fetal Bovine Serum) containing and FBS-deprived meidum of each cell population studied. Additionally, we examined the changes in both protein and mRNA levels of caspase-3 upon capsaicin exposure as well as conducted a series of experiments through which the relatively long term effect of capsaicin on the growth rate of the cells was assessed. As a result, the reduced Cyclin E obtained through the absence of FBS in growth medium was found not only to decrease IC_{50} values for all cell types dramatically ($p<0.05$) but also to cause a considerable difference between the values determined for cancerous and non-cancerous populations ($p<0.05$), which had not been observed in regular medium. Moreover, along with the fact that capsaicin exposure did not have an impact on the cell growth in long term in most cases, caspase-3 levels also remained the same when exposed to capsaicin, suggesting a mechanism of cell death independent of caspases.

Key words: Capsaicin; Mesothelioma; CRL-5946; AG07086; T4067; Cyclin E; FBS-deprived medium.

Introduction

Mesothelioma is the cancer of the mesothelial tissue which covers many internal organs such as lung, heart and stomach. Albeit rare, it is one of the most deadly cancers and its epidemiology varies greatly across countries. While intdustrialized countries such as Australia, New Zealand and UK have the highest incidence of the disease, the lowest rates were reported from Slovenia, Japan and other countries located in central Europe (1). Turkey, despite having a 1.1/100,000 and 0.6/100,000 incidence rates for males and females, respectively, according to 2015 statistics reported by the country's Cancer Control Department of the Ministry of Health, presents a very unusual epidemic in certain villages of Cappadocia, where nearly 50% of the death are linked to mesothelioma (2).

A discovery made about Malignant Mesothelioma in 2012 revealed that a mutation in BRCA Associatiated Protein 1 (BAP1) gene, which impairs the nuclear localization of the encoded protein, a deubiquitylase associated with many important pathways, such as the DNA damage response (DDR), the cell divison cycle, and cell death, increases the susceptibility of the individuals to develop the disease (3,4). Besides, this mutation has been shown to reduce the threshold of asbestos needed for the tumor formation in mice (5). Regardless of the increasing understanding of the mechanism laying behind it, mesothelioma is still rather difficult to treat such that cisplatin is still the only chemotherapy given to the people with unoperable tumor. In addition to the fact that the median survival of the patients remains around 12months and the median progression-free survival (PFS) does not even reach up to 6 months, no treatment has yet been proven to be beneficial to survival of relapsed malignant pleural mesothelioma patients (6). Consequently, an urgent need for novel therapeutic approaches has become evidentiary.

Capsaicin, as a pungent component of the pepper, captured significant attention since its anti-cancer potency shown in vitro as well as in vivo. A great number of cancer cell lines including breast (7), human leukemic cells (8), bladder (9), prostate (10), and hepatic carcinoma cells (11) has been demonstrated to be eliminated by the application of capsaicin, mostly around 200-300 µM IC50 value (12); although in some cases concentrations as low as 5 nM have also been reported (13). Moreover, not only has this elimination been proven to occur via apoptosis but it was also shown that when capsaicin was applied to the healthy tissue or the cells, it caused no harm whatsoever (14). Additionally, through the investigations of the molecular pathway of the effect of the compound, it was discovered that in many cases, capsaicin arrests cells at G1 phase of cell cycle, which later triggers the apoptotic pathways in cells (7,15,16).

Cell division cycle is the combination of the phases that all cells proliferating via mitosis proceed through their life lime. The cycle is usually seperated into three

steps as G1, G2 and S before reaching the mitotic site where the nucles and the cytoplasm of the cell is divided to produce two virtually identical cells. In the first step of the cycle, G1, a cell grows and controls the enviromental conditions as well as the quality and the integrity of DNA before leading to S phase where DNA is replicated. It has been known that the amount of Cyclin E protein is increased in a cell when it transiates between G1 and S phases in order to promote S phase entry and progress by activating CDK2 through bindging to it (17). The check point between G1/S phase of the cycle is named as Restriction Point and the decision machinery functioning at this point is so essential to normal differentiation that it is highly dysregulated in most cancer cells (18).

Upon considering these facts together, we intended to explore how cytotoxic capsaicin would be against normal, immortalized and cancerous cells of mesothelium *in vitro* as well as whether the lowering Cyclin E levels of the cells by growing them in FBS-deprived medium would modify its impact on these populations.

Materials and Methods

Cells and media

Cancerous cells, CRL-5946 , were purchased from American Type Culture Collection (ATCC), USA; immortalized cells (Catalog ID : AG07086) were kindly provided by Coriell Institute NJ, USA while primary mesothelial cells (T4067) were bought from abm, Canada. Mesothelioma cell line was cultured in T25 and T75 flasks in 10 % (v/v) FBS (Fetal Bovine Serum) (Cat. No : 10270-106; Thermo Scientific, USA) and 1% (v/v) Pennycilin-Streptomycin (Cat. No : 15140-122; Thermo Scientific, USA) consisting in DMEM (Dueblo's Modification on Eagle's Medium) (Cat. No: 11965-092; Thermo Scientific, USA) Immortalized cells were grown in a 1:1 Ham's F-12 : M199 media (Cat. No's : 11765-054 and 11150-059, respectively; Thermo Scientific, USA) supplemented with 1% Pennycilin+Stretomycin, 1% (v/v) L-Glutamine (Cat. No : 25030-024, Thermo Scientific, USA) 1% (v/v) Hydrocortisone (Cat. No : sc-300810) and 10 ng/mL EGF (Cat. No : PHG0314; Thermo Scientific, USA). For normal cells, the manifacture suggested to use PriGrow Medium I (Cat. No : TM001; abm, Canada) supplemented with 10% (v/v) FBS and 1% (v/v) Pennycilin-Streptomycin on a extracellular matrix (Cat. No : G422; abm, Canada) coated flask and culture plates.

For FBS-deprived media, FBS was excluded when the media were prepared. All media were sterilized through 0.2 µm filter in a vacuum system (Cat. No : SCGPT05RE, Milipore, Germany). Cells were incubated at 37°C under 5 % CO_2 pressure with incubator (Panosonic, Gunma, Japan). All experiments included living cells were performed inside Class II Biosafety Cabinet (Metis, Ankara, Turkey).

Capsaicin

Capsaicin was purchaed from Sigma (Cat. No : M2028; Taufkirchen, Germany) and was dissolved in absolute ethanol (Cat No.: 100983; Merck, Taufkirchen, Germany) at the concentration of 65 mM as a stock. Stock solution was kep at -20 for a storage exceeding two weeks while daily-used stock tubes were stored at 4°C.

Cell viability assay for regular medium experiments

Appropriate numbers of cancerous, immortalized and primary cells (5×10^3/well) were seeded in 96-well plate and the next day, the cells were treated with capsaicin of indicated concentrations of 200 µL in triplicates. After incubation for different time points (6, 12, 24, 48, or 72 hrs), 10 µL/well MTS Assay reagent, [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (Cat.No: G3582; Promega, Fitchburg, WI, USA) 1:1 diluted in molecular biology grade water was added and the mixture was incubated at 37°C, 5% CO_2 for 4 hours. Then the absorbances at 490 nm were measured by a plate-reader (SpectraMax Plus 384, Sunnyvale, CA, USA). In the experiments where FBS-deprived medium was used, the following day of the seeding, the medium of the cells were changed to FBS-free medium and the cells were kept in this condition for 24 hours prior to the treatment. Capsaicin solutions for these treatments were also prepared in FBS-free medium.

In order to eliminate the background absorbances caused by the medium, the solvent and the pungent molecule, only medium, onyl ethanol containing medium and only capsaicin solved in ethanol containing medium were plated along with the experimental set up. At the end of the experiment, required calculations were made to exclude background readings made by these materials.

Western blotting

Cells were trypsinizated and collected by centrifugation at 1000 rpm for 5 mins. The proteins were isolated with Mammalian Protein Extraction Reagent (ThermoScientific, USA) containing protease cocktail (Roche, Germany). After the determination of protein concentrations using the Bradford assay (Bio-Rad, PA, USA), proteins were separated through a hand-made % 12 acrylamid-bisacryl amid gel and transferred to a polyvinylidene difluoride membrane (BioRad, PA, USA). Later, the membranes were blocked in 5% (w/v) grade blocker dissolved in TBS-Tween 20, followed by their incubation with specific primary antibodies overnight at 4°C. All primary antibodies were pruchased from abcam (Cambridge, UK) and used in 1:1000 dilution in blocking buffer. Once the incubation was complete, the membranes were washed five times with TBST, and then incubated with HRP-conjugated secondary antibodies at room temperature for 2 hours. Then they were washed again with TBST and the protein bands were visualized using ECL Chemiluminescence reagent (Bio-Rad, PA, USA) via UVP EC3 Chemi HR 410 Imaging System (Upland, CA, USA) in dark.

Lowered Cyclin E levels

The cells were first counted in a T25 flask as equals to 5000 cels per well in a 96 well plate. The next day FBS-deprived medium was applied. After 24, 48 and 72 hours, the cell pellets were collected to check Cyclin E protein levels by Western Blotting. Low Cyclin E levels were regarded as a sign of G0/G1 arrest of cells. In all conditions 24 hours appeared as the best time point keep

most cells in the step. Following the determination of the best duration, the experiments were conducted to see the effect of capsaicin on IC_{50} values by Cell Viability (MTS) Assay.

cDNA preparation and analysis by real-time quantitative PCR

Total RNA from the treated and control cells were isolated with GF-1 Total RNA Extraction Kit (Vivantis, Selangor Darul Ehsan, Malaysia) according to the manufacturer's protocol. Total RNA was converted to cDNA by using BioLabs Protoscript-I cDNA Synthesis Kit (New England, USA) For the detection of caspase-3, 1 µL of cDNA was used in 10 uL PCR reaction in which 5 uL syBer green containing master mix, and 100 pmol of each primer of the targeted molecule was added. Ct values of each tube was calculated by using Light Cycler Nano (ROCHE, Germany). Accumulated PCR products were detected directly by monitoring the increase of the reporter dye (SYBR®). The expression levels of Caspase-3 in the exposed cells were compared with those in control cells The following primer sequences were used: Caspase-3 forward, 5′- AGAGGGGATCGTTGTAGAAG -3′; Caspase-3 reverse, 5′-TTGCCACCTTTCGGTTAAC-3′; β-actin forward: 5'CCCTGGACTTCGAGCAAGAG-3', β-actin reverse: 5′ATCTTCATTGTGCTGGGTGC-3'. Reaction conditions were set as 95C for 1 min following 45 cyles of 94°C for 30 seconds and 53°C for 1 min. Ct values were calculated as described in the instrument manual and normalized to the amount of β-actin, a housekeeping gene. The following formulas were used for determining relative mRNA levels of capsase-3 in treated and untreated groups as result of which the control group became adjusted to 1.00. Ct_{cap} represents Ct value of capsaicin treated cells while Ct_{none} is the same for control. For ethanol treated cells, Ct_{etoh}, instead of Ct_{cap}, can also be used in this algorithm. Considering each primer set used will present a different Ct value, the name of the mRNA checked was added to subscript as examplified in $Ct_{cap\_caspase3}$ where Ct values of caspase 3 mRNA levels in cap treated cells were presented.

Relative expression of caspase 3 in capsaicin treated cells = $2^{-\Delta\Delta Ct_{cap}}$

$\Delta\Delta Ct_{cap} = \Delta Ct_{cap} - \Delta Ct_{none}$

$\Delta Ct_{cap} = Ct_{cap\_caspase\text{-}3} - Ct_{cap\_Bactin}$; $\Delta Ct_{none} = Ct_{none\_caspase\text{-}3} - Ct_{none\_Bactin}$

Statistical analysis

All IC_{50} calculations and the statistical analysis were performed by GraphPad Prism 6.0. All the quantitative data are expressed as mean values ± standard deviation and represented as dots and error bars on the graphs, respectively. The statistical significance of the differences between the effect of capsaicin on cells' viability were determined by Two-Way Anova. Because of the dissimilarities in the absorbances measured for each cell type grown in distinct media, the values were converted the percentage of the viable cells assuming 100% of the cells were alive in only medium and cell containing wells at the end of the experiment. The effect of capsaicin on different cell types was accepted as statistically significant only when the p values of coloumn factor (cell type) and interaction (cell typeXcell viability) were both calculated to be lower than 0.05. To determine the statistical difference between treated and control groups at each time point of the experiment where relatively long term effect of capsaicin was evaluated, Student's t was used and a probability value of $P < 0.05$ was considered as significant.

Results

As it can be seen in Figure 1, 24 hours-growth in FBS-deprived medium reduced the Cylin E levels dramatically in all cell types. This observation was used as a proof of G0/G1-arrest in cells. Once it was established, the impact of reduced Cyclin E levels in capsaicin treatment on cancerous, immortalized and primary cells of mesothelium was investigated in *in vitro* conditions.

For this purpose, we determined the IC50 values of capsaicin in both conditions (FBS-containing and FBS-free) for all types of the cells studied. In order to achieve this goal, we used cell viability assay (MTS Assay) as described in Material and Method section. As a result, we found that when the cells were grown in their regular medium, the IC_{50} values calculated for each cell type on 12 hour exposure to capsaicin did not differ among the cell types while in FBS-deprived medium, immortalized and primary cells presented a significantly lower ($p<0.05$) values compared to canceorus cells. Furthermore, the cells grown in both media revealed remarkably distinct IC50 values, usually two fold higher when FBS is present in the medium for the same cell type. These results were displayed in Figure 2.

Next, we evaluated the long term effect of capsaicin treatment in all cells in both medium conditions. As visualized in Figure 3, the growth rate of the cells did not appear to be affected by the presence of capsaicin at IC_{50} concentration up to 72 hours in most conditions studied. Only statistically significant differences were observed in cancerous and primary cells at 24 hours in the medium without FBS, which quickly disappeared at 48 hours.

Since cell viabilities were determined to change when exposed to high concentrations of capsaicin, we wondered whteher IC_{50} concentration of capsaicin would indicate a apoptotic cell death in these cells. Relative protein and mRNA levels of caspase-3, the exe-

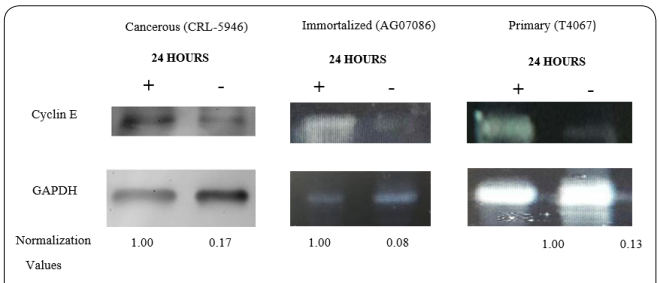

Figure 1. Western Blotting Pictures of Cyclin E Protein Levels In All Cell Lines Grown With and Without FBS for 24 Hours. + represents the conditions where FBS was present in the growth medium while – indicates its lacking. Albeit weaker, Cyclin E bands were present for each celly type even after the cells were grown in FBS-deprived (-) medium. However, when the insentisty of the bands was normalized with with loading control (GAPDH), at least 83% reduction (1.00 vs 0.17) in the production of the targeted protein was detected.

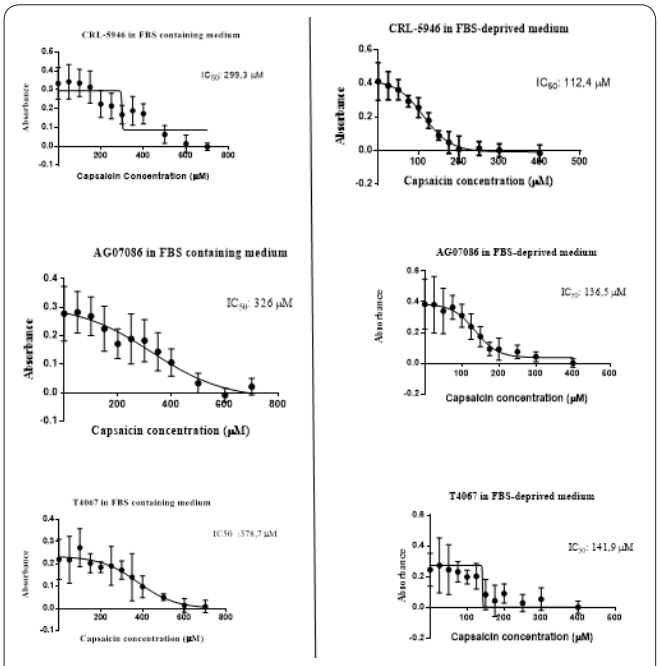

Figure 2. Capsaicin concentration versus absorbance graphs of all cell types in cell viability assay. Left pannel presents the cells in FBS containing medium whereas the cells in FBS-free medium were displayed on the right pannel. On each side, cancerous, immortalized and primary cells were located from top to the bottom. 5000 cells were cultured in 96 well plate and the next day, the cells were treated with increasing concentrations of capsaicin for 12 hours. For the cells grown in FBS-deprived medium, they were kept in the appropriate medium for 24 hours before the capsaicin treatment. Once the treatment is complete, 10 µL of MTS reagent was put into the medium and the absorbances at 490 nm were measured at the 4th hour. Each experiment was replicated three times with four wells for each conditions; mean values and standard deviations were presented on the graphs as dots and error bars, respectively.

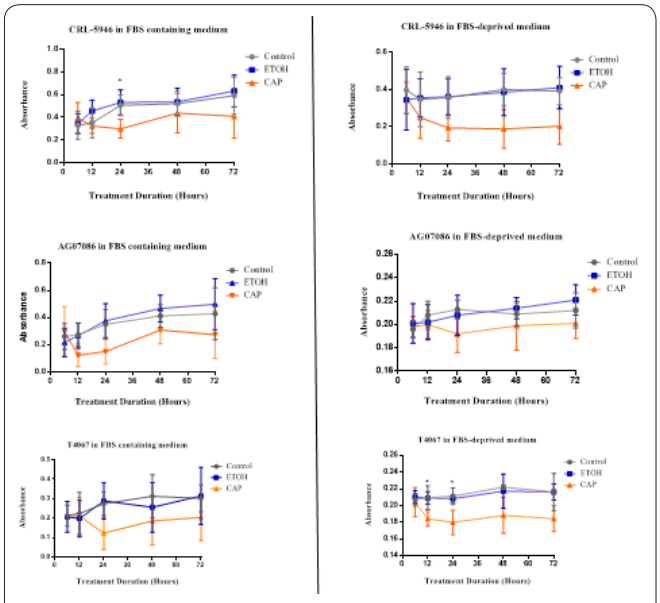

Figure 3. Long term effects of capsaicin treatment at IC_{50} value of each cell. Left pannel presents the cells in FBS containing medium whereas the cells in FBS-free medium were displayed on the right pannel. On each side, cancerous, immortalized and primary cells were located from top to the bottom. 5000 cells were cultured in 96 well plate and the next day, the cells were treated with capsaicin at IC50 concentration determined for each cell type (CAP), or only with ethanol (EtOH). One control group without any treatment (Control) was also included in each experimental set up. The cells grown in FBS-deprived medium were kept in the appropriate medium for 24 hours before the capsaicin treatment. 6, 12, 24, 48 and 72 hours after the treatment started, 10 µL of 1 :1 MTS reagent-water mixture was put into the medium and the absorbances at 490 nm were measured at the 4th hour. Growth rates were mostly not affected by the presence of capsaicin except for the 24th hour in cancerous and primary cells, which were indicated by asterisks on the graph.

cutioner of all apoptotic pathways, were detected in all cell types. As presented in Figure 4 and Figure 5, no variation of this kind was in untreated, ethanol treated and capsaicin treated cells in both media.

Altogether, we concluded that FBS absence in the medium might be increasing the efficiency of the cells since IC50 values were dropped nearly in half in all cell types and that cancerous cells of mesothelium appeared to be more sensitive to exposure than its immortalized and primary counterparts in FBS-deprived enviroment. Besides, despite the fact that the cell viability suggested possible death of the cells upon exposure to capsaicin, caspase-3 levels did not appear to be affected by its presence giving no indication for apoptosis in these cells.

Discussion

The present paper reports the results of first known investigation of capsaicin's effect on canceorus, immortalized and primary cells of mesothelium. Although there are many cell types whose behaviors were evaluated under the presence of capsaicin, it has been a surprise to encounter no such study for a disease so difficult to treat and desperately needs novel approaches to help the patients. Therefore, we believe the cancer type we investigated can easily provide our study with novelty. Additionaly, the fact that we evaluated not only cance-

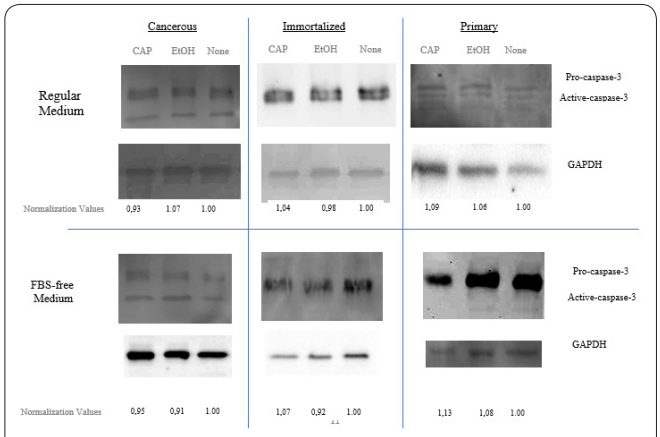

Figure 4. Western Blotting Pictures of Caspase-3 Protein Levels In All Conditions Studied. Upper pannel displays the cells experimented in regular (FBS-containing) medium while the cells examined in FBS-free medium were presented at the lower pannel. In both pannels, cancerous, immortalized and primary cells were placed from left to right. Band numbers observed in the blots appear to be cell-type and treatment dependent. According to the data sheet of the product, upper band/s is/are considered as the inactive form of caspase-3 (pro-caspase) and the lower band as its active form. Althouh some slight changes were observed in the protein levels depending on the treatment, when normalized with loading control (GAPDH), the difference seemed to be inconsequential. Where the both (pro- and active forms) were present, only active forms were considered for normalization.

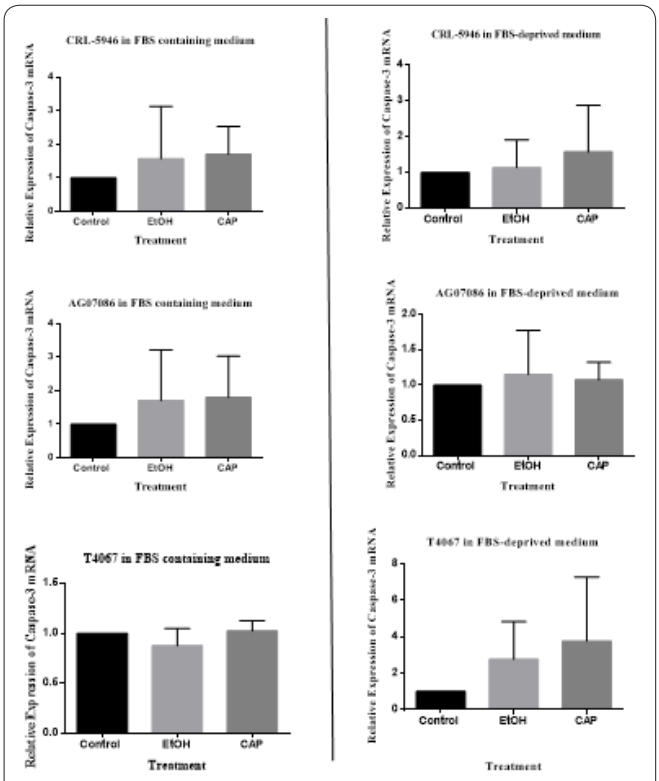

Figure 5. Relative Expression of Caspase-3 mRNA In All Conditions Studied. Cells were treated with capsaicin at IC concentration (CAP) or only equal amount of ethanol in medium with or without FBS. Untreated group (CAP) was also included in each experimental set up. After 24 hours, the cells were collected and mRNAs were isolated. mRNAs were converted to cDNA and Real-Time PCR with SyberGreen was performed with Caspase-3 and β-Actin primers in seperated tubes. β-Actin was used as the normalization factor. Each experiment was repeated two times with two replicates. Normalization values were graphed and analyzed. No statistically significant difference between the control and the treatments was detected in any cell type or condition. Left side of the pannel presents the results obtained from the cells grown in regular (FBS-containing) medium while the cells examined in FBS-free medium were displayed at the lower pannel. In both pannels, cancerous, immortalized and primary cells were placed from top to bottom.

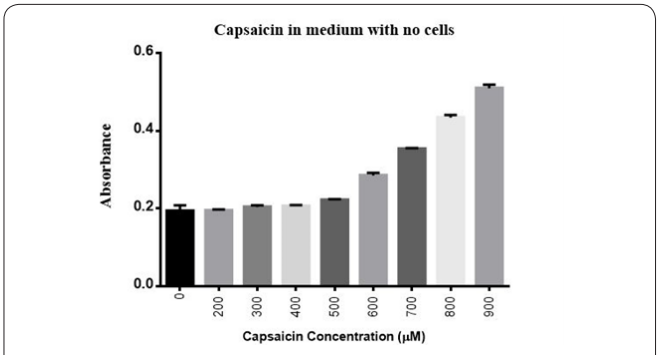

Figure 6. The absorbances of only media with increasing concentrations of capsaicin (0-900 μM) at 490 nm were measured with MTS Assay reagent after 4 hours of incubation.

rous and immortalized but also primary cells of the tissue from which the disease is formed, also redounds our approach with a dimension which is not really commonly seen in the literature. Considering that the untargeted drugs can be interacting with any type of cells once in the circulatory systems of an organism, the use of primary cells becomes crucial for *in vitro* studies of any potential substance since its impact on healthy cells is as important as its affect on the cancer targeted. To our view, the results obtained only for the cancerous cells will always be biased for its potential harm to the healthy cells.

For the purpose highlighted above, we used one cell population of each cell type of the tissue whose malignancy was in our focus; CRL-5946 as a malignant mesothelioma cell line with epithelial morphology established in 1990 from a male patient, AG07086, as immortalized cell line obtained from a 26 yearl old female and T4067 as primary healthy mesothelial cells whose donor did not agree on releasing her/his information by the provider. The fact that the cells were obtained from different individuals with different age and gender and that only a single population of each kind was investigated through our studies might be considered as one of the limitations of our approach. Because it has been known that people with distinct genetic profile and life styles may react to the same compound differently even in a highly controlled settings (19,20). However, reconginizing that we included primary and immortalized cells along with the canceorus cell line of the same tissue, it presented an unusual approach to the problem since most published reports investigated the effect on the cancerous cells only (9,11,16,21).

Our experiments have demonstrated the potential of use of LP9 (AG07086) immortalized cells as a model in understanding the short term effect of capsaicin on normal cells of mesothelioma because they presented a rather similar results to normal (primary) cells in most experiments, which agreed on the studies where these cells are considered as normal mesothelial cells (22,23). In our case, however, there was only one exception; the long term effect of capsaicin that at the 24th hour of FBS-deprived medium. While cell viability was found to be significantly lower in capsaicin treated primary cells compared to its ethanol and untreated counterparts, no such difference was observed in AG07086 cells in these experiments. Consequently, we recommend to choose the cell type cautiously when worked on the long term effect of capsaicin since immortalized cell lines may not be presenting the actual behavior of normal healthy cells, thus the results might be skewed in this manner.

Moreover, the readers must also be warned about the fluctations of the absorbances read in many experiments here. Although the same cell type in same conditions were studied, the error bars on the graphs clearly indicated the difference of the absorbances obtained in each experimental set up. Despite the fact that in some single experiments the difference we observed between the conditions were remarkable, once their repetations were added to the analysis, the difference weakened dramatically. These observation pointed the importance of the repetations of the experiments before reaching a conclusion.

Another important finding we gathered about out viability assays was that capsaicin itself in medium also caused absorbances at the wavelength studied. When we used only medium with increasing concentration of capsaicin, we found the concentrations 500 and upper caused a significant increase in the absorbance ($p<0.005$) (Figure 6). Therefore, every time we set up an experiment we included only capsaicin containing medium at

each concentration to normalize the background reading at 490 nm. Given that capsaicin has been shown to be an antioxidant (24), our findings were in accordance with the results suggesting that oxidative compounds might affect the MTS efficiency (25). When a similar approach was used for ethanol containing medium with and without cells, we figured that ethanol affected neither the viability nor the absorbance of the medium at the concentrations used in the study (data not shown).

The fact that we investigated the behavior of the cells in FBS-free medium was an innovative concept in the field since, albeit there are some studies in which capsaicin was applied to the cells in serum-deprived medium such as the one carried by Shin et al. (26), we have not encountered with an investigation where cells were kept in serum free medium both prior to and during the treatment. Surprisingly, our experimental design turns out to have a significant revelance to the effect of capsaicin on the cell types studied since it increased not only the efficiency (by reducing the IC_{50} value to nearly half) but also the specificity (canceorus cells presented significantly lower levels of IC_{50} compared to the others) of it.

Additionally, the observation of essentially no difference in viability of the cells in control and treated groups in long term caught us with surprise since the time and dose dependence of the effect of capsaicin has been repeatedly reported (8,15). Our result may be seemingly contrary to the previous reports; however in each experiment we conducted we also found the mean value of our capsaicin treated samples to be lower than ethanol treated and untreated groups even though, with standard deviations, these values reflected a statistical significance in only two conditions; FBS-free primary cells and FBS-free mesothelioma cell line at 24 hour. Besides, we would like to emphasize the fact that we have not changed the medium during the experiment, therefore capsaicin might have been decomposed at the culture conditions. Lastly at this topic, we would like to discuss the possibility of that slight differences in cells of the populations we studied might have led us to these findindgs; such that capsaicin-sensitive cells, which can be associated with their TRPV1 expression levels whose activation can be triggered by the binding of the compound (27), may have been eliminated in the first 12 hour of the capsaicin exposure while the rest has overcome the treatment and kept growing. Besides, when the fact fact that most mesothelioma tumors are polyclonal in origin (28) and the probability that capsaicin-sensitive cells might also be slow to grow, thus their contribution to the cell population can easily be dominated by the less-sensitive cells, are taken into the account, our speculation becomes reasonable and is open to be checked experimentally.

Cyclin E is known to be one of the many regulators of cell cyle by which CDK-2 is activated and G1-S transition of the cells is progressed (29). We are aware that decrease in cyclin E levels only is not sufficient to declare a G0/G1 arrest in cells since its level is also expected be reduced in late G2 and M stages of the cells (30). However, considering that the cell proliferation rate in FBS-deprived cells were reduced remarkably while the cell size did not seem to change dramatically (data not shown), we assume that our inference has a solid ground. Therefore, we speculate that these observations can be intpreted as the indication of G0/G1 arrest, which resulted in an increase in capsaicin efficiency against mesothelioma.

The IC_{50} values we calculated for the cells, were little higher than the avarage value presented in the review by Biley et al (12), however, this result is not unexpected when the fact that mesothelioma is one of the most aggresive tumors with poor prognosis is considered (31). Readers also need to pay attention to that such comparision could not be made for the results obtained in FBS deprived medium because of the lack of the results of this kind.

Despite the fact that some promising results have been collected about the potential effect of capsaicin on the cells of mesothelium in the present study, it has to be reminded that only one cell population of each kind was used, therefore more comprehensive studies including different cell lines are needed to reach a conclusion about the effect of capsaicin on mesothelioma. Further studies are also needed to investigate the actual type of cell death triggered by capsaicin exposure to be able to scrutinize its real potential to be used as a anti-tumor drug in patients.

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Conflict of interest
The authors declare no interest of conflict.

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