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OXIDATIVE STRESS ADAPTATION IN AGGRESSIVE PROSTATE CANCER MAY BE COUNTERACTED BY THE REDUCTION OF GLUTATHIONE REDUCTASE

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Running title: Glutathione reductase in prostate cancer therapy

ABSTRACT

Oxidative stress has been associated with prostate cancer development and progression due to an increase of reactive oxygen species (ROS). However, the mechanisms whereby ROS and the antioxidant system participate in cancer progression remain unclear.

In order to clarify the influence of oxidative stress in prostate cancer progression, we performed this study in two human prostate cancer cell lines, PC3 and HPV10 (from metastasis and from cancer in situ, respectively) and RWPE1 cells derived from normal prostate epithelium. Cells were treated with hydrogen peroxide (H_2O_2) and PC3 cells were also treated with diethyl maleate (DEM). The effect on cell growth, viability, mitochondria membrane potential and oxidative stress was analyzed. Oxidative stress was evaluated based on ROS production, oxidative lesion of lipids (MDA) and on determination of antioxidants, including enzyme activity of glutathione peroxidase (Gl-Px), glutathione reductase (Gl-Red) and on the quantification of glutathione (GSH), glutathione-s-transferase (GST) and total antioxidant status (TAS).

PC3 shows higher ROS production but also the highest GSH levels and Gl-Red activity, possibly contributing to oxidative stress resistance. This is also associated with higher mitochondrial membrane potential, TAS and lower lipid peroxidation. On the other hand, we identified Gl-Red activity reduction as a new strategy in overcoming oxidative stress resistance, by inducing H_2O_2 cytotoxicity. Therefore these results suggest Gl-Red activity reduction as a new potential therapeutic approach, in prostate cancer.

KEY WORDS: Prostate cancer, oxidative stress (OS), reactive oxygen species (ROS), glutathione (GSH), glutathione reductase (Gl-Red), cell lines

1. INTRODUCTION

Prostate cancer is still the most frequently diagnosed malignant disease and the second leading cause of cancer-related mortality in men in most Western countries [1, 2].

Although the causes of the high incidence of prostate cancer are poorly understood, epidemiological, experimental and clinical studies, suggest that oxidative stress (OS) plays a major role in explaining prostate cancer development and progression [3-12]. OS, defined as an imbalance between reactive oxygen species (ROS) production and antioxidant defences [13, 14] has been linked to some prostate cancer risk factors including diet intake [15-18], recurrent inflammation and aging [19-21].

ROS, generated *in vivo*, include free radicals and non-radicals. Free radicals are molecules containing unpaired electrons as superoxide anion ($^{\cdot}O_2^-$), hydroxyl radical ($^{\cdot}OH$) and peroxide radicals [22]. Non-radicals such as singlet molecular oxygen (O_2), nitrogen oxide (NO_x) and hydrogen peroxide (H_2O_2) can easily give rise to oxygen radicals. Namely, H_2O_2 leads to $^{\cdot}OH$ formation, in the presence of transition metals [22, 23]. ROS play an essential role in signal transduction pathways [24], cell cycle progression [25-30], gene transcription [31], cell differentiation [28, 32] and death (for review see Martindale and Holbrook [33]). However, an increase in ROS production and/or a decrease in antioxidant network may induce to severe OS leading to biomolecules damage such as DNA, proteins and lipids [3-12, 34, 35]. On the other hand, oxidative damage of DNA is thought to play a critical role in all stages of carcinogenesis [36].

Moreover, the major feature of radiation therapy that is a standard treatment of prostate cancer is based in ROS generation leading to oxidative damage [37]. However, metastatic prostate cancer cells may be resistant to radiotherapy, suggesting that the antioxidant system may play an important role in circumventing radiation cytotoxicity [34], thus, contributing for therapy failure. Therefore new therapeutic approaches related, in part, with OS modulation, have been suggested, namely, by Freitas *et al* [38].

The antioxidant network comprises the enzymes superoxide dismutase (SOD), catalase, glutathione peroxidase (Gl-Px), glutathione reductase (Gl-Red) and glutathione-s-transferase (GST) that play an important role in prostate cancer prevention, protecting cells from genomic damage mediated by carcinogens and ROS generated during inflammation. Other molecules, as

vitamins E and C and reduced glutathione (GSH) complement the antioxidant enzymes and are capable of neutralising ROS [22]. GSH plays a critical role in cellular redox maintenance. Gl-Px catalyzes the reduction of peroxides and the formation of oxidized glutathione (GSSG) [7]. Gl-Red uses NADPH and H^+ to reduce the GSSG back to GSH [9]. This paper investigates whether ROS (peroxides) and antioxidant defences contribute to prostate cancer progression and how the OS modulation may be a new prostate cancer therapeutic approach.

2. MATERIALS AND METHODS

2.1. Cell culture conditions

Human prostate cancer cell lines derived from localized adenocarcinoma, from (HPV10) [39], from bone metastasis (PC3) [40] and from the normal prostate epithelium (RWPE1) [41] were purchased from the American Type Culture Collection (ATCC) and cultured in optimum growth conditions.

RWPE1 and HPV10 cells were grown in keratinocytes medium (Gibco) supplemented with 5ng/ml of human recombinant epidermal growth factor (rEGF) (Gibco) and 0.05 mg/ml of bovine pituitary extract (BPE) (Invitrogen, formely Gibco-BRL). PC3 cells were grown in RPMI 1640 medium (Sigma) with 10 % (v/v) heat-inactivated fetal bovine serum (FBS) (Biochrom) and 2 mM L-glutamine (Sigma). Both medium, were supplemented with 100U/ml Penicillin, 100µg/ml Streptomycin and with 5µg/ml Kanamycin (Sigma).

Cells were maintained in a 95% humidified incubator with 5% CO_2 at 37°C and were passaged with trypsinization every fourth day. For assays RWPE1 and HPV10 were plated at a density of $5x10^5$cells/ml whereas PC3 were seeded at a density of $3x10^5$ cells/ml. After being cultured for 24h, the cells were washed once with fresh assay medium and treated for 24 to 72 hours with hydrogen peroxide (H_2O_2)(10nM-500µM). PC3 were also treated with diethyl maleate (DEM) (Sigma).

2.2. Cell proliferation analysis

Cell proliferation was measured by the colorimetric MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (Sigma) assay method that quantifies the reduction of the yellow tetrazolium salt to purple formazan crystals by the mitochondria of viable cells [42]. Briefly, untreated and treated cells were washed with PBS (Gibco) that was replaced by MTT (0.5mg/ml) supplemented with 1mM $CaCl_2$ (Sigma). The cells were then incubated at 37ºC for 2 h. Formazan crystals were dissolved with HCl 0.04 M in isopropanol. Absorbance from the resultant coloured solution was measured at 570 nm [38].

2.3. Flow Cytometry studies

Each 24h of incubation, 1 x 10^6 of treated cells and corresponding controls, were collected by trypsinization and washed two times in PBS buffer, by centrifugation, for further acquisition and analysis in a FACScalibur (488nm and 635nm), using the Cellquest and Paint-a-gate software (BD Bioscience). Attached cells are considered as viable and were selected for. mitochondrial membrane potential (MMP) and ROS analysis. For cell viability and death analysis we also collected the suspension cells.

Specimens were prepared in triplicate and at least 10000 events were collected.

2.3.1. Cell viability and death: detection of apoptosis or necrosis using Annexin V/Propidium iodide incorporation

For identification of cell death by apoptosis or necrosis, suspension and attached cells were collected for the assay. After washed, the collected cells (1 x 10^5) were resuspended in 100µL of binding buffer (0.025 M CaCl2, 1.4 M NaCl, 0.1 M Hepes) containing 5 µL annexin-V APC and 2µL propidium iodide 3µM (PI) (Invitrogen-Molecular Probes). Samples were kept in the dark at room temperature for 15 minutes, according to manufacturer's instruction [43] and Freitas *et al*. [38].

2.3.2. Mitochondrial membrane potential (MMP) analysis

In order to detect MMP, the cells were labelled with the fluorescent probe 5,5´,6,6´-tethrachloro-1,1´3,3´-tetraethylbenzimidazolylcarbocyanine iodide (JC1) (Cell Technology) according to manufacture's instructions as previously performed by Freitas *et al*. [38]. Briefly $5x10^5$ cells were resuspended in 0.5 ml of 1x JC-1 reagent solution and then incubated for 15 minutes, at 37ºC in a 5% CO_2 chamber. The cells were washed two times with 2 ml 1 x assay buffer under centrifugation at 1500 rpm, resuspended in 0.5 ml 1x assay buffer and were analyzed by flow cytometry.

The lipophilic cationic probe JC1, developed by Cossariza *et al*. [44] is able to selectively enter in the intact mitochondria, forming J-aggregates (J-A), which are associated with a large shift in emission (590 nm). However, in lower polarized mitochondrial membrane, JC1 accumulates in the cytoplasm in the monomeric form (J-M), emitting at 527 nm after excitation at 490 nm. Therefore the ratio J-M/ J-A is inversely correlated with MMP.

2.4. OS evaluation

OS was evaluated by measuring ROS production, antioxidant capacity and lipid peroxidation.

2.4.1. Reactive oxygen species measurements

We evaluate ROS levels by labelling $5x10^5$ cells with 5µM 2,7 dichlorodihydrofluorescein diacetate ($DCFH_2$-DA) (Sigma) according to adaptations of previously procedures [38, 45-47]. The cells were incubated during 1h at 37ºC, in the dark, washed 2 times in 0.5 ml phosphate-buffered saline (PBS) and were collected through centrifugation at 1500 rpm. The cells were then resuspended in 0.5ml PBS for flow cytometry analyses. This methodology is based on the conversion of ($DCFH_2$-DA) in $DCFH_2$ by intracellular esterases and consequent formation of the highly fluorescent 2,7-dichlorofluorescein (DCF) by ROS. The resultant green fluorescence is proportional to the intracellular level of ROS, upon excitation at 488 nm. Moreover total population was considered for ROS measurements.

2.4.2. Antioxidant capacity

2.4.2.1. Cell lysates preparation

After 24h, treated cells were washed 2 times with PBS, scraped off the flasks and resuspended in 1 ml PBS. Cells were then subjected to 3 pulses of sonication for 10 seconds with 1 minute intermittent cooling on ice in a Bandelin Sonorex.

Protein concentration was assessed using bicinchonic acid assay kit (Sigma) according to manufacture's instruction. The lysates had been stored at -80ºC before usage.

2.4.2.2. Reduced glutathione assay

Reduced glutahione (GSH) was performed using a kit from Oxisresearch according to manufacture's instructions. This method is based on the formation of a chromophoric thione that is proportional to GSH concentration at 420 nm [48]. Results are expressed as μmol of GSH per gram of protein (μmol/g prot).

2.4.2.3. Antioxidant enzymes determinations

2.4.2.3.1. Glutathione peroxidase

Glutathione peroxidase (Gl-Px) activity was evaluated by spectrophotometry using tert-butylperoxide as a substrate [49], monitoring the formation of oxidized glutathione, through the quantification of the oxidation of NADPH to $NADP^+$ at 340 nm. Results are expressed in international units of enzyme per gram of protein (U/g prot).

2.4.2.3.2. Glutathione reductase

Glutahtione reductase (Gl-Red) activity was determined using GSSG as a substrate and monitoring its reduction to GSH through quantification of NADPH oxidation at 340 nm [50] in a thermostatized spectrophotometer UVIKON 933 UV/ Visible, at 37ºC. Gl-Red activity was expressed in international units of enzyme per gram of protein (U/g prot)

2.4.2.3.3. Glutathione-s-transferase

Glutathione-s-transferase (GST**)** levels, namely from the Pi subgroup (GST-Pi), were quantified by an Enzyme Immuno Assay (EIA) according to manufacture's instructions (Immunodiagnostick) at 450nm. This method is based in the competition of GST samples and GST from plate for a rabbit antibody binding. After a washing step, the detection of the bound rabbit antibody is performed by a peroxidase labeled goat antibody anti rabbit (POD-antibody). The amount of converted substrate (TMB) is indirectly proportional to the amount of GST antigen in the sample [51]. The results are expressed as μmol of GST per gram of protein (μmol/g prot).

2.4.3. Lipid peroxidation evaluation

Oxidative lesion of lipids was evaluated by the formation of a thiobarbituric acid (TBA) adduct of malondialdehyde (MDA) and then separated by HPLC [52, 53]. Cell lysates were boiled during 60 min with TBA and phosphoric acid, then were deproteinized with methanol / NaOH 1M (10:1) and centrifuged. The supernatant (20 l) was injected into a Spherisorb ODS2 5 m (250 x 4.6mm) column. Elution was performed with 60% (v/v) potassium phosphate buffer 50 mM, pH 6.8, and 40% (v/v) methanol at a flow rate of 1 ml/min. The TBA-MDA adducts were detected at 532 nm and quantified by extrapolating the area of the peaks from a calibration curve of 1,1,3,3-tetraetoxipropane (TEP) standard solutions. Results are expressed as μmol of MDA per gram of protein (μmol/g prot).

2.4.4. Total Antioxidant Status (TAS) determination

TAS was determined by a chromogenic method (Randox Laboratories Crumham's, North Ireland) with briefly adaptations. This methodology is based on the capacity to inhibit the formation of the $ABTS^{+}$ radical cation (2,2'-azino-di- [3-etilbenzotiazolin sulfonate]) and detection at 600 nm in a spectrophotometer UVIKON 933-UV / Visible, thermostatized and computerized [54]. Results are expressed as μmol of TAS per gram of protein (μmol/g prot).

2.5. Statistics

Statistical analyses were carried out using t-tests. Significance was assessed for P values<0.05.

3. RESULTS

3.1. Metastatic prostate cancer cells are more resistant to H_2O_2 then the other cell lines

3.1.1. Effect of H_2O_2 on cell growth and viability

Fig.1 represents the proliferative effect of H_2O_2 (10nM-500μM) on a cell line derived from human normal prostate epithelium (RWPE1) and on prostate cancer cells, derived from localized and metastatic carcinoma (HPV10 and PC3 respectively), during 72h. As we observe H_2O_2 induces different effects according to cell type, time of incubation and concentration exposure. Therefore H_2O_2 inhibits RWPE1 cell growth for all tested concentrations whereas PC3 seems to maintain a cell proliferation rate above the half maximal inhibitory concentration **(**IC50**).** On the other hand we found that low H_2O_2 concentrations induce an increase in HPV10 cells proliferation.

We observed that the effect of H_2O_2 on cell proliferation inhibition, namely at IC50 concentration is associated to cell death mainly by necrosis, in HPV10 and RWPE1 cells. Data not shown, also indicate that lower ROS concentration, namely 100μM H_2O_2, induced necrosis in RWPE1 and HPV10. However, PC3 maintain cell viability besides a decrease in cell proliferation (Fig. 2). These results suggest a more efficient adaptation to peroxides in PC3.

3.1.2. Influence of H_2O_2 on MMP

To evaluate the role of mitochondria on prostate cancer progression/metastasization and the effect of H_2O_2 on MMP we used the JC1 assay. Fig. 3 shows that PC3 cells had the highest basal MMP and RWPE the lowest, which is in line with viability and proliferative results. However, in the presence of H_2O_2, we observe a significant decrease in MMP, in RWPE1 and HPV10 cells, as

304 demonstrated by the increase of monomers/aggregates (M/A) ratio that also agree with viability
305 and proliferative results.

306 **3.1.3. Metastatic prostate cancer cells are resistant to ROS (peroxides) by an increase in**
307 **GSH content and Gl-Red activity**
308
309 To evaluate the role of H_2O_2 on ROS (peroxides) production we used the 2,7
310 dichlorodihydrofluorescein diacetate ($DCFH_2$-DA) probe, a dye that fluoresces in the presence of
311 peroxides (H_2O_2) [46].
312 This study shows that prostate cancer cells, particularly the metastatic cells (PC3), exhibit
313 significant higher ROS levels compared with the others (Fig.4). On the other hand the sensitivity
314 to cytotoxicity induced by ROS (H_2O_2) in RWPE1 and HPV10 is confirmed by an increase in
315 lipid peroxidation as observed in Fig. 5. In opposite we found a decrease in lipid peroxidation
316 and increase of TAS in PC3 (Fig. 5). These results suggest that PC3 cells are resistant to ROS
317 which may contribute to a more aggressive phenotype, related with prostate progression and
318 metastasization. In order to determine the contribution of the antioxidant system in cells
319 adaptation to ROS, we analysed GSH and GST content and Gl-Red and Gl-Px activities,
320 simultaneously in the three cell lines. Results represented in Fig. 6 indicate a significant decrease
321 in GST (Fig. 6D) content and Gl-Px (Fig. 6B) activity in the malignant cells, particularly in PC3,
322 which may be related with higher H_2O_2 levels. On the other hand, PC3 have the highest GSH
323 content and Gl-Red activity that could contribute to resistance to OS (Fig. 6C and 6A
324 respectively).
325
326 **3.1.4. Adaptation to peroxides (ROS) is reverted by DEM**
327
328 In order to evaluate the modulation of ROS resistance by GSH content, the major thiol in
329 mammalian cells**,** and Gl-Red activity, PC3 cells were treated with a thiol depleting agent
330 (DEM), during 24h.
331 As we can observe in Fig. 7 (A1 and A2), DEM induces a decrease in GSH content and Gl-Red
332 activity concomitantly with a decrease in cell proliferation (Fig. 7 B1) and an increase of
333 cytotoxicity mediated by H_2O_2 (ROS) in PC3 cells (Fig. 7C).

In fact, the association of DEM and 500µM H_2O_2 induced a decrease in cell growth (Fig. 7 B2) and viability (Fig. 7C).when compared with 500µM H_2O_2 alone. This is accompanied by an increase in cell death mainly by apoptosis and late apoptosis/necrosis after 24h treatment with DEM (Fig. 7C).

4. DISCUSSION

Development of efficient therapies requires a better understanding of the mechanisms underlying prostate carcinogenesis. Although OS has been associated with prostate cancer development and progression due to an increase of ROS [7, 55], the mechanisms whereby ROS and antioxidants may induce cancer progression remain unclear [12]. As a result, greater understanding of OS may be of considerable importance for fighting prostate cancer.

In line with existing literature, we observed that the effect of H_2O_2 on cell proliferation is dose, time and cell type dependent. In particular, low levels of H_2O_2 (10-100nM) induced cell growth, in HPV10, derived from localized carcinoma, suggesting that small increments of ROS may be important in progression of prostate cancer. Interestingly, Sikka *et al*. [7] previously demonstrated that low levels of H_2O_2 (30pM to 300nM), induce cell growth on benign prostate hyperplasia (BPH), characterized by an intense cell proliferation rate and considered as potential precursor of prostate cancer [56]. On the other hand, chronic inflammation of prostate epithelium, due to persistent ROS production, has been associated with increase of OS and DNA damage leading to neoplastic transformation (for a good review of this literature see Nelson *et al*. [19]). However, an excess of ROS is expected to be harmful and to induce apoptosis in several human tumour cell lines [19]. Here we found that 500µM H_2O_2 inhibits cell proliferation in all the tested cells lines, but the effect was less pronounced in PC3. These differences may be notably observed after 72h of treatment.

Moreover it was accompanied by cell death, essentially by necrosis, in HPV10 and in the normal epithelium cell line, RWPE1 just after 24h treatment. Similar results were found by Sika *et al*. [7] in BPH cell lines with 300µM H_2O_2. However, higher H_2O_2 concentrations did not affect PC3 viability that appears to be more resistant to OS. It would be interesting to evaluate whether H_2O_2 causes some cycle phase-specific blockade, since drug-provoked oxidative stress causes cell cycle disruption as well as cell death. This may also be important in cancer treatment.

We observed the highest basal levels of ROS in PC3 (Fig. 4) which may be related to cells resistance to H_2O_2 induced cytotoxicity and to a more aggressive phenotype, although others have found that increase of ROS is related with cell injury, resulting from oxidative damage of biomolecules such as DNA, proteins and lipids [3-12, 34, 35]. It is also important to realize that tumor progression is based on DNA mutations which promote cancer cells proliferation and surveillance [57]. In part, these mutations may be acquired as a result of an increase of ROS which require an antioxidant system adaptation [58].

Viability results are in line with MMP detected in cells. In fact, at basal conditions, PC3 cells show the highest MMP whereas RWPE1 cells show the lowest MMP. These results may be related with cell vulnerability to ROS induced cytotoxicity. In the same way, when PC3 were treated with 500μM H_2O_2 we didn't observe a significant decrease in MMP, in contrast to other cell lines. Based on this evidence we also suggest that variation in MMP is related to cell growth but not in a directly proportional ratio.

In order to better understand the effect of OS in prostate cancer progression we evaluated the levels of ROS and antioxidant defences among the distinct cell lines. We found that ROS levels are lower in RWPE1 and substantially higher in PC3 as we have previously referred. These findings are in agreement with those observed by Kumar *et al*. [55]. The same authors show that the degree of ROS generation is directly proportional to aggressive phenotype. They also found higher levels of ROS in PC3 compared to RWPE1, although, they did not analyse localized prostate cancer cell lines as performed in our work. Likewise we demonstrate that HPV10 cells show intermediate ROS levels. These cells also represent an intermediate stage (localized cancer) between RWPE1 and PC3 cells. Kumar *et al*. [55] also demonstrated that ROS, mainly generated by the Nox system (trans-membrane proteins called the NADPH oxidases), is essential for deregulated growth, colony formation, cell migration and invasion and contribute to tumour metastization. Besides the increase of ROS levels in PC3 cells, we found lower levels of lipid peroxidation (MDA) and higher levels of TAS that are consistent with cell resistance to OS. Therefore, these results suggest that PC3 cells counteract OS by expressing particular free radical scavengers. In fact, we found significant increase of GSH and Gl-Red activity in PC3 that may protect these cells under persistent ROS (peroxides) production, induced by radiotherapy and chemotherapy. H_2O_2 treatment induced a clear decrease in TAS that is in agreement with the increase of MDA. These observations may be due to the absence of a significant increase in the

antioxidant defences, in face of the H_2O_2 insult. On the other hand it may be explained by the high basal levels of GSH content and Gl-Red activity observed in PC3. However, we may consider other explanations for the observed decrease of TAS and increase of MDA. Namely, as TAS evaluation is based on the antioxidants capacity to inhibit $ABTS^+$ radical cation (2,2'-azino-di- [3-etilbenzotiazolin sulfonate])[42] and that the antioxidant system may be recruited to face the increase of H_2O_2, we admit that the antioxidant defences are unavailable to inhibit $ABTS^+$, leading to TAS reduction observations. Moreover, the antioxidant system comprises other antioxidant defences like catalase, playing an important role in neutralizing H2O2, or superoxide dismutase and α-tocopherol (that protects from lipid peroxidation), which we didn't analyzed that may also contribute to explain the decrease of TAS and increase of MDA.

In contrast, Gl-Px activity and GST are depleted in PC3 suggesting that GST depletion may facilitate prostate cancer progression as described by others [8]. The same assumption may be addressed to Gl-Px activity.

GSH, the major thiol in mammalian cells, maintains an optimum cellular redox potential trough the inactivation of H_2O_2. The contribution of GSH and Gl-Red in PC3 cells protection against ROS was also suggested by Lim *et al*. [9]. These authors compared two metastatic prostate cancer cells, LNCaP, and PC3 and found lower ROS levels in PC3 that could be explained by an increase of GSH content, Gl-Red, thioredoxin reductase (TR) and GST activities. However, Kumar *et al*. [55] contradict those results showing higher ROS levels in PC3 comparing to LNCaP. In our study, PC3 Gl-Red activity is in the range values found by Jung *et al*. [3]. These authors also reported an increase in Gl-Red and a decrease in GST and Gl-Px activities in metastatic prostate cancer cells (PC3, LNCaP and DU145) comparing with primary cell cultures of benign and malignant human prostatic tissue. They also show higher Gl-Px and lower Gl-Red activities in PC3 cells comparing to other metastatic cell lines, reinforcing our observations that Gl-Px is decreased and Gl-Red is increased in advanced tumours cells. Therefore Gl-Red may contribute to protection against ROS.

To confirm the role of GSH content and Gl-Red activity in the adaptation to OS by metastatic prostate cancer cells, we treat the cells with DEM, a thiol depletion agent. Our study revealed that cells treated with 50μM DEM alone show a decrease in Gl-Red activity, additionally to a decrease in GSH content (Fig 7A1) with no interference on cell proliferation and death. However, in the presence of H_2O_2 the decrease in Gl-Red activity and GSH content was

accomplished by a decrease in cell proliferation and increase of cell death preferentially by apoptosis and late apoptosis/necrosis. GSH reduction in the presence of DEM is in agreement with the results obtained by Coffey *et al*. [59,60]. These authors show an increase of apoptosis induced by radiation, in the presence of DEM, in PC3 and in other metastatic prostate cancer cells, LNCaP and DU145. They also found that apoptosis is accompanied by an efflux of GSH from the nucleus to the cytosol and admit that the presence of GSH in the nucleus may offer resistance to apoptosis.

Here we strongly proved that Gl-Red activity reduction participates in the increase of H_2O_2 toxicity in metastatic prostate cancer cells, suggesting a potential therapeutic approach. It may be also related with GSH reduction as observed here and by others [59,60]. These results also conduct to the possibility of using other Gl-Red activity reducing agents or to evaluate the combined effect of DEM with conventional chemotherapeutic drugs, namely docetaxel, expecting that the association may allow to lowering drug concentrations, therefore reducing drug side effects, related with systemic toxicity. In fact we previously found that sodium selenite,a thiol depleting agent, combined with docetaxel, play a synergistic effect on PC3 cells growth inhibition and induces cell death [38] Likewise our study warrants further evaluation of oxidative stress modulation in prostate cancer therapeutic approach. In particular, it would be interesting to evaluate the effect of DEM-produced GSH depletion and Gl-Red reduced activity in the RWPE cells, in order to evaluate the potential toxicity in non tumour cells.

5. CONCLUSIONS

As described above, other reports have compared the relative efficacy of antioxidant system in prostate cancer. However, this work develops a more graded study by including models representative of different stages of prostate malignancy.

To our knowledge, our study performed on cell lines representing normal prostate epithelium, localized and metastatic prostate cancer, all stages of prostate cancer progression, is the first demonstrating an increase of ROS (peroxides) along with prostate cancer progression, concomitantly with OS adaptation. Therefore we suggest that this duality may be necessary for prostate cancer progression and for a more aggressive malignant phenotype. On the other hand,

as normal epithelium cell line shows lower ROS levels in basal conditions, but greater sensitivity to cytotoxicity induced by ROS (500μM H_2O_2) and concomitantly lower GSH levels and Gl-Red activity, these conditions may contribute to a higher susceptibility to OS lesion by normal prostate epithelium.

The cell line model, presented here shows an important approach to understanding oxidative stress through the different stages of prostate cancer. However, it is possible that cell lines do not exactly reflect the true in vivo situation. We also realize that the present strategy, developed in a cell line model, may not be efficient when applied to in vivo experiments. Different culture conditions could influence the observed results. However, this study does propose oxidative stress modulation as a possible new therapeutic approach in prostate cancer. We intend to carry out further evaluation of this strategy to in vivo model.

Furthermore, as far as we are aware, we are the first to confirm the pivotal role of GI-Red's activity as a new target-directed therapeutic tool in the treatment of prostate cancer.

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8. FIGURE LEGENDS

FIGURE 1. **Dose-response curves**. The effect of different H_2O_2 concentrations (10nM–500µM) on proliferation of human normal prostate epithelium (RWPE1) and prostate cancer cells, derived from localized and metastatic carcinoma (HPV10 and PC3) are represented. Proliferation was evaluated through the formation of formazan products by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), each 24h, during 72 hours of incubation, as refereed in materials and methods. Results are expressed as percentage of MTT reduction relatively to control (cells not treated with H_2O_2) and correspond to the mean ± SD of at least 3 separate experiments.

FIGURE 2. **Effect of H_2O_2 on cell viability and death in RWPE1, HPV10 and PC3 cells.** Dot-plot profiles (1) were obtained after the acquisition of 10 000 events. Cells cultured in the absence (untreated) or in the presence of 500µM H_2O_2 during 24 hours. Cells were incubated with AnnexinV/IP as refereed in materials and methods. Alive cells (A) exclude propidium iodide and do not bind annexin-V. Apoptotic cells with intact membranes exclude propidium

iodide, externalize phosphatidylserine to the outside of the plasma membrane and therefore bind Annexin V (IA) emitting fluorescence. Propidium iodide stains nuclear DNA of necrotic (N) and late apoptosis/necrosis cells (LA/N).

Results were expressed as the percentage of cells staining with the respective molecular probe (2). The results represent the means ± SD of at least triplicate determinations. Significantly viability and necrosis differences are considered: ***$p<0.001$ vs untreated samples, $^{###}p<0.001$ for PC3 and HPV10 vs RWPE1 and $^{+++}p<0.001$ for PC3 vs HPV10.

FIGURE 3. **MMP evaluation in RWPE1, HPV10 and PC3 cells**. Dot-plot profiles (1) were obtained after the acquisition of 10 000 events. RWPE1, HPV10 and PC3 cells were cultured in the absence (untreated) or in the presence of 500µM H_2O_2 for 24 hours. The MMP was assessed by flow cytometry labelling the cells with the molecular probes JC1 as described in materials and methods. Results were expressed as the ratio of Monomers/Aggregates (M/A) (which are inversely proportional to MMP) and represent the means MMP ± SD of at least triplicate determinations (2). Significantly viability differences are considered: ***$p<0.001$ vs untreated samples, $^{###}p<0.001$ and $^{##}p<0.01$ for PC3 and HPV10 vs RWPE1 and $^{+}p<0.05$ for PC3 vs HPV10.

FIGURE 4. **Effect of H_2O_2 on ROS production in RWPE1, HPV10 and PC3 cells**. Dot-plot profiles (1) were obtained after the acquisition of 10 000 events. RWPE1, HPV10 and PC3 cells were cultured in the absence (untreated) or in the presence of 500µM H_2O_2 for 24 hours. ROS was assessed by flow cytometry labelling the cells with 2,7 dichlorodihydrofluorescein diacetate ($DCFH_2$-DA). The results were expressed as the means intensity fluorescence (MIF) ± SD of at least triplicate determinations (2). Significantly differences are considered: *$p<0.05$ vs untreated samples, $^{##}p<0.01$, $^{###}p<0.001$ for PC3 and HPV10 vs RWPE1 and $^{+++}p<0.001$ for PC3 vs HPV10.

FIGURE 5. **Evaluation of lipid peroxidation and TAS in RWPE1, HPV10 and PC3 cells** Cells were treated with 500µM H_2O_2 for 24 hours as described in materials and methods. Lipid peroxidation (A) was evaluated by MDA determination levels. MDA and TAS (B) levels were detected according with described in material and methods. Results were expressed as the means

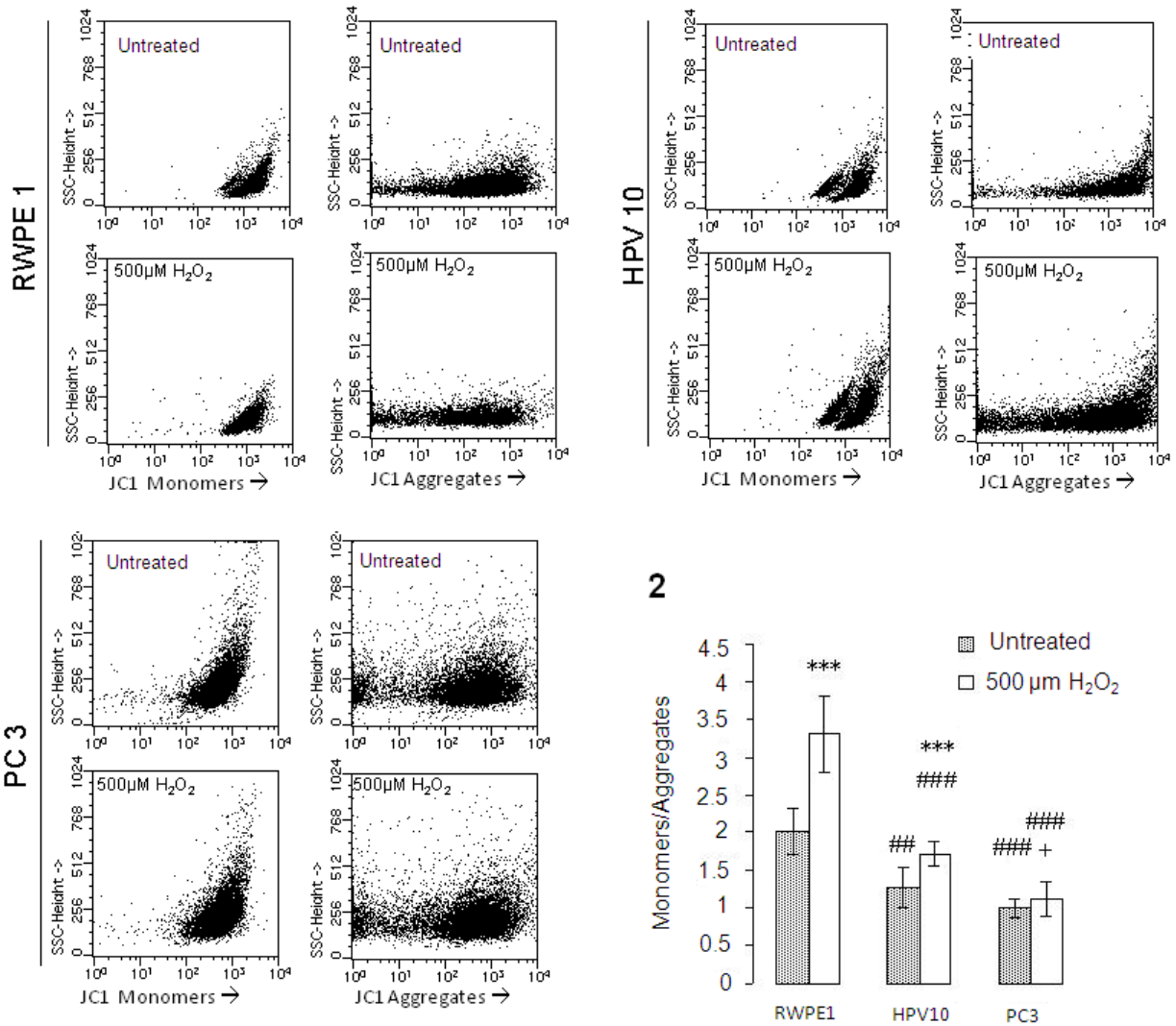
± SD of at least triplicate determinations. Significantly differences are considered: *p<0.05 *vs* untreated samples, $^{\#}$p<0.05, $^{\#\#}$ p<0.01, $^{\#\#\#}$p<0.001 for HPV10 and PC3 *vs* RWPE1 and $^{+}$p<0.05, $^{++}$p<0.01 for PC3 *vs* HPV10.

FIGURE 6. **Antioxidant defences in RWPE1, HPV10 and PC3 cells**. We evaluate Gl-Red (A) and Gl-Px (B) activities and GSH (C) and GST (D) content. Cells were treated with 500µM H_2O_2 for 24 hours as described in materials and methods. Results are expressed as the means ± SD of at least triplicate determinations. Significantly differences are considered for *p<0.05, **p<0.01 *vs* untreated samples, $^{\#}$p<0.05, $^{\#\#}$p<0.01, and $^{\#\#\#}$p<0.001 for HPV10 and PC3 *vs* RWPE1 and $^{+}$p<0.05 and $^{+++}$p<0.001 for PC3 *vs* HPV10.

FIGURE 7. **Effect of DEM on PC3 antioxidant defences -.relation with cell proliferation and viability**. GSH content (A1) and Gl-Red activity (A2) on PC3 were analysed after treating cells with 50µM DEM for 24 hours. The effect of DEM (50µM -1000µM) alone (B1) and combined with 500µM H_2O_2 (B2) on PC3 cells growth was evaluated during 72h. In (C) is represented the influence of DEM on PC3 cell viability and death. Cells were treated with 500µM H_2O_2, in the absence (control) and in the presence of 50µM DEM for 24 hours. Viability was assessed by flow cytometry as previously described. Viable cells are expressed by alive cells (A), initial apoptosis by (IA), necrosis (N) and late by apoptosis/necrosis (LA/N).
Results were expressed as the means ± SD of at least triplicate determinations. Significant differences are considered for *p<0.05, **p<0.01 and ***p<0.005 vs control. Cell viability and death statistical results are indicated for (IA) and (LA/N).

9. FIGURES

1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HPV 10
PC 3
Untreated
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SSC-Height ->
JC1 Monomers →
JC1 Aggregates →

2

Untreated
500 μm H_2O_2
Monomers/Aggregates
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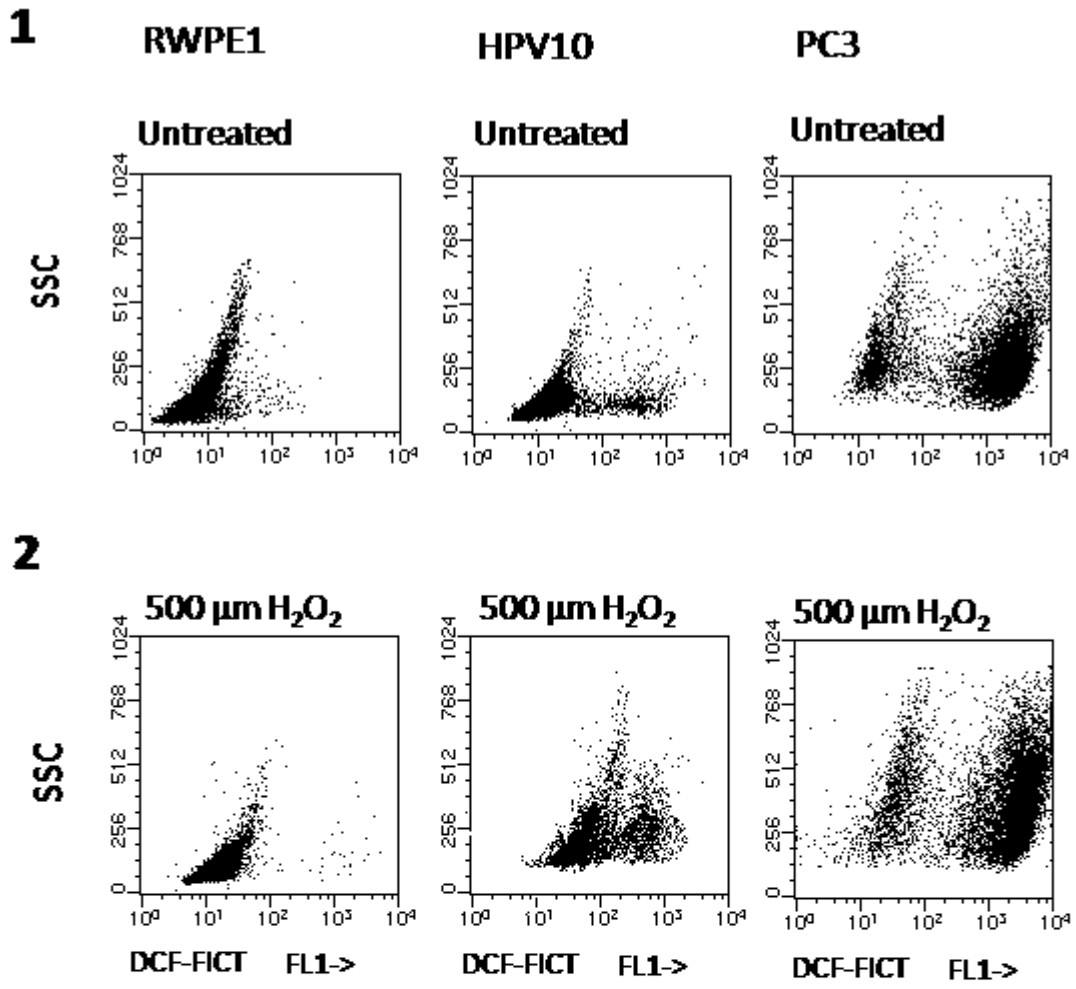
RWPE1
HPV10
PC3

758


1
RWPE1
HPV10
PC3
Untreated
Untreated
Untreated
SSC
2
500 µm H2O2
500 µm H2O2
500 µm H2O2
SSC
DCF-FICT FL1->
DCF-FICT FL1->
DCF-FICT FL1->
Untreated
500 µm H2O2
ROS (MIF)
3500
3000
2500
2000
1500
1000
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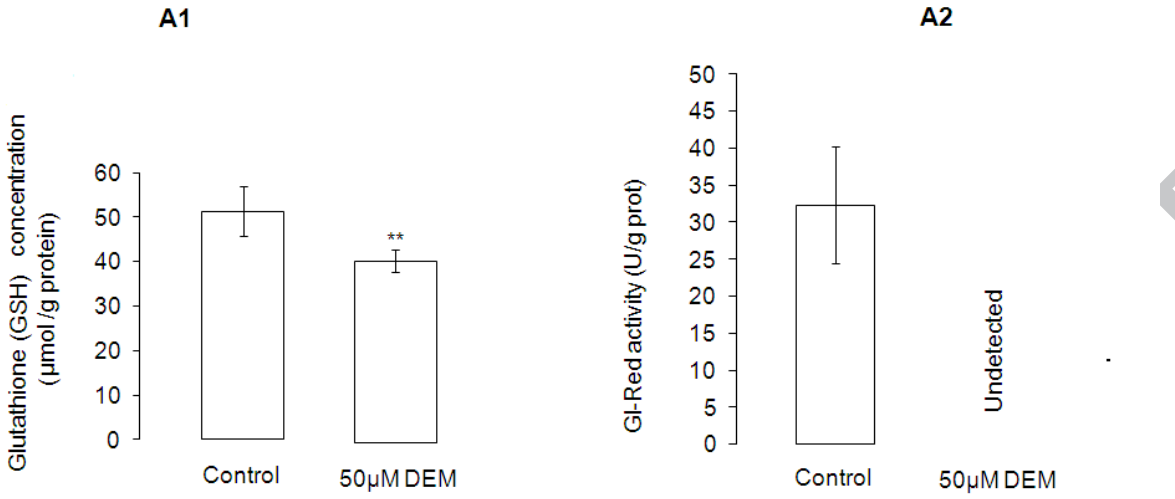
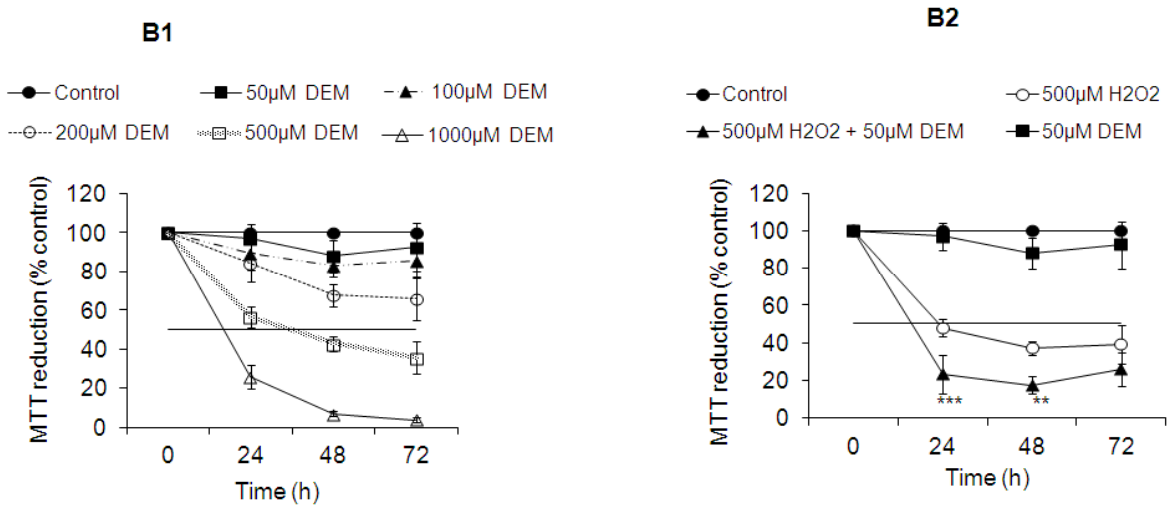
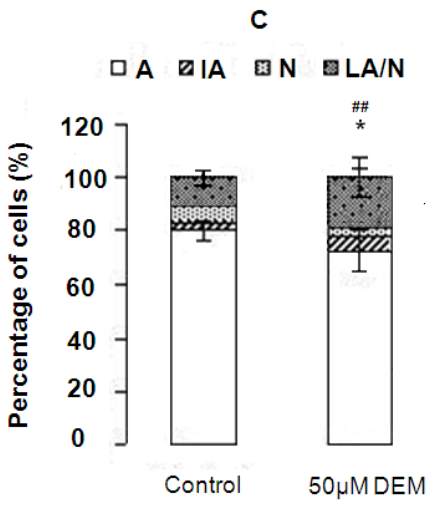
RWPE1
HPV10
PC3

759







761 **Highlights**

762 Oxidative stress was evaluated on a cell line model of prostate cancer progression

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764 Metastatic cell lines show the highest ROS, total antioxidant status and resistance to H_2O_2

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766 Metastatic cell lines show the highest levels of GSH levels and Gl-Red activity

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768 Decrease in GSH levels and Gl-Red activity induced a decrease in H_2O_2 resistance

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770 Gl-Red activity reduction may be a new therapeutic approach in prostate cancer

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