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Effect of Arsenic on Nitrosative Stress in Human Breast Cancer Cells and its Modulation by Flavonoids

Elio A. Soria[1,*], Guillermina A. Bongiovanni[2], Cintia Díaz Luján[1] and Aldo R. Eynard[1]

[1]*Instituto de Investigaciones en Ciencias de la Salud (INICSA-UNC-CONICET), Facultad de Ciencias Médicas, Universidad Nacional de Córdoba, Argentina;* [2]*Instituto Multidisciplinario de Investigación y Desarrollo de la Patagonia Norte (IDEPA-UNCO-CONICET), Universidad Nacional del Comahue, Argentina*

Abstract: Arsenic (As) is used in the treatment of leukaemia and breast cancer due to its strong oxidative cytotoxic action. However, it is also cytotoxic to normal cells. One proposed anticancer mechanism induced by As might be nitrosative stress (NS). Hence, it is believed that use of antioxidants such as flavonoids in combination with As might reduce its toxic action on normal cells without interfering with its antitumor action. In the present study, we evaluated the antineoplastic potential of As on breast human cancer lines MCF-7 and ZR-75-1 treated with redox-modulating flavonoids, such as quercetin (Q) and silymarin (S). It was noted that, even though both cell lines differed in their oxidative responsiveness, their viability was decreased by NS induction through γ-glutamyltranspeptidase inhibition. Arsenic triggered NS in both MCF-7 and ZR-75-1 cultures, with the formers being more sensitive without recovering their pre-treatment capacity. ZR-75-1 cells maintained their antioxidant status, whereas MCF-7 ones treated with S, As and As+Q did not. Silymarin did not interfere with the described As bioactivity. In summary, NS appears as an important anticancer mechanism exerted by As depending on the redox cellular response that could be differentially modified by dietary antioxidants. Hence, it is worthwhile to consider the use of dietary antioxidants as adjuvant in cancer chemotherapy, especially when using As.

Keywords: Arsenic, breast cancer, flavonoid, nitrosative stress, quercetin, silymarin.

INTRODUCTION

Arsenic (As) may be used as co-therapeutic strategy for certain human cancers due to its strong cytotoxicity. It is possible that molecular targets in leukemic cells may be different from those of mammary cancer [1], since they show distinct redox responses [2]. On the other hand, As is highly toxic to normal cells by direct binding to relevant thiol groups in peptides and proteins, thus inducing oxidative stress [3]. Co-treatments with redox-modulating agents could be beneficial if they do not interfere with the much desired anti-tumour effects of As. Epidemiological and experimental evidence indicate that plant polyphenols, broadly-named flavonoids, such as quercetin and silymarin, have chemopreventive effects [4].

Reactive nitrogen species (RNS) arise mainly from nitric oxide (NO), synthesized by NO synthase (NOS). Reactive oxygen species (ROS) include free radicals and non-radicals, such as superoxide anion and hydrogen peroxide, respectively [5]. Some antineoplastic drugs exert their effects by modulating free radical release in targeted cancer cells [6]. To establish NO roles in cancer development is not easy, since this molecule quickly reacts with free radicals [7]. However, NO is a cytoprotective molecule as well. For example, it can convert thiol radicals into nitrosothiols by acting as a chain-breaking agent [8]. This fact accounts for many of its antioxidant properties, although no direct repairing properties from radical damage are found, such as in the case of glutathione (GSH) [9]. Thus, nitric oxide may play bimodal redox activities in accordance with these particular conditions.

The aim of this work was: a. to evaluate whether the As nitrosative potential, previously described in other tissues [10], exerted cytotoxic activity on mammary tumour cells, b. to determine the role of redox-modulating flavonoids, such as quercetin and silymarin, in these responses.

*Address correspondence to this author at the Enrique Barros S/N (Ciudad Universitaria), Instituto de Biología Celular, Universidad Nacional de Córdoba, Córdoba 5014, Argentina; Tel: +543514334020;
E-mail: easoria@fcm.unc.edu.ar

MATERIALS AND METHODS

Chemicals. Quercetin (CAS nº 6151-25-3, $C_{15}H_{10}O_7$ $2H_2O$, MW= 338.3 g/mole) was obtained from E. Merck (Germany), and silymarin (CAS nº 22888-70-6, $C_{25}H_{22}O_{10}$, 31% silybinin, MW= 482.4 g/mole) from Sigma-Aldrich Inc. (USA). Sodium arsenite ($NaAsO_2$, MW= 130 g/mole) was purchased from Anedra (Argentina). Reagents for nitrite detection were purchased by Britania, and solvents by Cicarelli (Argentina). The γ-G-test kinetic AA kit™ was from Wiener Laboratories (Argentina). Other chemicals were obtained from Sigma-Aldrich Inc. (USA).

Cell culture and treatments. The human cancer cell lines were MCF-7 and ZR-75-1, which were obtained from mesothelial carcinomatosis exudates of two Caucasian 60–70 year-old women with human mammary duct adenocarcinoma (American Type Culture Collection n°: HTB-22 and CRL-1500, respectively). After 48 h post-seeding, cell monolayers were incubated under one of the following acute conditions: 200 μM $NaAsO_2$ (As), 5 μM silymarin (S), 50 μM quercetin (Q), 200 μM $NaAsO_2$ plus 5 μM silymarin (As+S), 200 μM $NaAsO_2$ plus 50 μM quercetin (As+Q), and controls having no treatment (C). Treatments were continued for 2 h to obtain *stressed cells*. Then, cells were allowed to recover for additional 2 h in free treatment medium to obtain *recovered cells*. Protein content of cells was assessed by the Bradford method, and it was used to normalize the different experimental parameters.

Tumour markers of stress response. In order to establish different correlations between membrane stress parameters (γ-glutamyltranspeptidase -GGT-, ganglioside content -GC-, conjugated dienes -CD-), results were calculated as percentages respect to controls, and then correlated with cellular viability and recovering response. After pre-treating stress cells with 1 % Triton X-100 for 30 min, specific GGT activity was assessed using a commercial kit under conditions required for cellular determinations [11]. After performing lipid extraction from cellular membranes as previously published [12], the upper layer was used for sialyl-lipid determinations (GC) at 580 nm in accordance with Miettinen and Takki-Luukkainen [13]. The lower layer was used for oxidized lipids determinations (CD) at 234 nm [2].

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Cellular viability. Viable cells after 8-h treatments without recovery were stained with 0.5% crystal violet in 50% methanol for 15 min. After washing with 50% methanol three times, the stained cells (attached in 96-well plates) were solubilised with 1% SDS in 60% ethanol. Results, consistent with cellular viability, were recorded by a BioRad 680 microplate reader using a 570 nm filter and were presented for relative absorbance. Then, percentage of living cells was calculated with respect to C as 100% of viability.

Markers of nitrosative stress. L-citrulline, a by-product of the nitric oxide biosynthetic reaction, was determined in stressed cells using a colorimetric assay at 530 nm [14]. L-citrulline concentrations in the sample were calculated from a citrulline standard curve, and normalized by cellular proteins. Then, nitrites, stable reaction products of nitric oxide and oxygen, were assayed in recovered cells using the Griess reaction for colorimetric quantification at 550 nm. Nitrite concentrations (% respect to C) were calculated from a sodium nitrite standard curve normalized by cellular proteins [15].

Cytoplasmatic reducing activity (CRA) after recovery. CRA was determined in protein-free cytosol of recovered cells by the Folin-Ciocalteau method [16], with 5 mM silymarin (in dimethylsulfoxide) being used as standard solution. Then, results were calculated as µM of reducing phenolic compounds (RPC) per mg of total cellular proteins.

Statistical analysis. Data were expressed as mean ± standard error (SE) from at least three separate experiments performed in triplicate, unless otherwise noted. ANOVA models were used to evaluate differences among the treatments (C, S, Q, As, As+S, As+Q), followed by Tukey tests for mean comparisons ($p<0.05$). Analytical probes, including Spearman coefficients, were done with the InfoStat 2012 software.

RESULTS

Cellular redox response. Correlation analysis using the Spearman coefficient (SC) showed that the closest variables linked to MCF-7 lost viability were L-citrulline rise and GGT inhibition, as also happened in ZR-75-1 cells in a lesser extent. Furthermore, nitrosative stress was further confirmed by the indirect relation between the increase of L-citrulline levels and the reduction of RPC/CRA after recovery, whereas oxidative stress (represented by the level of CD) was poorly associated to cellular viability. However, both cancer lines differed in their redox sensibility and recovery capacity (Table).

***In vitro* nitrosative stress.** In stressed cells, L-citrulline formation was increased in both cell lines treated with As ($p<0.05$), whereas the other treatments did not induce significant changes. Such increase was higher in MCF-7than in ZR-75-1 cells (Fig. **1**). In recovered cells, increased nitrite content was found in ZR-75-1 cells previously treated with S and Q, whereas As and As+Q treatments caused the described increase in MCF-7 cells ($p<0.05$). Control nitrite levels were found under the other treatments (Fig. **2**).

Cytoplasmatic reducing activity (CRA). After recovery, MCF-7 cells showed significant CRA reduction after S, As and As+Q treatments ($p<0.05$). ZR-75-1 cells generally showed upper CRA than those seen in the MCF-7 line (Fig. **3**).

DISCUSSION

Two concerns are essential before proposing As trivalent derivates for breast cancer treatment in patients [17,18]. First, putative carcinogenic effects on remnant mammary tissue should be avoided at the highest extent [19]. Secondly, it is important to know whether non-specific systemic cytotoxicity of As could be counteracted by nutraceutical co-adjuvants without losing the desirable anticancer activity [2,20]. In this work, among several anti-mammary cancer mechanisms proposed for As [21], we obtained some data suggesting that nitrosative stress induction was a plausible mechanism of action of As, which was defined by NO-related pathway increase with loss of the cytoplasm reducing activity.

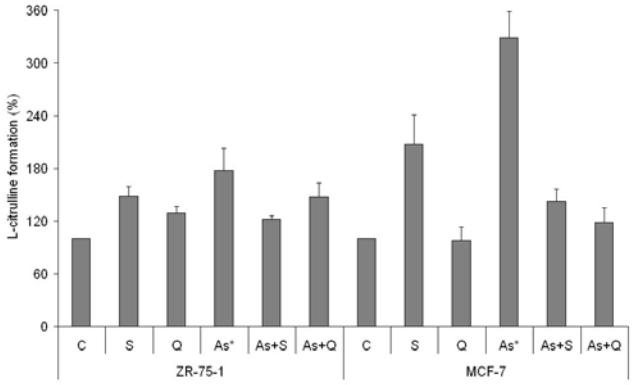

Fig. (1). L-citrulline formation in the breast cancer cells ZR-75-1 and MCF-7 incubated for 2 h under the following treatments: none (C), 5 µM silymarin (S), 50 µM quercetin (Q), 200 µM sodium arsenite (As), As+S and As+Q. Data were calculated as the average of three separate experiments ± SE and expressed as % respect to C (* $p< 0.05$).

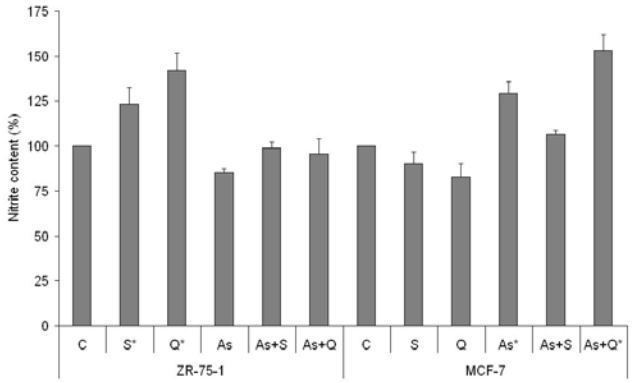

Fig. (2). Nitrite content in the breast cancer cells ZR-75-1 and MCF-7 incubated for 2 h in free treatment-medium after being exposed for 2 h to: none (C), 5 µM silymarin (S), 50 µM quercetin (Q), 200 µM sodium arsenite (As), As+S and As+Q. Data were calculated as the average of three separate experiments ± SE and expressed as % respect to C (* $p< 0.05$).

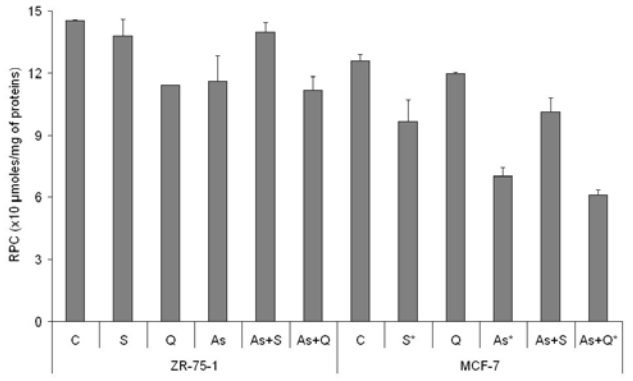

Fig. (3). Cytoplasmatic reducing activity (CRA by RPC, reducing phenyl-compounds) in the breast cancer cells ZR-75-1 and MCF-7 incubated for 2 h under the following treatments: none (C), 5 µM silymarin (S), 50 µM quercetin (Q), 200 µM sodium arsenite (As), As+S and As+Q. Data were calculated as the average of three separate experiments ± SE (* $p< 0.05$).

Table 1. Spearman correlations between stress-related markers found in breast cancer cells[a, b]

	Cit	CD	SA	GGT	N	RPC	CV
Cit	1.00	0.20	0.14	0.09	-0.37	-0.54	-0.60
CD	0.03	1.00	0.09	0.37	0.26	-0.09	-0.20
SA	-0.04	-0.01	1.00	-0.26	-0.14	-0.83	-0.31
GGT	-0.66	0.43	-0.16	1.00	0.77	-0.09	0.66
N	0.43	-0.66	0.56	-0.83	1.00	0.14	0.77
RPC	-0.60	0.60	-0.21	0.49	-0.71	1.00	0.26
CV	-0.94	0.09	0.07	0.83	-0.54	0.54	1.00

[a] CD: conjugated dienes, Cit: L-citrulline, GGT: γ-glutamyltranspeptidase activity, N: nitrites, RPC: reducing phenolic compounds/cytoplasmatic reducing activity, SA: lipid sialic acid. [b] Grey highlight: MCF-7: dark, ZR-75-1: light.

In vitro exposure to As resulted in different responses in both studied lines. MCF-7 cells exhibited sustained nitrosative stress, whereas ZR-75-1 cells retained their antioxidant status after NO up-regulation. Besides, these differences were reflected in the strength of the direct relation between early pathway induction (revealed by L-citrulline synthesis) and cell death triggering (MCF-7 > ZR-75-1). In this regard, the MCF-7 line showed poor ability to counteract redox imbalance [22]. Also, As impaired antioxidant defence due to GGT inhibition, a key enzyme for breast cancer viability and resistance [11,23]. On the contrary, GGT is over-expressed in the ZR-75-1 line [2], which could restore efficiently redox balance after recovery. These findings suggest that different cancer cell lines may have different yet distinct redox susceptibilities (sensitive: MCF-7, tolerant: ZR-75-1), as observed in the present study. We also observed that nitrosative pathway became toxic when its induction was not accompanied by the GGT response in MCF-7 cells, as actually did ZR-75-1 cells. Moreover, the MCF-7 line increases ganglioside synthesis to a lesser extent than ZR-75-1, with aberrant lipid membrane glycosilation being able to protect neoplastic cells against oxidative damage [2,24].

The balance between ROS, RNS and reactive sulphur species (RSS) are pivotal events for the final redox outcome. The fate of NO release depends on superoxide anion availability, which in turns allows peroxinitrite formation with the subsequent oxidative damage [7]. When superoxide anion is low free NO increases, which can be conjugated with GSH or to form nitrites/nitrates [25]. This may be the case of ZR-75-1 cells treated with S and Q, depending on GSH availability. Statistical association found between GSH-related and NO-related pathways might be because NO induces GGT to restore GSH and to prevent nitrosative death, as a feedback response [26]. Thus, RNS/RSS balance might be decisive for antioxidant and cytoprotective effects of NO and GSH, respectively, with their reactive derivatives possibly interacting each other forming S-nitroso-metabolites and reciprocally cancelling their oxidative potential. Consequently, GGT antagonism is an attractive molecular candidate to revert resistance to nitrosative/oxidative chemotherapy given its importance for cellular GSH restoration and cellular resistance [27].

In this context, deleterious effects induced by As might be partially avoided by the co-treatment with antioxidant flavonoids. Quercetin and silymarin prevented nitrosative induction in ZR-75-1 cells, and GGT was inhibited by As+S [2]. Thus, global toxic effect was not fully prevented by S. On the other hand, As-induced nitrosative stress was delayed but not totally prevented by quercetin addition in MCF-7 cells. Interestingly, S also promoted nitrosative stress in this cell line (intrinsic activity), but co-treatment with As did not, without avoiding death. Flavonoids did not seem to be accumulated by the breast cancer cells, since these phenolic compounds that contributed with RCP levels were not raised after recovery. These data might be related to the NO source, since NOS isoforms are distinctly up-regulated by the treatments, since epithelial isoform is inhibited by As and induced by flavonoids. On the contrary, the inducible form responds in a different way and is closely related to cellular stress [28]. Further studies should be encouraged in order to evaluate these complex pharmacological interactions between flavonoids, As and tumour cells.

CONCLUSION

In summary, As exhibited cytotoxic activity by nitrosative stress induction and impairment of the antioxidant defences in both human tumour cell lines ZR-75-1 and MCF-7, with the latter one being more sensitive to deleterious effect of As. Besides, silymarin consumption as a nutraceutical agent might be considered as a natural approach to prevent systemic toxicity of arsenic-related anticancer treatments.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

ACKNOWLEDGEMENTS

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