Nutrition and Cancer
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/hnuc20

Effect of Arsenite on Nitrosative Stress in Human Breast Cancer Cells and Its Modulation by Flavonoids

Elio A. Soria[a], Guillermina A. Bongiovanni[b], Cintia Díaz Luján[c] & Aldo R. Eynard[c]

[a] Instituto de Investigaciones en Ciencias de la Salud (INICSA-UNC/CONICET), Facultad de Ciencias Médicas, Universidad Nacional de Córdoba, Córdoba, Argentina

[b] Instituto de Investigación y Desarrollo en Ingeniería de Procesos, Biotecnología y Energías Alternativas (PROBIEN-UNCO/CONICET), Universidad Nacional del Comahue, Neuquén, Argentina

[c] Instituto de Investigaciones en Ciencias de la Salud (INICSA-UNC/CONICET), Facultad de Ciencias Médicas, Universidad Nacional de Córdoba, Córdoba, Argentina

Published online: 07 Apr 2015.

To cite this article: Elio A. Soria, Guillermina A. Bongiovanni, Cintia Díaz Luján & Aldo R. Eynard (2015): Effect of Arsenite on Nitrosative Stress in Human Breast Cancer Cells and Its Modulation by Flavonoids, Nutrition and Cancer, DOI: 10.1080/01635581.2015.1019637

To link to this article: http://dx.doi.org/10.1080/01635581.2015.1019637

Nutrition and Cancer, 0(0), 1–5

ISSN: 0163-5581 print / 1532-7914 online
DOI: 10.1080/01635581.2015.1019637

Routledge
Taylor & Francis Group

Effect of Arsenite on Nitrosative Stress in Human Breast Cancer Cells and Its Modulation by Flavonoids

Elio A. Soria
Instituto de Investigaciones en Ciencias de la Salud (INICSA-UNC/CONICET), Facultad de Ciencias Médicas, Universidad Nacional de Córdoba, Córdoba, Argentina

Guillermina A. Bongiovanni
Instituto de Investigación y Desarrollo en Ingeniería de Procesos, Biotecnología y Energías Alternativas (PROBIEN-UNCO/CONICET), Universidad Nacional del Comahue, Neuquén, Argentina

Cintia Díaz Luján and Aldo R. Eynard
Instituto de Investigaciones en Ciencias de la Salud (INICSA-UNC/CONICET), Facultad de Ciencias Médicas, Universidad Nacional de Córdoba, Córdoba, Argentina

Arsenic (As) is used in the treatment of leukemia and breast cancer due to its oxidative cytotoxic action. However, it is also toxic to normal cells. One proposed anticancer mechanism induced by As might be nitrosative stress (NS). It is believed that antioxidant 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). Even though both cell lines differed about 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 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. NS was an 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.

INTRODUCTION

The strong cytotoxicity of arsenic (As) has been proposed for therapy of certain human tumors, such as breast cancer (1). However, mammary cancer cells show distinct redox responses after As exposure, depending on their differentiation grades (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). Cotreatments with redox-modulating agents could be beneficial if they do not interfere with the much desired antitumor effects of As. Epidemiological and experimental evidence indicate that some plant polyphenols, broadlynamed flavonoids, such as quercetin and silymarin, have chemopreventive effects (4). These activities are related to counteract reactive nitrogen species (RNS) and reactive oxygen species (ROS). RNS arise mainly from nitric oxide (NO), synthesized by NO synthase (NOS). ROS include free radicals and nonradicals, 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 roles of nitrosative pathways in cancer development is not easy, because these molecules quickly react with free radicals (7). However, NO is a cytoprotective molecule as well, because 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, NO may play bimodal redox activities in accordance to these particular conditions.

The aims of this work were to evaluate the As nitrosative potential as cytotoxic on human mammary tumor cells, previously described in other tissues (10), and to determine the role of redox-modulating flavonoids, such as quercetin and silymarin, in this response.

Submitted 29 July 2014; accepted in final form 11 January 2015.
Address correspondence to Elio A. Soria, Instituto de Biología Celular, n/n Enrique Barros St., Ciudad Universitaria, Córdoba 5014, Argentina. Tele: +543514334020. E-mail: easoria@fcm.unc.edu.ar

MATERIALS AND METHODS

Chemicals

Quercetin (CAS n° 6151-25-3, $C_{15}H_{10}O_7$ $2H_2O$, MW= 338.3 g/mole) was obtained from E. Merck (Darmstadt, Germany), and silymarin (CAS n° 22888-70-6, $C_{25}H_{22}O_{10}$, 31% silybinin, MW= 482.4 g/mole) from Sigma-Aldrich Co. (St. Louis, MO). Sodium arsenite ($NaAsO_2$, MW= 130 g/mole) was purchased from Anedra (CABA, Argentina). Reagents for nitrite detection were purchased by Britania, and solvents by Cicarelli (CABA, Argentina). The γ-G-test kinetic AA kit™ was from Wiener Laboratories (Rosario, Argentina). Other chemicals were obtained from Sigma-Aldrich Co. (St. Louis, MO).

Cell Culture and Treatments

The human cancer cell lines were MCF-7 and ZR-75-1, which were obtained from mesothelial carcinomatosis exudates of 2 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 1 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

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 pretreating 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 sialyllipid determinations (GC) at 580 nm in accordance to Miettinen and Takki-Luukkainen (13). The lower layer was used for oxidized lipids determinations (CD) at 234 nm (2).

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 3 times, the stained cells (attached in 96-well plates) were solubilized 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 byproduct of the NO 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 NO 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 3 separate experiments performed in triplicate, unless otherwise noted. Analysis of variance 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 correlation analysis by the Spearman coefficient, were done with the InfoStat 2012 software.

RESULTS

Cellular Redox Response

The Spearman coefficient 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 1).

TABLE 1
Spearman correlations between stress-related markers found in breast cancer cells

	1	2	3	4	5	6	7
1. Cit	1.00	0.20	0.14	0.09	−0.37	−0.54	−0.60
2. CD	0.03	1.00	0.09	0.37	0.26	−0.09	−0.20
3. SA	−0.04	−0.01	1.00	−0.26	−0.14	−0.83	−0.31
4. GGT	−0.66	0.43	−0.16	1.00	0.77	−0.09	0.66
5. N	0.43	−0.66	0.56	−0.83	1.00	0.14	0.77
6. RPC	−0.60	0.60	−0.21	0.49	−0.71	1.00	0.26
7. CV	−0.94	0.09	0.07	0.83	−0.54	0.54	1.00

CD = conjugated dienes; Cit = L-citrulline; GGT = γ-glutamyltranspeptidase activity; N = nitrites; RPC = reducing phenolic compounds/cytoplasmatic reducing activity; SA = lipid sialic acid. Grey highlight: dark (below the diagonal) = MCF-7; light (above the diagonal) = ZR-75-1.

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-7 than 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).

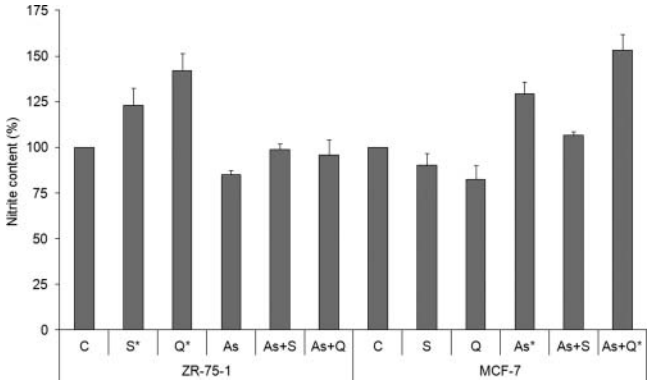

FIG. 2. Nitrite content in the breast cancer cells ZR-75-1 and MCF-7 incubated for 2 h in free treatment-medium after being exposed for 2 h to: none (C), 5 μM silymarin (S), 50 μM quercetin (Q), 200 μM sodium arsenite (As), As + S and As + Q. Data were averages of 3 separate experiments $\pm$ SE (% respect to C; $^*P < 0.05$).

DISCUSSION

Two concerns are essential before proposing As trivalent derivates for breast cancer treatment in patients (17). First, putative carcinogenic effects on remnant mammary tissue should be avoided at the highest extent (18). Secondly, it is important to know whether nonspecific systemic cytotoxicity of As could be counteracted by nutraceutical coadjuvants without losing the desirable anticancer activity (2,3). Among several antimammary cancer mechanisms proposed for As (19), 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.

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

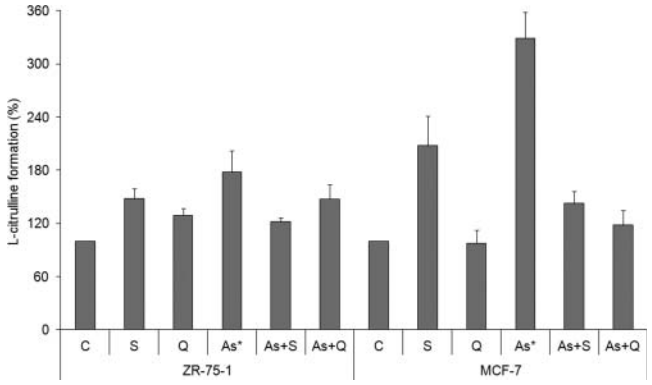

FIG. 1. L-citrulline formation in the breast cancer cells ZR-75-1 and MCF-7 incubated for 2 h under the following treatments: none (C), 5 μM silymarin (S), 50 μM quercetin (Q), 200 μM sodium arsenite (As), As + S and As + Q. Data were averages of 3 separate experiments $\pm$ SE (% respect to C; $^*P < 0.05$).

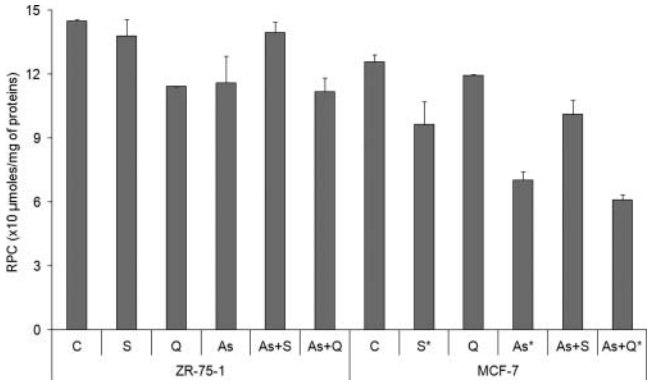

FIG. 3. Cytoplasmatic reducing activity [CRA by RPC (reducing phenyl-compounds)] in the breast cancer cells ZR-75-1 and MCF-7 incubated for 2 h under the following treatments: none (C), 5 μM silymarin (S), 50 μM quercetin (Q), 200 μM sodium arsenite (As), As + S and As + Q. Data were average of 3 separate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

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 (20). Also, As impaired antioxidant defence due to GGT inhibition and glutathione depletion, a key enzyme for breast cancer viability and resistance (11,21). On the contrary, GGT is overexpressed 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 (2,22).

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 (23). 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 (24). 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 (21).

In this context, deleterious effects induced by As might be partially avoided by the cotreatment 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, because these phenolic compounds that contributed with RCP levels were not raised after recovery. These data might be related to the NO source, because NOS isoforms are distinctly upregulated by the treatments, because 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 (25). Further studies should be encouraged to evaluate these complex pharmacological interactions between flavonoids, As, and tumor cells, which can exhibit phenotypes with different responsiveness to these compounds.

Arsenic triggered cytotoxicity by nitrosative stress induction and antioxidant defense impairment in both human tumor cell lines ZR-75-1 and MCF-7, with the later one being more sensitive to these deleterious effects. Besides, the silymarin use as a pharmaconutrient might be considered to prevent systemic toxicity of arsenic-related anticancer treatments.

FUNDING

This study was supported by the funds provided by the National University of Cordoba (SECYT-UNC 162/2012, Arg.). The authors declare that they have no conflict of interest. The experiments comply with the current laws of the country in which they were performed.

REFERENCES

1. Guilbert C, Annis MG, Dong Z, Siegel PM, Miller WH Jr, et al.: Arsenic trioxide overcomes rapamycin-induced feedback activation of AKT and ERK signaling to enhance the anti-tumor effects in breast cancer. *PLoS One* **8**, e85995, 2013.
2. Soria EA, Eynard AR, Quiroga PL, and Bongiovanni GA: Differential effects of quercetin and silymarin on arsenite-induced cytotoxicity in two human breast adenocarcinoma cell lines. *Life Sci* **81**, 1397–1402, 2007.
3. Soria EA, Eynard AR, and Bongiovanni GA: Cytoprotective effects of silymarin on epithelial cells against arsenic-induced apoptosis in contrast with quercetin cytotoxicity. *Life Sci* **87**, 309–315, 2010.
4. Husein AI, Ali-Shtayeh MS, Jondi WJ, Zatar NA, Abu-Reidah IM, et al.: In vitro antioxidant and antitumor activities of six selected plants used in the Traditional Arabic Palestinian herbal medicine. *Pharm Biol*, in press.
5. Rahal A, Kumar A, Singh V, Yadav B, Tiwari R, et al.: Oxidative stress, prooxidants, and antioxidants: the interplay. *Biomed Res Int* **2014**, e761264, 2014.
6. Wang HJ, Wei XF, Jiang YY, Huang H, Yang Y, et al.: Silibinin induces the generation of nitric oxide in human breast cancer MCF-7 cells. *Free Radic Res* **44**, 577–584, 2010.
7. Pacher P, Beckman JS, and Liaudet L: Nitric oxide and peroxynitrite in health and disease. *Physiol Rev* **87**, 315–424, 2007.
8. Kevil CG and Patel RP: S-Nitrosothiol biology and therapeutic potential in metabolic disease. *Curr Opin Investig Drugs* 11, 1127–1134, 2010.
9. Rozza AL, de Mello Moraes T, Kushima H, Nunes DS, Hiruma-Lima CA, et al.: Involvement of glutathione, sulfhydryl compounds, nitric oxide, vasoactive intestinal peptide, and heat-shock protein-70 in the gastroprotective mechanism of Croton cajucara Benth. (Euphorbiaceae) essential oil. *J Med Food* **14**, 1011–1017, 2011.
10. Ma N, Sasoh M, Kawanishi S, Sugiura H, Piao F: Protection effect of taurine on nitrosative stress in the mice brain with chronic exposure to arsenic. *J Biomed Sci* **17**(Suppl 1), S7, 2010.
11. Quiroga A, Quiroga PL, Martinez E, Soria EA, and Valentich MA: Anti-breast cancer activity of curcumin on the human oxidation-resistant cells ZR-75-1 with γ-glutamyltranspeptidase inhibition. *J Exp Ther Oncol* **8**, 261–266, 2010.
12. Soria EA, Eynard AR, and Bongiovanni GA: Modulation of early stress-related biomarkers in cytoplasm by the antioxidants silymarin and

quercetin using a cellular model of arsenic acute poisoning. *Basic Clin Pharmacol Toxicol* **107**, 982–987, 2010.
13. Miettinen T and Takki-Luukkainen IT: Use of butyl acetate in the determination of sialic acid. *Acta Chem Scand* **13**, 856–858, 1959.
14. Boyde TR and Rahmatullah M: Optimization of conditions for the colorimetric determination of citrulline, using diacetylmonoxime. *Anal Biochem* **107**, 424–431, 1980.
15. Green LC, Wagner DA, Glogowski Skipper J, Wishnok PL, and Tannenbaum SR: Analysis of nitrite, nitrate, and [15N] in biological fluids. *Anal Biochem* **126**, 131–138, 1982.
16. Cittadini MC, Canalis AM, Albrecht C, and Soria EA: Effects of oral phytoextract intake on phenolic concentration and redox homeostasis in murine encephalic regions. *Nutr Neurosci*, in press.
17. Si L, Jiang F, Li Y, Ye X, Mu J, et al.: Induction of the mesenchymal to epithelial transition by demethylation-activated microRNA-200c is involved in the anti-migration/invasion effects of arsenic trioxide on human breast cancer cells. *Mol Carcinog*, in press.
18. Aballay LR, Díaz MD, Francisca FM, and Muñoz SE: Cancer incidence and pattern of arsenic concentration in drinking water wells in Córdoba, Argentina. *Int J Environ Health Res* 22, 220–231, 2012.
19. Kim MJ, Jung JH, Lee WS, Yun JW, Lu JN, et al.: Arsenic hexoxide enhances TNF-α-induced anticancer effects by inhibiting NF-κB activity at a safe dose in MCF-7 human breast cancer cells. *Oncol Rep* **31**, 2305–2311, 2014.
20. O'Shea M, Stanton C, and Devery R: Antioxidant enzyme defence responses of human MCF-7 and SW480 cancer cells to conjugated linoleic acid. *Anticancer Res* **19**, 1953–1959, 1999.
21. Li J, Zhang D, Jefferson PA, Ward KM, and Ayene IS: A bioactive probe for glutathione-dependent antioxidant capacity in breast cancer patients: implications in measuring biological effects of arsenic compounds. *J Pharmacol Toxicol Methods* **69**, 39–48, 2014.
22. Varki NM and Varki A: Diversity in cell surface sialic acid presentations: implications for biology and disease. *Lab Invest* **87**, 851–857, 2007.
23. Alderton WK, Cooper CE, and Knowles RG: Nitric oxide synthases: structure, function and inhibition. *Biochem J* **357**, 593–615, 2001.
24. Huseby NE, Asare N, Wetting S, Mikkelsen IM, Mortensen B, et al.: Nitric oxide exposure of CC531 rat colon carcinoma cells induces gamma-glutamyltransferase which may counteract glutathione depletion and cell death. *Free Radic Res* **37**, 99–107, 2003.
25. Wu LY, Dang XQ, He XJ, and Yi ZW: Effects of clearance of superoxide anion by catechin on the expression of iNOS and eNOS and apoptosis in endothelial progenitor cells induced by angiotensin II. *Zhongguo Dang Dai Er Ke Za Zhi* **11**, 476–480, 2009.