Effects of N-acetyl cysteine, vitamin E and vitamin C on liver glutathione levels following amiodarone treatment in rats

Esin Akbay[1], Beril Erdem[1], Ahmet Ünlü[2], Ahmet Barış Durukan[1,2], Mehmet Ali Onur[1]

[1]Department of Cardiology, Faculty of Science, Hacettepe University, Ankara, Turkey
[2]Department of Cardiovascular Surgery, Medical Park Usak Hospital, Usak, Turkey

Abstract

Introduction: Amiodarone, a pharmaceutical extensively used to suppress atrial and ventricular tachyarrhythmias, is also known to cause many side effects on many tissues. N-acetyl-cysteine (NAC), vitamin E and vitamin C are known as anti-oxidants for their ability to minimize oxidative stress. In the peer-reviewed literature, there is no study reporting on the protective effects of these antioxidant agents against its hepatotoxicity.
Aim: We investigated the oxidative effects of NAC, vitamins E and C on liver tissue after amiodarone treatment.
Material and methods: Rats were randomly assigned to: control; amiodarone group; amiodarone + NAC treated group; amiodarone + Vit. E group and amiodarone + Vit. C group. Liver tissues were isolated from animals and total glutathione levels were measured.
Results: In all time intervals, the level of glutathione increased. When all time intervals were compared, the amiodarone group revealed the lowest levels. The antioxidant co-administered group was studied; the glutathione levels were statistically significantly higher than the sole amiodarone group. When vitamins E, C or N-acetyl cysteine were examined, there was no statistically significant difference among them.
Conclusions: In this study we found that hepatotoxicity capacity of amiodarone may be reduced by taking up antioxidants. In addition, the effect documented here may be reproducible and may be applied to clinical settings.

Key words: amiodarone, toxicity, N-acetyl cysteine, vitamin E, ascorbic acid, hepatotoxicity, rats.

Streszczenie

Wprowadzenie: Amiodaron – lek powszechnie stosowany w tachyarytmii przedsionkowej i komorowej – wywołuje wiele skutków ubocznych w różnych tkankach. N-acetylocysteina (NAC), witamina E i witamina C znane są jako przeciwutleniacze, ponieważ mają zdolność redukcji stresu oksydacyjnego. W piśmiennictwie nie ma badań opisujących ich działanie ochronne w odniesieniu do hepatotoksyczności amiodaronu.
Cel: Zbadanie wpływu oksydacyjnego NAC, witaminy E i witaminy C na tkanki wątroby po leczeniu amiodaronem.
Materiał i metody: Szczury losowo przydzielono do grupy kontrolnej, grupy otrzymującej amiodaron, grupy otrzymującej amiodaron i NAC, grupy otrzymującej amiodaron i witaminę E oraz grupy otrzymującej amiodaron i witaminę C. Od zwierząt pobrano tkanki wątroby i zbadano całkowite stężenie glutationu.
Wyniki: We wszystkich przedziałach czasowych stężenie glutationu zwiększało się. Gdy porównano wszystkie przedziały czasowe, najmniejsze jego stężenie stwierdzono w grupie leczonej amiodaronem. W grupach przyjmujących jednocześnie przeciwutleniacze stężenia glutationu były statystycznie istotnie większe niż w grupie leczonej tylko amiodaronem. Nie stwierdzono statystycznie istotnych różnic między grupami otrzymującymi witaminę E, witaminę C i NAC.
Wnioski: Zaobserwowano, że przyjmowanie przeciwutleniaczy może zmniejszać hepatotoksyczność amiodaronu. Opisany efekt jest powtarzalny, zatem może być wykorzystany w warunkach klinicznych.

Słowa kluczowe: amiodaron, toksyczność, N-acetylocysteina, witamina E, kwas askorbinowy, hepatotoksyczność, szczury.

Introduction

Amiodarone is a type 3 anti-arrhythmic agent that is widely used and has many toxic effects on different tissue functions, including hepatic toxicity, steatosis, gastrointestinal upset, hepatitis, bradycardia, liver and pancreas fibrosis. It was shown that amiodarone has cytotoxic effects on different cell types in cell culture studies. It is most extensively prescribed for the benefits as a drug used in

atrial fibrillation (AF). Amiodarone can cause many adverse effects especially on the liver [1]. Many studies have concluded that amiodarone generates free radicals *in vitro* and *in vivo* which are the main causes of oxidative stress [2, 3]. Oxidative stress manifests by producing reactive oxygen species (ROS) in the cell [4]. Superoxide anion is a reactive and superoxide dismutase that converts superoxide to hydrogen peroxide, which is less reactive than oxygen anion. Glutathione peroxidase detoxifies hydrogen peroxide to water [4]. Reduced glutathione (GSH) regulates the activity of glutathione peroxidase by giving hydrogen. Therefore, GSH is considered as a detoxifying molecule. Glutathione is present mostly in reduced form (GSH) in the cell. It can be oxidized to GSSG (oxidized glutathione), which is approximately 1% of total glutathione in the cell in normal conditions [5]. GSH has an important role in detoxification and it can help regenerating some antioxidants such as vitamin C and E, which are also capable of detoxifying hydrogen peroxide [4]. In addition to them, N-acetyl cysteine (NAC) is another antioxidant molecule. It is a cys residue with acetylation and in this way it becomes a part of the cysteine pool in the cell to maintain the biosynthesis of GSH [6]. Deficiency of GSH or the ratio of GSH/GSSG could be a marker for several pathological problems [5]. As a result of AF, in myocardial tissues, high levels of superoxide and hydrogen peroxide are observed [7–10]. Also, as an oxidative stress marker, the ratio of GSSG to GSH (reduced glutathione) was found increased in blood samples of patients with AF [11]. Both AF and using amiodarone as a drug for AF can be obvious causes of increased ROS and consequently change of total concentration of GSH in cells and related tissues. Many studies have shown that using antioxidants such as NAC, vitamin C and vitamin E could be helpful to minimize the effects of oxidative stress in the cell [3]. With this information, using antioxidants could reduce oxidative stress, which is a result of amiodarone as a drug for AF.

Recently, especially amiodarone's toxic effects on the liver have not been well studied and no clinical agents are available for prevention. Oxidative stress has been proved to be the major pathogenic mechanism of amiodarone's tissue damage effects. From this perspective, the aim of this study is to investigate toxic effects of amiodarone and the prevention potential of NAC, vitamin E and vitamin C in a rat experimental model.

Aim

We studied the level of glutathione in rat liver tissue, following the oral administration of amiodarone and antioxidants.

Material and methods

Agents used

Amiodarone hydrochloride (Cordorone, 200 mg, Sanofi-Aventis, Istanbul, Turkey), vitamin E (Evicap, 400 I.U., İlaç ve Kimya Sanayi A.Ş., Çerkezköy, Tekirdağ, Turkey), ascorbic acid (Redoxon, 500 mg/tablet, Bayer Türk Kimya San

Ltd. Şti., Istanbul, Tukey), and N-acetyl cysteine (NAC, 200 mg/tablet, Bilim İlaç San ve Tic. A.Ş., Gebze, Kocaeli, Turkey) were purchased from a pharmacy. Glutathione Assay Kit (CAS number: CS0260) was obtained from Sigma-Aldrich (St. Louis, MO).

Animals

Thirty Wistar albino male rats were used with the approval of the Animal Care and Use Committee of Hacettepe University (Permit no. 2013/5-10) and carried out in accordance with the National Guidelines for the Use and Care of Laboratory Animals. After all animals were purchased from Animal Laboratory Units of Hacettepe University, they were allowed to become adapted to the conditions of the laboratory for seven days. All animals were housed randomly selected as three animals per cages, using a 12 h light-dark cycle.

Design of the study

All rats were randomly assigned to five groups as follows: control group (untreated group, $n = 6$), amiodarone treated group (100 mg/kg/day, $n = 6$), amiodarone + Vit. E treated group (50 mg/kg/day, $n = 6$), amiodarone + Vit. C treated group (50 mg/kg/day, $n = 6$), amiodarone + NAC treated group (50 mg/kg/day, $n = 6$). Amiodarone and all other substance were given orally for 7 days. The amiodarone dose is higher compared to the dose used in human beings due to the fact that the metabolism of rats is higher compared to human beings [12]. All antioxidant agents were given 1 h after administration of amiodarone. All the animals were sacrificed under anesthesia on the 8th day. Liver tissues were taken from the animals, homogenized in 0.9% NaCl and 10% (w/v) homogenates were prepared. Total glutathione was determined in the liver homogenates. Experiments were carried out at the temperatures given in the procedures.

Glutathione assay

The level of total glutathione (GSH and GSSG) in the cell was measured using the Glutathione Assay Kit (Sigma-Aldrich, USA) according to the manufacturer's instructions. Liver samples was firstly deproteinized with 5% 5-sulfosalicylic acid solution and then centrifuged to remove the precipitated protein. Supernatants of the samples were used for the determination of the glutathione according to the manufacturer's protocol. Samples were read with kinetically at 1 min intervals for 5 min at 412 nm using an ELISA reader (EZ Read 400 Micro-plate Reader, Biochrom).

Statistical analysis

Data are presented as mean ± SD in Table I and mean ± SEM in Figure 1. Differences between groups were analyzed using analysis of variance (ANOVA) for triplicate measurements. The level of significance was taken as $p < 0.05$ in all instances. GraphPad Prism 5.01 for Windows (GraphPad Software) was used for statistical analysis. In cases of sig-

Table I. Total glutathione data of all groups (µM)

t [min]	Control	Amiodarone	P-value**	Amiodarone + Vit. C	Amiodarone + NAC	Amiodarone + Vit. E
1	584.2 ±91.2	150.5 ±87.1	< 0.0001	321.4 ±126.1 p*** < 0.0001	332.8 ±118.7 p*** < 0.0001	372.7 ±92.0 p*** < 0.0001
2	607.7 ±91.4	162.2 ±96.3	< 0.0001	334.3 ±134.1 p*** < 0.0001	345.7 ±125.6 p*** < 0.0001	386.8 ±95.2 p*** < 0.0001
3	627.0 ±88.2	176.3 ±104.7	< 0.0001	347.7 ±142.7 p*** < 0.0001	360.1 ±132.8 p*** < 0.0001	402.3 ±99.2 p*** < 0.0001
4	644.3 ±83.6	188.8 ±113.8	< 0.0001	363.6 ±151.5 p*** < 0.0001	375.5 ±140.0 p*** < 0.0001	419.7 ±104.4 p*** < 0.0001
5	659.4 ±77.4	205.4 ±122.5	< 0.0001	380.0 ±157.7 p*** < 0.0001	392.8 ±147.4 p*** < 0.0001	440.6 ±109.3 p*** < 0.0001
P-value*	< 0.0001	< 0.0001		< 0.0001	< 0.0001	< 0.0001

*2-way ANOVA, **comparison between control vs. amiodarone groups/Bonferroni adjusted Mann-Whitney U, ***comparison between groups vs. amiodarone group/Bonferroni adjusted Mann-Whitney U. All data are presented as mean ± SD. The level of significance was taken as p < 0.05. t – time, Vit. C – vitamin C, NAC – N-acetyl cysteine, Vit. E – vitamin E.
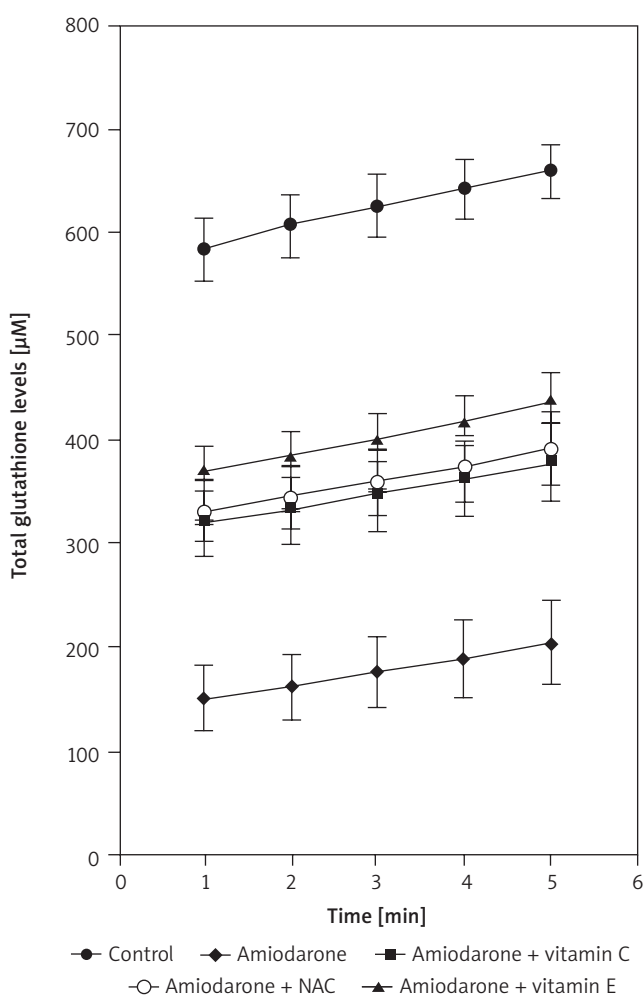
Fig. 1. Results of total glutathione levels (µM) in rat liver tissues with kinetic read at 1-minute intervals for 5 min

nificance, Bonferroni adjusted Mann-Whitney U test was used to determine the efficient group.

Results

Total glutathione amount of the liver samples of rats was measured spectrophotometrically with kinetic read at 1-minute intervals for 5 minutes. When the groups were examined individually there was a significant increase in glutathione levels with time (p < 0.0001) (Table I). When separate time intervals were compared, the amiodarone treated group had the lowest levels compared to control and each antioxidant co-administered group in all time intervals (p < 0.0001), which may demonstrate the protective potential of antioxidants (Fig. 1). When the antioxidants were compared, there was no difference between amiodarone + NAC, amiodarone + Vit. C and amiodarone + Vit. E (p > 0.05 for each).

Discussion

Hepatic injury is a common manifestation of drug toxicity and may be reflected as different clinical features [13]. Mitochondrial dysfunction is one of these features and clinical studies scrutinizing mitochondrial dysfunction came a long way for decades [14]. Mitochondrial dysfunction manifests itself in different ways such as membrane permeabilization, oxidative phosphorylation impairment, inhibition of fatty acid oxidation and mitochondrial DNA depletion [14]. Within the cell, mitochondria are the source of ROS production through the mitochondrial respiratory chain. In this respiratory chain, electrons enter the system and directly react with oxygen to generate superoxide anion radical. Then this radical is converted to hydrogen peroxide (H_2O_2) by superoxide dismutase. Hydrogen peroxide is detoxified to water by glutathione peroxidase which uses reduced glutathione (GSH). In healthy tissues, all reactive oxygen species are detoxified properly by mitochondrial systems. However, in different pathophysiological situations, GSH depletion was seen in liver mitochondria, which reduce the capacity of H_2O_2 detoxification. Hydrogen peroxide accumulation causes mitochondrial dysfunction. Amiodarone can also induce hepatotoxicity due to the mitochondrial dysfunction [15–18]. Also, many studies have documented that it has long-term effects on different tissues. After discontinuation of the drug, the effects of amiodarone were still detected in patients' different tissues including liver, lung and adipose tissue [19]. In our study, we measured total glutathione level

(total GSSG and GSH in µM) of rat livers which were administered with amiodarone. Compared to the control group (which has high levels of glutathione), the amiodarone administered group showed dramatically reduced glutathione levels at each time interval, which shows the reduced detoxification capacity of mitochondria. On the other hand, it was shown that treatment with antioxidants such as vitamin C, vitamin E and NAC has preventive effects on reduced detoxification capacity of mitochondria [20–23]. Both cell culture studies and animal model experiments on amiodarone in relation to several antioxidants show similar beneficial effects of antioxidants to reduce toxicity of amiodarone [24–27]. These antioxidants have similar mechanisms in antioxidant action via scavenging ROS [28, 29]. Also, NAC has importance for production of glutathione in the cell because its cysteine part together with glutamate and glycine is a precursor of glutathione [30]. Glutathione is already an antioxidant in the cell; therefore, NAC administration has a kind of backup. To analyze the antioxidant effects of vitamin C, vitamin E and NAC on amiodarone toxicity, we treated rats with both amiodarone and these antioxidants separately as described above. All antioxidants that we used showed increased glutathione levels when they were coadministered with amiodarone compared to the reduced glutathione level of the amiodarone group. They could not produce glutathione like in the control group but when compared to the effect of amiodarone only, we can say that antioxidant usage is successful in producing glutathione in the cell. The group receiving vitamin E coadministered with amiodarone had the highest level of glutathione, followed by the NAC + amiodarone group and Vit. C + amiodarone group. It is already known that separate treatments of NAC, vitamin C and vitamin E on cultured cells have antioxidant effects [3].

Conclusions

Our results are important in terms of understanding the toxic effects of amiodarone and its prevention via use of antioxidants. In our previous study, we demonstrated the decrease of amiodarone cytotoxicity using antioxidants in cell culture [3]. Now, we showed the toxicity reducing capacity of antioxidants in animal experiments. To make a comprehensive conclusion, further analysis such as separate measurement of GSSG and GSH amounts, glutathione peroxidase enzyme activity and measurement of lipid peroxidation products could be supportive. In addition, we believe that this study is reproducible, and clinical studies may be performed based on these findings.

Disclosure

The authors report no conflict of interest.

References

1. Mason JW. Amiodarone. N Engl J Med 1987; 316: 455-466.
2. Vereckei A, Blazovics A, Gyorgy I, Feher E, Toth M, Szenasi G, Zsinka A, Foldiak G, Feher J. The role of free radicals in the pathogenesis of amiodarone toxicity. J Cardiovasc Electrophysiol 1993; 4: 161-177.
3. Durukan AB, Erdem B, Durukan E, Sevim H, Karaduman T, Gurbuz HA, Gurpinar A, Yorgancioglu C. May toxicity of amiodarone be prevented by antioxidants? A cell culture study. J Cardiothor Surg 2012; 7: 61.
4. Burton GJ, Jauniaux E. Oxidative stress. Best Pract Res Clin Obstet Gynaecol 2011; 25: 287-299.
5. Camera E, Picardo M. Analytical methods to investigate glutathione and related compounds in biological and pathological processes. J Chromatogr B Analyt Technol Biomed Life Sci 2002; 781: 181-206.
6. Kerksick C, Willoughby D. The antioxidant role of glutathione and N-acetyl-cycteine supplements and exercise-induced oxidaive stress. J Int Soc Sports Nutr 2005; 2: 38-44.
7. Chang JP, Chen MC, Liu WH, Yang CH, Chen CJ, Chen YL, Pan KL, Tsai TH, Chang HW. Atrial myocardial nox2 containing NADPH oxidase activity contribution to oxidative stress in mitral regurgitation: potential mechanism for atrial remodeling. Cardiovasc Pathol 2011; 20: 99-106.
8. Kim YM, Guzik TJ, Zhang YH, Zhang MH, Kattach H, Ratnatunga C, Pillai R, Channon KM, Casadei B. A myocardial Nox2 containing NAD(P)H oxidase contributes to oxidative stress in human atrial fibrillation. Circ Res 2005; 97: 629-636.
9. Kim YM, Kattach H, Ratnatunga C, Pillai R, Channon KM, Casadei B. Association of atrial nicotinamide adenine dinucleotide phosphate oxidase activity with the development of atrial fibrillation after cardiac surgery. J Am Coll Cardiol 2008; 51: 68-74.
10. Zhang J, Youn JY, Kim AY, Ramirez RJ, Gao L, Ngo D, Chen P, Scovotti J, Mahajan A, Cai H. NOX4-dependent hydrogen peroxide overproduction in human atrial fibrillation and hl-1 atrial cells: relationship to hypertension. Front Physiol 2012; 3: 140.
11. Neuman RB, Bloom HL, Shukrullah I, Darrow LA, Kleinbaum D, Jones DP, Dudley SC Jr. Oxidative stress markers are associated with persistent atrial fibrillation. Clin Chem 2007; 53: 1652-1657.
12. Nair AB, Jacob S. A simple practice guide for dose conversion between animals and human. J Basic Clin Pharm 2016; 7: 27-31.
13. Larrey D. Drug-induced liver diseases. J Hepatol 2000; 32: 77-88.
14. Labbe G, Pessayre D, Fromenty B. Drug-induced liver injury through mitochondrial dysfunction: mechanisms and detection during preclinical safety studies. Fundam Clin Pharmacol 2008; 22: 335-353.
15. Berson A, Descatoire V, Sutton A, Fau D, Maulny B, Vadrot N, Feldmann G, Berthon B, Tordjmann T, Pessayre D. Toxicity of alpidem, a perpheral benzodiazepine receptor ligand, nut not zolpidem, in rat hepatocytes: role of mitochondrial permeability transition and metabolic activation. J Pharmacol Exp Ther 2001; 299: 793-800.
16. Berson A, Fau D, Fornacciari R, Degove-Goddard P, Sutton A, Descatoire V, Haouzi D, Lettéron P, Moreau A, Feldmann G, Pessayre D. Mechanisms for experimental buprenorphine hepatotoxicity: major role of mitochondrial dysfunction versus metabolic activation. J Hepatol 2001; 34: 261-269.
17. Larosche I, Letteron P, Fromenty B, Vadrot N, Abbey-Toby A, Feldmann G, Pessayre D, Mansouri A. Tamoxifen inhibits topoisomerases, depletes mitochondrial DNA, and triggers steatosis in mouse liver. J Pharmacol Exp Ther 2007; 321: 526-535.
18. Waldhauser KM, Torok M, Ha HR, Thomet U, Konrad D, Brecht K, Follath F, Krähenbühl S. Hepatocellular toxity and pharmacological effect of amiodarone and amiodarone derivatives. J Pharmacol Exp Ther 2006; 319: 1413-1423.
19. Begriche K, Massart J, Robin MA, Borgne-Sanchez A, Fromenty B. Drug-induced toxicity on mitochondria and lipid metabolism: mechanistic diversity and deleterious consequences for the liver. J Hepatol 2011; 54: 773-794.
20. Carnes CA, Chung MK, Nakayama T, Nakayama H, Baliga RS, Piao S, Kanderian A, Pavia S, Hamlin RL, McCarthy PM, Bauer JA, Van Wagoner DR. Ascorbate attenuates atrial pacing-induced peroxynitrite formation and electrical remodeling and decreases the incidence of postoperative atrial fibrillation. Circ Res 2001; 89: E32-E38.
21. Ozaydin M, Peker O, Erdogan D, Kapan S, Turker Y, Varol E, Ozguner F, Dogan A, Ibrisim E. N-acetylcysteine for the prevention of postoperative atrial fibrillation: a prospective, randomized, placebo-controlled pilot study. Eur Heart J 2008; 29: 625-631.
22. Rodrigo R, Vinay J, Castillo R, Cereceda M, Asenjo R, Zamorano J, Araya J, Castillo-Koch R, Espinoza J, Larraín E. Use of vitamins C and E as a prophylactic therapy to prevent postoperative atrial fibrillation. Int J Cardiol 2010; 138: 221-228.
23. Korantzopoulos P, Kolettis TM, Kountouris E, Dimitroula V, Karanikis P, Pappa E, Siogas K, Goudevenos JA. Oral vitamin C administration reduces early recurrence rates after electrical cardioversion of persistent atrial fibrillation and attenuates associated inflammation. Int J Cardiol 2005; 102: 321-326.

24. Golli-Bennour EE, Bouslimi A, Zouaoui O, Nouira S, Achour A, Bacha H. Cytotoxicity effects of amiodarone on cultured cells. Exp Toxicol Pathol 2012; 64: 425-430.
25. Bolt MW, Racz WJ, Brien JF, Massey TE. Effects of vitamin E on cytotoxicity of amiodarone and N-desethylamiodarone in isolated hamster lung cells. Toxicology 2001; 166: 109-118.
26. Card JW, Racz WJ, Brien JF, Massey TE. Attenuation of amiodarone-induced pulmonary fibrosis by vitamin E is associated with suppression of transforming growth factor-beta1 gene expression but not prevention of mitochondrial dysfunction. J Pharmacol Exp Ther 2003; 304: 277-283.
27. Ágoston M, Örsi F, Fehér E, Hagymási K, Orosz Z, Blázovics A, Fehér J, Vereckei A. Silymarin and vitamin E reduce amiodarone-induced lysosomal phospholipidosis in rats. Toxicology 2003; 190: 231-241.
28. Viswanatha Swamy AH, Wangikar U, Koti BC, Thippeswamy AH, Ronad PM, Manjula DV. Cardioprotective effect of ascorbic acid on doxorubicin-induced myocardial toxicity in rats. Indian J Pharmacol 2011; 43: 507-511.
29. Oyewole AO, Birch-Machin MA. Mitochondria-targeted antioxidants. FASEB J 2015; 29: 4766-4771.
30. Sansone RA, Sansone LA. Getting a knack for NAC: N-acetyl-cysteine. Innov Clin Neurosci 2011; 8: 10-14.