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(*as of April 25, 2009*)

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Hokkaido University Hospital, Sapporo, Japan

Hokkaido University is located in Sapporo, Hokkaido a northern island of Japan. Hokkaido has been developed in earnest since 19 century. Pioneer spirits live on today with words by William S. Clark, an American who was the first vice president of Sapporo Agricultural College (now Hokkaido University), "Boys, be ambitious!". The photo shows Hokkaido University Hospital dedicating for clinical practice, medical education, and local health development. (*Photo by Xiaoguang Li*)

Review

Bidi smoking and lung cancer

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Summary **This article discusses the role of bidi smoking as a risk factor for lung cancer. A review of the documented evidence is presented. The literature from Pubmed has been searched using the key words 'beedi smoking', 'bidi smoking' and 'lung cancer'. The bibliographies of all papers found were further searched for additional relevant articles. After this thorough search, eight studies were found. The evidence suggests that bidi smoking poses a higher risk for lung cancer than cigarette smoking and risk further increases with both the length of time and amount of bidi smoking. The focus of tobacco control programs should be expanded to all types of tobacco use, including bidis, to reduce the increasing problem of lung cancer.**

Keywords: Smoking, bidi, cigarette, lung cancer, India

1. Introduction

The "epidemic" of lung cancer mortality has been identified as a major health issue confronting both developed and developing countries. It is the leading cause of cancer deaths in developed countries and is also rising at alarming rates in developing countries. Almost half (49.9%) of the cases occur in the developing countries of the World – a big change since 1980, when it was estimated that 69% were in developed countries (*1*). Worldwide, it is by far the most common cancer in men.

In India, lung cancer has been considered to be an infrequent entity, but an increased rate of diagnosis of bronchogenic carcinoma was recognized in the early 1960s by Vishvanathan *et al*. (*2*). Population-based as well as hospital-based data from the Cancer Registry of the Indian Council of Medical Research (ICMR) and the Cancer Atlas Programme of the ICMR revealed that lung cancer has increased in India during the last few years (*3-5*).

Tobacco smoking is the most important etiologic factor for the development of lung cancer. 90% of all lung cancers in men and 79% in women are directly attributed to smoking (*6*). Smoking habits in India differ from other countries like USA. In India out of total tobacco consumption, smoking accounts for 72% of the total, 73% is related to bidi and the other 27% is due to cigarettes (*7*). Reports of an increase in prevalence of bidi consumption have also emerged from other countries in Asia, as well as other parts of the world, such as USA, France, Canada and Australia (*8-13*).

In India, tobacco smoking in general and bidi smoking in particular have long enjoyed social acceptance and respectability in some parts of the culture like wedding ceremonies in the rural area (*14*). The leaf-wrapped appearance of bidis and absence of health warnings on their package lead to the perception among young people that bidis are "safe, herbal" cigarettes. Even though, a number of worldwide, population based case controlled studies as well as cohort studies have proved the association of tobacco smoking to the lung cancer, very few studies have been carried out to demonstrate the association between bidi smoking and lung cancer.

2. Data Sources

The Pubmed medical literature database was searched for published articles that had the key words 'bidi-smoking', 'beedi-smoking' and 'lung cancer'. The bibliographies of all papers found were searched for further relevant

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articles. Any study, whether case-control, retrospective, cross-sectional or longitudinal, was included.

3. Results

After this thorough search, there were only 8 studies where the association between bidi smoking and lung cancer had been specially analyzed. A review of this study is presented.

The first study was done by Notani and Sanghvi (*15*). This retrospective study of 520 patients with lung cancer conducted at Tata Memorial Hospital, Bombay revealed the relative risk of all types of smokers to nonsmokers was 2.45, of cigarette smokers 2.23 and bidi smokers 2.65.

In another study by Notani *et al*. (*16*), the relative risks for bidi and cigarette smoking were assessed from analysis of 683 male lung cancer cases and 1,279 male non-cancer patients from Mumbai. The relative risk of 3.38 for bidis was higher than the 2.36 relative risk for cigarette smoking, compared to non-smokers.

In another retrospective study by Jussawalla *et al*. (*17*), 792 male lung cancer patients for whom detailed smoking history is available, were matched for age, and community with randomly selected controls. All smokers appear to be at high risk (16.8) compared with non-smokers. The relative risk in bidi smokers of 19.3 was, however, even higher than in cigarette smokers.

Prasad *et al*. (*18*) from Lucknow conducted a hospital-based case-control study comparing 52 cases of lung cancer with 156 healthy controls. They were able to demonstrate that bidi smokers had an odds ratio of 5.05 (2.21-11.7). The authors were also able to show a significant length of time smoking and dose-relationship between bidi smoking and lung cancer. The risk of lung cancer in bidi smokers of 11-20 bidis per day was seven times that of nonsmokers (7.06; 5.41-11.24) while that of smokers of more than 20 bidis per day, the risk was more than 10 times (10.60; 6.82-15.36). The effect of increasing duration of bidi smoking is reflected by the increasing odds ratios from 3.43 (1.62-6.84) for bidi smokers of 1-39 years duration to 14.24 (8.34-24.31) that of bidi smokers of more than 40 years.

Gupta *et al*. (*19*) from Chandigarh conducted a case-control comparing 265 cases of lung cancer with 525 matched controls from 1995 to 1997. The odds ratio for bidi smoking was 5.76 (3.42-9.70) while that for cigarette smoking was 3.86 (2.11-7.06).

A study from Bhopal (*20*) revealed that the risk of developing lung cancer was higher for bidi smokers when compared to cigarette smokers. The odds ratio for bidi smoking was 11.6 (6.4-21.3) while that for cigarette smoker was 7.7 (3.2-18.4). The authors were also able to demonstrate that the risk of lung cancer increases with the increase in duration of cigarette smokers.

Gajalakshmi *et al*. (*21*) from Chennai and Thirvananthapuram conducted a case-control study comparing 778 lung cancer cases with 3,430 controls. The odds ratios were 4.54 (2.96-6.95) and 6.45 (4.38-9.50) for more than 30 years of exclusive cigarette smoking and exclusive bidi smoking, respectively. The study also concluded that the lung cancer risk of former cigarette smokers drops more quickly after they quit smoking than it does for former bidi smokers.

In a study from Kolkata (*22*), 217 new patients with lung and larynx cancer along with 200 matched controls were recruited. Adjusted odds ratios observed for smokers for a duration of more than 40 years of smoking and smoking more than 40 bidi/cigarettes per day were 4.3 and 3.9, respectively. This study did not analyze the odds ratio of bidi smoking separately.

4. Why bidi is more hazardous than cigarettes

Although bidi contains about one-fourth the quantity of tobacco as a cigarette, the mainstream smoke of bidi contains a much higher concentration of several toxic agents including hydrogen cyanide, carbon monoxide, ammonia, other volatile phenols, and carcinogenic hydrocarbons such as benz(a)anthracene and benzopyrene (*23*). Bidis typically deliver 3-5 times more nicotine, tar and carbon monoxide as compared to conventional cigarettes (*24*). It has been reported that bidi contains 1.5 times more carcinogenic hydrocarbons than American cigarettes (*25*). The relatively low combustibility and non-porous nature of the tendu leaves require more frequent and deeper puffs by the smokers to keep bidi lit, and it is therefore harder on the smoker's lungs than cigarettes rolled in paper (*26*). Bidi smokers were found to take almost five puffs per minute compared to cigarette smokers who smoked two puffs per minute (*23*). All these facts are responsible for a greater deleterious effect of bidi in comparison to cigarettes.

5. Conclusion

A review of the literature strongly suggests that bidi smoking should be considered an important risk factor for the development of lung cancer. All eight studies on bidi smoking and lung cancer demonstrated a direct relationship and seven studies showed that bidi smoking is more hazardous than cigarette smoking. Two studies also demonstrated that the rate of developing lung cancer increases was directly proportional to the duration of smoking. One study showed that lung cancer risk of former cigarette smokers drops more quickly after they quit smoking than it does for former bidi smokers.

It can be concluded that bidi smoking also poses a very high risk of lung cancer. Traditionally, tobacco control programs have focused on reducing cigarette consumption. Effective strategies are now needed to expand the focus of tobacco control programs to all types of tobacco use, including bidis (*27,28*). Countries that adopted comprehensive tobacco control programs

with a mix of interventions (including bans on tobacco advertising, strong warnings on packages, controls on the use of tobacco in indoor locations, high taxes on tobacco products, and health education and smoking cessation programs) have had considerable success in decreasing the prevalence of cigarette smoking (*29*). A similar policy framework with a mix of interventions will have to be implemented to control bidi use in India and other Southeast Asian countries where bidi use is prevalent, as well as in countries like USA where the bidi market is relatively new and expanding.

References

1. Behera D. Managing lung cancer in developing countries: difficult and solutions. Indian J Chest Dis Allied Sci. 2006; 48:243-244.
2. Viswanathan R, Gupta S, Iyer PVK. Incidence of primary lung cancer in India. Thorax. 1962; 17:73-76.
3. National Cancer Registry Programme. An epidemiological study. Indian Council of Medical Research, Biennial Report, 1988-1989. New Delhi: ICMR; pp. 3-42.
4. National Cancer Registry Programme. Consolidated Report of the Population-based Cancer Registries 1990-1996. New Delhi: Indian Council of Medical Research. 2002.
5. Nandkumar A, Gupta PC, Gangadharan P, Visweswara RN. Development of an Atlas of Cancer in India: First All India Report 2001-2002. National Cancer Registry Programme 2001-2004. Bangalore; Indian Council of Medical Research. 2004.
6. Prasad R. Bidi smoking and bronchogenic carcinoma in India. JAMS. 2003; 14:7-10.
7. Chaudhry K, Rath GK. Multisectoral and intersectoral approach to national tobacco control. Paper commissioned by the World Health Organization on the occasion of the WHO International Conference on Global Tobacco Control Law: Towards a WHO Framework Convention on Tobacco Control. 2000 Jan 7-9, New Delhi, India.
8. Celebucki C, Turner-Bowker DM, Connolly G, Koh HK. Bidi use among urban youth – Massachusetts. MMWR. 1999; 48:796-799.
9. Centers for Disease Control and Prevention. Tobacco use among middle and high school students – United States 1999; MMWR. 2000; 49-53.
10. Indian hand-rolled cigarettes outpacing Sri Lankan brands. Hoover's online. 2001 Aug 31 [cited 2004 Jun 5]. Available from: *http://news.globalink.org 119354.shtml.*
11. Ansari KM. French women blow hot with Indian bidi. Hindustan Times, 2003 Nov 10 [cited 2004 Jun 5]. Available from: *http://news.globalink.org 220832.shtml.*
12. Barbara McLintock/The Province. Liberals move to ban bidis. National newspapercanada.com network. 2003 Feb 13 [cited 2004 Jun 5]. Available from: *http://news.globalink.org/157122.shtml.*
13. Australian customs seize smuggled cigarettes from India. Wall Street Journal 2001 Jul 17 [cited 2004 Jun 5]. Available from: *http://news.globalink.org 116019.shtml.*
14. Gupta PC, Asma S (eds.) Bidi smoking and Public Health, New Delhi: Ministry of Health and Family Welfare, Government of India, 2008.
15. Notani P, Sanghvi LD. A retrospective study of lung cancer in Bombay. Br J Cancer. 1974; 29:477-482.
16. Notani PN, Rao DN, Sirsat MV, Sanghvi LD. A study of lung cancer in relation to bidi smoking in different religious communities in Bombay. Indian J Cancer. 1977; 14:115-121.
17. Jussawalla DJ, Jain DK. Lung cancer in Greater Bombay: correlations with religion and smoking habits. Br J Cancer. 1979; 40:437-448.
18. Prasad R, Tandon S, Kumar S, Pant MC, Sinha KN, Mukerji PK. A case control study on tobacco smoking and lung cancer. Lung India. 1998; 16:60-64.
19. Gupta D, Boffetta P, Gaborieau V, Jindal SK. Risk factors of lung cancer in Chandigarh, India. Indian J Med Res. 2001; 113:142-150.
20. Dikshit RP, Kanhere S. Tobacco habits and risk of lung, oropharyngeal and oral cavity cancer: a population-based case-control study in Bhopal, India. Int J Epidemiol. 2000; 29:609-614.
21. Gajalakshmi V, Hung RJ, Mathew A, Varghese C, Brennan PK. Tobacco smoking and chewing, alcohol drinking and lung cancer risk among men in southern India. Int J Cancer. 2003; 107:441-447.
22. Roychowdhury S, Roychowdhury G, Sen U. Assessment of awareness level on tobacco and smoking habits as risk factors for cancer among lung and laryngeal cancer patients in Kolkata-a case control study. Asian Pac J Cancer Prev. 2005; 6:332-336.
23. Jayant K, Pakhale SS. Toxic constituents in bidi smoke. In: Sanghavi LD, Notani P (eds). Tobacco and Health: The Indian Scene. Bombay: Tata Memorial Centre 1989; pp. 101-110.
24. Rickert WS. Determination of yields of "tar", nicotine and carbon monoxide from Bidi, cigarettes: final report. Ontario, Canada: Labstat International, Inc., 1999.
25. Hoffman D, Sanghvi LD, Wynder EL. Comparative chemical analysis of Indian bidi and American cigarette smoke. Int J Cancer. 1974; 14:49-55.
26. Bhonsle RD, Murti PR, Gupta PC. Tobacco habit in India. In: Gupta PC, Hammer JE, Murti PR, editors. Control of tobacco related cancers and other disease. Proceedings of an International Symposium; 1990 Jan 15-19; Bombay, India. Bombay: Oxford University Press. 1992; pp. 25-46.
27. The Global Youth Tobacco Survey Collaborative Group. Differences in worldwide tobacco use by gender: findings from the Global Youth Tobacco Survey. J Sch Health. 2003; 73:207-215.
28. The Global Youth Tobacco Survey Collaborative Group (US Centers for Disease Control and Prevention; the World Health Organization, the Canadian Public Health Association, and the U.S. National Cancer Institute). Tobacco use among youth: a cross country comparison. Tobacco Control. 2002; 11:252-270.
29. World Health Organization. Reducing risks, promoting healthy life. In: The World Health Report, 2002. Geneva, 2002; pp. 225.

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Brief Report

Characteristics of reporting diabetes mellitus research results in Japanese newspapers

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Summary **This study aims to characterize the coverage of research on diabetes mellitus by Japanese newspapers. Newspaper articles with the key words diabetes mellitus, diabetes disease, or blood sugar in the headline were selected from four major Japanese newspapers for the period 1988-2007 using the ELNET database and coded by two researchers. Of 152 newspaper articles examined, 92 (60.5%) were based on journal articles, and published in English. The remaining 60 (39.5 %) were based on academic meetings, of which 51 (85.0%) were conducted in Japan. Seventy-two articles covered non-human studies (47.4%), but only 26 used a word such as animal, mouse, or cell in the headline to describe the study subjects. Publications in countries where the native language is not English may have language and geographical barriers that affect the reporting of research results.**

Keywords: Health information, mass media, communication, newspapers, Japanese

1. Introduction

With increase of the interest in evidence-based medicine, both health care professionals and journalists who write health-related articles need to have appropriate health literacy (*1*). This is because the mass media are an important source of information for the general public, who may use the results of medical research as evidence to decide their health care practices (*2*). However, most journalists lack training in assessing the validity of evidence bearing on research, and in translating the results for the general public. In addition to lack of training, competition and commercialism, limited newspaper column space, and a shortage of time have been also identified as obstacles by journalists (*3,4*).

Several researchers have studied the reporting of medical research by newspapers. Their main interests are the quality of reporting and the method of selection of research articles. Regarding the former, inaccuracy and misleading statements are important issues (*5-7*). It is true that some newspaper articles accurately convey the results of scientific journal articles. However, journalists generally prefer sensationalism to scientific objectivity in reporting scientific articles (*8-11*).

The other main interest is how journal articles are selected for news coverage. Medical journal press releases may affect the selection process of medical articles (*12-15*). Bartlett *et al*. reported that the selective process introduced by newspaper journalists was stronger than that operating in the issuing of press releases (*13*). Thus, journalists assume important roles in the process of selecting medical articles. In addition to selection bias of scientific articles, it has been pointed out that the mass media fail to capture disparities in health information availability. Minorities and individuals living in rural communities are subject to disadvantages (*16*). The language issue is an especially important cause for disparities in accessibility of health information. As the English language is used in most academic research, English itself might be an obstacle for non-English speaking journalists. However, it is not

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well known how research results are covered in mass media in non-English countries like Japan and other Asian countries.

This study examined how Japanese newspapers in report medical research. Diabetes mellitus is one of the most common diseases in Japan, yet only a handful of peer-reviewed publications have examined the coverage of diabetes by the mass media (*17*).

2. Methods

We searched for newspaper articles using the ELNET database (*http://www.elnet.co.jp/index.html*), which is the largest electronic database of news articles in Japan. We searched the headlines of four national Japanese newspapers (Asahi, Yomiuri, Mainichi, and Nihon Keizai) for the period January 1988-May 2007 using the keywords: diabetes mellitus, diabetes disease, and blood sugar. We selected articles based on publications in scientific journals, and based on scientific meetings, separately.

The following characteristics of the selected articles were coded: *i*) demographics of the newspaper articles (date published, newspaper company, morning or evening paper, page, article length expressed as the numbers of letters and paragraphs, whether the article contained photographs, tables, or figures, and whether the same article was reported in other newspapers); *ii*) characteristics of the research reported (whether the journal paper/meeting was published/held in Japan or another country, and whether the research subjects included Japanese people); and *iii*) basic study facts and newspaper reporting style based on the methods of Woloshin and Schwartz (*18*) (research topic of type 1 or type 2 diabetes; animal, cell, or human study subjects, if human subjects, the study design and study size, if animal or cell subjects, whether the headline included the words 'animal' or 'cell' when the news report was with regard to a non-human study, and whether the limitations of the study were noted).

Coding was performed independently by two researchers. When there were discrepancies, we discussed the issue until a consensus was reached. Article characteristics were analyzed as frequencies, cross-tabulations, and chi-square tables using SPSS version 13 for Windows.

3. Results

3.1. *Demographics of the newspaper articles*

The keyword searches resulted in 1,073 articles. Of these, 152 (14.2%) articles covering scientific works published in professional journals or presented at scientific meetings were selected manually. The number of articles selected from each newspaper was 47 (30.9%) from Asahi, 42 (27.6%) from Yomiuri, 32 (21.1%) from Nihon Keizai, and 31 (20.4%) from Mainichi. The percentage of articles selected out of all available articles published in each newspaper was 24.4% (47/193), 13.2% (42/318), 9.3% (32/344), and 14.2% (31/218), respectively. The number of articles reported in any 1 year was < 10 until 2000 (except in 1992, when 10 articles were reported). The number of articles tended to increase after 2001 (18 articles were reported in 2002). The number of articles that appeared in morning papers and in evening papers was 87 (57.2%) and 65 (42.8%), respectively. The median number of letters and paragraphs in the articles was 487 (25% = 397.3, 75% = 606) and four (25% = 3, 75% = 6), respectively. Photographs, tables, or figures were included in 17 of the 152 (11.2%) articles.

Four articles appeared on the front page, three of which covered research published in journals (*The American Journal of Human Genetics*, *Nature Medicine*, and *Nature*), and one of which covered a scientific meeting (*The Japanese Diabetes Society*). All articles but the one published in *The American Journal of Human Genetics* involved animal studies. The median number of letters and paragraphs for front-page articles was 752 (25% = 507, 75% = 1,141), and 6 (25% = 3.3, 75% = 10.3).

Four of the research studies/meetings were reported by three newspapers simultaneously, and 13 by two newspapers. Only one study was reported by all four newspapers; it was originally published in the journal *Nature Medicine* (2002, Vol. 8, pp. 1288-1295). The study, which was conducted by a Japanese research group, was titled "Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase." All newspaper articles regarding this study appeared in morning papers on 31 July 2001.

3.2. *Characteristics of the research reported in the newspaper articles*

Of the 152 articles, 60 (39.5%) cited presentations at academic meetings, whereas 92 (60.5%) cited findings published in scientific journals. No (0.0%) Japanese scientific journals were cited, whereas 51 (85.0%) academic meetings conducted in Japan were cited ($\chi^2(1) = 117.1$, $p < 0.01$). The major meetings covered were *the Japan Diabetic Society* ($n = 10$), *the American Diabetes Association* ($n = 5$), and *the Japan Society for Transplantation* ($n = 4$). The major journals covered were *Nature Medicine* ($n = 18$), *Nature Genetics* ($n = 10$), *Nature* ($n = 9$), and *Science* ($n = 9$). The name of one (1.7%, 1/60) article from a meeting and 16 (17.4%, 16/92) articles from scientific journals were not clearly identified.

Japanese research was covered more frequently than was foreign research (meetings: 54/60, 90.0%; journals: 25/64, 72.8%). Furthermore, the names of

Japanese researchers (107/121, 88.4%) were more often mentioned than those of foreign researchers (11/31, 35.5%; $\chi^2(1) = 39.8$, $p < 0.001$).

3.3. *Basic study facts and newspaper reporting style*

In general, newspapers tended to cover more articles on type 2 diabetes (22.4%, 34/152) than on type 1 diabetes (5.9%, 9/152). However, more than half of the articles (69.7%, 106/152) did not mention the specific type. Three articles mentioned both types.

Nearly half of the articles (46.1%, 70/152) covered studies of humans (non-human: 47.4%, 72/152; not stated: 6.6%, 9/152). Human studies had a variety of study designs such as case studies (20.0%, 14/70), cross-sectional studies (34.3%, 24/70), cohort studies (35.7%, 25/70), and controlled trials (5.7%, 4/70); three designs were undetermined. The median study size was 1,275.5 subjects (25% = 42, 75% = 14,525). Of the 72 newspaper articles that covered studies of non-humans, 36.1% (26/72) used a word such as animal, mouse, or cell to describe the study subjects in the headline.

Important limitations regarding the study design were rarely noted (19.1%, 29/152). Although articles that covered non-human studies tended to report limitations more than those that covered human studies, the difference was not significant (non-human: 22.2%, 16/72; human: 14.3%, 10/70; $\chi^2(4) = 3.7$, *ns*).

4. Discussion

We analyzed how four major Japanese newspapers reported the results of research on diabetes. Many of our results can be linked to the fact that Japan is not an English-speaking country.

Newspaper articles that covered journal publications focused more on research published in Western countries, whereas most articles that covered scientific meetings focused on meetings conducted in Japan. These results clearly show selection bias. Press releases by scientific journals are not popular in Japan because Japanese journalists cannot easily obtain information from Japanese scientific journals. In addition, Japanese journalists might consider international journals superior to Japanese ones.

Whereas all newspaper articles that reported findings published in scientific journals cited international journals, almost all articles that referred to scientific meetings (85.0%) were based on meetings conducted in Japan. This is because Japanese journalists attend these meetings and collect the news themselves (*19*). This may be because of both a language barrier and a geographical barrier in that it may be difficult for Japanese journalists to attend or gather news from scientific meetings conducted abroad. To confirm these speculations, interviews with Japanese journalists are necessary.

We also found that journalists sought newsworthy medical research that they felt would be appealing to readers. They preferred breakthrough stories such as new medical scientific developments, as well as discoveries by domestic researchers (*1,9*). Our results agree with those of previous studies that indicated that research involving Japanese researchers was frequently covered in Japanese newspaper articles and that Japanese researchers' names were mentioned more often than those of foreign researchers. More interestingly, we found that the majority of newspaper articles (and all front page stories) reported on non-human studies. Overall, there were significant gaps between the basic findings and the application of those findings. It is important for the general public to know that there is a tendency for journalists of the mass media to value sensational stories more than information that is practical to human lifestyles. Without such awareness, the general public may be misled about diabetes.

Few articles mentioned the limitations of the studies. Such reporting may give a favorable impression of the findings to the general public, but may also lead to misinterpretations regarding how research results are used in practice. In medical journals, structured abstracts that facilitate the communication of the contents of research articles in a limited number of words are popular, and these often include a statement of the study's limitations (*20*). Previous studies have also emphasized the importance of describing in media reports the limitations of studies (*18*).

Not all news articles that reported on non-human studies made this clear in the headline. Although further studies are needed to confirm how readers interpret the reported research, our results suggest that articles that cover research should be read critically and carefully. In addition, it is necessary to educate journalists on how to cover medical research, because we found 17 articles that did not clearly mention their sources.

Our study has a few limitations. First, we did not know the total number of studies reported or published in scientific meetings and journals between 1988 and 2007. Second, we may have overlooked articles that did not include words such as journal, meeting, or conference. Despite these limitations, our results indicate some important findings about research reporting in newspapers published in a non-English-speaking country. Further studies are necessary to confirm our findings.

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References

1 Entwistle VA, Watt IS. Judging journalism: how should the quality of news reporting about clinical interventions

be assessed and improved? QHC. 1999; 8:172-176.
2 Brunt ME, Murray MD, Hui SL, Kesterson J, Perkins AJ, Tierney WM. Mass media release of medical research results: an analysis of antihypertensive drug prescribing in the aftermath of the calcium channel blocker scare of March 1995. J Gen Inter Med. 2003; 18:84-94.
3 Entwistle V. Reporting research in medical journals and newspapers. BMJ. 1995; 310:920-923.
4 Larsson A, Oxman AD, Carling C, Herrin J. Medical messages in the media – barriers and solutions to improving medical journalism. Health Expect. 2003; 6:323-331.
5 Oxman AD, Guyatt GH, Cook DJ, Jaeschke R, Heddle N, Keller J. An index of scientific quality for health reports in the lay press. J Clin Epidemiol. 1993; 46:987-1001.
6 Molnar FJ, Man-Son-Hing M, Dalziel WB, Mitchell SL, Power BE, Byszewski AM, St John P. Assessing the quality of newspaper medical advice columns for elderly readers. CMAJ. 1999; 161:393-395.
7 Petersen A. Biofantasies: genetics and medicine in the print news media. Soc Sci Med. 2001; 52:1255-1268.
8 Bubela TM, Caulfield TA. Do the print media "hype" genetic research? A comparison of newspaper stories and peer-reviewed research papers. CMAJ. 2004; 170:1399-1407.
9 Shuchman M, Wilkes MS. Medical scientists and health news reporting: a case of miscommunication. Ann Intern Med. 1997; 126:976-982.
10 Ransohoff DF, Ransohoff RM. Sensationalism in the media: when scientists and journalists may be complicit collaborators. Eff Clin Pract. 2001; 4:185-188.
11 Cooper CP, Yukimura D. Science writers' reactions to a medical "breakthrough" story. Soc Sci Med. 2002; 54:1887-1896.
12 de Semir V, Ribas C, Revuelta G. Press releases of science journal articles and subsequent newspaper stories on the same topic. JAMA. 1998; 280:294-295.
13 Bartlett C, Sterne J, Egger M. What is newsworthy? Longitudinal study of the reporting of medical research in two British newspapers. BMJ. 2002; 325:81-84.
14 Stryker JE. Reporting medical information: effects of press releases and newsworthiness on medical journal articles' visibility in the news media. Prev Med. 2002; 35:519-530.
15 Woloshin S, Schwartz LM. Press releases: translating research into news. JAMA. 2002; 287:2856-2858.
16 Hoffman-Goetz L, Shannon C, Clarke JN. Chronic disease coverage in Canadian aboriginal newspapers. Health Commun. 2003; 8:475-488.
17 Rock M. Diabetes portrayals in North American print media: a qualitative and quantitative analysis. Am J Public Health. 2005; 95:1832-1838.
18 Woloshin S, Schwarts LM. Media reporting on research presented at scientific meetings: more caution needed. MJA. 2006; 184:576-580.
19 Japanese Association of Science & Technology Journalists. Journalism in science. Kagakudojin, Kyoto, Japan, 2004. (in Japanese)
20 Nakayama T, Hirai N, Yamazaki S, Naito M. Adoption of structured abstracts by general medical journals and format for a structured abstract. J Med Libr Assoc. 2005; 93:237-242.

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Original Article

Protective effect of anti-intercellular adhesion molecule-1 antibody on global cerebral ischemia/reperfusion injury in the rat

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Summary **The present study aimed to clarify the protective effect of administration of an anti-intercellular adhesion molecule-1 (ICAM-1) antibody (1A29) on neurological damage after global cerebral ischemia/reperfusion in rats. Global cerebral ischemia/reperfusion was produced by four-vessel occlusion for 30 min followed by reperfusion for 24 h. Animals were randomly divided into four groups: PC group (n = 10), PI group (n = 10), PR group (n = 10), and PM group (n = 10). Rats in the PC group were administered isotype-matched control antibody at a dose of 1 mg/kg IV. Rats in the PI group, PR group, and PM group were infused with 1A29 at a dose of 1 mg/kg IV before ischemia, upon reperfusion, and 4 h into reperfusion, respectively. All animals were sacrificed after reperfusion for 24 h. Cerebral sections were stained with hematoxylin and eosin for histological evaluation. The brain wet-to-dry ratio and neurological deficits were evaluated. In comparison to the PC group, the counts of polymorphonuclear leukocytes (PMNLs) and macrophages (MΦ) decreased significantly in the PI, PR, and PM groups ($P < 0.01$). In comparison to the control antibody group, the brain wet-to-dry ratio and the percent infarct volume were significantly reduced in rats receiving 1A29 antibody ($P < 0.05$ and $P < 0.01$, respectively). In comparison to the PC group, with a median neurological score of 2.5, mild deficits were noted in the PI, PR, and PM groups (median neurological scores were 1.6 to 1.8) ($P < 0.05$). 1A29 antibody decreased the counts of PMNLs and MΦ and the neurological score and it reduced the brain wet-to-dry ratio and the infarct volume, suggesting that anti-ICAM-1 antibody provides neuroprotection after global cerebral ischemia/reperfusion injury in rats.**

Keywords: Global cerebral, ischemia/reperfusion, intercellular adhesion molecule-1, antibody, rat

1. Introduction

Inflammatory response and oxidative stress are known to exacerbate the damage caused by acute cerebral ischemia/reperfusion injury (*1,2*). Cytokines formed immediately after ischemia stimulate the expression of adhesion molecules on endothelial cells and leukocytes, leading to leukocyte adherence and extravasation into brain parenchyma (*3,4*). Extravasated polymorphonuclear leukocytes (PMNLs) release reactive oxygen species and lipid peroxidation products and promote blood-brain barrier disruption, vascular plugging, edema, and cerebral infarction (*5,6*).

The migration of leukocytes into injured tissue is regulated in part by a specific cell-surface integrin known as the CD18 receptor complex (*7*). Intercellular adhesion molecule-1 (ICAM-1) is a cell surface glycoprotein that is expressed on vascular endothelium and other cells. ICAM-1 expression facilitates leukocyte adhesion to endothelium (*8*).

Previous studies demonstrated that drugs designed to inhibit recruited leukocytes/microglia markedly curtailed inflammation and oxidative stress-related apoptosis and consequently provided neuroprotection in cerebral I/R injury (*9,10*). Previous studies showed a significant decrease in middle cerebral artery

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occlusion (MCAO)-induced brain damage in ICAM-1 knockout mice and anti-ICAM-1 antibody-treated rats (*11*). Treatment with the anti-ICAM-1 antibody reduces neurological deficits after spinal cord injury and embolic stroke in the rabbit (*12*). In rodents, hypothermic neuroprotection against focal ischemia is associated with attenuation of ICAM-1 induction and PMNL infiltration (*13,14*). Thus, these data support the hypothesis that neutrophils contribute to ischemic cell damage and blocking of ICAM-1 expression reduces ischemic cell damage. However, the optimal point in time for antibody administration has yet to be determined. The present study evaluated the efficacy of anti-ICAM-1 antibody used at various times to prevent infarct development and neurological deficiency after global cerebral ischemia/reperfusion injury in adult rats.

2. Material and Methods

2.1. *Animals*

Sprague-Dawley rats (weight, 180 to 200 g; $n = 40$) used in this study were cared for in accordance with the NIH guidelines for the ethical use of laboratory animals. The Research Animal Resources and Care Committee of Nanjing University approved the surgical procedures. Animals fasted for 8 h before surgery and were allowed free access to water. All animals were anesthetized with ketamine (80 mg/kg, i.p.).

2.2. *Four-vessel occlusion model*

A four-vessel occlusion model as described earlier was used to induce global cerebral ischemia/reperfusion (*14*). Under ketamine anesthesia, a pin 0.5 mm in diameter was inserted through each alar foramen and both vertebral arteries were cauterized and permanently occluded. Through a ventral mid-cervical incision, each carotid artery was isolated and a 9-0-nylon ligature was looped around it. After 24 h, global brain ischemia/reperfusion was induced by traction on both carotid ligatures for 30 min and then loosening of both ligatures. During ischemia/reperfusion, body temperature (37°C to 38°C) and cranial temperature (36°C to 37°C) were maintained at the physiological level.

2.3. *Grouping*

Animals were randomly divided into four groups: PC group ($n = 10$), PI group ($n = 10$), PR group ($n = 10$), and PM group ($n = 10$). Rats in all groups were subjected to ischemia for 30 min and reperfusion for 24 h. Rats in the PC group were administered isotype-matched control antibody at a dose of 1 mg/kg IV. Rats in the PI group were infused with 1A29 at a dose of 1 mg/kg IV before ischemia. Rats in the PR group and PM group were infused with 1A29 at the same dosage upon reperfusion and 4 h into reperfusion, respectively. Antibody to rat ICAM-1, designated 1A29 (*15*), reacts with the 85- to 89-kD epitope present on cytokine-activated rat endothelial cells. The endotoxin level of the anti-ICAM-1 antibody is less than 0.35 eu/mg. The control antibody has an endotoxin level of less than 1.0 eu/mg.

2.4. *Determination of degrees of brain injury*

All rats were sacrificed after reperfusion for 24 h. Tissues of the right cerebrum were processed and embedded in paraffin, and 4 μm-thick paraffin sections were stained with hematoxylin-and-eosin for histopathological evaluation. Six random high-double views (magnification, ×100) were taken to count PMNLs and macrophages (M*Φ*).

The volume of the ischemic lesion was computed by the numeric integration of data from 12 to 14 sections with respect to the sectional interval, as described earlier (*16*). The infarct volume was corrected to account for edema and shrinkage due to processing. The injury volumes were corrected using the following formula: corrected injury volume = contralateral hemisphere volume – (ipsilateral hemisphere volume – measured injury volume). The indirect method for calculating infarct volume, in which the intact area of the ipsilateral hemisphere was subtracted from the area of the contralateral hemisphere, was used. The infarct volume is presented as the percentage of the infarct lesion of the contralateral hemisphere.

2.5. *Measurement of the brain wet-to-dry ratio*

After the rats were sacrificed, cerebral tissues of the left hemisphere were removed and immediately weighed. The cerebral tissues were dried in an oven at 80°C for 12 h and reweighed. The brain wet-to-dry ratios were obtained by dividing the mass of the initial specimen by the mass of the dried specimen.

2.6. *Neurological evaluation*

Global cerebral ischemia/reperfusion–induced neurological deficit was evaluated on a 6-point scale following 1 day of reperfusion (before the animals were sacrificed) by an investigator blinded to the study groups, as described earlier (*17*). A score of 0 suggests no neurological deficit (normal), 1 suggests a mild neurological deficit (*e.g.* failure to fully extend the right forepaw), 2 suggests a moderate neurological deficit (*e.g.* circling to the right), 3 suggests a severe neurological deficit (*e.g.* falling to the right), and 4 suggests a very severe neurological deficit (*e.g.* failing to walk spontaneously and having a reduced level of consciousness).

2.7. *Statistical analysis*

All values are presented as mean ± standard error. Statistical evaluation was performed with the use of ANOVA followed by an unpaired *t* test. Significance was indicated by $P < 0.05$, and a high level of significance was indicated by $P < 0.01$.

3. Results

3.1. *Infiltration of PMNLs and MΦ*

Table 1 shows the PMNLs and MΦ counts in the 1A29 groups and the groups treated with control antibody. In comparison to the PC group, the counts of PMNLs and MΦ decreased significantly in the PI, PR, and PM groups ($P < 0.01$). No significant difference in the counts of PMNLs and MΦ was detected among the 1A29 groups.

3.2. *Wet-to-dry ratio of injured cerebral tissue*

Disruption of the blood-brain barrier was assessed by measuring water and fluid content in the brain. In comparison to the control antibody group, the brain wet-to-dry ratio was significantly reduced in rats receiving 1A29 antibody ($P < 0.05$) (Figure 1).

3.3. *Determination of infarct volume*

Table 2 shows representative infarct areas in rats with control antibody and 1A29 infusion. Total infarct volume did not differ significantly among the groups. In comparison to the control antibody group, the percent infarct volume decreased significantly in the 1A29-treated group after global cerebral ischemia/reperfusion injury ($P < 0.01$).

3.4. *Effect of anti-ICAM-1 antibody on neurological deficits*

The neurological deficits analyzed at 24-h reperfusion were severe in the PC group, with a median neurological score of 2.5, compared with mild deficits in the PI, PR, and PM groups (median neurological scores were 1.6 to 1.8) (Figure 2).

Table 1. PMNLs and MΦ infiltration on cerebral in various groups

Groups	Infiltration[a,b]	
	PMNLs	MΦ
PC	12.3 ± 2.5	2.1 ± 0.4
PI	5.3 ± 2 .0*	1.2 ± 0.3*
PR	6.0 ± 1.8*	1.1 ± 0.6*
PM	7.2 ± 2.4*	1.4 ± 0.2*

[a] Data are represented as the mean ± SE; [b] Comparison with PC group, * $P < 0.01$.

Table 2. Absolute hemisphere and lesion volumes and percent lesion volume of the contralateral hemisphere in various groups

Groups	Volumes (mm^3)[a,b]		% Lesion volume[b]
	Hemisphere	Lesion	
PC	438.7 ± 16.4	265.9 ± 7.8	60.6 ± 6.5
PI	425.1 ± 21.3	156.5 ± 11.7*	36.8 ± 4.8*
PR	429.9 ± 28.5	144.2 ± 12.4*	33.5 ± 7.5*
PM	434.5 ± 18.8	168.7 ± 9.6*	38.8 ± 3.9*

[a] Data are represented as the mean ± SE; [b] Comparison with PC group, * $P < 0.01$.

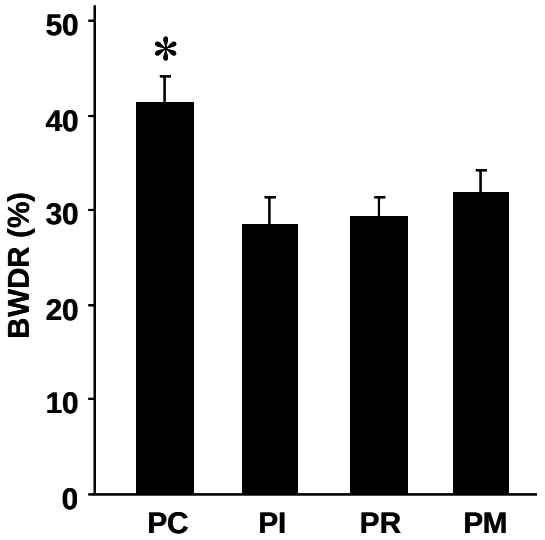

Figure 1. Brain water content in rats with global cerebral ischemia/reperfusion injury. Brain water content was determined by measuring the brain wet-to-dry ratio (BWDR) in rats with global cerebral ischemia/reperfusion injury. In comparison to the PC group, BWDR was significantly reduced in the PI, PR, and PM groups. * $P < 0.05$. Data were analyzed with the unpaired t test and are shown as mean ± SE.

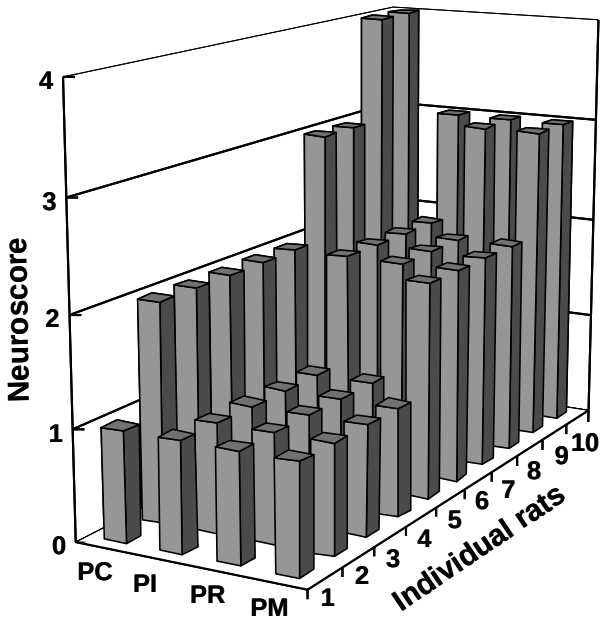

Figure 2. Individual neurological scores upon 24 h of reperfusion after global cerebral ischemia in rats with control antibody and rats with 1A29 infusion (n = 10 per group). In comparison to the PC group, with a median neurological score of 2.5, mild deficits were noted in the PI, PR, and PM groups (median neurological scores were 1.6 to 1.8). * $P < 0.05$.

4. Discussion

The current data indicate that intravenous administration of an anti-ICAM-1 antibody (1A29) significantly reduces the volume of infarcts and the brain wet-to-dry ratio and it decreases the subject's neurological score and the counts of PMNLs and MΦ. However, no significant difference in the above indices was observed among the 1A29 groups.

The protective effect of anti-ICAM-1 antibody against ischemic/reperfusion injury is attributed to the blockage of leukocyte adhesion, transendothelial migration, and improvement of blood flow during reperfusion (*18*). Ferulic acid provides neuroprotection against oxidative stress-related apoptosis after cerebral ischemia/reperfusion injury by inhibiting ICAM-1 mRNA expression in rats (*19*). Previous findings by the current authors revealed a new mechanism of hypothermia brain protection *via* inhibition of ICAM-1 expression and blocking of PMNL and MΦ infiltration in a rat global cerebral I/R injury model (*14)*. Studies have shown that prevention of ICAM-1 protein expression by antisense infusion significantly decreases transient focal ischemia–induced infarct size and neurological deficits (*20,21*). However, the choice of using antisense versus antibodies depends on the state of the patient. Antisense can be used as a preventive measure to bind to mRNA and inhibit ICAM-1 protein formation. Anti-ICAM-1 antibody may be more appropriate during ischemia/reperfusion injury by binding to ICAM-1 protein and halting its action.

The present study supports the contention that anti-ICAM-1 antibody administered either before ischemia or after reperfusion has a neuroprotective effect. The anti-ICAM-1 antibody is effective in reducing ischemic cell damage when administered during the reperfusion period and specifically 4 h after the initiation of reperfusion. This has positive implications for use of this form of therapeutic intervention in a clinical environment, where delayed intervention may be needed. Further study is required to determine the last point after reperfusion at which this therapy can be used.

In conclusion, the administration of the anti-ICAM-1 antibody significantly reduced global cerebral ischemic/reperfusion injury, regardless of whether it was before ischemia or after reperfusion.

References

1. Shin DH, Bae YC, Kim-Han JS, Lee JH, Choi IY, Son KH, Kang SS, Kim WK, Han BH. Polyphenol amentoflavone affords neuroprotection against neonatal hypoxic-ischemic brain damage *via* multiple mechanisms. J Neurochem. 2006; 96:561-572.
2. Iadecola C, Alexander M. Cerebral ischemia and inflammation. Curr Opin Neurol. 2001; 14:89-94.
3. Khan M, Elango C, Ansari MA, Singh I, Singh AK. Caffeic acid phenethyl ester reduces neurovascular inflammation and protects rat brain following transient focal cerebral ischemia. J Neurochem. 2007; 102:365-377.
4. Imai H, Graham DI, Masayasu H, Macrae IM. Antioxidant ebselen reduces oxidative damage in focal cerebral ischemia. Free Radic Biol Med. 2003; 34:56-63.
5. Zoppo GJ, Becker KJ, Hallenbeck JM. Inflammation after stroke: is it harmful? Arch Neurol. 2001; 58:669-672.
6. Ding Y, Young CN, Li J, Luan X, Clark JD, Diaz FG. Reduced inflammatory mediator expression by pre-reperfusion infusion into ischemic territory in rats: a real-time polymerase chain reaction analysis. Neurosci Lett. 2003; 353:173-176.
7. Caimi G, Canino B, Ferrara F, Montana M, Musso M, Porretto F, Carollo C, Catania A, Lo Presti R. Granulocyte integrins before and after activation in acute ischaemic stroke. J Neurol Sci. 2001; 186:23-26.
8. Arumugam TV, Salter JW, Chidlow JH, Ballantyne CM, Kevil CG, Granger DN. Contributions of LFA-1 and Mac-1 to brain injury and microvascular dysfunction induced by transient middle cerebral artery occlusion. Am J Physiol Heart Circ Physiol. 2004; 287: H2555-H2560.
9. Kao TK, Ou YC, Kuo JS, Chen WY, Liao SL, Wu CW, Chen CJ, Ling NN, Zhang YH, Peng WH. Neuroprotection by tetramethylpyrazine against ischemic brain injury in rats. Neurochem Int. 2006; 48:166-176.
10. Storini C, Rossi E, Marrella V, Distaso M, Veerhuis R, Vergani C, Bergamaschini L, De Simoni MG. C1-inhibitor protects against brain ischemia-reperfusion injury *via* inhibition of cell recruitment and inflammation. Neurobiol Dis. 2005; 19:10-17.
11. Frijns CJ, Kappelle LJ. Inflammatory cell adhesion molecules in ischemic cerebrovascular disease. Stroke. 2002; 33:2115-2122.
12. Zhang RL, Chopp M, Jiang N, Tang WX, Prostak J, Manning AM, Anderson DC. Anti-intercellular adhesion molecule-1 antibody reduces ischemic cell damage after transient but not permanent middle cerebral artery occlusion in the Wistar rat. Sroke. 1995; 26:1438-1442.
13. Wang GJ, Deng HY, Maier CM, Sun GH, Yenari MA. Mild hypothermia reduces ICAM-1 expression, neutrophil infiltration and microglia/monocyte accumulation following experimental stroke. Neuroscience. 2002; 114:1081-1090.
14. Gao JP,Xu JG, Li WY, Liu J. Influence of selective brain cooling on the expression of ICAM-1 mRNA and infiltration of PMNLs and monocytes/macrophages in rats suffering from global brain ischemia/reperfusion injury. BioScience Trends. 2008; 2:241-244.
15. Tamatani T, Miyasaka M. Identification of monoclonal antibody reactive with the rat homologue of ICAM-1, and evidence for differential involvement of ICAM-1 in the adherence of resting versus activated lymphocytes to high endothelial cells. Int Immunol. 1993; 2:166-172.
16. Raghavendra Rao VL, Dogan A, Bowen KK, Dempsey RJ. Ornithine decarboxylase knockdown exacerbates transient focal cerebral ischemia-induced neuronal damage in rat brain. J Cereb Blood Flow Metab. 2001; 21:945-954.
17. Rao VL, Dogan A, Todd KG, Bowen KK, Kim BT, Rothstein JD, Dempsey RJ. Antisense knockdown of the glial glutamate transporter GLT-1, but not the neuronal

glutamate transporter EAAC1, exacerbates transient focal cerebral ischemia-induced neuronal damage in rat brain. J Neurosci. 2001; 21:1876-1883.
18. Zhang RL, Chopp M, Li Y, Zaloga C, Jiang N, Jones ML, Miyasaka M, Ward PA. Anti-ICAM-1 antibody reduces ischemic cell damage after transient middle cerebral artery occlusion in the rat. Neurology. 1994; 44:1747-1751.
19. Chin YC, Shan YS, Nou YT, Tin YH, Su YC, Ching LH. Ferulic acid provides neuroprotection against oxidative stress-related apoptosis after cerebral ischemia/reperfusion injury by inhibiting ICAM-1 mRNA expression in rats. Brain Res. 2008; 1209:136-150.
20. Rao VL, Dogan A, Bowen KK, Todd KG, Dempsey RJ. Antisense knockdown of the glial glutamate transporter GLT-1 exacerbates hippocampal neuronal damage following traumatic injury to rat brain. Eur J Neurosci. 2001; 13:119-128.
21. Vemuganti R, Dempsey RJ, Bowen KK. Inhibition of intercellular adhesion molecule-1 protein expression by antisense oligonucleotides is neuroprotective after transient middle cerebral artery occlusion in rat. Stroke. 2004; 35:179-184.

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Original Article

Inhibitory effects of short hairpin RNA against caspase-8 on apoptosis of murine hepatoma Hepa1-6 cells

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Summary **Caspase-8 plays an important role in death-receptor-mediated apoptosis of hepatocytes. We constructed short hairpin RNAs (shRNAs) against caspase-8 and investigated the effects of caspase-8 targeting shRNAs on apoptosis of Hepa1-6 cells induced by TNF-α. Oligonucleotides coding for four shRNAs against caspase-8 were cloned into mammalian expression vector Pgenesil-1 containing U6 promoter, which were then introduced into Hepa1-6 cells using liposome-mediated transfection. Effects of caspase-8-shRNAs on apoptosis of Hepa1-6 cells induced by TNF-α were detected by PI apoptosis detection kit. Effects of caspase-8-shRNAs on caspase-8 mRNA expression in apoptosis Hepa1-6 cells induced by TNF-α were detected by real-time fluorescent RT-PCR. Of the four caspase-8-shRNAs, Pgenesil-caspase-8-1 and Pgenesil-caspase-8-2 were successfully constructed. The apoptosis of Hepa1-6 cells induced by TNF-α was significantly inhibited by either Pgenesil-caspase-8-1 or Pgenesil-caspase-8-2 ($p < 0.05$). Caspase-8 mRNA expression levels in apoptosis Hepa1-6 cells induced by TNF-α were significantly decreased by either Pgenesil-caspase-8-1 or Pgenesil-caspase-8-2 ($p < 0.05$). This study suggested that shRNAs against caspase-8 could effectively inhibit apoptosis of Hepa1-6 cells induced by TNF-α by suppressing caspase-8 mRNA expression.**

Keywords: Caspase-8, RNA interference, apoptosis, Hepa1-6 cells

1. Introduction

Acute liver failure (ALF) is a dramatic clinical syndrome with high mortality rates in which a previously normal liver fails within days or weeks. A major concern in ALF therapy is protection of hepatocytes to prevent apoptosis and maintain liver function (*1*). In ALF, signals released from the cell membrane of hepatocytes trigger suicide pathways, leading to the activation of caspase cascades that subsequently kill hepatocytes through apoptosis. Death receptors such as Fas trigger death signals when bound to their natural ligands, leading to recruitment of the adaptor protein, Fas-associated death domain, which in turn recruits caspase-8 zymogens to form death-inducing signaling complexes (DISCs) (*2,3*). Caspase-8 molecules become activated at DISCs and subsequently activate proapoptotic downstream molecules (*4*). Hence caspase-8, a downstream target of all known death receptors, may be a more suitable target than Fas to achieve future successful ALF therapy.

RNA interference (RNAi) is a potent gene silencing mechanism conserved in all eukaryotes, in which double-stranded RNAs suppress the expression of cognate genes by inducing degradation of mRNAs or by blocking translation of mRNAs (*5-7*). Short hairpin RNAs (shRNAs) are RNA sequences that makes a tight hairpin turn that can be used to silence gene expression *via* RNAi (*8,9*). Conditional gene silencing in mammalian cells, *via* the controlled expression of shRNAs, is an effective method for studying gene function, particularly if the gene is essential for cell survival or development (*10*). Also, efficient delivery of small interference RNAs (siRNAs) into hepatocytes

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in vivo and *in vitro* has already been reported (*11,12*). Since activation of caspase-8 is the central event in the hepatocyte apoptotic pathway, shRNA against caspase-8 might be useful to control the apoptosis of hepatocytes. In this study, we constructed shRNA eukaryotic expression vector for caspase-8 and investigated the inhibitory effects of caspase-8-shRNAs on apoptosis of Hepa1-6 cells induced by tumor necrosis factor (TNF)-α.

2. Materials and Methods

2.1. *Materials*

The plasmid vector Pgenesil-1, which contains U6 promoter and the reporter gene of enhanced green fluorescence protein (EGFP), was purchased from Wuhan Genesil Biotechnology Co., Ltd., Wuhan, China. TRIzoL reagents and SuperScript™ First-Strand Synthesis System for RT-PCR were obtained from Invitrogen, Carlsbad, CA, USA. Magnetic bead plasmid extract kit, reverse transcription kit, restriction enzyme *BamH* I, *Hind* III, T4 DNA ligase, and Taq DNA polymerase were obtained from Promega, Madison, WI, USA. Annexin V-FITC Apoptosis Detection Kit was purchased from IMGENEX Corporation, CA, USA. DNA marker was obtained from Takara Bio, Shiga, Japan. Murine hepatoma Hepa1-6 cells were obtained from Mount Sinai Medical Center, Miami Beach, FL, USA.

2.2. *Construction of caspase-8-shRNAs*

Following the rules of Tuschl T (*13*), shRNAs were designed according to Mus caspase-8 sequence in the GenBank (NM_009812), which showed no homology to any other sequences by a blast search. Eight oligodeoxyribonucleotides (66 bp) encoding four shRNAs against caspase-8 were synthesized by Wuhan Genesil Biotechnology Co., Ltd., Wuhan, China. The shRNAs sequences are shown in Figure 1. The four DNA templates, named successively caspase-8-1 to caspase-8-4, contained *BamH* I and *Hind* III restriction sites which can ligate with Pgenesil-1 at the 5' extreme of positive-sense and antisense strands. The map of Pgenesil-1 vector containing the U6 promoter region is shown in Figure 2.

To construct caspase-8-shRNAs, two corresponding oligodeoxyribonucleotides encoding a shRNA were dissolved in annealing buffer, kept at 100°C for 5 min, and cooled gradually to room temperature to anneal. Pgenesil-1 was digested with *BamH* I and *Hind* III, blunt-ended with T4 DNA polymerase, and then ligated with the annealed oligodeoxyribonucleotides. Pgenesil-GAPDH and Pgenesil-HK were used as positive and negative controls, respectively. Then, 5 μL ligation mixtures (Pgenesil-caspase-8-1 to caspase-8-4) were transformed to *E. coli* DH5α. Positive kanamycin-resistant clones were selected and expanded. The recombinant plasmids were extracted according to the manufacturer's magnetic bead plasmid extract kit protocol and verified by *Sal* I digestion analysis. All the constructed plasmids were confirmed by DNA sequencing (Invitrogen).

2.3. *Transfection*

Hepa1-6 cells were transiently transfected using lipofectamine 2000 in accordance with the manufacture's protocol. In brief, cells at 80-90% confluence in a 6-well plate were incubated for 6 h with a mixture of 4 μg caspase-8-shRNAs or Pgenesil-GAPDH, or Pgenesil-HK, and 8 μL lipofectomine 2000 in serum-free DMEM medium (*14*). The transfection medium was then replaced with DMEM medium including 10% FCS. All transfections were performed in triplicate. The transfection efficiency was determined by calculating the rate of positive EGFP staining cells under fluorescence microscopy.

2.4. *Effect of caspase-8-shRNAs on apoptosis of Hepa1-6 cells induced by TNF-α*

To induce Hepa1-6 cell apoptosis, TNF-α was added to the cells to a final concentration of 20 ng/mL and normal saline was used as the negative control. Cells were then harvested and transfected with Pgenesil-caspase-8-1 and Pgenesil-caspase-8-2. The cells were then cultured for 48 h and apoptosis levels were detected with Annexin V-FITC Apoptosis Detection Kit using FACScan® flow cytometer (Becton Dickinson, San Jose, CA, USA) and WinMDI 2.8 software (Scripps Institute, La Jolla, CA, USA).

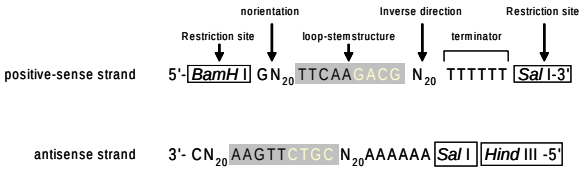

Figure 1. Structure of shRNAs oligonucleotide chains.

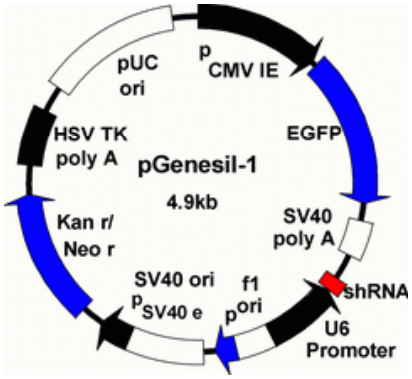

Figure 2. Map of Pgenesil-1 vector containing the U6 promoter region.

2.5. *Effects of caspase-8-shRNAs on caspase-8 mRNA expression levels in Hepa1-6 cells*

Levels of caspase-8 mRNA expression levels before and after caspase-8-shRNA treatment in apoptosis of Hepa1-6 cells induced by TNF-α were detected by quantitative RT-PCR (*15*). Briefly, after TNF-α was added to induce apoptosis, Hepa1-6 cells were transfected with caspase-8-shRNAs. Total RNA was isolated from Hepa1-6 cells using TRIzoL reagents according to the manufacturer's protocol. Isolated total RNA was first reverse transcribed into cDNA using random primers and SuperScript™ II reverse transcriptase. Then cDNA was used as the template in real-time RT-PCR. The sequences of the primers were as follows: caspase-8-primer, sense: 5'-GCC ACA GGG TCA TGC TCT TTA-3', antisense: 5'-TGC CAG CAT GGT CCT CTT CT-3'. beta-Actin-primer, sense: 5'-CAT CAT GAA GTG TGA CGT TGA CAT-3', antisense: 5'-GCT CAG GAG GAG CAA TGA TCT T-3'. Cycle threshold (Ct) values of samples were analyzed by Sequence Detection System software during the PCR procedure. Inhibition ratios were calculated by the following equation: inhibition ratio (shRNA on caspase-8 mRNA) = [1 – experiment group $2^{Ct\ (GAPDH)-Ct\ (caspase-8)}$/ control group $2^{Ct\ (GAPDH)-Ct\ (caspase-8)}$]·100.

2.6. *Statistical analysis*

SPSS 11.5 software was used for data analysis. The experimental results were recorded as mean ± S.D. Differences between group means were analyzed by *t* test. Differences were considered significant when $p < 0.05$.

3. Results

3.1. *Construction of caspase-8-shRNAs*

The multiclone sites of plasmid Pgenesil-1 were as follows: 5'-*Hind* III-ShRNA-*BamH* I-U6 Promoter-EcoRI-*Sal* I-XbaI-DraIII-3'. A *Sal* I site for plasmid Pgenesil was designed in the inserted fragments between the sites of *BamH* I and *Hind* III. Correct insertion was evaluated by a production of about 400 bp-band by *Sal* I digestion. As shown in Figure 3, digestion of the four recombinant DNA vectors with restriction endonucleases gave fragments at the expected position. However, by means of automated DNA sequencing, the insert sequence was verified in two shRNAs, Pgenesil-caspase-8-1 and Pgenesil-caspase-8-2, but not in the other two (data not shown).

3.2. *Transfection of caspase-8-shRNAs*

The transfection rates of Pgenesil-caspase-8-1 and Pgenesil-caspase-8-2 were evaluated under fluorescence microscopy. A typical example of EGFP positive cells is shown in Figure 4. The transfection rates were about 35%-40% according to the expression of EGFP in Hepa1-6 cells.

3.3. *Inhibition of Hepa1-6 cells apoptosis by caspase-8-shRNAs*

The effect of caspase-8-shRNAs on apoptosis of Hepa1-6 cells induced by TNF-α was examined (Figure 5). Apoptotic rates of Hepa1-6 cells were calculated using WinMDI 2.8 software. TNF-α successfully induced Hepa1-6 cells' apoptosis and the apoptotic rate was significantly increased from 1.20 ± 0.32% (panel *A*) to 17.40 ± 2.21% (panel *B*; $p < 0.05$). After the Pgenesil-caspase-8-1 or Pgenesil-caspase-8-2 treatment, apoptotic rates were significantly decreased from 17.40 ± 2.21% (panel *B*) to 4.70 ± 0.89% (panel *C*; $p < 0.05$) and 10.23 ± 2.56% (panel *D*; $p < 0.05$), respectively. This result suggests that Pgenesil-caspase-8-1 and Pgenesil-caspase-8-2 can significantly inhibit the apoptosis of Hepa1-6 cells induced by TNF-α. The inhibitory effect was more obvious in the Pgenesil-caspase-8-1 group than in the Pgenesil-caspase-8-2 group ($p < 0.05$).

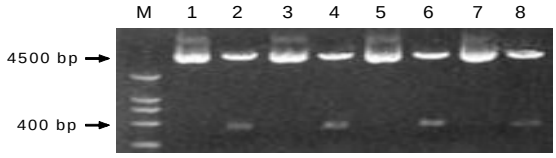

Figure 3. Restriction digestion analysis of eukaryotic recombinant vectors Pgenesil-caspase-8-1 through caspase-8-4. M, DNA marker; 1, 3, 5, and 7, negative control; 2, 4, 6, and 8, Pgenesil-caspase-8-1 through caspase-8-4, respectively.

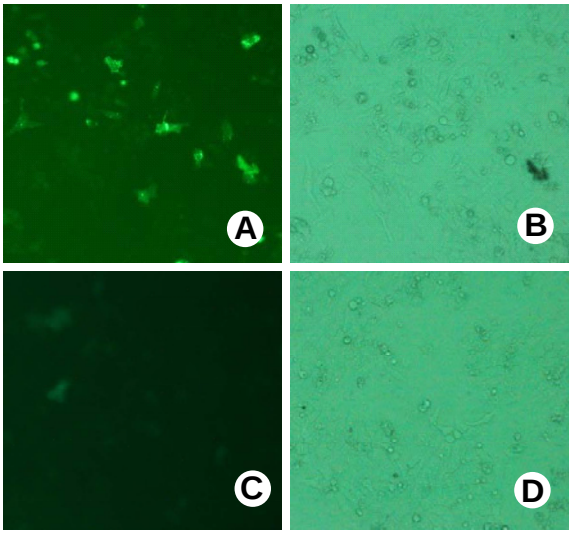

Figure 4. EGFP expression in Hepa1-6 cells transfected by eukaryotic expression vectors. A, Hepa1-6 cells transfected by Pgenesil-Caspase-8; B, Distribution of Hepa1-6 cells observed by light microscope in the same area as A; C, Hepa1-6 cells that were not transfected by Pgenesil-Caspase-8; D, Distribution of Hepa1-6 cells observed by light microscope in the same area as C. (×200)

3.4. *Inhibition of caspase-8 mRNA expression by caspase-8-shRNAs*

The effect of caspase-8-shRNAs on caspase-8 mRNA expression in Hepa1-6 cell apoptosis induced by TNF-α was measured by fluorescence RT-PCR. The results showed that the expression of caspase-8 mRNA was significantly increased from 0.050 ± 0.006 to 0.286 ± 0.063 ($p < 0.05$) after the treatment with TNF-α (Table 1). After transfection by Pgenesil-caspase-8-1 or Pgenesil-caspase-8-2, caspase-8 mRNA expression was significantly decreased to 0.098 ± 0.037 ($p < 0.01$) and 0.127 ± 0.043 ($p < 0.05$), respectively. The inhibitory effect was more obvious in the Pgenesil-caspase-8-1 group than in the Pgenesil-caspase-8-2 group ($p < 0.05$).

4. Discussion

Due to its high efficiency and specificity, RNAi is now being widely used as a method to knock down target genes, to study gene function, or to explore experimental treatments for certain diseases (*16-18*). The siRNAs can be produced by four different ways: chemical synthesis, *in vitro* transcription, enzymatic digestion of dsRNAs, and transfection of DNA vectors encoding siRNAs or shRNAs, which are converted to siRNAs in cells (*19*). Of the four ways, transfection of DNA vectors offers advantages in silencing longevity, delivery options, and cost. Since the central event in the hepatocyte apoptotic pathway is the proteolysis activation of caspase-8 (*20,21*), in the present study, we constructed four shRNAs against caspase-8 and against

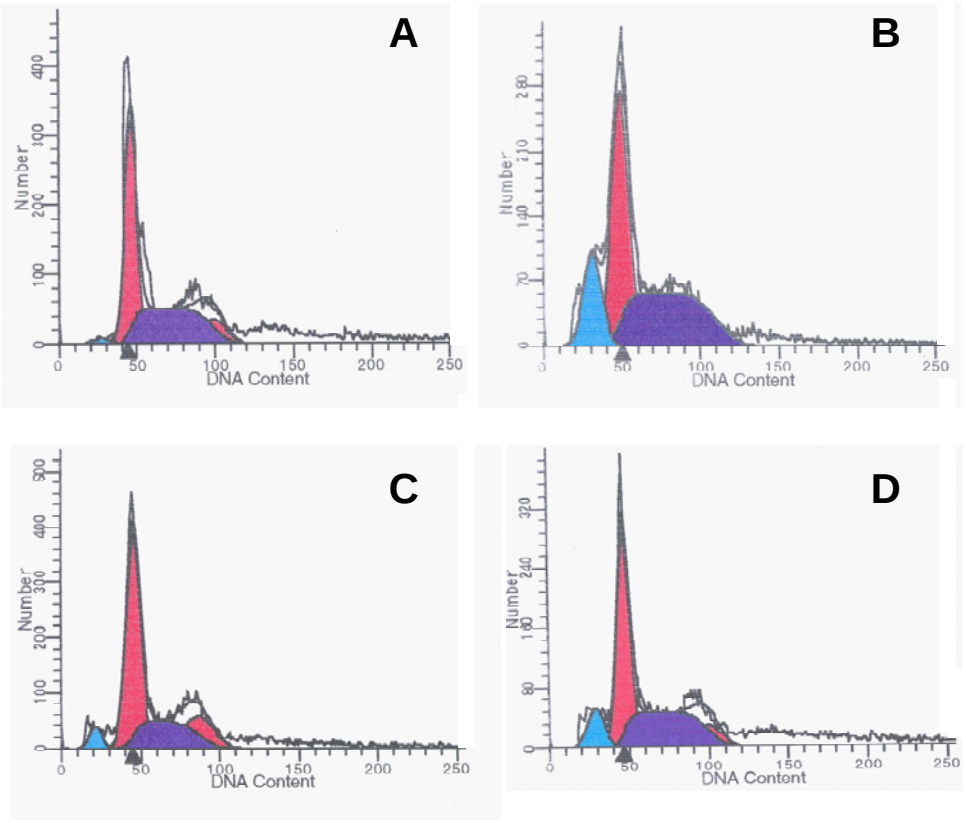

Figure 5. The effect of caspase-8-shRNAs on Hepa1-6 cells apoptosis induced by TNF-α. A, untreated Hepa1-6 cells; B, Hepa1-6 cells treated with TNF-α; C, Hepa1-6 cells treated with TNF-α followed by Pgenesil-caspase-8-1 transfection; D, Hepa1-6 cells treated with TNF-α followed by Pgenesil-caspase-8-2 transfection.

Table 1. Effects of caspase-8-shRNAs on caspase-8 mRNA expression levels in Hepa1-6 cells induced by TNF-α

Pre-treatment	Transfection	Caspase-8 mRNA levels (mean ± S.D.)	Inhibitory rates (%)
None	None	0.050 ± 0.006	–
TNF-α	None	0.286 ± 0.063[a]	–
TNF-α	Pgenesil-caspase-8-1	0.098 ± 0.037[b,d]	76
TNF-α	Pgenesil-caspase-8-2	0.127 ± 0.043[c]	52

[a] $p < 0.05$, compared with Hepa1-6 cells without TNF-α pre-treatment; [b] $p < 0.01$, [c] $p < 0.05$, compared with Hepa1-6 cells with TNF-α pre-treatment; [d] $p < 0.05$, compared with Hepa1-6 cells with Pgenesil-caspase-8-2 treatment.

Pgenesil-caspase-8-1 through caspase-8-4.

One problem with using siRNA to knock down gene expression is target sequence selection: siRNAs target different sites of the same gene, producing different effects varying from strong to no inhibition of gene expression. The mechanism of this selection is not well known. Therefore, the design of the most effective siRNAs is still empirical, although some principles have been put forward and some software products have been developed to facilitate the selection process (*22,23*). In the present study, four target sites were chosen according to these criteria, but only half of them turned out to be effective; these two effective shRNAs against caspase-8 were used in subsequent experiments.

Since TNF-α and TNF-related apoptosis-inducing ligand are important mediators of apoptosis in hepatocytes (*24*), we used TNF-α to induce apoptosis of mouse Hepa1-6 cells in this study. It was shown that TNF-α could significantly increase the apoptosis rate and caspase-8 mRNA expression of Hepa1-6 cells. After introducing the Pgenesil-caspase-8-1 or Pgenesil-caspase-8-2 into Hepa1-6 cells, both the apoptosis rate and caspase-8 mRNA expression were significantly decreased. This result suggests that caspase-8-shRNAs could effectively inhibit apoptosis of murine hepatoma Hepa1-6 cells induced by TNF-α. Further studies are needed to verify if the two shRNAs can also reduce caspase-8 protein levels efficiently and lead to growth arrest and/or apoptosis of hepatic cells *in vivo*.

In summary, this study suggested that the two shRNAs against caspase-8 could effectively inhibit apoptosis of Hepa1-6 cells induced by TNF-α by suppressing caspase-8 mRNA expression. The results of our study provide a basis for future research to utilize RNAi in induction of hepatocytes apoptosis.

References

1. Zender L, Hutker S, Liedtke C, Tillmann HL, Zender S, Mundt B, Waltemathe M, Gosling T, Flemming P, Malek NP, Trautwein C, Manns MP, Kuhnel F, Kubicka S. Caspase 8 small interfering RNA prevents acute liver failure in mice. PNAS. 2003; 100:7797-7802.
2. Kondo T, Suda T, Fukuyama H, Adachi M, Nagata S. Essential roles of the Fas ligand in the development of hepatitis. Nat Med. 1997; 3:409-413.
3. Kuhnel F, Zender L, Paul Y, Tietze MK, Trautwein C, Manns M, Kubicka S. NFkappaB mediates apoptosis through transcriptional activation of Fas (CD95) in adenoviral hepatitis. J Biol Chem. 2000; 275:6421-6427.
4. Mundt B, Kühnel F, Zender L, Paul Y, Tillmann H, Trautwein C, Manns MP, Kubicka S. Involvement of TRAIL and its receptors in viral hepatitis. FASEB J. 2003; 17:94-96.
5. Dykxhoorn DM, Novina CD, Sharp PA. Killing the messenger: short RNAs that silence gene expression. Nat Rev Mol Cell Biol. 2003; 4:457-467.
6. Downward J. RNA interference. BMJ. 2004; 328:1245-1248.
7. Agrawal N, Dasaradhi PV, Mohmmed A, Malhotra P, Bhatnagar RK, Mukherjee SK. RNA interference: biology, mechanism, and applications. Microbiol Mol Biol Rev. 2003; 67:657-685.
8. Hannon GJ, Rossi JJ. Unlocking the potential of the human genome with RNA interference. Nature. 2004; 431:371-378.
9. Tomari Y, Zamore PD. Perspective: machines for RNAi. Genes Dev. 2005; 19:517-529.
10. Fukuda Y, Kawasaki H, Taira K. Construction of microRNA-containing vectors for expression in mammalian cells. Methods Mol Biol. 2006; 338:167-173.
11. Ray R, Keyser B, Benton B, Daher A, Simbulan-Rosenthal CM, Rosenthal DS. Sulfur mustard induces apoptosis in cultured normal human airway epithelial cells: Evidence of a dominant caspase-8-mediated pathway and differential cellular responses. Drug Chem Toxicol. 2008; 31:137-148.
12. Thorpe JA, Christian PA, Schwarze SR. Proteasome inhibition blocks caspase-8 degradation and sensitizes prostate cancer cells to death receptor-mediated apoptosis. Prostate. 2008; 68:200-209.
13. Tuschl T. Expanding small RNA interference. Nat Biotechnol. 2002; 20:446-448.
14. Saito K, Meyer K, Warner R, Basu A, Ray RB, Ray R. Hepatitis C virus core protein inhibits tumor necrosis factor alpha-mediated apoptosis by a protective effect involving cellular FLICE inhibitory protein. J Virol. 2006; 80:4372-4379.
15. Lu D, Johnson C, Johnson S, Tazik S, Ou XM. The neuroprotective effect of antidepressant drug *via* inhibition of TIEG2-MAO B mediated cell death. Drug Discov Ther. 2008; 2:289-295.
16. Takei Y, Kadomatsu K, Yuzawa Y, Matsuo S, Muramatsu T. A small interfering RNA targeting vascular endothelial growth factor as cancer therapeutics. Cancer Res. 2004; 64:3365-3370.
17. Jacque JM, Triques K, Stevenson M. Modulation of HIV-1 replication by RNA interference. Nature. 2002; 418:435-438.
18. McCaffrey AP, Nakai H, Pandey K, Huang Z, Salazar FH, Xu H, Wieland SF, Marion PL, Kay MA. Inhibition of hepatitis B virus in mice by RNA interference. Nat Biotechnol. 2003; 21:639-644.
19. Guo Y, Liu J, Li YH, Song TB, Wu J, Zheng CX, Xue CF. Effect of vector-expressed shRNAs on hTERT expression. World J Gastroenterol. 2005; 11:2912-2915.
20. Saito F, Yokota H, Sudo Y, Yakabe Y, Takeyama H, Matsunaga T. Application of RNAi inducible TNFRI knockdown cells to the analysis of TNF-α-induced cytotoxicity. Toxicol In Vitro. 2006; 20:1343-1353.
21. Buck M, Chojkier M. C/EBPbeta associates with caspase 8 complex proteins and modulates apoptosis in hepatic stellate cells. J Clin Gastroenterol. 2007; 41:S295-S299.
22. Chalk AM, Wahlestedt C, Sonnhammer EL. Improved and automated prediction of effective siRNA. Biochem Biophys Res Commun. 2004; 319: 264-274.
23. Reynolds A, Leake D, Boee Q, Scaringe S, Marshall WS, Khvorova A. Rational siRNA design for RNA interference. Nat Biotechnol. 2004; 22:326-330.
24. Luedde T, Trautwein C. Intracellular survival pathways in the liver. Liver Int. 2006; 26:1163-1174.

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Original Article

Effects of gastrodin on the dopamine system of Tourette's syndrome rat models

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Summary **Gastrodin is used in traditional Chinese medicine to treat Tourette's syndrome (TS). This study evaluated the effects of gastrodin on the dopamine system. TS rat models were established by intraperitoneal injection of apomorphine. After intervention by gastrodin, stereotyped behaviors of TS rats were significantly inhibited and levels of homovanillic acid (HVA) were significantly increased. We conclude that gastrodin effectively inhibited stereotyped behaviors and controlled TS symptoms by promoting dopamine metabolism, thereby increasing levels of HVA in sera.**

Keywords: Gastrodin, Tourette's syndrome rat model, homovanillic acid, dopamine D2 receptor

1. Introduction

Tourette's syndrome (TS) is a neuropsychiatric disorder characterized by stereotypic, involuntary, purposeless, and repetitive movements. These motor tics include headshakes, violent clonic tics consisting of thrusting head jerks and orofacial tics such as facial grimacing, eye blinking, and throat clearing (*1*). The prevalence of this syndrome is estimated to be between four and six per 1,000 children and adolescents (*2*). Initial symptoms of TS often occur around the age of 7 years. It occurs three to four times more frequently in males than in females (*3*).

The pathophysiology and etiology of TS are unclear. It is widely believed that dopamine system abnormalities play a primary role in the pathophysiology of TS (*4*). Dopamine modulates striatal neuron activity by stimulating dopamine receptors (*5*). There are two families of dopamine receptors, called D1-like (DRD1) and D2-like (DRD2) receptors (*6*). DRD2 receptors have been found in increased densities in the frontal cortex of TS patients, compared to matched controls (*7*). Fachinetto's study showed that the densities of prefrontal DRD2 were greater than 140% of their matched control (*8*). Autopsies also indicate that DRD2 activity was increased in TS patients (*9*). As the main metabolite of dopamine in the central nervous system, homovanillic acid (HVA) and its levels are generally regarded as a major indicator of dopamine activity (*10*). HVA levels were obviously lower in the patients than in the control group (*11*). All these facts indicate that TS is associated with dopamine activity and DRD2 density and sensitivity in striatum.

Haloperidol is a U.S. Food and Drug Administration-approved treatment for the symptoms of TS and other tic disorders. It can selectively curb the activity of postsynaptic dopamine receptor and inhibit the excitability of cortical motor areas through restraining activity on the dopamine receptor, alleviating TS symptoms (*12*). Although haloperidol is efficacious for the treatment of TS, a very high proportion of patients eventually discontinue the therapy because of the side effects, which include sedation, weight gain, extrapyramidal symptoms, and prolongation on the electrocardiogram of the QT interval (*13*). Therefore, development of new drugs for treatment of TS is urgently needed.

Traditional Chinese medicine has been developed and refined by the Chinese people over the course

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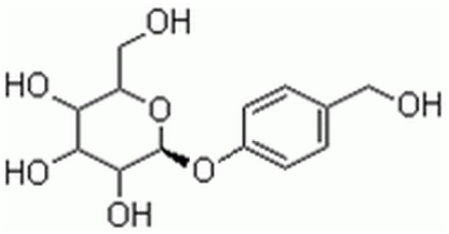

Figure 1. The chemical structure of gastrodin.

of thousands of years for use in the prevention and treatment of disease. Gastrodin (Gas, Figure 1), extracted from the Chinese herb *Gastrodia elata Blume*, has been shown to promote dopamine metabolism and to have obviously sedative, anticonvulsive, and antiepileptic properties (*14*). Our aim in this study was to explore the effect of gastrodin on TS and analyze the possible mechanisms.

2. Materials and Methods

2.1. *Materials*

Male wistar rats were purchased from Shandong Experimental Animal Center, Jinan, China. Apomorphine (Apo) was purchased from Sigma-Aldrich, St Louis, MO, USA. Haloperidol (Hal) was purchased from Shanghai Pharmaceutical Group Co., Ltd., Shanghai, China. Gastrodin was purchsed from Youcare Pharmaceutical Group Co., Ltd., Beijing, China. Trizol was purchased from Invitrogen, Carlsbad, CA, USA. RevertAid™ First Strand cDNA Synthesis Kit was purchased from Fermentas UAB, Vilnius, Lithuania, USA. TaKaRa TaqTM Hot Start Version was purchased from Takara Biotechnology (Dalian) Co., Ltd., Dalian, China, and Enzyme Immunoassay Kit from Adlitteram Diagnostic Laboratories, San Diego, CA, USA.

2.2. *Animals and experimental groups*

Rats (male, 4 weeks old, 100 ± 20 g) were housed in an air-conditioned animal room with a 12 h light/dark cycle, at a temperature of 22 ± 2°C and a humidity of 50 ± 10%. Rats were provided with a laboratory diet and water ad libitum, and maintained for 1 week before the start of the experiment. After 1 week, the rats were randomly divided into four groups: Control group (n = 16), Apo group (n = 16), Gas+Apo group (n = 16) and Hal+Apo group (n = 16). TS rat models were established by intraperitoneal injection of apomorphine (2 mg/kg) in rats in the Apo, Hal+Apo, and Gas+Apo group. Rats in the control group were intraperitoneally injected with normal saline (0.9%) (5 mL/kg). After injection, rats were intragastrically injected with gastrodin at 20 mg/kg (Gas+Apo group), haloperidol at 1.0 mg/kg (Hal+Apo), and normal saline (0.9%) at 10 mL/kg (control group and Apo group), respectively, once a day for 12 weeks. The rats' behaviors were observed by people who were familiar with the stereotypy actions, but were blind to the group. The stereotypy action included sniffing, body raising, licking, hyper-locomotion not associated with grooming, walking, eating, *etc*. Immediately following the treatment, the rats were observed for 1 min every 10 min for 60 min (a total of 6 observation periods). The standard was as follows (*15*): 0, asleep, resting or normal activity in place; 1, increased sniffing and head raising associated with hyper-locomotion; 2, discontinuous increased sniffing with body raising with hyper-locomotion; 3, discontinuous increased sniffing with head bobbing and body raising primarily in one place, with occasional rapid burst of locomotor activity (2-5 steps); 4, continuous sniffing, head bobbing and repetitive body raising/wall climbing; 5, continuous sniffing, licking, head bobbing, and continuous body raising/wall climbing. After 12 weeks, the rats were sacrificed. The blood was collected and striatum was isolated according to Paxinos and Watson's stereotaxic atlas of the rat brain (*16*). Animal handling for the experiments was in accordance with the American Physiological Society's "Guiding Principles in the Care and Use of Animals."

2.3. *HVA levels*

The levels of HVA in sera were measured by a sandwich enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions. Briefly, dispensed antigen standards and sera were added to each well of 96-well plates pre-coated with first antibodies. After adding Biotin Conjugate Reagent and Enzyme Conjugate Reagent into each well, the plates were incubated at 37°C for 60 min. Then the plates were rinsed 5 times with distilled water. After chromogenic reaction, the absorbance was measured at 405 nm by Microtiter plate reader within 30 min.

2.4. *DRD2 mRNA expression*

Expression of DRD2 mRNA in striatum in each rat was determined by reverse transcriptase-polymerase chain reaction (RT-PCR). The striatum samples were homogenized, and total RNA was extracted with Trizol reagent according to the manufacturer's instructions. The primers for DRD2 were forward primer 5'-CTG GTA ATG CCG TGG GTT-3' and reverse primer 5'-CAG GGT GGG TAC AGT TGC-3' (487 bp) (AJ347728). The primers for β-actin were forward primer 5'-CCT GTG GCA TCC ATG AAA CTAC-3' and reverse primer 5'-CTT CTG CAT CCT GTC AGC AAT-3' (134 bp) (NM031144). The PCR protocol consisted of an initial denaturation step at 94°C for 30 sec, followed by 30 cycles of amplification. For amplification of cDNA, the cycles consisted of denaturation at 94°C for 30 sec,

annealing at 58°C for 30 sec, and elongation at 72°C for 30 sec. For amplification of β-actin cDNA, the cycles included denaturation at 94°C for 30 sec, annealing at 56°C for 30 sec, and elongation at 72°C for 30 sec. To verify the specificity of amplification, PCR products were subjected to electrophoresis on a 1.5% agarose gel, which was then stained with ethidium bromide and examined with AlphaImager 2200 image analysis system (Alphaimager 2200 Pharmacia Biotech Co., San Francisco, CA, USA). β-Actin was used as an internal control in each sample.

2.5. *Statistical analysis*

Data were expressed as mean ± standard deviation. Statistical analysis was performed by one-way analysis of variance. All analyses were performed using the SPSS statistical software package (Version 13.0, SPSS Inc., Chicago, IL, USA), and $p < 0.05$ was considered statistically significant.

3. Results

3.1. *Behavior study*

As shown in Figure 2, treatment with gastrodin and haloperidol can inhibit the stereotyped behavior induced by apomorphine in TS rats. Apomorphine continued to induce stereotyped behavior without intervention and at the start of treatment ($p > 0.05$). Comparison of the three groups at the end of the experiment showed that the scores were conspicuously lower in the Gas+Apo group and the Hal+Apo group than in the Apo group ($p < 0.05$), while gastrodin had the same effect as haloperidol on stereotyped behavior.

3.2. *HVA levels in sera*

As shown in Figure 3, the HVA content was significantly lower in the Apo group than in the control group (3.49 ± 0.66 mg/mL *vs*. 4.76 ± 0.85 mg/mL, $p < 0.01$). The HVA content was significantly higher in the Gas+Apo group and the Hal+Apo group than in the Apo group (4.69 ± 1.30 mg/mL *vs*. 3.49 ± 0.66 mg/mL, 4.86 ± 1.48 mg/mL *vs*. 3.49 ± 0.66 mg/mL, $p < 0.05$). There was no significant difference between the Hal+Apo group and Gas+Apo group ($p > 0.05$).

3.3. *DRD2 mRNA expression*

Striatum DRD2 mRNA expression was analysed by RT-PCR. As shown in Figure 4, DRD2 mRNA expression in striatum of the Apo group was significantly increased compared with the control group ($p < 0.01$). In the Hal+Apo group, a significant decrease of DRD2 mRNA expression was observed compared with that of the Apo group ($p < 0.05$). Gastrodin had no effect on DRD2 mRNA expression ($p > 0.05$). DRD2 mRNA expression was significantly lower in the Hal+Apo group than in the Gas+Apo group ($p < 0.05$).

4. Discussion

Hyperfunction of the dopamine system plays an important role in the etiopathogenesis of TS. Dopamine

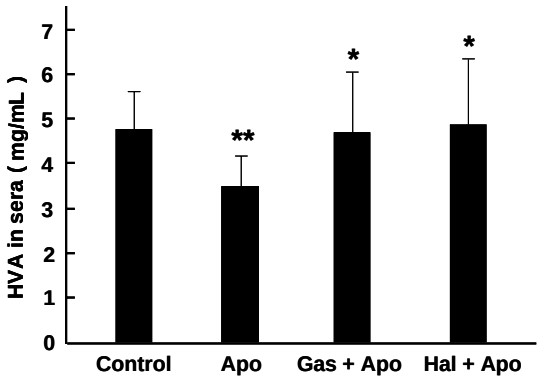

Figure 3. Effect of gastrodin on HVA levels in sera. Data are expressed as the mean ± S.D. ($n = 16$ rats/group). ** $p < 0.01$ versus the control group. * $p < 0.05$ versus the Apo group.

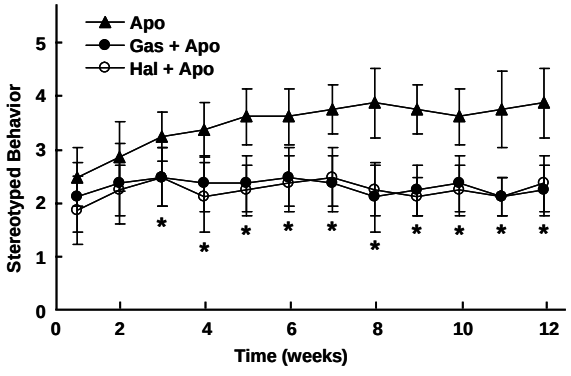

Figure 2. Stereotyped behaviors of rats in the three experimental groups during a 12-week period. The behaviors were recorded the last day of each week. Data were expressed as the mean ± S.D. ($n = 16$ rats/group). * $p < 0.05$ versus the Apo group.

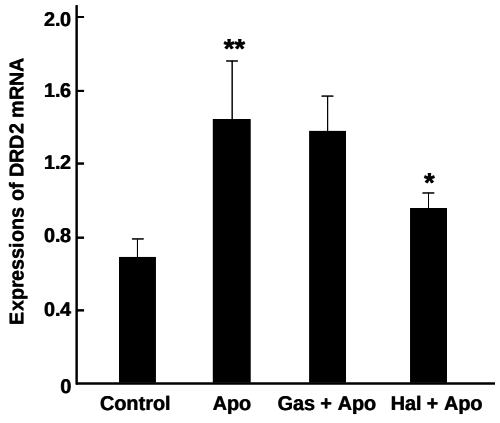

Figure 4. Effect of gastrodin on the expression of DRD2 mRNA in striatum. ** $p < 0.01$ versus the control group. * $p < 0.05$ versus the Apo group.

produced a remarkable effect only after combining with DRD2. After reuptake by dopamine transporters, the dopamine was transformed into HVA in neurons and released into the blood. Evidence shows that DRD2 activity is increased and HVA levels are decreased in TS patients (*9,11*). Promoting dopamine metaboly and inhibiting DRD2 activity may control tics.

Gastrodia elata Blume (Orchidaceae) is a medicinal plant and used in China as a crude drug for the treatment of epilepsy, infantile convulsions, headache, and dizziness. Gastrodin is one major active component of this herb. Gastrodin was found to penetrate the blood-brain barrier, enter the central nervous system, and protect from nerve lesions (*17*). Gastrodin reduces the seizure score in seizure-prone gerbils, modifies noradrenergic, dopaminergic, and serotonergic nervons in the rat brain, and have mitigative, antidepressant, anticonvulsive, and neuroprotective effects (*14,18*). Ju reported that gastrodin could inhibit activity of dopaminergic neurons, decrease dopamine reuptake, promote dopamine metaboly, and accommodate catecholamine in the nervous system (*19*). In the present study, we used gastrodin-treated TS rats and compared its effects with the effects of haloperidol. Confirming previous reports, our findings demonstrated that gastrodin significantly increases dopamine metabolism in TS rats and promotes its transformation into HVA, alleviating TS symptoms.

Haloperidol, a common medication for TS, reduces tic frequency and severity and elevates levels of HVA (*20*). In this study, we found that gastrodin also increases the levels of HVA and alleviates the stereotyped behaviors of apomorphine-induced TS rats, and that gastrodin has effects on HVA and on stereotyped behaviors similar to the effects of haloperidol. Both promote dopamine transformation into HVA. This observation indicates that gastrodin and haloperidol have the same effect on dopamine metabolism. DRD2 is a G-protein-coupled receptor. Excitable activity occurred after dopamine combined with DRD2. High DRD2 activity is associated with tics. In this study, we found the level of DRD2 mRNA in striatum was significantly lower in the Hal group than in the Apo group. Our data are consistent with other studies (*21*). However, we failed to observe any effect of gastrodin on DRD2 mRNA expression; this might indicate that gastrodin cannot act on DRD2 mRNA expression.

In summary, the results of this study provide evidence that gastrodin effectively inhibits the stereotyped behaviors and controls the symptoms of TS by promoting dopamine metabolism and increasing levels of the metabolic product HVA in sera.

Acknowledgements

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References

1. Grimaldi BL. The central role of magnesium deficiency in Tourette's syndrome: causal relationships between magnesium deficiency, altered biochemical pathways and symptoms relating to Tourette's syndrome and several reported comorbid conditions. Med Hypotheses. 2002; 58:47-60.
2. Cortese S, Lecendreux M, Bernardina BD, Mouren MC, Sbarbati A, Konofal E. Attention-deficit/hyperactivity disorder, Tourette's syndrome, and restless legs syndrome: the iron hypothesis. Med Hypotheses. 2008; 70:1128-1132.
3. Peterson BS, Leckman JF. The temporal dynamics of tics in Gilles de la Tourette syndrome. Biol Psychiatry. 1998; 44:1337-1348.
4. Wong DF, Brasić JR, Singer HS, Schretlen DJ, Kuwabara H, Zhou Y, Nandi A, Maris MA, Alexander M, Ye W, Rousset O, Kumar A, Szabo Z, Gjedde A, Grace AA. Mechanisms of dopaminergic and serotonergic neurotransmission in Tourette syndrome: clues from an *in vivo* neurochemistry study with PET. Neuropsychopharmacology. 2008; 33:1239-1251.
5. Centonze D, Gubellini P, Usiello A, Rossi S, Tscherter A, Bracci E, Erbs E, Tognazzi N, Bernardi G, Pisani A, Calabresi P, Borrelli E. Differential contribution of dopamine D2S and D2L receptors in the modulation of glutamate and GABA transmission in the striatum. Neuroscience. 2004; 129:157-166.
6. Emilien G, Maloteaux JM, Geurts M, Hooqenberq K, Craqq S. Dopamine receptors: physiological understanding to therapeutic intervention potential. Pharmacol Ther. 1999; 84:133-156.
7. Yoon DY, Gause CD, Leckman JF, Singer HS. Frontal dopaminergic abnormality in Tourette syndrome: a postmortem analysis. J Neurol Sci. 2007; 255:50-56.
8. Minzer K, Lee O, Hong JJ, Singer HS. Increased prefrontal D2 protein in Tourette syndrome: a postmortem analysis of frontal cortex and striatum. J Neurol Sci. 2004; 219:55-61.
9. Singer HS, Hahn IH, Moran TH. Abnormal dopamine uptake sites in postmortem striatum from patients with Tourette's syndrome. Ann Neurol. 1991; 30:558-562.
10. Dhir A, Kulkarni SK. Involvement of dopamine (DA)/serotonin (5-HT)/sigma (σ) receptor modulation in mediating the antidepressant action of ropinirole hydrochloride, a D2/D3 dopamine receptor agonist. Brain Res Bull. 2007; 74:58-65.
11. Yao Y, Ma HW. Neurobiology development of Tourette syndrome. Foreign Medical Science (Pediatr). 2005; 32:137-140. (in Chinese)
12. Fachinetto R, Villarinho JG, Wagner C, Pereira RP, Avila DS, Burger ME, Calixto JB, Rocha JB, Ferreira J. Valeriana officinalis does not alter the orofacial dyskinesia induced by haloperidol in rats: role of dopamine transporter. Prog Neuropsychopharmacol Biol Psychiatry. 2007; 31:1478-1486.
13. Yoo HK, Kim JY, Kim CY. A pilot study of aripiprazole in children and adolescents with Tourette's disorder. J Child Adolesc Psychopharmacol. 2006; 16:505-506.
14. Jung JW, Yoon BH, Oh HR, Ahn JH, Kim SY, Park SY,

Ryu JH. Anxiolytic-like effects of gastrodia elata and its phenolic constituents in mice. Biol Pharm Bull. 2006; 29:261-265.
15. Napier TC, Istre ED. Methamphetamine-induced sensitization includes a functional upregulation of ventral pallidal 5-HT2A/2C receptors. Synapse. 2008; 62:14-21.
16. Paxinos G, Watson C. The rat brain in stereotaxic coordinates. Academic Press, London, England, 1996; pp. 1104.
17. Wang Q, Chen GS, Zeng S. Distribution and metabolism of gastrodin in rat brain. J Pharm Biomed Anal. 2008; 46:399-404.
18. Zeng X, Zhang Y, Zhang S, Zheng X. A microdialysis study of effects of gastrodin on neurochemical changes in the ischemic/reperfused rat cerebral hippocampus. Biol Pharm Bull. 2007; 30:801-804.
19. Ju GC. Progress in studies of pharmacological activities and clinical applications of preparations of Gastrodia elata Blume. China Pharmaceuticals. 2008; l7:64-66. (in Chinese)
20. Janhunen S, Mielikäinen P, Paldánius P, Tuominen RK, Ahtee L, Kaakkola S. The effect of nicotine in combination with various dopaminergic drugs on nigrostriatal dopamine in rats. Naunyn-Schmiedebergs Arch Pharmacol. 2005; 371:480-491.
21. Lima MM, Andersen ML, Reksidler AB, Silva A, Zager A, Zanata SM, Vital MA, Tufik S. Blockage of dopaminergic D2 receptors produces decrease of REM but not of slow wave sleep in rats after REM sleep deprivation. Behav Brain Res. 2008; 188:406-411.

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Original Article

Protective effect of montelukast on paraquat-induced lung toxicity in rats

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Summary **In the current study, the possible protective effect of montelukast (Mont, 50 mg/kg, *p.o.* given 2, 24, and 48 h after paraquat injection) against acute paraquat toxicity (PQ, 25 mg/kg, *i.p.*) in rats was evaluated. The effects of treatment on selected oxidative stress parameters in lung tissues as well as certain inflammatory markers in serum were evaluated. The obtained results revealed that Mont can ameliorate the biochemical alterations in the lung tissues which were induced by PQ in rats. This was evidenced by the significant reduction in lung tissues lipid peroxidation, protein carbonyl content and DNA fragmentation, as well as by normalization of glutathione and myeloperoxidase activities. Moreover, the elevated levels of serum TNF-α, and LDH induced by paraquat were reversed in Mont-treated rats. Meanwhile, lung paraquat concentration was significantly reduced after Mont treatment compared with PQ alone group. Additionally, a higher survival percentage was observed in rats treated with Mont (80%) compared with the PQ alone group (30%) during 7 days observation. The results indicate that Mont protects lung tissue by balancing oxidant-antioxidant status, inhibiting neutrophil infiltration, and by regulating the generation of inflammatory mediators. In conclusion, the obtained results emphasize the beneficial effect of Mont in ameliorating the toxicity signs of paraquat in rats. The current study greatly recommends the usage of Mont in management of paraquat toxicity.**

Keywords: Paraquat, montelukast, lipid peroxidation, glutathione, protein carbonyl, DNA fragmentation, myeloperoxidase, tumor necrosis factor (TNF-α)

1. Introduction

Paraquat (PQ) is a widely used nonselective contact herbicide and a highly toxic compound for humans and animals (*1,2*). Irrespective of its route of exposure, paraquat is rapidly distributed in most tissues and most highly concentrated in the lungs, producing pneumotoxicity and lung injury (*1,3*). The strong pneumotoxicity of paraquat is mainly due to its accumulation in the lung through a polyamine uptake system and to its ability to induce redox cycling, leading to oxidative stress-related cell death and inflammation (*4*). Death is usually associated with respiratory insufficiency injury due to an oxidative insult to the alveolar epithelium with subsequent obliterating fibrosis (*1,5*). The paraquat-induced lung injury is morphologically characterized by an early destructive phase, in which the alveolar type I and type II epithelial cells are damaged and a second proliferative phase defined by alveolitis, pulmonary edema and infiltration of inflammatory cells (*6*). Thus, research for the treatment of paraquat toxicity has mainly focused on alleviating the lung injury.

The mechanism of paraquat toxicity involves the generation of the superoxide anion, with subsequent formation of more toxic reactive oxygen species (ROS), such as hydrogen peroxide and hydroxyl radical and the oxidation of the cellular NADPH, the major source of reducing equivalents for the intracellular reduction of paraquat, which results in the disruption of NADPH-requiring biochemical processes (*7-10*).

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Management of paraquat poisoning has remained mostly supportive, as there is no specific antidote or effective treatment for PQ poisoning has been identified so far. This management has been directed towards the modification of the toxicokinetics of paraquat before its cellular uptake (*11*). Additional protective measures have also been adopted recently, to prevent the generation or to scavenge ROS (*1*) and to reduce the inflammation (*4,10,12*).

Cysteinyl leukotrienes, namely leukotriene LTC4, LTD4, and LTE4, are potent proinflammatory lipid mediators derived *via* the 5-lipoxygenase pathway from arachidonic acid. They are mainly secreted by eosinophils, mast cells, monocytes, and macrophages, and play a crucial role in inflammation, bronchoconstriction, edema formation, and airway remodeling of asthmatics (*13-16*).

Anti-leukotriene agents, including leukotriene receptor antagonists and synthesis inhibitors, are known to be effective in several inflammatory models. The selective LTD4 receptor antagonist, montelukast (Mont), is used mainly to reduce eosinophilic inflammation in the airways of asthmatic patients (*17-18*), and is also effective in management of allergic rhinitis, COPD and idiopathic pulmonary fibrosis (*16*). Moreover, montelukast was reported to have beneficial effects in management of experimental gastric mucosal ulceration (*19*), colitis (*20*), oxidative renal damage (*21*), hepatopathy (*22*), burn-induced multiorgan damage (*23*), and renal ischemia/reperfusion injury (*24*). The protective effects of montelukast against vital organs injury in these experimental models were attributed, at least in part, to its ability to inhibit neutrophil infiltration, and to regulate the generation of inflammatory mediators in addition to its pronounced antioxidant potential.

Accordingly, in the current study, montelukast was chosen as a candidate to study its putative protective effect on paraquat-induced lung toxicity in rats. The effect of treatment on a selected oxidative stress markers and neutrophile migration in the lung tissues in addition to certain inflammatory markers in the serum, after three doses administration of Mont to rats exposed to acute PQ toxicity, were studied. The overall aim of this study was to find a new and a possible effective treatment that can protect against PQ-induced lung toxicity.

2. Materials and Methods

2.1. *Chemicals and drugs*

Paraquat dichloride was purchased from Sigma (St Louis, MO, USA). Montelukast was from Merck Sharp & Dohme Ltd. (Hoddesdon, Hertfordshire, UK). All other chemicals and reagents used were of highest analytical grade and were obtained from Sigma.

2.2. *Animals*

This study was performed using adult male Wistar rats (225 ± 25 g) obtained from the animal facilities of King Saud University. Animals were kept under standard laboratory conditions (12/12 h light/darkness, 22 ± 2ºC room temperature, 50-60% humidity) for at least 1 week before starting the experiments. Animals were allowed free access to tap water and rat chow ad libitum during the entire experiment. All experiments were carried out in accordance with the recommendations of King Saud University Committee Acts for using experimental animals which are complied with the international acts.

2.3. *Experimental protocol*

Animals were divided into three groups of rats (n = 10). One group of animals, served as control, was treated with saline (0.5 mL, *p.o.*) for three days. Paraquat group was treated with a single dose of paraquat (PQ, 25 mg/kg, *i.p.*) followed by oral saline administration after 2, 24, and 48 h. The third group was given montelukast (Mont, 50 mg/kg, *p.o.*) 2, 24, and 48 h after single injection of PQ (25 mg/kg, *i.p.*). All animals were sacrificed under light ether anesthesia, in the third day, after 3 h from the last treatment. Blood samples were collected, from 16 h fasted animals, and serum was separated immediately and stored at -80ºC for biochemical analysis. Lungs were removed immediately after killing, separated from the other organs, washed in ice-cold saline solution, blotted and weighed before subjecting to homogenization. Relative lung weight of each animal was calculated as a percentage of the absolute body weight on the sacrifice day.

In another set of experiments, animals were kept under the same conditions and treated as described above. Abnormal findings, including weakness and dyspnea, were noted and recorded if present. The lethality was registered every day until day 7 from PQ injection.

Paraquat dose (25 mg/kg, *i.p.*) was chosen according to the dose used in previous studies and after preliminary studies carried out by the author of the current study. This dose is known to produce severe lung toxicity and death in rats within few days (*4,25,26*).

2.4. *Determination of lung PQ concentration*

Measurement of PQ contents in lung tissues gives an indication about the severity of toxicity. Determination of lung PQ concentration was carried out according to the method of Fuke *et al.* (*27*) with minor modifications. Briefly, lung samples were homogenized in ice-cold 50 mM phosphate buffer containing 0.1% (v/v) Triton X-100 (pH 7.4). The homogenate was kept on ice, centrifuged at 3,000 × g, 4ºC, for 10 min. Aliquots of the lung supernatants were treated with

5-sulfosalicylic acid (5% in final volume) and then centrifuged (13,000 × g, 4ºC for 10 min). The resulting supernatant fractions were alkalinized with NaOH 10 N (pH > 9) and then gently mixed with the reductant sodium dithionite to give the blue color, characteristic of the PQ cation radical. The absorbance was read at 490 nm and the results were expressed as percentage of PQ group value.

2.5. *Estimation of lung malondialdehyde (MDA) and glutathione (GSH) activities*

Tissue samples were homogenized with ice-cold 150 mM KCl for the determination of MDA and glutathione levels. The MDA levels were assayed for products of lipid peroxidation by monitoring thiobarbituric acid reactive substance formation by the spectrophotometric method, as described previously by Ohakawa *et al*. (*28*). Lipid peroxidation was expressed as nmol MDA/g tissue. Glutathione was determined by the spectrophotometric method, based on the use of Ellman's reagent as described by Ellman (*29*), and the results were expressed as μmol GSH/g tissue.

2.6. *Estimation of lung protein carbonyl contents*

Protein carbonyl contents (PCO, ketones and aldehydes) were estimated according to the method of Levine *et al*. (*30*) using 2,4-dinitrophenylhydrazine (DNPH). Lung samples (0.2 g) were rinsed in 10 mM HEPES buffer (pH 7.4) and homogenized in phosphate buffer (pH 7.4). After centrifugation at 10,000 × g for 10 min, 0.5 mL of the supernatant was taken into tubes. Then equal volume of DNPH in 2 M HCl was added to each tube. The blank contained 2 M HCl only. All tubes were vortexed every 10 min for 1 h in dark and then mixed with 30% trichloroacetic acid. After centrifugation, the pellet was washed three times with 1 mL of ethanol: ethylacetate (1:1, v/v). The final pellets were dissolved in 1 mL of 6 M guanidine HCl in 20 mM potassium dihydrogen phosphate (pH 2.3). The difference in absorbance between the DNPH-treated and HCl-treated samples was determined at 370 nm. Lung tissue PCO contents were expressed as percentage of control normal group value.

2.7. *Determination of lung DNA fragmentation*

Estimation of DNA fragmentation was determined by colorimetric diphenylamine assay as described by Burton (*31*). Lung samples from different groups were homogenized in chilled lysis buffer (10 mM Tris-HCl, 20 mM EDTA, 0.5% Triton X-100, pH 8.0). Homogenates were then centrifuged at 27,000 × g for 20 min to separate intact chromatin in the pellet from fragmented/damaged DNA in the supernatant fractions. Perchloric acid was added to the pellets and supernatant samples to reach a final concentration of 0.5 N. Samples were heated at 90ºC for 15 min and centrifuged at 1,500 × g for 10 min to remove protein. The supernatants were left to react with diphenylamine for 18 h at room temperature and the absorbance was measured at 600 nm. DNA fragmentation was expressed as a percentage of total DNA appearing in the supernatant fractions. Treatment effects were reported as percentage of control fragmentation.

2.8. *Determination of lung myeloperoxidase activity*

Myeloperoxidase (MPO) activity in tissues was measured according to the modified method of Bradley *et al.* (*32*). Samples of lung tissues were homogenized in 50 mM potassium phosphate buffer pH 6.0 (PB), and centrifuged at 40,000 × g for 10 min. The pellets were then suspended in 50 mM PB containing 0.5% hexadec yltrimethylammonium bromide. After three freeze-and-thaw cycles, the samples were centrifuged at 40,000 × g for 10 min. Aliquots (0.3 mL) were added to 2.3 mL of reaction mixture containing 50 mM PB, *O*-dianisidine, and 20 mM H_2O_2 solution. One unit of enzyme activity was defined as the amount of MPO present that caused a change in absorbance, measured at 460 nm, for 3 min. MPO activity was expressed as U/g tissue.

2.9. *Estimation of serum tumor necrosis factor (TNF)-α and lactate dehydrogenase (LDH) activities*

Rat serum tumor necrosis factor (TNF)-α was estimated using enzyme-linked immunosorbent assay (ELISA) kit specific for the rat TNF-α according to the instruction manual of R&D kit with absorbance at 450 nm. The concentration of TNF-α was expressed as pg/mL. The activity of serum lactate dehydrogenase (LDH) was assayed using the commercial kit of bioMerieux, SA, France. The absorbance was measured at 340 nm and the enzyme activity was expressed as U/L.

2.10. *Statistical analysis*

Statistical analysis was done using a GraphPad Prism 3.0 (Graph-Pad Software, San Diego, CA). All data were expressed as means ± SEM. Groups of data were compared with analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. Values of $p < 0.05$ were considered significant.

3. Results

Data of the current study showed that, there was no behavioral changes were noticed on day 1 after PQ injection, as compared to normal rats. However, starting from day 2 symptoms including rapid shallow respiration, dyspnea, loss of appetite, piloerection, and cyanosis were observed in paraquat-treated rats. These

symptoms were less apparent in the group treated with PQ + Mont. By the end of the 7 days post treatment, the survival was 100% in control normal group, while three of ten animals (30%) of PQ group and eight of ten animals (80%) of PQ + Mont group were survived.

Relative lung weight (RLW) was assessed to give an indication about the edema degree (Figure 1). No significant differences were obtained in RLW values among control and PQ + Mont group. However, in comparison to these groups, animals from the PQ group showed a significant RLW increase at 72 h after PQ exposure ($p < 0.001$, $p < 0.01$, respectively).

Measurement of PQ in lung tissues gives an indication about the severity of toxicity. Administration of 3 doses of Mont (50 mg/kg, *p.o.*) after PQ injection, produced a 60% reduction in lung concentration of PQ compared with PQ alone group (Figure 2).

Lung malondialdehyde levels, as an index of lipid peroxidation, were significantly increased in PQ-treated group (to 2 fold, $p < 0.001$, as compared to the control normal group). However, in montelukast treated rats, malondialdehyde levels were significantly reduced (to 31%, $p < 0.001$ compared with PQ-only group) (Figure 3).

Contents of lung GSH, a key antioxidant, were decreased significantly ($p < 0.001$) in PQ group compared with normal rats. Meanwhile, the lung GSH content was significantly restored back to near control normal levels upon montelukast treatment (to 102%, $p < 0.01$, compared with PQ group) (Figure 4).

Data in figure 5 showed that, administration of PQ produced a 2.3 fold increase in protein carbonyl content in the lung tissues compared with control normal rats ($p < 0.001$). On the contrary, post treatment with Mont prevented the increase in protein carbonyl content induced by PQ in lung tissues (to 40%, $p < 0.001$, compared with PQ group) (Figure 5).

Effect of Mont on PQ-induced DNA fragmentation in rat lung tissues is illustrated in Figure 6. The results showed that oral administration of PQ induced an increase of 2.9 fold in DNA fragmentation in the rat lung tissues compared with normal control, demonstrating tissue degeneration. Post administration

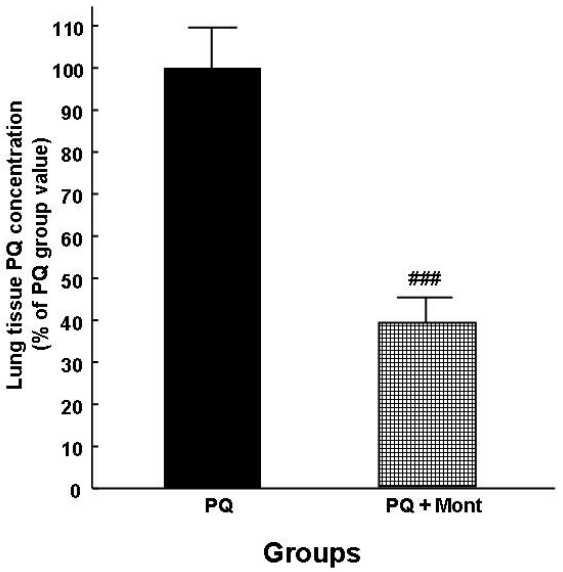

Figure 2. The lung paraquat concentration in paraquat (PQ), and paraquat + montelukast (PQ + Mont) treated rats. Values are expressed as percentage of paraquat treated rats ($n = 10$ rats). ### Significantly different from PQ treated group at $p < 0.001$.

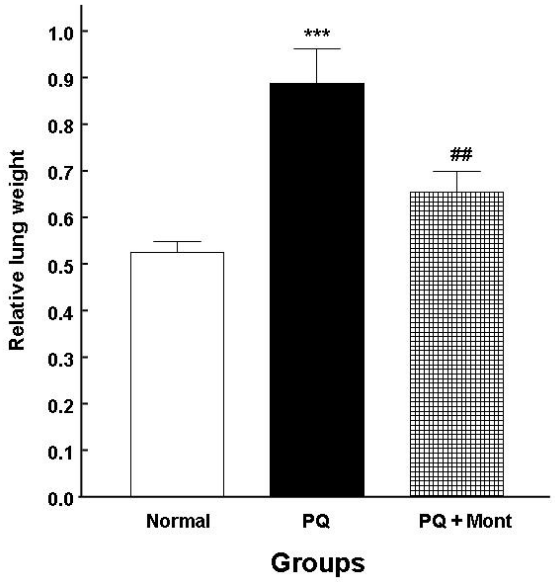

Figure 1. The relative lung weight in normal, paraquat (PQ), and paraquat + montelukast (PQ + Mont) treated rats. Values are expressed as mean ± SEM of ($n = 10$ rats). *** Significantly different from normal group (saline) at $p < 0.001$. ## Significantly different from PQ treated group at $p < 0.01$.

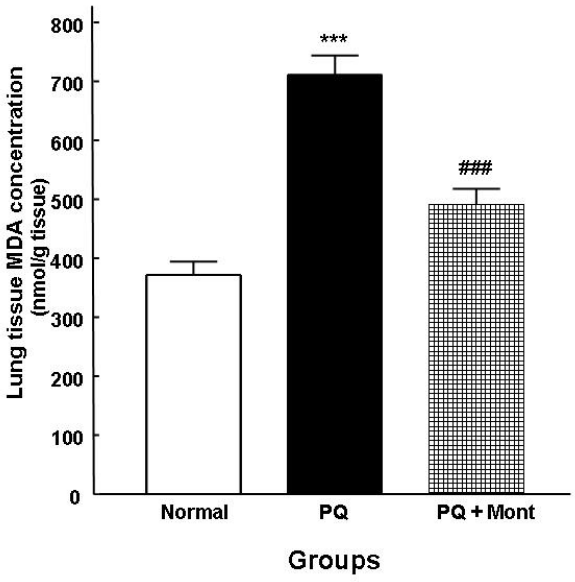

Figure 3. The lung malondialdehyde (MDA) concentration in normal, paraquat (PQ), and paraquat + montelukast (PQ + Mont) treated rats. Values are expressed as mean ± SEM of ($n = 10$ rats). *** Significantly different from normal group (saline) at $p < 0.001$. ### Significantly different from PQ treated group at $p < 0.001$.

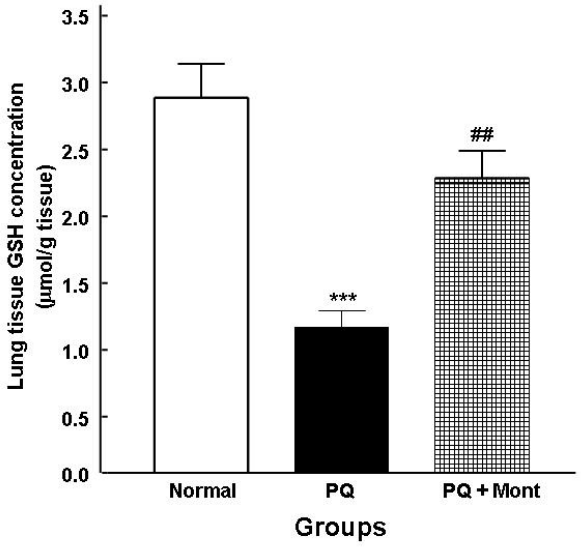

Figure 4. The lung glutathione (GSH) concentration in normal, paraquat (PQ), and paraquat + montelukast (PQ + Mont) treated rats. Values are expressed as mean ± SEM of (n = 10 rats). *** Significantly different from normal group (saline) at $p < 0.001$. ## Significantly different from PQ treated group at $p < 0.01$.

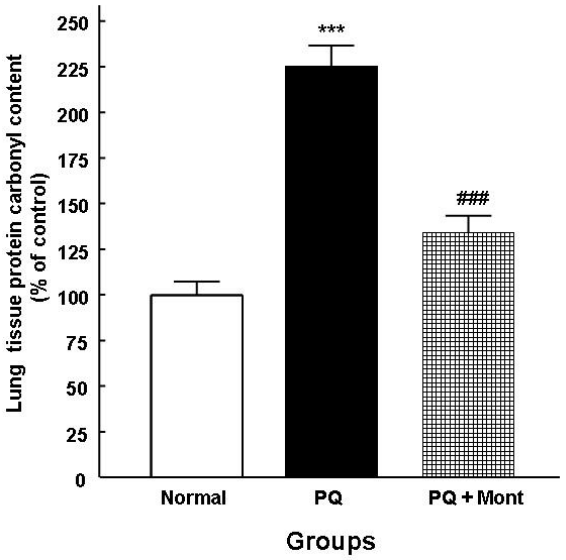

Figure 5. The lung protein carbonyl content in normal, paraquat (PQ), and paraquat + montelukast (PQ + Mont) treated rats. Values are expressed as percentage of control normal rats (n = 10 rats). *** Significantly different from normal group (saline) at $p < 0.001$. ### Significantly different from PQ treated group at $p < 0.001$.

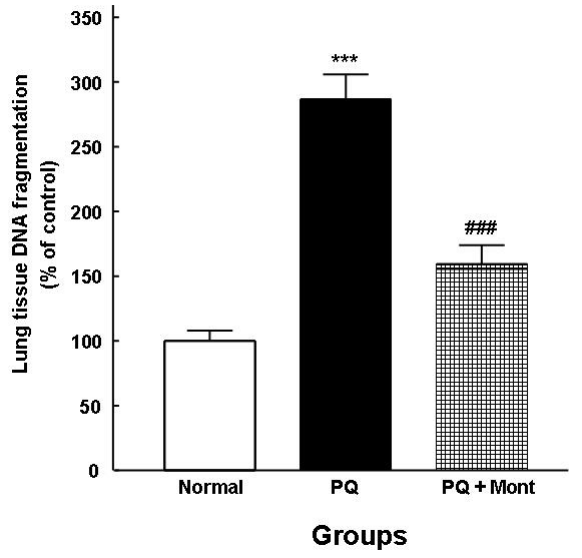

Figure 6. The lung DNA fragmentation in normal, paraquat (PQ), and paraquat + montelukast (PQ + Mont) treated rats. Values are expressed as percentage of control normal (n = 10 rats). *** Significantly different from normal group (saline) at $p < 0.001$. ### Significantly different from PQ treated group at $p < 0.001$.

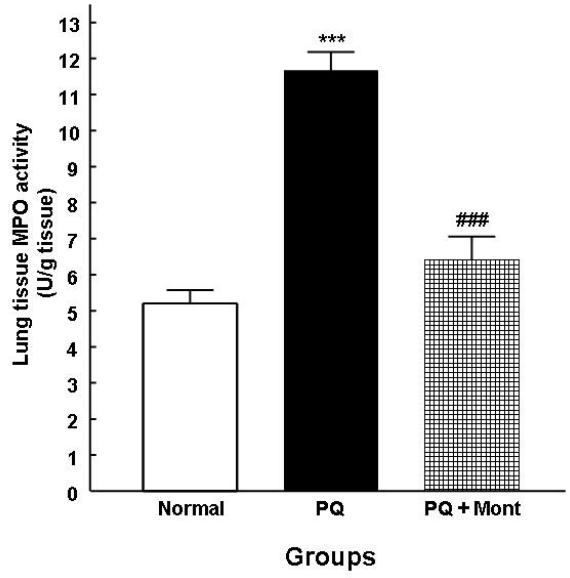

Figure 7. The lung myeloperoxidase (MPO) activity in normal, paraquat (PQ), and paraquat + montelukast (PQ + Mont) treated rats. Values are expressed as mean ± SEM of (n = 10 rats). *** Significantly different from normal group (saline) at $p < 0.001$. ### Significantly different from PQ treated group at $p < 0.001$.

of Mont significantly decreased PQ-induced lung DNA fragmentation (to 44%, $p < 0.001$, compared with PQ group). The results indicate the protective ability of Mont against PQ-induced DNA fragmentation in rat lung tissues.

Lung MPO activity is an indirect evidence of neutrophil infiltration into the tissue. As illustrated in Figure 7, Lung MPO activity of the PQ-exposed animals was significantly higher (about 2.2 fold, $p < 0.001$) compared with control normal group. Post-treatment of rats with Mont, completely prevented the increase of MPO activity and significantly reduced this parameter (to 45% compared with PQ group) (Figure 7).

Results of the present investigation showed that serum TNF-α level measured after 3 days from PQ injection was significantly higher than the control normal group ($p < 0.001$, Figure 8). Moreover, serum LDH level was significantly increased after PQ injection ($p < 0.001$, Figure 9) compared with normal rats. These findings verify generalized tissue damage.

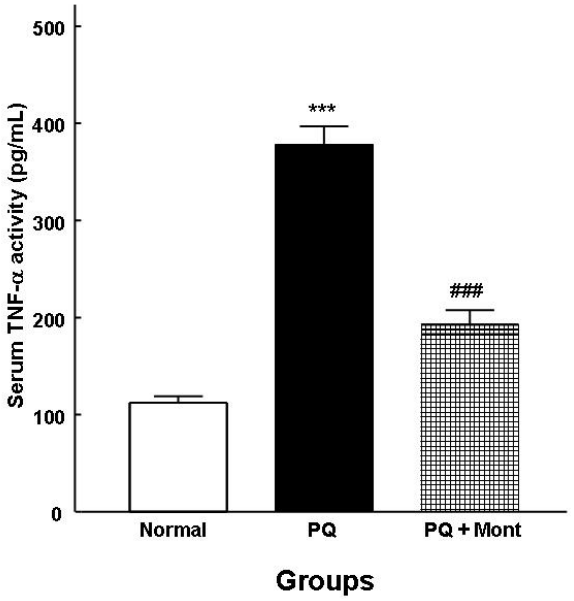

Figure 8. The serum TNF-α activity in normal, paraquat (PQ), and paraquat + montelukast (PQ + Mont) treated rats. Values are expressed as mean ± SEM of (n = 10 rats). *** Significantly different from normal group (saline) at $p < 0.001$. ### Significantly different from PQ treated group at $p < 0.001$.

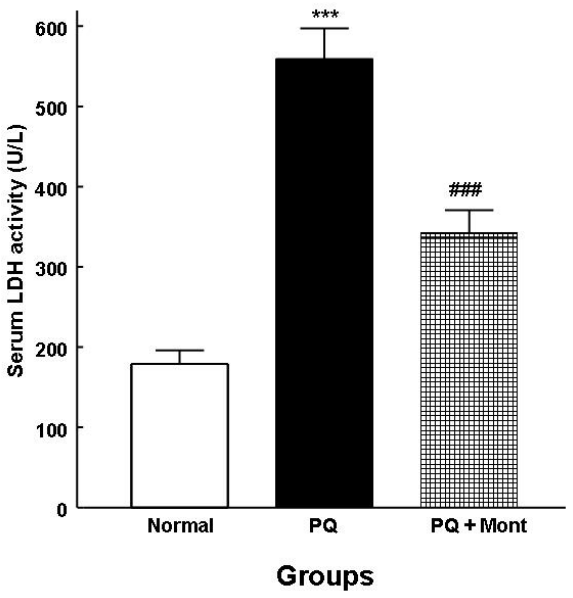

Figure 9. The serum lactate dehydrogenase (LDH) activity in normal, paraquat (PQ), and paraquat + montelukast (PQ + Mont) treated rats. Values are expressed as mean ± SEM of (n = 10 rats). *** Significantly different from normal group (saline) at $p < 0.001$. ### Significantly different from PQ treated group at $p < 0.001$.

On the contrary, PQ-induced rises in serum TNF-α and LDH levels were abolished and significantly reduced by montelukast treatment (to 49% and 39%, $p < 0.001$ compared with PQ group, respectively) (Figures 8 and 9).

4. Discussion

The current study revealed that montelukast produces a potent protection against paraquat-induced lung toxicity in rats. It was shown that administration of three doses of Mont (50 mg/kg, *p.o.*) 2, 24, and 48 h after PQ exposure (25 mg/kg, *i.p.*) resulted in a marked decrease in accumulation of PQ in the lung (about 60% compared to the PQ alone exposed rats). Consequently, the reduction in lung PQ contents resulted in prevention of PQ-induced lung toxicity. This was evidenced by the decrease in relative lung weight and the increase in the number of survived animals, to 80% compared with the 30% in PQ alone group. Additionally, the significant amelioration in the altered biochemical parameters induced by paraquat revealed the protection from toxicity.

The current findings also suggest that the PQ-induced death is mainly caused by rapid progress of respiratory failure. This assumption is supported by the observed symptoms including rapid shallow respiration, cyanosis, pulmonary edema, and the increase in relative lung weight. These findings are also concordant with the results of previous investigators (*2,33*).

It is well documented that the lung is a primary target organ of PQ toxicity because it has active polyamine uptake transport systems which concentrate paraquat rapidly into type II epithelial cells of the alveoli (*34*). The established mechanism of paraquat toxicity involves the cyclic reduction and re-oxidation of paraquat which results in oxidation of NADPH to $NADP^+$ with the production of oxygen free radicals. The free radicals generated by paraquat oxidation may interact with membrane lipids leading to cell damage (*35,36*). In the same time, depletion of NADPH results in the disruption of the pathways that are dependent on this nucleotide (*37,38*) specially, those involved in the production of pulmonary surfactants (*39,40*). Rats given a high dose of paraquat develop damage to the type I and II alveolar epithelial cells and within days large areas of the alveolar epithelium are completely lost (*41*). The lungs in these animals exhibit extensive alveolar fibrosis, which in association with residual edema, and reduced gas exchange may lead to death by anoxia (*8*). In association with the above mechanism, the oxygen free radicals generated by PQ also increase neutrophil adhesion and infiltration (*42*), and generate powerful hemotactic mediators including leukotrienes (*43*) leading to endothelial damage, increased vessel permeability and produced pulmonary edema (*2,44*).

Treatment with montelukast, a CysLT1 receptor antagonist, was reported to abolish significantly the increase in vascular permeability and to reduce edema formation in lung tissues (*24*), which are concordant with the findings of the current investigation.

According to the present results, Mont proved to protect the lungs, through antagonizing the biochemical parameters that were changed upon PQ-challenging. As a direct consequence of amelioration of the paraquat-induced toxicity, 80% survival was achieved in PQ + Mont exposed rats.

The current data demonstrate that paraquat causes

oxidative inflammatory response as evidenced by alterations in serum TNF-α, LDH, and in lung MPO, MDA and GSH levels. On the other hand, the results also demonstrate that montelukast, prevents the paraquat- induced inflammatory responses. This protection seems to occur through scavenging of ROS and inhibition of MPO and inflammatory mediators induced by PQ toxicity.

In accordance with the current results, other studies showed that, antagonizing the leukotriene CysLT1 receptors with montelukast ameliorates oxidative tissue injury and improves organs functions through the mechanisms that involve an inhibitory action on tissue neutrophil infiltration (*19,24*), reduction in the release of reactive oxygen species (*19,24*), and inhibition of the inflammatory cytokines (*21,22*).

The pulmonary toxicity caused by PQ is assumed to have a connection with the activation of neutrophils (*45*). Furthermore, various inflammatory mediators including TNF-α have been expected to be increased in the lung during PQ toxicity (*11*). It is known that, TNF-α triggers the synthesis of leukotrienes and prostaglandin E2 which then stimulate the infiltration of polymorphonuclear leukocytes into the lungs and produce lung injury (*9)*.

Concerning the effect of montelukast on TNF-α, a previous study (*21*) showed that TNF-α was increased significantly in the pyelonephritic rats exposed to *E. coli* and that montelukast treatment displayed an inhibitory effect on TNF-α release along with the alleviation of neutrophil mediated parenchymal destruction, The authors suggested that the anti-inflammatory effect of montelukast may be ascribed to an inhibition of TNF-α-mediated cytotoxicity.

Tissue myeloperoxidase (MPO) activity is a sensitive and specific marker of acute inflammation and reflects polymorphonuclear cell infiltration into the parenchyma. In the present study, MPO levels were significantly increased following paraquat administration, suggesting that oxidant-generated tissue injury involves the extracellular release of MPO by activated polymorphonuclear leukocytes. Previous studies recorded that neutrophils can recruit in the lungs during the inflammatory reaction generated by PQ leading to marked elevation in lung MPO activities (*9*). On the other hand, montelukast may attenuate neutrophil recruitment and promote the resolution of inflammation by antagonizing the effects of leukotrienes, which are potent stimuli for leukocyte infiltration. Similar explanation was given by other researchers (*21,24*) upon describing the protective effect of Mont in *E. coli* induced pyelonephritis and renal ischemia/reperfusion injury in rats, respectively. These findings greatly supported the assumption that the protective effects of Mont against PQ-induced lung toxicity are also related to less infiltration of inflammatory cells with the subsequent decrease in MPO activity.

In the present study, malondialdehyde, a good indicator of the degree of lipid peroxidation, was increased in the lung of PQ-intoxicated rats, indicating the presence of oxidative damage in lung tissues. This observation is in agreement with other reports showing that lipid peroxidation is a biomarker of PQ toxicity *in vitro* (*36*) and *in vivo* (*9,46,47*). Lipid peroxidation has been implicated in a number of deleterious effects such as increased membrane rigidity, osmotic fragility, decreased cellular and subcellular components, reduced mitochondrial survival, and lipid fluidity (*48*). The current results suggest that Mont may exert its protective effect against PQ toxicity through inhibition of lipid peroxidation in lung tissues. Similarly, recent studies indicate that Mont can protect against experimental organ damage through its marked antioxidant (reduce MDA, increase GSH) in addition to its anti-inflammatory effects (reduce TNF-α, LDH) (*22,24*). Additionally, another investigator (*19*) suggested that the gastroprotective effect of montelukast on indomethacin-induced ulcerations in rats was attributed to its ameliorating effect on oxidative damage.

In the current investigation, a severe depletion in lung GSH in PQ-exposed rats was observed. GSH, presents in high concentrations in lung epithelial cells (*49*). Depletion of GSH was shown to intensify lipid peroxidation and predispose alveolar cells to oxidative damage (*9,50*). Moreover, GSH, as a non-enzymatic radical scavenger, has the capability to interact with free radicals to yield more stable elements (*51*). As reported by Ross (*52*), cell injury and enhanced cell susceptibility to toxic chemicals are related to the diminished GSH biosynthesis. Accordingly, GSH plays a critical role in limiting the propagation of free radical reactions, which would otherwise result in extensive lipid peroxidation (*53,54*). On the other hand, montelukast, as an inhibitor of neutrophil infiltration, can reduce the oxidative injury of the lung and restore the GSH levels significantly. Similar explanation was previously discussed (*21,22*).

Moreover, GSH is also involved in numerous processes that are essential for normal biological function, such as DNA and protein synthesis (*55*). It is also considered to protect cells from toxic substances through conjugation resulting in a less toxic intermediate and thus, reducing the injury level of cells (*56*). However, its importance becomes particularly evident when the redox balance is disrupted due to excessive consumption of GSH this will greatly facilitate the development of the toxicity caused by pro-oxidant xenobiotics such as PQ (*54,57,58*) which is concordant with the obtained results in the current study.

In the present study, PQ administration increased both the carbonyl group content and DNA fragmentation in the lung. On the contrary, Mont treatment, by

antagonizing PQ effects, significantly reduced both parameters. The significant increase in carbonyl group content in the lung of rats exposed to PQ is in agreement with the previous studies (*4,9,11*). As previously reported, the cellular damage mediated by PQ is essentially due to its redox-cycling leading to continuous superoxide radical production (*35*) with simultaneous generation of the hydroxyl radical (*59*), which has been implicated in the initiation of membrane injury by lipid peroxidation (*60*) and cytotoxicity *via* mitochondrial dysfunction (*11,61*). The produced reactive oxygen species are also known to produce oxidative modification in DNA and proteins leading to fragmentation of polypeptide chains, increased sensitivity to denaturation and formation of protein–protein cross linkages as well as modification of amino acid side chains to hydroxyl or carbonyl derivatives (*62*).

In the current study, Mont was shown to protect against PQ-induced elevation of carbonyl groups contents. A plausible explanation for this protection can be conferred by the potent scavenging effect of Mont on hydroxyl radical. Among ROS, hydroxyl radical is thought to be the most damaging species and mainly responsible for protein oxidation and lipid peroxidation (*63*).

Similarly, the PQ-induced lung DNA fragmentation in rats was observed previously with other investigators (*4,64*). The mechanism by which paraquat damages DNA may involve the generation of oxygen free radicals (*35*) with subsequent alteration of the oxidative balance within the cell followed by increase in hydroxyl radicals leading to direct damage of DNA (*65,66*). On the other hand, Mont can protect against PQ-induced elevation of DNA fragmentation through antagonizing the previous cascade through inhibiting the reactive oxygen species and hydroxyl radicals generation and balancing oxidant-antioxidant status. Similar explanation was previously discussed (*24*).

The protective mechanism of Mont against PQ toxicity can also be explained through the ability of Mont to stabilize the mast cells followed by inhibition of the release of inflammatory mediators and cytokines. In this context, Mont was previously reported to protect against smoking-induced lung injury in rats through inhibition of the release of inflammatory mediators and cytokines from lung mast cells (*67*).

Although similar protecting effects against PQ toxicity can be attained with other anti-inflammatory drugs like dexamethasone (*11*), sodium salicylate (*4*), or cromolyn (*44*), the additional benefit which is demonstrated by Mont in the present work is the potent antioxidant potential.

5. Conclusion

Paraquat is a strong pnumotoxicant and the toxicity is chiefly due to free radical generation with subsequent increase in lipid peroxidation, protein carbonyl, DNA fragmentation, and depletion of glutathione in lung tissues. This effect is accompanied by neutrophile migration and increase in the myeloperoxidase activities in the lung tissues. Release of inflammatory mediators such as TNF-α is also increased during PQ toxicity.

Treatment with montelukast directly after paraquat intoxication increases the incidence of survival of the rats. This occur through antagonizing the effect of free radical generation, neutrophile migration, and reduction of the release of inflammatory mediators induced by paraquat. The overall effect of montelukast is a marked protection of the lung from injury. It is highly probable that the antioxidant in addition to the anti-inflammatory effects of montelukast could contribute to its protective effect against paraquat-induced lung toxicity.

This is the first experimental study, after searching in literature to investigate the use of montelukast for treatment of paraquat toxicity, the study greatly recommends the administration of montelukast directly after paraquat toxicity and concluded that montelukast may constitute an effective and promising treatment for management of paraquat poisonings.

References

1. Suntres ZE. Role of antioxidants in paraquat toxicity. Toxicology. 2002; 180:65-77.
2. Shopova VL, Dancheva VY, Salovsky PT, Stoyanova AM, Lukanov TH. Protective effect of U-74389G on paraquat induced pneumotoxicity in rats. Environ Toxicol Pharmacol. 2007; 24:167-173.
3. Rose MS, Smith LL, Wyatt I. Evidence for the energy-dependent accumulation of paraquat into rat lung. Nature. 1974; 252:314-315.
4. Dinis-Oliveira RJ, Sousa C, Remiao F, Duarte JA, Sanchez Navarro A, Bastos ML, Carvalho F. Full survival of paraquat-exposed rats after treatment with sodium salicylate. Free Radic Biol Med. 2007; 42:1017-1028.
5. Honore P, Hantson P, Fauville JP, Peeters A, Manieu P. Paraquat poisoning. "State of the art". Acta Clin Belg. 1994; 49:220-228.
6. Bus JS, Gibson JE. Paraquat: model for oxidant initiated toxicity. Environ Health Perspect. 1984; 55:37-46.
7. Ali S, Diwakar G, Pawa S. Paraquat induces different pulmonary biochemical responses in Wistar rats and Swiss mice. Chem Biol Interact. 2000; 125:79-91.
8. Mainwaring G, Lim FL, Antrobus K, Swain C, Clapp M, Kimber I, Orphanides G, Moggs JG. Identification of early molecular pathways affected by paraquat in rat lung. Toxicology. 2006; 225:157-172.
9. Dinis-Oliveira RJ, Remiao F, Duarte JA, Ferreira R, Sanchez Navarro A, Bastos ML, Carvalho F. P-glycoprotein induction: an antidotal pathway for paraquat-induced lung toxicity. Free Radic Biol Med. 2006; 41:1213-1224.
10. Franco R, Sanchez-Olea R, Reyes-Reyes EM, Panayiotidis MI. Environmental toxicity, oxidative stress and apoptosis. Mutat Res. 2009; 674:3-22.
11. Dinis-Oliveira RJ, Duarte JA, Remiao F, Sanchez-

Navarro A, Bastos ML, Carvalho F. Single high dose dexamethasone treatment decreases the pathological score and increases the survival rate of paraquat-intoxicated rats. Toxicology. 2006; 227:73-85.
12. Chen GH, Lin JL, Hunang YK. Combined methylprednisolone and dexamethasone therapy for paraquat poisoning. Crit Care Med. 2002; 30:2584-2587.
13. Samuelsson B. Leukotrienes: mediators of immediate hypersensitivity reactions and inflammation. Science. 1983; 220:568-575.
14. Busse WW, McGill KA, Horwitz RJ. Leukotriene pathway inhibitors in asthma and chronic obstructive pulmonary disease. Clin Exp Allergy. 1999; 29(Suppl 2):110-115.
15. Peters-Golden M. Do anti-leukotriene agents inhibit asthmatic inflammation? Clin Exp Allergy. 2003; 33:721-725.
16. Rubin P, Mollison KW. Pharmacotherapy of diseases mediated by 5-lipoxygenase pathway eicosanoids. Prostaglandins Other Lipid Mediat. 2007; 83:188-197.
17. Aharony D. Pharmacology of leukotriene receptor antagonists. Am J Respir Crit Care Med. 1998; 157: S214-S219.
18. O'Byrne PM. Asthma treatment: antileukotriene drugs. Can Respir J. 1998; 5:64A-70A.
19. Dengiz GO, Odabasoglu F, Halici Z, Cadirci E, Suleyman H. Gastroprotective and antioxidant effects of montelukast on indomethacin-induced gastric ulcer in rats. J Pharmacol Sci. 2007; 105:94-102.
20. Holma R, Salmenpera P, Virtanen I, Vapaatalo H, Korpela R. Prophylactic potential of Montelukast against mild colitis induced by dextran sulphate sodium in rats. J Physiol Pharmacol. 2007; 58:455-467.
21. Tuğtepe H, Sener G, Cetinel S, Velioglu-Ogunc A, Yegen BC. Oxidative renal damage in pyelonephritic rats is ameliorated by montelukast, a selective leukotriene CysLT1 receptor antagonist. Eur J Pharmacol. 2007; 557:69-75.
22. Cuciureanu M, Caruntu I-D, Paduraru O, Stoica B, Jerca L, Crauciuc E, Nechifor M. The protective effect of montelukast sodium on carbon tetrachloride induced hepatopathy in rat. Prostaglandins Other Lipid Mediat. 2009; 88:82-88.
23. Sener G, Kabasakal L, Cetinel S, Contuk G, Gedik N, Yegen B. Leukotriene receptor blocker montelukast protects against burn-induced oxidative injury of the skin and remote organs. Burns. 2005; 31:587-596.
24. Sener G, Sehirli O, Ogunc AV, Cetinel S, Gedik N, Caner M, Sakarcan A, Yegen BC. Montelukast protects against renal ischemia/reperfusion injury in rats. Pharmacol Res. 2006; 54:65-71.
25. Akahori F, Masaoka T, Matsushiro S, Arishima K, Arai S, Yamamoto M, Eguchi Y. Quantifiable morphologic evaluation of paraquat pulmonary toxicity in rats. Vet Hum Toxicol. 1987; 29:1-7.
26. Rocco PR, Souza AB, Faffe DS, Passaro CP, Santos FB, Negri EM, Lima JG, Contador RS, Capelozzi VL, Zin WA. Effect of corticosteroid on lung parenchyma remodeling at an early phase of acute lung injury. Am J Respir Crit Care Med. 2003; 168:677-684.
27. Fuke C, Ameno K, Ameno S, Kiriu T, Shinohara T, Songo K, Ijiri I. A rapid, simultaneous determination of paraquat and diquat in serum and urine using second-derivative spectroscopy. J Anal Toxicol. 1992; 16:214-216.
28. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem. 1979; 95:351-358.
29. Ellman G. Tissue sulfhydryl groups. Arch Biochem Biophys. 1959; 82:70-77.
30. Levine RL, Williams JA, Stadtman ER, Shacter E. Carbonyl assay for determination of oxidatively modified proteins. Methods Enzymol. 1994; 233:346-357.
31. Burton K. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem J. 1956; 62:315-332.
32. Bradley PP, Priebat DA, Christensen RD, Rothstein G. Measurement of cutaneous inflammation: estimation of neutrophil content with an enzyme marker. J Invest Dermatol. 1982; 78:206-209.
33. Hong SY, Yang DH, Hwang KY. Associations between laboratory parameters and outcome of paraquat poisoning. Toxicol Lett. 2000; 118:53-59.
34. Rose MS, Lock EA, Smith LL, Wyatt I. Paraquat accumulation: tissue and species specificity. Biochem Pharmacol. 1976; 25:419-423.
35. Bus JS, Aust SD, Gibson JE. Superoxide and singlet oxygen catalyzed lipid peroxidation as a possible mechanism for paraquat (methyl viologen) toxicity. Biochem Biophys Res Commun. 1974; 58:749-755.
36. Bus JS, Aust SD, Gibson JE. Lipid peroxidation: a possible mechanism for paraquat toxicity. Res Commun Chem Pathol Pharmacol. 1975; 11:31-38.
37. Fisher HK, Clements JA, Tierney DF, Wright RR. Pulmonary effects of paraquat in the first day after injection. Am J Physiol. 1975; 228:1217-1223.
38. Keeling PL, Smith LL. Relevance of NADPH depletion and mixed disulphides formation in rat lung to the mechanism of cell damage following paraquat administration. Biochem Pharmacol. 1982; 31:3243-3249.
39. Keeling PL, Smith LL, Aldridge WN. The formation of mixed disulphides in rat lung following paraquat administration. Correlation with changes in intermediary metabolism. Biochem Biophys Acta. 1982; 716:249-257.
40. Brigelius R, Dostal LA, Horton JK, Bond JR. Alteration of the redox state of NADPH and glutathione in perfused rabbit lung by paraquat. Toxicol Ind Health. 1986; 2:417-428.
41. Smith LL, Rose MS, Wyatt I. The pathology and biochemistry of paraquat. Ciba Found Symp. 1978; 65:321-341.
42. Cuzzocrea S, Riley DP, Caputi AP, Salvemini D. Antioxidant therapy: a new pharmacological approach in shock, inflammation, and ischemia/reperfusion injury. Pharmacol Rev. 2001; 53:135-159.
43. Al-Shabanah OA, Mansour MA, Elmazar MM. Enhanced generation of leukotriene B4 and superoxide radical from calcium ionophore (A23187) stimulated human neutrophils after priming with interferon-alpha. Res Commun Mol Pathol Pharmacol. 1999; 106:115-128.
44. Hemmati AA, Nazari Z, Motlagh ME, Goldasteh S. The role of sodium cromolyn in treatment of paraquate-induced pulmonary fibrosis in rat. Pharmacol Res. 2002; 46:229-234.
45. Hybertson BM, Lampey AS, Clarke JH, Koh Y, Repine JE. *N*-acetylcysteine pretreatment attenuates paraquat-induced lung leak in rats. Redox Rep. 1995; 1:337-342.
46. Burk RF, Lawrence RA, Lane JM. Liver necrosis and lipid peroxidation in the rat as result of paraquat and

diquat administration: effect of selenium deficiency. J Clin Invest. 1980; 65:1024-1031.
47. Dicker E, Cederbaum AI. NADH-dependent generation of reactive oxygen species by microsomes in the presence of iron and redox cycling agents. Biochem Pharmacol. 1991; 42:529-535.
48. Aydin S, Aral I, Kilic N, Bakan I, Aydin S, Erman F. The level of antioxidant enzyme, plasma vitamin C and E in cement plant workers. Clin Chim Acta. 2004; 341:193-198.
49. Cantin AM, North SL, Hubbard RC, Crystal RG. Normal alveolar epithelial lining fluid contains high levels of glutathione. J Appl Physiol. 1987; 63:152-157.
50. Maellaro E, Casini AF, Bello BD, Comporti M. Lipid peroxidation and antioxidant systems in the liver injury produced by glutathione depleting agents. Biochem Pharmacol. 1990; 39:1513-1521.
51. Rodriguez C, Mayo JC, Sainz RM, Antolin I, Herrera F, Martin V, Reiter RJ. Regulation of antioxidant enzymes: a significant role for melatonin. J Pineal Res. 2004; 36:1-9.
52. Ross D. Glutathione, free radicals and chemotherapeutic agents. Pharmacol Ther. 1988; 37:231-249.
53. Krall J, Speranza MJ, Lynch RE. Paraquat-resistant HeLa cells: increased content of glutathione peroxidase. Arch Biochem Biophys. 1991; 286:311-315.
54. Maran E, Fernandez M, Barbieri P, Font G, Ruiz MJ. Effects of four carbamate compounds on antioxidant parameters. Ecotoxicol Environ Saf. 2009; 72:922-930.
55. Meister A, Anderson ME. Glutathione. Annu Rev Biochem. 1983; 52:711-760.
56. Kanno S, Matsukawa E, Miura A, Shouji A, Asou K, Ishikawa M. Diethyldithiocarbamate-induced cytotoxicity and apoptosis in leukemia cell lines. Biol Pharm Bull. 2003; 26:964-968.
57. Pena-Llopis S, Pena JB, Sancho E, Fernandez-Vega C, Ferrando MD. Glutathione-dependent resistance of the European eel Anguilla anguilla to the herbicide molinate. Chemosphere. 2001; 45:671-681.
58. Seth V, Banerjee BD, Chakravorty AK. Lipid peroxidation, free radical scavenging enzymes, and glutathione redox system in blood of rats exposed to propoxur. Pestic Biochem Physiol. 2001; 71:133-139.
59. Youngman RJ, Elstner EF. Oxygen species in paraquat toxicity: the crypto-OH radical. FEBS Lett. 1981; 129:265-268.
60. Chen CM, Lua AC. Lung toxicity of paraquat in the rat. J Toxicol Environ Health A. 2000; 60:477-487.
61. Fukushima T, Yamada K, Hojo N, Isobe A, Shiwaku K, Yamane Y. Mechanism of cytotoxicity of paraquat. III. The effects of acute paraquat exposure on the electron transport system in rat mitochondria. Exp Toxicol Pathol. 1994; 46:437- 441.
62. Dean RT, Fu S, Stocker R, Davies MJ. Biochemistry and pathology of radical-mediated protein oxidation. Biochem J. 1997; 324:1-18.
63. Kaur H, Halliwell B. Detection of hydroxyl radicals by aromatic hydroxylation. Methods Enzymol. 1994; 233:67-82.
64. Dinis-Oliveira RJ, Sousa C, Remiao F, Duarte JA, Ferreira R, Sanchez Navarro A, Bastos ML, Carvalho F. Sodium salicylate prevents paraquat-induced apoptosis in the rat lung. Free Radic Biol Med. 2007; 43:48-61.
65. Nicotera TM, Block AW, Gibas Z, Sandberg AA. Induction of superoxide dismutase, chromosomal aberration and sister-chromatid exchanges by paraquat in Chinese hamster fibroblast. Mutat Res. 1985; 151:263-268.
66. Melchiorri D, Ortiz GG, Reiter RJ, Sewerynek E, Daniels WMU, Pablos MI, Nistico G. Melatonin reduces paraquat-induced genotoxicity in mice. Toxicol Lett. 1998; 95:103-108.
67. Yuksel H, Ozbilgin K, Coskun S, Tuglu I. Protective effect of leukotriene receptor antagonist montelukast against smoking induced lung injury in Wister rats. Acta Med Okayama. 2003; 57:13-19.

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Original Article

Comparison between endoluminal ultrasonography and spiral computerized tomography for the preoperative local staging of rectal carcinoma

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Summary **The aim of this study is to compare the efficacy of endoluminal ultrasonography (EUS) and spiral computerized tomography (SCT) in preoperative local staging of rectal carcinoma. EUS and SCT were performed prior to surgery in 78 patients with rectal carcinoma. After radical surgery, the preoperative findings were compared with histologic findings on the surgical specimen, and we assessed the values of EUS and SCT in staging the tumor. For T staging, accuracy was 84.6% for EUS, 70.5% for SCT ($p < 0.05$). For N staging, accuracy was 64.1% for EUS, 61.5% for SCT ($p > 0.05$). EUS is superior to SCT in judging tumor infiltrate depth, but neither could provide satisfactory assessments of lymph node metastases.**

Keywords: Rectal neoplasm, neoplasm staging, endo-luminal ultrasound, computed tomography

1. Introduction

Rectal cancer is a common form of digestive cancer and is responsible for significant morbidity and mortality rates. The decision about appropriate treatment for patients mainly depends on the knowledge of the exact stage. This has greatly increased the importance of accurate preoperative staging in providing information about tumor infiltration and lymph node metastasis. Recently, endoluminal ultrasound (EUS) and spiral computed tomography (SCT) have become one of the important methods for preoperative evaluation of rectal cancer as non-invasive instruments (*1-3*), but a comparison of EUS and SCT in patients with rectal cancer remains controversial (*4*). In this study, both EUS and SCT were performed in 78 patients with rectal cancer to compare the efficacy of EUS and SCT in preoperative local staging of rectal cancer.

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2. Patients and Methods

2.1. *Patient selection*

From October 2006 to June 2008, 78 patients with biopsy-proven rectal carcinoma underwent both SCT and EUS before their operation. There were 42 male and 36 female patients with a mean age of 61 years (range 32 to 78).

2.2. *EUS and SCT examination*

2.2.1. *Participants and procedures*

Before EUS and SCT examinations, all patients were prepared with an enema. We used Technos MPX DU8 (Esaote, Genoa, Italy) with double transducers for EUS. The 10-MHz transducer was used to estimate the invasion of depth of cancers and the 8-MHz transducer was used to detect lymph nodes. Five minutes before the start of SCT, the patients received a rectal enema with 500 mL of air. All patients underwent the same CT protocol using a GE HiSpeed-CT/i scanner (GE Healthcare, Waukesha, WI, USA) with 10-mm slice thickness, 5-mm increment at a table feed of 6 mm/0.75-sec scanner rotation, table speed of 10 mm/sec.

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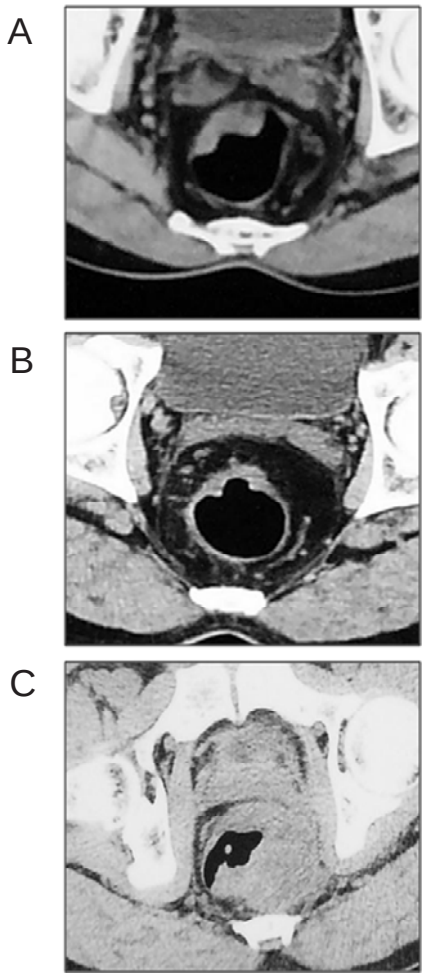

Figure 1. Different tumor stage by SCT. (A) Protrude type tumor in rectal area (T1-2). Tumors confined to the bowel wall. (B) Ulcer type tumor in rectal area (T3). Tumor infiltrates into the perirectal fat. (C) Rectal tumor classified as T4. Tumor infiltrates the left side of pelvic wall.

2.3. *Image analysis*

The depth of tumor infiltration and regional lymph nodes status were assessed as follows. As shown in Figure 1, tumors on SCT were classified by a modified TNM stage. Since SCT could not discriminate wall layers, tumors confined to the bowel wall were classified as T1 or T2. An indistinct or speculated border between the outer rectal wall and the surrounding fat at the level of the tumor was considered as evidence of perirectal invasion (T3). Tumor infiltration into adjacent organs was considered stage T4. Lymph nodes were considered to be positive for metastases if at least one perirectal lymph node with a short-axis diameter of more than 5 mm was found. For EUS, the invasion depth was classified into the following five tumor invasion categories: uT1, into the mucosa or submucosa; uT2, into the muscularis propria; uT3, into the serous membrane; uT4, through the muscularis propria into the adjacent organs. Metastatic lymph nodes were defined as mass lesions over 5 mm in diameter. Figure 2 shows EUS images of different stages according to our criteria.

EUS and SCT staging was compared with both surgical and histopathological results using UICC/AJCC 5th TNM stage (*3*). We defined the clinicopathological features of cancer, including depth of wall invasion and lymph node metastasis. The sensitivity, specificity and accuracy rates were calculated.

2.4. *Statistical analysis*

A chi-square test was performed to assess reliability. A *p* value of less than 0.05 was considered a statistically significant difference.

3. Results

3.1. *Histopathological data*

The histopathological examination showed stage pT1 tumors in 7 patients, pT2 tumors in 25 patients, stage pT3 tumors in 33 patients, and stage pT4 tumors in 13 patients. N staging showed 45 patients without lymph

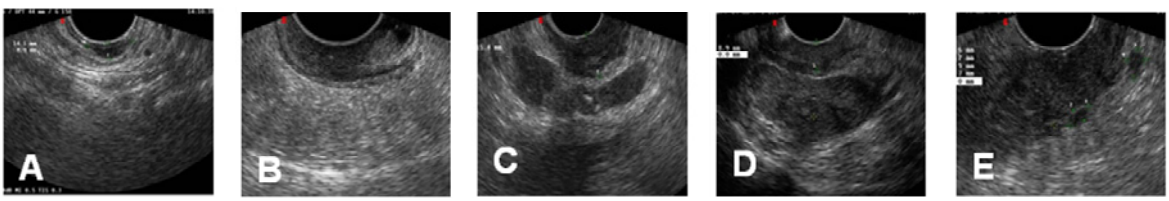

Figure 2. Different tumor stage by EUS. (A) Intramucosal carcinoma in rectal area (uT1). The high-echo band of submucosa was unbroken. (B) Tumor infiltrating into muscle (uT2). The low-echo band of proper muscle layer was broken. (C) Tumor infiltrating the whole rectal wall (uT3). (D) Tumor invading bladder (uT4). (E) Lymph node matastasis (uN1). Multiple low-echo nodules around the rectal wall.

node metastases, whereas 33 patients were classified as stage pN positive.

3.2. *T staging*

As shown in Table 1, the accuracy of EUS was 100% (7/7) for T1, 84.0% (21/25) for T2, 81.8% (27/33) for T3, and 84.6% (11/13) for T4. The accuracy of SCT was 71.9% (23/32) for T1-2, 66.7% (22/33) for T3, and 76.9% (10/13) for T4. The overall accuracy was 84.6% (66/78) and 70.5% (55/78) for EUS and SCT, respectively. There were statistically significant differences between EUS and SCT in diagnosing depth of tumor invasion ($p < 0.05$).

3.3. *N staging*

The result of N staging is summarized in Table 2, the sensitivity of EUS was 54.5% (18/33) and specificity was 71.1% (32/45). For SCT, the sensitivity was 60.6% (20/33) and specificity was 62.2% (28/45). The overall accuracy was 64.1% (50/78) and 61.5% (48/78) for EUS and SCT, respectively. There were no statistically significant differences between EUS and SCT in diagnosing lymph node metastasis ($p > 0.05$).

4. Discussion

Accurate staging of rectal cancer is necessary to provide the optimal treatment strategy. Recently, the benefit of preoperative radiochemotherapy for advanced colorectal cancer had been proven (*6*,*7*), and local resection or laparoscopic surgery can be performed for early colorectal cancer. These advances have greatly increased the importance of accurate preoperative staging in providing information about tumor location, size, configuration, and local infiltration. At present, EUS and SCT as non-invasive instruments have been widely used to provide useful information in assessing rectal wall invasion, infiltration of the mesorectum, and infiltration of the adjacent organs or vessels.

Table 1. A comparison of EUS and SCT in depth of tumor invasion

Histopathological staging (*n*)	EUS staging				SCT staging		
	T1	T2	T3	T4	T1-2	T3	T4
pT1 (7)	7	0	0	0	7	0	0
pT2 (25)	2	21	2	0	16	9	0
pT3 (33)	0	3	27	3	7	22	4
pT4 (13)	0	0	2	11	0	3	10

Table 2. A comparison of EUS and SCT in lymph node metastasis

Histopathological staging (*n*)	EUS staging		SCT staging	
	N(–)	N(+)	N(–)	N(+)
N(–) (45)	32	13	28	17
N(+) (33)	15	18	13	20

Initial studies have reported that accuracy rates of T staging were 53~92% for SCT and 81~93% for EUS (*8-10*). In our study, accuracy was 84.6% for EUS, while SCT had an accuracy rate of 70.5%, suggesting that EUS is superior to SCT in T staging as reported in previous studies. Due to the lack of detailed spatial and contrast resolution, SCT has some limitations for discriminating the rectal wall layers, leading to diminished accuracy for early-stage lesions confined to the rectal wall. Accuracy of SCT for T3 was 66.7%, where 7 patients were underestimated and results failed to reveal depth invasion. SCT showed an accuracy of 76.9% for T4, which suggest that SCT improves T staging accuracy in more localized advanced tumors. The accuracy of EUS was 100% for T1 and 84.0% for T2, indicating that EUS was superior to that of SCT for early stage rectal cancer. In EUS, two T2 patients with tumors of the ulcer type were overstaged as T3. The misinterpretation may be due to fibrosis caused by scarring and inflammation. Accuracy of EUS for T3 was 81.8%, in which peritumoral reaction, comprising fibrosis, inflammation, and congestive changes may have caused the overstaging. There is no significant difference in accuracy of EUS and SCT for T4 (84.6% *vs.* 76.9%), indicating that both can identify invasion into adjacent organs efficiently.

Lymph node involvement is also important for prognosis and treatment planning of rectal cancer. Pervious studies showed accuracy of EUS for N stage ranged from 58% to 83%, and accuracy of SCT ranged from 58% to 83% (*8-10*). In this study, there were no statistically significant differences between EUS and SCT in diagnosing lymph node metastasis (64.1% *vs.* 61.5%). The size and localization of the lymph nodes are important signs for the differentiation of lymph node metastases from reactive lymph nodes. Sensitivity for detecting lymph nodes was usually evaluated according to nodal size. When a lymph node is larger than 10 mm in diameter, the metastatic rate is thought to be higher. However, in some cases, the mean diameter of the metastatic nodes were under 5 mm (*11*), which indicates that dependence on size of the node only would reduce sensitivity for detection of lymph node metastasis. In EUS, a lymph node appears as a low-echo outside the rectal wall, but it is difficult to estimate whether or not it includes a metastatic locus. Morphologic characteristics suggestive of malignant involvement include a hypoechoic appearance, peritumoral location, and irregular shape or uneven echo levels (*12*), therefore, we should combine size with shape and density to estimate whether the lymph node is metastatic or not. Some studies have shown that endoscopic ultrasound-guided fine-needle aspiration demonstrated a trend toward more accurate nodal staging (*13*,*14*). Kim *et al.* found that 3D EUS showed greater accuracy than 2D

EUS or CT in rectal cancer staging and lymph node metastases (*14,15*). There is still a controversial issue in the preoperative staging of lymph node metastasis, and another new criterion should be clarified in a further study.

In conclusion, for the local staging of rectal cancer, our study shows EUS is superior to SCT in judgment for tumor infiltrate depth, but neither method could provide satisfactory assessment of lymph node metastases for rectal cancer.

References

1. Mackay SG, Pager CK, Joseph D, Stewart PJ, Solomon MJ. Assessment of the accuracy of transrectal ultrasonography in anorectal neoplasisa. Br J Surg. 2003; 90:346-350.
2. Siddiqui AA, Fayiga Y, Huerta S. The role of endoscopic ultrasound in the evaluation of rectal cancer. Int Semin Surg Oncol. 2006; 3:36.
3. Chiesura-Corona M, Muzzio PC, Giust G, Zuliani M, Pucciarelli S, Toppan P. Rectal cancer: CT local staging with histopathologic correlation. Abdom Imaging. 2001; 26:134-138.
4. Low G, Tho LM, Leen E, Wiebe E, Kakumanu S, McDonald AC, Poon FW. The role of imaging in the pre-operative staging and post-operative follow-up of rectal cancer. Surgeon. 2008; 6:222-231.
5. Sobin LH, Wittekind C. TNM Classification of Malignant Tumours. 5th ed. Wiley-Liss, New York, NY, USA, 1997.
6. Glimelius B, Holm T, Blomqvist L. Chemotherapy in addition to preoperative radiotherapy in locally advanced rectal cancer - a systematic overview. Rev Recent Clin Trials. 2008; 3:204-211.
7. Delaney CP, Lavery IC, Brenner A, Hammel J, Senagore AJ, Noone RB, Fazio VW. Preoperative radiotherapy improves survival for patients undergoing total mesorectal excision for stage T3 low rectal cancers. Ann Surg. 2002; 236:203-207.
8. Heriot AG, Grundy A, Kumar D. Preoperative staging of rectal carcinoma. Br J Surg. 1999; 86:17-28.
9. Matsuoka H, Nakamura A, Masakki T, Sugiyama M, Takahara T, Hachiya J, Atomi Y. Preoperative staging by multidetector-row computed tomography in patients with rectal carcinoma. Am J Surg. 2002; 184:131-135.
10. Adams DR, Blatchford GJ, Lin KM, Ternent CA, Thorson AG, Christensen MA. Use of preoperative ultrasound staging for treatment of rectal cancer. Dis Colon Rectum. 1999; 42:159-166.
11. Fukuya T, Honda H, Hayashi T, Kaneko K, Tateshi Y, Ro T, Maehara Y, Tanaka M, Tsuneyoshi M, Masuda K. Lymph-node metastases: efficacy with helical CT in patients with gastric cancer. Radiology. 1995; 197:705-711.
12. Bhutani MS. Recent developments in the role of endoscopic ultrasonography in diseases of the colon and rectum. Curr Opin Gastroenterol. 2007; 23:67-73.
13. Shami VM, Parmar KS, Waxman I. Clinical impact of endoscopic ultrasound and endoscopic ultrasound-guided fine-needle aspiration in the management of rectal carcinoma. Dis Colon Rectum. 2004; 47:59-64.
14. Miller L, Smith C, Canto MI. Endoscopic ultrasonography (EUS) and EUS-guided fine needle aspiration for accurate staging of rectal cancer: explanation of tumor staging and a case report. Gastroenterol Nurs. 2000; 23:97-101.
15. Kim JC, Cho YK, Kim SY, Park SK, Lee MG. Comparative study for t hree-dimensional and conventional ultrasonography used in rectal cancer staging. Surg Endosc. 2002; 16:1280-1285.
16. Kim JC, Kim HC, Yu CS, Han KR, Kim JR, Lee KH, Jang SJ, Lee SS, Ha HK. Efficacy of 3-dimensional endorectal ultrasonography compared with conventional ultrasonography and computed tomography in preoperative rectal cancer staging. Am J Surg. 2006; 192:89-97.

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