Protective Effects of *Citrullus vulgaris* on Irradiated Lymphocyte Sub-membrane

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ABSTRAK

Satu kajian telah dijalankan untuk menentukan kesan perlindungan radioaktif Citrullus vulgaris pada bawah membran limfosit terutama sekali di lapisan aktin. Sejumlah 30 ekor tikus jantan dewasa Sprague-Dawley telah bahagikan kepada tiga kumpulan yang terdiri daripada kawalan positif, negatif dan rawatan. Kumpulan kawalan positif dan negatif diberi secara paksa air salina sebanyak 40 ml/kg berat badan setiap hari manakala kumpulan rawatan pula diberikan air jus C. vulgaris segar sebanyak 40 g/kg berat badan setiap hari. Seminggu selepas bermulanya rawatan, kumpulan kawalan positif dan rawatan menerima radiasi gamma sebanyak 90 rad. Limfosit yang hidup ditentukan dengan menggunakan pewarnaan propidium iodin dan akridina jingga dan dilihat dibawah mikroskop floresen. Peratusan limfosit hidup kumpulan rawatan (71.0%; p=0.03) adalah lebih signifikan berbanding kumpulan kawalan negatif. Keputusan ini menunjukkan bahawa C. vulgaris mempunyai kesan perlindungan terhadap radioaktif kerana lapisan aktin pada limfosit tidak musnah. Kesan perlindungan terhadap radiasi ini mungkin disebabkan oleh kehadiran antioksidan dalam C. vulgaris.

Kata kunci: Perlindungan radiasi, Citrullus vulgaris, radikal bebas, limfosit, aktin, antioksidan.

ABSTRACT

A study was conducted to determine the radioprotective effects of Citrullus vulgaris on the lymphocyte sub-membrane particularly the actin layer. A total of 30 adult male Sprague-Dawley rats were divided into three equal groups of positive control, negative control and treatment. The positive and negative control groups were force fed with 40 ml/kg body weight of normal saline while the treatment group received 40 g/kg body weight of fresh juice of C. vulgaris daily. After a week the positive control and treatment groups were irradiated with 90 rad gamma radiation. Viable lymphocytes were determined using propidium iodine and acridine orange stain and observed under a fluorescent microscope. The percentage of viable lymphocytes of the treatment group (71.0%; p = 0.03) was significantly higher than the positive control group. The results showed that C. vulgaris possessed radioprotective effects because the lymphocyte actin was not damaged. The radioprotection effects could be due to the presence of antioxidants in C. vulgaris.

Key words: Radioprotection, Citrullus vulgaris, free radical, actin, lymphocytes, actin, antioxidant

INTRODUCTION

In general radiation therapy is commonly used as a treatment for blood and metastasized cancers (Allal et al. 2004). Despite its wide use by oncologists the treatment itself would cause various side effects. One of the most notable effects is low immunity. Recent findings have indicated that the immunity of rats exposed to 2.0 Gy silicon ions (28Si) was significantly reduced due to the declining amount of lymphocytes in circulation (Gridley et al. 2002). Current hypothesis feel that this phenomenon is particularly due to the inability of the lymphocytes to withstand above average radiation (Verastegui et al. 2003).

Until now it has been suggested that lymphocytes destruction is mainly due to the destruction of DNAse inhibitor I (Proomwichit et al. 1982). Due to this reduction, DNAase activity within the lymphocyte's nucleus increases dramatically. This will then lead to DNA destruction and eventually initiating the apoptosis mechanism (Proomwichit 1983; Zimowska et al. 1997). Recent findings by our group have shown that the destruction of the lymphocytes might also be due to the loss of integrity of the cell membrane (Yuen 2002). Initial study had indicated that when gamma radiation was given, water molecules within the body would break up and form various reactive free radicals. Among those that had been identified were hydroxyl, superoxide, alkyl, alpha-hydroxyalkyl, beta-dihydroxyalkyl and proxyl radicals (Shadyro et al. 2003). All these radicals are very reactive and have been known to specifically react on the polyunsaturated fatty acid (PUFA) components of cell membrane and eventually form pores that would allow cytosol components to escape. Recently it was indicated that free radicals were also capable of immobilizing cells by attacking their sub-membrane components particularly the actin layer (Allani et al. 2004). When extensive damage has occurred to the layer, the apoptosis pathway will be activated through a non-nucleated specific second messenger pathway (Shojaee et al. 1999).

Destruction of lymphocytes can be reduced through suppression of free radical formation within the body and this can be achieved by using radioprotective chemicals. In general various –SH based chemical like trypanothione and amifostine have been used (Awad et al. 1992; Giannopoulou et al. 2002). Although they are able to produce some radioprotection they are also highly toxic. Natural products that contain high amounts of antioxidants particularly vitamin A and C, but much less toxic can be used as alternatives. Preliminary studies had indicated that the adult male Sprague-Dawley rats which had consumed 40 g/kg body weight of *Morinda citrifolia*, *Mangifera indica*, *Averrhoa carambola* juices and were later exposed to 90 rads of gamma showed higher percentage of viable lymphocytes than those that were in the positive control group (Rachel 2003). Recently *Citrullus* sp. was shown to contain a high amount of antioxidants particularly lycopene and carotenoids (Edwards et al. 2003). In this study we would like to identify the radioprotective effects of *Citrullus vulgaris* on the lymphocyte sub-membrane specifically the actin layer.

MATERIAL AND METHOD

A total of 30 adult male Sprague-Dawley rats were obtained from the Animal Unit, Faculty of Medicine, Universiti Kebangsaan Malaysia. The rats were then divided into three equals groups of positive control, negative control and treatment. The positive

control and negative control groups were force fed with normal saline of 40 ml/kg of body weight while the treatment group received 40 g/kg body weight of fresh juice of *Citrullus vulgaris* daily. After a week, the positive control and treatment groups were irradiated with 90 rad of gamma radiation. After three hours, a minimum of 5 ml blood was extracted through a myocardium puncture. The lymphocytes viability was determined by propodium iodide-acridine orange staining. This was carried out by mixing 10 µl 1.5 mM propidium iodide, 10 µl 1.5 mM acridine orange and 20 µl of fresh whole blood in an eppendorf tube. They were then left at 20^{o}C for one minute to ensure proper absorption of the stains into the cell. The blood was then transferred into a capillary tube and centrifuged for 10 minutes (Michie et al. 2003). The buffy layer was then transferred on to a glass slide and the cells were then observed under a Leitz LaborLux S fluorescent microscope (Germany) and documented using Kodak EasyShare CX4230 (USA). Viable lymphocytes were determined by an absence of florescent orange in the nucleus and the presence of a fluorescent green on the membrane wall. Non-viable lymphocyte cells were determined by the presence of florescent orange in the nucleus and fluorescent green on the membrane wall.

The lymphocyte actin layer was determined by putting the lymphocyte on a glass slide as mentioned in the above. The cells were then fixed in –20^{o}C methanol for 3 minutes and later washed in Tris buffer saline (TBS) for another 3 minutes. Later TBS 0.5% Triton-X was added for 10 minutes and followed by washing with the solution thrice (5 minutes per wash). Antibody dilution solution (AbDil) (Molecular Probes Inc., Eugene, Oregon, USA) was then added and left for another 10 minutes. Conjugated actin Alexa Fluor 488 obtained from Molecular Probes Inc. and diluted with AbDil in dilution of 1:200 was then added (Schmid et al. 2002). The slide was then left for 2 hours before it was then washed again 5 times with TBS 0.1% Triton X (2 minutes per wash). Finally the slides were then washed in TBS and left to dry in the dark at room temperature for 30 minutes. Prior to documentation the slides were soaked in normal saline. Documentation was done using the same equipment mentioned above. All test were done in triplicate for every animal. Significant changes in cell count was determine using one way ANOVA with a level of significant of $p < 0.05$.

RESULTS AND DISCUSSION

The results showed that the percentages of viable lymphocyte cells for the positive control, negative and treatment groups were at 33.5, 80.3 and 71.0%, respectively (Fig 1). Statistical analysis using one way ANOVA confirmed that the changes seen in the treatment group were significant compared to the positive and negative controls. The actin intensity was much more dominant in the negative group, followed in descending order by the treatment group and the positive control (Fig. 2).

The results indicated that *Citrullus vulgaris* at 40 g/kg body weight was able to retain lymphocyte by more than 37.5% when compared to the positive control group ($p=0.03$). A study on the lymphocyte membrane had also showed that free radicals were attacking the sub-membrane layer particularly the actin. Radioprotective effects produced by *Citrullus vulgaris* observed in this study could be due to the presence of three major

antioxidants – vitamin A, vitamin C and lycopene (Edwards et al. 2003; Rimando & Perkins-Veazie 2005).

During gamma radiation, both vitamin A and C quickly scavenge free radicals that are produced from water molecules. In this process vitamin A and C will form carotenoid radical cation and ascorbic acid radical, respectively (Mortensen et al. 1997; Polidori et al. 2004). Although these by-products are classified as free radicals themselves, their reactivity were much lower than the previous radical species. The reduction of reactive species will then result in a much lower rate of attack on the lymphocyte membrane PUFA layer. Apart from the direct action, vitamin C could also play a direct role in reducing cells own production of free radical by increasing cellular redox processes mediated by the up-regulation of the glutathione pathway (Harapanhalli et al. 1996).

As with vitamin A, lycopene is suspected to work by a direct action through its ability to scavange free radicals. In this process the formation of rhodamine 123, a radical from dihydrorhodamine 123, could have been partially inhibited (Panasenko et al 2000; Yeum et al. 2003; Black & Lambert 2001; Gupta & Kumar 2002). The radioprotection produced by the *Citrullus vulgaris* juice could possibly be contributed mainly by the antioxidant effects of lycopene. Lycopene is well known to quench singlet oxygen twice more effectively than beta-carotene (Rousseau et al. 1992).

The very low intensity of the actin expression in the lymphocyte sub-membrane of the positive control clearly indicated that free radicals had indeed played a role in the destruction of the actin (Fig 2) . This observation confirmed earlier results published by other workers (Butterfield 2002; Rimando & Perkins-Veazie 2005). It was proposed that when pores had formed in the cells membrane, the free radicals would then attack the actin layer that allows the cells to move. Immobilization and very likely myosin translocation to the cytoskeleton would then cause cell death by apoptosis (Petersen et al. 2000).

Despite this encouraging results, the role of other constituents in the juice of *Citrullus vulgaris* in radioprotection should not be ignored. Significant changes in the actin florescent intensity should also be identifed statically by utilizing quantitation software. In conclusion, *C. vulgaris* showed protective effects on irradiated lymphocyte sub-membrane. Its possible use as a radioprotection during cancer therapy must be further studied since recent report has indicated that use of antioxidants in some cancers would only create a much more resistant cancer cells (Panasenko et al. 2000).

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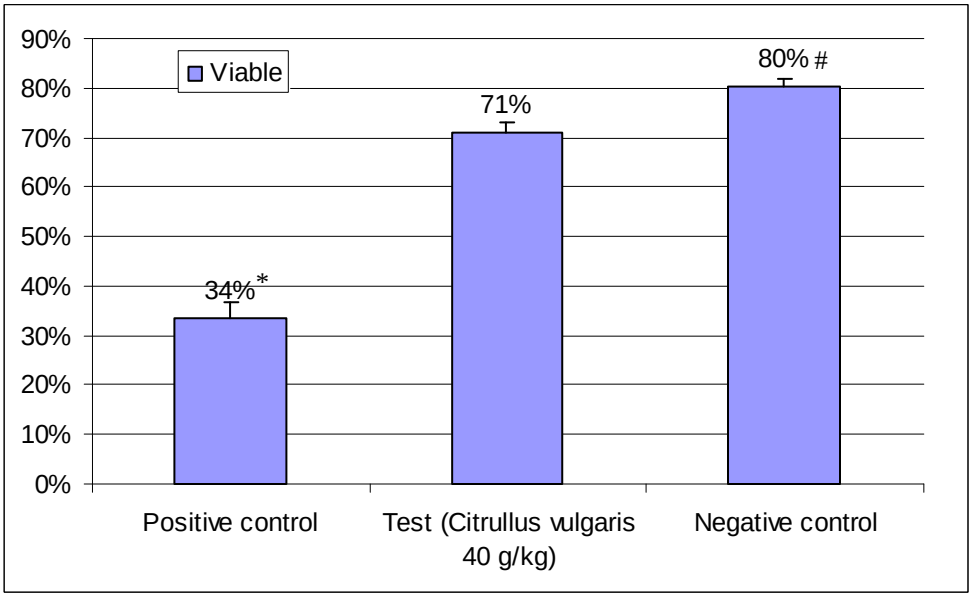

* : Significant difference (p = 0.03) compared to test group.
# : Significant difference (p = 0.04) compared to test group.

FIGURE 1 Percentage of viable lymphocyte based on group.

FIGURE 2 Actin intensity based on group. Magnification 40 x.