Journal of Life Science **2004**, Vol. 14. No. 2. 291~295 ©JLS

Neuronal Protection by Rooibos (*Aspalathus linearis*) Tea Infusions in a Hypoxic Model of Cultured Rat Cortical Neurons

Il Soo Moon* and Bok Hyun Ko

Synaptech Inc. and Department of Anatomy, College of Medicine, Dongguk University, Gyeongju 780-714, Korea.

Received November 21, 2003 / Accepted February 15, 2004

Rooibos (*Aspalathus linearis*) (RB) is a leguminous shrub native to the mountainous areas of the northwestern Cape Province in South Africa. RB tea infusions are the fermentation products of its leaves and fine stems, and known to have a high antioxidative activity due to the presence of flavonoids and phenolic acids. We investigated the effects of RB tea on the alleviation of oxidative stress on cultured rat cortical neurons in a hypoxic model. Measurement of lactate dehydrogenase (LDH) released into culture media revealed that RB increased cell viabilities in both normoxia (6-18%) and hypoxia (2-24%) dose-dependently (10-100 μg/ml) on 16 days *in vitro* (3 days after treatment). Visualization of cell morphology by expression of GFP-Hsc70 fusion protein showed that RB (50 μg/ml) reduced the average vacuolated soma from 55.4±4.59% (no RB addition) to 40.9±6.3% (RB addition) on 5 days after hypoxia. Our results proves efficacy of RB in the neuroprotection of hypoxic neurons and extend application for RB into the prevention and/or treatment of neuronal damages.

Key words – cortical culture, hypoxia, LDH, neuron, Rooibos

Rooibos (*Aspalathus linearis*) (RB) is a leguminous shrub native to the mountainous areas of the northwestern Cape Province in South Africa[1]. The Rooibos plant is recognized as one of the relatively few economic plants that has made the transition from a local wild resource to a cultivated crop. Its leaves and fine stems are used for the production of Rooibos tea; the leaves and stems are cut into 3-4 mm lengths, rolled, fermented by leaf enzymes, and dried in the sun[2]. Rooibos tea is a beverage rich in volatile compounds, polyphenols, flavonoids, minerals, caffeine-free with a low tannin content (as gallic acid)[3,4]. Because of these characteristics, RB tea is rapidly gaining in popularity as a health beverage.

Previous research on the presence of flavonoid compounds in RB tea revealed the occurrence of the flavonol quercetin and its 3-O-β-D-glucopyranoside derivative (isoquercitrin) and the quercetin-3-O-rutinoside (rutin)[4]. The presence of the aglycons, dihydrochalcones, and the flavones and their 4'-deoxy analogues has also been described[4]. Contents of catechin, procyanidin B3, and a profistinidin triflavanoid are extremely low, and underline the claim that RB tea has a low tannin content[4]. The phenolic acids present in RB tea are caffeic acid, ferulic acid, p-coumaric acid, p-hydroxybenzoic acid, vanillic acid, and protocatechuic acid[4], and syringic acid[5].

RB tea infusions have a good antioxidative activity[6], which can be attributed to the presence of flavonoids and phenolic acids. These compounds are known to play an important role in reducing the formation of reactive oxygen species (ROS) by catalyzing redox reactions and chelating metal ions such as Cu^{2+} and Fe^{2+}, involved in ROS production[7]. Despite the high antioxidative activity of RB tea, studies on its effects on neuronal cells are not available. We reasoned that RB tea would have a good neuroprotective effect in hypoxia, in which condition ROS are significantly formed. For this purpose, we took advantage of neuronal cultures and show that RB alleviates oxidative stresses and helps neuronal survival in hypoxia.

Materials and Methods

Preparation of RB

The concentrated water infusion of RB fermentation product for tea manufacture was a kind gift from Rooibos Korea (Seoul, Korea). The infusion was centrifuged at 15,000 rpm in a lap-top microcentrifuge. The supernatant was diluted in water and stored at -20°C in small aliquots. Dry weight was measured by averaging three freeze-dried 1 ml solutions.

***Corresponding author**
Tel : +82-54-770-2414, Fax : +82-54-770-2447
E-mail : moonis@dongguk.ac.kr

Dissociated rat cortical cell cultures

Embryonic day 18 (E18) rat (Sprague-Dawley) cortical neurons were dissociated and maintained as described in Brewer *et al.*[8] with some modifications. In brief, E18 cerebral cortices were dissected and chopped into small pieces in Hank's Balanced Salt Solution (HBSS) w/o Ca^{2+} and Mg^{2+} (Gibco BRL, Grand Island, NY) supplemented with 1 mM sodium pyruvate and 10 mM HEPES, pH 7.4. Individual cells were isolated by trituration using a fire-polished Pasteur pipette. Divalent cations were restored by dilution with 2 volumes of HBSS containing Ca^{2+} and Mg^{2+}. Non-dispersed tissues were let to settle for 3 min and the supernatant containing individual cells was transferred to a 15 ml tube. Cells were collected by centrifugation for 1 min at 200×g. Pellets were resuspended and plated in the poly-D-lysine coated wells with Neurobasal/B27 (Gibco BRL) containing 0.5 mM glutamine, 25 mM glutamate, and various concentrations of TCDD (0-50 nM). Cells were maintained in a humidified CO_2 incubator (95% air/5% CO_2, 37°C) and, one-third of the medium was replaced with new Neurobasal/B27 without glutamate every 3 days.

Treatment of RB and hypoxia

RB was added on 13 days in vitro (DIV) at various concentration directly to culture media (total medium was 300 μl in 24-well culture plates) and hypoxic shock was given on 16 DIV by incubating the culture plates in 2% O_2/5% CO_2, at 37°C for 3 hr. After shock, the plates were returned to the normoxic incubator and further incubated.

Neuronal transfection

On 13 DIV, media was changed with 400 μl of new Neurobasal/B27. Lipofectamine 2000™ (2 μl; Invitrogen Inc.) was diluted in 50 μl of OptiMEM (Invitrogen Inc.) and 1 μl (0.5 μg/μl) of pC1 (control GFP plasmid) or pC1-Hsc70 plasmid DNA was diluted in 50 μl OptiMEM. Diluted solutions were immediately mixed by a brief vortex. After 2-3 min at RT, the two solutions were combined and mixed by a brief vortex. After 15 min, the combined solution (100 μl) was added to the culture wells. The fluorescence images were observed in a CCD-camera-mounted fluorescence microscope (Leica, Germany) and images were acquired with 20 × or 40 × objective lens. Acquired images were processed in Adobe Photoshop software for optimization.

Viability and statistics

Cell viability was assessed by measuring lactate dehydrogenase (LDH) released into culture broth (LDH assay) on the indicated days and expressed in percentages compared to control cultures. The statistical significance was analyzed by Mann-Whitney *U*-test. Values are expressed in mean±SD.

Results

Neuronal protection by RB in normoxia

Since living *in vitro* itself is a stress for dissociated neurons, it is expected that neurons tend to die after long-term incubation *in vitro*. Therefore, we first investigated whether RB would have a positive effect on neuronal survival in normoxic culture conditions. In our experience neurons tend to deteriorate after 2 weeks *in vitro*. RB was added on 13 DIV at various concentration (0-150 μg/ml) and cell viabilities were assessed on 16 DIV by LDH assay. As shown in Fig. 1, RB increased cell viabilities by 6-18% dose-dependently. The best effectiveness was achieved at the range of 50-100 μg/ml ($p<0.05$) with statistical significance. At the 100 μg/ml concentration, RB had no positive effect but exhibited no toxicity. These results indicate that RB has a positive effect on neuronal survival in normoxic culture conditions.

Neuronal protection by RB in hyoxia

Next, we investigated whether RB could mitigate hypoxic stress. RB was added to culture media on 13 DIV at various concentrations (0-150 μg/ml). After 3 days (16

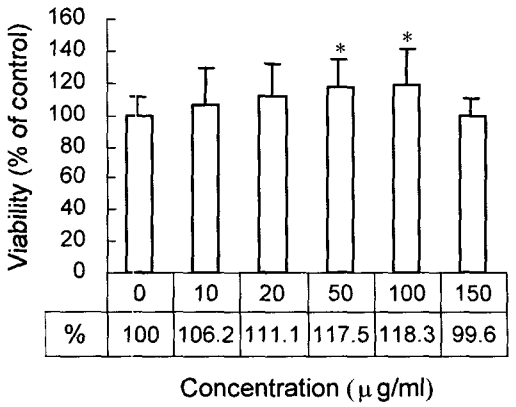
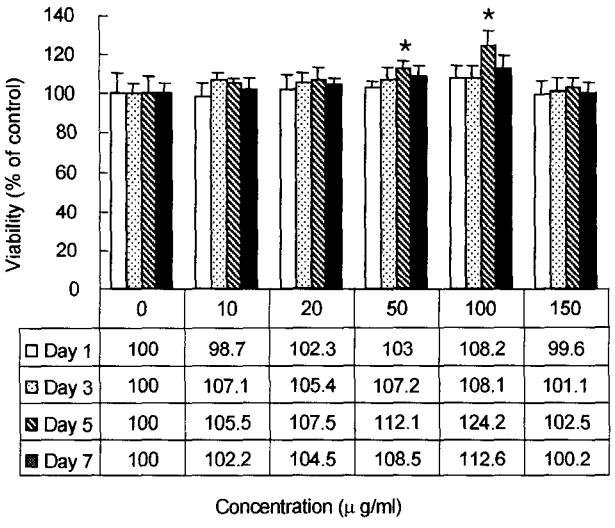


Fig. 1. Effects of RB on neuronal viability in normoxia. E18 cortical cells were grown in the Neurobasal medium with B27 supplements. RB was added on 13 DIV and neuronal viability was assessed on 16 DIV by LDH assay. *, $p<0.05$.

DIV), cells were given a hypoxic shock (2% O_2/5% CO_2, 37°C, 3 hr), and further incubated in normoxic conditions. Cell viabilities were assessed on 1, 3, 5, 7 days after shock. As shown in Fig. 2, RB exhibited dose-dependently positive effects on cellular viabilities. The most positive effect (24.2±7.9%, $p<0.05$) was shown at 100 μg/ml on 5 days after shock. In general, better effects were associated with 50-100 μg/ml and the effect was maintained until 7 days after shock which was the longest day we tested. Again, high concentration (100 μg/ml) was not effective but had no negative effects. These results indicate that RB can significantly reduce the hypoxic stress in cultures.

Direct visualization of neuronal protection by RB in hypoxia

Since RB exhibited positive effects on neuronal protection in hypoxia, we carried out direct visualization of morphological protection in hypoxia. For this purpose we took advantage of green fluorescent protein (GFP) to visualize the detailed morphology of neurons. Since transfection itself is another stress, cells transfected by pC1 (GFP control) plasmids die in about 2 days after transfection (data not shown). Since this time window is too narrow to observe the effect of RB, which exhibits best effectiveness on 5 days after hypoxic shock, we overcame this time window by transfecting pC1-Hsc70 plasmids. Before addition of RB, cultures were transfected with pC1-Hsc70 on 13 DIV and hypoxic shock was given on 16 DIV. Although overexpression of Hsc70 itself had neuroprotection effects, relative effects of RB could also be detected in this protocol. On 5 days after hypoxia, transfected neurons in wells with no RB addion exhibited vacuoles frequently in soma (Fig. 3A, long arrowhead). The dendritic filopodia and spines became much shorter (Fig. 3 inset, short arrowheads). When hypoxic shock was given in the presence of RB (50 μg/ml), there appeared vacuolated soma in much less frequency (Fig. 3B) and dendritic filopodia and spines remained robust (Fig. 3 inset, small arrowheads). Apparently, axons were not affected in morphology, regardless of RB addition in this condition (Fig. 3, arrows). Statistical analysis indicated that addition of RB (50 μg/ml) reduced the average vacuolated soma from 55.4±4.59% (no RB addition) to 40.9±6.3% (RB addition) (Fig. 4). This result confirms the neuroprotective effects of RB in hypoxia.



	0	10	20	50	100	150
Day 1	100	98.7	102.3	103	108.2	99.6
Day 3	100	107.1	105.4	107.2	108.1	101.1
Day 5	100	105.5	107.5	112.1	124.2	102.5
Day 7	100	102.2	104.5	108.5	112.6	100.2

Concentration (μ g/ml)

Fig. 2. Neuroprotective effects of RB in hypoxia. E18 cortical cells were grown in the Neurobasal medium with B27 supplements. RB was added on 13 DIV at the indicated concentrations, a hypoxic shock was given on 16 DIV (2% O_2/5% CO_2, 37°C, 3 hr), and further incubated in normoxic conditions. Cell viabilities were assessed by LDH assays on 1, 3, 5, 7 days after shock, and expressed as % of control. *, $p<0.05$.

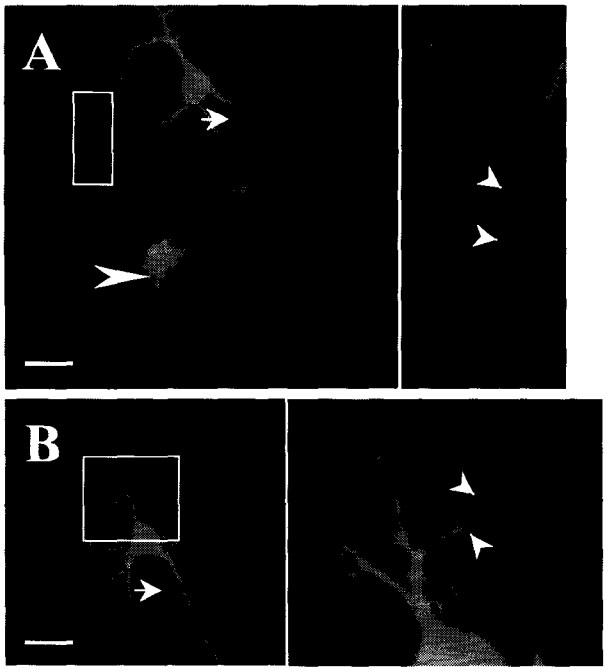


Fig. 3. Direct visualization of neuronal protection by RB in hypoxia. Before addition of RB (50 μg/ml) on 13 DIV, cultures were transfected with pC1-Hsc70 for 5 hr and hypoxic shock (2% O_2/5% CO_2, 37°C, 3 hr) was given on 16 DIV. A, RB added. B, not added. Insets show magnified images of the corresponding area (boxed). Dendritic filopodia or spines (small arrowheads), axons (arrows), and vacuoles formed in soma are marked. Bar, 20 μM.

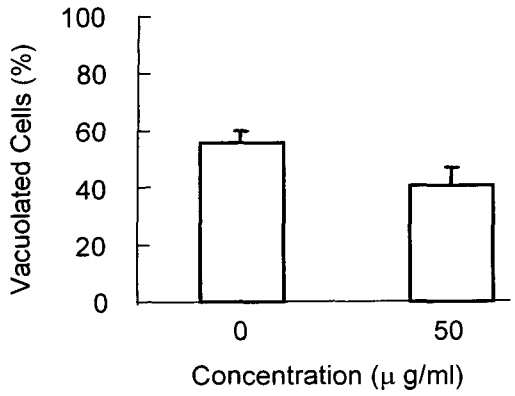


Fig. 4. Reduction of vacuolated soma by RB. Transfected neurons in the experiment for Fig. 3 were statistically analysed. The percentages of vacuolated soma from three sister wells were calculated from about 40 transfected neurons. The number represents average of three independent experiments.

Discussion

In this study, we have shown the neuroprotective effects of RB in a hypoxic model of rat cortical cultures. In addition, we have shown that RB has a supportive function in neuronal survival in normoxia and the morphological robustness of neurons in hypoxic conditions were directly visualized by expressing GFP-Hsc70 fusion proteins.

Our results indicated that RB has dose-dependent efficacy for neuroprotection in both normoxia and hypoxia in cultures. Formation of reactive oxygen species (ROS) is well documented in hypoxia (see a recent review by Hermes-Lima and Zenteno-Savin[9]). At present we do not know how RB reduced the oxidative stress. The term ROS includes superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\cdot}OH$), singlet oxygen, ozone, lipid peroxides, nitric oxide (NO) and peroxynitrite ($ONOO^-$). Endogenous antioxidant defenses are crucial for the control of ROS-mediated oxidative damage of biomolecules, including proteins, RNA, DNA and membrane lipids. When the rate of ROS formation is excessive, such as the hypoxic model used in this study, it can overwhelm the antioxidant capacity of organisms, creating oxidative stress [10]. Organisms are able to adapt themselves to ROS by increasing the expression of antioxidant enzymes and many other forms of defense/response and repair of oxidative damage[11]. Indeed, it is currently known that over 100 genes are activated upon exposure of mammalian cells to ROS[12]. Therefore, RB may have induced such genes that resulted in the alleviation of the oxidative stress.

Another possibility, which is more likely, for the protection mechanism of RB is direct scavenge of ROS by the tea infusions. It is well known that RB have a good antioxidative activity[6], which can be attributed to the presence of flavonoids and phenolic acids. These compounds are known to play an active role in reducing the formation of ROS[7]. In addition, the highly oxidizing ROS are reduced by flavonoids, which are transformed in less aggressive carboxyl radicals[13]. Recently, Bramati *et al.* [14] carried out quantitative characterization of flavonoid compounds in RB tea. They developed an HPLC method using a C18 reversed phase column for the assay of 10 flavonoids in aqueous and methanolic infusions. They found the main compounds to be the dihydrochalcone aspalthin, rutin, and orientin in the range of 1.0 to 1.3 mg/g. High contents of flavonoids and phenolic acids in RB tea may remove the ROS produced in hypoxia, and may explain the neuroprotective effects exhibited in the present study.

RB tea is consumed for calming digestive disorders and various stomach problems, for reducing nervous tension, and alleviating allergies[1]. It is used also for topical treatment of dermatological diseases, such as Behcet's disease and photosensitive dermatitis[15]. Recently, RB has gained much attention for clinical purposes. Our results provide a framework for its application in the prevention and/or treatment of neuronal disease such as delayed neuronal death after stroke or perinatal hypoxia.

Acknowledgment

This work was supported by the Dongguk Universtiy Research Fund. We thank S.-J. Cho for neuronal cultures.

References

1. Morton, J. F. 1983. Rooibos tea, Aspalathus linearis, a caffeinless, low-tannin beverage. *Econ. Bot.* **37**, 164-173.
2. Joubert, E. 1988. Effect of batch extraction condition on yield of soluble solids from Rooibos tea. *Int. J. Food Sci. Technol.* **23**, 43-47.
3. Habu, T., R. A. Flath, T. R Mon and J. F. Morton. 1985. Volatile components of Rooibos tea (Aspalathus linearis). *J. Agric. Food Chem.* **33**, 249-254.
4. Rabe, C., J. A. Steenkamp, E. Joubert, J. F. W. Burger and D. Ferreira. 1994. Phenolic metabolites from Rooibos tea

(Aspalathus linearis). *Phytochemistry.* **35**, 1559-1565.
5. Ferreira, D., C. Marais, J. A. Steenkamp and E. Joubert. 1995. Rooibos tea as a likely health food supplement. *In* Proceedings of Recent Development of Technologies on Fundamental Foods for Health; Korean Society of Food Science and Technology: Seoul, Korea. pp. 73-88.
6. Von Gadow, A., E. Joubert and C. F. Hansmann. 1997. Comparison of the antioxidant activity of Rooibos tea (Aspalathus linearis) with green, oolong and black tea. *Food Chem.* **60**, 73-77.
7. Kameoka, S., P. Leavitt, C. Chang and S. M. Kuo. 1999. Expression of antioxidant proteins in human intestinal Caco-2 cells treated with dietary flavonoids. *Cancer Lett.* **146**, 161-167.
8. Brewer, G. J., J. R. Torricelli, E. K. Evege and P. J. Price. 1993. Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-free medium combination. *J. Neurosci. Res.* **35**, 567-576.
9. Hermes-Lima, M. and T. Zenteno-Savin. 2002. Animal response to drastic changes in oxygen availability and physiological oxidative stress. *Comp Biochem Physiol C Toxicol Pharmacol.* **133**, 537-56.
10. Sies, H. 1986. Biochemistry of oxidative stress. *Angew. Chem. Int. Ed. Engl.* **25**, 1058-1071.
11. Demple, B. 1999. Radical ideas: genetic responses to oxidative stress. *Clin. Exp. Pharmacol. Physiol.* **26**, 64-68.
12. Allen, R. G. and M. Tresini. 2000. Oxidative stress and gene regulation. *Free Radical Biol. Med.* **28**, 463-499.
13. Pietta, P. G. 2000. Flavonoids and antioxidants. *J. Nat. Prod.* **63**, 1035-1042.
14. Bramati, L., M. Minoggio, C. Gardana, P. Simonetti, P. Mauri and P. Pietta. 2002. Quantitative characterization of flavonoid compounds in Rooibos tea (Aspalathus linearis) by LC-UV/DAD. *J. Agric. Food Chem..* **50**, 5513-5519.
15. Shindo, Y. and K. Kato. 1991. Effect of Rooibos tea on some dermatological diseases, pp. 385-389, *In* Proceedings of the International Symposium on Tea Science; The Organizing Committee of ISTS: Shizuoko, Japan.

초록 : 흰쥐 대뇌세포배양의 저산소증모델에서 루이보스차 침제에 의한 신경세포 보호작용

문일수* · 고복현
(동국대학교 의과대학 해부학교실)

루이보스(*Aspalathus linearis*; RB)는 콩과식물로서 남아프리카 Cape Province의 북서부 산악지역에 자생한다. 루이보스차 침제는 가는 가지와 잎의 발효산물로서 플리보노이드류와 페놀산이 있어 강한 항산화활성을 갖는 것으로 알려져 있다. 본 연구에서는 저산소증모델에서 루이보스차 침제가 배양한 흰쥐 대뇌세포의 스트레스를 완화하는지에 대한 연구하였다. 배지로 누출된 LDH의 정량실험에 의하면 루이보스는 정상산소환경 및 저산소증에서 함량 의존적으로(10-100 μg/ml) 각각 6-18% 및 2-24%의 세포생존율을 증가시켰다(16 DIV 세포, 처리 후 3일째). GFP-Hsc70 단백질을 표현시킨 신경세포의 모양을 관찰하였을때 루이보스(50 μg/ml)는 저산소처리 후 5일에 세포체에 수포가 있는 세포의 수를 대조군(55.4±4.59%)에 비하여 유의하게 감소시켰다(40.9±6.3%). 이러한 결과들은 루이보스차가 저산소증에서 신경세포를 보호함을 의미하며, 신경세포 손상을 예방 또는 치료하는데 응용될 수 있을 것으로 보인다.