Turk J. Pharm. Sci. 5 (2) 67-74, 2008

Original Article

INHIBITORY EFFECTS OF ESSENTIAL AMINO ACIDS ON THE PEAK CHEMILUMINESCENCE OF THE CELL-FREE SYSTEMS

Funda AYDEMİR, Nilüfer N. TURAN, Feyza ADEMOĞLU, Bilgen BAŞGUT*, Meltem SARIAHMETOĞLU, İclal ÇAKICI

Gazi University, Faculty of Pharmacy, Department of Pharmacology,
06330 Etiler - Ankara,TURKEY

Abstract

The inhibitory effect of essential amino acids in aqueous solutions, on the peak chemiluminescence of hydrogen peroxide (H_2O_2), hypochlorous (HOCl) (derived from NaOCl) or peroxynitrite ($ONOO^-$) was evaluated by using flow injection analysis (FIA)-luminol chemiluminescence (CL) method. The chemiluminescence was measured as the luminometer output in mV and the effects of amino acids were considered by the depression of the signal from its uninhibited level and expressed as the percentage attenuation of the peak chemiluminescence due to the oxidant. The luminol-H_2O_2, HOCl or $ONOO^-$-induced CL signal was inhibited by either amino acids or ascorbate (well characterized, chain-breaking antioxidant) in a concentration-dependent manner. The log IC_{50} values of the inhibitor effect of amino acids were calculated and found that histidine and tryptophan displayed the most potent inhibitory activity against H_2O_2 or HOCl induced peak chemiluminescence, respectively. Results were concluded that the inhibitory effect of the essential aminoacids on the peak chemiluminescence might represent their free radical scavenger activity.

Key Words: *Antioxidant effect, Luminol chemiluminescence, FIA, Amino acids, Ascorbate*

Esansiyel Aminoasitlerin Hücresiz Sistemlerde Pik Kemiluminesansi Üzerine İnhibitör Etkileri

Esansiyel aminoasitlerin hidrojen peroksit (H_2O_2), hipokloröz (HOCl), ve peroksinitritle ($ONOO^-$) oluşan pik kemiluminesansı üzerindeki inhibitör etkileri akışa injeksiyon analiz (FIA)-luminol kemiluminesans yöntemi kullanılarak incelenmiştir. Kemiluminesans milivolt (mV) cinsinden ölçülmüş ve antioksidanların etkileri sinyalin tepe noktasından, inhibe edilmediği seviyeye kadar olan kısmı ölçülerek, oksidana bağlı olarak oluşan maksimum kemiluminesansın yüzde değişimi olarak ifade edilmiştir. Esansiyel aminoasitler ve askorbat, H_2O_2, HOCl ve $ONOO^-$ ile indüklenen luminol kemiluminesans sinyalini konsantrasyona bağımlı olarak inhibe etmişlerdir. Aminoasitlerin inhibitör etkilerine ilişkin log IC_{50} değerleri hesaplanmış ve H_2O_2 ile indüklenen pik kemiluminesansına karşı en potent inhibitor etkiye sahip aminoasit olarak histidin bulunurken, HOCl ile indüklenen kemiluminesansı en potent olarak inhibe eden aminoasit olarak triptofan bulunmuştur. Bu sonuçlar, esansiyel aminoasitlerin pik kemiluminesansı üzerindeki inhibitor etkisinin, serbest radikal süpürücü etkilerinin bir sonucu olabileceğini gösterir.

Anahtar Kelimeler: *Antioksidan etki, Luminol kemiluminesansı, FIA, Aminoasitler, Askorbat*

***Correspondence:** Phone: +90 312 2023123; Fax: +90 312 2235018
E-mail: bilgenbasgut@gmail.com

INTRODUCTION

A principal characteristic of living organism is their capability of actively protect themselves against uncontrolled oxidation. Detoxification of pro-oxidative molecules is ensured by enzymatic and non enzymatic compounds. A number of studies have shown that amino acids are protective against reactive oxygen species (1-3). Although the antioxidant activity of amino acids was investigated by using some direct and indirect methods, there was no study in the literature investigating the antioxidant activity of essential amino acids by using flow injection analysis (FIA) coupled to luminol chemiluminescence (CL). CL is a term used to describe the light emission that is produced by a chemical reaction (4). It has been widely used as a sensitive detection method for production of reactive oxygen species from cell, enzyme and organ systems (5-8). Associated with FIA, CL detection allows the determination of a wide variety of biological parameters (9,10).

The aim of the present study is to investigate the effects of amino acids on HOCl, H_2O_2 and $ONOO^-$-induced CL and to compare the inhibitory efficacy with a standart antioxidant agent. In the present study, the effects of essential amino acids have been evaluated by using FIA-CL method. FIA- CL is a well established tecnique for rapid and quantitative analysis of the antioxidant activity of the chemicals (11).

EXPERIMENTAL

Apparatus

The flow injection manifold used is shown in Figure. 1. The peristaltic pump was a Gilson Minipuls 2 and the injection valve was a Rheodyne RH-5020, obtained from Anachem (Luton, Bedfordshire, UK). The pump tubing was Elkay Accurated, of suitable internal diameter to deliver the required flow-rate, obtained from Elkay Laboratory Products, (Basingstoke, Hampshire, UK). The remainder of the flow-injection manifold was constructed from PTFE tubing, obtained from Fisher Scientific (Loughborough, Leicestershire, UK), joined with low-pressure fittings from Anachem. CL detection was carried out using a luminometer consisting of a PTFE T-piece, a flat-coil 250 µl flow cell and a photomultiplier tube (Chrono-log (USA) Model Lumi-Flo). Results were recorded on a chart recorder (Chrono-log (USA) Model 706-707).

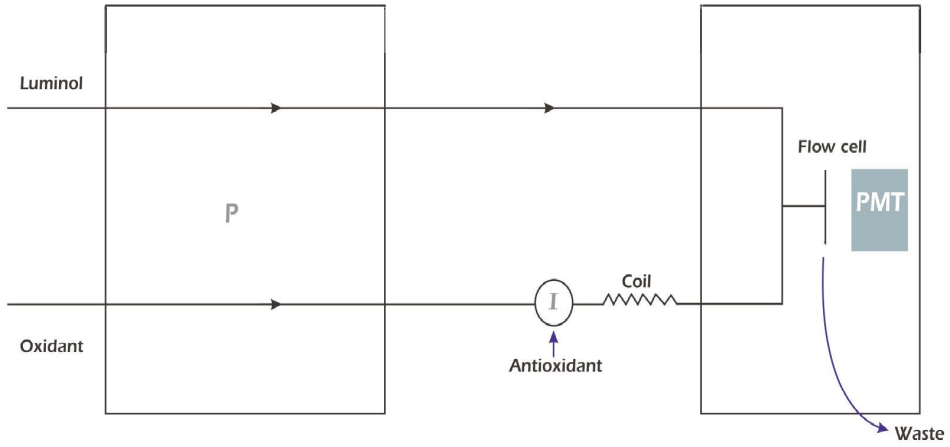

Figure 1. Flow- injection analysis system coupled to luminol chemiluminescence.
PMT: Photomultiplier tube, P: Peristaltic pump, I: Injection valve

Reagents

All chemicals were of analytical-reagent grade and obtained from the following sources: Luminol (5-amino-2,3-dihydro-phthalazinedione), ascorbic acid, hexadecyltrimethyl ammonium bromide (HTAB), cobalt(II) chloride, L-Histidine, L-Lysine and catalase from Sigma; sodium hypochlorite from Aldrich; hydrogen peroxide from Merck; L-Methionine, L-isoleucine, L- Leucine, L-Threonine, L-Phenylalanine, L-Valine and L-Tryptophan from Biological Industries (Israel).

Luminol stock solution was prepared by dissolving luminol in 2M NaOH and diluted by phosphate-buffered saline (PBS: 10 mM KH_2PO_4 and 150 mM NaCl, pH 7.4). HTAB (as a surfactant, final concentration was 10^{-5} M) was added into the working solution of 10^{-4} M luminol in PBS for maintaining luminol in basic environment (and 10^{-5} M in Co^{2+} when the oxidant in use was hydrogen peroxide). It was stored at 4°C protected from light by a foil wrapper.

All the amino acid solutions were freshly prepared and diluted in distilled water.

Hydrogen peroxide solutions were prepared daily by serial dilution of 100-volume hydrogen peroxide and, protected from light by a foil wrapper. HOCl was prepared as described previously by Vissers et al (12). Briefly, NaOCl was diluted with PBS and the pH of the solution readjusted to 7.4. At this pH, the solution contains approximately 1:1 HOCl and OCl^-.

$ONOO^-$ was synthetised according to the method described by Beckman et al. and diluted with 0.1 N NaOH (13).

Procedure and Statistics

The oxidant stream was merged with a luminol/buffer reagent immediately before the flow cell, which was situated in a dark box, adjacent to the window of the photomultiplier. The total flow rate was 1 ml min^{-1}, shared equally between the luminol and the oxidant channels; the oxidant channel includes an injection valve in the middle which allows to make successive nominally 20 µl injections of the antioxidant solutions. The length of connecting tubing is approximately the same as the length of the coil. The CL flow cell has an internal volume of 250 µl; in addition there is ~25 µl dead space from the point of mixing of oxidant/antioxidant with luminol/buffer and the entrance to the flow cell.

The CL is measured as the photomultiplier output in mV; the effects of antioxidants were measured by the depression of the signal from its uninhibited level and were expressed as a percentage attenuation of the maximum CL due to the antioxidant. Results are given as mean±SEM, n referring to the number of experiments. IC_{50} values of inhibitor effects of aminoacids and ascorbate were calculated by using probit regression analysis. If significant differences were detected by ANOVA, then individual means were compared with a control by using Dunnett test. Differences were considered to statistically significant when the *p* values were less then 0.05.

RESULTS

Hydrogen peroxide-luminol chemiluminescence

A continuous CL signal from H_2O_2 (10^{-4} M) (in the presence of 10^{-4} M luminol and 10^{-5} M Co^{2+} in PBS at pH 7.4) was obtained. The H_2O_2-dependent CL signal was inhibited by L-Histidine (10^{-6}-10^{-1} M) (n=6), L-Lysine (10^{-6}-10^{-1} M) (n=6), L-Methionine (10^{-6}-10^{-1} M) (n=6), L-isoleucine (10^{-4}-10^{-1} M) (n=7), L-Threonine (10^{-6}-10^{-1} M) (n=6), L- Leucine (10^{-4}-10^{-1} M) (n=6), L-Phenylalanine (10^{-5}-10^{-1} M) (n=6), L-Valine (10^{-4}-10^{-1} M) (n=6) and L-Tryptophan (10^{-5}-10^{-2} M) (n=6). Ascorbic acid (chain -breaking reference antioxidant) (10^{-9}-10^{-5} M) (n=6) also inhibited the CL signal in a concentration-dependent manner. The IC_{50} values were 1.4x10^{-}

^{4}±8.9x10^{-6}, 5.4x10^{-4}±3.3x10^{-5}, 1.5x10^{-3}±4.4x10^{-4}, 1.6x10^{-3}±3.3x10^{-4}, 2.6x10^{-3}±2.8x10^{-4}, 4.0x10^{-3}±1.4x10^{-4}, 2.4x10^{-2}±1.6x10^{-3}, 4.1x10^{-2}±7.8x10^{-3}, 4.2x10^{-2}±6.6x10^{-3} and 2.5x10^{-8}±5.0x10^{-9} for L-Histidine, L-Methionine, L-Lysine, L-Tryptophan, L-Phenylalanine, L-Threonine, L-Leucine, L-isoleucine, L-Valine and ascorbic acid respectively (Figure 2).

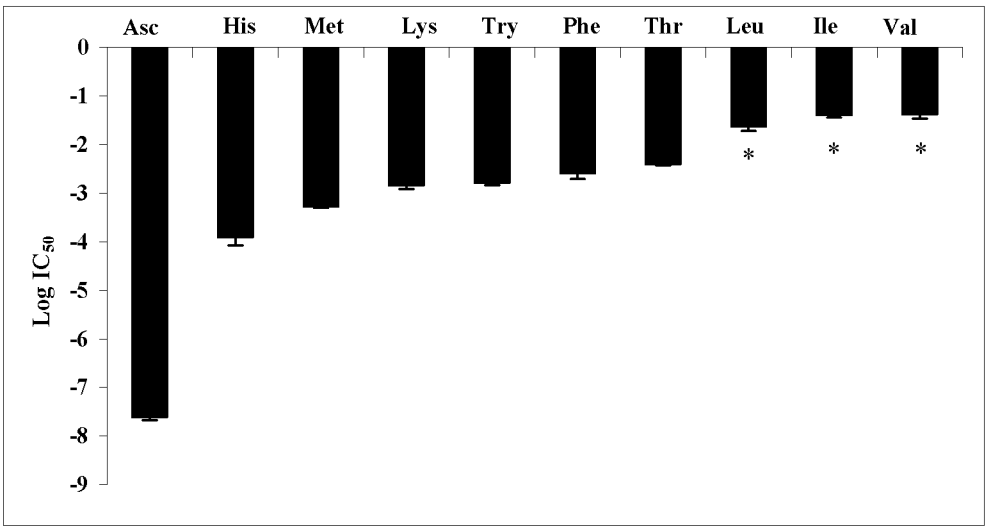

Figure 2. Log IC_{50} values of the inhibitory effect of the essential aminoacids and ascorbate on H_2O_2-induced chemiluminescence signal. Data are shown as mean ± S.E.M, *p<0.05; significantly lower than that of ascorbate.

Hypochlorous-luminol chemiluminescence

A continuous CL signal from NaOCl (10^{-4}M), (in the presence of 10^{-4} M luminol in PBS at pH 7.4) was obtained. The HOCl-dependent CL signal was inhibited by L-Histidine (10^{-6}-10^{-1} M) (n=6), L-Lysine (10^{-6}-10^{-1} M) (n=6), L-Methionine (10^{-5}-10^{-3} M) (n=4), L-isoleucine (10^{-5}-10^{-1} M) (n=6), L-Threonine (10^{-5}-10^{-1} M) (n=6), L- Leucine (10^{-5}-10^{-1} M) (n=6), L-Phenylalanine (10^{-6}-10^{-1} M) (n=6), L-Valine (10^{-6}-10^{-1} M) (n=6) and L-Tryptophan (10^{-6}-10^{-3} M) (n=6). Ascorbic acid (10^{-4}-10^{-1} M) (n=6) also inhibited the CL signal in a concentration-dependent manner. The IC_{50} values were 1.8x10^{-5}±2.0x10^{-6}, 1.0x10^{-4}±8.7x10^{-7}, 2.1x10^{-4}±2.7x10^{-5}, 3.0x10^{-4}±3.3x10^{-5}, 3.2x10^{-4}±8.7x10^{-5}, 3.4x10^{-4}±1.0x10^{-5}, 5.5x10^{-4}±2.6x10^{-5}, 6.7x10^{-4}±2.1x10^{-5}, 9.0x10^{-4}±1.6x10^{-5} and 4.0x10^{-5}±8.6x10^{-6} for L-Tryptophan, L-Methionine, L-Phenylalanine, L-Leucine, L-Lysine, L-Threonine, L-isoleucine, L-Valine, L-Histidine and ascorbic acid, respectively (Figure 3).

Peroxynitrite-luminol chemiluminescence

A continuous CL signal from $ONOO^-$ (10^{-4} M), (in the presence of 10^{-4} M luminol in PBS at pH 7.4) was obtained. The $ONOO^-$ -dependent CL signal was inhibited by L-Methionine (10^{-3}-10^{-1} M) (n=6), L-Threonine (10^{-5}-10^{-1} M) (n=4), L-Tryptophan (10^{-3}-10^{-1} M) (n=6) and, ascorbic acid (10^{-7}-10^{-3} M) (n=6) (Figure 4). However, L-Histidine (10^{-4}-10^{-1} M) (n=6), L-Phenylalanine (10^{-3}-10^{-1} M) (n=6),), L-Valine (10^{-3}-10^{-1} M) (n=6) and L-Lysine (10^{-3}-10^{-1} M) (n=6) were potentiated the $ONOO^-$ -dependent CL signal. Although L-Isoleucine has no effect on the signal at 10^{-6}-10^{-2} M concentrations, L- Leucine inhibited the signal at 10^{-4}-10^{-2} M (n=4). The IC_{50} values were 3.2x10^{-6}±8.8x10^{-7}, 4.6x10^{-2}±6.6x10^{-4}, 8.8x10^{-2}±1.7x10^{-3}, 4.8x10^{-1}±6.8x10^{-2} for ascorbic acid, L-Tryptophan, L-Methionine and, L-Threonine, respectively (Figure 4).

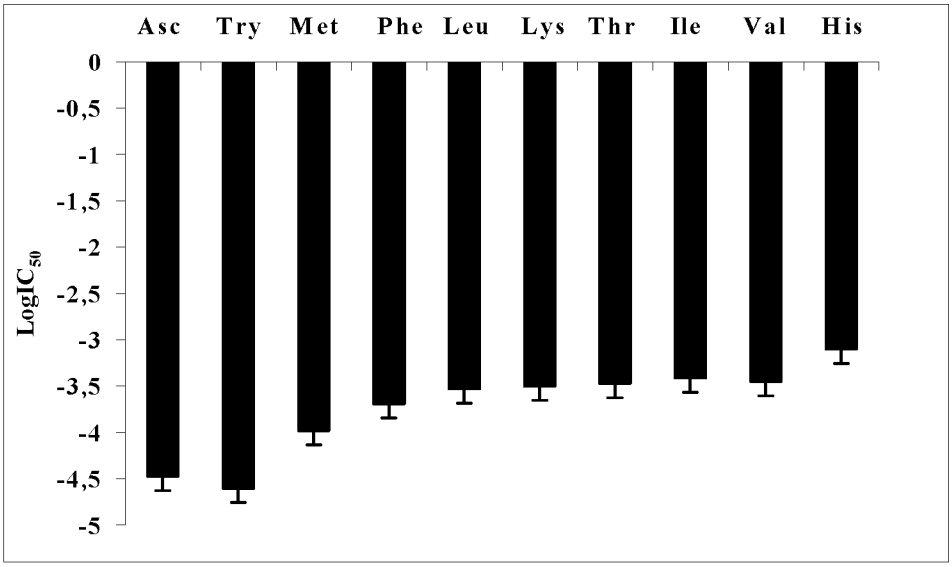

Figure 3. Log IC_{50} values of the inhibitory effect of the essential aminoacids and ascorbate on HOCl-induced chemiluminescence signal generated by NaOCl. Data are shown as mean ± S.E.M, *p<0.05; significantly lower than that of ascorbate.

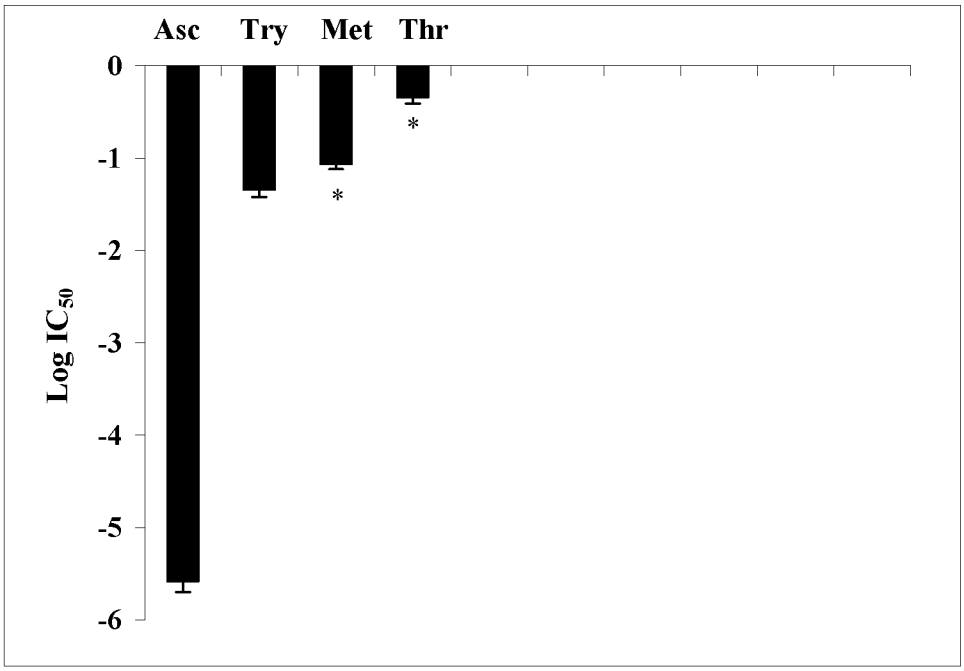

Figure 4. Log IC_{50} values of the inhibitory effect of L-Tryptophan, L-Methionine, L-Threonine and ascorbate on $ONOO^-$-induced chemiluminescence signal. Data are shown as mean ± S.E.M, *p<0.05; significantly lower than that of ascorbate.

DISCUSSION

It has been reported that reactive oxygen species contribute to various pathophysiologic conditions and, endogenous defense mechanisms have evolved to offer protection in these conditions. Increase in the antioxidant reserves of the organism can reduce oxidative stress and, some of the exogenous agents can help to reduce it (14). Antioxidant capacity of amino acids has been investigated in various studies and compared with the antioxidant activity of known compounds as ascorbic acid and Trolox. Charita et al, demonstrated that methionine has a significant effect on the myocardial antioxidant enzyme activities. They explained their results as, methionine was a methylating agent for proteins, and such methylation might be involved in the regulation of catalase and SOD enzyme activities. In addition, it has been concluded that methionine was an amino acid containing a sulfhydryl group under oxidative stress condition and, therefore it might also offers some direct protection against reactive oxygen species (2).

Proline is another amino acid which has been shown to reduce free radical generation (3). Alia et al. have also shown that proline reduced the production of singlet oxygen very effectively (3). Lastly as it was shown before, L- arginine was found to have only weak and non-specific antioxidant effects (15).

In the studies that have directly assessed the free radical scavenging potential of taurine have been shown minimal direct chemical scavenging actions against many oxygen derived radicals (16,17). In contrast to the previous reports, there are some studies establishing taurine as the major cellular scavenger for HOCl (18). However, in the study of Mehta and Dawson taurine was found a weak scavenger of $ONOO^-$ (19).

In the present study, by using flow-injection analysis-luminol chemiluminescence method we have demonstrated for the first time that these essential amino acids have significant inhibitory effects on the peak chemiluminescence signal produced by luminol-hydrogen peroxide, luminol-hypochlorous or luminol-peroxynitrite systems. This is the first systematic study that clearly identified the direct antioxidant potential of essential amino acids against a spectrum of oxidants (H_2O_2, HOCl and $ONOO^-$), by using FIA coupled to luminol chemiluminescence. Reactive oxygen species react with luminol via a succession of radical and peroxide intermediates prior to the formation of the final light emitting product. Amino acids may be involved in the reaction of the luminol-H_2O_2 system and, under the experimental conditions of the present study, they may inhibit the light emission by competing oxidants (H_2O_2, HOCl or $ONOO^-$) with luminol and consuming part of them. It is possible that the reaction of amino acids with oxidants was faster than that of luminol with oxidants. There might be a similar competitions between amino acids and hypochlorous anion with luminol or ONOO with luminol. Resulting interaction due to; either direct effect of amino acids on oxidants (H_2O_2, HOCl or $ONOO^-$) or, the interference of amino acids on oxidants to interact with luminol can inhibit the chemiluminescence signal. Our data cannot distinguish among these possibilities and additional studies to explore the mechanism of this effect were beyond the scope of our original study design.

Overall, the present study demonstrated that although valin and isoleucine had low intrinsic antioxidant activity to prevent the oxidation of luminol by H_2O_2 or HOCl. Histidine and tryptophan displayed the most potent inhibitory activity against H_2O_2 or HOCl induced peak chemiluminescence, respectively. Histidine was found to have antioxidant activity lower than those valin and isoleucine against HOCl-induced oxidation of luminol. In addition, tryptophane, methionine and threonine were found to have weak effects to block luminol-$ONOO^-$- induced peak chemiluminescence signal. It appears unlikely that these amino acids play a major role as a direct scavenger of $ONOO^-$. On the other hand, histidine, phenylalanine and valine, were found to act in an additive manner to potentiate the peak chemiluminescence of luminol- $ONOO^-$. On the other hand, the inhibitory effects of essential amino acids were shown at the milimolar concentrations; thus the question remains; "Could the weak or modest antioxidant activity

presented by amino acids (relative to well-known antioxidant, ascorbic acid) have biologic relevance to reduce oxidative stress?"

CONCLUSION

In the present study, the inhibition of peak chemiluminescence by amino acids is partly due to the direct reaction between the oxidants and amino acids and, also interference of the amino acids with the luminol-oxidants chemiluminescence reaction. The direct interaction between amino acids and the oxidants might have a physiologic importance.

ACKNOWLEDGEMENT

This project (Code:02/2001-23) was supported by the Division of the Projects of Scientific Research of Gazi University (Ankara, Turkey).

REFERENCES

1. **Shug A.L, Madsen D., Dobbie R., Paulson D.J., "**Protection of mitochondrial and heart function by amino acids after ischemia and cardioplegia" *Life Sci.,* 54 (8), 567-577, **1994**.

2. **Charita S.K., Li T., Khaper N., Singal P.K.,** "Effects of methionine on endogenous antioxidants in the heart" *Am. J. Physiol.(Heart Circ Physiol 46),* 277, H2124-H2128, **1999**.

3. **Alia, Mohanty P., Matysik J.,** "Effect of proline on the production of singlet oxygen" *Amino Acids,* 21, 195-200, **2001**.

4. **Robards K., Worsfold P.J.,** "Analytical applications of liquid-phase chemiluminescence" *Anal. Chim. Acta.,* 266, 147-73, **1992**.

5. **Archer R., Nelson D.P., Weir E.,** "Detection of activated O_2 species in vitro and in the rat lungs by chemiluminescence" *J. Appl. Physiol.,* 67, 1912-1921, **1989**.

6. **Kikuchi K., Nagano T., Hayakawa H., Hirata Y., Hirobe M.,** "Detection of nitric oxide production from perused organ by a luminol H_2O_2 system" *Anal. Chem.,* 65, 1794-1799, **1993**.

7. **Radi R., Cosgrove T.P., Beckman J.S., Freeman B.A., "**Peroxynitrite-induced chemiluminescence" *Biochem. J.,* 290, 51-57, **1993**.

8. **Janesek D., Spohn U., Beckman D.,** "Novel chemiluminometric H_2O_2 sensors for the selective flow injection analysis" *Sensors and Actuators B.* 51, 107-113, **1998**.

9. **Burguera J.L., Townshend A.,** "Flow injection analysis for monitoring chemiluminescent reactions" *Anal. Chim. Acta,* 114, 209-214, **1980**.

10. **Dodeigne C., Thunus L., Lejeune R.,** "Chemiluminescence as diagnostic tool" *Talanta,* 51, 415-439, **2000**.

11. **Fletcher P., Andrew K.N., Calokerinos A.C., Forbes S., Worsfold P.J.,** "Analytical applications of flow injection with chemiluminescence detection-a review" *Luminescence,* 16, 1-23, **2001**.

12. **Vissers M., Stern A., Kuypers A., Van Den Berg J., Winterbourn C.,** "Membran changes associated with lysis of red blood cells by hypochlorous acid" *Free Rad. Biol. Med.,* 16, 703-712, **1994**.

13. **Beckman J.S., Beckman T.W., Chen J., Marshall P.A., Freeman B.A.,** "Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide" *Proc. Natl. Acad. Sci,* 87, 1620-1624, **1990**.

14. **Reilly P.M., Schiller H.J., Bulkley G.B.,** "Pharmacologic approach to tissue injury mediated by free radicals and other reactive oxygen metabolites" *Am. J. Surg.* 161, 488-503, **1991**.

15. **Adams M.R., Phu C.V., Stocker R., Celermajer D.S.,** "Lack of antioxidant activity of the antiatherogenic compound L-argine" *Atherosclerosis,* 146, 329-335, **1999**.

16. **Aruoma O.I., Halliwell B., Hoey B.M., Butler J.,** "Antioxidant action of taurine, hypotaurine and their metabolic precursors" *Biochem. J.,* 256, 251-255, **1988**.

17. **Shi X., Flynn D.C., Porter D.W., Leonard S., Vallyathan V., Castranova V.,** "Efficacy of taurine based compounds as hydroxyl radical scavengers in silica induced peroxidation" *Ann. Clin. Lab. Sci.* 27, 365-374, **1997**.

18. **Marcinkiewicz J.**, "Neutrophil chloramines: missing links between innate and acquired immunity" *Immunol. Today,* 18, 577-580, **1997**.

19. **Mehta T.R., Dawson R.,** "Taurine is a weak scavenger of peroxynitrite and does not attenuate sodium nitroprusside toxicity to cells in culture" *Amino Acids,* 20, 419-433, **2001**.

Received: 26.09.2007
Accepted: 10.10.2007