Tohoku J. Exp. Med., 2006, **210**, 239-245

Glutamine Administration Enhances the Healing of Lung Parenchymal Injuries and Reduces Air Leakage in Rats

AYDIN SANLI, AHMET ONEN, SULEN SARIOGLU,[1] BANU SIS,[1] ENSARI GUNELI,[2] BANU GOKCEN, SAMI KARAPOLAT and UNAL ACIKEL

Department of Thoracic Surgery, [1]Department of Pathology, and [2]Department of Animal Science Laboratory, Faculty of Medicine, Dokuz Eylul University, Izmir, Turkey

SANLI, A., ONEN, A., SARIOGLU, S., SIS, B., GUNELI, E., GOKCEN, B., KARAPOLAT, S. and ACIKEL, U. *Glutamine Administration Enhances the Healing of Lung Parenchymal Injuries and Reduces Air Leakage in Rats*. Tohoku J. Exp. Med., 2006, **210** (3), 239-245 —— Beneficial effects of glutamine on wound healing are well known. Parenchymal injuries in the lung cause air leakage that resolves with wound healing. We aimed to determine the effect of glutamine on the healing of lung injuries. Wistar albino female rats were randomized in three groups. One group (control, $n = 7$) received intraperitoneal injection of 0.9% sodium chloride (1.5 ml /day), while other group (GLN, $n = 7$) received glutamine (1.5 g/kg/day), beginning two days prior to the operation for total four days. After thoracotomy, a lung parenchymal lesion was made with a scalpel in the right upper lobe. Only thoracotomy was performed to sham group ($n = 4$). Air leakage was observed in the isolated lungs of control group, but not GLN and sham groups, at 5 cm H_2O of positive airway pressure ($p < 0.001$). The threshold of positive airway pressure for air leakage was 4.85 ± 0.37 and 19.42 ± 4.54 cm H_2O for control and GLN groups, respectively ($p < 0.001$). For measurement of collagen content in the healing parenchyma, digital images were processed to calculate the stained area percentage (SAP). SAP for immature collagen, a marker for wound healing, was 0.36 ± 0.18% and 1.48 ± 0.83% ($p = 0.02$) in control and GLN groups, respectively, but no significant difference was noted in SAP for mature collagen. The grade of inflammation was not significantly different between control and GLN groups. We conclude that glutamine enhances lung parenchymal healing by increasing immature collagen secretion. ———— glutamine; collagen; air leakage; lung
© 2006 Tohoku University Medical Press

In clinical practice, damage of the lung parenchyma results in air leakage that causes prolonged hospitalization. Application of radiotherapy and/or chemotherapy, surgical procedures for infective lesions, decortication of pleural thickenings as after tuberculosis are often the causes of prolonged air leakage (PAL). In order to prevent or reduce these complications, thoracic surgeons have studied the use of various agents as fibrin glues, talc, cyanoacrilate, polytetrafluorethylene

Received May 17, 2006; revision accepted for publication September 22, 2006.
Correspondence: Aydin Sanli, Dokuz Eylul Universitesi, Tip Fakultesi Hastanesi, Gogus Cerrahisi AD, 35340 Balcova, Izmir, Turkey.
e-mail: aydin.sanli@deu.edu.tr

plaques, or autologous hemopatch (Vaughn et al. 1997; Catalyurek et al. 2002; Fabian et al. 2003). All these studies focused on the healing capacity of the local use of these agents at the site of air leakage.

Glutamine is the most abundant amino acid in the circulation accounting for 30-35% of the plasma amino acid content. It is mainly produced in lungs and skeletal muscles. While it is the most important non-essential amino acid under normal conditions, glutamine becomes essential when the requirement increases as in trauma and sepsis (Wilmore 2001). Fibroblasts, lymphocytes and macrophages make abundant use of glutamine for nucleotide synthesis regulating cell proliferation as well as energy source. Decrease in glutamine content causes decelerated fibroblast growth while supplementation in glutamine stimulates growth and cellular proliferation (Miller 1999).

Metabolic responses to wound healing in trauma or surgery are formed through the loss of nitrogen in the body glutamine, which constitutes the main reservoir of amino acid nitrogen content of the plasma (Wilmore 2001). Glutamine requirement increases in trauma or surgery and it can be given safely in a wide dose range (0.2 - 1.5 g/kg/day) and only diarrhea appears to be the most important side effect in glutamine replacement therapy.

Systemic glutamine replacement enhances wound healing (Miller 1990; Wilmore 2001; Costa et al. 2003). The presence of air leakage is related to the speed of wound healing process in the lung parenchyma (Fabian et al. 2003). In this study, we aimed to determine the effect of systemic glutamine administration on parenchymal healing of the lung and on the prevention of air leakage.

Materials and Methods

Animals

The experimental protocol was approved by the Ethics Committee of Dokuz Eylul University Medical School (Project number: 04/08/32). Eighteen Wistar albino female rats weighting 200 – 270 g were used in this study. The rats were maintained in accordance with the Guide for the Care and Use of Laboratory Animals. The animals were housed in the Department of Animal Science Laboratory, University of Dokuz Eylul. They were kept at 21°C to 23°C with controlled humidity and a dark-light cycle of 12 to 12 hrs. Food and water were available ad libitum.

Surgical procedure and experiment

Rats were randomly assigned into three groups: 1) 0.9% Sodium chloride (control, $n = 7$), 2) Glutamine (GLN, $n = 7$), and 3) Sham ($n = 4$). The rats were anesthetized with ether and they were supported with nasal oxygen. The right chest wall was shaved and disinfected. Thorocatomy was performed in left lateral position through the 5th intercostal space using an anterolateral incision in all rats. In order to standardize the parenchymal damage in control and GLN group rats, a clip was placed horizontally on a scalpel. The scalpel was plunged into the lung parenchyma vertically forming a damage of 1 mm width and 2 mm depth in the right upper lobe of the right lung. No parenchymal damage was performed to rats in the sham group. Lungs were completely expanded through nasal insufflations. Thorocatomy was closed without pleural drainage after injection of 0.5 mg/kg bupivacaine (Marcaine flacon®, 0.5%, 20 ml, Astra Zeneca, Istanbul, Turkey) subcutaneously. Sodium chloride 0.9% and glutamine were administered by daily IP injections from two days before to two days after the thoracotomy procedure. While sodium chloride was administered at a dose of 1.5 ml/day in control group, glutamine (Dipeptiven®, 20%, 100 ml, Fresenius Kabi, Graz, Austria) was administered at a dose of 1.5 g/kg/day in GLN groups (1.3 to 1.5 ml /day depending on the weight). The rats were sacrificed on the postoperative 3rd day. Median sternotomy was performed in order to avoid any new lung damage. Dissection was continued till trachea and the whole lung was removed together with the trachea. A canule was placed into the trachea and tightly bound to prevent air leakage. Using a triple tap, one orifice of the canule was connected to the pressure monitor (KMA 250, Petas, Istanbul, Turkey) while the other orifice was connected to an injector. Positive airway pressure was applied to the lungs placed into 0.9% sodium chloride solution. The pressure level at which air leakage began to occur in the damaged area was termed “the threshold air leakage pressure”. Lung biopsy specimens were taken from the damaged regions and histopathological examination was performed.

Histological examinations

Lung specimens were fixed in 10% formalin, processed for paraffin embedding and sectioned at 5 μm. Hematoxylin-Eosin stained sections were used to evaluate histopathological findings via light microscopy. Inflammation involving subpleural areas was scored on a scale from 0 to 3 as follows; no inflammation (score 0), mild inflammation (few cells, score 1), moderate inflammation (cells involving most of the interstitial area, score 2), severe inflammation (diffuse infiltration and expansion of the interstitium with many inflammatory cells, score 3).

Image analysis

The amount of mature and immature collagen in granulation tissue was measured by computerized digital histochemistry image analysis of sections stained with Masson's trichrome and Sirius red, respectively. For Sirius red staining, the sections were deparaffinized, rehydrated, and then stained in saturated picric acid with 0.1% Sirius red (Francis 1990).

Digital images were obtained from Sirius red and Masson's trichrome stained sections using a 3 CCD color video camera (Olympus® DP70, Olympus Optical Co. Ltd., Tokyo), connected to the light microscope (Olympus® BX51, Olympus Optical Co. Ltd.) at an original magnification of × 40. Images were processed with Mediscope Image Analysis Software (Mediscope, Dokuz Eylul University, Clinical Engineering Department, Izmir, Turkey) (Kavukcu et al. 2003; Demiral et al. 2004; Sarioglu et al. 2004; Sis et al. 2005). For each case, after detection of the most severely scarred areas by eye, 5 high power fields were selected for digital acquisition (total area: 79,390 μm^2).

For each captured field, the percentage of the marked area with the stain of selection was determined semi-automatically. Sirius red stained sections were examined under polarized light. The software for image processing required the selection by the pathologist of the areas stained with Masson's trichrome (green) and with Sirius red (white) on the image. Subsequently, the system selected the areas with the same configurations of stainings and converted them into pixel density units after what it presented the selected areas as a percentage within each field. This analysis is repeated for each of the five selected fields. In order to achieve thorough analysis, each field was examined by the pathologist before running the software analysis (Fig. 1A-B in GLN group, Fig. 2A-B in Sham group). Stained Area Percentage (SAP) was defined as the average of the percentage of the five selected fields.

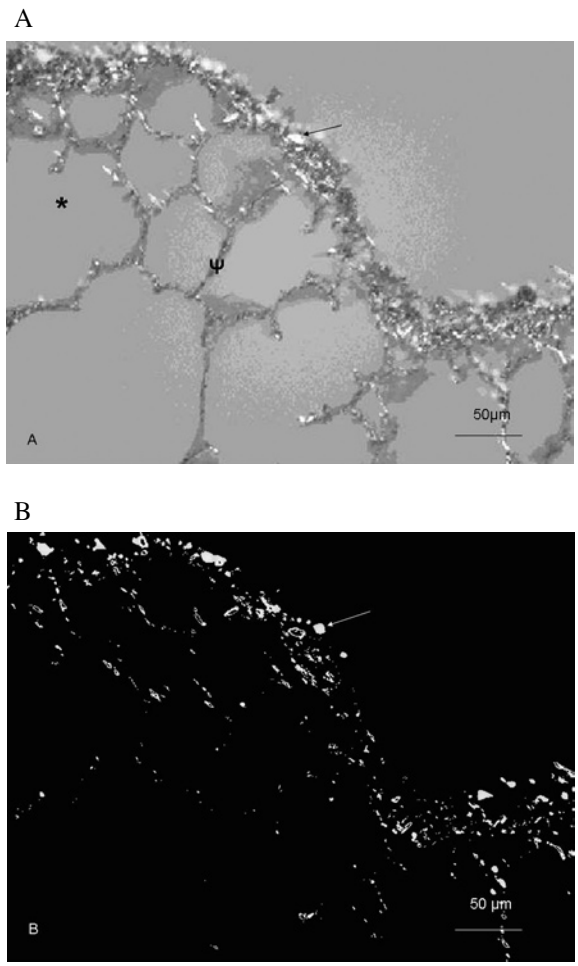
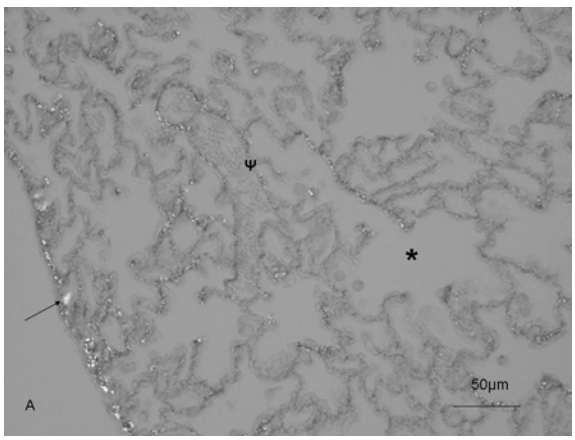


Fig. 1. Histopathological appearance of collagen formation on the damaged lung tissue under polarized light in the GLN group.
A: Intense collagen formation is monitored particularly in the subpleural field (Sirius red × 40).
B: The same image after processing by the image analysis software (Mediscope, Dokuz Eylul University, Clinical Engineering Department, Izmir, Turkey), for the measurement of the SAP. Only white colored collagens are shown.
*Alveolar space; arrow, pleural field; Ψ, interalveoler septum.

Reproducibility and reliability analyses have previously showed that the measurement of SAP by computer assisted image analysis is a highly reliable method (the coefficient of intraclass correlation = 0.98) (Sis et al. 2005).

A



B

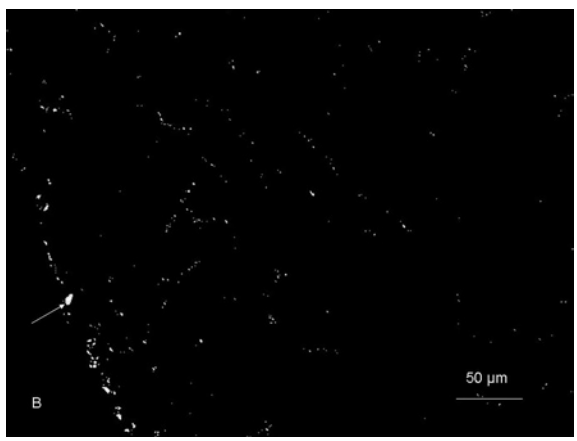


Fig. 2. Histopathological appearance of non-damaged lung tissue under polarized light in the sham group (normal appearance).
A: Alveolar septal and subpleural collagens are visible (Sirius red × 40).
B: The same image after processing by the image analysis software (Mediscope, Dokuz Eylul University, Clinical Engineering Department, Izmir, Turkey), for the measurement of the SAP.
White colored collagens are distributed in the alveolar septal and subpleural areas.
*Alveolar space; arrow, pleural field; Ψ, interalveoler septum.

As Sirius red stains both mature and immature collagen while Masson's trichrome only stains mature collagen, the amount of immature collagen was calculated by subtracting Masson's trichrome SAP values from Sirius red SAP values (Francis 1990).

Statistical analyses

Data were analyzed using SPSS 11.0 statistical program. Mann Whitney's U-and Fisher Exact Chi-Square tests were used to determine the significance of the difference between the groups. Results are given as mean ± S.D. $P < 0.05$ was considered as significant.

Results

Evaluation of air leakage

While air leakage was observed in all rats of the control group at 5 cm H_2O, no air leakage was demonstrated in GLN and sham groups at this pressure level ($p < 0.001$). Mean threshold air leakage pressure was 4.85 ± 0.37 cm H_2O for the control group and 19.42 ± 4.54 cm H_2O for the GLN group. The threshold air leakage pressure levels were significantly higher in the GLN group compared to control group ($p < 0.001$). No air leakage was observed in sham group until the pressure level of 50 cm H_2O.

Evaluation of the immature collagen

Immature collagen SAP values were 0.36 ± 0.18% in the control group, 1.48 ± 0.83% in the GLN group and 0.50 ± 0.28% in the sham group. Significant difference on the amount of immature collagen was detected between control and GLN groups ($p = 0.02$). No significant difference was detected between control and sham groups ($p = 0.527$).

Evaluation of the mature collagen

Mature collagen SAP values were 2.54 ± 0.83% in the control group, 1.79 ± 0.66% in the GLN group and 4.58 ± 2.56% in the sham group. No significant difference was detected between control and GLN ($p = 0.180$) and control and sham groups ($p = 0.190$).

Evaluation of the inflammation

Predominantly, lymphocyte and plasmocyte infiltration was observed. Inflammation scores were 1.71 ± 1.10 in control group, 2.14 ± 1.00 in GLN group and 0.25 ± 0.50 in sham group. There was no significant difference between control and GLN group ($p = 0.453$) but significant difference was detected between control and sham groups

TABLE 1. Results of the study.

Groups	Air leakage at 5 cm H_2O pressure	Air leakage threshold Pressure (cm H_2O)	Mature collagen (SAP)	Immature collagen (SAP)	Inflammation score (score 0 – 3)
Control ($n = 7$)	Positive	4.85 ± 0.37 [c]	2.54 ± 0.83	0.36 ± 0.18	1.71 ± 1.10
GLN ($n = 7$)	None	19.42 ± 4.54	1.79 ± 0.66	1.48 ± 0.83	2.14 ± 1.00
Sham ($n = 4$)	None	-	4.58 ± 2.56	0.50 ± 0.28	0.25 ± 0.50
P values between control and GLN groups	$p < 0.001$ [a]	$p < 0.001$ [b]	$p = 0.180$ [b]	$p = 0.02$ [b]	$p = 0.453$ [b]
P values between control and sham groups			$p = 0.190$ [b]	$p = 0.527$ [b]	$p = 0.004$ [b]

[a] Fisher Exact Chi-Square test was used to determine the significance of the difference between the groups.

[b] Mann Whitney's U-test was used to determine the significance of the difference between the groups.

[c] Results are mean ± S.D.

($p = 0.004$).

The results of our study are shown in Table 1.

Morbidity and mortality

There was no mortality during the study period. Respiratory distress, lung collapse and pleural adherence did not occur in any of the rats. Side effects due to glutamine were not observed in our study.

DISCUSSION

Until now, agents used to prevent air leakage were not consistently effective. Although cyanoacrilate is a highly adhesive agent in vitro tests, its use is not appropriate in lung surgery due to its non-biological, non-absorbable and penetrating nature (Ennker et al. 1994). Glues containing collagen polymerized with polysaccharides have failed to treat the PAL sooner than the control group (Feito et al. 2000). Fibrin glues did not demonstrate any advantage compared to other agents used in many studies and even caused anaphylactic reactions with serious hypotension (Mitsuhata et al. 1994; Wong et al. 1997). The ideal agent should not cause any tissue reaction nor should increase the infection risk. Moreover, it should have penetrating nature, with rapid and permanent effect. Furthermore, the ideal agent should be applicable in PAL occurrence with the application of positive pressure with mechanical ventilation treatment.

For the period of catabolic stress, intracellular level of glutamine decreases by 50% and plasma concentration decreases by 30%. While the concentration of all the other amino acids return to normal, glutamine concentration continues to decrease and replacement treatment becomes necessary (Miller 1999; Wilmore et al. 2001).

Various studies have shown that glutamine treatment enhances wound healing, increases intestinal permeability and supports immune system by regulating negative nitrogen balance after trauma or surgery. Glutamine is an energy source for fibroblasts that have an important role on wound healing. Prolin, which is one of the final products of glutamine metabolism, is effective on wound healing because of its essential role in collagen synthesis (Costa et al. 2003). The healing of the lesions of lung parenchyma is the result of collagen synthesis as in other tissues.

Although air leakage was observed in all of the rats in the control group at a pressure of 5 cm H_2O, this was not the case in the GLN group ($p < 0.001$). This result shows the significant preventive effect of glutamine on air leakage at low pressures. The threshold air leakage pressure

level was significantly higher in the GLN group (19.42 ± 4.54 cm H_2O) than in the control group (4.85 ± 0.37 cm H_2O) ($p < 0.001$). It can be suggested that glutamine decreases the incidence of air leakage in rats by enhancing wound healing in the lung parenchyma as early as the postoperative 3rd day. The higher threshold air leakage pressure that was determined in the GLN group is considered as an indicator of wound healing. It can be suggested that there is a correlation between the threshold air leakage pressure and the healing process. Air leakage due to surgical procedure may lead to misleading results in air leakage tests. It is known that pleural lacerations may occur in the normal lung tissue especially at the hilar regions at a threshold air leakage pressure level of 50 cm H_2O (Otani et al. 1999). The absence of an air leakage at a pressure of 50 cm H_2O in the sham group showed that the surgical procedure did not cause any pleural lacerations and subsequent air leakage in rats.

Although mean inflammation score for GLN group is higher than control group, there was no significant difference between the inflammation scores of both groups ($p = 0.453$). It can be suggested that these results showed the presence of standardized parenchymal damage in all the rats of the study. However there was significant difference between the control and sham groups ($p = 0.004$). This reason can be related to 0.9% sodium chloride effect.

Wound healing consists of inflammatory, proliferative and mature phases. Proliferative phase begins on the postoperative 3rd days and continues for approximately 2 weeks postoperatively. In this phase, the immature collagen secreted by fibroblasts is predominant. The secreted collagen migrates to wound tissue (Hattori et al. 2000; Tagaki et al. 2001). In our clinical practice, air leakage disappears on the postoperative 3rd day of thoracic surgery. Therefore, this study was terminated on the postoperative 3rd day.

During the maturation phase, there is a gradual decrease in the number of fibroblasts and the collagen fibers evolve to a thick and regular structure (Hattori et al. 2000). In this study, amounts of immature collagen was determined to be significantly higher in the GLN group ($p = 0.02$) on the postoperative 3rd day and there was no difference on the amounts of mature collagen between two groups due to termination of the study at the beginning of the proliferative phase. On the other hand, the significant increase in the amount of immature collagen on the postoperative 3rd day seems to be the result of time-maturation correlation. If glutamine administration is prolonged to the mature phase in postoperative period, a significant increase in the amount of mature collagen is expected. In the study of Costa et al. (2003), the effect of glutamine replacement therapy on the healing of intestinal anastomosis was studied and it has been reported that mature collagen on the anastomosis line significantly increased on the postoperative 8th day paralelly to the increase in resistance at the anastomosis line in the traction tests. The fact that there is no significant difference between the collagen values of the control and sham groups, strengthens the effectiveness of glutamine.

Limitations

First, the present study was performed on healthy animals while thoracic surgery is performed on unhealthy and poorly vascularized lungs. This situation has a negative effect on wound healing. Secondly, the dose of glutamine was the same as in the study of Costa et al. (2003) and the response to different doses was not evaluated in this study. Finally, the present study is the first to determine the effects of the use of glutamine in lung surgery. Further experimental and clinical investigations are needed to determine the effects of glutamine on the prevention of air leakage and wound healing of the operated lung.

We conclude that glutamine administration promotes lung parenchymal healing by increasing immature collagen, thereby reducing air leakage in the early postoperative period in rat lung.

References

Catalyurek, H., Silistreli, E., Hepaguslar, H., Kargi, A. & Acikel, U. (2002) The role of autologous blood injection on postoperative air leak at lung resections. *J. Cardiovasc.*

Surg. (Torino), **43**, 135-137.

Costa, M.A., Campos, A.C., Coelho, J.C., Barros, A.M. & Matsumoto, H.M. (2003) Oral glutamine and the healing of colonic anastomosis in rats. *J. Parenter. Enteral Nutr.*, **27**, 182-186.

Demiral, A.N., Sarioglu, S., Birlik, B., Sen, M. & Kinay, M. (2004) Prognostic significance of EGF receptor expression in early glottic cancer. *Auris. Nasus. Larynx*, **31**, 417-424.

Ennker, I.C., Ennker, J., Schoon, D., Schoon, H.A., Rimpler, M. & Hetzer, R. (1994) Formaldehyde-free collagen glue in experimental lung gluing. *Ann. Thorac. Surg.*, **57**, 1622-1627.

Fabian, T., Federico, J.A. & Ponn, R.B. (2003) Fibrin glue in pulmonary resection: a prospective randomized, blinded study. *Ann. Thorac. Surg.*, **75**, 1587-1592.

Feito, B.A., Rath, A.M., Longchampt, E. & Azorin, J. (2000) Experimental study on the in vivo behavior of new collagen glue in lung surgery. *Eur. J. Cardiothorac. Surg.*, **17**, 8-13.

Francis, R.J. (1990) *Theory and Practice of Histological Techniques*. Churchill Livingstone, Edinburgh, 155-175.

Hattori, H., Otani, H., Omiya, H., Tabata, S., Nakao, Y., Yamamura, T., Osako, M., Saito, Y. & Imamura, H. (2000) Fate of fibrin sealant in pericardial space. *Ann. Thorac. Surg.*, **70**, 2132-2136.

Kavukcu, S., Soylu, A., Turkmen, M., Kuralay, F., Yilmaz, O. & Sarioglu, S. (2003) Unilateral ureteroperitoneostomy in the management of hypoproteinemia in nephrotic rats with normal renal function. *Tohoku J. Exp. Med.*, **201**, 67-73.

Miller, A.L. (1999) Therapeutic consideration of L-glutamine: a review of the literature. *Altern. Med. Rev.*, **4**, 239-248.

Mitsuhata, H., Horiguchi, Y., Saitoh, J., Saitoh, K., Fukuda, H., Hirabayasi, Y., Togashi, H. & Shimizu, R. (1994) An anaphylactic reaction to topical fibrin glues. *Anesthesiology,* **81**, 1074-1077.

Otani, Y., Tabata, Y. & Ikada, Y. (1999) Sealing effect of rapidly curable Gelatin- Poly (L - Glutamik Acid) hydro gel glue on lung air leak. *Ann. Thorac. Surg.*, **67**, 922-926.

Sarioglu, S., Celik, A., Sakar, M., Sonmez, D. & Tekis, D. (2004) Methenamine silver staining quantitative digital histochemistry in chronic allograft nephropathy. *Transplant Proc.*, **36**, 2991-2992.

Sis, B., Sarioglu, S., Sokmen, S., Sakar, M., Kupelioglu, A. & Fuzun, M. (2005) Desmoplasia measured by computer assisted image analysis: an independent prognostic marker in colorectal carcinoma. *J. Clin. Pathol.*, **58**, 32-38.

Takagi, M., Akiba, T., Yamazaki, Y., Nariai, K. & Iwaki, T. (2001) The wound - healing effect of fibrin glue for tracheal anastomosis in experimental pulmonary surgery. *Surg. Today*, **31**, 845-847.

Vaughn, C.C., Wolner, E., Dahan, M., Grunenwald, D., Vaughn, C.C., Klepetko, W., Filaire, M., Vaughn, P.L. & Baratz, R.A. (1997) Prevention of air leaks after pulmonary wedge resection. *Ann. Thorac. Surg.*, **63**, 864-866.

Wilmore, D.W. (2001) The effect of glutamine supplementation in patients following elective surgery and accidental injury. *J. Nutr.*, **131**, 2543-2549.

Wong, K. & Goldstraw, P. (1997) Effect of fibrin glue in the reduction of post thorocatomy alveolar air leak. *Ann. Thorac. Surg.*, **64**, 979-981.