CHAPTER III

RESULTS

3.1 TESTING AND VALIDITY OF METHODOLOGY

In this study has tested all methods for analysis of the GSH, MDA, alpha-tocopherol, hyaluronic acid, and urea concentrations in the blood and tracheal aspirates. Conclusion of the optimized method were as following.

The DTNB-method; Linearity has been achieved over the ranges of 10-60 μ g/ml. The recoveries at three levels were 91.0-91.6 % and the minimal quantity that could be detected was 3 μg/ml. The within-run and between-run coefficients of variation at three times showed 2.38 to 3.97% and less than +/-2 standard deviation of the mean values of the control samples (see appendix, Figure 37). For the tracheal aspirates testing were performed as same as the blood, and use added external standard GSH (20 μg/ml).

The TBARs-assay; linearity has been achieved in the ranges of 5-40 μmol/L The recoveries at three levels were 92.4-100% and the minimal quantity that could be detected was 2 μmol/L. The within-run and between run coefficients of variation at three times showed 4.3-5.2 % and less than ± standard deviation of the mean value of the control samples (see appendix, Figure 36).

The alpha-tocopherol analysis with HPLC; Linearity has been achieved in the ranges of 5-60 ng/ml, whereas the linearity of standard alpha-tocopherol at 2-5 mg/L were not as good as a high alpha-tocopherol concentrations. The minimal satisfied volume for injection was should be equal or more than 50 μL. The recoveries at three levels were 86.45-93.66% and the minimal quantity that could be detected was 2 mg/L, The within-run and between-run coefficients of variation at

less than 5-6 % and less than $\pm$ 2 standard deviation of the mean of the standard alpha-tocopherol at 20 mg/ml. The mean of retention time (RT) of alpha-tocopherol peak was averaged 3.79 second (range 3.75-3.90 second). In the tracheal aspirates were extracted and dissolved with absolute ethanol and added external standard alpha-tocopherol (100 mg/L) before injection compared with standard alpha-tocpherol (100 mg/L) (see appendix, Figure 38)

The hyaluronic acid (HA) analysis with ELISA; The manual method was tested for validity and the percentages of recovery from the HA standard laboratory. Linearlity has been achieved in the ranges of 20-10,000 ng/ml. The within-run and between-run coefficients of variations were 3.35% and 6.02% resepctively. The recoveries at three levels were 92.76%. (see appendix, Figure 37)

3.2 THE CHARACTERISTIC OF PEDIATRIC PATIENTS

In the 40 pediatric patients, Group A (n=20) and Group B (n=20) in the acute care unit had been studied since July, 2000 until June, 2001. All patients were transferred to chest physical therapy and studied with the problems of secretion and/or lung atelectasis. The previous primary diseases of the most patients, lesion problem, medical treatment, etc., (see appendix, Table 15). The most lung diseases were bronchopulmonary dysphasia (BPD) with preterm neonates. About a half of all subjects found that the previous primary diseases which were related with heart disease such as congenital heart disease, septum defect disease, or heart dysphasia, but all subjects were supported and kept in the stable condition from the doctors. All patients were diagnosed for the lung problem and treated with ventilator before transferring to physical therapy. Therefore all patients in the study were suffered with pulmonary disease directly.

Table 4. Characteristic of pediatric patients in group A and group B pediatric patients in the 1st day of treatment.

	Group A	**Group B**
Ages (months) (n=20)	5.3±0.62 (2-12)	5.4±0.63 (1-12)
Required problems (n=40)	(n=20)	(n=20)
Atelectasis (n)		
Right upper lobe (RUL)	2	6
Right lower lobe (RLL)	1	2
Left upper lobe (LUL)	1	1
Left lower lobe (LLL)	2	1
Secretion accumulation (n)	14	11
Chemistry Data		
Sodium (mmol/L) (n= 16)	139.1±1.14 (135-150)	138.0+1.7 (128-158)
Potassium (mmol/L) (n=16)	4.6±0.2 (3.6-6.2)	4.2±0.3 (2.0-6.1)
Choride (mmol/L) (n=14)	99.7±2.2 (82-114)	104.7±4.1 (80-160)
Complete Blood count		
Hct (%) (n=20)	38.4±0.9 (31-45)	41.3±1.5 (32-58)
Lymphocytes (cells/ml)(n=19)	31.9±2.7 (12-57)	27.4±2.5 (9-56)
Neutrophil (cells/ml) (n=19)	67.1±3.1 (40-84)	71.6±3.6 (32.4-93)
White blood cell (cells/ml) (n=19)	12714.2±1455.4 (4400-31300)	13454.0±793.3 (9030-23700)
Lung injury score (n=20)		
Mild lung injury (0.1-2.5)	1.098 (0.66-1.33)	1.099 (1.00-1.33)

Data represent with Mean±SE (range) from total 40 patients. mmol/L = millimole per liter, Hct = Hematrocit

Before study, data from laboratories showed no difference in the characteristic of both groups (Table 4). They were in the matched of ages, GSH, HA, MDA, plasma Vit E in blood and TA, sodium, chloride, potassium, lymphocytes, neutrophil, white blood cells in both groups in the 1st day. Severity of the lung injury in both groups were not statistically different by which evaluated from lung injury score.

3.3 CHANGES OF MDA, GSH, VIT E, AND HA LEVELS IN BLOOD AND TRACHEAL ASPIRATES (TA) AFTER PHYSICAL THERAPY.

The results of two physical therapy in six days were shown in the Table 5-8, MDA, GSH, Vit E and HA concentrations in the first and sixth day in both treatments, The statistically different levels of concentrations were shown in the Figures 15-18. All concentrations in TA were calculated with total volume of TA in each day that collected from the patients. The final concentrations were measured in the amount per liter (or milliliter).

For this study , the clinical improvement had shown by the change in the lung injury score and oxygenation index (PaO_2/FiO_2). The lung injury score was calculated from parameters in which recorded from clinicians, for example, arterial blood gases, ventilator setting, etc.

Table 5. The concentrations of MDA in the serum and Tracheal aspirates (TA) of the 1st day and the 6th day in group A (n=20) and B (n=20) treatments.

	Serum (μmol/L)		**Tracheal aspirates** (μmol/L)	
	Group A	Group B	Group A	Group B
The 1st day	21.58±1.3 (11.92-33.75)	21.43±.9 (14.36-28)	32±14 (14-71.56)	31.5±14.5 (15-75)
The 6th day	20.56±1.6 (10.6-36.5)	20.68±.9 (14-30)	28.2±14.7 (16.12-71.64)	30.5±12.5 (14.16-51.48)

TA= tracheal aspirates, MDA = malondialdehyde, Data represent in mean±SE. (range)

Table 6. The concentrations of GSH in the serum and Trachea aspirates (TA) of the 1st day and the 6th day in group A (n=20) and B (n=20) treatments.

	Whole Blood (mg/dl erythrocytes)		**Tracheal aspirates** (mg)	
	Group A	Group B	Group A	Group B
The 1st day	66.5±.3 (27.15-94.55)	52.9±2.7 (37.13-75.28)	3.06±1.8 (1-7.72)	2.132±1.2 (1-5.9)
The 6th day	73.9±3.0 (51.25-97.13)	61.1±3.1 (40.25-89)	6.0±2.5 (1.6-9.8)	4.4±2.3 (1.48-9.4)

TA = tracheal aspirates, GSH = glutathione, Data represents in mean±SE. (range)

Table 7. The concentrations of alpha-tocopherol (Vit E) in the serum and trachea aspirates (TA) of the 1st day and the 6th day in group A (n=20) and B (n=20) treatments.

	Plasma (mg/L)		**Tracheal aspirates** (mg/L)	
	Group A	Group B	Group A	Group B
The 1st day	6.3±1.1 (0.29-17.61)	2.4±0.7 (0.12-11.64)	19.8±15.8 (2.1-63.88)	20.2±16.5 (2.5-51.52)
The 6th day	6.8±1.7 (0.29-29.55)	4.0±0.9 (0.29-17)	27.5±25.2 (2.3-89.08)	26.67±19.4 (20.2-56.8)

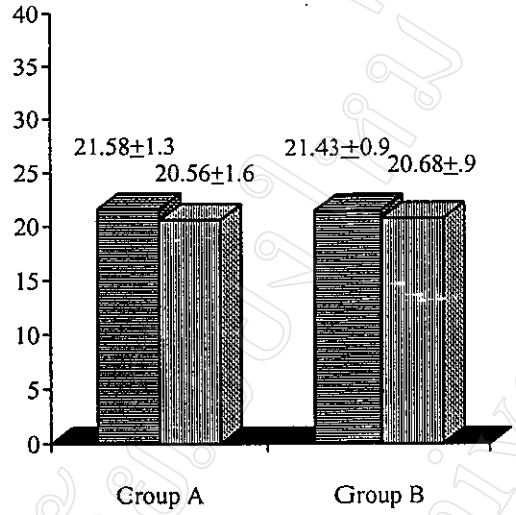
TA = trahceal aspirates, Vit E = alpha-tocopherol, Data represents in mean±SE. (range)

Table 8. The concentrations of HA in the serum and Trachea aspirates (TA) of the 1st day and the 6th day in group A (n=20) and B (n=20) treatments.

	Serum (ng/ml)		**Tracheal aspirates** (X 10^2 ng/ml)	
	Group A	Group B	Group A	Group B
The 1st day	3545.0±733.3 (412.9-13668.99)	3402.1±720.9 (732.3-12540.5)	780.1±1119 (24.68-387.22)	640.4±665.4 (45.95-855.56)
The 6th day	1663.9±347.2 (252.13-4875.02)	2402.7±480.9 (458.2-9905.1)	550.6±1003 (343.4-1301.6)	178.4±366 (329.4-1027.68)

TA= tracheal aspirates, HA = hyaluronic acid , Data represents mean±SE. (range)

A. Serum MDA (μmol/L)



B. TA MDA (μmol/L)

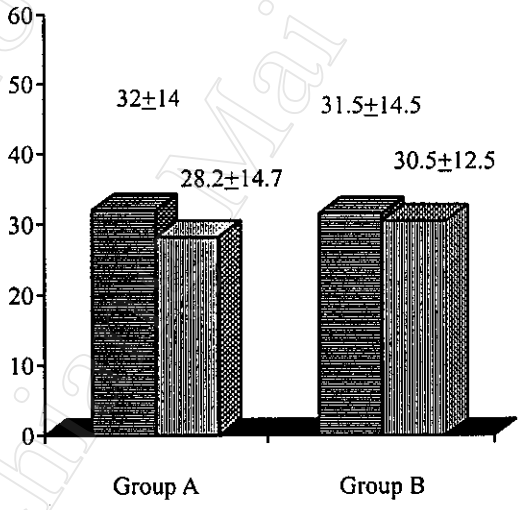
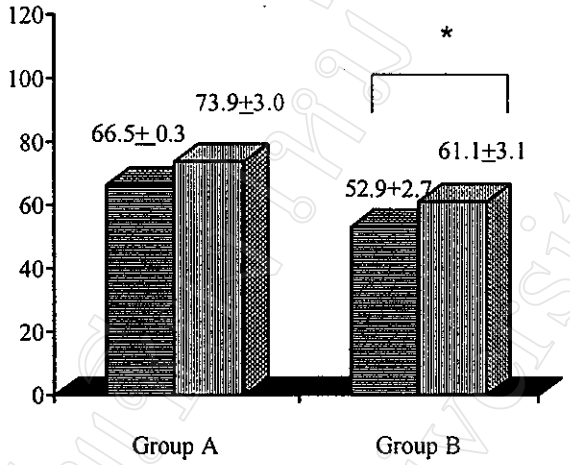


Figure 15. The concentrations of malondialdehyde (MDA) in serum (A) and in tracheal aspirates (TA) (B) which were detected by TBARs assay, Data represents mean±SE. MDA levels within groups & days, between days in each groups were analyzed using two way ANOVA and paired **t**-test respectively.

Note = the 1st day of treatment = the 6th day of treatment

A. GSH (mg/dl erythrocytes)



B. TA GSH (mg/dl)

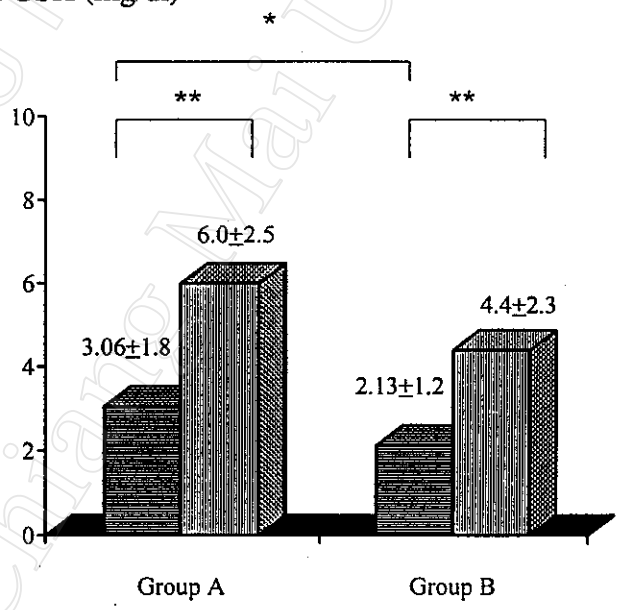
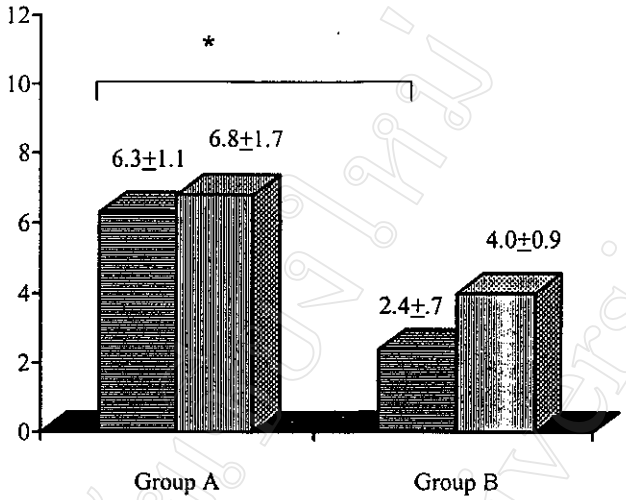


Figure 16. The concentrations of GSH in the erythrocytes (A) and Tracheal aspirates (TA) (B) with DTNB-method in the 1st day and 6th day between group A and group B treatment. Data represent mean± SE. GSH levels within groups & days, between days each groups were analyzed by two way ANOVA and paired t-test respectively. (* $P<0.05$, ** $P<0.01$)

Note = the 1st day of treatment = the 6th day of treatment

A. Plasma alpha-tocopherol (mg/L)



B. TA Alpha-tocopherol (mg/L)

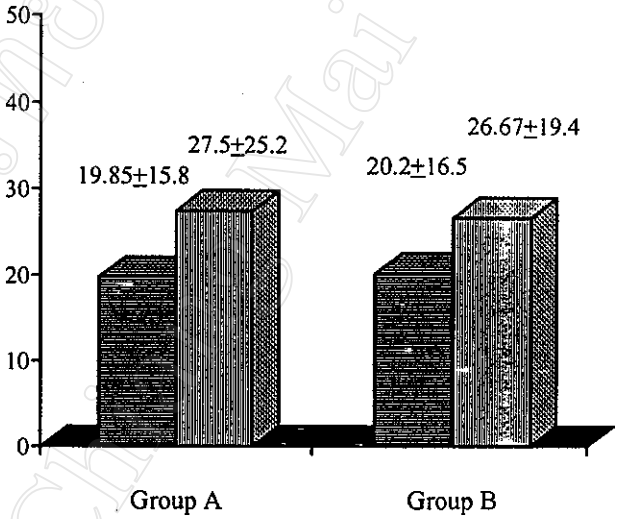
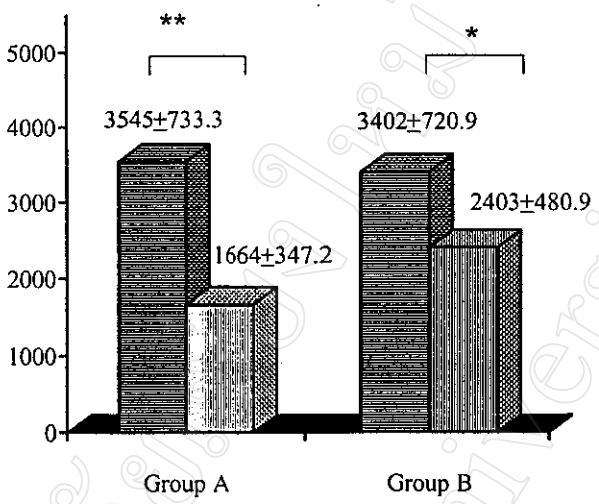


Figure 17. The concentrations of alpha-tocopherol in plasma (A) and Tracheal aspirates (TA) (B) with HPLC method, in the 1st day and 6th day between group A and group B treatment. Data represent mean± SE. Vit E levels within groups & days, between days in each groups were analyzed using two way ANOVA and paired t-test respectively. (* P<0.05, ** P<0.01)

Note = the 1st day of treatment = the 6th day of treatment

A. Serum HA (ng/ml)



B. TA HA (X 10^2 ng/ml)

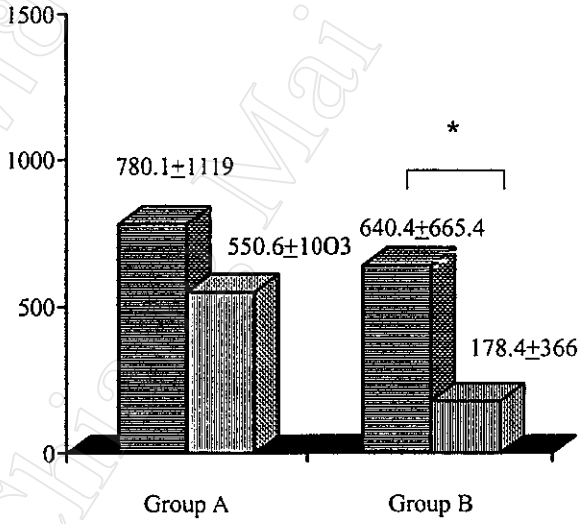


Figure 18. The concentrations of hyaluronic acid (HA) in serum (A) and in tracheal aspirates (TA) (B) with ELISA based-assay, in the 1st day and 6th day between group A and group B treatment. Data represent with mean± SE. HA levels within groups & days, between days in each groups were analyzed using two way ANOVA and paired t-test respectively. (* $P<0.05$, ** $P<0.01$)

Note = the 1st day of treatment = the 6th day of treatment

From the results showed the MDA, GSH, alpha-tocopherol, and HA concentrations in the blood and tracheal aspirates in the Tables 5-8 and Figures 15-18. The statistically comparative of concentrations in the 1st day and the 6th day of group A and B treatments showed an improvement; as following,

In the 6th day, MDA levels in blood and tracheal aspirates were slightly reduced when compared with MDA levels in the 1st day in both groups (Table 5, Figure 15).

Statistically difference of GSH concentration within groups A and B were not found. But the GSH concentrations in the 6th day of the group B treatment showed significantly increased in blood and tracheal aspirates, whereas the GSH levels in tracheal aspirates of group A treatment showed increasing significantly. (Table 6, Figure 16)

Alpha-tocopherol concentrations in blood and tracheal aspirates showed general increasing in the 6th day, when compared with level in the 1st day of both groups. Although in the 1st day, group B represented lower concentration than in group A significantly. (Table 7, Figure 17).

HA levels in blood of both group of treatments were not different in the 6th day compared with the 1st day. In the tracheal aspirates, HA concentrations were significantly decreased only in group B treatment, while HA levels slightly decreased after group A treatment. (Table 8, Figure 18)

CLINICAL IMPROVEMENT AFTER PHYSICAL THERAPY

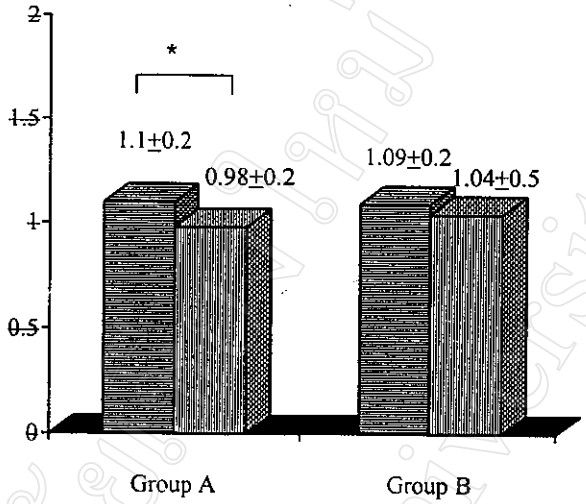
The effects of physical therapy in clinical improvement showed the reduction of lung injury score (LIS) and the improvement of oxygenation index (PaO_2/FiO_2 ratio) showed only significantly in the 6th day of group A that compared with levels in the 1st day. From observation the values in the 6th day of group B showed slightly reduced in lung injury score and increased in oxygenation index as same as in the group A treatment. (Table 9, Figure 19)

Table 9. The lung injury score and oxygenation index (PaO_2/FiO_2) on the 1st day and 6th day in group A (n=20) and B (n=20) treatments.

	Lung injury score		**Oxygenation index** (PaO_2/FiO_2)	
	Group A	Group B	Group A	Group B
The 1st day	1.09±0.2 (0.66-1.33)	1.09±0.2 (1.0-1.33)	122.1±14.9 (53.11-250)	143.9±14.0 (84-255)
The 6th day	0.98±0.2 (0.33-1.33)	1.04±0.5 (0.66-1.33)	169.1±13.1 (74-282.4)	149.9±11.1 (88-250)

Data represents mean±SE. (range)

A. Lung injury score



B. PaO_2/FiO_2 ratio

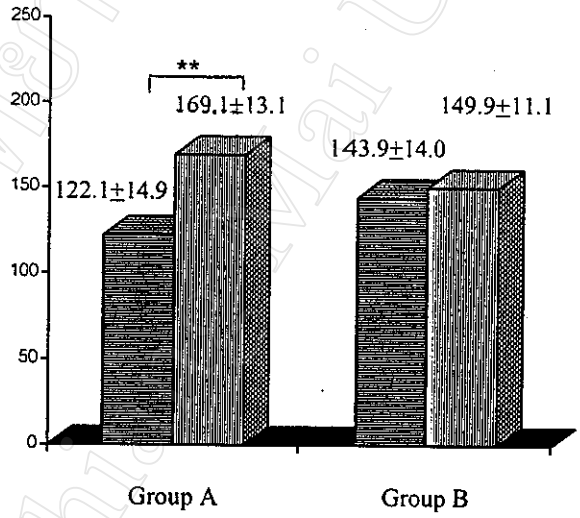


Figure 19. The lung injury score (A) and oxygenation index (PaO_2/FiO_2 ratio) (B) from the arterial blood analyzer and medical ventilator setting in the 1st day and 6th day between group A and group B treatment. Data represent mean±SE. Lung injury score and PaO_2/FiO_2 within groups & days, between days in each groups were analyzed using two way ANOVA and paired t-test respectively. (* $P<0.05$, ** $P<0.01$)

Note = the 1st day of treatment = the 6th day of treatment

The effects of physical therapy in six days on tested substances showed very interesting. From the TA samples of 40 patients, groups A and B were determined for the concentrations of MDA, GSH, Vit E and HA. The levels were shown in the mean±SE (Table 10). The data showed the decline of MDA and HA (Figures 20 and 23), where as increase GSH and Vit E (Figures 21 and 22). For the clinical outcome of physical therapy were found that lung injury score has decline change and PaO_2/FiO_2 ratio incline change as in the figures 24 and 25.

Table 10. The concentrations of MDA, GSH, Vit E, and HA in tracheal aspirates (TA) of six days. Data represent mean±SE.

	Treatment day	Group A	Group B
Glutathione (GSH) (mg/day)	1	3.1±1.7	2.1±1.2
	2	3.5±1.7	2.3±1.7
	3	3.3±1.6	2.8±1.5
	4	4.5±1.7	2.7±0.9
	5	5.2±2.9	2.6±1.4
	6	6.0±2.5	4.4±2.3
Malondialdehyde (MDA) (μmol/L)	1	32±14	31.5±14.5
	2	37.8±14	31.3±8.2
	3	39.5±14	31.4±11.9
	4	37.1±11.3	32.9±14.3
	5	36.17±15.1	31.5±11.0
	6	28.2±14.7	30.5±12.5

Table 10. (continued)

	Treatment day	Group A	Group B
Vit E (mg/L)	1	19.8±15.8	20.2±16.5
	2	21.2±13.3	22.4±13.4
	3	24.5±15.3	24.1±11.6
	4	22.0±14.9	24.3±12.11
	5	19.2±20.1	22.6±17.5
	6	27.5±25.2	26.67±19.4
Hyaluronic acid (HA) ($X10^2$ ng/ml)	1	780.1±1119	640.4±665.4
	2	621.1±780	417.7±577.7
	3	512.0±534	404.7±499.6
	4	521.7±609.9	242.2±421.1
	5	752.9±860.2	326.9±541.3
	6	550.6±1003	178.4±366
Lung injury score Classes: No in jury (<0.1) Mild in jury (0.1-2.5) Severe injury (>2.5)	1	1.09±0.2	1.09±0.2
	2	1.05±0.2	1.08±0.1
	3	1.03±0.2	1.04±0.1
	4	1.00±0.2	1.04±0.1
	5	0.99±0.2	1.07±0.1
	6	0.98±0.2	1.04±0.5
Oxygenation index (PaO_2/FiO_2)	1	123.8±68	135.1±58
	2	127.5±47	136.2±46
	3	123.8±20	129.7±33
	4	145.4±52	130.6±33
	5	136.7±57	135.5±36
	6	172.2±58	149.9±49

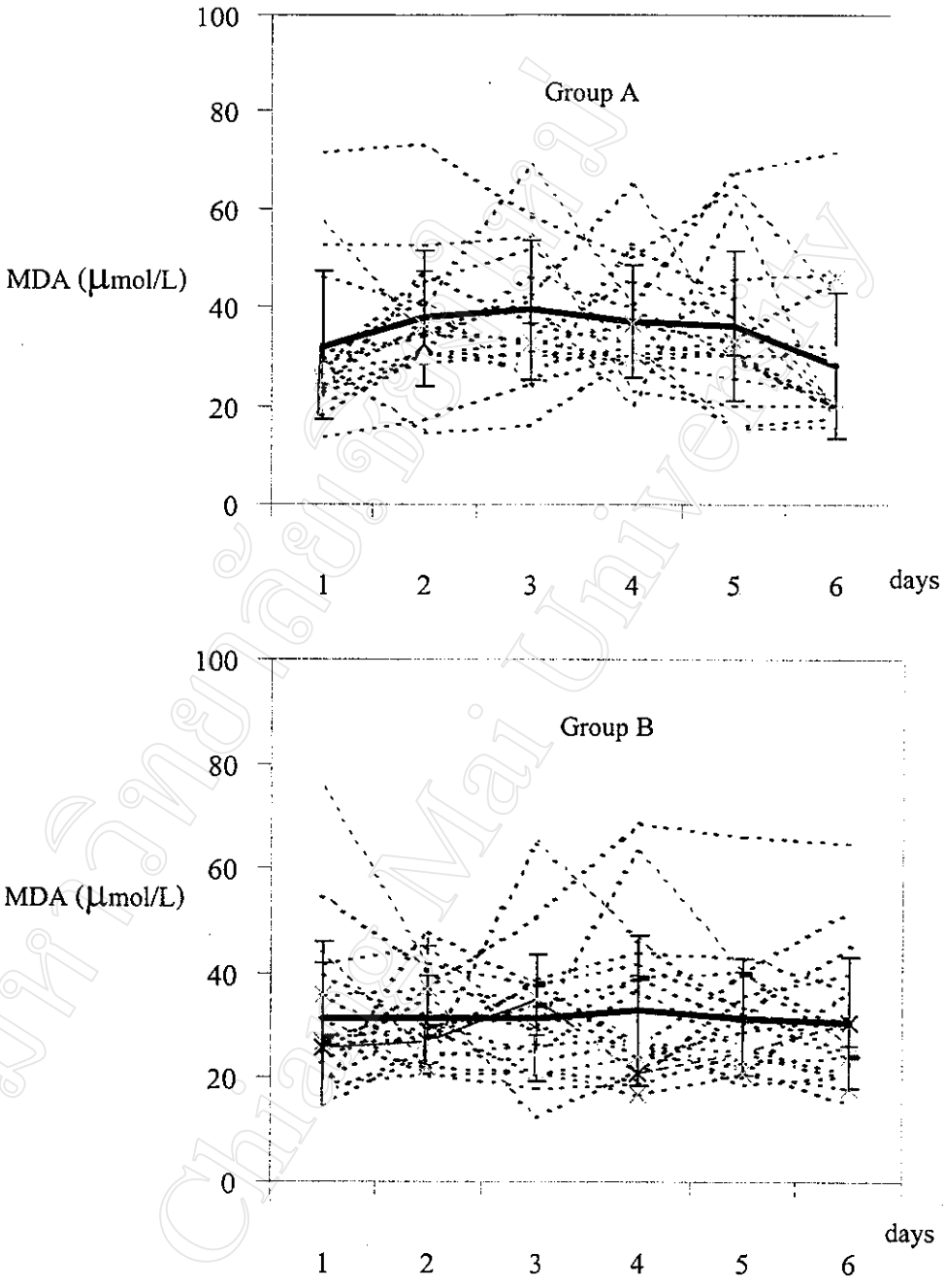


Figure 20. The changes of malondialdehyde (MDA) levels in tracheal aspirates (TA) of group A and group B within six days of physical therapy. Each dash lines represent individual cases and bold line represents the mean±SE.

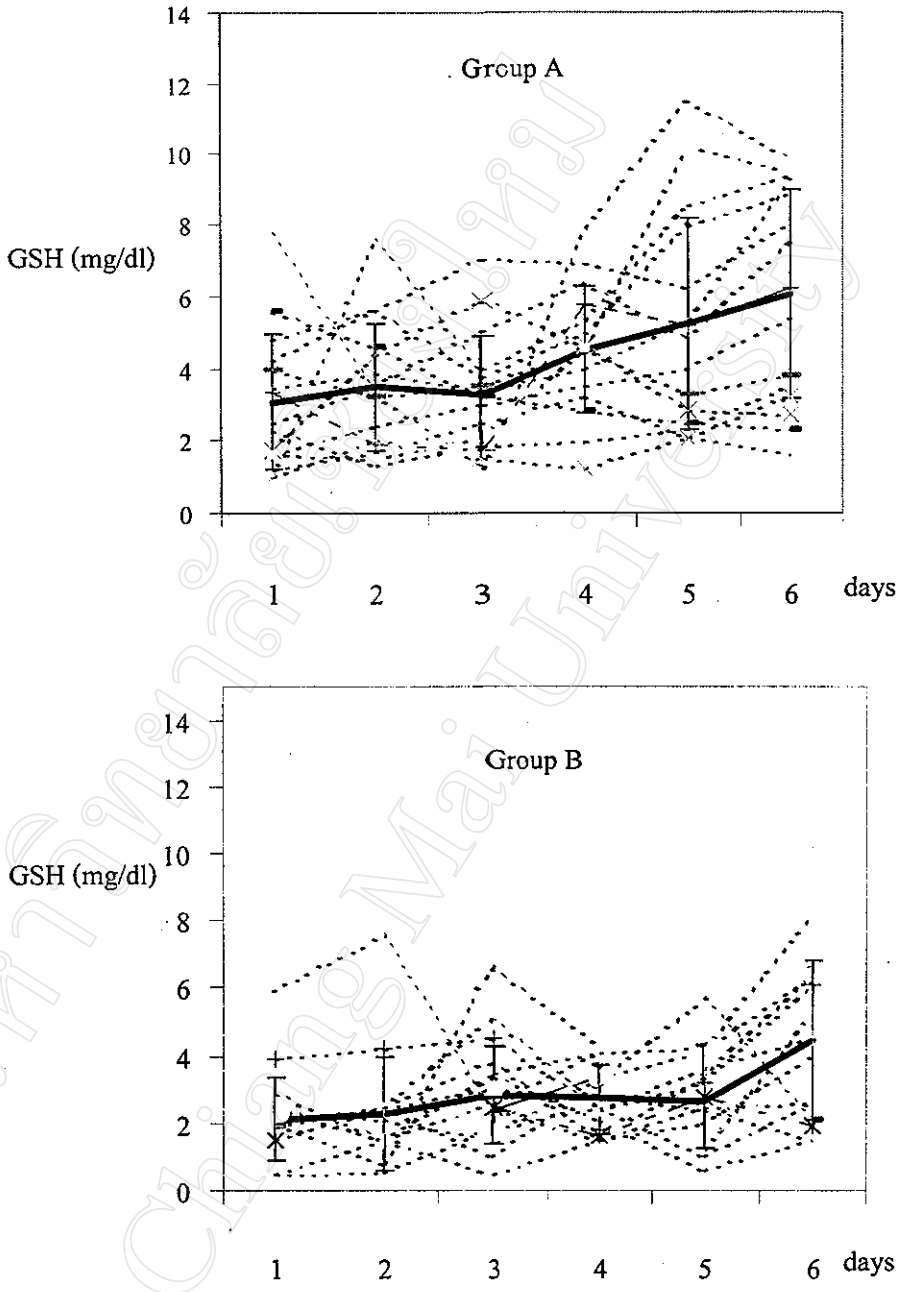


Figure 21. The changes of glutathione (GSH) levels in tracheal aspirates (TA) of group A and group B within six days of physical therapy. Each dash lines represent individual cases and bold line represents the mean±SE.

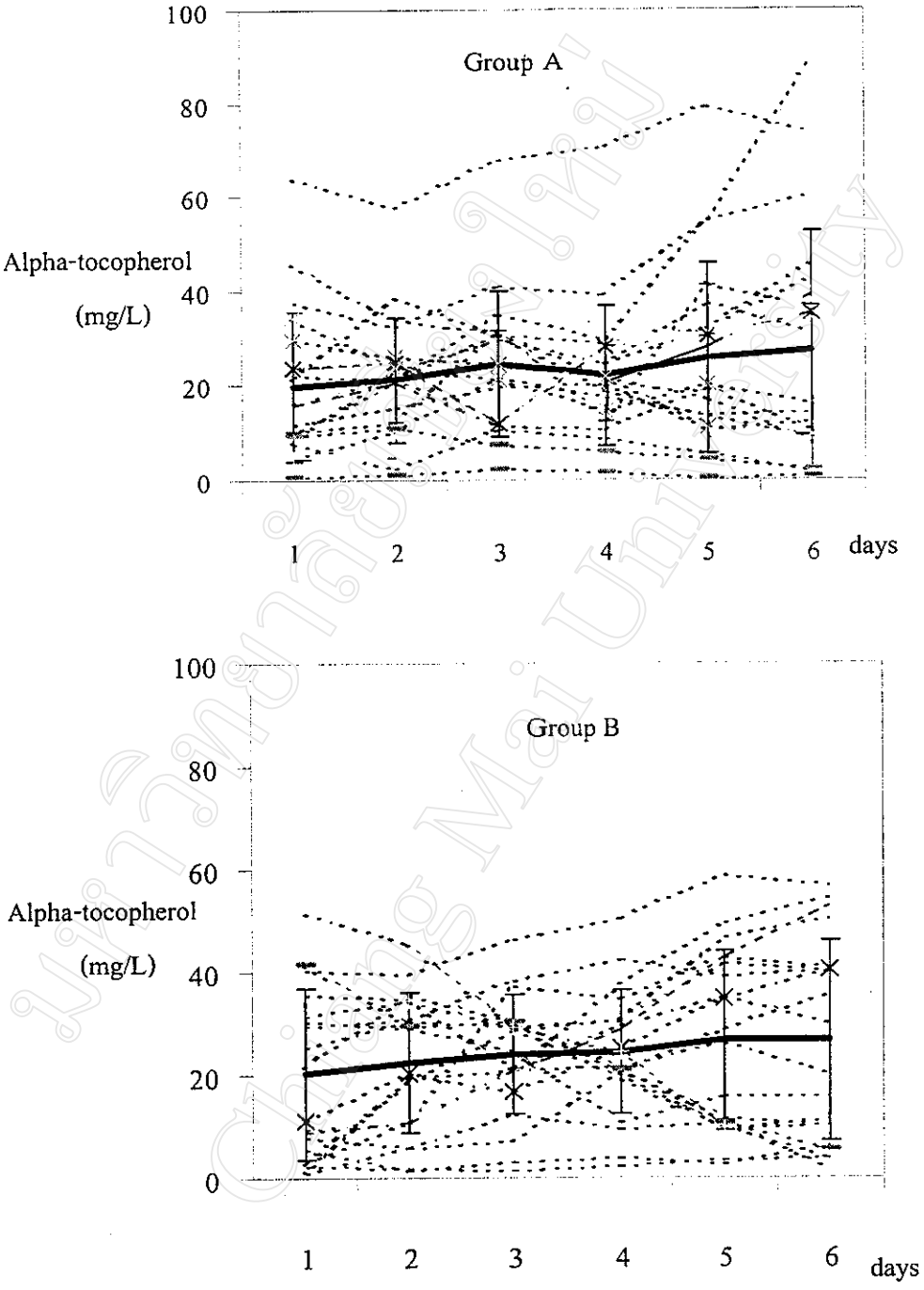


Figure 22. The changes of alpha-tocopherol (Vit E) levels in tracheal aspirtes (TA) of group A and group B within six days of physical therapy. Each dash lines represent individual cases and bold line represents the mean±SE.

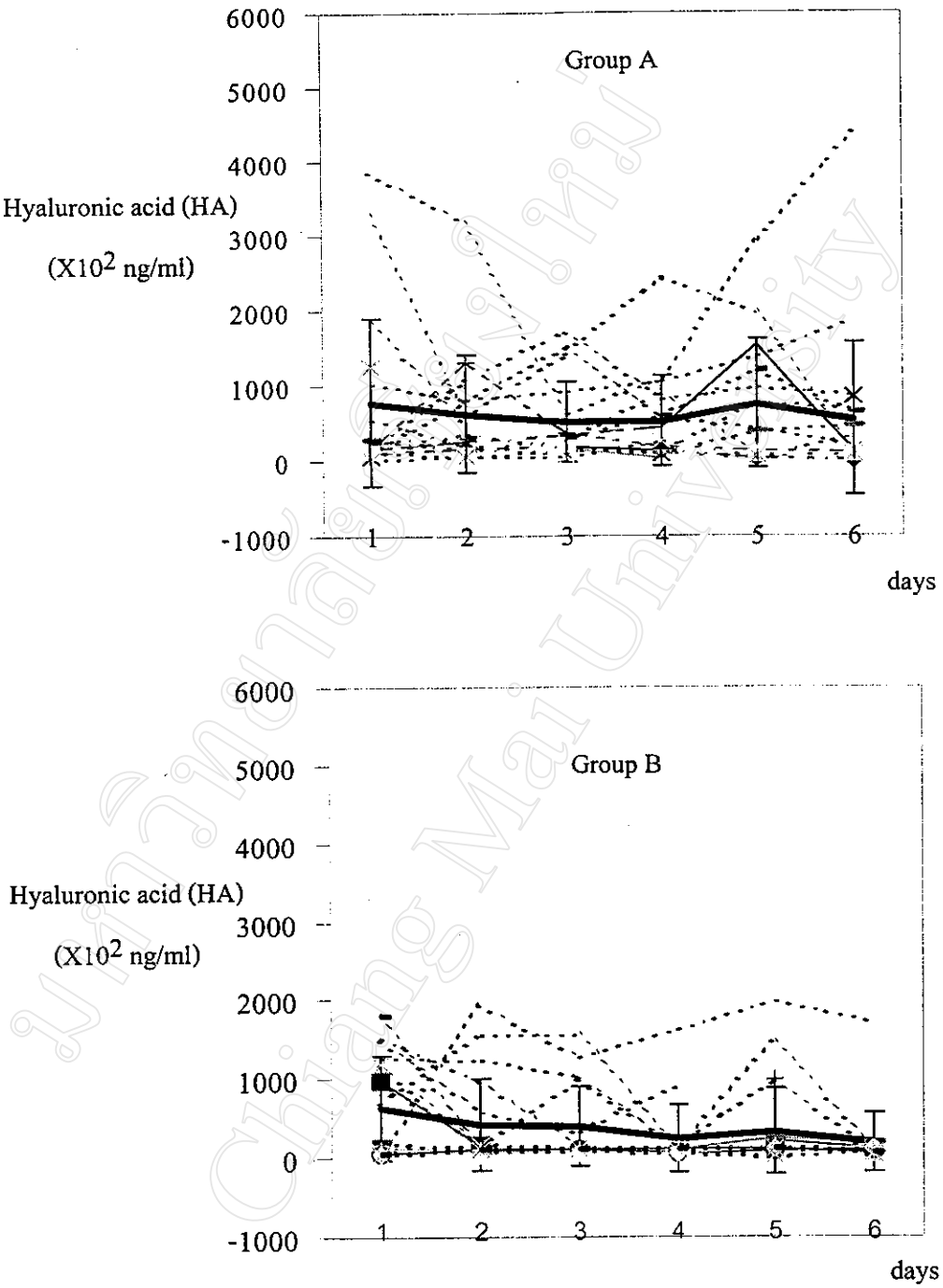


Figure 23. The changes of hyaluronic acid (HA) levels in tracheal aspirates (TA) of group A and group B within six days of physical therapy. Each dash lines represent individual cases and bold line represents mean±SE.

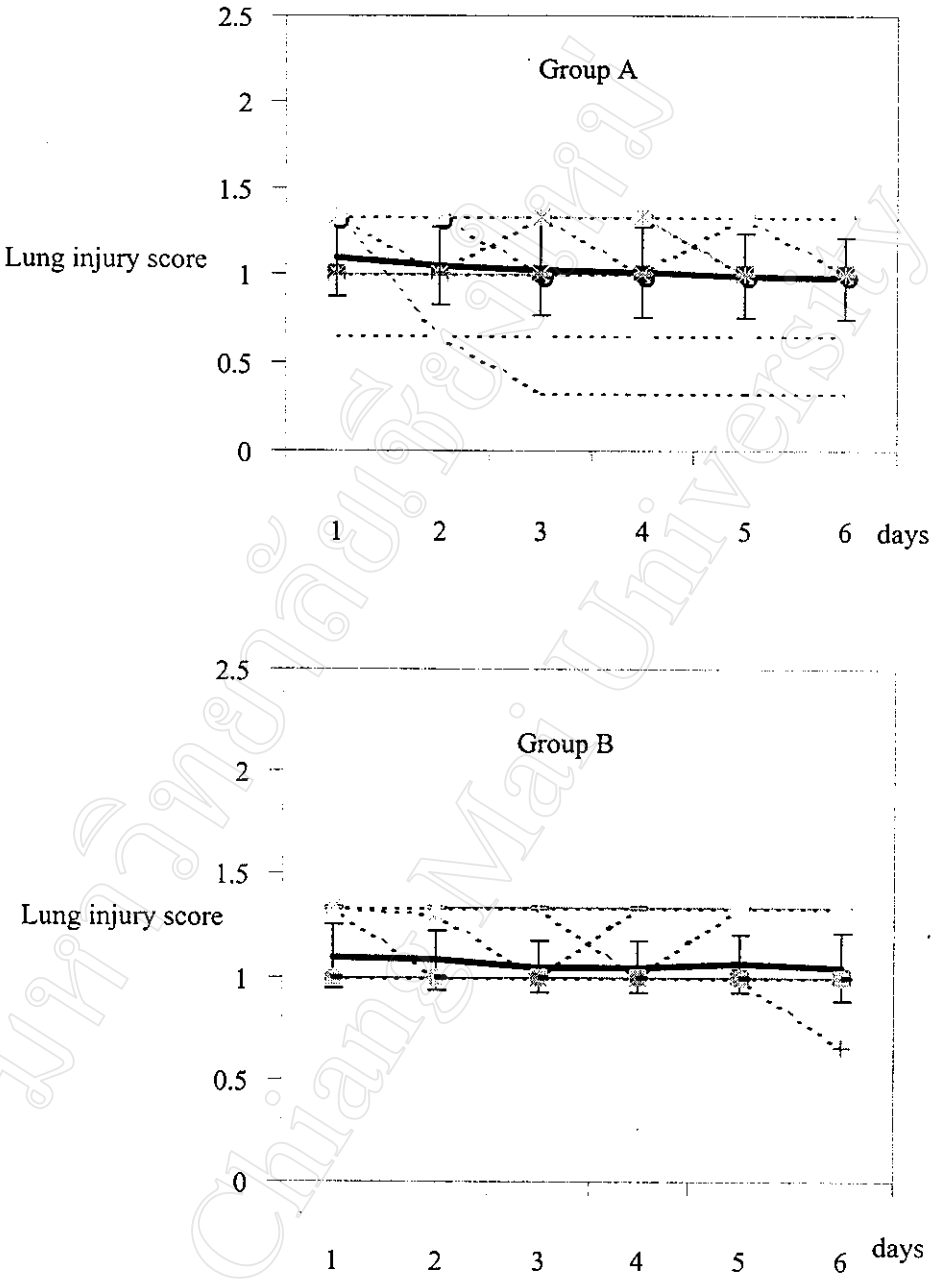


Figure 24. The changes of lung injury score (LIS) of group A and group B within six days of physical therapy. Each dash lines represent individual cases and bold line represents the mean±SE.

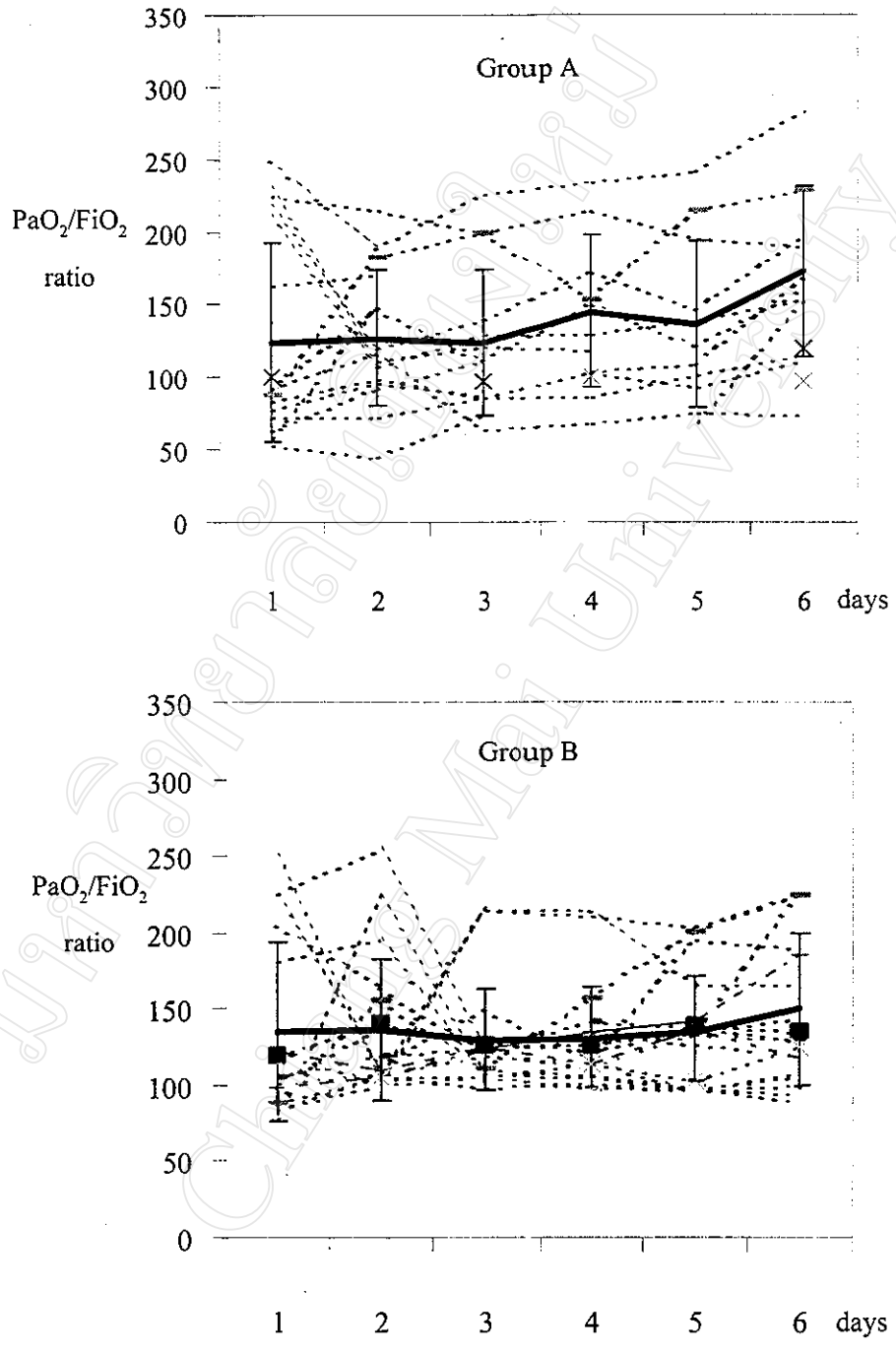


Figure 25. The changes of oxygenation index (PaO_2/FiO_2 ratio) levels of group A and group B within six days of physical therapy. Each dash lines represent individual cases and bold line represents mean±SE.

3.4. THE CORRELATION OF MDA, GSH, ALPHA-TOCOPHEROL, LUNG INJURY SCORE AND OXYGENATION INDEX

The MDA, GSH, alpha-tocopherol, HA levels in blood and TA, lung injury score, and oxygenation index (PaO_2/FiO_2 ratio) of the 1st day were analyzed for statistic correlation. The results of each group (A or B) and whole group were found, as following:

Table 11. The correlation values of glutathione (GSH), malondialdehyde (MDA), alpha-tocopherol (Vit E), hyaluronic acid (HA) in blood and tracheal aspirates (TA), Lung injury score, PaO_2/FiO_2 ratio in the 1st day of group A treatment (n= 20).

(Pearson correlation coefficients)

N=20		Blood				TA					
		GSH[t]	Vit E	MDA	HA	GSH	Vit E	MDA	HA	LIS	PaO_2/FiO_2
Blood	GSH	.895**	.153	-.435	.059	-.219	.099	.054	-.237	.034	.125
	GSH[t]		.264	-.423	.071	-.236	.170	.177	-.086	-.009	.241
	Vit E			-.164	-.102	.033	.070	-.256	-.062	.043	.083
	MDA				.326	-.279	-.077	.122	.166	-.393	-.215
	HA					-.346	.134	.238	-.133	-.108	-.217
TA	GSH						.466	-.339	.032	.037	.338
	Vit E							.046	.106	-.029	.197
	MDA								.494*	-.236	-.506*
	HA									-.339	-.086
	LIS										-.229

Data represent in the correlation coefficients (r). TA = Tracheal aspirates, LIS = Lung injury score, HA = hyaluronic acid, MDA = Malondialdehyde, GSH = glutathione (mg/dl whole blood), GSH^t = glutathione (mg/dl erythrocytes), * $P<0.05$, ** $P<0.01$

Table 12. The correlation values of glutathione (GSH), malondialdehyde (MDA), alpha-tocopherol (Vit E), hyaluronic acid (HA) in blood and tracheal aspirates (TA), Lung injury score, PaO_2/FiO_2 ratio in the 1st day of group B treatment. (n=20)

(Pearson correlation coefficients)

N = 20		Blood				TA					
		GSH^t	Vit E	MDA	HA	GSH	Vit E	MDA	HA	LIS	PaO_2/FiO_2
Blood	GSH	.628**	-.033	.070	.626**	.374	.099	-.031	-.049	-.073	-.180
	GSH^t		-.232	-.375	.232	.024	.095	-.096	-.185	-.098	.114
	Vit E			.178	.025	.188	-.221	.078	.413	-.320	-.393
	MDA				.542**	-.081	-.145	.064	.165	.163	-.281
	HA					-.029	-.028	.099	.038	.077	-.314
TA	GSH						.379	-.065	-.076	-.343	-.029
	Vit E							-.613**	-.805**	.207	.569**
	MDA								.805**	.004	-.642**
	HA									-.230	-.714**
	LIS										.180

Data represent in the correlation coefficients (r). TA = Tracheal aspirates, LIS = Lung injury score, HA = hyaluronic acid, MDA = Malondialdehyde, GSH = glutathione (mg/dl whole blood), GSH^t = glutathione (mg/dl erythrocytes), * P<0.05, ** P<0.01

Table 13. The correlation values of glutathione (GSH), malondialdehyde (MDA), alpha-tocopherol (Vit E), hyaluronic acid (HA) in blood and tracheal aspirates (TA), Lung injury score, PaO_2/FiO_2 ratio in the 1st day of group A and B treatment (n=40)

(Pearson correlation coefficients)

N=40		Blood				TA					
		GSH^t	Vit E	MDA	HA	GSH	Vit E	MDA	HA	LIS	PaO2/FiO2
Blood	GSH	.522**	-.017	-.111	.412**	.086	.093	-.014	-.108	-.027	-.054
	GSH^t		.271	-.365**	.131	-.012	.123	.113	-.096	-.035	.098
	Vit E			-.043	-.036	.212	-.042	-.072	.084	-.067	-.161
	MDA				.410**	-.715	-.104	.100	.166	-.207	-.238
	HA					-.193	.053	.175	-.064	-.032	-.263
TA	GSH						.396*	-.195	.008	-.124	.155
	Vit E							-.262	-.254	.069	.371*
	MDA								.600**	-.143	-.320*
	HA									-.304	.573**
	LIS										-.064

Data represent in the correlation coefficients (r). TA = Tracheal aspirates, LIS = Lung injury score, HA = hyaluronic acid, MDA = Malondialdehyde, GSH = glutathione (mg/dl whole blood), GSH^t = glutathione (mg/dl erythrocytes), * P<0.05, ** P<0.01

From the results of correlation analysis were shown that correlation between MDA, GSH, alpha-tocopherol, HA levels in blood and tracheal aspirates, lung injury score and oxygenation index (PaO_2/FiO_2 ratio) as following:

In group A; The results were found in three couples of significant correlation; erythrocytes GSH and whole blood GSH, MDA levels and HA levels in tracheal aspirates, including MDA levels in tracheal aspirates and PaO_2/FiO_2. (Table 11)

In group B; The results were found in nine couples of significant correlation. In blood there are three couples of correlation; (i) erythrocytes GSH and whole blood GSH; (ii) serum HA and serum MDA; (iii) serum HA and erythrocytes GSH. In tracheal aspirates showed three couples of significance correlation; (i) MDA and Vit E; (ii) HA and Vit E; and (iii) HA and MDA. The results were also showed the significance correlation between PaO_2/FiO_2 ratio with alpha-tocopherol, MDA, and HA. (Table 12)

For the whole group: The antioxidant of erythrocytes GSH was significantly inversely correlated with serum MDA, and also correlation with serum HA, and whole blood glutathione. The HA levels in serum were significantly converse correlated with serum MDA levels. In the tracheal aspirates showed the converse correlation between alpha-tocopherol and glutathione levels, and between HA and MDA levels. The PaO_2/FiO_2 ratio showed significantly positive correlated with alpha-tocopherol levels, and inverse correlated with HA and MDA levels in tracheal aspirates. (Table 13)

3.5. THE CORRELATION BETEWEEN MDA, GSH, VIT E, AND HA WITH OXYGENATION INDEX.

From the correlation values in the table 13 between MDA, GSH, Vit, HA, lung injury level score, and oxygenation index of all patients from the 1st day. The data showed three correlated couples; (i) MDA and PaO_2/FiO_2 ratio, (ii) Vit E and PaO_2/FiO_2 ratio, and then (iii) HA and PaO_2/FiO_2 ratio.

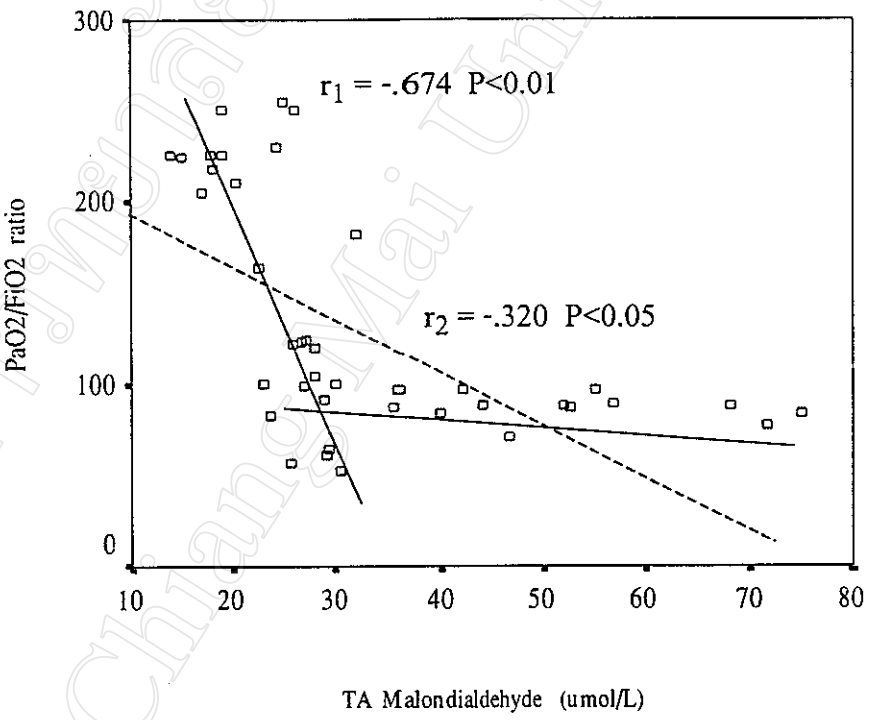


Figure 26. Scatter plots showing relationship between malondialdehyde (MDA) in tracheal aspirates and oxygenation index (PaO_2/FiO_2 ratio) in 40 patients of the 1st day. Each point represents data from a single case. Solid regression line for the all data. r_2 represent the correlation coefficients values of all 40 patients, r_1 represents only in group which showed MDA concentration less than 35 μmol/L.

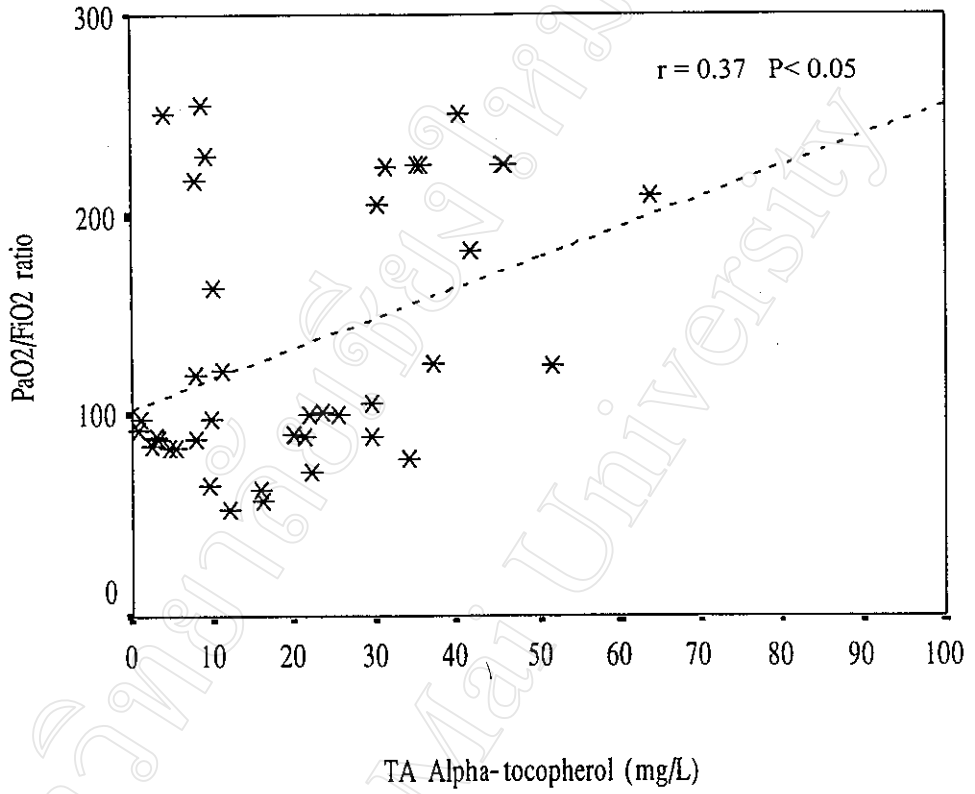


Figure 27. Scatter plots showing relationship between alpha-tocopherol (Vit E) in tracheal aspirates and oxygenation index (PaO_2/FiO_2 ratio) in 40 patients of the 1st day. Each point represents data from a single case.

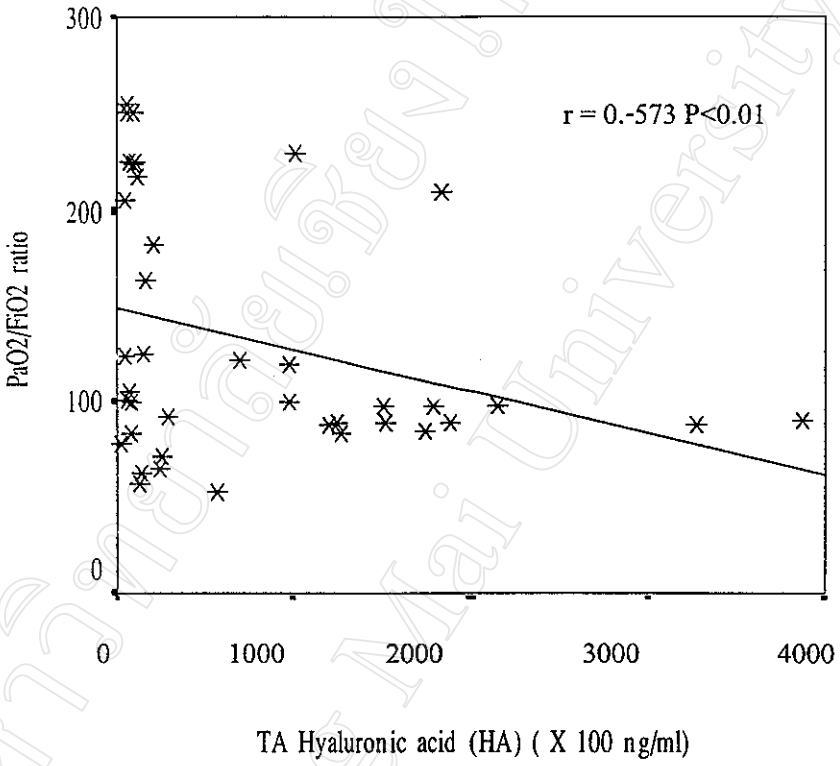


Figure 28. Scatter plots showing relationship between hyaluronic acid in tracheal aspirates and oxygenation index (PaO_2/FiO_2 ratio) in 40 patients of the 1st day. Each point represents data from a single case.