The Evaluation of the Role of the Cytokines TNF- alfa and IL 6 in the Production of Hypoalbuminemia in Patients Undergoing Major Surgical Interventions

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This article presents an observational randomized prospective study done on 65 patients, who underwent major surgical interventions in the field of orthopedic surgery-total hip replacement or general surgery – total colectomy. The level of albuminemia in these cases were determined before the surgical intervention, after 6 hours of the intervention and after 24 h of the intervention. The measurements of the plasmatic concentration of the pro-inflammatory cytokines Tumor Necrosis factor -alpha (TNF-alpha) and interleukin 6 (IL6) were simultaneously done with the determination of the plasmatic levels of albumin. Values of hemoglobin and hematocrit were determined 24 h after the surgical procedure in order to exclude hemodilution, which could lead to a possible drop in the levels of plasmatic albumin. After the collection of the data, the statistical work was done and it consisted of descriptive statistics, correlation and comparison tests as well as statistical validation tests. Obtained results indicate that IL-6 plays a major role comparatively with that of TNF-alfa, regarding the decrease of the plasmatic level of albumin, and due to this, the primordial cause for hypoalbuminemia is an acute hepatic phase reaction. Supplemental permeability of the capillary wall under the action of TNF alpha has a secondary role, but could lead to a faster decrease in plasmatic albumin in the first hours after the surgical procedure.

Keywords: hypoalbuminemia, TNF-alpha, plasmatic protein, hemoglobin, hematocrit, albuminemia, IL 6

Mechanisms which lead to the decrease plasmatic level of albumin in case of patients with systemic inflammatory response syndrome (SIRS) produced by surgical trauma are represented by the permeability of the capillary wall in the conditions in which the hepatic synthesis of albumin lowers, during the acute hepatic phase[1]. The albumin is considered a negative acute phase protein [2,3].

In the same time we can take into consideration another possible mechanism of lowering the concentration of plasmatic albumin-hemodilution, iatrogenic, induced by the perfusion solutions [4,5].

Regarding the permeability of the capillary wall, this phenomenon takes place under the simultaneous action (direct and indirect) of multiple mediators [6], which can be divided in two groups:

-Cellular mediators which are represented by peptide molecules with a pro-inflammatory role – TNF-alpha, IL 6, interleukin 8 (IL 8) and interleukin 1 (IL 1) which are mainly secreted by macrophages, that are found in interstitial tissue[7] The main role of this intercellular messengers is to stimulate the innate immune system so it can react to SIRS [8,9]. Out of all these cellular mediators, TNF-alpha is the most important mediator because it has prolonged action in the induced inflammatory response and it also has the most important role in the synthesis of endothelial glycoproteins [10,11].

-Plasmatic mediators are represented by complement fragments C5a, bradykinin products of the degradation of fibrinogen [12]. The main role is played by complement fragment C5a, the activation of the complement cascade taking place in the case of patients with SIRS appearing secondary to surgical interventions (surgical trauma) is mainly through the positive hepatic phase reaction protein lectin- mannose [13].

In this moment, it is considered that cellular mediators TNF-alpha simultaneously acts with C5a inducing in the capillary wall (interendothelial junction) for the activation of neutrophils [14]. Neutrophils release proteolytic enzymes and oxidation products, both having a role in the destruction of junctional proteine-cadeine. This mechanism is considered the main way in which the permeability of the wall occurs [15-17].

The secondary role is considered to be held by plasmatic mediators (bradykinin, serotonin and histamine) which acts specifically through receptors found in the capillary wall [18] Due to this, wall permeability is achieved through enzyme kinase, which produce the phosphorylation with degradation of junctional proteins [19,20].

Regarding the acute hepatic phase, it is considered an adaptation reaction meaning that under the action of IL6 and possible IL1 [21,22], the stimulation of positive proteins occur in the detriment of negative ones [23,24,]. If a plasmatic value of a protein is modified by at least 25% then it is considered an acute phase protein [8,9]. The main role of a positive protein is to activate the complement cascade and bacterial opsonization [25-27].

Experimental part

Ethical Committee of The Arad Clinical County Hospital gave approval for this study. After obtaining the written consent, 68 patients who underwent surgical procedures such as total hip replacement and partial or total colectomy were initially introduced in the study. The main exclusion criteria was the presence of associated pathology which

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can lead to decrease of the plasmatic albumin level such as hepatic or renal pathology, diarrheal disease with protein loss. One patient was excluded after 6 h due to high level of of transaminases and bilirubin and other two patients were excluded after obtaining extremely high TNF-alpha levels, which can be due to a errors during blood withdrawing procedures (hemolysis occurred after blood centrifugation).

The determination of albuminemia was done through dry chemistry methods based on bounding of the albumin with a pigment substance in an acidic environment. This bounding produces a changing in color from yellow to purple. This change is quantified using spectrophotometry. The reaction that stands at the basis of determining the albuminemia level is:

Albumin + Brooches purple (BCP)----Ph. acid--------->

--------->Complex BCP-Albumin

Regarding the determination of total proteins, this was based of the reaction of these proteins with Cu+2 and measuring with spectrophotometry of the formed complex.

Protein totale+Cu+2---OH_------→Complex Prot.-Cu

The determination of hepatic transaminases (ALT and AST) was based on the colorimetric method and bilirubin levels were measured using the enzyme oxidation method. The precision of the measurements were evaluated according to the standard guidelines issued by the Institution of Clinical Studies. The precision was evaluated using BIO-RAD level 1-3 and the result showed that the variation coefficient was less than 5% in each determination.

The measuring of plasmatic level of hemoglobin was based on dilutions with lyzer and analyzed photometrically by an automatic analyzer. The principle of measuring the hematocrit consists of measuring the height of the impulse of the cell that went through the aperture, this height is directly proportional with the volume of the cell. The hematocrit is measured by numerical integration of the cell volume (MCV).

Regarding the determination of the cytokines TNF-alpha and IL-6, these were done using the ELISA reaction on a chromogen solution, using specific kits such as IL-6 ELISA Kit and TNF-alpha ELISA Kit.

The blood samples were drawn using a holder, centrifugation took places at around 30-45 min after the blood draw to prevent hemolysis and they were immediately stored at -80 degrees Celsius.

The determination is based on the quantitative imunoenzymatic reaction of IL-6 and TNF alpha from the serum, having a good precision. Calibrators and samples react to monoclonal antibodies tied to the wall of the wells. The measuring of these complexes (antibody-enzyme) is done through a chromogenic reaction after adding the substrate and the changing of colors and were measured using spectrophotometry. The reference level for IL 6 Is 7 pg/mL and for TNF alpha is 8.7 pg/mL.

Statistical tests were done using the program called Jamovi.

Descriptives

	IL 6 / 0	IL 6 / 3	IL/6	IL 6 / 24
N	65	65	65	65
Missing	934	934	934	934
Mean	3.65	9.30	45.6	190
Median	3.10	5.10	40.0	184
Standard deviation	2.81	10.7	36.2	55.4
Variance	7.90	115	1312	3067
Range	16.4	54.8	218	281
Minimum	1.10	1.20	10.0	101
Maximum	17.5	56.0	228	382
25th percentile	1.90	2.80	20.0	151
50th percentile	3.10	5.10	40.0	184
75th percentile	5.00	13.0	61.0	210

Table 1
VALUES FOR IL 6

Descriptives

	TNF / 0	TNF / 3	TNF / 6	TNF / 24
N	65	65	65	65
Missing	934	934	934	934
Mean	4.12	11.4	5.54	4.58
Median	3.60	11.7	4.60	3.20
Standard deviation	2.40	4.95	5.17	6.69
Variance	5.75	24.5	26.8	44.7
Range	10.6	20.6	33.0	44.3
Minimum	1.10	1.75	1.00	0.700
Maximum	11.7	22.3	34.0	45.0
25th percentile	2.20	8.20	2.10	1.50
50th percentile	3.60	11.7	4.60	3.20
75th percentile	6.10	14.5	6.90	5.10

Table 2
VALUES FOR TNF ALPHA

Results and discussions

In the tables below the descriptive statistics are shown for TNF alpha and IL6 which consists in calculating the medium values, medians, and standard deviations and also the dispersions of the groups.

We can observe an increase of medium IL 6 values a little bit higher than the reference limit at approximately 3 h post surgery, then high and very high values at 6 and 24 h. In the case of TNF-alpha only the values availed at 3 h were over the reference level. The rest of the values meaning those recorded at 6 and 24 h post surgery, were below the reference level.

On the other hand we observed a very high level of the standard deviation and variance in the case of IL 6 and high levels of the same parameters in the case of TNF alpha. These very high values are caused by the variation of the plasmatic concentration of these cytokines in the moment when blood was drawn.

Statistical validation tests of the differences between medium values calculated in case of TNF-alpha.

	Sum of Squares	df	Mean Square	F	p	η^2	$\eta^2 p$	ω^2
fix 0-3	1719	1	1719.2	114	<.001	0.470	0.470	0.464
Residuals	1936	128	15.1					

Table 3
ANOVA test validates the difference between 1 and 3 of TNF- alpha resulting a F= 114 at a p<0.001

	Sum of Squares	df	Mean Square	F	p	η^2	η^2p	ω^2
0-6 tnf	65.3	1	65.3	4.02	0.047	0.030	0.030	0.023
Residuals	2081.0	128	16.3					

Table 4. ANOVA test validates nearly at the limit the differences between 0 and 6 of TNF- alpha resulting in an F of only 4.02 at a P=0.047 (all the other tests are highly significant statistically p<0.001)

	Sum of Squares	df	Mean Square	F	p	η^2	η^2p	ω^2
fix 0 -24	6.78	1	6.78	0.269	0.605	0.002	0.002	-0.006
Residuals	3231.14	128	25.24					

Table 5. ANOVA test does not validate the difference between 0 and 24 resulting the value of F=0.269 at a p>0.05.

In case of TNF-alpha, the single ANOVA test which validates this difference at a p<0.001 is the test done in the first 3 h post surgery. The other tests do not validate these differences (from 6 to 24 h) or marginally validates them (from 3 to 6 h). Based on this results, values below the reference level, non-validated statistical tests and also the decreasing trends of TNF-alpha after 3 h, we can state that the only time interval when TNF- alpha acts is from 0 to 3 h, meaning right after the surgical trauma occurs.

Validation tests of the differences between medium values calculated in the case of IL 6.

ANOVA

	Sum of Squares	df	Mean Square	F	p	η^2	η^2p	ω^2
fix 0-3	1037	1	1037.2	16.9	< .001	0.117	0.117	0.109
Residuals	7834	128	61.2					

Table 6. ANOVA test validates the differences between 0 and 3 of IL-6 representing F =16.9 at a p < 0.001.

	Sum of Squares	df	Mean Square	F	p	η^2	η^2p	ω^2
fix 3 - 6	42858	1	42858	60.1	< .001	0.319	0.319	0.312
Residuals	91303	128	713					

Table 7. ANOVA test validates the differences between 3 and 6 resulting F = 60.1 at a p < 0.001

	Sum of Squares	df	Mean Square	F	p	η^2	η^2p	ω^2
factor fix	1.49e+6	3	496775	441	< .001	0.838	0.838	0.836
Residuals	288118	256	1125					

All this three ANOVA tests validate this difference regarding the variation of IL 6. Based on this tests and on values from descriptive statistic, we can state that this cyokine begins its action just before the 3 h mark from the production of the surgical trauma and this action could go on after 24 h, taking into account the high level of the medium value at this moment.

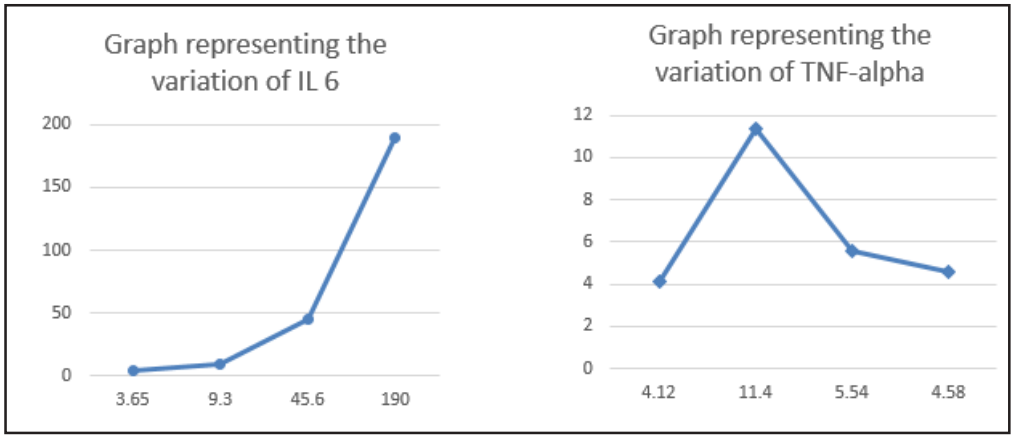
After the statistical validation of both cytokines we can draw the graph below for IL 6 as well as for TNF-alpha. The very high increase of medium values from 6 to 24 h of the IL 6, sustains the hypothesis of *flow-stream*, meaning the secretion of this cytokine by the macrophage, takes place under the action of the other pro-inflamatory cytokines(TNF- alpha, IL 1, IL 8).

ANOVA test validates this hypothesis.

Table 8.ANOVA test validates the differences between 0 and 24 resulting F=441 at a p < 0.001

	Sum of Squares	df	Mean Square	F	p	η^2	η^2p	ω^2
6-24	680980	1	680980	311	< .001	0.708	0.708	0.705
Residuals	280285	128	2190					

Table 9. ANOVA test validates the differences between 6 and 24 h resulting F = 311 at a p<0.001.



This chart represents the variation of medium values of plasmatic concentration of both cytokines (L 6 and TNF-alpha).

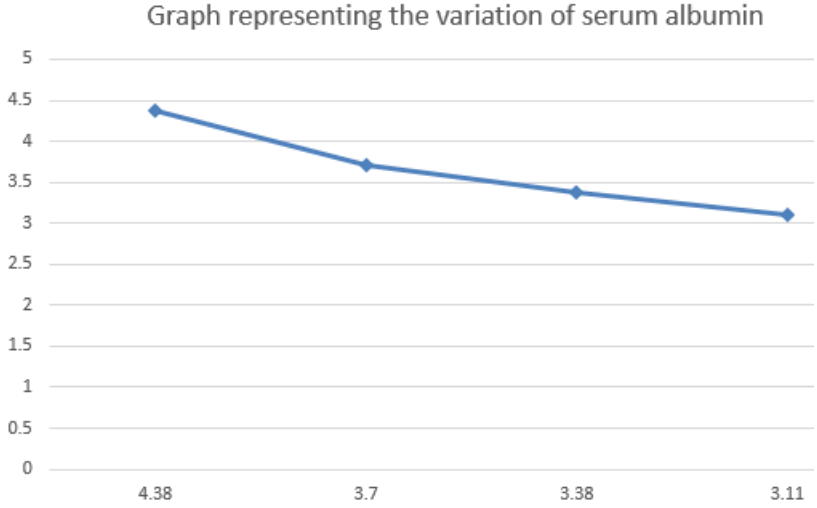
Descriptive statistics of albumin

	ALBUMINA 0	ALBUMINA 3	ALBUMINA 6	ALBUMINA 24
N	65	65	65	65
Missing	934	934	934	934
Mean	4.38	3.70	3.38	3.11
Median	4.40	3.70	3.30	3.10
Standard deviation	0.483	0.451	0.466	0.448
Variance	0.234	0.203	0.217	0.201
Range	2.30	2.00	1.60	1.70
Minimum	3.10	2.70	2.60	2.20
Maximum	5.40	4.70	4.20	3.90
25th percentile	4.10	3.30	3.10	2.80
50th percentile	4.40	3.70	3.30	3.10
75th percentile	4.70	4.00	3.80	3.50

We can observe the tendecy of continous decrease of the plasmatic level of albumin also very low values of standard deviation and its variance.

	Sum of Squares	df	Mean Square	F	p	η^2	η^2_p	ω^2
0-3 alb	15.2	1	15.233	69.7	< .001	0.353	0.353	0.346
Residuals	28.0	128	0.219					

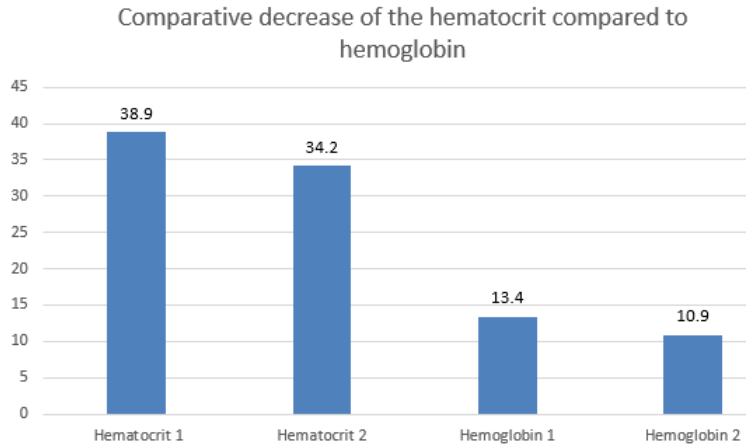
ANOVA test validates the difference between 0 and 3 resulting in a $F = 60.97$ at $p < 0.001$. All the other validation tests are statistically significant because, considering descriptive statistic, values of the albuminemia at 6 and 24 h are lower than the value at 3 h.



This chart represents the variation of plasmatic levels of albuminemia.

Taking in the consideration all the mechanisms which could lead to the decreasing of albuminemia, for the exclusion of a possible hemodilution, we calculated the medium value of hemoglobin and hematocrit both at 0 and at 24 h.

	AD	HEMATOCRIT	AF	HEMOGLOBINA
N	65	65	65	65
Missing	934	934	934	934
Mean	38.9	34.2	13.4	10.9
Median	39.0	35.0	14.0	11.0
Minimum	30.0	23.0	10.0	7.00
Maximum	49.0	42.0	16.0	13.0



We can see a percentual drop of hemoglobin higher than the hematocrit, 19 to 13%, which excludes a possible hemodilution which can lead to decrease of albuminemia.

Correlation tests were done and are shown in the table below. We tried to determine a possible correlation between the plasmatic level of albumin, total plasmatic proteins, IL 6 and TNF alpha.

Model	Predictor	Estimate	SE	t	p
1	Intercept	4.32142	0.1205	35.864	< .001
	TNF / 0	0.01460	0.0253	0.576	0.567
2	Intercept	4.30876	0.1375	31.328	< .001
	TNF / 0	0.01384	0.0258	0.536	0.594
	IL 6 / 0	0.00432	0.0220	0.196	0.845
3	Intercept	1.65015	0.6389	2.583	0.012
	TNF / 0	0.00250	0.0230	0.109	0.914
	IL 6 / 0	0.00872	0.0195	0.446	0.657
	PROT TP0	0.41649	0.0982	4.239	< .001

This table represents the calculation of Pearson coeficient between the variable stated.

The only correlation statistically validated is between albumin and total plasmatic protein, resulting an Pearson coeficient of 0.41 at $p < 0.001$. There are 2 possible hypothesis which explain the lack of correlation between IL 6, TNF-alpha and plasmatical levels of albumin.

The first hypothesis consists of statistical errors and it's based on the really high and high values of the dispersion and also the standard deviations in the case of IL6 and TNF alpha (uneven lots), compared to those on which the album was tested.

The second hypothesis consists of the physiopathological mechanism which produces hypoalbuminemia, a direct interaction between the molecule of the albumin and the polipeptide molecules of the cytokines does not exist, as in this case it is necessary the intervention of intermediate mediators. In the case of total proteins and albumin, it is known that albumin determins aproximatively 60% of the plasmatic concentration of the total proteins, and hence exists in this way a direct interaction.

The way in which the variation of the plasmatic proteins depend on the variation of the plasmatic album was analysed using the linear regression method.

Model Fit Measures

			Overall Model Test					
Model	R	R^2	F	df1	df2	p	BF_{10}	±%
1	0.479	0.229	18.7	1	63	< .001	376	2.27e-5

Based on this statistical evaluation, we find a determination factor R2 = 22 at a $p < 0.001$

In order to validate the hypothesis that there prevails a relationship between the increase of plasmatic values of these cytokines and the decrese of the plasmatic concentration of the albumin. We used the multiple comparison ANOVA test.

Within Subjects Effects

	Sum of Squares	df	Mean Square	F	p	η^2	partial η^2	ω^2
RM Factor 1	565379	1	565379	736	< .001	0.457	0.852	0.456
RM Factor 1 ✻ timp 0 -24	574237	1	574237	747	< .001	0.464	0.854	0.463
Residual	98343	128	768					

Note. Type 3 Sums of Squares

The first ANOVA test calculated between the increase at 0-24 h of the cyokine IL 6 and the decrease of the albuminemia in the same interval, indicates a variation coefficient of F = 736 at a $p<0.001$.

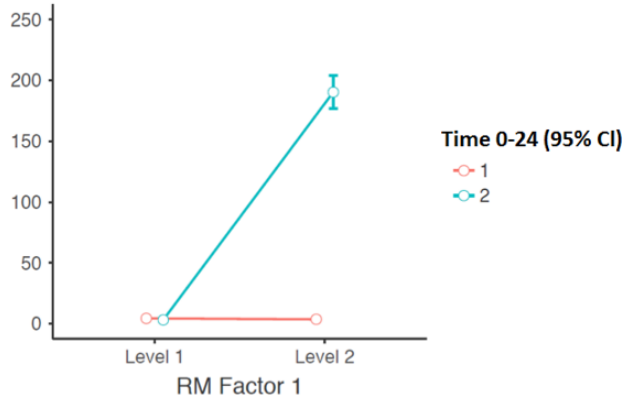
	Sum of Squares	df	Mean Square	F	p	η^2	partial η^2	ω^2
timp 0 -24	558851	1	558851	726	< .001	0.850	0.850	0.848
Residual	98499	128	770					

Note. Type 3 Sums of Squares

The second ANOVA test calculated between IL6 and blood albuminemia in the same interval, indicates a variation coefficient F = 726 at a $p < 0.001$.

Post Hoc Comparisons - RM Factor 1 ✻ timp 0 -24

Comparison									
RM Factor 1	timp 0 -24		RM Factor 1	timp 0 -24	Mean Difference	SE	df	t	ptukey
Level 1	1	-	Level 1	2	1.268	4.86	256	0.261	0.994
		-	Level 2	1	0.728	4.86	128	0.150	0.999
		-	Level 2	2	-185.988	4.86	256	-38.237	< .001
	2	-	Level 2	1	-0.540	4.86	256	-0.111	1.000
		-	Level 2	2	-187.255	4.86	128	-38.513	< .001
Level 2	1	-	Level 2	2	-186.715	4.86	256	-38.387	< .001



The graph shown above indicate an increase of the plasmatic values of IL6 simultaneously with the decrease of the plasmatic albumin values at the interval 0-24h.

The first multiple comparison ANOVA test between TNF-alpha in the interval 0-3h and the decrease of plasmatic albumin in the same interval, indicates a F of only 136 at a $p<0.001$.

Within Subjects Effects

	Sum of Squares	df	Mean Square	F	p
RM Factor 1	897	1	897.45	119	< .001
RM Factor 1 ✻ timp 0-3	1029	1	1029.03	136	< .001
Residual	968	128	7.56		

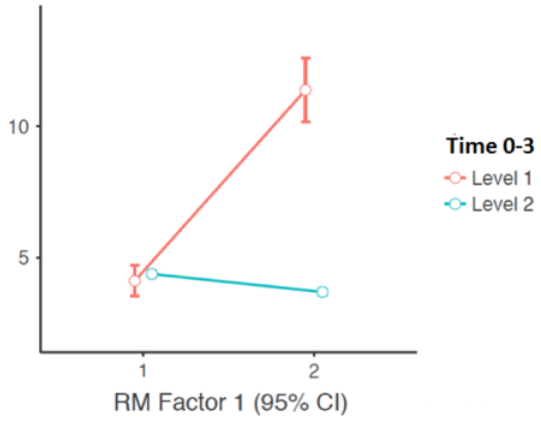
Note. Type 3 Sums of Squares

Between Subjects Effects

	Sum of Squares	df	Mean Square	F	p
timp 0-3	705	1	705.38	90.7	< .001
Residual	996	128	7.78		

Note. Type 3 Sums of Squares

The second multiple comparison ANOVA test indicate an F of only 90.7 at a $p<0.001$.



The graph represented above represents an increase of TNF-alpha with the decrease of plasmatic albumin at the 0-3 h interval.

Post Hoc Comparisons - RM Factor 1 * timp 0-3

	Comparison								
RM Factor 1	timp 0-3		RM Factor 1	timp 0-3	Mean Difference	SE	df	t	pscheffe
Level 1	1	-	Level 1	2	-7.273	0.486	256	-14.970	< .001
		-	Level 2	1	-0.263	0.482	128	-0.545	0.998
		-	Level 2	2	0.422	0.486	256	0.868	0.980
	2	-	Level 2	1	7.010	0.486	256	14.429	< .001
		-	Level 2	2	7.695	0.482	128	15.949	< .001
Level 2	1	-	Level 2	2	0.685	0.486	256	1.409	0.851

Based on this multiple comparison ANOVA tests, we can affirm that there is a relationship, statistically validated, between the increase of plasmatic concentration of cytokines and the decrease of the plasmatic albumin values. The variation coefficient F is really high in case we compare the increase of the IL 6 with the decrease of albumin, this phenomenon is due to very high concentrations of IL6 in the last hours.

Conclusions

Interferential statistics tests - ANOVA (simple or multiple), along with data from descriptive statistic, validate the hypothesis that IL6 holds the most important role in the decrease of plasmatic albumins compared to TNF-alpha role, in the patients sample that participated in this study. We can also state that there is no statistic correlation validated between the values of the inflammatory cytokines and plasmatic albumin.

According to this study, the rise of TNF-alpha produces a decrease of albumin only in the initial hours (0-3 h), after the surgical trauma. IL6 begins to increase in the first 3 h and acts just before the end of this period, the increase of IL6 is associated with the decrease of albuminemia in the interval 3-24 h , this decrease being higher, if we compare it to the decrease of plasmatic level of albumin, in the time interval of 0-3 hours. The high medium values of the concentration of IL6 at 24 h indicate a possible action of this cyokine even after this moment, being the only inflammatory cytokine with a prolonged action [28,29]

Taking into consideration the fact that IL6 acts only on the hepatic tissue during the acute hepatic phase, which leads to a decrease of the synthesis of albumin, and also this cytokine is the only one that has a prolonged action [30,31]. We can affirm that this mechanism plays a crucial role in the decrease of albumin levels [32,33].

Future studies should demonstrate the precise mechanism of action in case of IL6, at level of hepatic tissue. It stimulates only the synthesis of positive acute phase proteins in detriment of negative ones, or there is a simultaneous inhibition of negative phase proteins [34].

The next experimental studies will focus on determining the role of hypertonic solution of albumin, given for the correction of hypoalbuminemia, whether this administration blocks the increase of IL6 by the possible anti-inflammatory effects of this solution. Another possibility is IL6 will remain high and this increase of serum IL6 is due to surgical trauma. The decrease of IL6 should demonstrate a curve of positive feedback, in which high IL6 caused by the surgical trauma induces the decrease of serum albumin and is maintained by hypoalbuminemia, in this case the administration of hypertonic albumin solution should interrupt this vicious cycle.

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