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Systemic effects of locally injected Platelet Rich Plasma in a rat model: an analysis on muscle and bloodstream

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SUMMARY

Abundant evidences suggest that growth factors, contained in platelets alpha granules, may play a key role in the early stages of the muscle healing process with particular regard to the inflammatory phase. Although the contents of the platelet-rich plasma preparations have been extensively studied, the biological mechanisms involved as well as the systemic effects and the related potential doping implications of this approach are still largely unknown. The aim of the present study was to investigate whether local platelet-rich plasma administration may modify the levels of specific cytokines and growth factors both in treated muscle and bloodstream in rats. Additional aim was to investigate more deeply whether the local platelet-rich plasma administration may exert systemic effects analyzing contralateral lesioned but untreated muscles. The results of the present study showed that platelet-rich plasma treatment induced a modification of certain cytokines and growth factor levels in muscle but not in the bloodstream, suggesting that local platelet-rich plasma treatment influenced directly or, more plausibly, indirectly the synthesis or recruitment of cytokines and growth factors in the site of injury. Moreover, the observed modifications of cytokine and growth factor levels in contralateral injured but not treated muscles, strongly suggested a systemic effect of locally injected platelet-rich plasma.

INTRODUCTION

The repair response of the musculoskeletal tissue generally starts with the formation of a blood clot and the following degranulation of platelets, which release locally growth factors (GFs) and cytokines (1). This microenvironment results in chemotaxis of inflammatory cells as well as activation and proliferation of local progenitor cells. Alpha granules are storage units within platelets, which contain pre-packaged GFs in an inactive form. Abundant evidences suggest that GFs, contained in platelet alpha granules, may play a key role in the early stages of the healing process with particular regard to the inflammatory phase being able of modulating the recruitment, duplication, activation and differentiation of the cells involved in the healing process (2-6). The efficacy of those GFs should be, in theory, directly proportional to their local concentration. This hypothesis is at the base of the use of platelet-rich plasma (PRP) in several circumstances, all of them characterized by the need of activating, modulating, speeding up or ameliorating the process of tissue repair.

With regard to sport medicine, doping related issues are still matter of debate when considering this therapeutic approach for the treatment of sport-related injuries, in particular because of the Insulin-like growth factor-1 (IGF-1) content in the platelets alpha granules as well as the blood manipulation procedures. With particular regard to the muscle injection of platelets derived GFs, several issues still need to be clarified. Assuming that this procedure, as it has been demonstrated by several studies, is able to ameliorate the muscle tissue repair processes, it is still unclear whether the locally injected

concentrated amount of GFs may exert systemic effects. Indeed, although the contents of the PRP preparations themselves have been extensively studied (7-12), the biological mechanisms involved in treatment with PRP as well as its systemic effect with the related potential doping implications are largely unknown. With this regard, it has to be underlined that the World Anti-Doping Agency (WADA) prohibited the use of intramuscular injection of PRP in the 2010 list (13), then allowing its use in the 2012 list (14). This modification has been introduced despite the suggested systemic effect of locally injected GFs described by some authors (7, 15). Banfi et al. measured the levels of some cytokines and GFs in the serum obtained from five male subjects 30 minutes, three hours and 24 hours after the treatment with PRP in order to evaluate the eventual systemic effect of this local injection. Authors reported significant modifications of Vascular Endothelial Growth Factor (VEGF), Epidermal Growth Factor (EGF) and Chemokine (C-C motif) Ligand 2 (CCL2) levels 30 minutes after the treatment followed by a gradual return near to basal values 24 hours after the injection (15). Wasterlain et al. measured the levels of some GFs in the serum obtained from 25 subjects treated with leukocyte-rich PRP (LR-PRP). Authors reported significant increases of Insulin-like Growth Factor 1 (IGF-1), basic Fibroblast Growth Factor (bFGF) and VEGF levels following the local PRP injection (7).

With the exception of these two studies, the possible systemic effect of locally injected PRP is largely unknown. Conceivably, the systemic effect should be studied observing whether the treatment with PRP is able to modify

the healing process in contralateral, but untreated with PRP, injured muscle, as suggested by Borrione et al in a recent study (2).

The aim of the present study was to investigate whether the local PRP administration may modify the levels of specific cytokines and GFs both in treated muscle and bloodstream. The hypothesis at the base of this speculation was that the injection of cytokines and GFs naturally present in platelets and included in the WADA prohibited list could generate an increase of normal function, strength and capacity in non injured muscles if a systemic effect is really present.

MATERIALS AND METHODS

Animals and Surgery

Wistar male adult rats (n=60), 8-9 weeks old, weighing approximately 250g, were used. Twenty-seven animals were sacrificed 2 days after surgery: seven rats were subjected to muscle injury on the right flexor muscles and immediately treated with PRP (treated group: TR), eight animals, used as controls, were subjected to the same muscle injury and left untreated (untreated group: UT), seven rats were subjected to muscle injury in both anterior limbs: the right limb was treated with PRP while the injury on the left limb remained untreated (contralateral group: CL). Five rats, left untreated and uninjured, were used as controls (C). Twenty-five animals were sacrificed 5 days after surgery (seven treated, seven untreated, six contralaterals and five controls). Eight animals were analyzed 30 days after surgery (two treated, two untreated, two contralaterals and two controls).

Animals were kept in cages in a room with controlled temperature and humidity, with light/dark cycle of 12/12h, and fed with food and water *ad libitum*. Animals underwent surgery under general anesthesia by intramuscular injection of tiletamine + zolazepam (Zoletil) 3mg/kg. The decision of treating all animals with Finadyne administered at a dose of 2.5 mg/kg/12h, independently of the presence of signs of suffering, was determined by the intention of obtaining the same condition in the different experimental settings since the use of anti-inflammatory drugs may affect both the healing process and the first inflammatory response (16). The surgical procedures were performed with the aid of a surgical microscope (Zeiss OPMI7, Jena, Germany). A longitudinal incision was performed on the right arm (or both arms) from the elbow region to the wrist in order to access the flexor sublimis muscles of the upper joint of the fingers. The muscle was then injured transversely and medially using a scalpel. The wedge-shaped lesion had a length of approximately 3 mm, a width of 2 mm and a depth of 3 mm. After the incision, the injury sites of the treated animals were immediately filled with PRP. The flexor muscles were withdrawn after 2, 5 or 30 days and analyzed. Animals were daily monitored to assess their state of wellness, to prevent self-mutilation, skin ulcers, muscle contractures and suffering. All procedures were carried out in accordance with the Local Ethical Committee and the European Communities Council Directive of 24 November 1986 (86/609/EEC). All procedures were approved by the local Animal Care Committee and supervised by a veterinary.

Blood Collection and PRP preparation

Blood was collected by intracardiac drawing. Briefly the needle (21G) was inserted at the base of the sternum at 20° angle just lateral of the midline. 3-3.5 ml of blood were slowly aspirated in a syringe containing 1 ml of 3.8% sodium citrate as anticoagulant in order to avoid platelet activation and subsequent degranulation. Blood was then transferred in sterile tubes containing sodium citrate and underwent a first centrifugation at 220 g for 15 minutes. The top layer plasma was transferred, in another sterile tube without anticoagulant. To objectively determine the number of platelets and investigate the presence of other cells, before proceeding with the second centrifugation, a complete blood count was performed using a cell counter ADVIA 2021 (Bayer, Leverkusen, Germany) on a small amount of the plasma layer obtained after the first spin centrifugation (platelets content: 361.43±32.48 $x10^3/\mu l$; white blood cells content: 0.01±0.082 $x10^3/\mu L$). A second centrifugation at 1270 g for 5 minutes allowed the platelets to fall to the bottom of the tube. The most of acellular plasma was removed and discarded. The pellet was re-suspended in 100 µL of plasma to obtain a concentration of 4 times greater than the initial condition. This platelets enriched preparation was activated with 20 µL of 10% calcium chloride (Braun, Melsungen, Germany, 1000 IE / ml CaCl2-2SG) room temperature and after jellification, immediately inserted through tweezers into the injured muscle of the same animals from which blood has been drawn. The wound was then sutured and washed with saline solution (17-18).

Cytokines and growth factors

Blood samples were collected before surgery and animal sacrifice. Muscle samples (200-300 mg) were lysed in RIPA buffer (25 mmol/L Tris-HCl pH 7.6 , 150 mmol/L NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS; Sigma Aldrich) supplemented with Halt™Protease Inhibitor Cocktail (Sigma Aldrich). Plasma and muscle tissue samples were analyzed by Milliplex plus kit (Millipore, Billerica, Massachusetts) to quantify the levels of the following 10 molecules: Granulocyte-colony stimulating factor (G-CSF), Granulocyte-macrophage colony-stimulating factor (GM-CSF), Epidermal growth factor (EGF), Tumor necrosis factor alpha (TNF-α), Interleukin 1 alpha (IL1-α), Interleukin 4 (IL-4), Interleukin 5 (IL-5), Interleukin 6 (IL-6), Interleukin 10 (IL-10), Interleukin 13 (IL-13).

Statistical analysis

Cytokines and Growth factors data were analyzed by two-way ANOVA analysis. Multiple comparisons were further performed with Bonferroni's post hoc test. The effects of the time for each cytokines and growth factors in each experimental condition were analyzed by one-way ANOVA. Differences were considered statistically significant when $p\text{-value} < 0.05$. All data are presented as mean ± SD. The little variation of number of rats in 2 day group and 5 day group depended by accidental deaths, probably caused by intracardiac drawing or anesthesia.

RESULTS

Cytokines and growth factors analysis

In blood samples, the levels of all the investigated molecules remained unmodified in all the experimental conditions as well as in all the time points considered (data not showed).

IL-4, IL-6, IL-10, IL-1α, TNF-α and EGF showed significant modifications in treated and contralateral muscle samples. On the contrary, G-CSF, GM-CSF, IL-5 and IL-13 showed no modifications when compared to basal values.

IL-4. At day 2, TR, CL and UT showed lower values in comparison to C. The reduction was statistically significant only in TR (Table I). At days 5 and 30, TR showed a significant increase of IL-4 levels when compared to C and UT (Table I) while cytokine values in CL and UT remained lower in comparison to C. The increase in time of IL-4 levels in TR was statistically significant ($p<0.05$, one way-ANOVA).

The evaluation of data was supported by two-way ANOVA analysis that revealed significant effects for interaction ($F=5.975$; $p<0.001$), treatment ($F=7.346$; $p<0.001$) and time ($F=8.064$; $p<0.001$).

IL-10. 2 days after surgery, IL-10 showed a significant peak in TR (Table I). The cytokine in CL showed a behavior similar to what observad in TR with higher values when compared to C, but the difference was not statistically significant. At 2 days, TR and CL values were higher in comparison to UT whereas UT showed lower values when compared to C (Table I).

At day 5, IL-10 levels in TR and CL decreased showing values lower than C (significant difference in CL). UT continued to show values lower than C (Table I).

Interestingly, IL-10 showed a trend, even if not statistically significant, toward a progressive increase in TR, CL and UT from day 5 to day 30 after surgery. Two-way ANOVA analysis showed significant effects for interaction ($F=5.618$; $p<0.001$), treatment ($F=20.39$; $p<0.0001$) and time ($F=13.14$; $p<0.0001$).

IL-1α showed a significant peak level at day 2 in TR in comparison to all the other groups. UT showed values lower than C while CL revealed values similar to C (Table I).

At 5 days after surgery, IL-1α levels decreased in TR maintaining otherwise higher values when compared to all the other groups (Table I).

At day 30 IL-1α values in TR reached values found in C. The decrease of IL-1α values from day 2 to day 30 was statistically significant ($p<0.05$, one way-ANOVA). Two-way ANOVA analysis confirmed significant effects for interaction ($F=49.17$; $p<0.001$), treatment ($F=146.0$; $p<0.0001$) and time ($F=30.40$; $p<0.0001$).

IL-6 showed a significant peak in TR and CL at day 2 when compared to C and UT respectively (Table I). IL-6 values in UT did not differ from those observed in C.

At day 5, the values in TR remained higher than in C and UT (Table I).

At 30 days after surgery, the cytokine values in TR decreased showing values similar to all the other groups. The IL-6 decrease in TR and CL from day 2 to day 30 was statistically significant ($p<0.05$, one way-ANOVA). The evaluation of data was supported by two-way ANOVA analysis that showed

significant effects for interaction ($F=95.24$; $p<0.001$), treatment ($F=179.2$; $p<0.0001$) and time ($F=277.0$; $p<0.0001$).

At day 2, **TNF-α** showed UT levels lower than in C ($p<0.05$). At day 5 TNF-α in TR was characterized by a significant peak level when compared to the other experimental groups. At day 30, UT, TR and CL presented values lower than in C (Table I). The variations in time of the values of TNF-α in TR and UT resulted significant ($p<0.05$, one way-ANOVA). Two-way ANOVA analysis showed significant effects for interaction ($F=11.16$; $p<0.001$), treatment ($F=14.72$; $p<0.0001$) and time ($F=29.07$; $p<0.0001$).

EGF levels in all injured muscles were significantly lower when compared to C values in all considered time points. In particular, at day 2 TR, CL and UT presented values lower than in C. UT levels were higher than those observed in TR and CL (Table 1).

At day 5, the values detected in all injured muscles continued to remain lower than those observed in C without significant differences among them. These results were still present 30 days after surgery (Table I).

The comparison between EGF values in time in TR and UT groups showed two different trends. The UT values at day 30 were lower than those observed at days 2 and 5 ($p<0.01$, one way-ANOVA). On the contrary, EGF values in TR presented a progressive increase in the same time interval ($p<0.001$, one way-ANOVA). Two-way ANOVA analysis confirmed significant effect for interaction ($F=3.422$; $p<0.01$), treatment ($F=51.74$; $p<0.0001$) but not for the time.

DISCUSSION

The results of the present study showed that PRP treatment induced a modification of certain cytokines and GFs in muscle but not in the bloodstream, suggesting that local PRP treatment influenced directly or, more plausibly, indirectly the synthesis or recruitment of cytokines and GFs in the site of injury.

Since cytokines and GFs have a short half-life (15), we can hypothesize that cytokines and GFs present in the site of injury after 2, 5 and 30 days from PRP application, were not directly derived from PRP application (data confirmed by the absence of relevant data in bloodstream). Conceivably, the variation of cytokines and GFs concentration observed in muscles resulted from biosynthetic activity of other cells (e.g.macrophages/monocyte) recruited in the site of injury.

Moreover, the observed modifications of cytokines and GFs levels in contralateral injured but not treated muscles, strongly suggested a systemic effect of locally injected PRP. Indeed, several of the analyzed molecules, namely IL-1α, IL-4, IL-6, IL-10, TNF-α and EGF, showed a different behavior in the three different experimental conditions: treated, untreated and contralateral. In particular IL-1α, IL-4, IL-6, IL-10 and TNF-α showed a significant modification 2 and 5 days after PRP treatment.

The local increase of cytokines can be determined by different factors. It is known that muscle healing process progresses through a constant series of overlapping phases (degeneration and inflammation, regeneration, remodelling) resulting in the restoration of the anatomic continuity and

function (19). The first stage usually starts with the formation of a blood clot followed by the degranulation of platelets that release locally GFs and cytokines. The following phases are controlled by complex and dynamic molecular mechanisms involving local and systemic factors interacting with many different cell types recruited to the site of injury from the surrounding tissues and/or circulation (1). Generally, during the acute phase, following a muscle injury, polymorphonucleated leukocyte are the most abundant cells presents in the lesion site (19-22) and they are replaced by monocytes within the first days. Monocytes are then activated into macrophages and involved in the proteolysis and phagocytosis of the necrotic material (19, 23, 24). Previous studies demonstrated that PRP treatment increases the leukocyte infiltration in the injured muscle (2). Conceivably, the higher concentration of macrophages in the muscles treated with PRP may easily explain the observed increased concentration of IL-1α, in the treated group (25, 26). These data were confirmed by a recent study carried out by our group in which it has been observed a significant increase of NF-κB-p65 at 2-day post-injury. On the contrary, at 5-day post-injury, while in the PRP group the level of NF-κB-p65 was still significantly higher than in the C group, in the UT group the NF-κB-p65 protein returned to approximately the same level as in the C group. The trend of NF-κB-p65 was directly correlated to the IL-1α trend (18).

The amplification and modulation of the first inflammatory phase induced by PRP treatment may also be explained by the observed reduction of IL-10 levels following PRP injection. Indeed, the reduction of IL-10 levels results in

an increased macrophage recruitment, enhancing consequently the inflammatory condition during the first 2 days following the treatment (27).

The observed increase of IL-4 and TNF-α levels at day 5 is a direct consequence of the amplification of the early inflammatory response. Indeed, macrophages recruited in the site of injury are subsequently activated, producing additional chemoattractors, thus resulting in an increased leukocyte recruitment in the site of injury. Conceivably, when considering the short half life of these cytokines, it could be assumed that the observed increased levels of IL-4 and TNF-α at day 5 after PRP treatment is the result of the synthesis of these cytokines by the leukocytes recalled in the site of injury. This hypothesis may explain the persistence of high levels of cytokines until 30 days after the treatment. Indeed, IL-4 is able to protect lymphoid cells from apoptosis (28, 29), thus favoring the persistence of the amplified inflammatory response. Furthermore, IL-6 levels were found increased only in treated and contralateral muscles but not in untreated samples. Conceivably, this finding supports the hypothesis of a stimulated IL-6 production by infiltrating lymphocytes and excludes its lesioned muscle origin. This observation strongly supports the conclusions of previous studies indicating that PRP injection induces an amplification and modulation of the early inflammatory response resulting in an increase of the inflammatory infiltration in the site of injury, data further confirmed by present study (2, 18). Indeed, changes in cytokine values were predominantly recorded at day 2 and day 5 with gradual reduction at day 30 after surgery with the exception of IL-4 in treated group. This evidence suggests that the inflammatory response

in the treated group continued beyond 30 days after surgery, though to a lesser extent.

The hypothesis of a possible systemic effect of locally injected PRP preparation was effectively also confirmed by the results of the present study since several analyzed molecules such as IL-10, IL-6, TNF-α and EGF in contralateral muscles showed an intermediate behavior between treated and untreated samples. The observation that none of the analyzed molecules showed any statistically significant modification in the bloodstream following PRP local administration reinforces the hypothesis that certain, not yet identified, locally produced molecule may exert systemic effects being able of modifying the inflammatory response of contralateral injured but not treated muscles. The peculiarity of this investigation was to analyze "contralateral" muscles (the same animal was injured on both limbs and only one was treated with PRP while the other, the contralateral one, was left untreated) and observe whether the treatment with locally injected PRP, may influence the healing process even far from the site of injury. The results obtained using this experimental model strongly suggested that local PRP treatment may influence inflammatory responses even far from the site of injection. When considering its application on athletes, no evidence supports the hypothesis of possible action on non injured muscles, thus excluding possible muscle performance enhancing properties. Certainly, the issue of the systemic effect of locally injected PRP preparation needs further investigations.

In conclusion, the results of the present study confirmed that PRP treatment influences the early inflammatory phase of the healing process. This

observation may have an immediate clinical translation. Indeed, the demonstrated modulation of the inflammatory response may explain the pain reduction usually observed after PRP administration and accounting for the early mobilization of the patients (30). Moreover, suggests that an early treatment after the injury may results in better clinical responses.

In literature, PRP has been studied *"in vitro "* and *"in vivo"* in the field of maxillofacial surgery and general surgery, and more recently in muscle and tendon healing but little is known about a possible systemic effect deriving by the local use of PRP. Further experimental studies are needed in order to understand the biological mechanisms at the base of the inflammatory process following the local treatment with PRP preparations, focusing on which mediators exert systemic effect. For example a microarray analysis could be useful to investigate how IL-1α, IL-4, IL-6, IL-10, TNF-α, EGF, IL-5 and IL-13 expression could be modulated in muscles. The easy reproducibility achieved in this study has allowed us to create a solid foundation on which future studies will be carried out in order to deeply understand the molecular dynamics of the inflammatory process modulated by PRP administration. A potential limitation of the present study was represented by the low number of animals analyzed 30 days after surgery (2 animals in each experimental condition). These data should be considered the result of a pilot study carried out in order to obtain useful indications for future analysis aimed to analyze the long-term effects of PRP preparations.

The little variation of number of rats in 2 day group and 5 day group depends by accidental deaths probably caused by intracardiac drawing or anesthesia.

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Attachment 1. Cytokines and growth factors in bloodstream at 2, 5 and 30 days after surgery.

IL-4	**2 days**	**5 days**	**30 days**	**IL-10**	**2 days**	**5 days**	**30 days**
C	<3.1	<3.1	<3.1	**C**	11.85±7.99	18.73±11.36	14.93±5.77
UT	<3.1	<3.1	<3.1	**UT**	14±8.34	16.74±10.93	17.75±10.22
TR	<3.1	<3.1	<3.1	**TR**	11.01±4.92	18.48±10.48	13.72±6.3
CL	<3.1	<3.1	<3.1	**CL**	12.68±5.70	16.75±11.31	13.38±9.94
IL-1α	**2 days**	**5 days**	**30 days**	**IL-6**	**2 days**	**5 days**	**30 days**
C	< 6.1	<6.1	<6.1	**C**	72.33±34.5	75.32±12.01	109.24±50.5
UT	<6.1	<6.1	<6.1	**UT**	50.02±10.93	82.88±35.53	90.17±40.20
TR	<6.1	<6.1	<6.1	**TR**	113.14±100.2	117±12.73	108±45.12
CL	<6.1	<6.1	<6.1	**CL**	97.59±80.90	58.30±21.48	112.41±19.2
TNF-α	**2 days**	**5 days**	**30 days**	**EGF**	**2 days**	**5 days**	**30 days**
C	7.82±4.09	6.26±1.01	6.55±2.82	**C**	<0.17	<0.17	<0.17
UT	4.47±2.89	5.80±1.62	4.65±1.22	**UT**	<0.17	<0.17	<0.17
TR	7.39±5.34	6.51±0.01	7.36±3.76	**TR**	<0.17	<0.17	<0.17
CL	7.34±1.18	4.61±1.41	6.06±0.64	**CL**	<0.17	<0.17	<0.17
G-CSF	**2 days**	**5 days**	**30 days**	**GM-CSF**	**2 days**	**5 days**	**30 days**
C	< 2.61	< 2.61	< 2.61	**C**	< 6.73	< 6.73	< 6.73
UT	< 2.61	< 2.61	< 2.61	**UT**	< 6.73	< 6.73	< 6.73
TR	< 2.61	< 2.61	< 2.61	**TR**	< 6.73	< 6.73	< 6.73
CL	< 2.61	< 2.61	< 2.61	**CL**	< 6.73	< 6.73	< 6.73
IL-5	**2 days**	**5 days**	**30 days**	**IL-13**	**2 days**	**5 days**	**30 days**
C	< 8.67	< 8.67	< 8.67	**C**	<4.77	<4.77	<4.77
UT	< 8.67	< 8.67	< 8.67	**UT**	<4.77	<4.77	<4.77
TR	< 8.67	< 8.67	< 8.67	**TR**	<4.77	<4.77	<4.77
CL	< 8.67	< 8.67	< 8.67	**CL**	<4.77	<4.77	<4.77

Table I. Cytokines and growth factors in muscles at 2, 5 and 30 days after surgery.

IL-4	**2 days**	**5 days**	**30 days**	**IL-10**	**2 days**	**5 days**	**30 days**
C	4.05±0.95	4.13±0.33	3.96±1.7	**C**	0.63±0.34	0.64±0.34	0.60±0.23
UT	2.30±0.61*	3.41±0.72	3.29±1.33	**UT**	0.06±0.05**	0.02±0.01	0.30±0.03
TR	1.77±0.45*	6.24±1.92*##	7.06±2.78*##	**TR**	1.09±0.41**##	0.46±0.21	0.61±0.04
CL	3.45±0.61*	3.63±0.91	2.78±0.82	**CL**	0.93±0.26*##	0.16±0.08*	0.29±0.17
IL-1α	**2 days**	**5 days**	**30 days**	**IL-6**	**2 days**	**5 days**	**30 days**
C	0.96±0.21	0.74±0.24	1,2±0,20	**C**	6.08±2.17	6.07±3.1	5.99±1.9
UT	0.14±0.08	0.23±0.08	1,32±0,06	**UT**	17.53±4.61	1.59±0.53	0.85±0.5
TR	6.66±1.33**##	3.41±0.51**##	1,27±0,07	**TR**	327.02±40.9**##	165.22±65.11**##	0.69±0.43
CL	1.32±0.54	0.94±0.38	1,05±0,18	**CL**	298.51±6.4**##	1.55±0.93**	0.47±0.39
TNF-α	**2 days**	**5 days**	**30 days**	**EGF**	**2 days**	**5 days**	**30 days**
C	0.11±0.03	0.10±0.03	0.12±0.02	**C**	1.66±0.05	1.66±0.07	1.65±0.06
UT	0.03±0.01*	0.09±0.03	0.02±0.02*	**UT**	1.19±0.12**	1.14±0.18**	0.88±0.07**
TR	0.13±0.03#	0.31±0.10**##	0.02±0.02*	**TR**	0.73±0.08**##	0.86±0.11**	1.16±0.11**
CL	0.09±0.05	0.14±0.08	0.06±0.02	**CL**	0.79±0.13**##	0.94±0.12**	0.75±0.06**

C: control group, UT: untreated group, TR: treated group, CL: contralateral group.

Multiple comparison Bonferroni's test: *p<0,05 vc C; **p<0,01 vs C; # p<0,05 vs UT; ## p<0,01 vs UT