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N-3 Fatty Acid Supplementation After Burn Supports Wound Healing and Prevents Systemic Inflammation. An Experimental Study in Rats

Yanık Sonrası Destek Tedavisinde N-3 Yağ Asidi Kullanımı Yara İyileşmesini Destekler ve Sistemik Enflamasyonu Engeller. Sıçanlarda Yapılan Deneysel Çalışma

ABSTRACT

Objective:
Burn may cause hypersensitivity towards infection, systemic inflammatory response syndrome, respiratory distress syndrome and multiple organ failure. Anti-inflammatory and resistance towards infection effects of omega-3 (n-3) fatty acids was shown before. But their effect on systemic inflammation and wound healing after burn was not investigated.

Material and Methods:
Seventy Wistar albino rats were used in the study. Fourteen rats without burn composed the control group. Cutaneous burn was created on remaining 56 rats which received 50 mg/kg/day intraperitoneal ringer lactat solution in two equal amounts. Twenty-eight rats with burn received injections of n-3 1 mg/kg/day throughout the study. Rats in control group were sacrificed on day 4 and rats in treatment groups were sacrificed on days 4 and 8. Leukocyte and mast cell infiltration, epidermal and collagen thickness measurements were used in histological evaluation. White blood cell, hematocrite, total protein, albumin, fibronectin, TNFα, IL1β and IL6 levels were measured for biochemical evaluations.

Results:
Elevation of leukocyte and mast cell infiltration was lower and collagen thickness was higher in n-3 receiving group on day 4. There was no difference in leukocyte infiltration on the 8th day whereas mast cell infiltration was lower and collagen thickness was higher in n-3 receiving group. Epidermal thickness was higher in n-3 receiving group on both evaluation days. White blood cell counts were higher in 8th day than 4th day groups. Total protein on 8th, IL-6 on 4th, and albumin levels on both days was lower than control.

Conclusion:
N-3 fatty acids used after burn enhances wound healing and prevents systemic inflammation.

Key Words:
Nutrition, Inflammation, N-3 Fatty Acids, Burn, Wound Healing

ÖZ

Amaç:

Yanık enfeksiyon, sistemik enflamatuar yanıt sendromu, solunum sıkıntısı sendromu ve çoklu organ yetmezliği yatkınlığını artırır. Omega-3 (n-3) yağ asitlerinin anti-enflamatuar ve enfeksiyona karşı direnç artırma etkileri daha önce gösterilmiştir. Ancak yanık sonrası sistemik enflamasyon ve yara iyileşmesi üzerine etkileri bilinmemektedir.

Gereç ve Yöntemler:

Çalışmada 70 Wistar albino sıçan kullanıldı. Yanık olmayan 14 sıçan kontrol grubunu oluşturdu. Geriye kalan 56 sıçanda yanık modeli oluşturuldu ve 50 mg/kg/gün olacak şekilde periton içi ringer laktat solüsyonu eşit iki dozda verildi. Yanığı olan 28 sıçana ayrıca 1 mg/kg/gün dozunda n-3 enjekte edildi. Kontrol grubundaki sıçanlar dördüncü günde, tedavi gruplarındaki sıçanlar dört ve sekizinci günlerde sakrifiye edildi. Histolojik incelemede lökosit ve mast hücre infiltrasyonu, epidermal ve kollajen kalınlık ölçümleri değerlendirildi. Biyokimyasal incelemede beyaz küre, hematokrit, total protein, albumin, fibronektin, TNFα, IL1β ve IL6 seviyeleri ölçüldü.

Bulgular:

Dördüncü günde n-3 uygulanan grupta lökosit yüksekliği ve mast hücre infiltrasyonu daha düşük, kollajen kalınlığı daha yüksek bulundu. Sekizinci günde n-3 uygulanan grupta lökosit infiltrasyonu açısından fark yok iken mast hücre infiltrasyonu daha düşük ve kollajen kalınlığı daha yüksek bulundu. N-3 uygulanan grupta her iki değerlendirme zamanında da epidermal kalınlık daha yüksekti. Sekizinci günde beyaz küre sayısı dördüncü güne göre daha yüksek bulundu. Sekizinci günde total protein, dördüncü günde IL-6 ve her iki değerlendirme zamanında albumin seviyeleri kontrol grubuna göre daha düşük bulundu.

Sonuç:

Yanık sonrası n-3 yağ asidi tedavisi yara iyileşmesini destekler ve sistemik enflamasyonu önler.

Anahtar Klimeler:

Beslenme, Enflamasyon, N-3 Yağ Asidi, Yanık, Yara iyileşmesi

INTRODUCTION

Burn is a type of trauma that may cause infections, shock, multiple organ failure and death (1, 2). The cytokines released from leukocytes following injury may enhance host resistance. Furthermore, they may cause systemic inflammation and response of immune system cells and disturb remote organ functions. These types of effects in patients with major burns may result in susceptibility to infections, systemic inflammatory response syndrome, adults' respiratory distress syndrome, multiple organ dysfunction syndrome and even death (2, 3).

Early initiation of nutrition support effects the cytokine induced stress response thus minimizes metabolic abnormalities and tissue damage. Enhancement of immune response in critical patients by enrichment of standard nutritional solutions with specific nutrients came into question recently.

For this purpose, glutamine, arginine, nucleotide and n-3 fatty acids are supplemented to the treatment regimen particularly or in combinations (4). It is also known that additional usage of n-3 fatty acids has anti-inflammatory function in critical care patients (5). The aim of this study is to investigate the effect of n-3 fatty acid rich emulsion on inflammatory cytokines and wound healing in experimental burn model.

MATERIAL and METHODS

This study is conducted in Experimental Medical Investigation Laboratory of Mersin University after approval of animal studies ethics committee (Application number: 40, Approval date: 23.01.2009). All procedures on animals were conducted according to global "Guide for the Care and Use of Laboratory Animals" rules. Study was also conducted according to research and publishing ethics.

Seventy female albino Wistar rats weighing 250-300 grams were used. All rats were housed in standard cages and room temperature, in 12 hours light-dark cycle, and fed with standard rodent diet (240 kcal/day approximately) and water. Rats were divided randomly into 5 groups:

Group K (n=14): Control group

Group R4 (n=14): Treated with ringer lactate solution (RLS) after burn and sacrificed in day 4.

Group RO4 (n=14): Treated with RLS and Omegaven® after burn and sacrificed in day 4.

Group R8 (n=14): Treated with RLS after burn and sacrificed in day 8.

Group RO8 (n=14): Treated with RLS and Omegaven® after burn and sacrificed in day 8.

Omegaven® is an emulsion which is enriched with n-3 fatty acids. Rats in groups RO4 and RO8 received 1 ml/kg (5) of Omegaven® daily until sacrifice. In order to prevent dehydration rats received 50 ml/kg of RL intra-peritoneally after burn twice daily.

In order to acquire standard area of burn a mold with a window was formed (6). The dimensions of this window were calculated according to the 40 % of rats' surface area.

Rats were anesthetized with ketamine and xylazine. Dorsal side of the rats were shaved and placed in the mold. Molds holding the rats were immersed into the 96°C hot water for 10 seconds. Rats in the control group in the molds were immersed into 21°C water for 2 seconds. Pain control was achieved with subcutaneous injections of morphine (2,5 mg/kg) in 8 hour intervals.

Rats were sacrificed by cardiac punction. Blood samples were reserved for hematological and biochemical analyses. Skin samples from burn area borders were taken and placed in formaline for further investigation.

Histological investigations

Tissues were fixed in 10% neutral formaline, routinely processed for light microscopy evaluation and embedded in paraffin. Sections (5 µm) were cut using a microtome and stained with hematoxylin-eosin to assess inflammatory changes with the number of leukocytes, epidermal thickness and toluidine blue for mast cells. Slides were examined with a light microscope and photographed by a digital camera.

Randomly selected ten areas were used for leukocytic infiltration, the total number of mast cells and epidermal thickness measurement. Epidermal thickness measurements were performed using commercially available software. Leukocytic infiltration was assessed using the following scale.

•No extravascular leucocyte; 0
•<20 leucocyte; 1
•20-45 leucocyte; 2
•>45 leucocyte; 3

For electron microscopic investigations, tissues were fixed with 2.5% glutaraldehyde. Samples were postfixed in 1% osmium tetroxide, processed routinely for electron microscopy and embedded in resin kit. Ultrathin sections (50-70 nm) were cut by ultramicrotome and contrasted with uranyl acetate and lead citrate and were examined with an electron microscope. Randomly distributed collagen fibers were examined and cross sections were selected for measurement. These areas were photographed by a digital camera attached to the electron microscope. Photographes were transferred to a commercially available software and diameters of 500 collagen fibers of each animal were measured by this software.

Biochemical investigations

White blood cell (WBC) counts and hematocrite concentrations were analyzed in blood samples collected in tubes with EDTA. Samples were centrifuged with 5000 rpm for 10 minutes. Plasma was stored in -20ºC for further quantification of IL-6, IL-1β, fibronectin, TNF- α, total protein and albumin levels.

Statistical analyses

Comparisons regarding epidermal thickness and collagen diameter variables were made with Kruskal Wallis and Mann-Whitney U tests with Bonferroni correction. Cross-table analyses were conducted for comparisons of epidermal leukocyte counts. Chi-square tests were used for comparisons of mast cell counts. P values under 0.05 were considered significant.

Comparisons of hematocrite, IL-1β, IL-6, TNF-α, fibronectin values were made with Kruskal Wallis tests. ANOVA were used for comparisons of WBC, total protein, albumin values.

RESULTS

Histological findings

Necrosis was evident macroscopically on forth day after burn. At day 8 epithelized areas were seen beneath the necrotic tissues.

Calculations of epidermal width and collagen diameter are given in Table I.

Table I: Mean epidermal width (EW) and collagen diameter (CD)

Groups	EW (µm)	CD (nm)
K	11.10 ± 3.60	71.31 ±15.86
R4	7.65 ± 2.59	62.67 ± 12.95
RO4	8.40 ± 2.36	73.88 ± 16.15
R8	8.36 ± 2.56	91.83 ± 15.89
RO8	9.37 ± 2.63	75.07 ± 14.17

Epidermal width was lower in all groups of burn than control group (p=0.001). Groups R4 and R8 had lower epidermal width than groups RO4 and RO8, respectively (p=0.007 and p<0.001, respectively). Epidermal widths in groups R8 and RO8 were higher than groups R4 and RO4, respectively (p<0.001 and p=0.001, respectively).

While the collagen diameter calculations were lower in group R4 than control (p=0.001), it was higher than control in groups RO4, R8 and RO8 (p=0.016, p=0.001 and p=0.001, respectively). Collagen diameter was lower in group R4 than group RO4 (p=0.001), and was higher in group R8 than group RO8 (p=0.001). While groups receiving ringer lactate only (R4 and R8) had higher collagen diameters on day 8 than day 4, (p=0.001), 4 and 8th day comparison of collagen diameters were similar in groups receiving omegaven (RO4 and RO8) (p=0.210). Results of leucocyte infiltration calculations are given in Table II.

Table II: Evaluations of leucocyte infiltration.

Leucocyte count	Group K	Group R4	Group RO4
<20	86	40	53
20-45	54	81	64
>45	0	9	3
		Group R8	**Group RO8**
<20		33	25
20-45		105	111
>45		2	4

Leucocyte counts were lower than 20 in most of Group K cross-sections. The number of cross-sections which had leucocyte counts below 20 were lower in group R4 than group RO4 and lower in group RO4 than group RO8 (p=0.027 and p=0.001, respectively). The number of cross-sections which had leucocyte counts between 20-45 were lower in group R4 than group R8 and lower in group RO4 than group RO8 (p=0.0024 and p=0.001, respectively). The number of cross-sections which had leucocyte counts more than 45 were lower in group R8 than group R4 (p=0.0024). Results of mast cell infiltration calculations are given in Table III.

Table III: Evaluations of mast cell infiltration.

Mast cell count	Group K	Group R4	Group RO4
<20	127	49	90
20-45	13	80	30
		Group R8	**RO8**
<20		79	116
20-45		61	24

In all study groups the number of cross-sections which had mast cell infiltration below 20 were lower than control group (p<0.05). The number of cross-sections which had mast cell infiltrations below 20 were higher in group R8 than group R4, higher in group RO4 than group R4 and higher in group RO8 than group R8 (p=0.002, p=0.000 and p=0.000, respectively).

Biochemical results

Blood samples of 3 rats from group RO8 and 4 rats from group R8 were excluded because of hemolysis. IL-6, IL-1β and TNF α evaluation of 1 rat from group RO8, TNF α evaluation of 3 rats and IL-1β evaluation of 1 rat from group RO8 could not be completed because of insufficient serum centrifugation.

Serum WBC, albumin, total protein results are given in Table-IV.

Table IV: Mean serum white blood cell (WBC), albumin (Alb.) and total protein (T.Prot.) levels.

Groups	WBC (10^3/µl)	Alb. (g/dl)	T.Prot. (g/dl)
K	5.49 ± 1.64	4.11 ± 0.31	6.94 ± 0.36
R4	4.46 ± 1.90	3.46 ± 0.30	6.54 ± 0.42
RO4	4.84 ± 1.45	3.73 ± 0.26	6.97 ± 0.26
R8	8.44 ± 2,06	3.68 ± 0.34	6.01 ± 0.42
RO8	7.27 ± 2.36	3.49 ± 0.29	5.80 ± 0.37

WBC values of group R8 were higher than control group (p=0.004). Groups R8 and RO8 had higher WBC values than R4 and RO4, respectively (p=0.00 and p=0.026, respectively). Serum albumin levels were lower in all rats with burn than control group (p<0.05). Albumin levels did not show any difference in comparisons of evaluation days and treatment groups (p>0.05). Total protein values were similar with control in groups R4 and RO4, but lower in groups R8 and RO8 (p=0.00). Hematocrite values were similar in all groups (p>0.05). Fibronectin, IL-1β and TNF-α values were also similar (p>0.05) (Figure 1A, B and C). IL-6 values were lower in groups R4 and RO4 than control group (p=0.021 and p=0.009, respectively). Slight elevated values of IL-6 in groups R8 and group RO8 did not reach statistical significance (Figure 1D).

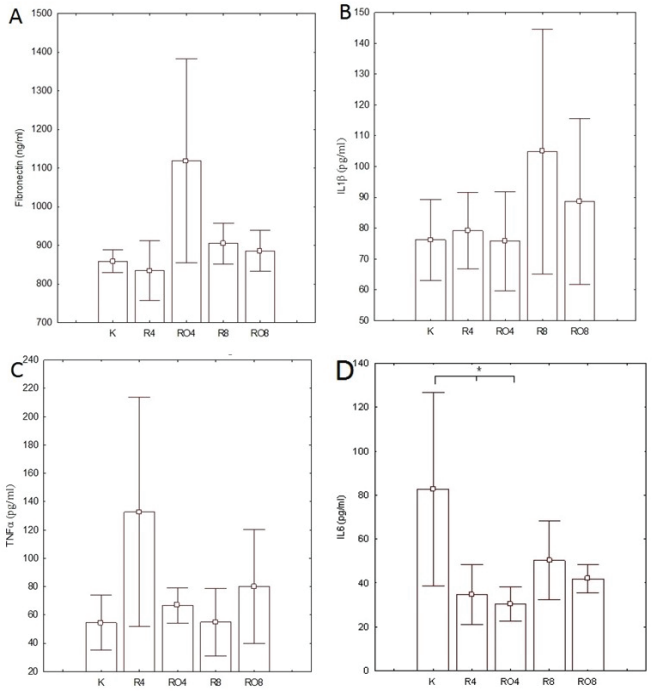

Figure 1: (A) Serum fibronectin, (B) IL-1 β, (C) TNF- α, and (D) IL-6 levels.
* Group R4 and Group RO4 vs Group K (p<0,05).

DISCUSSION

Protein denaturation after burn causes tissue loss and release of inflammatory mediators and migration of trombocytes, eritrocytes, leucocytes and macrophages occurs subsequently (7). The degree of inflammation is proportional with duration of exposure and degree of heat, and area of tissue exposed. In our study we made comparisons between groups for leucocyte and mast cell infiltration during inflammatory phase of burn and saw that rats treated with RLS and Omegaven® had lower values 4 days after burn. We think that this result is achieved by inhibition of proinflammatory cytokines by immunomodulatory effects of n-3 fatty acid alimentation (8). Similar effects are reported in pseuriasis and atopic dermatitis patients treated with parenteral n-3 fatty acids (9-11). Conversely, while leucocyte infiltrations did not differ between groups receiving and not receiving Omegaven®, mast cell infiltration was lower in groups treated with Omegaven at 8th day. Both treatment groups at day 8 had slightly elevated leucocyte levels than control. Therefore, it can be speculated that cellular inflammatory response required for tissue healing is not fully inhibited by n-3 fatty acids.

Reactive oxygen species (ROS), which are known to be responsible from local and systemic damage in burn pathophysiology, are produced mostly in burn area and in distant organs also (12). In their thermal damage study in rats, Friedl et al., reported elevated histamine secretion from activated mast cells in burned tissue and ROS in blood and organs as a result (13). Santos et al., believed that elevated ROS may be responsible from secretion of mediators from mast cells by degranulation as a result of their burn model study in rats (14). We found decreased infiltration of mast cells at burn area of rats treated with n-3 fatty acids. We also believe that they may also diminish production of ROS after burn.

Epidermis remodels by degredation and re-epithelisation of granulation tissue following inflammatory phase of wound healing. Epidermal healing after burn in rats is evaluated by Jeschke et al., and they reported that treatment consisting enriched vitamins, proteins, amino acids and n-3 fatty acids had an enhancement effect on healing (15). In their study Gerçek et al., evaluated the remodelation of incision wounds in rats and reported that Omegaven® and dexamethasone treatment had significant effect on epidermal width (5). In our study epidermal widths were larger in groups receiving both Omegaven® and RLS than groups treated with RLS only. In conjunction with histological findings discussed above, we think that n-3 fatty acids have an enhancement effect on wound healing along with controlling cellular inflammation.

Collagen formation is important during the initial phase of wound healing by their fibroblast stabilizing and barrier forming action. This granulation tissue initially (2nd-3rd day) contains type-3 collagen which is thinner, and type-1 collagen after, which is thicker and stiffer (16). Mast et al., realized that collagen fibrils in wounds are thinner and longer than the ones in normal tissue and related this with more type-3 collagen in wounds (17). Conversely, Peacock et al., interpreted that thin and long collagen fibrils are the result of pulling force during

wound contraction (18). Collagen diameters were found higher in group treated with Omegaven® plus RLS than group receiving only RLS on day 4. It can be speculated that n-3 fatty acids prevented harmful effect of hypermetabolic phase of wound healing on collagen synthesis. Thus, a healthy wound cover with a good collagen support should be able to act as a barrier towards infection during initial phase of healing. Conversely, collagen diameters were lower in group treated with Omegaven® plus RLS than group receiving only RLS on day 8. While elevated collagen fibril content and size is found protective in early wound healing, elevation of type-1/type-3 collagen ratio causes a risk of hypertrophic scar formation subsequently (16, 19). We think that n-3 fatty acids have controlled effect on collagen production similar to controlled effect on leucocyte infiltration.

Cytokines are important mediators in pathophysiological pathways after burn. They have complex interactions with each other where they take place as soon as burn occurs and inflammatory phase begins. It is important to recognize the dynamics of active cytokines because of their subsequent metabolic effects during wound healing in order to detect possible factors which may have effect on these dynamics. TNF-α is a strong mediator of shock and induces synthesis of cytokines like IL-1 and IL-6 and some humoral factors (20). IL-6 and IL-1 levels alter after burn (21, 22). Many of these cytokines are being used to detect the severity of burn damage (23-25). Agay et al., reported that cytokine levels varied according to body surface area and evaluation times in their rat burn damage study (26). Gauglitz et al., found that IL-6 levels start to increase soon after burn, takes peek at 6th hour and decrease gradually until a second increase after day 4 or 5 (27). IL-6 levels increase proportional with surface area of burn (26, 27). Our study did not include investigations soon after burn but we observed that IL-6 levels' being low at day 4 congruently reported in other studies, and elevated to values similar to control group at day 8. IL-1β increases acutely during first 3 hours after burn, reaches its peak level at 12th hour and after 48 hours it starts to decrease and normalize to basal levels at 4th day (27). Some studies show that even when IL-1β levels have no alteration in serum, high IL-1β levels can be observed in tissue samples from lung (26, 28). This is thought to be among mechanisms of pulmonary complications after burn. Our study showed normal levels of IL-1β at days 4 and 8 similar with the literature. Studies which quantify TNF-α levels after burn have variable results. TNF-α levels showed critical elevations at days 3 and 7 in a study of burn including 20% of body surface area (29). When larger areas are burnt TNF-α levels increased more rapidly (30, 31). There are also studies which report similar results of serum TNF-α levels with control groups after burn (26, 27). Likewise, we observed no difference of serum TNF-α from control. Complexities and unknown interactions of factors affecting cytokines during inflammatory phase after burn cause these contradictions between studies even with high levels of standardization. In our study we investigated the effect of n-3 fatty acids on cytokines after burn and observed that they did not change the normal course. One of the reasons (and handicap of the study) of this may be that we did not take samples during initial hours or days after burn in which serious alterations of cytokine levels occur. Another handicap of our study is that we did not evaluate tissue samples. Hence we can only speculate that metabolic advantages of n-3 fatty acids on wound healing found in histological findings may be the result of alterations of cytokine levels during acute phase after burn.

Hypovolemia is a clinical problem that must be addressed soon after burn. It is caused by inflammatory response which increases vascular permeability. Fluid replacement is mandatory for resuscitation in burn patients. Volume expanders and protein solutions are also infused for institution of osmotic pressure during first 24 hours (32). We observed lower levels of albumin in rats with burn. Total protein levels were low on day 8. Earlier decrease of albumin than protein is a finding which is compatible with the literature.

Fibronectin is an important protein found in granulation tissue as soon as after burn. The function of TGF-β1 is dependent on fibronectin presence in extracellular matrix (33, 34). Fibronectin also have an opsonisation function in removal of bacteria remnants and debris in wound (34). Severe damage, burn or sepsis decreases the fibronectin level acutely during initial hours. At the end of the first day it returns to normal levels (35). Our study results showed normal levels of fibronectin as expected. We believe that decreased protein in conjunction with normal fibronectin levels was due to lack of protein containing dietary supplement rather than ongoing inflammatory process in our study.

CONCLUSION

In conclusion, n-3 fatty acid supplementation in rats after burn supports wound healing and prevents systemic inflammation.

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Ethics Committee Approval:
This research complies with all the relevant national regulations, institutional policies and is in accordance with the tenets of the Helsinki Declaration, and has been approved by the Local Ethical Committee for Animal Studies of University of Mersin, (approval number: 2009/40).

Author Contributions:
Concept – C.A.C., N.D., S.A.; Design - C.A.C., N.D., S.A.; Supervision - C.A.C., N.D.; Resources - C.A.C.; Materials - C.A.C.,; Data Collection and/or Processing - C.A.C., N.D., S.A.; Analysis and/ or Interpretation - C.A.C., N.D., S.A.; Literature Search - C.A.C., N.D., S.A.; Writing Manuscript - C.A.C., N.D., S.A.; Critical Review - C.A.C., N.D., S.A.

Conflict of Interest:
The authors have no conflict of interest to declare.

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