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ORIGINAL ARTICLE

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Lipid mediated modification of rat heart allograft survival

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Abstract The effect on allograft survival of intravenous fat emulsions that differed in the ratio of functionally important n-3 and n-6 fatty acids was studied in a heterotopic cardiac transplant model in rats. Twenty percent fat emulsions were administered by continuous infusion at a dosage of 9 g fat/kg body weight per day, starting immediately after transplantation and continuing until complete rejection. The n-6 and n-3 fatty acids represent 75 %, 43 %, 60 %, and 59 % of all fatty acids in safflower oil, fish oil, soybean oil, and a 1:1 mixture of safflower and fish oil, respectively. The n-6 fatty acids predominate in safflower oil (370/1) and soybean oil (6.5/1), while the n-3 fatty acids dominate in the fish oil (7.6/1). The 1:1 mixture of safflower and fish oil has the balanced composition (n-6/n-3 = 2.1/1) recommended by Kinsella and served as oil-treated controls. Continuous infusion of safflower oil, fish oil, and soybean oil prolonged graft survival time to 13.3, 12.3, and 10.4 days, respectively, compared to 6.8 days in the oil-treated controls ($P < 0.01$ for all comparisons). Another control group infused with saline rejected the allografts after 7.8 days (P = NS compared to oil-treated controls; $P < 0.01$ for all other comparisons). The data suggest that intravenous administration of polyunsaturated fat emulsions results in an immunosuppressive effect that seems to be dependent on the n-3/n-6 fatty acid ratio of the fat emulsion. The n-6 fatty acids turned out to be just as immunosuppressive as the n-3 fatty acids if each fatty acid family was applied as the main polyunsaturated fatty acid source. Soybean oil with a n-3/n-6 fatty acid ratio, coming closer to the ratio of the oil-treated controls, was significantly less immunosuppressive than safflower oil.

Key words Lipid/emulsion, rat, heart · Heart, rat, lipid emulsion · Immunosuppression, lipid emulsion, rat, heart

Introduction

In 1987 Perez et al. reported that the oral administration of supplementary linoleic acid (n-6 fatty acid) or fish oil (rich in n-3 fatty acids) to allotransplanted rats resulted in an increased allograft survival time [14], a finding that has since been confirmed by Tchervenkov et al. [15] and Otto et al. [13]. In all these studies the heterotopic heart allograft model was used. A possible explanation for an effect of dietary fat on the immune response could lie in the fact that polyunsaturated n-3 and n-6 fatty acids are precursors of prostaglandins, thromboxanes, and leukotrienes, all compounds that are reported to influence the rejection process [5, 12].

In our laboratory we have studied the effect on rat heart allograft survival of fat emulsions given by continuous intravenous infusion in the post-transplant period. Infusions of fat emulsions may influence the immune system more effectively than administration per os because they can be given continuously in a standardized manner during the entire post-transplant period. To assess the impact of the n-3/n-6 ratio on the immunosuppressive effect of fat emulsions, three emulsions having different n-3/n-6 fatty acid ratios were tested and compared to a fat emulsion in which the n-3/n-6 fatty acid ratio was ideally balanced according to Kinsella et al. [9].

Table 1 Fat composition of R3-EWOS-ALAB brood stock feed for rats

	Percentage
Crude fat	5
Fatty acids percentage of TEA	
Palmitic acid (16:0)	21
Palmitoleic acid (16:1)	2
Stearic acid (18:0)	4
Oleic acid (18:1)	22
Linoleic acid (18:2)	42
Linoleic acid (18:3)	7
Others	2

Materials and methods

Animals

Inbred PVG rats (RT 1 ^{c}, male, 100–150 g) served as donors and Wistar/Kyoto rats (RT 1 ^{l}, male, 200–250 g, Mollegaard Breeding Center, Skensved, Denmark), as recipients. The rats were housed in plastic cages with stainless steel wire bottoms in a laboratory with a controlled temperature (20°C), humidity (50%), and a 12-h light-dark cycle. The animals were allowed to adapt to the environment for at least 1 week prior to transplantation. They were fed R3-EWOS-ALAB Brood Stock Feed (ALAB, Sollentuna, Sweden). The fat composition of the feed is given in Table 1.

Intravenous catheter

A 5-cm long, spiral-shaped piece of PE 10 (polyethylene) catheter (Clay Adams, Parsippany, N.J., USA) attached to a silicon tube (Silastic, 0.012 in × 0.025in, No. 602-105 HH 061999, Dow Corning, Midland, Mich., USA) was fused to a 30-cm piece of a PE 20 catheter. The silicon part of the catheter was placed in the animal's left jugular

Table 2 Fatty acid composition of safflower, fish, soybean, and the 1:1 mixture of safflower and fish oil

Percentage of total fatty acids				
Fatty acid	Safflower oil	Fish oil	Soybean oil	1:1 Mixture of safflower + fish oil
12:0		0.2		0.1
14:0	0.1	5.5	0.1	2.8
16:0	7.6	14.2	11.0	10.9
16:1	0.1	6.5	0.1	3.3
17:0			0.1	
18:0	2.9	3.2	4.4	3.05
18:1 (n-9)	13.6	10.1	23.3	11.85
18:1 (n-7)		3.1	1.55	
18:2 (n-6)	73.6	2.6	51.6	38.1
18:3 (n-6)		0.2		0.1
18:3 (n-3)	0.2	0.7	6.6	0.45
18:4 (n-3)		2.5		1.25
20:0	0.3	0.2	0.3	0.25
20:1 (n-9)	0.3	1.5	0.3	0.9
20:1 (n-7)		1.8		0.9
20:1 (n-5)		0.2		0.1
20:2 (n-6)			0.5	
20:3 (n-6)			0.04	
20:4 (n-6)	0.1	1.6	0.1	0.85
20:5 (n-3)		18.1		9.05
22:0			0.4	
22:1 (n-11)		2.0		1.0
22:1 (n-9)		0.5		0.25
22:1 (n-7)		0.1		0.05
22:5 (n-3)		2.7		1.35
22:6 (n-3)		12.9		6.45
22:6 (n-6)			0.3	

Table 3 PVG heart survival in Wistar/Kyoto rats treated with polyunsaturated fat emulsions with different n-3/n-6 ratios (*SO/FO* safflower/fish oil 1:1 mixture, *SO* safflower oil, *FO* fish oil, *SBO* soybean oil)

Group	Treatment	n-3/n-6	Graft survival (days)	Mean ± SEM	*P* values
1	Saline		7, 7, 7, 7, 7, 8, 8, 9, 9, 9	7.8 ± 0.3	
2	SO/FO	1/2.1	5, 5, 5, 5, 6, 7, 7, 9, 9, 9	6.7 ± 0.56	NS vs 1
3	SO	1/360	9, 11, 11, 12, 13, 13, 13, 15, 16, 20	13.3 ± 1.0	< 0.01 vs 1,2
4	FO	7.6/1	10, 11, 12, 12, 12, 12, 12, 13, 14, 15	12.3 ± 0.4	< 0.01 vs 1,2
					NS vs 3
5	SBO	1/6.5	8, 8, 8, 9, 10, 11, 12, 12, 13, 13	10.4 ± 0.7	< 0.01 vs 1.2
					< 0.05 vs 3,4

vein as described elsewhere [16] and the PE 20 end was diverted to the exterior immediately prior to transplantation. This catheter was connected to a SAGE pump enabling 24-h continuous infusion.

Surgical technique

The recipients were anesthetized with phentanylcitrate 0.315 mg/kg body weight, given IM (Hypnorm, Janssen, Belgium). The abdomen was opened with a midline incision. The left kidney was removed and the kidney vessels were cuffed as described elsewhere [7]. The donors were anesthetized with pentobarbital, 60 mg/kg body weight i.p. (Mebumal vet., Nord Vacc, Sweden), and 300 IU of heparin was injected IV before the harvesting of the heart. The grafts were flushed with cold Ringer lactate solution containing 50 IU of heparin/ml. Immediately after the harvesting, the graft was anastomosed with the cuffed vessels; the cold ischemia time was less than 5 min.

Fat emulsions

Emulsions containing 20% oil were prepared using safflower oil, fish oil, soybean oil, or a 1:1 mixture of safflower and fish oil. The fatty acid composition of these oils is given in Table 2. The isotone water phase contained distilled water, purified egg phospholipids, and glycerol. It was heated to 60–70 °C and the lipid phase was added in a mixer at high speed. The emulsions contained the antioxidant vitamin E at a concentration of 1 mg/ml. A fine emulsion was created in a valve homogenizer at high pressure. The emulsion was dispensed on glass vials and heat-sterilized.

Treatment groups

Animals in groups of ten were given any one of the four fat emulsions. Nine grams of fat per kg body weight per day was administered by continuous intravenous infusion over 24 h. Animals that were infused with the 1:1 mixture of safflower and fish oil served as oil-treated controls, since in this emulsion the n-3/n-6 ratio was optimally balanced according to Kinsella et al. [9]. Another control group was infused with a corresponding volume of sodium chloride.

Assessment of graft rejection

The transplanted hearts were palpated twice daily. Rejection was considered to be complete when no pulsations were palpable and electrocardiography showed no activity of the transplant. Following removal, the grafts were weighed and the rejection diagnosis was then verified histologically.

Statistics

Differences between the groups were analyzed with Student's *t*-test after a normal distribution was confirmed by the Kolmogorov-Smirnov test. Significance was assumed when *P* was less than 0.05. Values are presented as mean ± SEM.

Results

Animals in both control groups rejected their allografts around day 7 (saline-treated controls 7.8 ± 0.3 days, oil-treated controls 6.8 ± 0.56 days, *P* = NS).

Continuous infusion of safflower, fish, or soybean oil prolonged the graft survival time significantly. With safflower oil, the graft survival time was 13.3 ± 1.0 days, with fish oil it was 12.3 ± 0.4 days, and with soybean oil 10.4 ± 0.7 days ($P < 0.01$ vs controls for all three groups, Table 3). Treatment with safflower oil ($P < 0.01$) and with fish oil ($P < 0.05$) resulted in a significantly longer graft survival than did the infusion with soybean oil. The difference in survival time between the fish oil and the safflower oil-treated groups did not reach statistical significance.

As a rule, the hearts beat perfectly for a number of days; then the contractions became weaker. In the saline-treated controls, the heart function weakened after an average of 6.4 days; in the oil-treated controls after 5.9 days; in the safflower oil group after 8.2 days, in the fish oil group after 7.9 days, and in the soybean oil group after 8.2 days. The hearts then beat for an additional 1.4 days (saline controls), 0.9 days (oil controls), 5.1 days (safflower oil), 4.4 days (fish oil), and 2.2 days (soybean oil).

When the weights of the removed grafts were compared with the weights at implantation there was, on the average, a 3.01-fold increase in the saline-treated controls and a 2.9-fold increase in the oil-treated controls. In the safflower oil, fish oil, and soybean oil-treated animals, the increase was 2.11-fold ($P < 0.01$), 2.34-fold ($P < 0.05$), and 2.15-fold ($P < 0.05$), respectively.

The animals' food consumption was highest in the saline-treated control group (mean 59.85 g/kg body weight per day), which was to be expected since these ani-

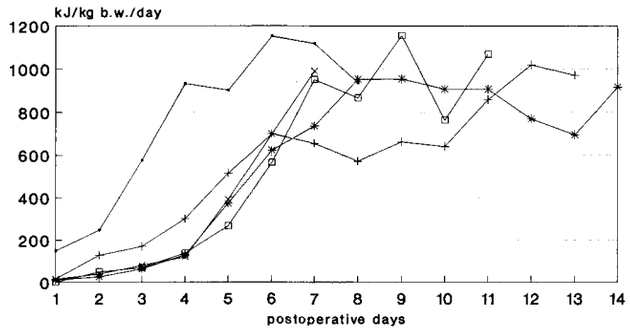

Fig. 1 Daily oral energy uptake in the control (-•-), fish oil (+), safflower oil (*), soybean oil (-□-), and fish oil/safflower oil 1:1 mixture (*) groups

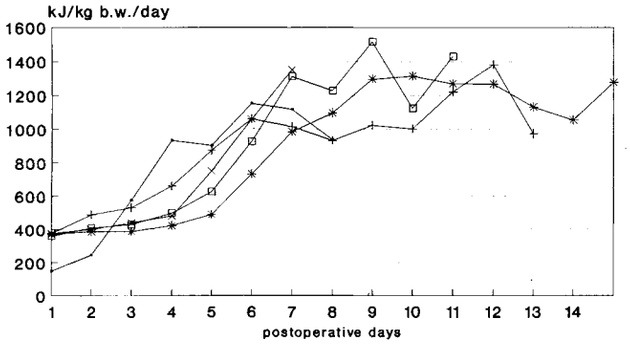

Fig. 2 Daily oral plus parenteral energy uptake in the control (-•-), fish oil (+), safflower oil (*), soybean oil (-□-), and fish oil/safflower oil 1:1 mixture (*) groups

mals had to satisfy their entire energy need by oral intake. In comparison, the animals given 9 g fat/kg body weight per day parenterally consumed approximately one-third less food, i.e., 35 g/kg body weight per day in the safflower oil group, and 36.2 g/kg body weight per day in the oil-treated control group (Fig. 1). The animals given parenteral fat downregulated their oral consumption to such a degree that there was no significant difference in the average daily energy intake (oral + parenteral energy) between the saline-treated control animals and the animals given different fat emulsions (saline-treated controls 778.05 kJ/kg body weight per day, oil-treated controls 790.21 kJ/kg body weight per day, safflower oil group 815 kJ/kg body weight per day, fish oil group 780.55 kJ/kg body weight per day, soybean oil group 845 kJ/kg body weight per day; $P = 0.61$; Fig. 2). All of the rats lost weight after transplantation. Despite a similar energy uptake, the animals that were given fat emulsions lost slightly, but significantly, more weight than the animals in the saline-treated control group ($P < 0.05$). Among the groups treated with fat emulsions, there was no significant difference in weight loss. The mean weights on day 7 after transplantation were 93% of the weight on day 0 in the control group and 86%, 85%, 84%, and 85% in the fish oil, safflower oil, soybean oil, and safflower/fish oil 1:1 mixture groups (oil-treated controls), respectively.

Discussion

Intravenous infusion of both safflower oil, fish oil and soybean oil after transplantation caused a significant prolongation of graft survival in our rat heart allotransplant model. Thus, the time to rejection was delayed and the intensity of the rejection was decreased. Also, the weight gain of the rejected grafts was lower in the rats treated with these three fat emulsions. There was no aspecific immunosuppressive effect of the fat; the safflower/fish oil 1:1 mixture group did not prolong graft survival compared to the saline-treated controls. Polyunsaturated n-3 and n-6 fatty acids are likely to account for these effects by virtue of being precursors of immunomodulating prostaglandins, thromboxanes, and leukotrienes. The fatty acids in safflower, fish, soybean, and the safflower/fish oil 1:1 mixture group consist, respectively, of 75%, 43%, 60%, and 59% polyunsaturated n-3 or n-6 fatty acids. However, the composition of the oils varies. Thus, in safflower oil the n-6/n-3 fatty acid ratio is 370/1, while it is 6.5/1 in soybean oil and 2.1/1 in the safflower/fish oil mixture. In fish oil the ratio is inverse, the n-3/n-6 ratio being 7.6/1. The safflower/fish oil mixture group with a n-3/n-6 ratio of 1/2.1, termed "ideally balanced" by Kinsella et al. [9], actually turned out to be immunologically neutral in our model. When the polyunsaturated fatty acids in the feed were also taken into account, the rats in the safflower oil, soybean oil, and safflower/fish oil mixture groups received n-6 and n-3 fatty acids in a ratio of 49/1, 6.4/1, and 2/1. The inverse ratio in the fish oil group was 3.3/1. Saline-treated control rats got polyunsaturated fatty acids only via the feed at an n-6/n-3 ratio of 6/1.

Safflower oil (high n-6/n-3 ratio) and fish oil (high n-3/n-6 ratio) were equally immunosuppressive. Apparently, the predominance of n-6 fatty acids must be markedly higher than the predominance of n-3 fatty acids to unfold an equivalent immunosuppressive effect. Soybean oil, with a more balanced n-6/n-3 ratio than safflower oil, was significantly less immunosuppressive ($P < 0.01$ vs safflower oil; $P < 0.05$ vs fish oil). The safflower/fish oil 1:1 mixture group showed no immunosuppressive effect at all.

The mechanism of immunosuppression induced by n-6 fatty acids (safflower oil) is thought to be due to an increase in prostaglandin synthesis. Prostaglandin E2 is known to inhibit the cell-mediated immune response [6, 10, 11]. Other n-6 derivatives, i.e., thromboxane A2 and leukotriene B4, have prorejection properties [5]. A diet high in linoleic acid (n-6) has been shown to increase the prostaglandin E2 but decrease the thromboxane A2 release from rat macrophages [4]; in humans, Hornstra et al. [8] demonstrated reduced platelet aggregation, also probably due to

a lower thromboxane A2 formation. Such a selective increase in prostaglandin E2 production after a high supply of n-6 fatty acids could be the reason for the prolonged allograft survival in our safflower oil-treated animals.

The immunosuppressive effet of n-3 fatty acids (fish oil group) could have its explanation in an increased production of eicosapentaenoic acid. As a result of this, the synthesis of the immunosuppressive prostaglandins E3 and I3 is enhanced. Also, thromboxane A2 and leukotriene B4 should be replaced by thromboxane A3 and leukotriene B5, these compounds having a weaker prorejection effect [5].

In our material post-transplant infusion of fish oil was sufficient to prolong allograft survival. In the study done by Otto et al. [13], graft survival could be prolonged only by administering fish oil diets to the donors and to the recipients both pre- and post-transplantation. Perez et al. [14] achieved a prolongation by force-feeding the rats with fish oil starting after transplantation. Both infusion and force-feeding make it possible to administer a standardized quantity of fat from the very 1st day after transplantation. This might be important in view of the fact that, early after transplantation, the spontaneous oral fat intake is low. It seems possible that Otto's dietary pretreatment compensates for the low oral fat uptake during the 1st days after transplantation.

It is noteworthy that soybean oil, with a more balanced n-6/n-3 fatty acid ratio, had a smaller immunosuppressive effect than safflower oil and that the safflower/fish oil 1:1 mixture had no immunosuppressive effect at all. Apparently, n-3 and n-6 fatty acids have no additive immunosuppressive effect; instead, the n-3/n-6 fatty acid ratio is decisive. Boudreau et al. [1] claim that the more immunosuppressive a fat emulsion is, the higher its n-3/n-6 fatty acid ratio. Beyond that, our data indicate that the immunosuppressive properties of fat emulsions are highest when the n-3/n-6 fatty acid ratio is unbalanced, no matter whether n-3 or n-6 fatty acids are markedly predominant.

The route of administration may also influence the immunosuppressive mechanism of fat. Only so can we explain why intravenous soybean oil resembling the n-6/n-3 ratio of the feed (6.5/1 vs 6/1) caused a prolongation of graft survival compared to the saline-treated control group. Fat emulsions consist, for the most part, of fatty acid triglycerides, but they also contain free fatty acids. It has been shown in vitro that free fatty acids directly inhibit lymphocyte function and proliferation, killer cell activity, and macrophageal phagocytosis via a mechanism independent of prostaglandins, thromboxanes, and leukotrienes [2, 3, 17]. In the intravenous, but not the oral, route of administration, these free fatty acids enter the bloodstream directly and may unfold an additional immunosuppressive effect.

The reports on the effects of polyunsaturated fatty acids on allograft survival are, however, not uniform, and while the n-6 fatty acid studies of Tchervenkov et al. [15] and Perez et al. [14] support our findings, Otto could not prolong graft survival by administering corn oil (rich in n-6). It is obvious that the effects of the polyunsaturated fatty acids and the impact of the route of administration, as well as their potential interaction with conventional immunosuppressive drugs, deserve further investigation.

In summary, our data indicate that the infusion of fat emulsions rich in n-3 or n-6 fatty acids has an immunosuppressive effect in the rat heart allograft model. The immunosuppressive properties of a fat emulsion seem to be maximal when either n-3 or n-6 fatty acids are markedly predominant and minimal when the n-3/n-6 ratio approximates a ratio of 1/2. Apparently, the effect of the fatty acid families is not additive although the immunosuppression due to n-3 or n-6 fatty acids is brought about by different pathways. The infusion of polyunsaturated fat emulsions seems to have a greater immunosuppressive effect than oral administration, as shown by soybean oil.

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