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Original Research Article

Indomethacin inhibits PGE2, regulates inflammatory response, participates in adipogenesis regulation, and improves success rate of fat transplantation in C57/B6 mice

Hongyu Xue[1], Hongyi Zhao[2]*, Huiying Wang[3], Song Zhang[1]
[1]Department of Plastic Surgery, Peking University Third Hospital, [2]Department of Plastic Surgery, Beijing Hospital, National Center of Gerontology, [3]Department of Burns and Plastic Surgery, Beijing Luhe Hospital, Capital Medical University, Beijing, PR China

For correspondence:* ***Email:* *ethj78@163.com*

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Abstract

Purpose: *To investigate the effect of indomethacin on prostaglandin E2, regulation of inflammation and adipogenesis, and success of fat transplantation in mice.*
Methods: *The mice were randomly divided into 4 groups: group A (free fat group), group B (free fat + stromal vascular fragments group (SVF)), group C (free fat + 200 μM indomethacin group), and group D (free fat + 200 μM indomethacin + SVF group), with 21 mice in each group. Expression levels of adipogenic genes CEBP-α, FABP4 and LPL in each group were determined. Changes in PGE2 level in transplanted adipose tissue, and changes in the expression of NF-κB in apoptotic stem cells induced by different pro-inflammatory treatments were assayed.*
Results: *Compared with group B, the expression levels of adipogenic genes CEBP-α, FABP4 and LPL significantly decreased in groups A, C and D, with group A as the lowest ($p < 0.05$). Compared with the indomethacin treatment group, the level of inhibition of PGE2 in mice adipose tissue in the indomethacin-free group increased significantly ($p < 0.01$). The expression of NF-κB in the adipose stem cells from the indomethacin-treated group was significantly lower than that in the indomethacin-treated group after pretreatment with IL-17 or INF-γ + TNF-α.*
Conclusion: *Indomethacin regulates adipogenesis by inhibiting the production of COX2 metabolite, PGE2. It also regulates the local microenvironment, inhibits the inflammatory process, and protects various stem cells. Therefore, it may improve the success rate of fat transplantation.*

Keywords: *Indomethacin, PGE2, Wnt signaling pathway, adipogenic regulation, fat transplantation success*

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INTRODUCTION

Fat transplantation refers to the technology of sucking fat from a fat-rich parts of the human body such as abdomen, lateral thigh or buttock, through negative pressure liposuction, and injecting into the affected area purified fat particles so as to improve its shape [1]. The source of fat for transplantation is abundant and easily accessible, but the survival rate of fat

transplantation is low, and the results are difficult to predict accurately. With continuous improvement in science and technology, the outcome of fat transplantation has improved significantly, although it still faces problems of insufficient donor sites and low success rate [2].
It has been reported that indomethacin significantly promotes adipogenesis of adipose-derived stem cells and increases the expression of adipogenic genes [3]. Indomethacin is a non-steroidal anti-inflammatory drug and a non-specific inhibitor of cyclooxygenase (COX) which blocks the production of prostaglandin E2 (PGE2). It has anti-inflammatory, antipyretic and analgesic effects. Peroxisome proliferator-activated receptors (PPAR) affect adipogenic activity [4]. The product of COX2 i.e. PGE2 functions as an assimilator in promoting bone formation and inhibiting adipogenesis [5].

Indomethacin upregulates the expression of PPAR-γ2 and activates adipogenic process in pre-adipocytes and bone marrow-derived mesenchymal stem cells [6]. In addition, indomethacin blocks the downstream effector molecule beta catenin in adipogenic transcription through suppression of PGE2 signaling pathway. Aggregation improves the integrity of adipose tissue structure and enhances the percentage of active fat [7]. This study investigated the effect of indomethacin on PGE2, Wnt signaling pathway regulation of adipogenesis, and degree of success of fat transplantation in C57/B6 mice.

EXPERIMENTAL

Animals

A total of 84 healthy female C57/B6 mice were obtained from Shanghai Ruitai Moss Biotechnology Co. Ltd {production license SCXK (Shanghai) 2016-0001)}. The mice were 8 weeks old, with mean body weight of 36 ± 5 g, and were allowed free access to feed and clean water. This research was approved by the Animal Ethical Committee of Beijing Hospital (approval no. 20187162) and performed according to Principles of Laboratory Animal Care [8].

Equipment and reagents

The major equipment and reagents used, and their makers (in brackets) were: centrifuge (Hunan Kaida Instrument Co. Ltd, model: GL21M); constant temperature incubator (Shanghai Precision Instrument Co. Ltd, model: LRH-70); refrigerator (Hengtai Epp Group Co. Ltd, model: AP-86-160LA); optical microscope (Olympus, model: BX53); DMEM cell culture medium (Shanghai Thermo Fisher Scientific Co. Ltd), and anhydrous ethanol (Qingdao Jielong Chemical Co. Ltd). The others were fetal bovine serum (Shanghai Yuchuang Biotechnology Co. Ltd, Specification: Z7185FBS-100); phosphate buffer (Wuhan Punosei Life Technology Co. Ltd), and Yan Meixin (Shanghai Jinbui Lankao Pharmaceutical Co. Ltd., production batch number: 41021631, specification: 25mg x 100 pieces).

Treatment and grouping

Animal model of fat transplantation was first established. The mice were anesthetized and placed on a console. The hair on their backs was shaved off, and the skin was disinfected. Then, 200 µl free adipose tissue was mixed with stromal vascular fragments (SVF) and indomethacin using 1-mL syringe. The mixture was injected on both sides of the back to form a raised fat mass on the skin. The mice were randomly divided into 4 groups: group A (free fat group), group B (free fat + SVF), group C (free fat + 200 µM indomethacin), and group D (free fat + 200 µM indomethacin + SVF). Each group contained 21 mice.

On the 3^{rd}, 5^{th}, and 11^{th} weeks after fat transplantation, 7 mice in each group were subjected to skin disinfection. The subcutaneous fat, skin and bottom muscles were peeled off to remove the transplanted fat. The excised transplanted fat was then immersed in isotonic saline, and its volume was measured.

H & E staining

The transplanted adipose tissue samples were fixed in 4 % formaldehyde, dehydrated with xylene, embedded in paraffin, sliced with a paraffin slicer, oven-dried, dewaxed, rehydrated, and stained with hematoxylin and eosin (H & E staining). The histological morphology of each group was examined under the microscope.

Cell culture

Healthy adipose-derived stem cells were seeded in 24-well plates at a density of 2×10^4 cells/well, and cultured in complete medium to 80 % cell aggregation. Subsequently, the culture medium used was either complete medium (a), Zuk medium) (b), indomethacin + complete medium (c), or indomethacin + adipose tissue-derived adipogenic induction medium (d). The stem cells were then cultured in their respective media for 7 days, after which the expression levels of the adipogenic genes CEBP-α, FABP4 and LPL in each medium were measured.

ELISA

One week after establishment of the animal fat transplantation model, 2 mL of blood was taken from the tail vein of each mouse and centrifuged. The supernatant was taken and stored in a refrigerator. Part of the fat samples obtained 3, 5, and 11 weeks after fat transplantation were placed in sterile containers. The tissue homogenate of each specimen was prepared in phosphate buffer 4 ^{o}C, centrifuged, and the supernatant was taken. The levels of PGE2 in supernatants from adipose tissue homogenates and peripheral blood of mice treated with indomethacin and indomethacin- free mice were determined using enzyme-linked immunosorbent assay (ELISA).

Western blotting

Western blotting was used to assay the expression of NF-κB in adipose-derived stem cells treated with different pro-inflammatory factors *in vitro*. The transplanted adipose tissue specimens were collected at 5, 10, and 15 days after fat transplantation, and semi-quantitative counts were used to compare the expression levels of caspase-3 and caspase-8 in the indomethacin-free and indomethacin-treated groups at different time points.

Statistical analysis

Measurement data were compared between two groups using independent sample *t*-test. Comparison between multiple groups was done with single factor multi-sample test; while comparison of count data was performed using χ^2-test. SPSS 21.0 software package was used for all statistical data analyses. Differences were deemed significant at $p < 0.05$.

RESULTS

Changes in graft volume

The volume of grafts in each group gradually decreased with time. Changes in graft volume were most obvious in group A, while changes in the volume of grafts in mice treated with indomethacin were the lowest. These results are shown in Table 1.

Histological changes in mouse adipose tissue

At 3 weeks, there were more vacuoles in adipose tissue, and some inflammatory infiltration appeared. From 5 to 11 weeks, the vacuoles in adipose tissue gradually decreased, the degree of inflammatory infiltration was significantly reduced, and the adipose tissue structure tended to be stable. The tissue integrity and inflammatory infiltration of adipose tissue in the indomethacin group were significantly better than those in group A. These results are presented in Figure 1.

Table 1: Changes in graft volume (mean ± SD, μL)

Group	0 week	3 weeks	5 weeks	11 weeks
A	200.00 ± 0.00	121.22 ± 2.06	87.23 ± 6.25	29.84 ± 2.26
B	200.00 ± 0.00	157.38 ± 4.18[a]	102.33 ± 5.29[a]	49.88 ± 5.41[a]
C	200.00 ± 0.00	150.14 ± 4.27[a]	123.36 ± 3.97[a]	57.69 ± 5.15[a]
D	200.00 ± 0.00	163.68 ± 4.49[a]	142.19 ± 5.54[a]	75.27 ± 4.43[a]

[a]$P < 0.05$, compared with group A

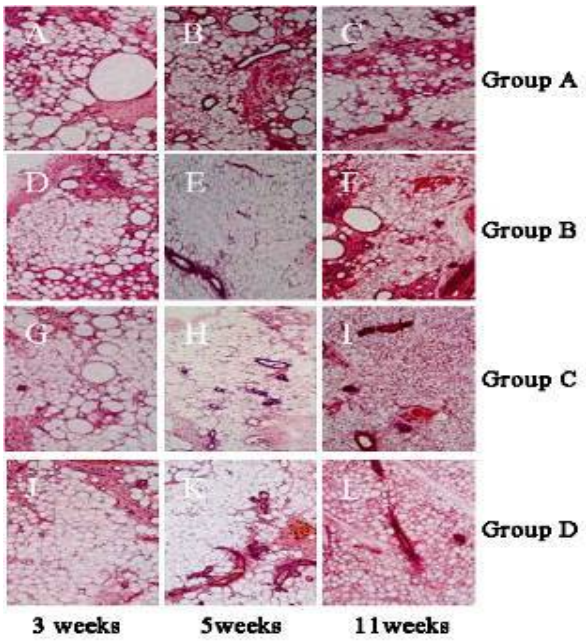

Figure 1: Histological changes in mice adipose tissue. A: adipose tissue at 3 weeks in group A; B: adipose tissue at 5 weeks in group A; C: adipose tissue at 11 weeks in group A; D: adipose tissue at 3 weeks in group B; E: adipose tissue at 5 weeks in group B; F: adipose tissue at 11 weeks in group B; G: adipose tissue at 3 weeks in group C; H: adipose tissue at 5 weeks in group C; I: adipose tissue at 11 weeks in group C; J: D: adipose tissue at 11 weeks in group C

Active adipocytes in mouse transplanted tissues

Compared with group A, the percentage of active fat in mice transplanted tissues from groups B, C and D increased significantly, with group D accounting for the highest proportion. However,

there were no significant difference amongst the three groups B, C and D ($p < 0.05$; Table 2).

Table 2: Active adipocytes in mice transplanted tissues (mean ± SD)

Group	Active adipocytes (%)
A	43.87 ± 3.54
B	60.38 ± 2.44[a]
C	62.88 ± 2.01[a]
D	65.43 ± 2.57[a]

Expressions of CEBP-alpha, FABP4 and LPL

Compared with group B, the expressions of CEBP-alpha, FABP4 and LPL in groups A, C and D were significantly lower, with group A as the lowest ($p < 0.05$). The expression levels of CEBP-a, FABP4 and LPL in groups C and D were significantly higher than those in group A ($p < 0.05$). These results are displayed in Table 3.

Table 3: Expression levels of adipogenic genes CEBP-alpha, FABP4 and LPL (mean ± SD)

Group	CEBP-α	FABP4	LPL
A	1.52 ± 0.07[a]	1.04 ± 0.02[a]	2.54 ± 1.27[a]
B	45.26 ± 6.74	852.16 ± 47.82	202.25 ± 14.14
C	6.57 ± 0.62[ab]	53.57 ± 11.26[ab]	15.53 ± 2.38[ab]
D	14.92 ± 3.13[ab]	384.32 ± 22.63[ab]	121.35 ± 15.67[ab]

[a]$P < 0.05$, compared with group B; [b]$p < 0.05$, compared with group

PGE2 levels in peripheral blood and transplanted adipose tissue

Table 4 shows that, compared with the indomethacin treatment group, the level of inhibition of PGE2 in mice adipose tissue in the indomethacin-free treatment group was increased significantly ($p < 0.01$), but there was no significant difference in the level of PGE2 in peripheral blood between the two groups ($p > 0.05$).

Table 4: PGE2 levels in peripheral blood and transplanted adipose tissue (mean ± SD)

Group	Peripheral blood (ng/mL)	Transplanted adipose tissue (pg/mL)
Indomethacin treatment	1679.59 ± 514.15	24.29 ± 5.33
Indomethacin-free treatment	1726.59 ± 527.75	217.27 ± 68.53
T	0.285	12.556
P-value	0.777	< 0.001

Expression of apoptotic signaling pathway NF-κB in adipose stem cells treated with different pro-inflammatory factors

As shown in Figure 2, pretreatment with IL-17 or INF-γ in combination with TNF-α significantly decreased the expression of NF-κB in adipose stem cells treated with indomethacin.

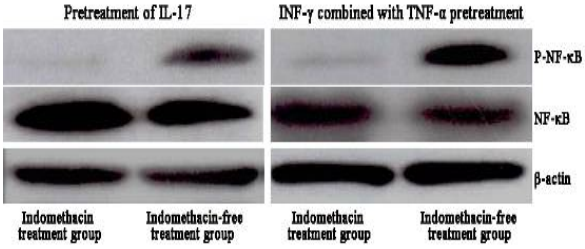

Figure 2: Expression of apoptotic signal pathway NF-κB in adipose stem cells treated with pro-inflammatory factors

Expression levels of caspase-3 and caspase-8 in transplanted adipose tissues in indomethacin-treated and indomethacin-free groups at different time points

The expression levels of caspase-3 and caspase-8 in adipose transplanted adipose tissue in the indomethacin-free group were significantly lower than those in the indomethacin-free group at different time points ($p < 0.05$). These results are presented in Table 5.

Table 5: Expression levels of caspase-3 and caspase-8 in transplanted adipose tissue in indomethacin-treated and indomethacin-free groups at different time points

Group		Caspase-3	Caspase-8
Indomethacin	5 days	3.14 ± 0.12	2.96 ± 0.13
	10 days	5.03 ± 0.22	6.02 ± 0.25
	15 days	15.24 ± 3.37	14.97 ± 4.21
Indomethacin-free	5 days	8.41 ± 1.58[a]	8.32 ± 1.57[a]
	10 days	12.58 ± 1.44[b]	13.74 ± 1.26[b]
	15 days	22.15 ± 4.48[c]	21.38 ± 3.72[c]

[a]$P < 0.05$, [b]$p < 0.05$; [c]$p < 0.05$, compared with indomethacin at 5 and 10 days

DISCUSSION

Adipose-derived stem cells are a family of hepatocytes with multiple differentiation potential isolated from adipose tissue. They restore the repair function of tissue cells and promote cell

regeneration. Adipose stem cells are of great significance in the development of regenerative medicine [9].

Studies have shown that regulation of fat regeneration by adipose stem cells is partly affected by the local microenvironment. Proinflammatory T cells inhibit the regulation of fat regeneration by adipose stem cells. This effect may be mediated through INF-γ-induced down-regulation of adipogenesis regulator and enhancement of TNF-α signaling pathway [10]. Indomethacin is a non-steroidal anti-inflammatory and analgesic drug. It reduces the synthesis of PGE2 by inhibiting COX, thereby preventing inflammation and painful nerve impulses (including inhibition of leukocyte chemotaxis and lysosomal enzyme release) [11]. Some scholars have found that mixed transplantation of indomethacin and encapsulated hydrogel materials regulate the local microenvironment of the graft, alleviate the apoptotic process induced by pro-inflammatory cytokines, and improve and protect the activity of adipose stem cells in the transplanted materials [12].

It has been reported that indomethacin significantly inhibits the deposition of collagen on fibrocyte layer and transplantation surface at the late stage of transplantation, and also enhances the activity and function of adipose precursor stem cells in plants [13]. Studies by Kumar *et al* have suggested that INF-γ and TNF-α activate the NF-κB signaling pathway and increase COX levels, leading to significant increases in PGE2 levels, thereby mediating apoptosis and tissue necrosis [14]. The Wnt inhibitors usually act on multiple sites in the Wnt signaling pathway. Non-steroidal anti-inflammatory drugs (NSAIDs) inhibit the Wnt signaling pathway, and block the accumulation of β-catenin and the transcription of downstream effector molecules through inhibition of COX [15].

Fat regeneration and remodeling consists of inflammation, proliferation and remodeling stages. A large number of regulatory factors, cytokines and extracellular matrix are involved in each complex process at the inflammation and proliferation stages [16]. It has been found that the immune response affects tissue regeneration regulated by adipose-derived stem cells [17]. Indomethacin has some antagonistic effects on the pro-inflammatory cytokine-mediated NF-kB signaling pathway. It downregulates the related pro-apoptotic signaling pathways of caspase-3 and caspase-8, thereby reducing apoptosis of active cells such as stem cells [18]. Furthermore, some scholars have shown that pro-inflammatory cytokines activate apoptotic signaling pathway by activating the NF-kB signaling pathway, leading to apoptosis and cell damage [19].

The results of this study show that infiltration of inflammatory cells and fibrous tissue of adipose tissue in mice transplanted with indomethacin were significantly reduced; the percentage of active adipocytes was significantly increased, and the level of PGE2 in the indomethacin-treated group was significantly lower than that in the indomethacin-free group. However, there was no significant difference in the level of PGE2 in peripheral blood between the two groups.

Treatment with IL-17 or INF-γ + TNF-α significantly lowered the expression of apoptotic signal pathway NF-kB in adipose stem cells treated with lipid, relative to adipose stem cells treated with indomethacin. The expression levels of caspase-3 and caspase-8 in transplanted adipose tissue in the indomethacin-free group were significantly lower than those in the indomethacin-free group at different time points. These results suggest that indomethacin significantly inhibits inflammation and PGE2, and downregulates caspase-3 and caspase-8, thereby slowing down apoptosis while enhancing the activity of adipocytes after transplantation.

CONCLUSION

Indomethacin participates in the regulation of adipogenesis by inhibiting the production of PGE2 (the product of COX2 activity). At the same time, indomethacin regulates the local microenvironment by inhibiting inflammatory responses and protecting the viability of various stem cells, thereby improving the success rate of fat transplantation. In this way, indomethacin may also participate in the regulation of adipogenesis by blocking the Wnt signaling pathway.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. All authors read and approved the manuscript for publication. Hongyi Zhao conceived and designed the study, Hongyu Xue, Hongyi Zhao,

Huiying Wang and Song Zhang collected and analysed the data, and Hongyu Xue wrote the manuscript.

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