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ORIGINAL ARTICLE

Enhancement of vascular endothelial growth factor's angiogenic capacity by the therapeutic modulation of notch signalling improves tram flap survival in rats submitted to nicotine

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ABSTRACT

Background: Smoke of cigarettes, and specifically nicotine, has been shown to diminish pedicled transverse rectus abdominis musculocutaneous (TRAM) flap survival. Considering that Notch signalling through its ligand Delta-like 4 (Dll4) functions as anti-angiogenic factor by inhibiting the pro-angiogenic effects of vascular endothelial growth factor (VEGF), it is hypothesised that inhibition of the Notch would promote angiogenesis and increase TRAM flap survival in rats submitted to nicotine.

Methods: Twenty rats were treated with nicotine for 28 days preoperatively. Thereafter, a pedicled TRAM flap was created in all animals. The Notch inhibitor N-[N-(3,5-difluorophenacetyl)-1-alanyl]-S-phenylglycine-t-butyl-ester was administered in animals of the treatment group. Animals in the control group were given the same amount of solvent. Five days after the surgery, viable flap areas were determined. Skin samples were evaluated for *VEGF* and *Dll4* mRNA levels. Immunohistochemical analysis was used for the assessment of endothelial Dll4 expression. Vascular density was determined histologically. Plasma levels of VEGF and Dll4 were measured.

Results: A significant improvement in TRAM flap surviving area was observed in the treatment group (53.50 ± 14.25%) compared with the controls (32.20 ± 9.15%). Immunohistochemical analysis revealed a significant increase in the number of Dll4 stained vessels in animals of the treatment group (9.2 ± 1.6) in comparison with the controls (5.7 ± 1.9). *VEGF* mRNA levels (0.22 ± 0.08) in the treatment group were significantly lower than those in the control group (0.36 ± 0.09).

Conclusion: Notch inhibition significantly improved TRAM flap survival in animals exposed to nicotine by promoting VEGF-induced angiogenesis.

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Notch; Dll4; TRAM; nicotine; survival; angiogenesis

Introduction

Since it was introduced in 1982, the pedicled transverse rectus abdominis musculocutaneous (TRAM) flap has been a workhorse of autologous breast reconstruction after mastectomy [1]. The main advantage of this procedure is that it avoids the complications inherent in the use of alloplastic implants [2]. On the other hand, the major disadvantage of TRAM flap is associated with the loss of muscle from the abdominal region that can result in herniation [3]. Despite an overall greater experience with pedicled TRAM flap, insufficient vascularisation of the flap remains a substantial problem and results in complications such as partial flap loss and fat necrosis [4]. For this reason, several methods have been proposed to prevent TRAM flap necrosis [5–7]. Ischaemia related complications are especially important in patients with risk factors such as smoking, obesity, diabetes, and previous abdominal surgeries [8–10]. In particular, smoking has a well-documented detrimental effect on flap survival [11]. Among women undergoing breast reconstruction with pedicled TRAM flap, the risk of ischaemia-related complications is significantly higher in smokers than in non-smokers [12]. For this reason, heavy smoking is generally considered to be a contraindication for breast reconstruction with pedicled TRAM flap.

The smoke of tobacco contains over 4000 toxic chemicals, of which nicotine is the most harmful to skin perfusion [13]. Several studies have proven the deleterious effects of nicotine in perfusion experiments in skin flaps in rats [14–16]. The mechanism of nicotine-related skin ischaemia is multifactorial and complex. Nicotine is a potent vasoconstrictor that diminishes tissue perfusion by stimulating the release of thromboxane A_2 and catecholamines, and reducing the synthesis of vasodilating prostacyclin [17]. Nicotine further compromises microcirculation by raising the risk of thrombotic microvascular occlusion secondary to its ability to activate

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thrombocytes and stimulate their aggregation [18]. Pharmacological modulation of these detrimental effects could improve the safety of surgeries in nicotine addicted patients.

Several growth factors with angiogenic activities were identified with the aim of increasing tissue perfusion by inducing functional angiogenesis [7,19]. Among these, vascular endothelial growth factor (VEGF) is a potent endothelial cell mitogen that affects circulation by promoting angiogenesis [20]. Extended skin paddle survival of the TRAM flap has been observed in many experimental studies following administration of VEGF [21,22]. However, the rate of successful translation of this experimental strategy into clinical practice remains low. The main barrier to efficient clinical translation could arise from the fact that the application of a single factor represents a simplification of complex system that involves highly regulated orchestration of multiple signalling pathways. This situation prompted interest in the development of therapies targeting pro- and anti-angiogenic signals originating from the tissue environment.

The Notch pathway is an evolutionarily conserved ligand-receptor signalling system that regulates cell proliferation and differentiation [23]. Notch proteins are four different transmembrane-spanning receptors (Notch 1, Notch 2, Notch 3, Notch 4) that interact with one of five membrane bound ligands (Delta-like 1 (Dll1), Delta-like 3 (Dll3), Delta-like 4 (Dll4), Jagged 1 and Jagged 2) through direct cell–cell contact [24]. It is now evident that the Notch pathway through its ligand Dll4 is critically important for the proper construction of vascular network through the regulation of angiogenic effects of VEGF [25]. During angiogenesis, VEGF activates endothelial cells on the main vessel to promote the formation of new vascular sprouts. Coincidentally with initiating sprouting, VEGF also upregulates the expression of Dll4 on tip endothelial cells that lead to new sprouts. The subsequent interaction of Dll4 with Notch receptors on the adjacent endothelial cells triggers a series of intracellular events that lead to inhibition of VEGF receptor expression. This restricts the emergence of excessive sprouting through decreasing responsiveness to VEGF [26]. This crosstalk is critical in order to form a vascular network with the appropriate density of branches.

Considering the harmful effects of nicotine on microcirculation and the search for strategies that can provide smokers with a better healing condition, the current study aims to evaluate whether therapeutic modulation of the Notch pathway in rats previously exposed to nicotine would induce angiogenesis and improve TRAM flap survival. Since Notch signalling functions as a negative regulator of VEGF, we hypothesised that pharmacological inhibition of the Notch would promote angiogenesis by potentiating the angiogenic activity of VEGF.

Methods

Experimental animals

The current study was approved by Kobay DHL Ethical Committee for Experimental Research on Animals (Application number 123) and supported by Ahi Evran University Research Fund (Project number PYO.TIP. 4001.15.005). Twenty adult male Sprague Dawley rats, weighing 250–300 g, were purchased from a commercial company (Kobay DHL A.S., Ankara, Turkey). Animals were housed in individual cages for 1 week before the experiment, with free access to water and standard laboratory food.

Pharmacological inhibition of notch signalling

For the pharmacological inhibition of Notch signalling, the gamma-secretase inhibitor (GSI) N-[N-(3,5-difluorophenacetyl)-1-alanyl]-S-phenylglycine t-butyl ester (DAPT) (Selleck Chemicals, Houston, TX) was used [27]. DAPT was prepared according to the manufacturer's instructions by dissolving in the solvent dimethlysulphoxide (DMSO).

Experimental protocol and groups

All animals were treated with nicotine (Nicotine Sulphate L-1 Metil-2 (3 Piridil)-Pirrolidine Sulphate; grade II; MW 422–6; Sigma-Aldrich, Germany) injected into the subcutaneous tissue in the dorsal midline in a dose of 2 mg/kg, twice a day, for 28 days preoperatively [28]. A TRAM flap, based on the inferior epigastric vessels as the vascular pedicle, and the right rectus abdominis muscle as the carrier, was created in all rats [21].

Surgeries were performed under general anaesthesia using a mixture of 100 mg/kg Ketamine and 10 mg/kg Xylazine. A skin paddle measuring 3×5 cm was outlined 1 cm caudally to the xiphoid process. The margins of the skin paddle were incised, and the flap was first detached from the left side to the linea alba. On the right side, the skin was raised to the lateral border of the right rectus muscle. Next, longitudinal incisions were performed along the linea alba and the lateral border of the right rectus abdominis muscle. The muscle was divided cranially, and the skin-muscle complex was elevated from the posterior rectus sheath down to the pubic level, preserving the attachments between the muscle and skin. Finally, the abdominal defect was closed primarily, and the flap was sutured to its original bed.

After the surgery, the animals were subdivided randomly into two groups consisting of 10 rats each, according to nature of the postoperative treatment:

1. *Treatment group* ($n = 10$): For each animal, DAPT was injected daily into the subcutaneous plane of the skin paddle (5 mg/kg/day).
2. *Control group* ($n = 10$): For each animal, the same amount of the solvent DMSO was injected daily into the subcutaneous plane of the skin paddle.

The animals were returned to their individual cages for 5 days, after which they were anaesthetised again. After gross measurement of flap survival, tissue specimens were obtained from the skin paddles for subsequent genetic, histologic, and immunohistochemical evaluations.

Table 1. Primers used for real-time polymerase chain reaction analysis.

Gene	Primer
VEGF	
Forward	5' ACg AAA gCg CAA gAA ATC CC 3'
Reverse	5' TTA ACT CAA gCT gCC TCg CC 3'
Dll4	
Forward	5' TAC TgC Tgg TgT TgC Tgg TC 3'
Reverse	5' gCA gCA ggg ATT Agg TTg TC 3'
β-Actin	
Forward	5' Agg gAA ATC gTg CgT gAC AT 3'
Reverse	5' AAC CgC TCA TTg CCg ATA gT 3'

Thereafter, blood samples were obtained (2 cc for each rat) by cardiac puncture.

Assessment of flap survival

The viable flap area was grossly determined based on its appearance, texture, and colour. A template was drawn on the entire skin paddle, and the surviving area was outlined. The template was then scanned on a computer. The survival area was measured as a percentage of the total skin paddle using Adobe Photoshop CS5 (Adobe Systems, Inc., San Jose, CA).

Assessment of gene expression by real-time polymerase chain reaction assay

VEGF and *Dll4* mRNA levels in skin paddles were analysed by real-time polymerase chain reaction assay (RT-PCR). Whole RNAs were extracted from tissues using High Pure RNA Tissue Kit (Roche Diagnostics GmbH, Mannheim, Germany). Afterwards, extracted RNAs were reverse transcripted to cDNA using Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics GmbH, Mannheim, Germany). RT-PCR was carried out using Light Cycler® Fast Start DNA Master SYBR Green I Kit (Roche Diagnostics). Housekeeping gene β-actin served as an internal control. Table 1 shows primer sequences of the target genes.

Assessment of endothelial Dll4 expression by immunohistochemistry

The evaluation of Dll4 expression in endothelial cells was performed by immunohistochemical staining using specific anti-Dll4 antibodies, as previously described [29]. Briefly, sections were incubated with primary antibody (Bioss, Inc., Woburn, MA) overnight at 4 °C and appropriate secondary antibody (Bioss, Inc.) for 1 hour at room temperature. Immunoreactivity was observed using light and fluorescein microscopes.

Assessment microvascular density by hematoxylin and eosin staining

Tissue sections were harvested throughout the common viable part of the skin paddles. The tissues were fixed in formalin for 24 hours. The fixed skin tissues were embedded in paraffin for conventional hematoxylin and eosin (H&E) staining. Vascular density was assessed by measuring the number of capillaries in randomly selected 20 different fields under $\times$40 magnification using a light microscope [30].

Immunohistologic assessment of the vessels

Tissue sections were first deparaffinised and redehydrated. Then, immunohistochemical staining of endothelial cells was performed using antibodies against von Willebrand Factor (vWF) (Bioss, Inc.). The protocol was to follow manufacturer's instructions [5].

Assessment of VEGF and Dll4 blood levels by enzyme linked immunosorbent assays

Collected blood samples were stored at at 37 °C for 30 minutes, 6 hours at 4 °C, and then centrifuged at 2000 rpm for 20 minutes to obtain serum. The obtained serum was stored at −80 °C, until used. Measurement of circulating VEGF and Dll4 levels was performed by enzyme linked immunosorbent assays (ELISA) using specific VEGF (Ray Biotech, Inc., Norcross, GA) and Dll4 (Life Science, Inc., St Petersburg, FL) antibodies, following manufacturer's instructions [31].

Statistics

Data analyses were performed using the Statistical Package for the Social Sciences, version 21.0 (SPSS Inc., Chicago, IL). Kolmogorov–Smirnov and Shapiro–Wilk tests of normality were used to check the normality of variable distributions. Independent variables that showed normal distribution were compared using independent-samples *t*-test. A p-value <0.05 was considered statistically significant. The results of statistical analysis were expressed as mean ± standard deviation.

Results

Assessment of flap survival

All animals survived the experiment until sacrifice. No behavioural abnormalities or self-inflicted injuries were observed. At postoperative day 5, the boundaries between viable and necrotic areas were clearly demarcated in every flap, considering rosy colour, softness, and warmth as markers for viability. Animals of the treatment group demonstrated an average survival of 53.50 ± 14.25%. Meanwhile, the control group showed an average flap survival rate of 32.20 ± 9.15%. The results revealed that the treatment group demonstrated a higher rate of flap survival ($p < 0.001$) (Figure 1).

Assessment of gene expression

We examined *VEGF* and *Dll4* mRNA levels in skin paddles 5 days after the surgery. mRNA levels were expressed as a fraction of VEGF or Dll4 to β-actin. The mean value of VEGF expression in the control group (0.36 ± 0.09) was higher than

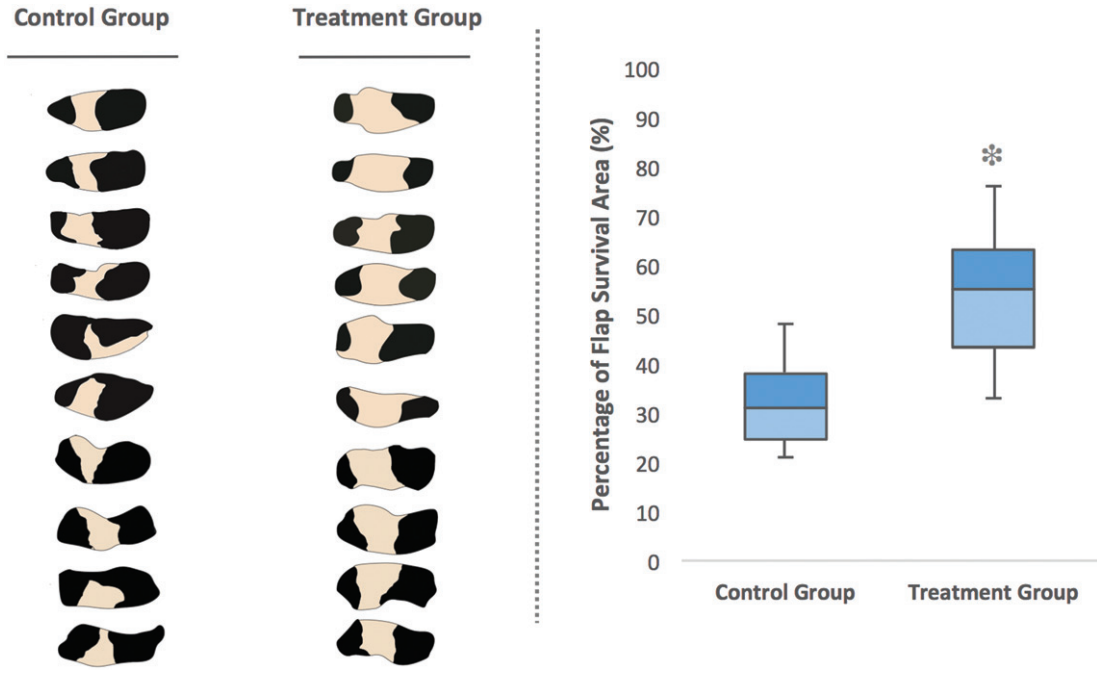

Figure 1. Paper templates measured to the area of the flaps to determine the percentage of surviving skin (left). Plot showing the mean percentage of flap survival area measured in each group. The sample size was $n = 10$ for each group. Data was presented as mean ± SD. $^{*}p < 0.001$.

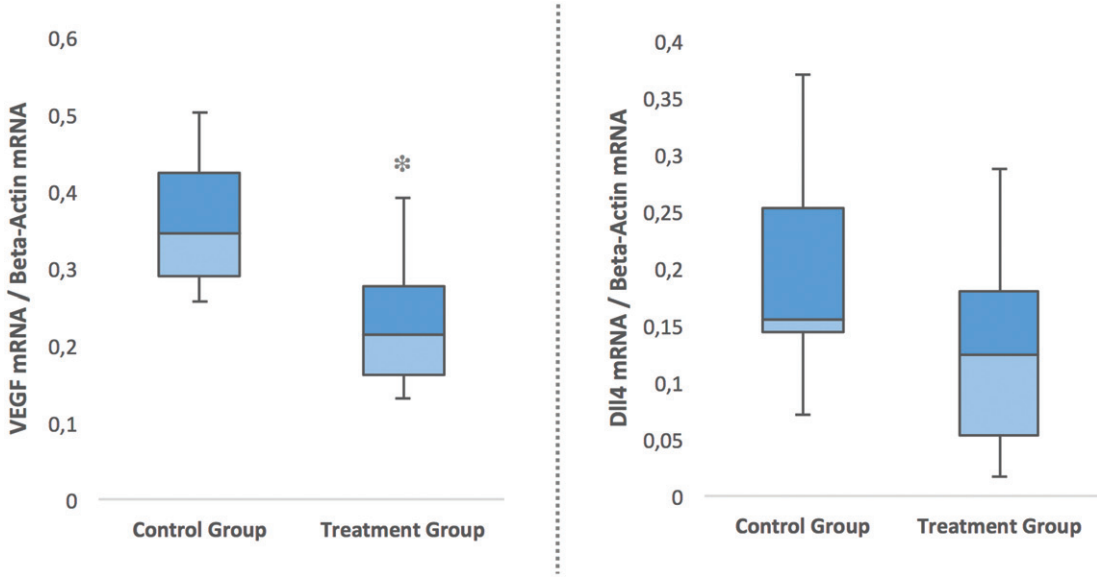

Figure 2. Quantitative analyses of VEGF and Dll4 expression levels, respectively. β-actin was used as the internal control. Results of gene expressions were represented as a fraction of VEGF or Dll4 to β-actin. The sample size was $n = 10$ for each group. Data was presented as mean ± SD. $^{*}p < 0.01$.

that in the treatment group (0.22 ± 0.08). The difference between two groups was statistically significant ($p < 0.01$). On the other hand, the mean values of Dll4 expression in the control and treatment groups were 0.20 ± 0.10 and 0.12 ± 0.09, respectively, without any significant difference between two groups ($p = 0.109$) (Figure 2).

Assessment of Dll4 expression by immunohistochemistry

To confirm the expression of Dll4 in endothelial cells of the skin paddles, immunohistochemical staining using specific anti-Dll4 antibodies was performed. In both groups, analysis of stained sections revealed strong immunoreactivity in capillary endothelial cells with the same pattern of distribution (Figures 3 and 4). Examination of sections revealed that immunoreactivity was present mainly in the proliferating endothelium of the newly-formed small caliber arteries, and relatively weaker staining was observed in the quiescent endothelium of the large vessels (Figure 5).

Assessment microvascular density

To document the effects on Notch inhibition on angiogenesis, vascular density was determined. To this end, capillaries

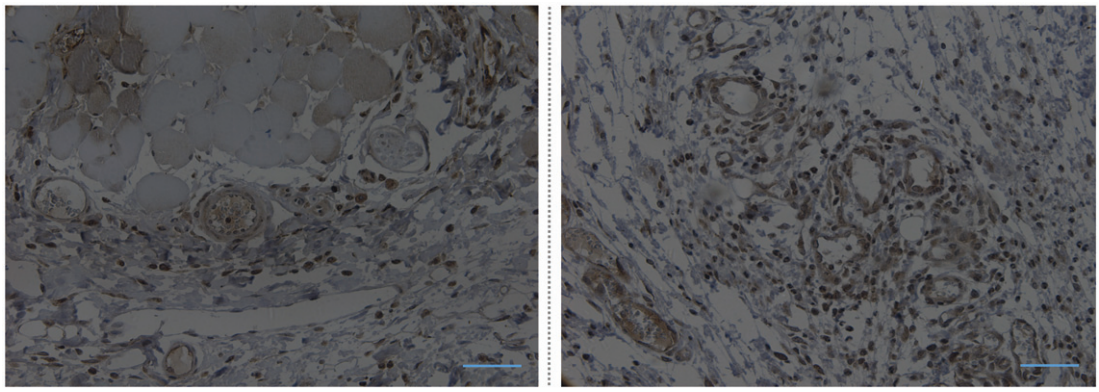

Figure 3. Immunohistochemical staining using specific anti-Dll4 antibodies revealed strong Dll4 expression in capillary endothelial cells of the control (left) and treatment (right) groups (×40 magnification). Scales = 100 μm.

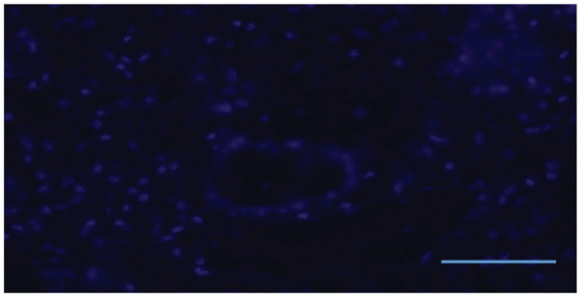

Figure 4. Representative image of immunofluorescent staining with antibodies against Dll4 revealed positive immunoreactivity in endothelial cells of tissues obtained from the treatment group (×40 magnification). Scale =100 μm.

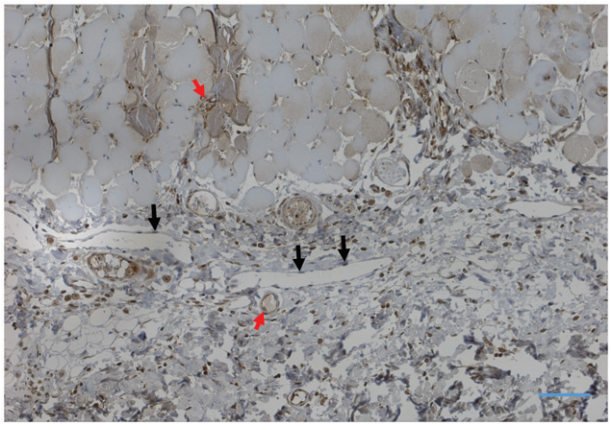

Figure 5. Immunohistochemical staining with antibodies against Dll4 revealed stronger staining in proliferating endothelial cells of the newly-formed small caliber arteries (*red arrows*) in comparison with the large ones (*black arrows*) (×40 magnification). Scale =100 μm.

were counted manually on H&E stained sections and were confirmed by immunostaining with antibodies against vWF, a specific marker for endothelial cells. The mean number of vessels was 9.2 ± 1.6 in the treatment group and 5.7 ± 1.9 in the control group (Figure 6). There was a significant difference between two groups ($p < 0.001$).

Assessment of VEGF and Dll4 blood levels by enzyme linked immunosorbent assays

The mean serum VEGF levels in animals of the control and treatment groups were 1.72 ± 0.34 pg/ml and 1.85 ± 0.29 pg/ml, respectively. The difference between two groups wasn't statistically significant ($p = 0.354$). On the other hand, the mean serum levels for Dll4 in the control and treatment groups were 0.28 ± 0.07 ng/ml and 0.24 ± 0.09 ng/ml, respectively, without any significant difference between the two groups ($p = 0.124$) (Figure 7).

Discussion

The association between nicotine exposure and delayed wound healing is well known [32]. In many experimental models, nicotine has been shown to increase the rate of flap necrosis [16,33,34]. This finding carries over to clinical practice as well [12]. For this reason, the current study sought to investigate a treatment strategy that could reduce the detrimental effects of nicotine on flap microcirculation. The Notch ligand Dll4 acts in feed-back fashion as a negative regulator of VEGF-initiated angiogenic sprouting, controlling this process to modulate over-exuberant angiogenic sprouting, thereby promoting the formation of a vascular network with the appropriate density of branches [35–37]. For this reason, we assumed that blockade of Notch activation can increase angiogenesis and improve TRAM flap perfusion in rats previously exposed to nicotine.

The smoke of tobacco contains over 4000 toxic chemicals, and ensuring the exposure of laboratory animals to all of these substances would be possible only with the use of smoke chambers [38]. However, the exposure to cigarette smoke in these chambers doesn't resemble that of smokers, and plasma levels of the byproducts of the cigarette smoke may not be similar to those in human counterparts [17]. Since the principle damaging effect of smoking on vascular structures originates from nicotine, the nicotinisation method was used in the current study to simulate the effects of smoking on flaps [39]. The dose of nicotine used in this study was designed to approximate closely those levels seen in heavy human smokers [17].

In the present study, we used the inferior epigastric vessel pedicled TRAM flap model, which resembles the conventional human TRAM flap [40]. The medication was given from the time of surgery until surviving areas were measured on day 5 to mimic the clinical scenario of a chronic smoker requiring TRAM flap reconstruction. Previous studies investigating the

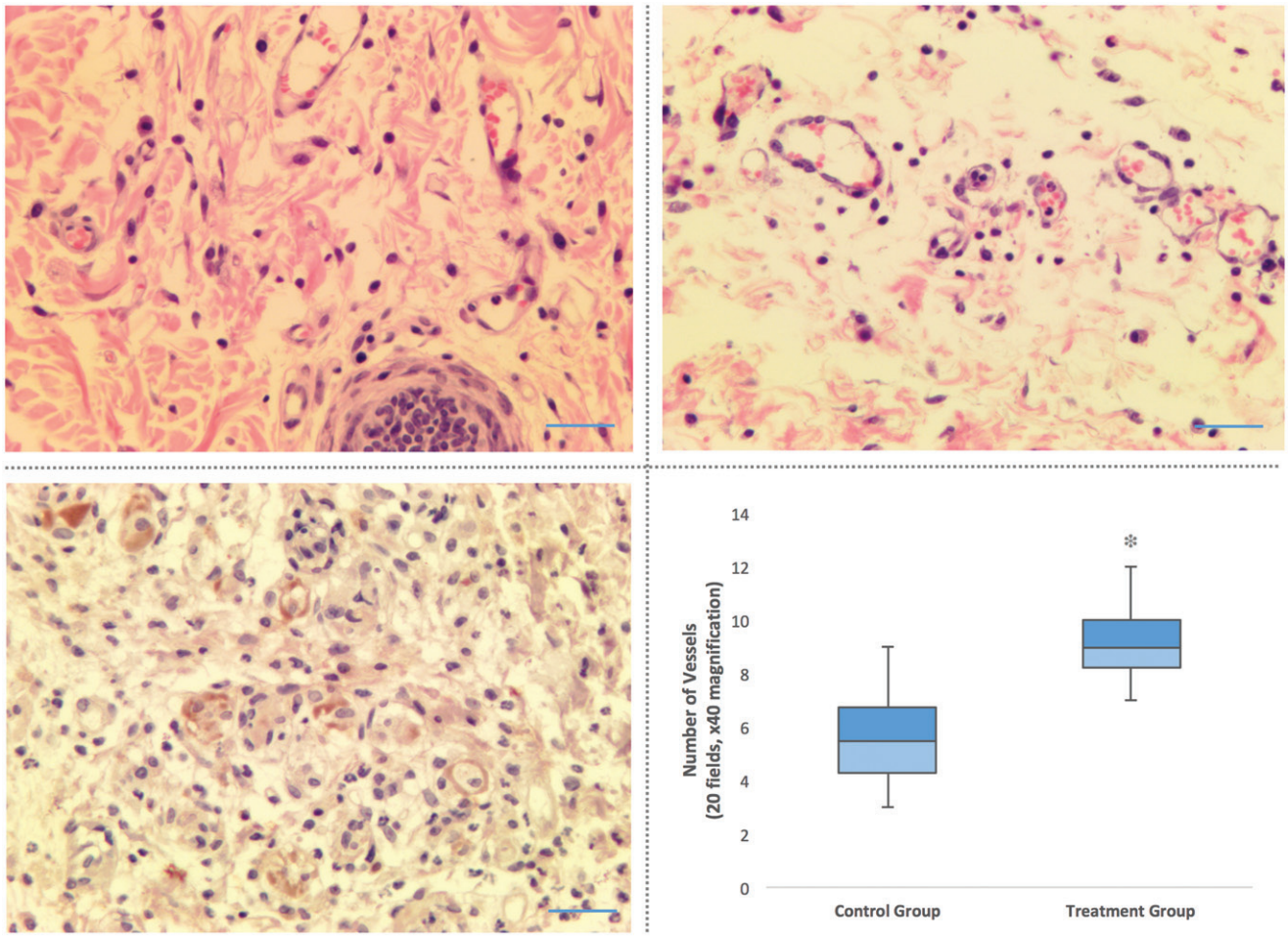

Figure 6. Hematoxylin and eosin staining revealed enhanced microvessel density in tissues obtained from animals of the treatment group (above, right) in comparison with the controls (above, left). Microvessel formation was confirmed by immunostaining with antibodies against vWF (below, left) (×40 magnification). Scales =100 μm. Plot showing the mean number of vessels counted in each group (below, right). The number of vessels is expressed as vessels in 20 different fields under ×40 magnification. The sample size was $n = 10$ for each group. Data was presented as mean ± SD. $^*p < 0.001$.

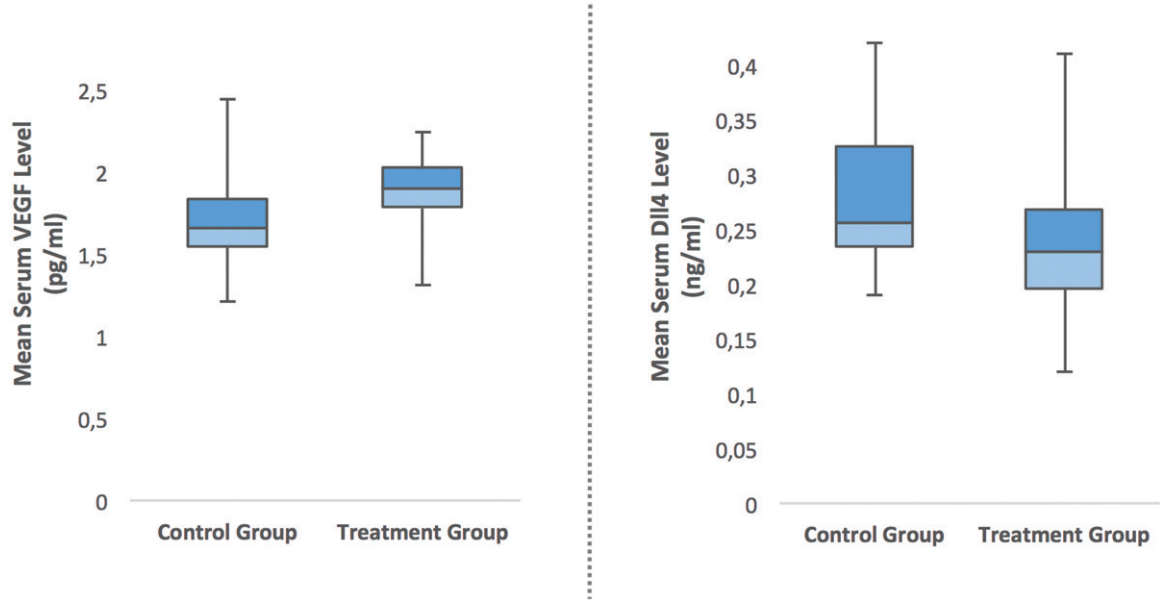

Figure 7. Quantitative analyses of VEGF and Dll4 serum level,s respectively. The sample size was $n = 10$ for each group. Data was presented as mean ± SD.

effects of nicotine on rat TRAM flap survival documented that necrotic areas had been demarcated as early as 48 hours after flap elevation [28,41]. In the current study, postoperative day 5 was chosen for the macroscopic, genetic, and histologic assessments based on the findings of our previous study revealing that maximus Dll4 transcription levels in the skin tissue were reached 5 days after ischaemia [29]. A moderate but statistically significant improvement in flap survival was observed in animals treated with Notch inhibitor. Subsequent immunohistochemical analysis of the skin paddles revealed a significant increase in the number of Dll4 stained vessels. The underlying mechanism of action is totally different from classic proangiogenic therapies that depend on the administration of exogenous proangiogenic growth factors [42]. The blockade of Dll4-mediated Notch signalling promotes flap perfusion by enhancing the angiogenic effects of endogenous VEGF.

An interesting finding of our study is that *VEGF* mRNA levels in the treatment group were significantly lower than those in the control group on day 5. We speculate that this observation is an indication of better tissue perfusion in animals of the treatment group since ischaemia acts as the primary stimulus for VEGF expression and higher degrees of ischaemia are associated with higher levels of expression [43]. In addition, pharmacological inhibition of Notch signalling augments the sprouting effect of VEGF by the elimination of negative feed-back mechanism, without any direct effects on its expression [35]. Another important finding of our study is that Dll4 expression levels were found to be the same in both groups. This situation may be explained by the fact that GSIs exert their function by blocking the activation Notch receptors without any direct inhibitory effect on Dll4 synthesis [44].

We found no significant difference between two groups with regard to circulating Dll4 levels. This may be related to the fact that Dll4 levels in the local flap environment may not reflect the plasma equivalent from as early as the first postoperative day. This is because endothelial cells expressing Dll4 proteins in their membranes activate Notch receptors on neighbouring endothelial cells through juxtacrine mechanism without any paracrine effect [24]. In a previous study by our group, we found that circulating Dll4 levels were significantly higher in rats who underwent flap surgery in comparison with non-operated controls [29]. We think that this elevation is a direct result of upregulated transcription after ischaemia. Because DLL4 ligands exert their function in the zone of ischaemia, we don't believe that free circulating DLL4 ligands have any function in other normoperfused tissues. However, they may provide a negative feed-back mechanism to control Notch pathway in the zone of ischaemia. In many models, soluble DLL4 molecules are used to block Notch function [45,46]. Elevated plasma levels for DLL4 may help in suppressing the transcription of DLL4 genes [47]. However, further studies are needed to determine the functional importance of these free circulating DLL4 proteins.

Previous studies have examined the effects of Notch signalling inhibition on tumoural angiogenesis. Consistent with Notch's anti-angiogenic function, inhibition of the Notch in tumours caused increased vascular density and vascular sprouting [45,48]. Surprisingly, this overgrowth resulted in an inhibitory effect on tumour growth. This is because the neovessels were immature, disorganised, and non-functional. This strategy stimulated interest in exploring the Notch pathway as a potential target for the treatment of malignancies. On the other hand, in non-tumour models of angiogenesis, including flap surgery, and wound healing, Notch signalling inhibition improved recovery from acute vascular ischaemia by promoting a functional vascular proliferation [31,46]. We believe that the non-functional nature of the neo-vessels obtained in tumour tissues after Notch inhibition is associated with the fact that tumour vessels differ structurally and functionally from their normal counterparts by being fragile, leaky, and chaotic [49].

One of the major limitations of our study is that GSIs are non-selective inhibitors of Notch. In addition to the Notch pathway, GSIs prevent the activation of many angiogenic mediators such as erbB-4, CD44, and E-cadherin. Although the inhibition of these mediators would affect the results, many angiogenic functions of the Notch have been documented using only GSIs [50,51].

Conclusion

Taken together, our findings provide evidence that the Notch signalling system is a potential target for angiogenic therapies through its ligand Dll4. Pharmacological inhibition of the Notch significantly improved TRAM flap survival in animals exposed to nicotine by creating a vascular network that has a significant increase in microvascular density. The possible underlying mechanism of action is totally different from classic proangiogenic therapies that depend on the administration of exogenous proangiogenic growth factors. The blockade of Dll4-mediated Notch signalling promotes flap perfusion by enhancing the angiogenic effects of endogenous VEGF. Further studies are needed to determine the safety of Notch inhibition in the clinical scenario.

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Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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