The protective effects of ascorbic acid and Tualang honey on reverse sural fasciocutaneous flaps in a smoking rabbit model




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Smoking is a risk factor for poor wound healing and flap failure due to oxidative damages produced by components in cigarette smoke. Reverse sural fasciocutaneous (RSF) flaps are commonly used for the reconstruction of the distal third of the leg. The authors compared the protective effects of two antioxidant agents: ascorbic acid (AA) and Tualang honey (TH) in improving the survival of RSF flaps in a smoking rabbit model. **Method:** 36 RSF flaps were raised on White New Zealand (Oryctolagus cuniculus) rabbits. The rabbits were divided into 3 groups and all groups were exposed to cigarette smoke, using smoke chambers for 2 hours daily for 4 weeks prior to the flap elevation and then continued for 1 week postoperatively. The control arm had exposure to cigarette smoke without intervention, the AA group was exposed to cigarette smoke along with AA intervention and the TH group was exposed to cigarette smoke along with TH intervention. Intramuscular AA and oral TH were given daily to the respective groups two weeks prior to flap elevation and continued until the end of the study. Temperature, capillary refill time, infection and haematoma were observed on postoperative day 1, 3 and 7. Flap survival was calculated on postoperative day 7 using 2D planimetry. Data were analysed using one-way ANOVA test and $p < 0.05$ was considered as significant. **Results:** The flap survival in the control arm was 62.0%, while in the AA arm it was 88.9% and 81.0% in the TH group ($p = 0.075$, CI 59.5 – 82.7).

Smoking is an established risk factor for poor wound healing and flap reconstruction failure. Cigarette smoke increases the activity of oxygen-derived free radicals leading to endothelial dysfunction (Eskurza et al, 2004). Reduction in nitric oxide production from vascular endothelium and increased oxygen free radicals leads to arterial wall injury, resulting in poor wound healing leading to flap failure (van den Heuvel et al, 2009). Nicotine in cigarette smoke is a potent inflammatory mediator that produces vasoconstriction, leading to irreversible tissue damage (Babayan, 2012). Wounds over the distal third of the leg imposes a great challenge for reconstructive efforts due to the scarcity of soft tissue in this region and the paucity of blood supply. RSF flaps have been extensively used for the reconstruction of distal leg and heel defects due to its ease of flap elevation and constant vascular anatomy (Masquelet, 1992; Akhtar and Hameed, 2006). Various anti-inflammatory agents have been used to reverse oxidative damages with variable success. AA and TH have shown to have high anti-inflammatory property (Bashkaran K et al, 2011; Babayan, 2012; Baynosa and Zamboni, 2012; Ozturk et al, 2012). However, to date there is no similar study that looked at the effects of both these agents in improving the survival of RSF flaps in a smoking rabbit model.

Materials and methods

This animal experimental study was conducted in accordance with the regulation stipulated by laboratory animal study and ethics committee of Universiti Sains Malaysia USM/Animal Ethics Approval/2010/ (54) (176). White New Zealand rabbits (Oryctolagus cuniculus) were used in this experimental study and divided into three groups. The sample size calculated using a free web-based programme "G-Power Version 3.0" from Heinrich-Heine University of Düsseldorf. The power of the study 0.95 and total sample size was 36. All the rabbits were obtained from Laboratory Animal Research Unit, Health Campus, Universiti Sains Malaysia. All

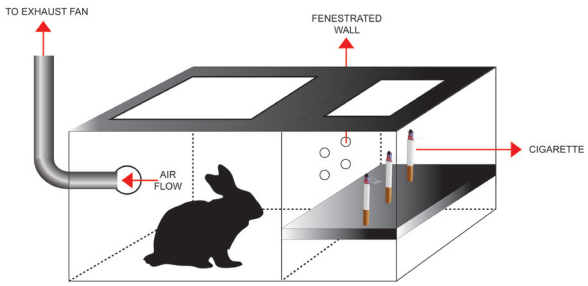

Figure 1. A sketch of the smoke chamber

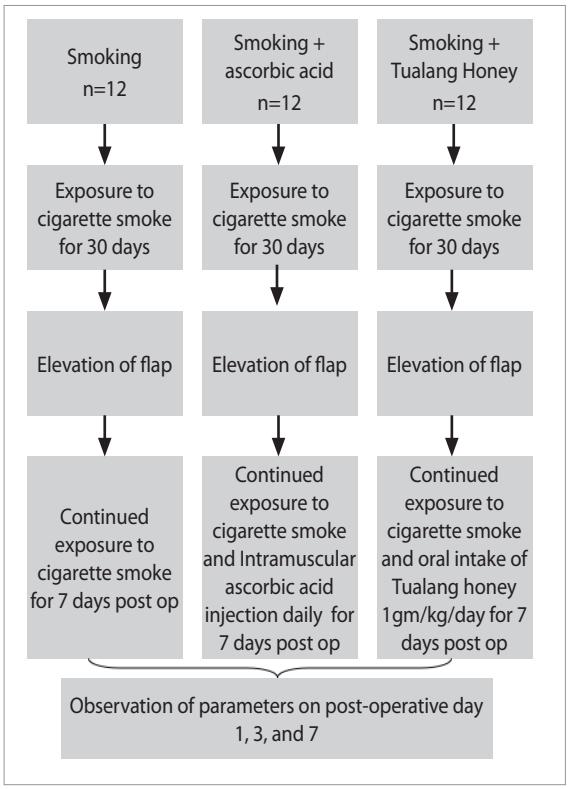

Figure 2. Flowchart of research methodology

the rabbits were housed and cared separately in accordance with the standard of care of laboratory animal.

Preparation of study group

The rabbits were categorized into three arms: the Smoking (control group), Smoking with AA and Smoking with TH arm. Cigarette smoke (John Black @Virginia Blend) exposure took place in specially designed smoke chambers *[Figure 1]* for 2 hours daily for a total of 37 days. Details of research methodology are given in the flow chart below *[Figure 2]*.

Flap elevation

The rabbits were anaesthetised using Ketamine (35 mg/kg) and Xylazine (5 mg/kg) and anaesthesia was maintained using Sevoflurane. A RSF flap was raised based on a technique developed by Chang et al (2003).

Flap assessment

An assessment of flaps was carried out first in the immediate post-operation period followed by days 1, 3 and 7 post flap surgery. Parameters assessed were colour, temperature, evidence of infection and haematoma were documented. Flap survival measured using 2D planimetry. The survival is documented in terms of percentage:

$$\text{FLAP SURVIVAL PERCENTAGE} = \frac{\text{AREA OF SURVIVING FLAP in mm}^2}{\text{TOTAL FLAP AREA in mm}^2} \times 100\%$$

In order to prevent observer bias and data variability, single investigator evaluated the flap tracing, total survival area determination and final survival percentage calculations.

Statistical analyses

Data were analyzed using Independent t test, Fisher exact test, repeated measure ANOVA to study group effect, post hoc comparison using Bonferroni procedure and survival percentage was analyzed with Oneway ANOVA using SPSS version 21 (SPSS Inc, Chicago, Illinois). Differences were considered statistically significant at $p < 0.05$.

Results

The rabbits were divided into three groups and the descriptive statistic are given in *Table 1*. The AA intervened group had a higher mean temperature compared to control and TH arm which was statistically significant, $p = 0.001$ *[Table 2]*. Improved flap perfusion was seen in AA intervened group. The smoking group had the highest flap congestion (91.7%) *[Table 3]*.

The TH arm had less infection rate compared to other groups, however, this was not statistically significant. Comparing haematoma rate among the groups throughout the study was also not statistically significant.

Mean survival was measured on day 7 postoperationly. The smoking only rabbit arm had a mean of 62.0% survival, while AA and TH intervened group had 88.9% and 81.0% mean survival. An improvement of 26.9% and 19.0% as compared to smoking group *[Table 4]*. One way ANOVA test was statistically not significant *[Table 4]*. However, the clinical improvement with regards to temperature, improvement in colour, reduction of haematoma and infection rates was seen in both groups intervened with AA and TH.

Discussion

Wounds over lower limb especially distal third of leg and foot are commonly seen following road traffic accident or complication of diabetes. Diabetes complications having

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Table 1. Descriptive statistic of the study groups

Group	Number (n)	Weight/Mean (SD)	Survival/Mean (SD)
Smoking	12	3.17 ± 0.58 kg	62.0 ± 34.0 %
Smoking and ascorbic acid	12	2.82 ± 0.20 kg	88.9 ± 11.6%
Smoking and Tualang honey	12	2.90 ± 0.23 kg	81.0 ± 39.1%

Table 2. Comparison of average in temperature between three groups

Day	Smoking/ Mean (SD)	Smoking and Ascorbic acid/ Mean (SD)	Smoking and Tualang honey/ Mean (SD)	F stat (df)[a]	*p* Value[a]
0	30.0 (1.55)	32.1 (1.45)	32.3 (1.45)		0.001[b]
1	35.8 (1.24)	36.9 (1.62)	37.3 (0.86)	8.94	
3	35.6 (1.50)	38.1 (0.54)	36.3 (1.25)	(2;33)	
7	35.6 (1.28)	37.3 (0.70)	36.4 (1.65)		

[a] Repeated measure ANOVA (group effect)

[b] Post hoc comparison for each pair of group was done with adjustment for multiple comparison using Bonferroni procedure showed temperature change for the smoking and ascorbic acid arm was statistically significant, $p = 0.001$

Table 3. Association between groups and flap colour on Day 7

Group	Congested frequency (%)	Pink frequency (%)	*p* value[a]
Smoking	11 (91.7)	1 (8.3)	62.0 ± 34.0 %
Smoking and ascorbic acid	2 (16.7)	10 (83.3)	88.9 ± 11.6%
Smoking and Tualang honey	4 (33.3)	8 (66.7)	81.0 ± 39.1%

[a]Chi square test

Table 4. Comparison of survival percentage between groups

Group	Mean (SD)	F Statistic (df)	*p* value[a]
Smoking	62.0 (34.01)		
Smoking and ascorbic acid	88.9 (11.61)	2.81(2;35)	0.075
Smoking and Tualang honey	81.0 (34.09)		

[a] Oneway ANOVA test

an exponential rise in Asian countries with many patients presenting with diabetic foot ulcers. These wounds require soft tissue coverage in the form of flap. Additional challenges are seen with injuries over the distal third of leg due to vascular scarcity and tissue paucity. Due to technical and resource limitation of free tissue transfer, the use of pedicled tissue such as RSF flap has gained popularity (Kneser et al, 2011). Cutaneous blood flow determines the survivability of skin paddle of the flap and it is controlled by myogenic tone, neural innervations, temperature and humoral factors.

Smoking is an established risk factor for poor wound healing and also flap failure. Cigarettes are made up of a cocktail of chemical agents that are carcinogenic and potent vasoconstrictors. Nicotine and carbon monoxide in cigarette smoke produce vasoconstriction that impairs cutaneous circulation through myogenic and humoral factor. Activation of anaerobic metabolism following vasoconstriction starts a cascade of events leading to ischemic injury (Ejaz and Lim, 2006; Babayan, 2012). Resultant oxygen free radicals produce endothelial dysfunction, leading to reduced nitric oxide production, a potent vasodilator produced by endothelial smooth muscles (Xanthoulea et al, 2013). This resulted in stiffer vessels that respond poorly to the metabolic need of the flap and wound healing. Smoking also produces endogenous AA depletion, inflammation, hypercoagulability, and cutaneous vasoconstriction that are all proponent for flap failure (Babayan, 2012; Talbot and Palmer, 2013). Various anti-oxidant agents were studied on their ability to reverse the oxidative damages produced by cigarette smoke resulting in variable success rates (Bashkaran et al, 2011; Bababyan, 2012; Baynosa and Zamboni, 2012; Ozturk et al, 2012). To the authors' knowledge, this is the first study comparing protective effects of AA and TH on flap microcirculation with the ongoing insult. The findings of this study suggested both these agents could overcome the oxidative and vasoconstrictive damage of cigarette smoke as evidenced by the increased survival of RSF flaps from 62% in control arm to 89.9 % in AA group and 81.0 % in TH group. In comparing both the agents, we found AA is more potent and showed better flap survival.

Supplementation with AA invariably provides protection to endothelium against oxidative damages mainly in centrally located large calibre vessels (Lehr, 1997). In this study, similar improvement was being demonstrated in smaller calibre and distally located vessels resulting in improved skin perfusion of RSF flap. It indirectly implies supplementation of exogenous AA has a beneficial effect in remodelling the endothelium of vessels damaged by cigarette smoke.

Impaired cutaneous circulation clinically manifested on the flap as pale colour, lower flap temperature and prolonged capillary refill time. Prolonged insult leads to skin necrosis and flap failure (Sørensen et al, 2009; Sørensen et al, 2010). AA and TH intervention improved flap circulation thus promoting improvement in the clinical parameters. In a reverse flow flap, significant

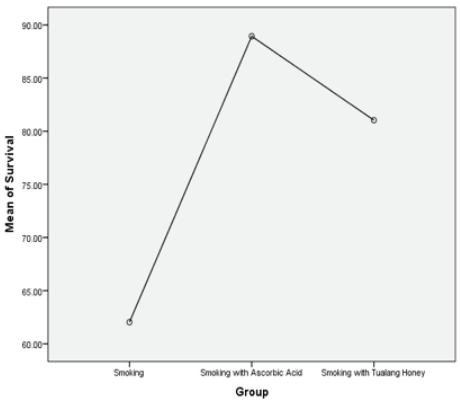

Figure 3. Graph depicting the improvement in mean survival of flap

dilatation of vessels and increased circulation pressure is required to direct the cutaneous flow to perfuse the flap. Intervention with both AA and TH showed improvement in the flap perfusion that is reflected by the improvement in clinical features and finally overall flap survival. AA intervention has a flap temperature nearing core temperature that was statistically significant. This suggests improvement in the flap perfusion, which is supplied by the reverse flow mechanism.

The mean survival of flaps in AA and TH intervened group showed marked improvement compared to control group. Intergroup comparison showed that AA is more potent compared to TH *[Figure 3]* in reversing the constant oxidative damages of cigarette smoke; however, it was statistically not significant. Based our study AA is capable to improve microvascular circulation despite the constant vasoconstriction insult produced by cigarette smoke. Extrapolation of our result to human model needs to take into consideration of inter species anatomy and physiological difference. A dose response study also needed to determine the dosage of TH deemed medically therapeutic. A larger sample with longer follow up may establish a better outcome that may produce statistically significant results. This study lack of histopathologic evidence to demonstrate the microscopic changes to vessel calibre and cellular components upon exposure

Conclusion

Wounds over the distal third of leg and foot impose a great challenge both for reconstructive surgeon and also wound care specialist. Smoking habits in patients with wounds in these regions further complicates the healing and recovery. RSF is a reliable and reproducible reconstructive option for injuries to those areas. This experimental study shows that AA produced significant clinical improvement in RSF flap survival compared to TH in the smoking rabbit model. These findings could provide an interventional option in augmenting the final wound healing outcome in smokers. Although this is an animal model study, the promising preliminary result may pave way to trying these solution model in human subjects. Besides that the authors could eventually save time, cost, reduce donor site morbidity and reduce the need for free tissue transfer. WAS

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