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Use of a Model of a Blood-Induced Bruise for the Evaluation of Formulations on Bruising

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Abstract

Esthetic treatments can induce swelling and bruises. Thus, a treatment that would prevent or hasten the resolution of bruising should be very useful. Generally, the regression of bruising was conducted with patients or animals models. So we decided firstly to develop an *ex vivo* model in order to test antibruising properties of topical formulations and secondly to evaluate a curative effect of a cream (mixture of arnica extract and apigenin) in comparison with a positive control (vulnerary cream) and also to estimate the preventive interest of this cream. The results showed that the injection of 25 µl of blood into the dermis of skin fragments was sufficient to create a model of induced-bruise. The duration of 24 hours was chosen to compare the effects of actives on the decrease in the size of the bruise. Joint effects of a pretreatment and a treatment of a mixture of arnica extract and apigenin decreased significantly the area of bruising compared to the treatment group, the control group and the positive control group. Many topical products claim to improve bruising on their package label. Our model can demonstrate their efficacy and determinate the best topical antibruising formulation. The mechanism involved in anti-inflammatory activity of active compounds of topical formulations is often not fully understood. Our blood-induced model may bring some responses through the study of mediators of the inflammation.

Keywords

Bruise, Apigenin, Arnica, Skin Explants

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1. Introduction

A bruise, also called a contusion or an ecchymosis, happens when a part of the body is struck and the muscle fibers and connective tissue underneath are crushed but the skin doesn't break. Bruises take days or weeks to resolve and they can be a form of temporary disfigurement. When this occurs, blood from the ruptured capillaries near the skin's surface escapes by leaking out under the skin. With no place to go, the blood gets trapped, forming a red or purplish mark that is tender to the touch. Firstly, it is kind of reddish. The red initial color of the bruise is the product of the human's natural skin pigmentation, the color of the pigments in the extravasated blood, and any color added by the inflammatory reaction (e.g. redness due to vasodilatation), but the color of the bruise changes over time. After the shock, the red color becomes bluish black. Then after a few days, it becomes rather green (after 4 to 7 days), yellow or brown (after 7 days) [1]. The destruction of red blood cells releases a pigment which gives its red color to blood, hemoglobin, whose different stages of degradation in the skin explain the different colors of the bruise [2]. Hemoglobin consists of four globins. Each globin encloses a heme (which contains iron and a binding site for oxygen), hence its name. Each heme is degraded into biliverdin (green/blue pigment), and bilirubin (yellow/brown pigment) by enzymes present in monocytes and macrophages. Both pigments are poorly soluble and therefore difficult to remove from the skin to the blood plasma, which explains the persistence of the color for several days in the skin. Published studies measuring the regression of bruising, were generally conducted with patients who have undergone surgery in order to demonstrate a preventive or a curative effect of oral or topical medication [3].

Few techniques are available for the assessment of actives on bruising. Animal models are commonly used to screen conventional non-steroidal antiinflammatory drug based on the use of carrageenan or arachidonic acid. An edema is induced by injection of carrageenan or arachidonic acid into the sole of the right posterior paw of rats [4] [5]. This model has also frequently been used to assess the *in vivo* anti-edematous effect of natural products [6].

Another model consists of the injection of autologous blood, which mimics a traumatic condition into the joint (typical of common sprains and bruises) and the development of inflammation lasting for a few hours. This model was used to test the power of homeopathic remedies [7] [8]. Our study was based on experiments performed by Lussignoli *et al.*, who induced an edema in rat paw by injection of blood made uncoagulable by the addition of heparin [7] [8].

The purpose of this study was 1) to create a model of bruising with fresh skin fragments maintained in survival conditions (*ex vivo* model) in order to be closer to *in vivo* conditions, mimicking the extravasated blood, and 2) to evaluate a curative effect of a cream (mixture of arnica extract and apigenin) in comparison with a positive control (vulnerary cream) and also to estimate the preventive interest of this mixture. In the present study, apigenin, flavonoid abundant in various vegetables and grapefruits, has been associated to arnica extract because it has been reported to possess anti-inflammatory effect [9] [10].

2. Materials and Methods

Skin explants obtained from discarded tissues (excised female abdominal skin) of eight healthy female patients (37 to 53 years old) were used in order to create a model of induced-bruise and to compare the action of creams on the evolution of bruising. Patients had given their written consents. Blood samples, made uncoagulable by the addition of heparin, were given by French Blood Agency (EFS, Besançon, France).

2.1. Creation of an *ex Vivo* Model of Induced-Bruises

Five skin explants were used to create this model. The skin surface was cleaned with bidistilled water. Skin fragments were maintained in survival conditions in phosphate buffer at 37˚C for 48 hours. Several volumes of blood (25, 150 and 500 µl) were injected into the dermis of skin explants (n = 8) with insulin syringes. Then the skin surface was massaged manually with moderate pressure for 20 seconds. The evolution of the area of the bruise was monitored for 48 hours. A rule was deposited on the bruised skin and photographs were taken immediately after the induction of the bruise (T0) and after 17, 24 and 48 hours. Pictures were analyzed and the area of bruising was calculated thanks to Axio Viso Rel 4.6 (Carl Zeiss) software. Data of each area was given in $pixel^2$ and was processed into cm^2 using a ruler as shown in **Figure 1**. The variation of the bruise (cm^2) at different times (x) was calculated as following (Tx − T0).

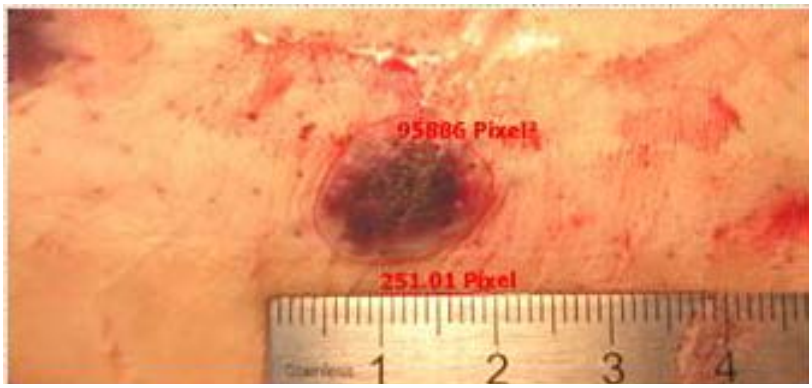

Figure 1. Determination of the area of the bruise in pixel^2 after injection of 25 µl of blood into the dermis of skin explants maintained in survival conditions.

2.2. Comparison of the Effects of Vulnerary Cream and a Mixture of Arnica and Apigenin

2.2.1. Tested Product

Vulnerary cream was used as positive control, it was composed of vulnerary alcoholate (10%) and aluminum acetate. The tested product was a mixture of arnica extract and apigenin (Cicabio Arnica+, Laboratoire Bioderma, France).

A total of 3 skin explants was used for these comparisons firstly as a treatment and secondly as the joint effects of a pretreatment and a treatment. Each of the 3 skin explants were cut into 4 fragments for the comparison of the two types of treatment.

a) For control group with no cream: the bruises were massaged manually for 20 seconds.

b) For control positive group: the bruise was massaged manually for 20 seconds with 2 mg/cm^2 of positive control after the injection of blood.

2.2.2. For Tested Mixture (Arnica Extract + Apigenin), Two Groups Were Studied

c) For the treatment group: the bruise was massaged manually for 20 seconds with 2 mg/cm^2 of tested mixture after the injection of blood.

d) For (pretreatment + treatment) group: the skin surface was massaged manually for 20 seconds with 2 mg/cm^2 of the tested mixture, two hours before the induction of the bruise. Then, 2 mg/cm^2 of the same tested mixture was again applied to the skin surface after the induction of the bruise and the skin surface was also massaged manually for 20 seconds.

Photographs were taken immediately after the induction of the bruise (T0) and after 24 hours.

2.3. Statistical Analysis

Data were expressed as mean ± standard deviation (SD). Groups were compared by using one way (time) or two ways (blood volume and time) variance analysis followed if necessary by Fisher test. Values were considered significantly different when $p < 0.05$.

3. Results

3.1. *Ex Vivo* Model of Induced-Bruises

3.1.1. Determination of the Volume of Blood Injection

Bruises were induced using an injection of blood into the dermis of skin fragments maintained in survival conditions. This model was used in order to observe the resolution of bruises in the presence of topical formulations. The first results showed that the injection of 25 µl of blood into the dermis of five skin fragments was sufficient to induce a significant increase in the size of a bruise after 24 hours in comparison to T0 value ($p < 0.001$). Indeed no significant difference in the increase of the size of bruises was observed between injections of 25 µl, 150 µl or 500 µl of blood. The volume of 25 µl of injected blood was chosen for the following experiments. In each case, the size of bruises increased significantly after 4, 8 and 24 hours (**Table 1**).

3.1.2. Evaluation of the Duration of the Experiment after the Injection of 25 µl of Blood

The size variation of bruises after an injection of 25 µl was compared at several times (T = 0, 17, 24 and 48 hours) on five skin explants. The size of the bruise was stable at T = 24 hours and tended to decrease after 48 hours (**Table 2**) so the duration of 24 hours was chosen to compare the effects of actives on the decrease in the size of the bruise.

3.2. Effects of a Mixture of Arnica and Apigenin. Evaluation of the Joint Effects of a Pretreatment and a Treatment

As the variation of the size of the bruise in cm^2 after injections of blood in the control group depends on each skin fragments of patients, the percentage of area variations was calculated to normalize the results. In the control group, a significant increase in the size of bruises (3 times greater than the initial size) was observed after 24 hours ($p < 0.001$, **Figure 2**, **Table 2**).

The positive control showed a significant decrease (>30%) in the variation of the size of bruising (T24-T0) compared to control group ($p < 0.001$, **Table 3**).

Treatment (>40%, $p < 0.001$) or the joint effects of a pretreatment and a treatment with a mixture of arnica extract and apigenin decreased the area of induced-bruises (>55%, $p < 0.001$) significantly compared to the control group (**Table 3**). No significant difference was observed between treatments with the positive control and the mixture of arnica extract and apigenin. It was observed that joint effects of a pretreatment and a treatment of a mixture of arnica extract and apigenin decreased significantly the area of bruising compared to the treatment group ($p < 0.05$), the control group ($p < 0.001$) and the positive control group ($p < 0.01$) (**Figure 3**).

Table 1. Evolution of the area of bruises, according to the time after injection of 25 µl, 150 µl or 500 µl of blood into the dermis of skin explants maintained in survival conditions. In each group, 8 bruises were induced by the injection of the amount of blood. The evolution of the area of the bruise was monitored for 24 hours. The variation of the size of the bruise in cm^2 after injections of blood at different times (x) was calculated as following (Tx − T0). $^{**}p < 0.01$ and $^{***}p < 0.001$ versus T0. $^{a}p < 0.05$ and $^{aaa}p < 0.001$ versus 25 µl.

Volume of blood injection	Area of bruises (% in comparison with T0)			
	T0	T4 hours	T8 hours	T24 hours
25 µl	100.0 ± 0.0	176.5 ± 8.3**	212.1 ± 15.3***	339.8 ± 35.5***
150 µl	100.0 ± 0.0	144.4 ± 14.5	184.4 ± 19.8**	224.2 ± 25.2***
500 µl	100.0 ± 0.0	138.7 ± 6.3	158.1 ± 8.7^{a}	203.9 ± 7.6^{***,aaa}

Table 2. Variation of the area of bruises (Tx − T0) after injection of 25 µl of blood into the dermis of skin explants. X = 0, 17, 24 or 48 hours ($^{**}p < 0.01$ and $^{***}p < 0.001$ versus T0; $^{a}p < 0.05$ and $^{aaa}p < 0.001$ versus 25 µl).

Area of bruises [variation of bruise areas (Tx − T0) in cm^2]			
T0	T17h	T24h	T48h
0.00 ± 0.00	0.28 ± 0.10	0.34 ± 0.13***	0.23 ± 0.11

Table 3. Measurements of the variation (T24h-T0h) of bruise areas (expressed as %/control) in the presence of tested mixture ($^{aa}p < 0.01$ versus positive control group; $^{b}p < 0.01$ versus treatment group; $^{***}p < 0.001$ versus control group).

Groups	Treatment	Variation of bruise area (%/control)	
		Mean	SD
Control	**None**	100.0	0.0
Positive control	**Treatment**	65.54***	2.4
Tested mixture (arnica extract + apigenin)	**Treatment**	59.87***	3.1
	Pretreatment + treatment	44.4^{***,aa,b}	8.2

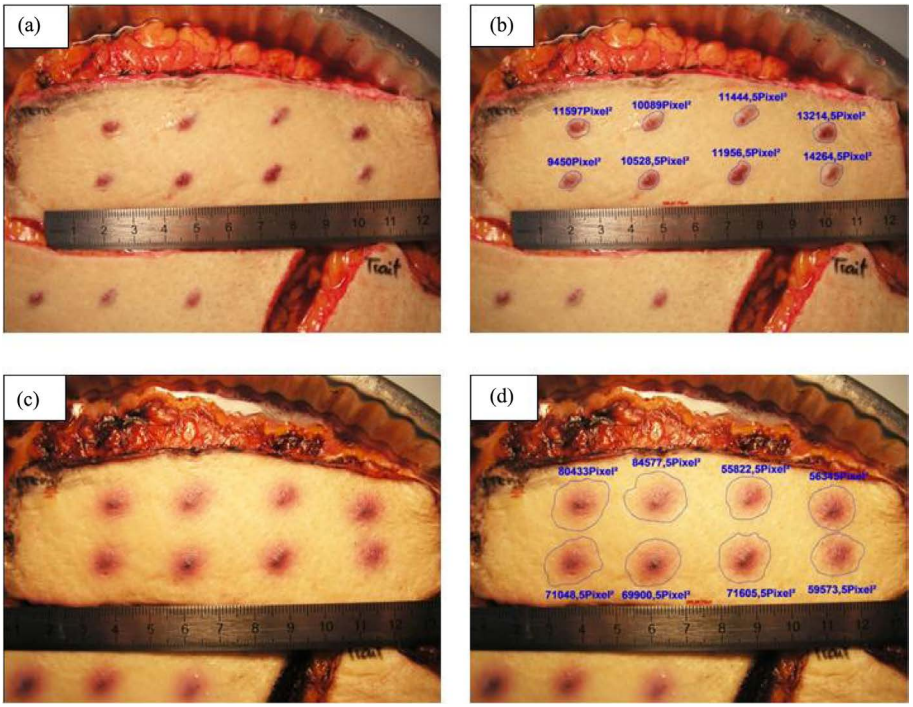

Figure 2. Measurement of the bruise areas in the control group. 25 µl of blood were injected into the dermis of skin explants maintained in survival conditions. (a) and (b) pictures at T = 0; (c) and (d) pictures at T = 24 h.

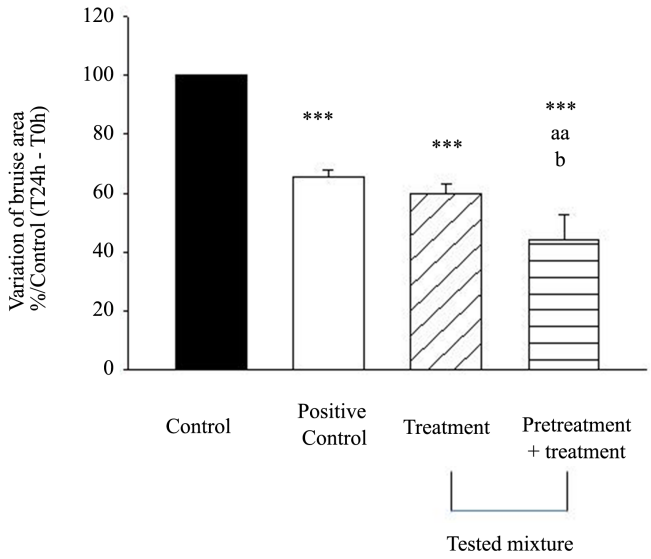

Figure 3. Variation of bruise areas (T24h-T0h) (expressed as %/control) in the presence or not of a pretreatment or a treatment with a mixture of arnica extract and apigenin. Comparison with positive control group. $^{***}p < 0.001$ versus control group; $^{aa}p < 0.01$ versus positive control group; $^{b}p < 0.05$ versus treatment group.

4. Discussion

The aim of the present study was to develop an *ex vivo* model in order to test antibruising properties of topical formulations. Up to now, the regression of bruising, was conducted with patients or animals models. In this work, an *ex vivo* model of human induced-bruises using human skin explants was used in order to be the closest to *in vivo* conditions and to screen different topical creams. Amongst potentially suitable surrogates for *in vivo* clinical tests and studies of bioequivalence of topical products, there are *in vitro* dermatopharmacokinetics me-

thods. The Franz cells system and *ex vivo* cutaneous microdialysis use respectively, dermal and epidermal layers of frozen skin fragments and a fresh fragment of the whole skin (skin explants) maintained in survival conditions in which the rate and extent of permeation through *ex vivo* skin, is measured [11] [12]. Previous studies showed that fresh skin explants maintained in survival conditions are useful for the *ex vivo* cutaneous absorption assessment of an active from a formulation [13] or the screening of the effects of topical corticosteroids [14].

It is why we decided to use fresh skin explants maintained in survival conditions as those used in microdialysis method in order to be closer to *in vivo* conditions. The results showed that the use of skin explants maintained in survival conditions allowed the observation of a decrease in the area of bruises by topical application of actives, 24 hours after the bruise induction by the injection of 25 µl of blood. Our model mimic the extravasated blood observed during bruises. Esthetic treatments can induce swelling and bruises. Thus, a treatment that would prevent or hasten the resolution of bruising should be very useful. The development of this *ex vivo* induced-bruises is useful to show and compare the efficiency of topical formulations. Even though that blood flow is not present, we can follow the decrease in the size of blood induced-bruises and evaluate the direct action of an active on bruises formation. Mixtures of plants are usually used to treat bruises such as those met in vulnerary cream (our positive control). Arnica is an herbal product, frequently recommended by homeopaths. Alcoholic preparations from flowers of *Arnica montana* are applied externally to treat bruises, contusions, sprains, rheumatic diseases and inflammations of the skin surface in traditional medicine [15]. In commercial products, oily extracts from *Arnica montana* flowers are also used as ointments and gel formulation [16]. Active components in arnica extract are sesquiterpene lactones that are known to reduce inflammation and decrease pain. Anti-inflammatory effects also are attributed to helenalin, whose actions include a marked antiedemic effect.

In the present study, we tested an association of apigenin to arnica extract because it has been reported that apigenin possesses anti-inflammatory effect. Apigenin is a flavonoid abundant in various vegetables and grapefruits.

The anti-inflammatory properties of apigenin are at different levels: at the cellular level, it reduces the infiltration of inflammatory cells *in vivo* [9] [10]. At the molecular level, apigenin inhibits the secretion of pro-inflammatory cytokines TNF α (tumor necrosis factor α) and IL-6 (interleukin-6) *in vivo* [17] and the inflammatory cascade at various stages: in macrophages through the inhibition of phospholipase A2 [18]. Apigenin inhibits the release of prostaglandins, the secretion of arachidonic acid, cyclooxygenase-2 (COX-2) (also in the keratinocytes) [19].

Some studies have demonstrated that apigenin could be also effective in the treatment of skin inflammatory process induced by free radicals [20]-[23].

Indeed apigenin inhibits the enzyme iNOS (nitric oxide synthase), responsible for the synthesis of free radical NO (vasodilator and inflammatory mediator) [24] [25].

The present study showed that a treatment with a mixture of arnica extract and apigenin decreased the area of induced-bruises significantly compared to the control group. A significant difference was also observed between a treatment including a pretreatment compared to the control bruise, the treatment with positive control or only a treatment with the tested mixture. A pretreatment with an arnica extract and apigenin linked with a follow-up a treatment may obtain optimal reduction of side effects of esthetic treatments such as bruises. Our results show that the mixture of arnica extract and apigenin promotes the natural biological mechanism of the absorption of bruises. The mechanism involved in anti-inflammatory activity of active compounds of topical formulations is often not fully understood. It is also hypothesized that apigenin through the induction of the expression of UGT1A1 in the skin might play an important role in the decrease in the size of induced-bruises. Even though the blood flow is not preserved in this *ex vivo* model, blood vessels are still present and apigenin might decrease the size of the bruise perhaps through the elimination of bilirubin after the induction of skin UGT1A1 and mostly when apigenin is used in pretreatment. However further experiments should be done in order to bring some responses through the study of mediators of the inflammation such as dosages of PGE_2, IL-1, IL-6, TNF α or UGT1A1 expression in the dermis. Indeed using *ex vivo* cutaneous microdialysis technique, an evaluation of inflammatory mediators or UGT1A1 expression could be done in the dermis dialysates collected after the induction of bruises in the presence or not of apigenin.

5. Conclusion

In conclusion, many topical products claim to improve bruising on their package label. Our *ex vivo* model can

demonstrate their efficacy and determinate the best topical antibruising formulation through the measurement of the decrease in the area of the induced-bruises.

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