Study of the cytotoxicity and evaluation on wound healing of K Ceutic

B. Hannand[1], F. Bouffard[2], Paul Richard[3], Clotilde Quelen[4]
1. Study Director 2. Quality Assurance Unit 3. Head Manager 4. Scientific Department

CYTOTOXICITY
Determination of cytotoxicity: Direct contact test

Aim and principle of the study

This study has the following objective:

Assessment of the cytotoxicity in **Direct Contact Test** using a protocol for testing biomaterialcytotoxicity, according to ISO 10993-5 (or EN 30993-05).

Type system

Human dermal fibroblasts (7th passage) free from mycoplasma, were used.

Methodology

Direct contact test

- Firstly, cells are seeded in a multiwell plates of 6 wells, with 35 mm in diameter, at the starting density of 30 x 10^3cells/cm^2 ; the culture medium (CM) is IMDM (Iscove's Modified Dulbecco's Medium) (Gibco) supplemented with 5 % (v/v) Fetal Calf Serum (FCS) (Gibco) and 0.2 % (v/v) antibiotics.
- Cultures are incubated at 37°C in a humidified atmos-phere containing 5 % (v/v) C02, for 24 hours.
- Then the cultures are examined with a microscope to verify that cells constitute a subconfluent monolayer and that their morphology is not altered. Culture medium is withdrawn and discarded.
- After that, fresh medium is added to each well.

Preparation of test samples

We set up 3 series with 3 replicates each one:

A- One serie of the test sample: 30mg, are spread in sterile conditions onto a plastic discs of 13 mm in diameter for cell culture (Thermanox).
B- One serie of negative control material that is a plastic disc of 13 mm in diameter for cell culture (Thermanox).
C- One serie of positive control material that is BSI disc of 13 mm in diameter of tin stabilized PVc.
Test sample is carefully placed in the centre of each replicate well, covering 1.3 cm^2 i.e. 13 % of the cell layer surface area (~ 10 cm^2) Negative and positive control are treated in the same way.
Then wells are incubated at 37° C in a humidified atmosphere containing 5 % (v/v) CO_2 over a 24 hour period.

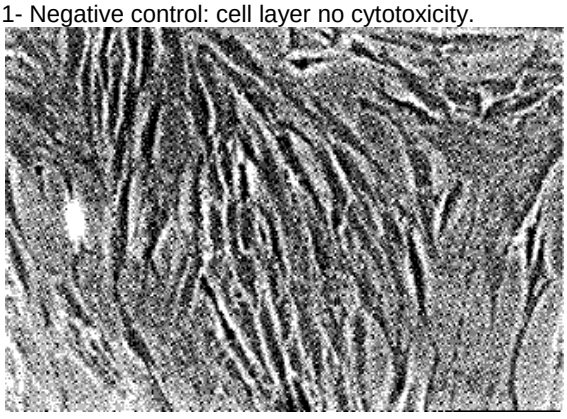
Cytotoxicity evaluation

At the end of the incubation period cells are examined microscopically. Thereafter the test substance is carefully removed, culture medium is withdrawn and discarded.
Cells are detached using 0.2 % (w/v) Trypsin in Hank's balanced salt solution Ca^{2+} and Mg^{2+} free, and incubated for 2 minutes with Trypan blue 0.2 % (w/v) in 0.15 M NaCI.
Living cells (uncoloured) are counted using a hemocytometer.

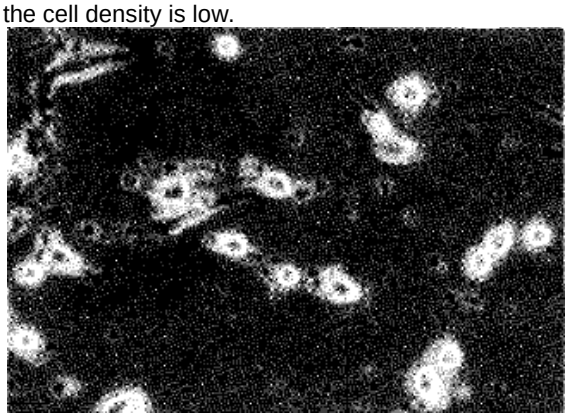
Results and interpretation

Qualitative results, taking into account the photographies (x320), **show the morphology of the culture corresponding to:**

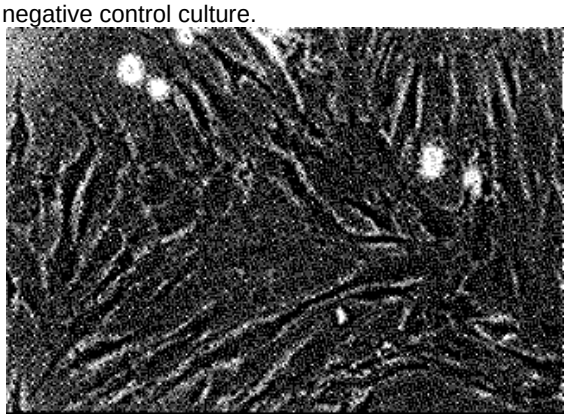
1- Negative control: cell layer no cytotoxicity.



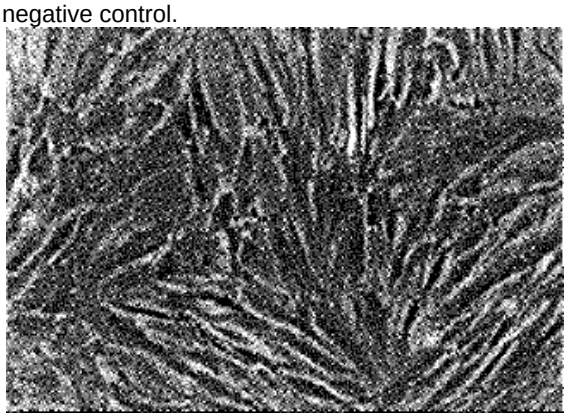
2- Positive control: the cell morphology is very affected. Cells are not spread, they are rounded and the cell density is low.



3- Cell layer under the K Ceutic: his morphology is unchanged; cell density is slightly lower than negative control culture.



4- Cell layer in the vicinity of the test substance: morphology and density are comparable to those of negative control.



Quantitative results are presented in tables 1A (raw data) and 1B (mean +/-SEM)

Statistical analysis is performed using the Student's test. The P values that are less than 0.05 are considered statistically significant.

- Comparison of cell densities of the test substance culture, negative and absolute negative control show a statistically significant decrease in the cell density of the cultures in contact with the test substance: - 8 %, $P < 0.01$.

- This decrease has no biological relevance, taking into account the morphology of the culture, especially under the test substance sample (photography 3).

- Positive control (- 66 % ($P < 0.001$) versus negative control) induces a cytotoxic effect which quotation is 4 on a scale from 0 to 5.

- Because of we can assure that the K Ceutic is not cytotoxic in Direct Contact Test with human dermal fibroblasts.

Table 1A

Series	Cell number (cell/cm²)
K Ceutic	92.500
	100.500
	98.500
Positive control	46.500
	48.000
	45.000
Negative control	100.000
	108.000
	108.000

Table 1B

Series	Cell/cm²	%*	P
K Ceutic	97.165 +- 4.165	92	<0,01
Positive control	46.500 +- 1.500	44	<0,001
Negative control	105.335 +- 4.620	100	

**versus negative control NS: Non statistically significant (P>=0,05)*

WOUND HEALING
"IN VITRO" study of the effect of K Ceutic on wound healing

Aim and principle of the study

We used an "in vitro" model of wound healing that is named *"Scratch Test",* the study is based on both qualitative and quantitative evaluation of the effect of the test substance on the wound closing through cell proliferation and migration.

A wound is mechanically created in a confluent monolayer of human dermal fibroblasts.

Type system

Human dermal fibroblast (foreskin) (7^{th} passage) checked free from mycoplasma, are used.

Methodology

Wound healing model establishment

- Firstly, fibroblasts are seeded in a culture medium of IMDM supplemented with 10 % (v/v) FCS, in a multiwall culture plates of 6 wells with 35 mm in diameter, at the starting density of 35 x 10^3cells/cm².
- The well's centre is covered with a plastic disc of 13 mm in diameter, for cell culture (Thermanox) and incubated 24 hours in a humidified atmosphere containing 5 % (v/v) C02.
- Then, cell layers are rinsed (1 x) with culture medium. Thereafter cells are incubated for 18 hours in serum-free culture medium.
- Cell morphology is checked by microscope examination. Discs are carefully removed. The few cells that migrated under the disc are eliminated using a scrapper and then the cell layer is washed twice with IMDM in order to remove loosened debris before incubation with the test substance.

Preparation of the test substance and series definition

We arranged 3 different series with 3 wells for each one:

A- One serie of absolute negative control: culture medium (CM).

B- One serie of positive control: rhPDGF-AB 20 ng/mL.

C- 30 mg of the K Ceutic are spread onto the wounded part of the CM. The CM is IMDM supplemented with 2 % (v/v) FCS and 0.2 % (v/v) antibiotics.

Wound repair assessment

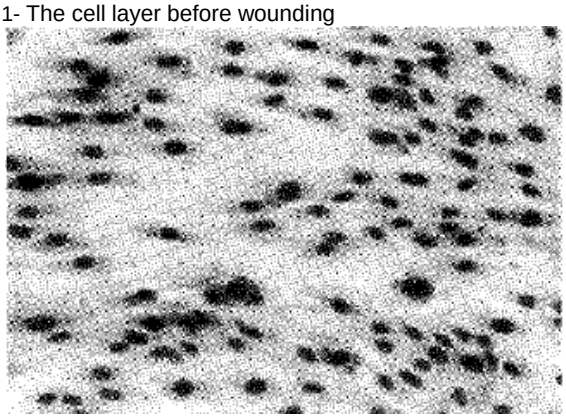
Series are incubated for 48 h. at 37° C in a humidified atmosphere containing 5% (v/v) $C0_2$.
At the end of incubation period, medium is discarded, and the cells washed twice with PBS. The cells are fixed with methanol and stained with 0,4% (v/v) Giemsa.

Results and interpretation

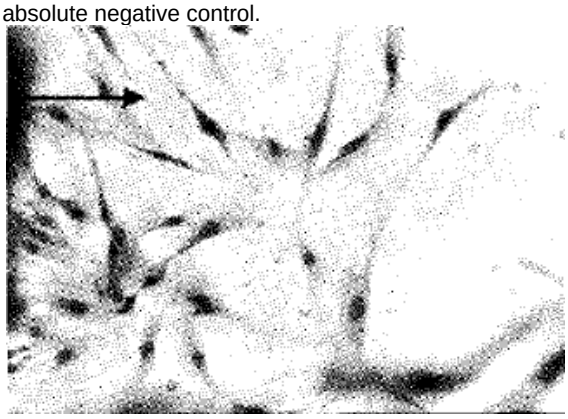
The cell number in the "wounded area" covered with the test substance is counted in 10 consecutive microscope fields (x 200) in each culture well. Results are expressed as mean ± SD. Statistical significance of differences between mean values are assessed with the Student's t test. A P value < 0.05 is used to indicate statistical significance.

Qualitative results are presented in different photomicrographs 1-4 (x200):

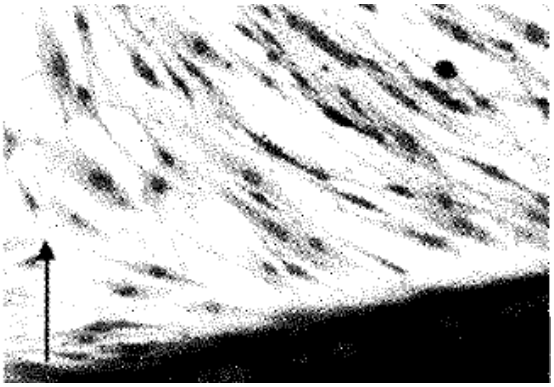
1- The cell layer before wounding



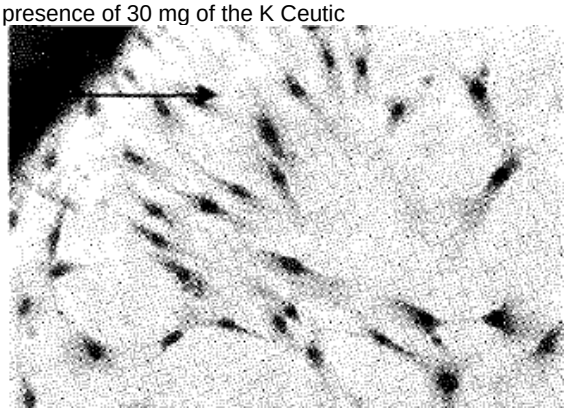
2- The fibroblast migration 48 h after wounding in the absolute negative control.



3- The fibroblast migration 48 h after wounding in the positive control



4- The fibroblast migration 48 h after wounding in the presence of 30 mg of the K Ceutic



Migration direction

Quantitative results are presented in table 1 and figure 1, we can appreciate that in:

A- The absolute negative control, the migration is of 42 cells/field into the wound area.

B- The positive control, the migration has increased to the wound area by 137 % ($P < 0.001$) compared with negative control. This result validates the wound healing model used in this experiment.

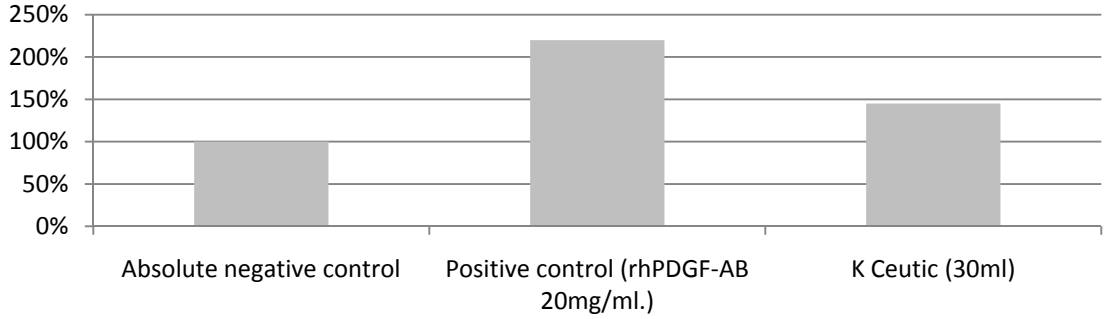
K Ceutic has a significant increase in cell migration in an attempt to fill the wound area: +41%, ($P<0.01$).

Table 1

Series	Absolute negative control	Positive control	K Ceutic (30ml)
Culture #1	42,1	97,9	60,2
Culture #2	42,2	99,4	56,2
Culture #3	40,8	99,2	60,2
Cell number / Field	41,7 +- 0,8	98,8 +- 0,8	58,9 +- 2,3
% Increase**	-	137	41
P***	-	<0,001	<0,01

** Mean of 10 microscope fields/culture well (x200)*
*** Percentage increase versus absolute negative control*
**** Statistical significance, not statistically significant if P>=0,05*

Figure 1



Conclusions

According to the experimental conditions adopted and taking into the grading scale established by the investigator centre, the product K Ceutic:

A- It is no cytotoxic, in Direct Contact Test ,with human dermal fibroblasts in the framework of ISO 10993-5 ("Biological evaluation of medical devices - Tests for cytotoxicity: in vitro methods").

B- Increased significantly human dermal fibroblast migration in a wound healing model (scratch test): + 41 % with the concentration of 30 mg/well.

Bibliography

Fujiwara Y, Kaji T, Sakurai S., Sakamoto M., Kozuka H. "Inhibitory effect of lead on the repair of wounded monolayers of cultured vascular endothelial cells". Toxicology fi: 193-198 (1997).

Oates T.W., Mumford J.H., Cames D.L., Cochran D.L. "Characterization of proliferation and cellular wound fill in periodontal cells using an in vitro wound model".

J. Periodontol 72: 324-330 (2001).

Mumford J.H., Cames D.L., Cochran D.L., Oates TW. "The effects of platelet-derived growth factor-BB on periodontal cells in an in vitro wound model".

J. Periodontol 72: 331-340 (2001).

Rieck P.w., Cholidis S., Hartmann Ch. "Intracellular signalling pathway of FGF-2- modulated comeal endothelial cell migration during wound healing in vitro".

Exp. Eye Res. 73: 639-650 (2001).

Mohajer B., Tamawksi A., Hoa N.T, Tran D., Chen J., Park H., Ma T.Y
"Low dose ethanol potentiates indomethacin induced inhibition of wound re-epithelialization in duodenal monolayers".
LifeSciences 70: 3143-3153(2002).