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Confidential

Report: In Vitro Clinical Study

Dosification of Collagen Neo-synthesis

Study of product effects on the production of collagen neo-synthesis in human fibroblasts.

Product being investigated:	Organic Silica (ref: Silicium G5)
Spanish I.E.C. Code:	E080043 130003
Experiment Protocol:	No. E080043PE, of February 26, 2008
Study:	No. E080043RD, of March 31, 2008
Commencement of Observations:	March 5, 2008
Conclusion of Observations:	March 21, 2008

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9 Page Document

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E u r o T e s t

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Study of the collagen synthesis in human fibroblasts culture after treatment by the product

*Study Code	Bio. IEC. 01/2008
* Report Date	March 28th 2008

Product: Organic Silica G5

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Authentication

This study, object of the present study has been conducted under my responsibility, conforms with the experiment protocol, and in accordance with the best practices of the laboratory. All of the observations and numerical data compiled during this trial are reflected in the present document.

After a second reading, I certify this data conforms with the actual results obtained.

Mr. R. ENNAMANY, Director of the Study

I certify I have reread this study and I am in agreement with the contents.

Mr. R. ANAME, Director of Quality Control

1. INTRODUCTION

At the request of the INSTITUT D'EXPERTISE CLINQUE – ESPAGNE, we have evaluated the effect of the product Organic Silica G5 in the following parameters:

- Cytoxicity in human fibroblasts
- Synthesis of collagens in human fibroblasts
-

2. EXPERIMENT PROTOCOL

2.1 Human fibroblast cultures

The fibroblast cultures are derived from human foreskin. They were placed in culture medium RPMI 1640 which contained fetal bovine serum, L-glutamine and gentamicin. The evaluation was made between the 2^{nd} and 4^{th} attempts, with the final to ensure reproducibility of the different experiments. The fibroblasts were cultivated to 10^6 cells per ml en a beaker of 25 cm^2, and later, were incubated for 24 hours.

2.2 Evaluation of cytoxicity

The objective of the first stage was to find the cytoxicity of the product with respect to the human fibroblasts in the culture. Cellular multiplication was performed to 3 concentrations with no toxicity.

2.2.1 Beginning

Tetrazolium salt (MTT) has the ability to reduce to blue formazon crystals by the succinate dehydrogenase of the mitochondrial cells. This enzyme, which plays an important role in Krebs cycle, catalyzes the dehydrogenation of succinate to fumarate. It is the activity of this enzyme, a flavoprotein very closely related to the mitochondrial inner membrane, which will be measured by the reduction of MTT. By a spectrophotometric test, it is possible to determine the toxicity of a particular cell population and, in fact, the absorbance will be directly related to succinate dehydrogenase activity, associated with cellular toxicity.

2.2.2 Distribution of Lots

The tests were performed by triplicate after 24 hours of treatment.

-Lot 1: Untreated negative control

-Lot 2: Tested with Orgono Silica G5 (0.2%)

-Lot 3: Tested with Orgono Silica G5 (.5%)

-Lot 4: Tested with Orgono Silica G5 (1%)

2.2.3 Evaluation of cytoxicity

After 24 hours of incubation of cells with different concentrations of the product to study, these are put in contact with MTT for 3 hours at 37C, after breaking with DMSO. The optical density at 570 nm was determined using a spectrophotometer after homogenization of the coloration.

2.3 Product effects on collagen neo-synthesis on human fibroblasts

After incubation, the fibroblasts were utilized after centrifugation. The cell was digested by collagenesa (1mg/cell depth) in acetic acid at 0.5ml/L for 24 hours at 4°C. After centrifugation at 10000g, the collagen is precipitated with 1M sodium chloride; the precipitate was suspended and later dialyzed. Primary amino acids are derived by phthalaldehyde acid thus eliminating possible interference. The hydroxyproline and proline are derived by the NBD-CL by coupling the amino groups. The NBD-Hyp is Identified by inverse phase HPLC.

Hydroxyproline is metered by measuring the fluorescence after separation by reverse phase HPLC.

- Automatic injection.
- Ultrasep C18 Column (30cm x 018cm),
- 6µ Porosity
- Flourescence detector

The mobile phase, consists of a mixture of acetonitrile / sodium phosphate buffer 0.1mol/1 with pH 7.1(9:91 v.v), the flow is regulated at 1ml/min, the action is done in static mode and the cycle is 10 minutes. The mobile phase is initially filtered and later degassed before use.

The solutions are prepared in the following manner:

- NBD-CI :24mmol dissolved in the methanol,
- OPA : 150mmol/l dissolved in the methanol,
- Phosphate Buffer : 0.4mmol.l, pH adjusted to 7.2.

The control pattern is prepared from a hydroxyproline solution of 50mg /l. Successive dilutions permitted to obtain solutions ranging from 0.5 to 40mg/l.

The derivatization and the establishment of the calibration curve are carried out from 10 µl standard solution at different concentrations mixed with 10 µl of buffer. After adding 5 µl OPA and agitated, the tubes were stored at room temperature for 5 minutes then 10 µl of the solution NBD-CI are added. The derivatization is performed in a water bath (60°C), for 3 minutes under lighting. Next, the tubes are removed and the orange coloration allows derivatization verification. Later, the tubes are placed in ice to ensure cooling. 50 µl of this mixture is injected into the column to obtain the curve of the control pattern which should be linear. The samples are treated in the same manner.

3 Results

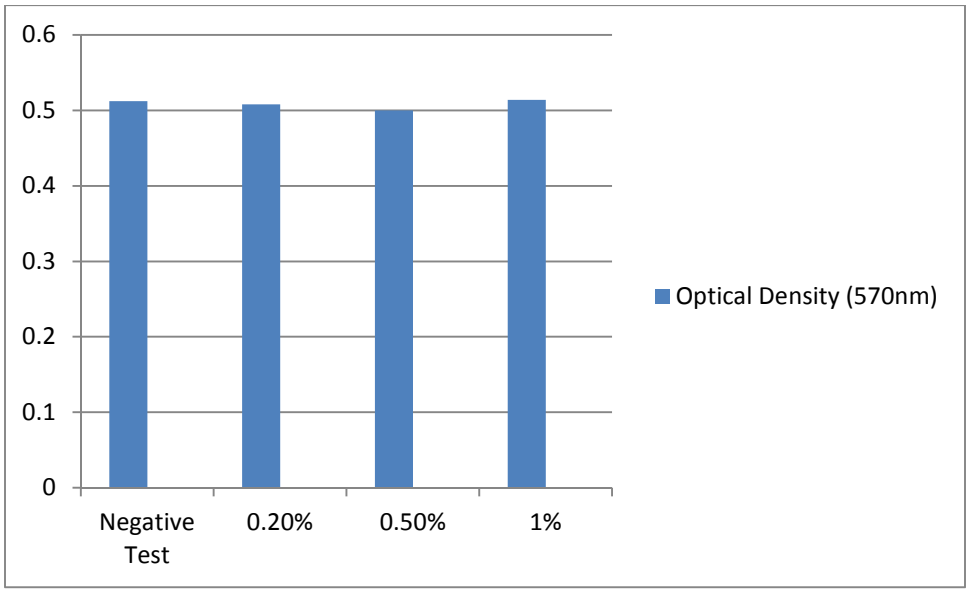
3.1 Evaluation of cytoxicity

The results are presented in the following table:

	Optical density (570nm)	%
Negative Test	**0.512 ± 0.03**	**-**
Organic Silica (0.2%)	**0.508 ± 0.04**	**ns**
Organic Silica (0.5%)	**0.500 ± 0.06**	**ns**
Organic Silica (1%)	**0.514 ± 0.04**	**ns**

ns: not significant

Histogram



The results show that the organic silicon G5 does not present cytoxicity with regard to any human fibroblasts in culture, when used to 0.2%, 0.5% and 1.%.

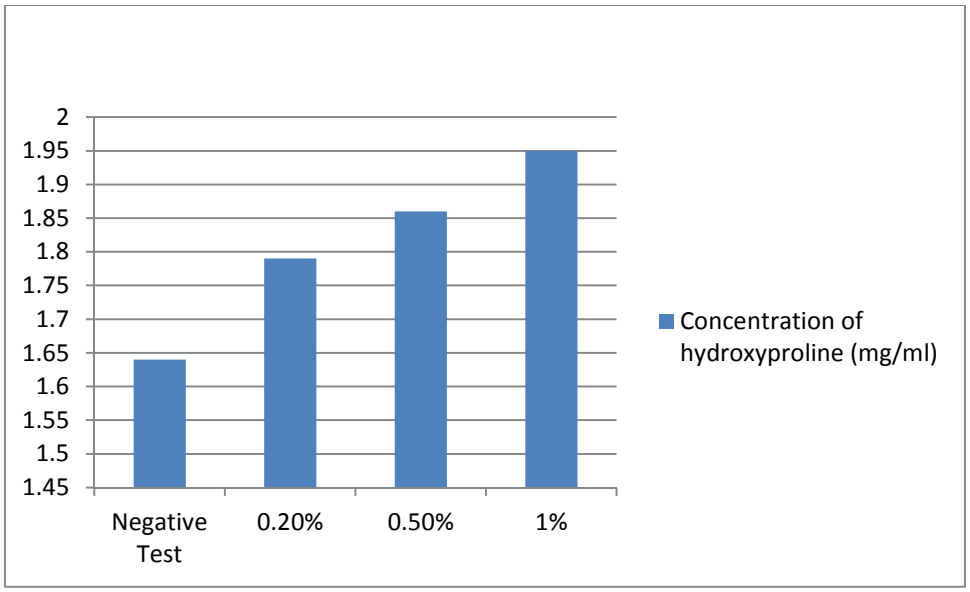
3.2 Evaluation of the collagen index

The separation and identification of hydroxyproline were performed by reversed-phase HPLC. Fluorescence peaks, after integration, used to calculate the concentration of hydroxyproline in the medical culture.

The results are presented in the following table:

	Concentration of hydroxyproline (mg/ml)	% increase
Negative Test	**1.64±0.10**	**-**
Organic Silica G5 0.2%	**1.79±0.13**	**9**
Organic Silica G5 0.5%	**1.86±0.08**	**13**
Organic Silica G5 1%	**1.95±0.10**	**19**

Histogram



The results show that the product Orgono Silica G5 significantly increment the level of collagen produced by the human fibroblast cultures by comparison with the control

4. Conclusion

In the experimental conditions adopted for the product Organic Silica G5:

- Does not induce cytoxicity in the concentrations (0.2%, 0.5%, and 1%)
- Induces a significant increase in collagen levels of 13% and 19% respectively for the concentrations of .5 and 1% in cultured human fibroblasts compared with the control.

In conclusion, the Organic Silica G5 product induces a significant increase in the index in cultured human fibroblasts.

May 13, 2011 Report facsimile translated into English and edited for clarity by Felix Corraliza

Translators note: Organic Silica G5 is the European designation and identical to Orgono Silica as distributed in North America.