Translation from Bulgarian

Official letterhead of the NATIONAL CENTER OF INFECTIOUS AND PARASITIC DISEASES

F I N A L R E P O R T

Subject: Performance of a scientific task assignment under a contract between A.V.S.T. TRADING Ltd. and the National Center of Infectious and Parasitic Diseases (NCIPD) on: Study of the cytotoxic effects of Inactivated Pepsin Fragment (IPF) – an active ingredient of the EnzoImmune Active preparation – on cell cultures. Determination of maximum Non-Toxic Concentration (MNC) and cytotoxic concentration 50% (CD_{50})

Scientists:

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I. Relevance of the problem

One of the biggest challenges facing modern biomedical science is the need to create new, highly effective and well-tolerated drugs for the treatment of viral infections. Despite the significant advantages of chemotherapy, it cannot be considered that any definitive results have been achieved at this stage in terms of the treatment of viral diseases. Low selectivity, significant toxicity, emergence of resistant mutants, a high number of deaths and the emergence of new zooanthroponotic viruses are among the main reasons for the search for new antiviral agents. In recent years, there has been an increase in research into the antiviral and immunomodulatory properties of biologically active molecules.

II. Materials and methods

Substance

Inactivated Pepsin Fragment (IPF) – an active ingredient of the EnzoImmune Active preparation provided by A.V.S.T. TRADING Ltd.

IPF was dissolved to an initial concentration of 50 mg/ml (background solution), of which *ex tempore* were prepared serial dilutions in a DMEM growth medium (Dulbecco's Modified Eagle Medium, Sigma-Aldrich, Germany), containing 2% fetal bovine serum (FBS, Gibco) and 1% antibiotic solution

(100 U/ml penicillin and 100 μg/ml streptomycin sulphate (Gibco, USA) (working solutions), which were filtered through sterile antibacterial filters with a pore diameter of 0.45 μm (Sartorius Stedium, Australia).

Cell culture

An African Green Monkey's (*Cercopithecus aethiops*) kidney monolayer cell line (Vero line) was used as a model system in the tests, kindly provided by the National Reference Laboratory for Cell Cultures, Rickettsiae and Oncogenic Viruses, NCIPD. The cells were cultured in DMEM growth medium with thermoinactivated 10% FBS, 1% sodium pyruvate (Sigma-Aldrich, Germany) and antibiotics (penicillin (100 U/ml), streptomycin sulphate (100 μg/ml)) (growth medium). The cell cultures were incubated at 37 °C with 5% CO_2 and the required humidity was made available. Vero cells were treated 1:3 - 1:5 at a density of about 2 x 10^5 cells/ml, then resuspended several times and poured into cell culture mattresses (25 cm^2) (Orange Scientific, Belgium). Prior to inoculation of the virus cells and/or the corresponding dilutions of IPF, the monolayer was washed three times with phosphate-buffered saline (PBS) at pH 7.4 for 1-2 minutes, after which the solution was removed. All experiments were performed during the exponential phase of the cell growth.

Treatment of the cell culture

The cells were seeded using a solution containing 0.05% trypsin (Trypsin) and 0.0025% ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich, Germany), pre-tempered at 37 °C. The cells were treated for about 3-10 minutes with this solution until the cells began to assume a round shape and dissociate. Subsequently the trypsin-versene solution was replaced with a small amount of growth medium, a trypan blue test was applied to differentiate the living from the dead cells, and they were counted using a hemocytometer, diluted to a certain volume with a density of 2 x 10^5 cells/mL and resuspended in cell culture mattresses. The cells were cultured at 37 °C with 5% CO_2 at the required humidity.

Test for staining of dead cells with trypan blue

Aliquots of cell suspension were mixed with an equal volume of 0.4% trypan blue dye solution, observed under an inverter light microscope and using a hemocytometer, and the live (unstained, clear cytoplasm) and dead (dark blue) cells were counted.

Freezing and thawing of the cells

Freezing and thawing of the cells was performed according to conventional methods, as described by Pegg D., 2007. Cells in the logarithmic growth phase were used for freezing. They were washed once with DMEM growth medium (5 - 1 0 min, 800 - 1500 rpm/min, 4 °C). The resulting cell depot was resuspended in culture medium and FBS (90%) and the cell suspension was cooled on ice (2 - 4 °C), then DMSO was added with stirring to a final concentration of 10%. The cells were dispensed into pre-cooled ampoules (0.5 - 1 mL/cell suspension/ampoule) and placed in a styrofoam box in a freezer at -80 °C. After a minimum of 24 hours, the ampoules were transferred into liquid nitrogen (-196 °C). The cells were frozen at a concentration of at least 1 x 10^6 cells / mL, and most often - 5 x 10^6 cells/ampoule.

Upon thawing of the cells, the ampoules were quickly placed in a vessel with water warmed to 37 - 40 °C and after thawing they were immediately transferred to a medium containing 10% FBS. In order to

prevent the toxic effects of DMSO, the culture medium was replaced with a new one immediately after the cells had adhered to the substrate (no later than 20 hours after seeding).

Determination of cell viability

Three methods were used to assess relative cell survival and proliferation: 1) microscopic observation of morphological changes in the monolayer of treated cells; 2) colorimetric MTT analysis and 3) study of the kinetics of the proliferative activity of the treated cells by the method of surface plasmon resonance (SPR analysis).

1) Microscopic observation of changes in the morphology of the cell layer

The cell monolayer was observed every 24 hours under an inverter light microscope for typical cytopathology, characterizing the toxic effect in the treated cells. The analysis of the observation of the cell morphology of Vero cells was synchronized with the MTT analysis of cell survival.

The MTT [[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] dye is a water-soluble tetrazolium salt that imparts a yellowish color to the solution. When added to the growth environment of the cells, MTT infiltrates the cellular cytosol and reaches the mitochondria, where mitochondrial dehydrogenases detach the tetrazolium ring and convert the soluble yellow salt into a water-insoluble blue formazan product. The ability of cells to reduce MTT is an indicator of mitochondrial integrity and activity, which is interpreted as a measure of cell viability and/or the number of viable cells. Dead cells do not participate in this transformation due to mitochondrial dysfunction.The amount of transformed MTT to insoluble formazan crystals is proportional to the number of living cells. To measure the formazan amount, the cells were treated with a solution that lyses them and simultaneously thereby dissolving the blue formazan crystals. The amount of the latter is measured spectrophotometrically at a wavelength of $\lambda = 540$ nm.

After cell counting, the latter were resuspended in growth medium supplemented with 2% FBS. The cell suspension was then dispersed at a concentration of 5×10^4 cells/well in sterile 96-well plates (Orange Scientific, Belgium) (0.2 mL/well). Due to experimental data that a decrease in volume was observed in the end rows and columns during longer cultivation, no cells were seeded in them. Only medium without FBS is added there. When the cell monolayer reached between 70 - 80% confluence (usually after 24 hours), the supernatant was decanted and 0.1 mL of supporting growth medium, and 0.1 mL of pre-prepared dilutions of IPF in the concentration range of 0.0001 - 20 mg / mL were added. A minimum of 3 wells were added with each IPF dilution. Only nutrient-free nutrient medium (0.2 mL) was added to several wells for the purpose of cell control. Because the volume of the substance used (with a given concentration) was diluted twice when diluted in the well, the actual concentration of each dilution added was twice as low as that previously prepared. The plates thus treated were incubated at 37 °C for 72 hours. At the end of the third day, 0.02 mL of MTT working solution (starting concentration 0.05 mg/mL) was added dropwise to each well (excluding end rows and columns), after which the plates were incubated at 37 °C for 3 hours. The culture medium with the MTT dissolved in it was removed after incubation, then 0.2 mL of the lysol solution containing ethanol: DMSO (v:v) was added. The plate thus treated was read spectrophotometrically at $\lambda = 540$ nm using an ELISA reader (Bio-Tek Instruments, Germany). Cell

survival was defined as a percentage (%) of the living cells in the wells treated with different concentrations of the test substance, as compared to untreated control cells.

The following formula was used for this purpose:

% cell survival – $OD_{\text{treated cells}} / OD_{\text{cellular culture}} \times 100$

The values of the Maximum Non-Toxic Concentration (MNC) and cytotoxic concentration of 50% (CD50) of the test substance relative to the Vero cell line were calculated based on the curve showing the dose (concentration) and the cell survival using GraphPad Prism software (GraphPad Software, San Diego, CA, USA).

- A cytotoxic concentration of 50% (CD50) was defined as the concentration of the test substance at which 50% of the cells die as a result of the toxic action of the substance.
- Maximum Non-Toxic Concentration (MNC) was defined as the highest concentration of the test substance that does not cause damage or death to the treated cells. The two values in the experiments were expressed in mg/mL.

3) Determination of the kinetics of proliferative activity of the IPF- treated cells by SPR analysis at different exposure times

Measurements were made every 2 hours until the 48th hour of viral infection development, the last hours coinciding with the initial cytopathic cellular changes. As a rule, the increase/decrease of the PPR signal is a consequence of the increase/decrease of the effective index of refraction of the cell monolayer as a result of the morphological changes in the cells caused in the process of viral replication. The stages of the replication cycle – adsorption, penetration of the virus into the cell, changes in cellular metabolism, as well as the production and release of daughter offspring, cause such changes and accordingly generate a PPR signal.

Statistical processing of the obtained results

The data obtained are presented with mean values ± standard error of the mean value (SEM) from at least two separate experiments, each performed in three to five replicates. Statistically significant differences in survival/proliferation between untreated controls and samples treated with different concentrations were determined by a single factor ANOVA (analysis of variance) and subsequent Dunnett's test. Statistically significant differences between untreated controls and treated samples were determined by t-test, where the values at $p < 0.05$ were defined as statistically significant.

III. Results

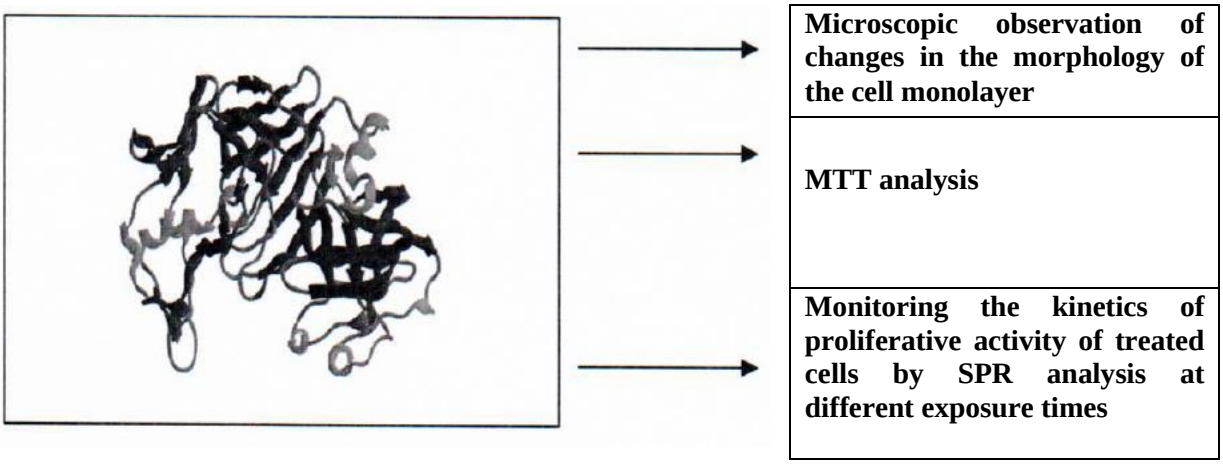
1. Influence of the Inactivated Pepsin Fragment (1PF) on the survival and proliferative activity of the cells cultured in laboratory conditions

The first important step in antiviral experiments involves determining the cytotoxicity of the test substance on laboratory-cultured Vero cells. In the screening analyzes for assessment of the relative cell survival and proliferation three methods were used in parallel: 1) microscopic observation of the changes in the morphology of the cell monolayer; 2) MTT analysis and 3) monitoring the kinetics of proliferative

activity of treated cells by SPR analysis (See Figure 1). Survival was reported at different exposure times after treatment with Inactivated Pepsin Fragment (IPF), as significant cell proliferation could be observed at different time intervals, direct toxic effects of the test substance leading to cell death could be assessed, and to monitor the morphological changes that occurred in the cells as a result of the treatment.

MTT analysis of cell survival and proliferation was performed over a wide concentration range of the studied Inactivated Pepsin Fragment (IPF). The limits within which the applied concentrations varied were selected on the basis of preliminary experiments involving larger concentration intervals in order to accurately determine the values of CD_{50} and MNC. The test substance IPF was administered in the concentration range of 0.0001 – 20 mg/mL, in the time interval 2 – 96 hours. Untreated Vero cells which survival was assumed to be 100%, were used as negative control. Dose-dependent curves of the applied IPF on the viability of the cell monolayer were obtained from the used MTT test. The analysis of cell morphology was synchronized with the MTT analysis of cell survival and the kinetics of proliferative activity of treated cells was assessed by SPR analysis at different exposure times.

Figure 1. Successive stages for the implementation of the experiments

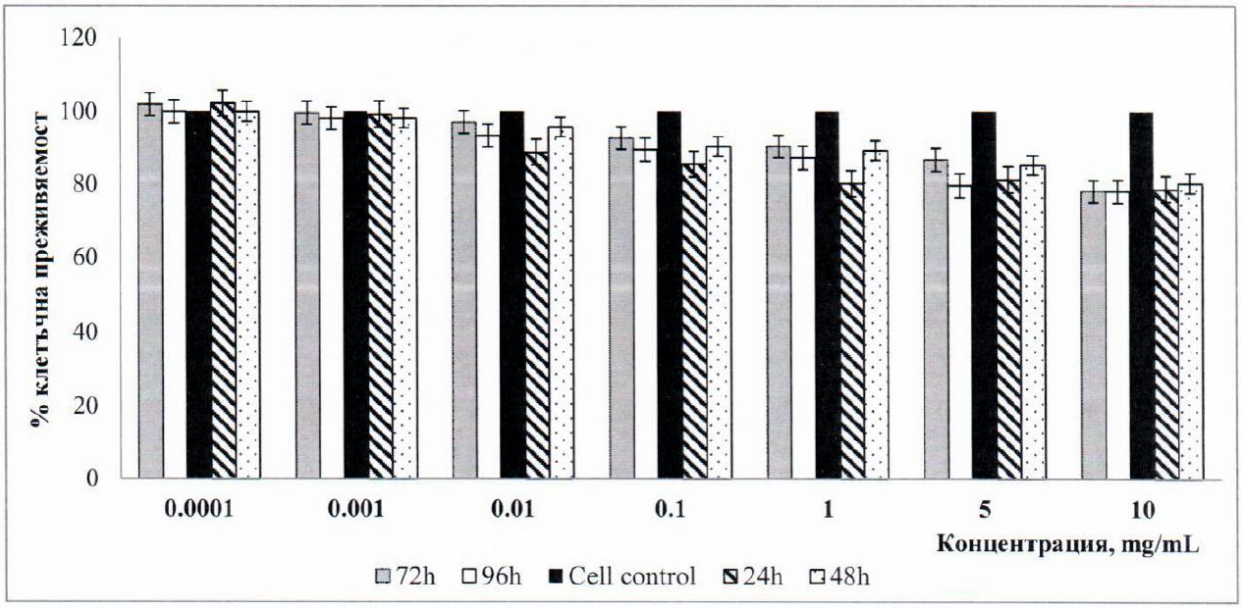


The obtained results show that in general the survival of the treated monolayer cells of the Vero line did not decrease dramatically under the influence of the tested Inactivated Pepsin Fragment (IPF). At the lowest incubation concentrations studied (0.0001 - 0.1 mg/mL), relatively low inhibition of survival (90.34 - 99.52%) was observed, while at subsequent increasing concentrations (from 5 mg/mL to 20 mg/mL) the value of cell survival decreased and reached a value close to 78%, which according to ISO 10993 for the study of cytotoxicity of medical devices in cell cultures is considered non-toxic ("non-toxic concentration is that at which cell survival does not fall below 75%"). Survival differences between untreated controls and treated samples at doses of 0.0001 mg/mL ($p < 0.001$) and 1 mg/mL ($p < 0.05$) were statistically significant (See Figure 2).

When comparing the experimental data on cell survival, it became clear that the following antiviral drugs widely used as positive controls in antiviral experiments: acyclovir (ACV), Remdesivir (REM), oseltamivir phosphate (Tamiflu) and hydroxychloroquine (HQV) are about 35 times more toxic than IPF (See Figure 2) (data not included in this report).

In conclusion, the tested IPF has shown no toxicity to Vero cells after 96 hours of treatment administered at concentrations ranging from 0.0001 mg/mL to 5 mg/mL.

Figure 2. Effects of IPF on the survival/proliferative activity of Vero cells as reported by MTT analysis after exposure time (24 h to 96 h).



Abscissa: Concentration, mg/mL; ordinate: Percentage (%) of cell survival

The decrease in the number of adherent living cells, as well as the rounding and decrease in the size of the cells follow the trend established in the MTT analysis (See Table 1).

Visualization of changes in the morphology of the cell monolayer after treatment is an accessible and reliable approach in the initial study of the cytotoxicity of various substances.

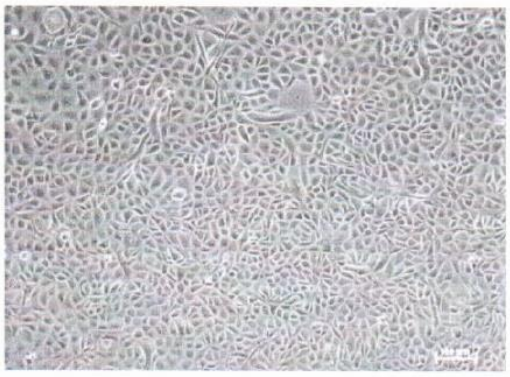
In this regard and in parallel with the MTT analysis, changes in the morphology of the cells exposed to the tested IPF and the ACV antiviral drug were observed under an inverter light microscope. Untreated control cells and IPF-treated cells in the concentration range of 0.0001 - 10 mg/mL retained their morphology and structure (See Figure 3).

A weak concentration-dependent decrease in proliferation was observed for the IPF-treated Vero cells, where the decrease was from 98.54% at a concentration of 0.0001 mg/mL down to 78.28% at the highest dose of 10 mg/mL.

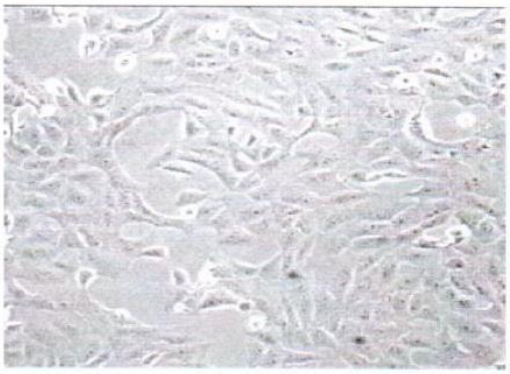
Table 1. Effects of IPF on the survival/proliferative activity of Vero cells, as reported by different methods after different exposure times with the test substance (24 h - 96 h)

Exposure time	Method	MNC (mg/mL)	CD_{50} (mg/mL)
24 h	Microscopic observation of changes in the morphology of the cell monolayer	0.1 ± 0.1	15 ± 0.05
	MTT test	0.1 ± 0.12	15 ± 0.22
48 h	Microscopic observation of changes in the morphology of the cell monolayer	0.1 ± 0.05	10 ± 0.5
	MTT test	0.1 ± 0.3	10 ± 0.03
72 h	Microscopic observation of changes in the morphology of the cell monolayer	0.1 ± 0.22	10 ± 0.01
	MTT test	0.1 ± 0.5	12.5 ± 0.05
96 h	Microscopic observation of changes in the morphology of the cell monolayer	0.1 ± 0.02	10 ± 0.5
	MTT test	0.1 ± 0.15	10 ± 0.03

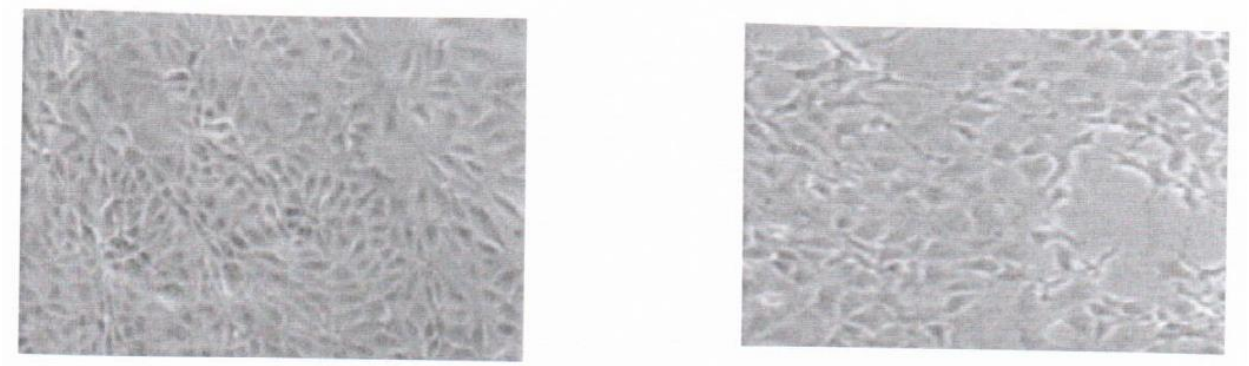
Figure 3. Cytotoxic effects of IPF on Vero cells after various exposure times (24 – 96 hours) (Photo A - D).



A. Untreated Vero cells



B. IPF-treated (1 mg/mL) Vero cells every 24 h



C. IPF-treated at MNC (0.1 mg/mL) Vero cells for 48 h

D. IPF-treated at MNC (1 mg/mL) Vero cells for 96 h

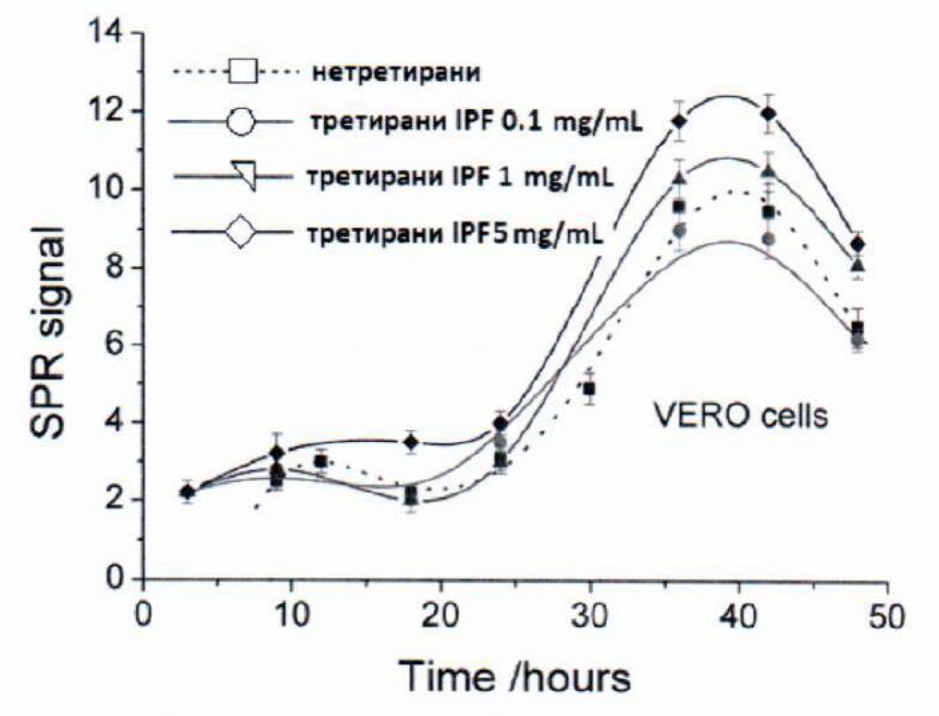
3) Determination of the kinetics of proliferative activity of IPF-treated cells by SPR analysis at different exposure times

Control SPR conditions were determined for the chip with an untreated monolayer of cells (cell control). Some of the chips were treated with IPF at concentrations of 0.1 mg/mL, 1 mg/mL and 5 mg/mL, determined by MTT analysis and corresponding to the MNC or near it. The results of the SPR analysis are shown in Figure 4. The curves show the kinetics of the proliferative activity of IPF-treated cells (at MNC or near it) and untreated (cell control) at different exposure times.

The exponential increase of the signal in the interval 24 - 40 hours from the treated and control cells is associated with an increase in the density on the chip, and hence with greater cell survival. This process was most intense around 24 hours and 40 hours, which coincides with the time of active cell division and doubling their number. As a result, there was an increase in cell density and compaction of the chromatin structure in their nuclei, which in turn led to an increase in the refractive index (in the interval 24 - 40 hours). It is noteworthy that the cell viability for the cells treated with Inactivated Pepsin Fragment (IPF) at a concentration of 0.1 mg/mL (MHC) or near it (at 1 mg/mL or at 5 mg/mL) followed the same tendency to increase the signal as for the cell control, which is proof of the lack of toxicity of the substance applied.

The interval 2 - 20 hours is not associated with definitive changes in cell density, therefore the change in the SPR signal is not significant. After 48 hours, there was no increase in the SPR signal, as the cell density on the chip decreased over time.

Figure 4. Comparison of non-treated and IPF-treated cells at various concentrations and exposure times carried out using a SPR analysis.



IV. Conclusion

The tested Inactivated Pepsin Fragment (IPF) – an active ingredient of the EnzoImmune Active preparation – has shown no toxicity to the Vero cell line at various exposure intervals (24 – 96 hours), as administered in concentrations ranging from 0.0001 mg/mL to 5 mg/mL.

V. References

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Summarized by: Signature: *illegible*

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Approved by: Signature: *illegible*

Prof. Dr. Iva Hristova

On **14 October 2021,** I, Tsvetelina Koleva, Assistant Notary with the Notary Public Gabriela Yordanova-Daskalova, acting in and for the area of the Sofia Regional Court, Reg. No. 343 with the Bulgarian Notaries' Chamber, hereby certify the signature placed on this document by:
Roza Videnova Bozhilova, residing in Sofia City,
and there were not any stricken out words, additions, corrections or other peculiarities on the original document.

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Assistant Notary by
replacement
TSVETELINA KOLEVA

I, the undersigned Ivan Petrov Tortorochev, hereby declare that this is, to the best of my knowledge and belief, a correct and complete translation from Bulgarian into English of the document enclosed. The translation comprises ten (10) pages.

Translator: Ivan Tortorochev