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Parvovirus H-1 induces cytopathic effects in breast carcinoma-derived cultures

Ghaffar Muharram¹, Emilie Le Rhun², Ingrid Loison¹, Pierre Wizla¹, Audrey Richard¹, Nathalie Martin¹, Annie Roussel¹, Agnes Begue¹, Patrick Devos⁴, Marie-Christine Baranzelli³, Jacques Bonneterre², Perrine Caillet-Fauquet¹ and Dominique Stehelin¹

¹CNRS-UMR 8161, Institut de Biologie de Lille, 59021 Lille, France

²Département de Sénologie, ³Département d'Anatomie Pathologique, Centre Oscar Lambret, 59020 Lille France

⁴Département des Biostatistiques, Centre Hospitalier Universitaire et Régional de Lille, 59037 Lille, France

Corresponding author

Ghaffar Muharram (PhD)
CNRS, UMR 8161 "Institut de Biologie de Lille"
1 rue du Professeur Calmette
59021 Lille cedex, France
Tel : +33 3 20 87 10 98
Fax: +33320871111
ghaffar.muharram@ibl.fr

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Abbreviations : MOI, multiplicity of infection; MTT, tetrazolium salts.

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Abstract

Parvovirus H-1 (H-1 PV) preferentially replicates in malignant cells resulting in their death by cytolysis. It has often been considered a potential candidate for use in novel anticancer therapy. To evaluate its potential in a model of natural tumors, we assayed in vitro the effect exerted by H-1 PV on short-term cultures derived from breast tumor samples freshly excised from patients. Our results show that H-1 PV effectively kills tumor-derived cells, whereas normal-tissue-derived cells showed no H-1 PV-induced cytopathic effects (CPE). We also determined that the H-1 PV sensitivity (up to 67% sensitive cultures) is related with the quantities of virus assayed. We further examined the expression and phosphorylation state of the parvoviral nonstructural protein 1 (NS1), known to be associated with parvoviruses-induced CPE. Both appear to be impaired in normal-tissue-derived cells and resistant cultures. Finally, we show that H-1 PV sensitivity in cultures correlates significantly with higher tumor grades (Nottingham combined histologic grade 2 or 3). This report confirms that H-1 PV can efficiently induce CPE in primary breast tumor cells in vitro. It identifies tumor characteristics representing potential criteria for recruiting patients for clinical evaluation of H-1 PV antitumor effects.

Introduction

Developing cancer-eradicating agents with low toxicity remains a prime goal. An attractive prospect is the use of oncolytic viruses, i.e. natural agents that act like tumor-cell parasites and are able to find and destroy malignant cells selectively [1]. Many different viruses of several viral families, i.e. Herpes simplex virus [2; 3], Reovirus [4], Adenovirus [5], and Parvovirus [6; 7], possess such properties. Those associated with limited or no toxicity are relevant to potential clinical use.

The rodent autonomous oncolytic parvovirus H-1 (H-1 PV) is a small (~25 nm), non-enveloped virus with single-stranded DNA (~5 kb). It replicates efficiently in human tumor cells in an episomal fashion, yielding infectious viral progeny. H-1 PV has never been linked to any pathology in humans [8]. Even retrospectively, no correlation has been found between H-1 PV serological detection and any pathology [9]. When H. Toolan in 1965 injected the virus into two patients (12 and 13 years old) severely affected by osteosarcomas that had metastasized, with no other therapeutic option left, the virus caused no detectable side effect [10]. Ultimately, nevertheless, only a proper phase-I clinical trial with H-1 PV will be able to establish its therapeutic interest and innocuousness.

H-1 PV-induced destruction of malignant human cells has often been reported, both *in vitro* (transformed cell lines) **and *in vivo* (human cell lines xenografted in animal models)** [11; 12]. As demonstrated with the closely related Minute Virus of Mice (MVM), the cytopathic effects (CPE) exerted on malignant cells result from expression of the parvoviral nonstructural protein 1 (NS1) [13; 14], its phosphorylation [15], and its interaction with cell proteins, resulting in cytoskeletal modifications and cell death [16].

The tropism of H-1 PV for neoplastic cells has also been dissected in many studies. The virus can enter both normal and transformed cells. In the former, it seems only to cause abortive infection without proper neovirion production [14]. In the latter, a complete viral cycle can occur, leading to release of new virions upon host cell lysis. Fibroblasts transformed by ionizing radiation, a chemical carcinogen, or a tumor virus [17] and spontaneously or tumor-virus-transformed keratinocytes [18] appear much more sensitive to H-1 PV-induced cell killing than their normal parental counterparts. The greater H-1 PV permissiveness of transformed cells might be attributable to changes in the nature and quantity of cell factors associated with cell cycling [19]. Activation of cell division might not suffice, since the differentiation status of the cell also appears to play a role in correct replication of H-1 PV [14]. Cell immortalization, resulting in permanent cell proliferation (a high proportion cells in

the S phase), plus cell transformation thus represent ideal conditions for efficient parvovirus replication.

Most information concerning effective killing of malignant cells by H-1 PV has been obtained in studies on cell lines. Despite their overall relevance thanks to repeated observations, this information pertains model systems where conditions differ considerably from the physiological reality encountered in patients' tumor tissues. **Only two preliminary studies have described H-1 PV-induced cell killing in primary breast tumor cells [20; 21]. The authors have notably demonstrated cytopathic effect induced by H-1 PV preferentially in tumor cells, as opposed to normal cells derived from the same patients [20].**

In this study we examine a large number of short-term breast-tumor-derived cultures in order 1) to quantify precisely the H-1 PV-induced cell killing, 2) to test if there is a H-1 PV dose-effect, 3) to analyse the viral cytotoxic protein NS1 expression, 4) to determine if tumors' histopathological characteristics are predictive of H-1 PV-induced cell killing.

Materials and Methods

All products were purchased from Invitrogen (Paisley, UK) unless otherwise specified.

HeLa-GFP xenografted-tumor-derived cell cultures and H-1 PV infection

SCID-mice (Institut Pasteur de Lille) were grafted with HeLa-GFP cells as described in [12]. After 10 days, tumors ($\approx 100\text{mm}^3$) were detectable. Mice were euthanized (in agreement with the ethical committee of our institution) and the excised tumors were minced as finely possible and disaggregated in a solution of dispase (10 mg/mL) and collagenase (5 mg/mL) at 37°C under gentle stirring for at least one hour. The enzymatic reaction was stopped by addition of OptiMEM supplemented with 10% FCS. The cells were centrifuged. The pellet was resuspended in DMEM medium supplemented with 10% FCS, L-glutamine (2 mM), gentamycin (8 $\mu\text{g}/\text{mL}$) and neomycine (900 $\mu\text{g}/\text{mL}$) and plated in 100-mm-diameter culture dishes. Once cultures were established, cells were counted and plated in 12-well plates at 20000 cells/well. The next day, cells were infected with H-1 PV at different multiplicities of infection (MOIs) or mock treated in control wells (Figure 1a).

Short-term breast-tumor-derived cell cultures and H-1 PV infection

Samples of invasive breast carcinomas (according to the World Health Organization classification) were collected at the Centre Oscar Lambret (Lille, France) in the Pathology Department in agreement with all ethical rules specified by the French Ministry of Research for use of patients' samples for research purposes. All patients were women and agreed for use of their tumor samples in our study. The tumor samples were obtained during surgical resection of newly diagnosed tumors, before any other anticancer treatment (chemotherapy, hormonotherapy, or radiotherapy). **Patients' tumor detailed histopathological examination reports, including the Nottingham combined histologic grade (differentiation status, mitotic count, nuclear pleomorphism), vascular and sentinel and/or axillary lymph node invasion status, estrogen and progesterone receptor expression, were collected (see Table 1).** Upon receipt immediately after surgery, the patients' tumor samples were dissected and analyzed by a pathologist. One part of the sample was transferred to a tube containing OptiMEM medium supplemented with 10% FCS, L-glutamine (2 mM), and gentamycin (8 $\mu\text{g}/\text{mL}$).

Upon arrival, the samples were weighed, decontaminated in Betadine® 10% dermal solution (Vitaris Manufacturing, Mérignac, France), and washed twice in PBS and disaggregated as described with HeLa-GFP tumors. Once monocellular suspensions were obtained, breast tumor cells were resuspended in serum-free Mammary Epithelial Cell Growth Medium

(PromoCell, Heidelberg, Germany) supplemented with bovine pituitary extract (0.052 mg/mL), epidermal growth factor (10 ng/mL), insulin (5 µg/mL) and hydrocortisone (0.5 µg/mL) (supplied by PromoCell with the medium) and plated in 35-mm-diameter culture dishes. After one night at 37°C, dead floating cells were eliminated and live adhering cells were left to proliferate (Figure 1b-picture 2).

After two to three passages, when sufficient cells were available, the cells were counted and plated in 24-well plates at 25000 cells/well. The next day, the cells were infected with H-1 PV at different MOIs or mock treated in control wells (Figure 1b).

Virus and cell lines

For infection, H-1 PV was produced in a human cell line, as described in [22], from a plasmid containing the wild-type H-1 PV genome (kindly provided by Pr J. Rommelaere, Heidelberg, Germany). Virus stock titers were determined according to plaque assays published methods [23]. The multiplicity of infection (MOI) is given by number of plaque-forming units (pfu) inoculated per cell.

SK-BR-7 and MDA-MB-231 cells (kindly provided by Dr P. Mehlen, Lyon, France) were cultured, respectively, in RPMI medium supplemented with 10% FCS and gentamycin (8 µg/mL) and DMEM medium supplemented with 10% FCS, L-glutamine (2 mM), and gentamycin (8 µg/mL).

Parvovirus-H-1-induced CPE assay

Seven days post-infection, H-1 PV-induced CPE were observed after Giemsa staining (Figure 1). Briefly, the culture medium was removed, the cells were washed twice with physiological water, fixed in methanol for 10 minutes, stained for 1 hour at room temperature with diluted (1/10 in tap water) Giemsa stain modified solution (Sigma-Aldrich, Steinheim, Germany), and dried after a final wash. The level of cytotoxicity was evaluated by direct microscopic observation, and pictures (representing ≈70% of the well) were taken. Cultures were considered sensitive to H-1 PV-induced CPE when massive cytolysis (> 50% of the cells) was observed as compared to the mock-treated well.

Parvovirus-H-1-induced CPE quantification

The H-1 PV-induced CPE were quantified 7 days post-infection by means of a tetrazolium salt (MTT) viability assay (as described in [24]) for n = 30 tumor-derived cultures. Each

condition was tested in duplicate. Briefly, the medium was removed and replaced with 500 μ L MTT solution (Sigma-Aldrich) diluted (final concentration: 0.5 mg/mL) in Mammary Epithelial Cell Growth Medium, then the culture was incubated for 4 h at 37°C. MTT salts were metabolized to formazan salts by viable cells and released in the supernatant after addition of a solubilization solution and incubation for 1 h at room temperature. A 100- μ L volume of supernatant was transferred to a well in a 96-well plate (in duplicate) and read with a microplate spectrophotometer-reader (Multiskan RC, Thermo LabSystems, Helsinki, Finland) at 540 nm and 620 nm. The background absorbency (at 620 nm) was subtracted from the value obtained at 540 nm. The viability of the infected cells was expressed as the ratio of the corresponding absorbency to that of non-infected cells set arbitrarily at 100%.

Western blot analysis

Forty-eight hours post-infection, before any H-1 PV-induced effect was visible, the cells were lysed in PY buffer [25] supplemented with 0.04% Complete EDTA-free™ Protease Inhibitor Cocktail (Roche, Mannheim, Germany). Cell lysates were stored at -20°C. Equal amounts of the various lysates were loaded. After 10% SDS-PAGE separation, the proteins were transferred onto nylon membranes and parvoviral NS1 was revealed with the NS1-specific Sp8 rabbit antiserum [26] diluted 1/1000 in PBS supplemented with 0.2% Aurora™ blocking reagent (MP Biomedicals, Illkirch, France) and 0.01% Tween 20 (Sigma-Aldrich). Expression levels were analyzed with a LAS-3000 imaging system (FUJIFILM, Quentin en Yvelines, France).

Statistical analysis

Breast tumor samples (n = 112) were obtained from the Centre Oscar Lambret. **Some samples were excluded from the statistical analysis because of bacterial contamination in culture or because of technical difficulties in deriving the cultures. Similarly a small number of samples of undifferentiated or epidermoid or mucinous breast cancer were also excluded in order to compare homogeneous sub-groups.** The statistical analysis was thus performed on 96 samples. First, a descriptive analysis (frequencies, box-plot, etc.) was performed in order to check and recapitulate the data. Then comparisons of subgroups were performed. In accordance with parameter distribution and subgroup size, comparisons of means were performed using the nonparametric Wilcoxon test. Comparisons of frequencies were

performed using the Chi-square test or Fisher's exact test if necessary. All analyses were carried out with the SAS software, V9.1. The significance level was set at 0.05.

Results

Parvovirus H-1 induces cytopathic effect on cultures from freshly excised xenografted HeLa-GFP tumors

Solid HeLa-GFP tumors were obtained in SCID-mice (Figure 1a-picture 1). The advantage of using HeLa carcinoma cells stably expressing GFP, enable us to visualize specifically the destruction of malignant cells in vitro when H-1 PV was added (Figure 1a, CONTROL well compared to MOI 1 and MOI 5).

This model experiment is a proof-of-concept of our in vitro assay showing H-1 PV induced CPE on short-term derived tumor cell cultures. This assay was then subsequently used with patients' tumors as described in the following sections.

Parvovirus H-1 induces cytolysis of short-term breast-tumor-derived cells, as opposed to normal cells

To examine H-1 PV induced cytopathic effects (CPE) on primary breast-tumor cells, short-term cultures were derived from breast tumor samples freshly excised from patients (Figure 1). As controls, cultures were also derived from normal peritumoral tissue taken from the same patients (6 samples). **To avoid stromal cell contamination in our cultures, we used a media specifically designed for the culture of mammary epithelial cells (confirmed by cytokeratin 19 labeling, data not shown).** The tumor types studied were mainly ductal carcinomas (82%) and invasive lobular carcinomas (18%). 84% of the samples were from undifferentiated tumors (differentiation grade 3). As for the Nottingham combined histologic grade, most of the samples were from high-grade tumors (51% grade-2 and 39% grade-3 tumors). Detailed histologic data are presented in Table 1.

Once primary cell cultures were successfully established in a media, they were infected with H-1 PV at varying multiplicity of infection (MOI) and incubated for one week, at which time CPE were evaluated. As illustrated in Figure 2, cultures derived from an invasive ductal carcinomas (grade 3, ER+, PR+) showed very severe H-1 PV-induced CPE, in contrast to the corresponding normal-cell cultures (Figure 2 rows B and A respectively). The latter showed an effect only at the highest dose tested (MOI 100), and even then the effect was weak.

H-1 PV-infected tumor-cell cultures showed a dose-dependent reduction in cell number (Figure 2, rows B: MOI 1, MOI 10 and MOI 100), in contrast to mock-treated tumor-cell cultures (Figure 2, CONTROL).

The level of sensitivity to H-1 PV-induced CPE varied among the different tumor samples examined.

The exploitation of these data was hampered by a major difficulty; patients' tumors are quite heterogeneous by nature, as opposed to tumor cell lines that appear very homogeneous in culture. A more refined and quantitative test was thus required as described in the next section.

Quantification and analysis of the differential effect of H-1 PV on normal and tumor-derived cells

The assay based on spectrophotometric detection of purple formazan produced by the metabolic action on tetrazolium salts (MTT) of mitochondrial dehydrogenases in living cells is commonly used to measure cell death induced by toxic chemicals or biological substances. Here the MTT viability assay was used to quantify H-1 PV-induced CPE.

To compare the results of this assay with those obtained by Giemsa staining, we first used two breast carcinoma cell lines, the MDA-MB-231 and SK-BR-7. The cells were infected (at MOI 1, 5, or 10 or with no virus in CONTROL wells) and incubated for 7 days. Giemsa staining (Figure 3a) and MTT quantification (Figure 3b) were done in parallel. The viable cell rates measured by the MTT assay appeared to tally quite accurately with the visual impression after cell staining.

After one week H-1 PV appears to affect MDA-MB-231 cells very severely, since cells infected at MOI 1, MOI 5, and MOI 10 showed, respectively, only $10.2\% \pm 0.8$, $8.7\% \pm 0.3$, and $9.1\% \pm 0.8$ survival. In CONTROL wells, in contrast, survival was $100\% \pm 5.3\%$. The effect of H-1 PV on SK-BR-7 cells was almost as drastic, leading to $28\% \pm 1.7\%$, $26.7\% \pm 1.8\%$, and $9.7\% \pm 1.66\%$ at MOI 1, 5, and 10 respectively (Figure 3b).

The same procedure was used to quantify H-1 PV-induced CPE on cultures derived from fresh normal and tumoral breast tissues (Figure 4b, A and B respectively). The former cultures displayed little or no H-1 PV-induced cell death, with $80\% \pm 6.5\%$ or $72\% \pm 5.2\%$ of the cells remaining viable 7 days post-infection at MOI 1 or MOI 100 respectively (Figure 4b, A). Of the tumor-derived cells, only $69\% \pm 3\%$ and $28\% \pm 1\%$ of the cells remained viable 7 days post-infection at MOI 1 or MOI 100 respectively (Figure 4b, B). The quantification test appeared as a decisive addition to the visual results obtained by Giemsa staining (Figure 4a).

Differential expression and phosphorylation of the NS1 cytotoxic protein in the normal and tumor-derived cells

To further investigate reasons for the differential response to H-1 PV infection in the above-described normal and tumor-derived cells, we measured expression of the cytotoxic protein NS1 by immunoblotting with a specific antiserum directed against the carboxy-terminal region of the protein [26] (Figure 4c). When expression profiles were compared 48 hours after infection with H-1 PV, cells derived from normal tissue showed much lower levels of both NS1 (lower band) and its active phosphorylated form (NS1-P, upper band) (Figure 4c, A) than the corresponding tumor-derived cells. More interestingly, the ratio of NS1-P to NS1 was very different in the two situations, the phosphorylated form being more abundant in tumor-derived cells and less abundant in normal cells than unphosphorylated NS1.

Tumor histopathologic characteristics predictive of sensitivity to H-1 PV induced CPE

Of the 96 tumors collected and considered for statistical analysis (see Material and Method section), 69 yielded successful short-term cultures (72%). Among these successfully cultured tumors, 48% yielded cultures sensitive to H-1 PV and 35% yielded resistant cultures. For 17% of the cultures the level of sensitivity was intermediate. To study the factors predictive of sensitivity toward H-1 PV oncolysis, only the sensitive and resistant groups were compared. Only cultures from high-grade tumors appeared significantly ($p = 0.03$) sensitive to H-1 PV-induced CPE (Figure 5). A trend ($p = 0.07$) towards higher sensitivity was also associated with a higher mitotic grade (data not shown).

Tumor-derived cells sensitivity to H-1 PV increase with higher doses

H-1 PV induced CPE was examined with increasing quantities of virus. On a total number of 79 tumor-derived cultures, 61 were treated with doses of H-1 PV up to MOI 25, 47% were sensitive, 11% showed intermediate sensitivity and 41% were resistant (Table 2). On the last 18 tumor-derived cultures assayed, we were able to reach doses up to MOI 100 resulting in 67% of H-1 PV sensitive cultures, 11% intermediately sensitive and 22% resistant cultures (Table 2).

Discussion

Parvovirus H-1 is often referred to as an ideal candidate for use in cancer therapy based on viral agents capable of selectively killing malignant cells [6]. With the Minute Virus of Mice (MVM), H-1 PV is known for its preferential replication in malignant cells (oncotropism) [14; 17] ultimately resulting in their death (oncolysis) [7; 16]. It also appears that H-1 PV injection is not associated with pathological side effects in patients [10]. Breast neoplasia appears as a potential target for H-1 PV-based anticancer virotherapy.

In this study we have evaluated the in vitro capacity of H-1 PV to destroy primary breast tumor cells. Our data clearly demonstrate that a certain number of short-term cultures derived from freshly excised breast tumors are sensitive to H-1 PV induced CPE (Figures 2, 4, 5 and 6). H-1 PV exerts its cytotoxic effects in a dose-dependent manner: cytolysis is much greater at MOI 10 and MOI 100 than at MOI 1 (Figures 2, 4). Most often, to obtain a significant effect, we had to use large quantities of virus (starting at MOI 10 up to MOI 100). This is in contradiction with many studies describing H-1 PV-induced CPE on a variety of cell lines, where low virus doses were sufficient to cause effective cell killing [7; 27]. Our own data show very efficient H-1 PV induced cytolysis in cultures derived from model HeLa-GFP tumors established in SCID-mice (Figure 1a). H-1 PV effect is also massive with MDA-MB-231 and SK-BR-7 cells starting at MOI 1, with almost no viable cells left at MOI 5 and 10 (Figure 3). **The use of primary cells might explain this discrepancy, such cells generally cycle less rapidly than cell lines in vitro.** It is known that full H-1 PV replication, resulting in final cell killing, strongly depends on cell factors available for efficient cells cycling [28]. In this respect, our statistical analysis applied to the histopathologic characteristics of the tumors from which H-1 PV-sensitive cell cultures were obtained, showed a trend towards a higher number of mitoses in such tumors ($p = 0.07$). **The need for higher quantities of H-1 PV to induce efficient cytolysis in tumor-derived cells might also be explained by the fact that viral propagation is limited (as measured for few tumor-derived cultures, data not shown). Although it was shown in [17] that H-1 PV sensitivity does not rely directly on the capacity of propagation of the virus i.e. HT1080 cells with low viral amplification are more severely killed by H-1 PV than KMST-6 cells with higher viral amplification rate.**

Of the successfully established cultures from breast tumor samples that were assessed in our study, 47% up to 67% were considered sensitive to H-1 PV-induced CPE. A previous study describing H-1 PV-induced CPE on short-term breast-tumor-derived cell cultures ($n = 19$) established the success rate at 32% [21]. Our better success rate is probably due to the larger quantities of virus we used. When comparing our own data between cultures where H-1 PV

doses up to MOI 25 were tested (table 2), with cultures assayed with doses reaching up to MOI 100 we obtained much more higher rate of H-1 PV induced CPE in the latter case. Thus this dose effect seems quite relevant and it was correlated with the levels of expression of NS1, with higher amounts of NS1 at MOI 100 rather than MOI 10 for example (data not shown).

If in vitro assays on short-term tumor-derived cell cultures reflect (and predict) the in vivo H-1 PV effects on breast tumors, our results suggest that with high doses of injected H-1 PV, most tumors may be sensitive to its cytotoxic effect.

Yet given the current H-1 PV production capacity, such doses would be difficult to reach in vivo. However if the virus is to be injected into or close to the tumor, a high number of virus particles per tumor cell could be reached for an average-sized tumor. Multiple injections might likewise be an appropriate way to reach effective doses [29].

The overall histopathologic characteristics of the tumors analyzed in our study are representative of invasive breast cancer in whole population. We sought to determine whether there was any correlation between sensitivity to H-1 PV-induced CPE and the histopathological subtype of the tumors from which a culture was derived. If so, any observed correlations might provide interesting criteria for the recruitment of patients into a future clinical trial. Van Pachterbeke *et al.* [21] have shown the presence of estrogen receptors to be a factor predictive of H-1 PV sensitivity. **In our study, we found no such correlation. Instead, we found that cultures established from higher tumor grades (Nottingham combined histopathologic grade 2 or 3) show significantly greater sensitivity toward H-1 PV-induced cytolysis ($p = 0.03$).** Such grades indicate that tumoral cells present in these samples are quite undifferentiated. Undifferentiated malignant cells [14] are often assumed to be particularly sensitive to H-1 PV-induced killing, though so far no transcription factor or growth factor or any other molecular element associated with the cell differentiation status has clearly surfaced.

NS1, a key multifunctional parvoviral protein, is particularly important in initiating replication of the viral genome [30] and producing new virions [31]. It is also critically involved in the cytotoxicity caused by the virus [15; 16]. Our data confirm this involvement, as we find NS1 expression, and especially the ratio NS1 to P-NS1, to differ in cells derived from breast tumors and normal breast tissue (Figure 4). In cultures of the latter (which are resistant to H-1 PV-induced CPE), NS1 was barely detectable at low MOI. At MOI 100 it was expressed, but seemed not to be correctly processed, since its phosphorylated (active) form was either absent or present at a much lower level than either NS1 or NS1-P in sensitive

cultures, even when corresponding blots were produced 48 hours after inoculation of H-1 PV at MOI 1.

NS-1 expression is under the control of the parvoviral promoter P4, the activation of which seems to depend on cell factors restricted to the S phase [19; 32]. Anouja *et al.* [33] have shown with a vector driving inducible, P4-independent NS-1 expression that only the non-phosphorylated form is expressed during the G₁/S transition. This suggests that NS-1 phosphorylation also depends on cell factors available during the S phase [33], hence the enhanced parvovirus activation observed in actively cycling cells. In our study, the tumors from which H-1 PV-sensitive cultures were derived showed higher mitotic grades than expected for normal breast tissue (not measured in the normal tissue samples we examined). This might explain the better production and phosphorylation of NS1 in tumor-derived cultures.

In conclusion, our study using an *in vitro* assay to estimate the effect of H-1 PV on cells derived from patients' tumor tissue constitutes a first validation step towards proof-of-concept that H-1 PV may be an effective viral anti-cancer agent. We confirm here what has been shown so far mostly with tumor cell lines, since up to 67% of the cultures successfully derived from breast tumors (such cultures are more representative of the heterogenic nature of patients' tumors) were sensitive to H-1 PV-induced CPE depending on the doses of virus assayed.

Our study also indicates that cells derived from tumors with a high mitotic grade (actively cycling cells), and more particularly those showing lower levels of differentiation (identified by higher Nottingham combined histologic grades; $p = 0.03$), are most sensitive to H-1 PV-induced CPE. Patients with such tumors could be given priority for inclusion in a phase I clinical study based on the use of H-1 PV.

Our study confirms H-1 PV as a serious candidate for use in viral anticancer therapy, especially if it is associated with standard chemotherapy or radiotherapy [6; 34].

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Figure 1: In vitro assay showing the effect exerted by H-1 PV on short-term tumor-derived cultures

a)- Short-term cultures were derived from HeLa-GFP tumor freshly excised from SCID-mice bearing tumors (picture 1). After 5 days post-infection, H-1 PV-induced cytotoxicity was

assessed in wells where doses of MOI 1 or MOI 5 virus were added as compared with mock-treated control wells (lower panel).

b)- Short-term cultures were derived from patients' freshly excised tumors (picture 1). Once sufficient amounts of cells were obtained (picture 2), they were seeded in equal number into different wells. Cells were mock-treated or inoculated with a defined quantity of virus. One week post-infection, either the plates were stained (pictures 3) or H-1 PV induced cytotoxicity was assessed with a MTT cell viability assay.

Figure 2: H-1 PV-induced cytopathic effect assay on primary breast tumor cells

By Giemsa staining and direct microscopic observation, H-1 PV-induced CPE were compared in mock-treated wells (CONTROL column) and wells where different doses of H-1 PV (columns MOI 1, MOI 10, and MOI 100) had been added. From the same patient, in (A) cultures derived from normal peritumoral breast tissue and in (B) cultures derived from invasive ductal carcinoma. The scale bar represents 200 μm .

Figure 3: Quantification of H-1 PV-induced CPE on MDA-MB-231 and SK-BR-7 cells in an MTT viability assay

a)- H-1 PV-induced CPE on MDA-MB-231 (A) and SK-BR-7 (B) cells 7 days post-infection, with increasing doses of virus (MOI 1, 5, and 10) or mock-treated cells (CONTROL) after Giemsa staining. Scale bar represents 500 μm .

b)- MDA-MB-231 and SK-BR-7 cell viability rates (\pm SD) measured with a MTT viability assay for each MOI, by comparison with the mock-treated CONTROL (viability=100%).

Figure 4: Comparison of the effects exerted by H-1 PV on cultures derived from tumoral and normal breast tissue

Cultures derived from normal breast tissue (A) and from an invasive ductal carcinoma (B).

a)- H-1 PV induced CPE 7 days post-infection (MOI 1 or 100) and mock-treated cells (CONTROL) after Giemsa staining. Scale bar represents 200 μm .

b)- Quantitative analysis of H-1 PV-induced oncolysis with an MTT viability assay.

c)- NS1 and β -actin western blot analysis on cell extracts from cultures inoculated with H-1 PV (lanes MOI 1 and MOI 100) or from mock-treated cultures (CONTROL lanes) 48 hours post-infection. The experiment was repeated at least 4 times with each type of cultures.

Figure 5: Tumor characteristics predictive of H-1 PV sensitivity

p = 0.03 when correlation between H-1 PV sensitivity and tumor histopathologic grade was made. Grade 1 tumors (lower grade) correspond to well-differentiated less-aggressive tumors, grade 2 tumors are quite undifferentiated and aggressive tumors and grade 3 tumors (higher grade) are the most undifferentiated and very aggressive tumors.

Table 1: Histopathologic characteristics of the tumor samples from which cultures were derived and assayed for H-1 PV induced CPE (*Material and Methods section*)

Invasive ductal carcinoma	82%
Invasive lobular carcinoma	18%
Tumor grade (Nottingham combined histologic grade)	Grade 1: 11% Grade 2: 51% Grade 3: 39%
Differentiation state	Grade 2: 16% Grade 3: 84%
Number of mitoses	Grade 1: 46% Grade 2: 35% Grade 3: 19%
Anisonucleosis	Grade 2: 35% Grade 3: 65%
Presence of vascular invasion	37%
Sentinel - axillary lymph nodes: positive	Sentinel : 42% Axillary : 60%
Estrogen receptor-positive	87%
Progesterone receptor-positive	67%

Table 2 : Breast carcinomas short-term derived cultures assayed with increasing doses of H-1 PV

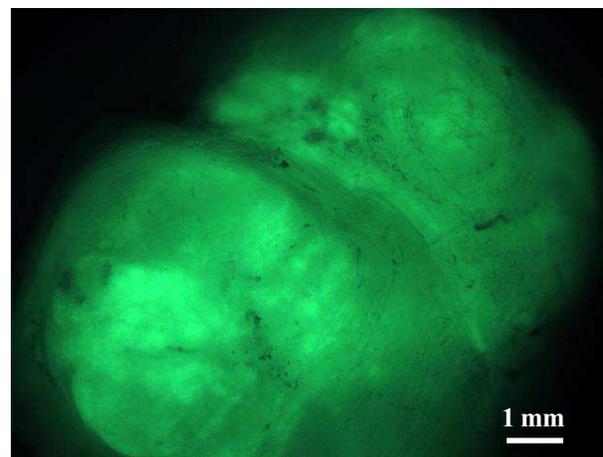
H-1 PV doses used	H-1 PV sensitive cultures	H-1 PV intermediate cultures	H-1 PV resistant cultures
Up to MOI 25 (n = 61)	48%	11%	41%
Up to MOI 100 (n = 18)	67%	11%	22%

H-1 PV induced CPE was examined visually after Giemsa coloration. Cultures were considered sensitive when more than 75% of the cells were lysed, intermediate when between 50% and 75% of cells were lysed and resistant when less than 50% cells were lysed.

Figure 1

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- Figure 1a -

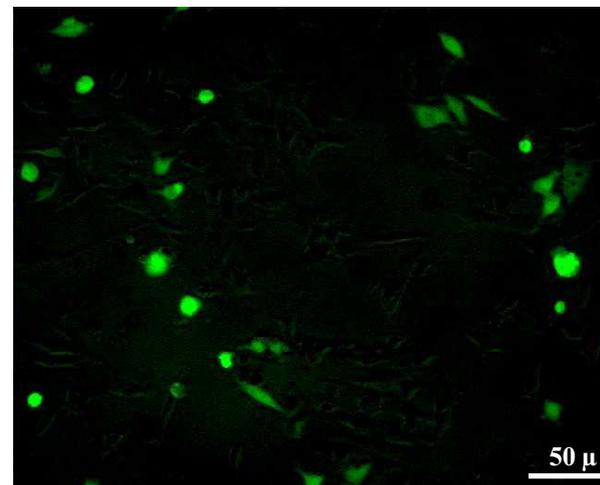
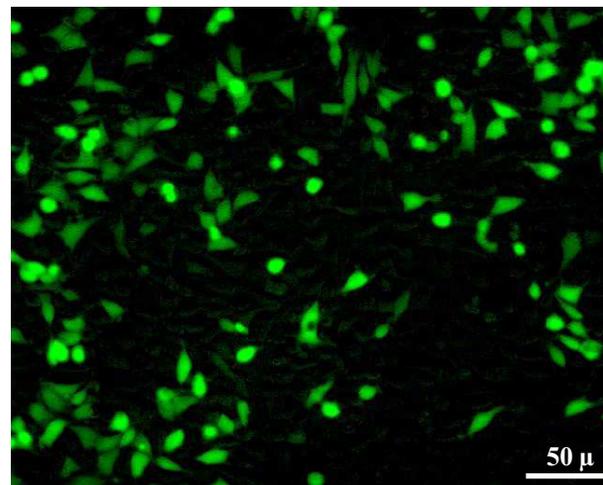
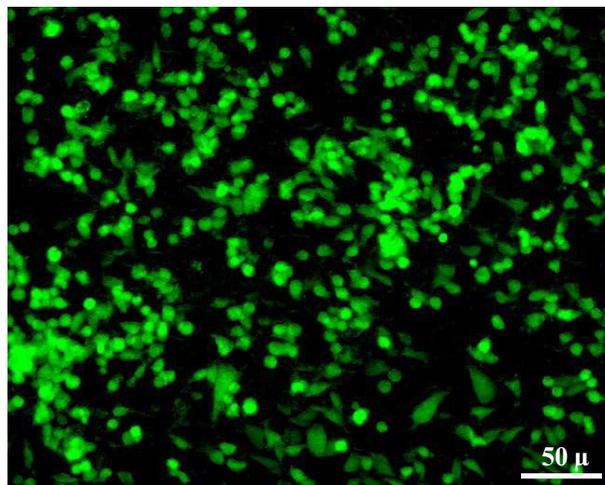


**Ex vivo tumour mechanical and enzymatic disaggregation
+
Tumor-derived cells H-1 PV infection**

CONTROL

MOI 1

MOI 5



- Figure 1b -

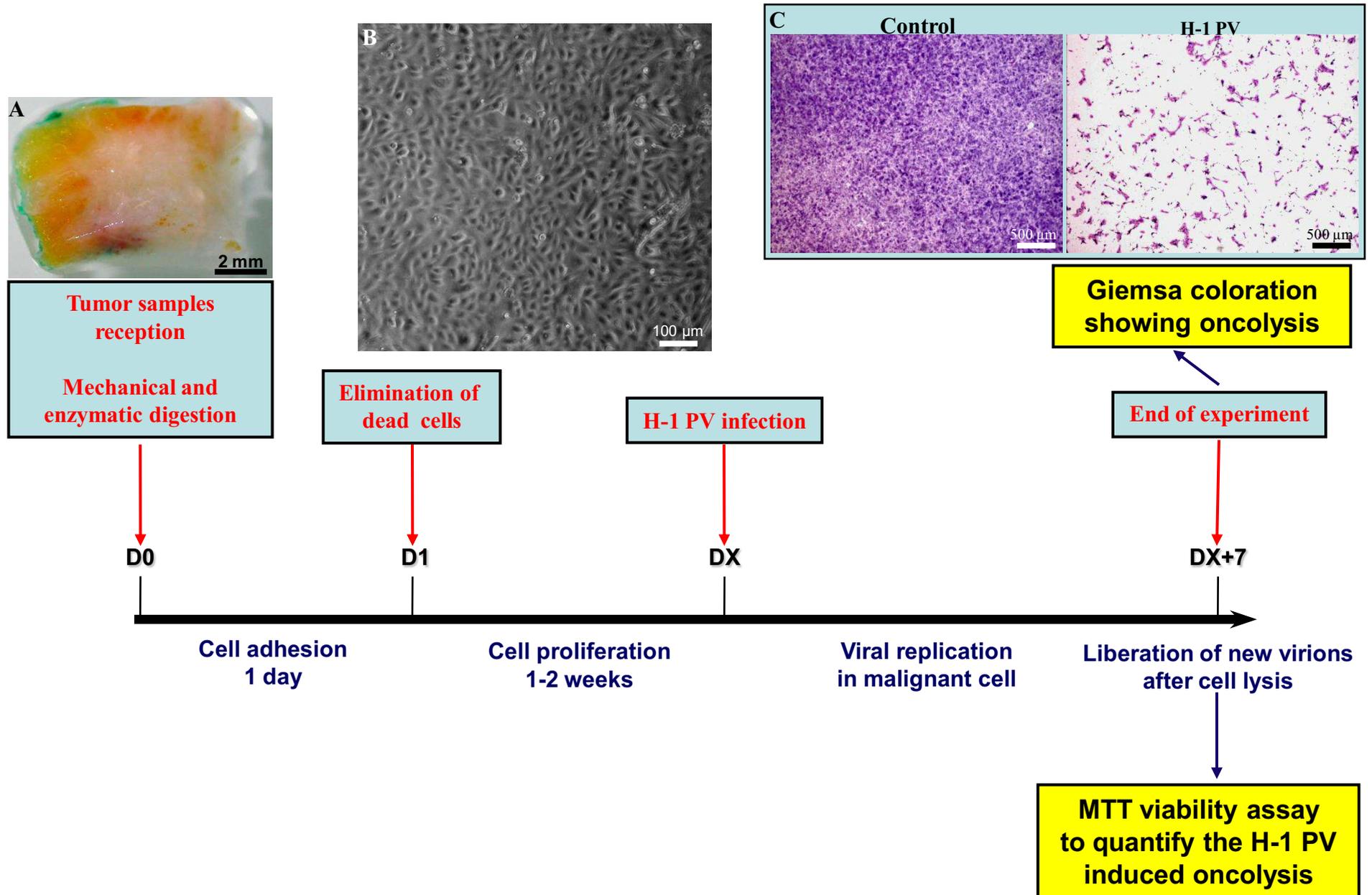


Figure 2

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- Figure 2 -

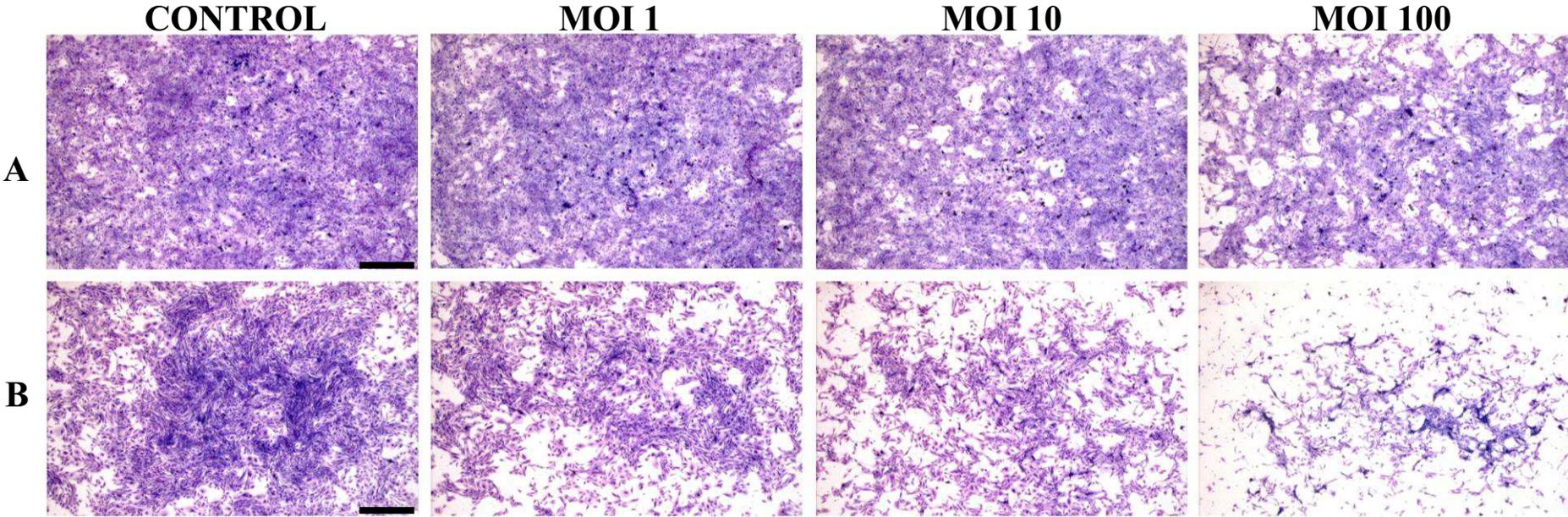
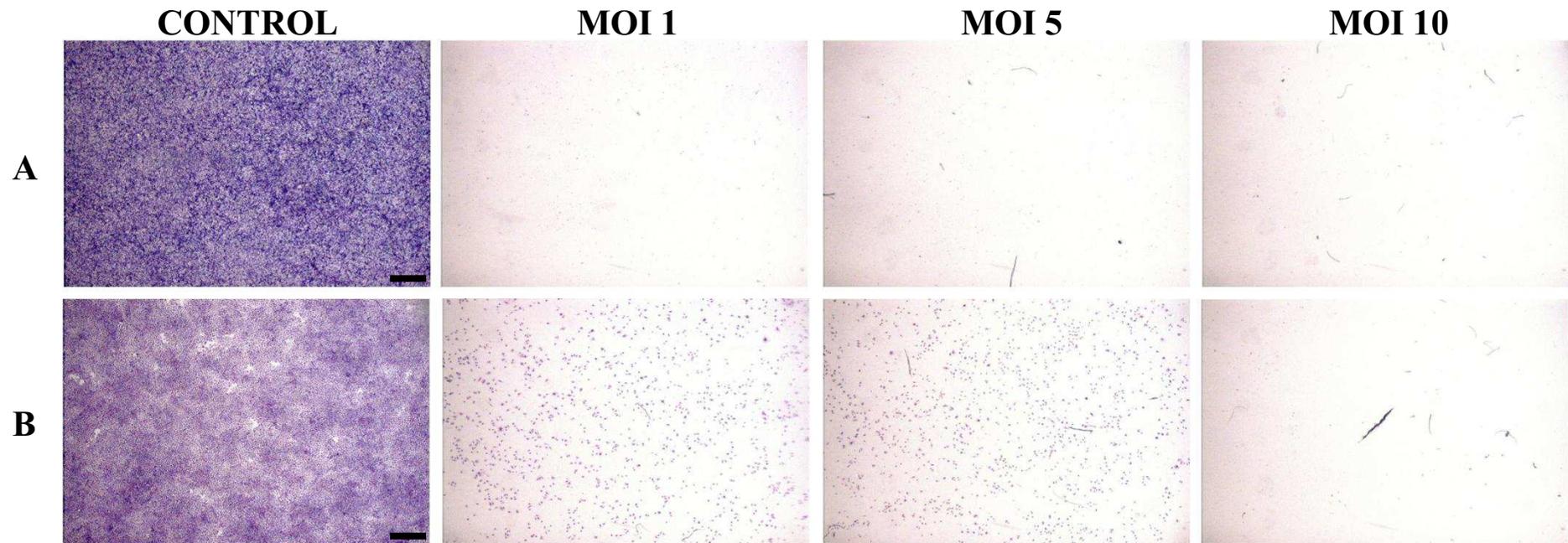


Figure 3

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- Figure 3a -



- Figure 3b -

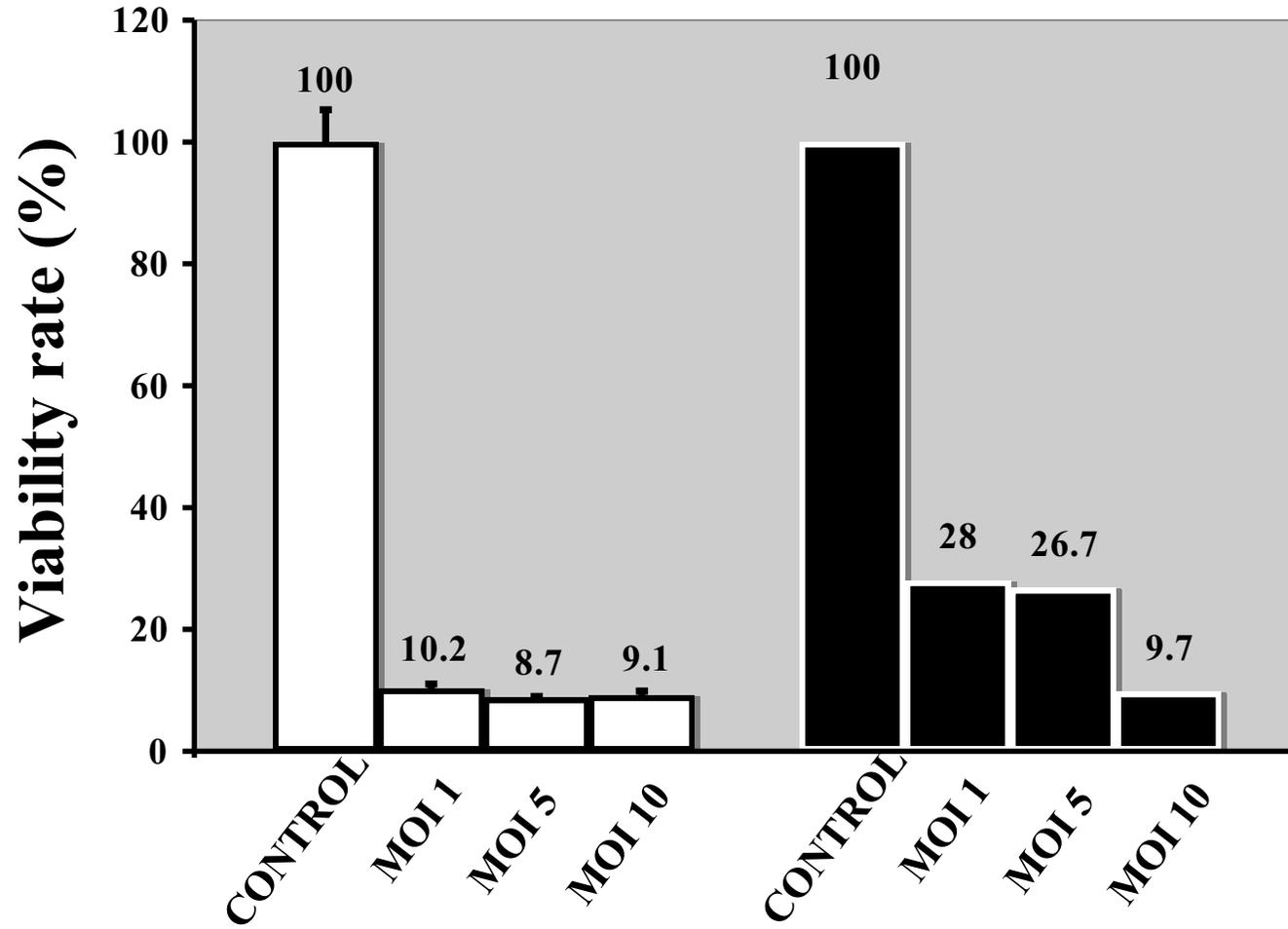
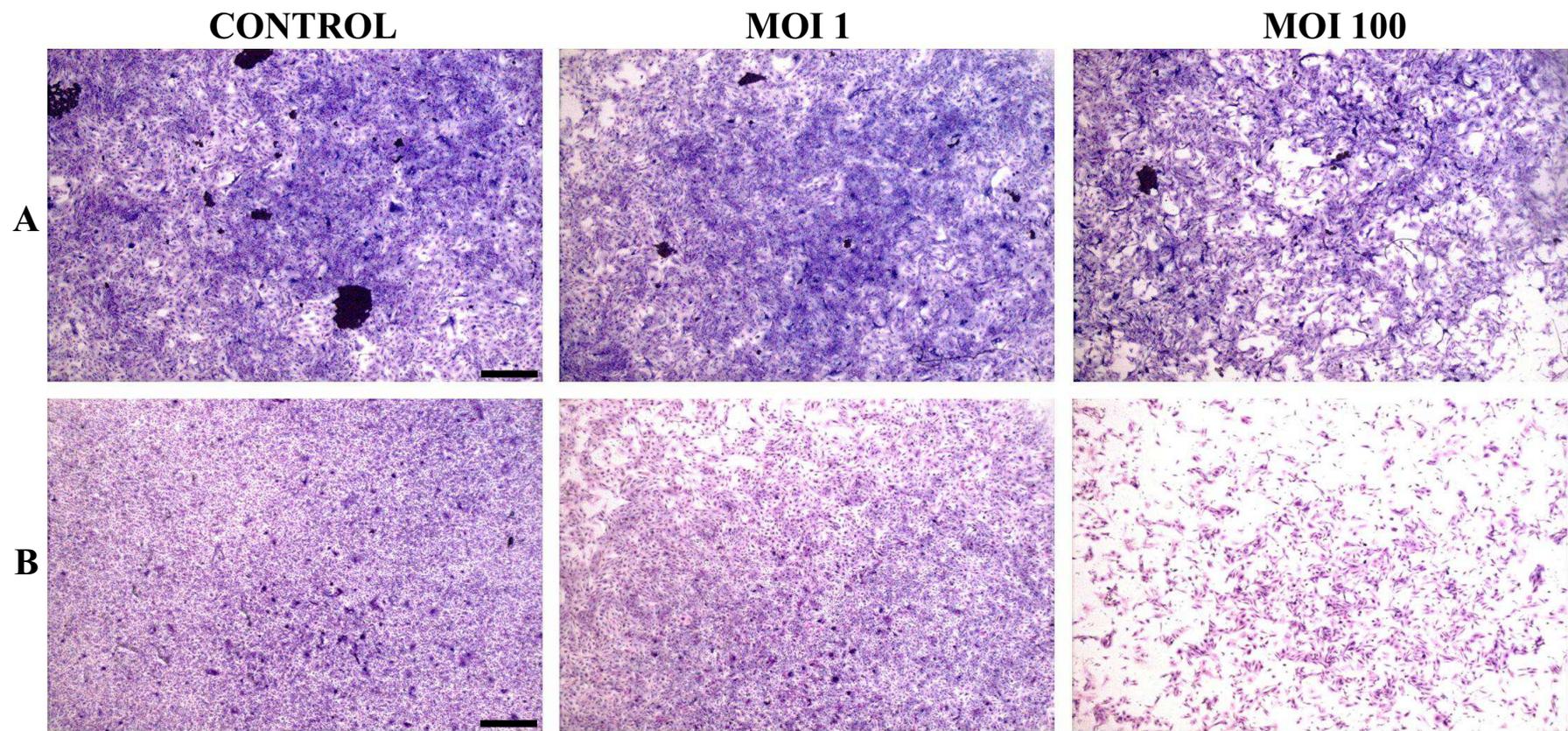
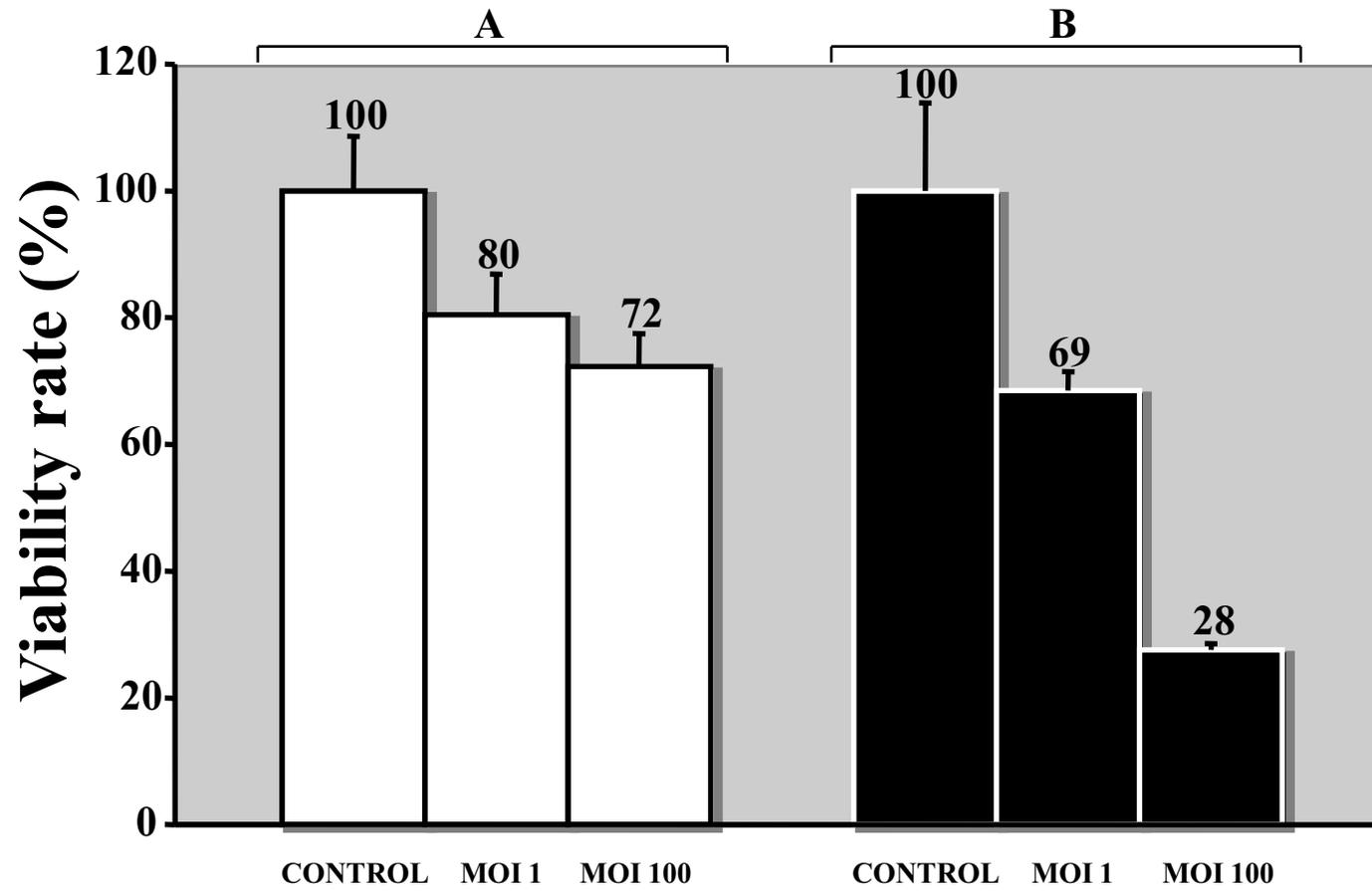


Figure 4
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- Figure 4a -



- Figure 4b -



- Figure 4c -

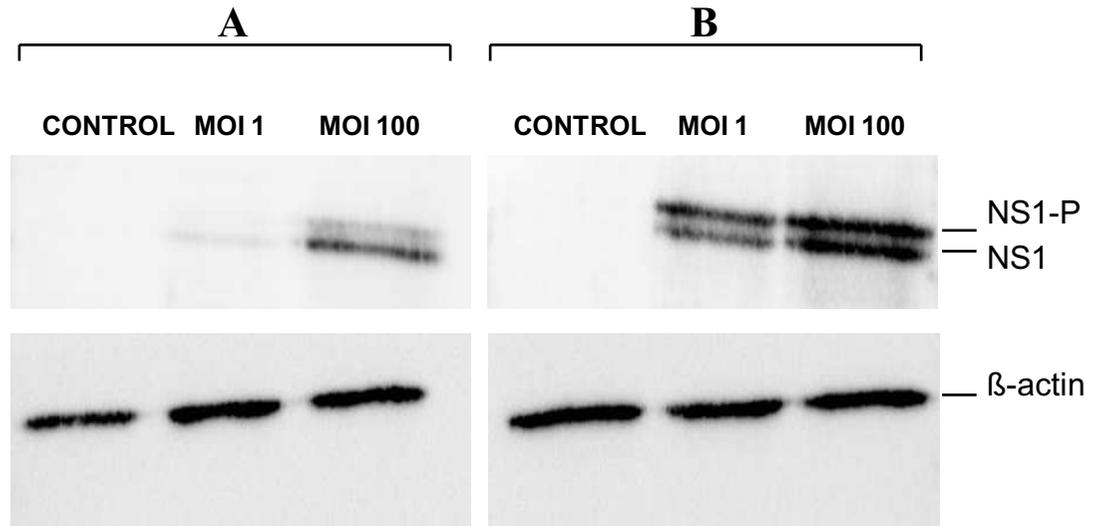


Figure 5

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- Figure 5 -

