

Epstein–Barr Virus *LMP1* Does Not Trigger Oxidative Stress or Caspase Activity under Transient Expression in NP69 Cells

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ABSTRACT

Objective: This study aimed to determine whether transient *LMP1* expression induces oxidative stress and caspase 3/7 activity in NP69 nasopharyngeal epithelial cells.

Material and Methods: NP69 cells were transiently transfected with *LMP1*-expressing (pTracer-*LMP1*, pcDNA-*LMP1*) or vector control plasmids. ROS levels were measured using DCFHDA or CellROX, and caspase 3/7 activity using Caspase-Glo®. Data were analysed using Independent Samples ttest and contingency analysis.

Results: Transient *LMP1* expression did not significantly increase ROS levels or caspase 3/7 activity in either pTracer or pcDNA systems ($p > 0.05$). Combined analysis also showed no significant association between *LMP1* expression and elevated oxidative stress or apoptosis.

Conclusion: These findings suggest that transient *LMP1* expression alone is insufficient to induce oxidative stress or apoptosis in normal nasopharyngeal epithelial cells. Additional cellular or viral factors may be required to trigger oxidative stress-related pathways implicated in NPC development.

KEY WORDS

LMP1, Epstein–Barr virus, nasopharyngeal carcinoma, oxidative stress, caspase 3/7, NP69, apoptosis

INTRODUCTION

Reactive oxygen species (ROS) arise as byproducts of cellular respiration when electrons leak during the electron transport process¹. Although ROS participate in normal signaling, excessive accumulation leads to oxidative stress, which damages DNA, proteins, and lipids and contributes to carcinogenesis²⁻⁴. Oxidative DNA lesions are primarily repaired through the Base Excision Repair pathway involving hOGG1 and XRCC1, and polymorphisms in these genes have been linked to increased nasopharyngeal carcinoma (NPC) risk⁵⁻⁷.

Oxidative stress can trigger apoptosis⁸, during which characteristic chromatin fragmentation occurs at matrix/scaffold attachment regions (MAR/SAR)^{9,10}. Caspase-activated DNase (CAD), released upon caspase3-mediated cleavage of ICAD, generates both highmolecular-weight (HMW) DNA fragments and internucleosomal DNA ladders^{11,12}. CAD-mediated DNA cleavage has been implicated in chromosome rearrangements in leukemia¹³ and is proposed to contribute to oxidative stress-induced chromosomal breaks in NPC, including recurrent breaks at AF9 on 9p22¹⁴⁻¹⁶.

Epstein–Barr virus (EBV), present in about 90% of the adult population¹⁷, plays a central role in NPC. In NPC tissues, EBV persists in a latent state expressing *LMP1/2*, EBNA1, EBEBs, and BARTs. *LMP1* promotes epigenetic alterations by activating DNA methyltransferase via JNK signaling¹⁸ and inducing BMI1¹⁹, while latent EBV genomes undergo host-mediated DNA methylation essential for maintaining latency²⁰. Stable EBV infection has been established in nasopharyngeal epithelial and NPC cell lines²¹, and *LMP1* can induce a stress-resistant phenotype²². Latent EBV infection in genetically altered epithelial cells drives clonal expansion, a key early event in NPC development²³. EBV

also expresses multiple antiapoptotic factors, aiding infected cell survival^{24,25}.

Collectively, oxidative stress, apoptosis-associated DNA cleavage, and EBV latency converge to create a cellular environment conducive to chromosomal rearrangements, contributing to NPC pathogenesis.

Table 1: Mean and Standard Deviation of Combination of Oxidative Stress and Caspase Activity in Transfected NP69

Transfection Plasmid DNA	N	Mean	Std. Deviation	Std. Error
pTracer	200	.43	.496	.035
pTracer - <i>LMP1</i>	200	.40	.490	.035
pcDNA	200	.47	.500	.035
pcDNA- <i>LMP1</i>	200	.44	.498	.035
pTracer & pcDNA	400	.41	.492	.025
pTracer - <i>LMP1</i> & pcDNA- <i>LMP1</i>	400	.45	.498	.025

Received on February 19, 2026 and accepted on February 27, 2026
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DOI: https://doi.org/10.82781/intmedj.33.3_242

Table 2: Association of Oxidative Stress and Caspase Activity with LMP1 Expression

Plasmid DNA		t-value	Df	Sig. (2-tailed)	95% Confidence Interval of the Difference	
					Lower	Upper
pTracer & pTracer- <i>LMP1</i>	Equal variances assumed	.609	398	.543	-.067	.127
	Equal variances not assumed	.609	397.950	.543	-.067	.127
pcDNA & pcDNA- <i>LMP1</i>	Equal variances assumed	.501	398	.617	-.073	.123
	Equal variances not assumed	.501	397.991	.617	-.073	.123
pTracer & pTracer- <i>LMP1</i>	Equal variances assumed	-1.213	798	.225	-.111	.026
	Equal variances not assumed	-1.213	797.886	.225	-.111	.026
pcDNA & pcDNA- <i>LMP1</i>						

MATERIALS AND METHODS

Plasmid Preparation and Bacterial Culture

pTracer, pTracer-*LMP1*, pcDNA, and pcDNA-*LMP1* plasmids were transformed into ampicillin-resistant *E. coli*. Bacteria were grown overnight on LBampicillin plates and subsequently cultured in LB broth with 50 µg/ml ampicillin. Plasmid DNA was extracted using the PureLink™ HiPure Midiprep Kit (Invitrogen) according to the manufacturer's protocol. Purified plasmids were eluted in TE buffer and sequence-verified.

Cell Line and Culture

NP69 nasopharyngeal epithelial cells^{26,27} were maintained in KeratinocyteSFM supplemented with rEGF, BPE, 2% FBS, and penicillin/streptomycin at 37°C, 5% CO₂. Cells were subcultured at 70% confluency using TrypLE Express.

Transient Transfection

Cells were seeded at 70-85% confluency and transfected with Lipofectamine 3000 (Thermo Fisher Scientific) following the manufacturer's protocol. DNA-lipid complexes were incubated with cells for 4 hours before replacement with fresh medium. GFP expression (pTracer constructs) was used to confirm transfection. Cells were collected at 24-72 hours for downstream assays.

Transfection for ROS and Caspase Assays

NP69 cells (2 x 10³/well) were seeded in 96well plates and transfected with 0.2 µg plasmid DNA. After 4 hours, the medium was replaced, and cells were incubated for 48 hours.

Measurement of Oxidative Stress

ROS levels were measured 48 hours posttransfection.

- **pcDNA / pcDNA-*LMP1*:** Cells were incubated with 10 µM DCFHDA for 30 minutes; fluorescence (Ex 485 nm/Em 530 nm) was recorded.
- **pTracer / pTracer-*LMP1*:** Due to GFP spectral overlap, ROS was measured using CellROX Deep Red (5 µM); fluorescence was measured at Ex 640 nm/Em 665 nm.

Each condition was assayed in 20 replicates.

Measurement of Caspase Activity

Caspase 3/7 activity was assessed using the CaspaseGlo® 3/7 Assay (Promega) 48 hours posttransfection. Cells were incubated with CaspaseGlo reagent for 30 minutes at room temperature, and luminescence was measured using a SpectraMax® iD3 reader.

Statistical Analysis

Data analysis was performed using SPSS version 23. The associa-

tion between *LMP1* expression and oxidative stress and caspase activity was evaluated using the Independent Samples t-test.

RESULTS

Table 1 summarizes the combined effects of *LMP1* expression on oxidative stress and caspase activity, using low (0) and high (1) categories based on earlier mean values. Across 400 transfection replicates, the differences between *LMP1*-expressing cells and vector controls were very small. In the pTracer system, *LMP1*-transfected cells had a mean score of 0.40 compared to 0.43 in controls. In the pcDNA system, the means were 0.44 for *LMP1* and 0.47 for controls. These minor differences, along with large standard deviations (~0.49-0.50), indicate that *LMP1* does not meaningfully alter combined oxidative stress and caspase activity. When both systems were combined, the overall mean was 0.45 for *LMP1*-transfected cells and 0.41 for vector controls, again showing only minimal variation. Overall, the results suggest that transient *LMP1* expression does not significantly increase oxidative stress or apoptosis under these conditions.

Table 2 examines whether *LMP1* transfection is associated with the combined levels of oxidative stress and caspase activity in pTracer-*LMP1* and pcDNA-*LMP1* cells. In the pTracer system, the association was not statistically significant ($t = 0.609$, $p = 0.543$) when compared with vector controls. Similarly, in the pcDNA system, no significant association was observed ($t = 0.501$, $p = 0.617$). When data from both systems were combined, *LMP1* expression still showed no significant association with the combined oxidative stress and caspase activity ($t = -1.213$, $p = 0.225$). These findings indicate that *LMP1* does not meaningfully influence these two parameters under the tested conditions.

DISCUSSION

Latent Membrane Protein 1 (*LMP1*) is a key EBV oncoprotein involved in the development of EBV-associated malignancies in both B cells and epithelial cells²⁸. Acting as a constitutively active mimic of CD40, *LMP1* activates multiple signaling pathways through its CTAR1 and CTAR2 domains, including NF-κB, PI3K/AKT, MAPK/ERK, JNK, JAK/STAT, and p38/MAPK^{29,30}. Although well characterized in B cell lymphomas, *LMP1* functions differ in epithelial cells where its expression is much lower than in EBV-transformed B cells³¹. *LMP1* is considered a major EBV oncogene in nasopharyngeal carcinoma (NPC)³².

LMP1 overexpression has been linked to increased ROS generation and oxidative stress (Feng *et al.*, 2021), a process also observed during EBV latency³³. *LMP1* can promote ROS production via pathways such as JNK/p38/AP1³⁴, while mitochondrial dysfunction in cancer cells further elevates ROS levels and contributes to DNA damage^{35,36}. Oxidative stress can trigger apoptosis and DNA damage, promoting tumor progression³⁷.

Apoptosis is generally considered tumorsuppressive, but apoptotic caspases can also induce genomic instability^{38,39}. Oxidative stress activates intrinsic apoptosis through mitochondrial release of cytochrome c, leading to apoptosome formation and activation of caspases 9, 3, and 7⁴⁰. Caspases respond to oxidative damage and regulate programmed cell death^{41,42}. During apoptosis, caspase-activated DNase (CAD) cleaves

chromatin at MAR/SAR sites, generating highmolecularweight DNA fragments and nucleosomal ladders^{12,15}. Such cleavage can contribute to chromosome breaks and genomic instability.

Genomic instability, including frequent copynumber variations and structural alterations, is a hallmark of NPC^{43,44}. Oxidative stress, DNA damage, and mitochondrial dysfunction can induce apoptosis and may contribute to chromosomal rearrangements in NPC^{16,45}. CAD may play a key role in cleavage events driven by oxidative stress-induced apoptosis.

However, in the present study, no significant association was observed between *LMP1* expression and combined oxidative stress or caspase activity. This may reflect the absence of significant oxidative stress induction by transient *LMP1* expression in NP69 cells. In contrast, previous studies have shown that oxidative stress can induce apoptosis in various cell types, including leukaemia cells⁴⁶, glioma cells⁸, and both normal and NPC nasopharyngeal epithelial cells¹⁵. ROS are also widely recognized as triggers of tumor cell death⁴⁷.

CONCLUSION

In this study, transient expression of *LMP1* in NP69 nasopharyngeal epithelial cells did not significantly alter oxidative stress or caspase 3/7 activity. These results contrast with previous reports linking *LMP1* to ROS production and apoptosis in EBV-associated malignancies, suggesting that the oxidative and proapoptotic effects of *LMP1* may depend on cell type, expression level, or duration of expression. Our findings imply that transient *LMP1* expression alone is insufficient to drive oxidative stress-induced apoptosis or chromosomal damage in normal nasopharyngeal epithelial cells. This supports the notion that additional cofactors—such as stable EBV latency, persistent *LMP1* expression, mitochondrial dysfunction, or tumor microenvironmental stress—may be necessary to initiate the genomic instability characteristic of NPC. Future work using stable *LMP1* expression models or EBV-infected nasopharyngeal epithelial cell systems may help clarify the mechanistic contributions of *LMP1* to NPC pathogenesis.

ACKNOWLEDGEMENTS

We would like to thank Universiti Malaysia Sarawak (UNIMAS) for the infrastructure provided. This work was supported by the Fundamental Research Grant Scheme (FRGS) from the Ministry of Higher Education, Malaysia with grant no. FA 052000-0708-0030.

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