Inflammatory mediator-induced bystander effects and genomic instability: role of tissue-specificity

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التأثيرات المشاهدة وعدم الاستقرار الجيني المستحث بالوسيط الالتهابي: دور خصوصية النسيج

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المستخلص

كثير من الدلائل أوضحت أن الالتهاب المستحث بالإشعاع يستطيع أن يسبب ضرر خلوي في الخلايا المعرضة للإشعاع بشكل مباشر و الخلايا المشاهدة التي تكون بالقرب من الخلايا المشعة تحت عنوان التأثيرات المشاهدة للإشعاع BE. كما إن عدم الاستقرار الجيني المستحث بالإشعاع GI قد برهن وبشكل وافي في الخلايا الأبناء للخلايا المعرضة للأشعة والخلايا المشاهدة كاستجابة متأخرة. حاولت هذه الدراسة أن تقلد التأثيرات المشاهدة وعدم الاستقرار الجيني للإشعاع باستخدام التأثير الالتهابي لليبوبوليساكرايد LPSعلي خلايا الإنسان المنتجة للألياف HF 19 والخلايا الطلائية للحبل السري HUVEC.

لاختبار التاثير البايايولجي لل LPS وإمكانيته على إحداث ضرر في DNA تم معاملة خلايا HF19 ب 1مايكروغرام/مل LPS, بينما استحث BE بتقنية نقل الوسط الزرعي لخلايا غير معاملة بال LPS. أستخدم اختبار المذنب لقياس التحطم الكلي لل DNA في الخلايا المعاملة ب LPS بشكل مباشر وكذلك الخلايا المشاهدة. كانت نتائج الفحص المبكر (الجيل الأول) للخلايا المعالجة مباشرة ب LPS والمشاهدة أعلى من نتائج مجاميع السيطرة وبشكل معنوي. إلا أن التأثيرات المتأخرة GI لم تلاحظ في أبناء الجيل الثلاثين لكلا المجموعتين. لذا الدراسة اقترحت إن LPS له تأثير مبكر على ال DNA لخلايا HF19 وليس تأثير متأخر.

كررت التجربة باستخدام نوعين من الخلايا HF19 و HUVEC حيث تم قياس التحطم الكروموسومي في المجاميع التجريبية لتلك الخلايا. أظهرت النتائج أن الخلايا المعاملة مباشرة بال LPS والخلايا المشاهدة لخلايا HF19 و HUVEC أعطت تحطم كروموسومي معنوي ($p\leq0.05$) مقارنة مع مجاميع السيطرة. غير أن عدم الاستقرار الكروموسومي (التأثير المتأخر) لوحظ فقط في أجيال الخلايا المعاملة بشكل مباشر من خلايا HUVEC فقط.

اقترحت النتائج أن LPS قادر على حث BE في خلايا HF19 و HUVEC. كما يعتقد ان السايتوكاينز الالتهابي المتسبب بوساطة LPS له دور في تلك العملية. علاوة على ذلك يعتقد أن LPS يشترك في ظاهرة عدم الاستقرار الجيني ولكن يكون محدد بخصوصية النسيج.

Abstract

Much evidence showed that radiation-induced inflammation can cause cellular damage in direct irradiated and bystander cells, which are in vicinity of irradiated cells underlying radiation-induced bystander effects (BE). As well as radiation-induced genomic instability (GI) is observed within the progeny of irradiated and bystander cells as a delayed damage responses. This study tried to mimic the effects

of radiation-induced GI and BE by exploring the inflammatory effect of lipopolysaccharide (LPS) on human primary fibroblast (HF 19) and umbilical vein endothelial (HUVEC) cells.

To prove the principles of LPS can cause DNA damage in HF 19; cells were treated with 1 µg/ml LPS, and BE was induced by media transfer. Comet assay was used to estimate DNA damage in the direct treated and bystander cells. The early DNA damage result was significantly observed in the direct treated and bystander HF 19 cells. However, these cells did not show a significant delayed DNA damage within their progeny. Comet data showed that LPS frequently induced only initial DNA damage in HF 19 cells. The experiment was repeated utilising HF 19 and HUVEC cells, in order to investigate whether LPS could be involved in the induction of GI in a tissue-specificity manner. Both HF 19 and HUVEC cells were treated with 1 µg/ml LPS. BE was also induced by media transfer. Chromosomal analysis was used for early and late chromosomal damage estimation. Data showed that LPS treatment could significantly cause early chromosomal aberrations in the direct treated and bystander HF 19 and HUVEC cells. However, delayed chromosomal instability was observed only in the direct treated HUVEC cells.

Our finding suggested that LPS frequently induced BE in HF 19 and HUVEC cells, which could be mediated by pro-inflammatory cytokines that stimulated by LPS treatment. Moreover, LPS could be involved in the GI induction in a tissue-specificity manner.

Keywords: Chromosomal instability, Bystander effects

Introduction

In the last 2 decades many studies have demonstrated that communication between irradiated and un-irradiated cells can induce cellular damage responses in the un-irradiated cells underlying radiation-induced bystander effects (1, 2). These un-irradiated/bystander cells receive bystander molecular signals that produced by irradiated cells through molecules in the irradiated cell condition media (ICCM) or via intercellular communication through cell gap junctions. In addition, much evidence has reported that both irradiated and bystander cells show a delayed damage responses within their progeny, in terms of radiation-induced genomic instability (GI). GI is thought to be a critical step in the onset and progression of cancer. (3-6).

Bystander cell populations demonstrate a wide range of biological responses, with many phenotypic similarities to GI. The nature of the soluble transmitting factor(s) is yet to be fully understood, but cytokines including interleukin (IL)-8 (7), transforming growth factor (TGF)-beta (8), and tumour necrosis factor (TNF)-alpha (9, 10), as well as calcium fluxes, nitric oxide (NO) (1), reactive oxygen species (ROS) (11) and exosomes (12) have been suggested as mediators of bystander responses. A role for plasma membrane-bound lipid rafts has also been indicated (13).

Lipopolysaccharide (LPS) is one of the major components of the outer membrane of Gram- negative bacteria and it has toxic properties (14, 15). As well as it can stimulate inflammation in humans (16). Furthermore, LPS has a group of proteins that are typically composed of a hydrophobic domain, the lipid A, which is an

endotoxin (17), and can cause damage to the cells components including DNA (18). LPS also stimulates macrophages and monocytes to produce pro-inflammatory cytokines (19).

The aim of this study therefore, was to mimic the effects of radiation-induced GI and BE by exploring the inflammatory effect of LPS on primary human fibroblast (HF 19) and umbilical vein endothelial (HUVEC) cell line, and test the hypothesis that inflammation responses of cells/tissues by LPS treatment could contribute to the induction of GI and BE.

Materials and methods

Cell culture

Primary human fibroblast (HF 19) and umbilical vein endothelial (HUVEC) cells were utilised in this study. The HF 19 cells were maintained as described by Kadhim, et al. 1998 (20). In brief, Cells were cultured in MEM media (Gibco, 21090) supplemented with 15 % FBS 2 mM L-Glutamine (Gibco, 25030) and 1% (v/v) penicillin/ streptomycin solution (Sigma, P0781). Whilst the HUVEC cells, which were purchased from the European Collection of Cell Cultures, were maintained endothelial basal media-2 (EBM-2, Lonza: CC-3156) that was supplemented with EGM-2 kit (Lonza: CC-4179) and foetal bovine serum (Sigma: F7524). Both of these cells were incubated in a humidified 5% CO2 incubator at 37 ºC.

LPS treatment and experimental design

The cells were treated with 1 µg/ml of LPS (Sigma, L2630) for 24 hours at 70% cell confluence for direct LPS treated cells. For bystander cells, cells were treated with of LPS for 1hour. Then the medium was removed and the cells were washed once with sterile PBS to remove the residual of LPS. Fresh medium was then added to the treated cells for 4 hours. The treated cell condition media (TCCM) was collected, filtered through 0.2µm filter and added to fresh cells to induce bystander effects. Initial DNA damage and chromosomal aberrations were measured in the cells after 1 population doubling following LPS treatment. Additionally, cells were propagated for 30 population doublings for chromosomal instability estimating.

Comet assay

The HF 19 cells were first subjected to Single-cell gel electrophoresis or comet assay, to investigate whether LPS can induce chromosome damage in the direct treated, bystander cells and the progeny of the direct treated and bystander cells. Comet assay is a sensitive method to quantify total DNA damage (double-strand breaks, single-strand breaks and base damage) in individual cells (21, 22). The comet assay was performed as described by (23, 24). Briefly, microscope slides were coated with 1% normal melting point agarose (NMPA) and allowed to dry overnight. The coated slides were then placed on a metal tray on ice. 2×10^4 cells were re-suspended with 200 µl of 0.6% low melting point agarose (LMPA) and placed immediately onto chilled pre-coated slides. The cell-LMPA suspensions were flattened with cover slips, which were removed after 5-10 minutes. The slides were then transferred to a Coplin jar, which was filled with cold lysis buffer (2.5 M NaCl, 100 mM EDTA pH 8.0, 10

mM Tris-HCl pH 7.6, and 1% Triton X-100, pH >10). The jar was kept at 4ºC over night. The slides were then moved to a horizontal electrophoresis tank filled with electrophoresis buffer (0.3 M NaOH and 1 mM EDTA, pH 13) at 4ºC for 40 minutes. The electrophoresis was run for 30 minutes, at 19V, 300A. Slides were neutralised with neutralising buffer (0.4 M Tris-HCl, pH 7.5), washed with distilled water, and immediately stained with a 1:10,000 dilution of SYBR Gold (Molecular Probes/Invitrogen, Carlsbad, CA). The slides were analysed using Komet 5.5 Image Analysis Software (Kinetic Imaging Technology/Andor, Germany).

Chromosomal analysis

To confirm the targeted and non-targeted effect of LPS on cells; as well as to investigate the tissue specificity of LPS, both HF 19 and HUVEC cells were analysed for a cytogenetic analysis using Giemsa solid staining technique. Cells were harvested for metaphase preparation at 60-70% confluence using our established method (25). Briefly, cells were treated with 20ng/ml demecolcine (Sigma, D0125) for 1.5 hours in a humidified 5% CO_2 incubator at 37ºC. Cells were centrifuged at 259g for 10 minutes. The supernatant was discarded, whilst the cell pellet was re-suspended with a hypotonic solution (75 mM potassium chloride solution (KCl, Sigma, P5405)) for 20 minutes at 37ºC. The hypotonic cell suspensions were centrifuged at 180g for 10 minutes. The supernatant was aspirated, and the cell pellet was fixed twice with 25% acetic acid in methanol. Fixed cells were then dropped onto clean slides, and stained using Giemsa solid staining technique. Slides were coded and at least 100 metaphases were analysed per group.

Statistical analysis

For analysis of comet assays at least 200 cells per group were analysed; statistical analysis was performed using the Mann-Whitney Test, utilising the median of raw data. The Mann-Witney test deals with the median of non-parametric data and it is an acceptable test for comet data (26, 27). Each experiment was carried out 3 times. Analysis showed no significant inter-experimental variation; therefore, data from these experiments was pooled. Chromosomal data represented as mean aberrations per cell, was subjected to Fisher's exact test. For each group, 100 metaphases were analysed.

Results and discussion

HF 19 cells were treated with 1 μg/ml LPS and subjected to comet assay measuring the total DNA damage, in order to prove that LPS has a biological effect on cells. The findings demonstrated a significant induction of DNA damage ($p \leq 0.0001$) in the directly treated HF 19 cells with LPS compared to the non-treated HF 19 (control cells) (Fig 1A). Data postulated that LPS could cause DNA damage induction through inflammation (28-30). In addition, LPS has a group of proteins that are typically composed of a hydrophobic domain, the lipid A, which is an endotoxin (17), which can induce damage to the DNA (18). The result demonstrated that LPS was able to cause BE in the HF 19 cells. Media transfer technique was used to achieve communication between treated and non-treated cells. The TCCM significantly ($p \leq 0.0001$) induced DNA damage in the bystander HF 19 cells (Fig 1A). The findings

have been supported by much evidence documented that tumour necrosis factor-α (TNF-α) and cytokines can play crucial roles in BE induction (31-33).

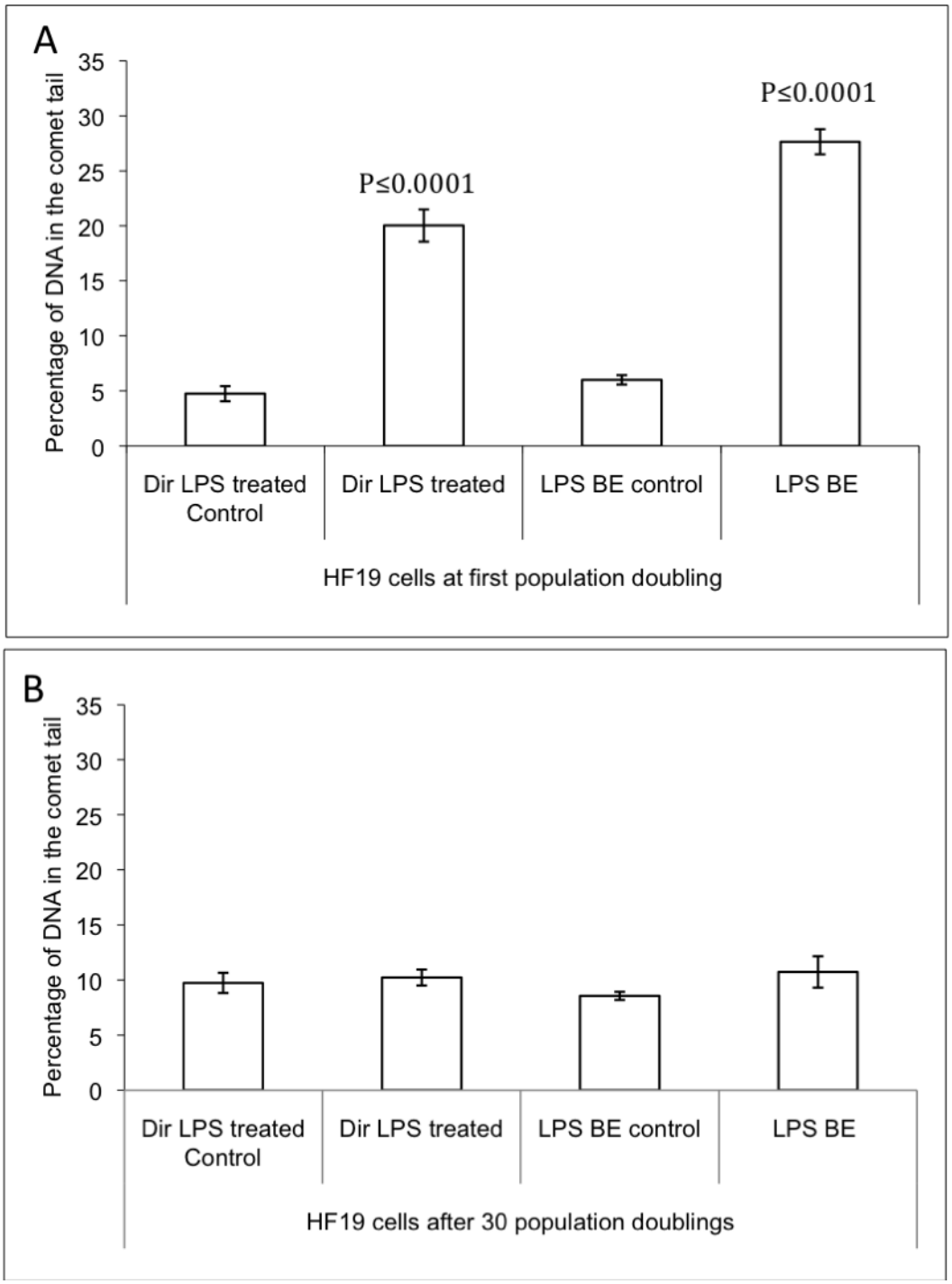

Figure (1): Total DNA damage in HF 19 after 1 and 30 population doublings following LPS treatment.

Panel A represented the initial DNA damage in the direct treated and bystander HF 19 cells. Cells were treated with 1µg/ml LPS for 24 hr, and considered as direct treated cells. BE was induced by incubating HF 19 cells with 1µg/ml for 1 hr. Then the media was removed and the cells were washed with PBS. Fresh media was added to the cells for 4 hr. The media then was collected and filtered through 0.2 µm filter and added to fresh/non-treated cells, which considered as bystander cells. Bystander cells were incubated for 24hr. Both direct and bystander HF 19 cells were subjected to comet assay to measure the

early response of DNA damage. The result showed that both direct treated and bystander HF cells demonstrated a significant initial DNA damage ($p \leq 0.0001$) after 1 generation following LPS treatment, compared to the corresponding controls. Panel B showed the delayed DNA damage in the progeny of the direct treated and bystander HF 19 cells. Both direct treated and bystander HF 19 cells were propagated up to 30 population doublings following irradiation, and then comet assay was carried out to estimate the delayed total DNA damage. The progeny of the direct and bystander cells did not demonstrate a significant DNA damage after 30 generations following LPS treatment. Data suggested that LPS could cause only initial DNA damage in direct treated HF 19 cells. Furthermore, the media from direct treated HF 19 cells was able to induce BE in fresh HF 19 cells.

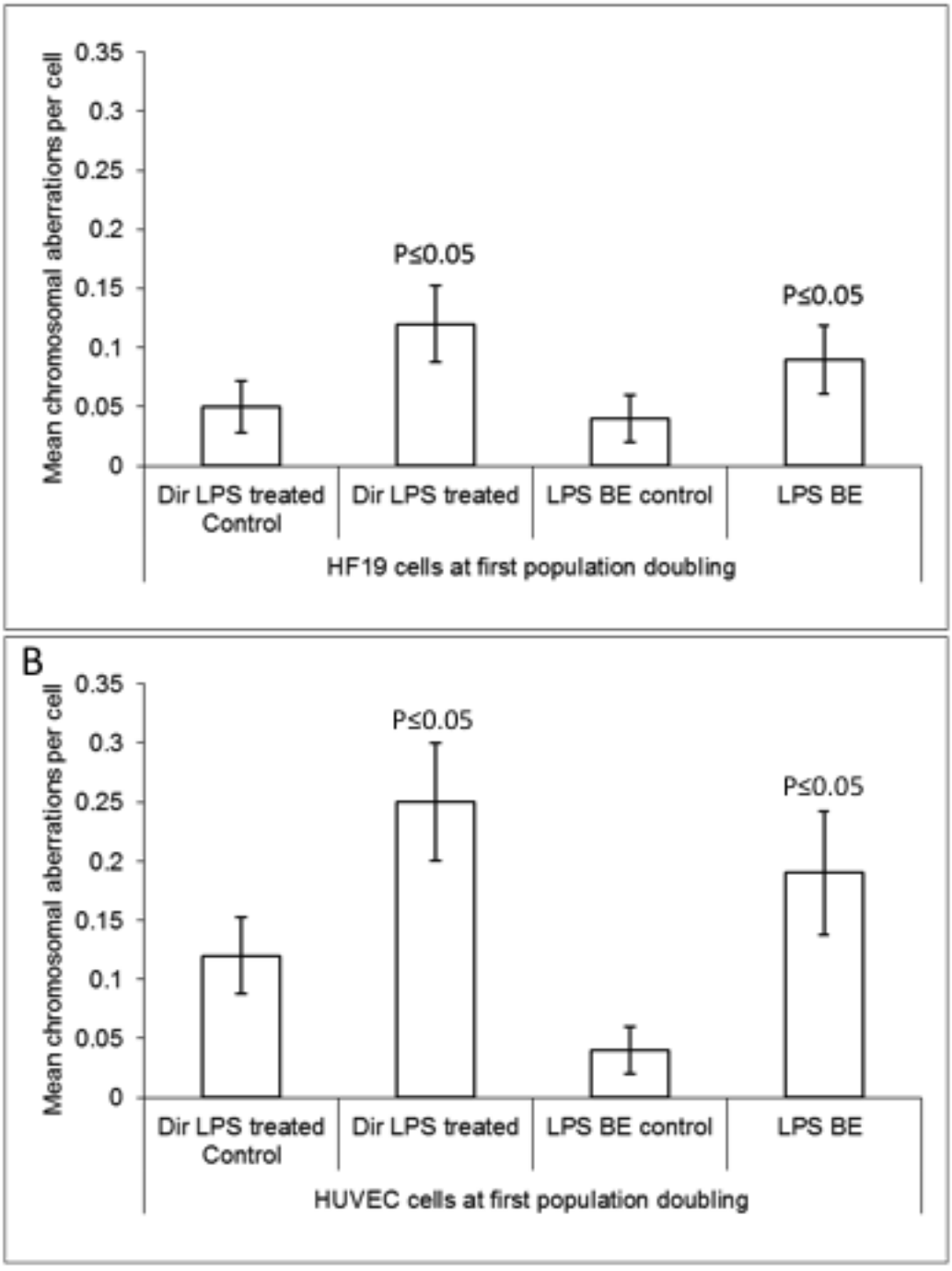

Figure (2): Chromosomal aberrations in the HF 19 and HUVEC cells after 1 population doubling following LPS treatment.

Both HF 19 and HUVEC cells were separately treated with 1µg/ml LPS for 24 hr, and considered as direct treated HF 19 and HUVEC cells. BE was separately induced in HF 19 and HUVEC cells by

incubating HF 19 and HUVEC cells with 1µg/ml for 1 hr. Then the media was removed and the cells were washed with PBS. Fresh media was separately added to HF 19 and HUVEC cells for 4 hr. The media then was separately collected and filtered through 0.2 µm filter and added to fresh/non-treated HF 19 and HUVEC cells, which considered as bystander cells. Bystander cells were incubated for 24hr. Chromosomal analysis was performed to measure the initial response in these groups of cells. Panel A showed the early chromosomal aberrations in the direct treated and bystander HF 19 cells, which displayed a significant chromosomal damage ($p \leq 0.05$) after 1 generation following treatment. Data suggested that LPS could cause chromosomal damage in the direct LPS treated HF 19. As well as the TCCM from HF 19 cells frequently induce BE in fresh HF 19 cells. Panel B showed the ability of LPS to induce chromosomal damage in the HUVEC cells. Similarly to the HF 19 chromosomal data, HUVEC cells demonstrated a significant chromosomal damage ($p \leq 0.05$) in both direct treated and bystander groups after 1-generation post LPS treatment. Data also suggested that LPS could mediate chromosomal damage in the direct treated HUVEC cells, and the media from treated cells could cause BE in fresh HUVEC cells.

Both treated and bystander HF-19 cell populations were cultured until 30^{th} generation to estimate the delayed damaged responses, which is GI, within the progeny of experimental cells; and whether LPS can be involved in the late DNA damage. The progeny of both direct treated and bystander HF 19 cells did not show a significant delayed DNA damage compared to the control (Fig 1B). Data suggested that the damaged cells repaired the damage or could not repair the damage and died; or LPS has a tissue specificity, which can induce GI in a different kind of tissue.

Therefore, the experiment was repeated using HF 19 and HUVEC cells. Chromosome analysis had been utilised to measure the effect of LPS in the direct treated, bystander and the progeny of direct treated and bystander cells. The treated and bystander HF 19 and HUVEC cells demonstrated a significant ($p \leq 0.05$) early chromosomal aberrations following LPS treatment as shown in figure 2 (A and B), in which Chromosomal data confirmed comet data in the first experiment (Fig 1). Our finding suggested that initial chromosomal damage could be mediated by inflammation, which was induced by LPS (34).

To investigate the delayed damage effect of LPS and GI in the progeny of treated and bystander HF 19 and HUVEC cells, cells were propagated up to 30 population doublings; then chromosomal analysis were utilised to estimate the level of chromosomal instability in these cells. The HF 19 cells did not show a significant chromosomal instability in the treated and bystander cells after 30 generations following LPS treatment compared to their controls (Fig 3A). However, the progeny of direct treated HUVEC cells demonstrated a high induction of chromosomal instability ($p \leq 0.0001$) as shown in figure 3B. Our chromosomal data confirmed our comet data suggestion, that LPS could be involved in the induction of GI, but in a tissue specificity manner. Kim and co-worker have documented that media from LPS-treated prostate epithelial cells has IL-1β, IL-6 and TNF-α. They suggested that LPS can stimulate pro-inflammatory cytokines secretion (35). These long lived signals (cytokines) can induce stress and injury, which can instigate GI and BE (36).

Surprisingly, the bystander HUVEC cells did not show a significant chromosomal instability in their progeny after 30 population doublings following TCCM transfer (Fig 3B). Data suggested that the high level of chromosomal damage in the bystander

HUVEC cells could lead to cell death leading to remove the cells with multi-chromosomal damage and abrogate GI.

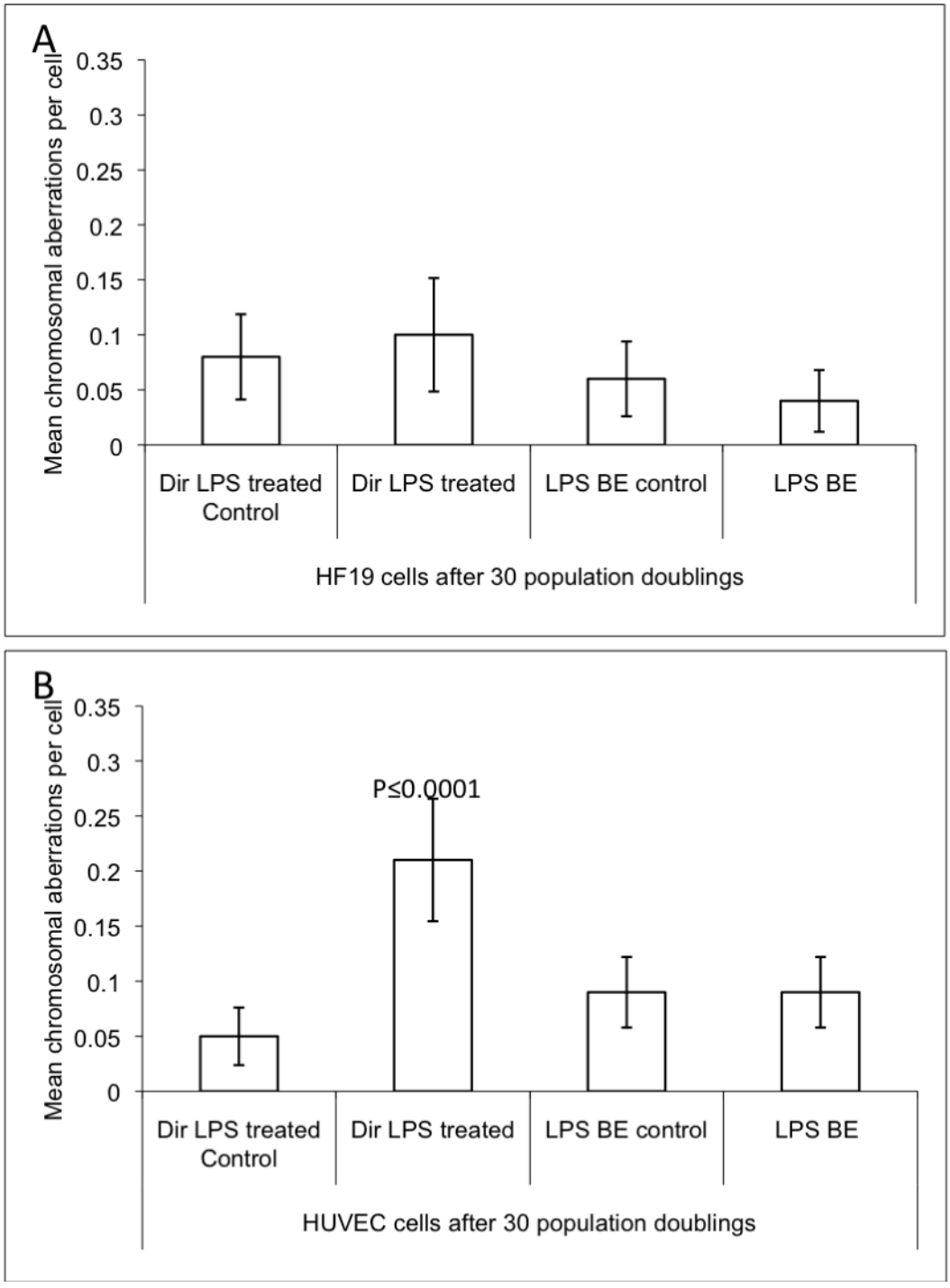

Figure (3): Chromosomal aberrations in HF 19 and HUVEC cells after 30 population doublings following LPS treatment.

The direct treated and bystander HF 19 and HUVEC cells were propagated up to 30 population doublings. The chromosomal analysis was carried out to estimate the level of chromosomal instability in these cells. Panel A displayed the progeny of the direct treated and bystander HF 19 cells, which did not showed a significant chromosomal instability after 30 generations post LPS treatment. Panel B illustrated the chromosomal damage response in the progeny of direct treated and bystander HUVEC

cells after 30 generations following LPS treatment. Only the progeny of the direct treated HUVEC cells showed chromosomal instability, which was statistically significant compared to the controls. The progeny of bystander HUVEC cells did not demonstrate a significant late chromosomal damage. The result suggested that LPS could be involved in the induction of GI, but in s tissue-specificity manner.

Conclusion

LPS can be considered as a inflammatory mediator, which can induce early chromosomal damaged responses in the direct treated HF 19 and HUVEC cells. As well as, the TCCM from treated HF 19 and HUVEC cells has a crucial role in BE induction in the non-treated/fresh HF 19 and HUVEC cells respectively. Furthermore, LPS can be involved in the induction of GI, but in tissue specificity manner, in which, LPS can induce GI in the HUVEC cells not in the HF 19 cells. As well as only the direct LPS-treated HUVEC cells showed GI within their progeny.

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