Nanosecond Pulsed Electric Fields (nsPEFs) Induce Socs1 and Socs3 Gene Expressions but not HeLa Cells

Nanosecond Pulsed Electric Fields (nsPEFs) Menginduksi Ekspresi Gen Socs1 dan Socs3 namun bukan pada Sel HeLa

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ABSTRACT
Nanosecond Pulsed Electric Fields (nsPEFs) is one of bioelectric technologies applied widely in a number of sciences. nsPEFs cause some biological responses and known to play a role as a novel cancer therapy. However, the nsPEFs molecular mechanisms have not been fully elucidated. This study determines the effects of nsPEFs in socs (Suppressor of Cytokine Signaling) genes which are target genes of JAK/STAT signaling pathway. Through a negative feedback mechanism, SOCS proteins can suppress both cytokine signal transduction and overgrowth factor, so the cell growth is controlled. In cervix cancer, the presence of E6 and E7 HPV’s oncoprotein is associated with methylation and inactivation of socs1 and socs3 genes. This mechanism is related to the increase of STAT expression and cancer prognostic. In this research, nsPEFs as much as 20 kV/cm for 80 ns was exposed over HeLa S3 cells in 4 mm cuvette. Socs1, socs2 and socs3 gene expressions were analyzed using real time PCR SYBR green and reverse transcription PCR (RT-PCR). This study shows that at 20 and 30 shots, nsPEFs significantly increase socs1 and socs3 but not socs2 gene expression. Effect of nsPEFs on socs1 and socs3 gene expression pattern is influenced by duration of post exposure incubation and each cell activity on internal cell condition. This research provides a new cancer therapy target for nsPEFs.

Keywords: Bioelectric, gene expression, nsPEFs, shot, socs gene

ABSTRAK
Nanosecond Pulsed Electric Fields (nsPEFs) merupakan salah satu teknologi bioelektrik yang secara luas diaplikasikan pada sejumlah bidang sains. NsPEFs dapat menyebabkan sejumlah respon biologis dan diketahui berperan sebagai salah satu novel terapi kanker. Meski demikian, mekanisme molekular nsPEFs belum banyak diketahui. Penelitian ini bertujuan untuk mengetahui efek nsPEFs pada gen socs (Suppressor of Cytokine Signaling) yang merupakan salah satu gen target jalan sinyal JAK/STAT. Melalui aktivitas umpan balik negatif, protein SOCS dapat menekan transduksi sinyal sitokin dan faktor pertumbuhan yang berlebih sehingga dapat mengontrol pertumbuhan sel. Pada kanker serviks, kehadiran onkoprotein HPV E6 dan E7 diasosiasikan dengan metilasi dan inaktivasi gen socs1 dan socs3. Mekanisme tersebut juga berkaitan dengan peningkatan ekspresi STAT dan peningkatan prognosivitas kanker. Pada penelitian ini, nsPEFs sebesar 20 kV/cm selama 80 ns dipaparkan pada sel HeLa S3 di dalam kuvet 4 mm. Ekspresi gen socs1, socs2 dan socs3 dianalisis menggunakan real time PCR SYBR green dan reverse transcription PCR (RT-PCR). Penelitian ini menunjukkan bahwa pada 20 dan 30 kali tembakan, nsPEFs mampu meningkatkan ekspresi gen socs1 dan socs3, namun bukan socs2. Efek nsPEFs pada kedua gen tersebut juga dipengaruhi lamaanya waktu inkubasi pasca paparan dan aktivitas masing-masing gen pada kondisi internal sel. Penelitian ini memberikan target terapi kanker baru bagi nsPEFs.

Kata Kunci: Bioelektrik, ekspresi gen, gen socs, nsPEFs, tembakan

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INTRODUCTION

Nanosecond pulsed electric fields (nsPEFs) are one of the bioelectric application that developed from conventional electroporations. Generally, electroporations are used to deliver plasmid, gene, antibody or other molecules to cell for some purposes especially for therapy (1). Unlike electroporations, nsPEFs are exposure with high electric fields (1-100 kV/cm) in very short duration (1-300 nanoseconds). It causes low energy density and non thermal effect (2). In cell membrane, nsPEFs cause nanoporeformation (1-1.5 nm) without disturb the membrane integrity (3). NsPEFs also activate voltage-gated Ca2+ channels and increases Ca2+ influx into cytoplasm (4).

Unlike conventional electroporations, nsPEFs raise some intracellular responses. In the high intensity, nsPEFs reach subcellular level and can be active in cellular system (5). In mitochondria, nsPEFs increase mitochondrial membrane permeability (mPTP) and cause cytochrome c release (6,7). NsPEFs also cause Ca2+ influx into mitochondria and induce dissipation of membrane potential (ΔΨm) that implicate to cell viability (7,8). NsPEFs also cause Ca2+ release from ER (9), involve in some signaling pathways (10-12), and induce stress responses (13). In the nucleus, nsPEFs increase some endogen gene expressions (14). In the high intensity (>60kV/cm), nsPEFs can cause actin filament depolymerization, disturb telomere attachment to the nucleus and disturb DNA stability (15).

Recently, application of electric fields in an extremely short duration with high intensity is widely used in various fields of the life science including for cancer therapy. NsPEFs can induce apoptosis via intrinsic and extrinsic pathways (16-18). NsPEFs also decrease cell proliferation and metastasis via suppression of NFκB and Wnt/β-catenin signaling (12), decrease viability of cancer cell (19), increase cell death (20), have antiangiogenic activity (21,22) and decrease viability of cancer cell (19). NsPEFs also activate voltage-gated Ca2+ channels and increases Ca2+ influx into cytoplasm (4).

Suppressor of Cytokine Signaling (SOCS) proteins are identified as tumor suppressor proteins and one of the cancer targeted therapies. In normal condition, the active SOCS have important role in negative feedback mechanism of JAK/STAT signaling pathway and inhibit cytokine and growth factor signal transduction (24). In some cancers, overexpressions and dysregulations of JAK/STAT signaling pathway are associated with high proliferation and low apoptosis. However, SOCS are methylated and silenced in many cancers (25-32). Dysregulations of SOCS are also associated with phosphorylation defect and mutation (33,34). SOCS1, SOCS2 and SOCS3 are reported as the three most active SOCS protein with some tumor suppression roles.

Cervical cancer is one of gynecological cancer that caused by HPV infection. Two oncoproteins of HPV, E6 and E7, can trigger carcinogenesis and genetic instability (35). The presences of HPV's genomes are associated with overexpression of STAT3 and STAT5, and are also implicated to prognosis of cancer (36,37). STAT3 and STAT5 overexpression are also associated with epigenetic control as well as methylation at promoter of socs1 gene (38). E6 and E7 can induce methylation in some tumor suppressor genes including socs1 and socs3 (39). Wherease, the activity of socs2 has not found in cervical cancer. However, the overexpression of STAT3 and STAT5 as the degradation target of socs2 is one reason to investigate socs2 gene expression (40).

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TCCCCCAAGAGCTATTAC-3'; R: 5' - TCCGACAGAGACTGAAAGTGT-3'; GAPDH (452 bp), F: 5' - A C C A C A G T C C A T G G C A T C A C - 3', R: 5' - TCCACCACCTGTGCTGTA-3'.

**Statistical Analysis**

The present data were expressed as mean ± SD. The distribution of data was evaluated using Kolmogorov-Smirnov analysis. The data were analyzed using one way analysis of variance (ANOVA) with HSD Tukey post hoc test, simple t-test, and correlation-regression test. Statistic significance was set at P value < 0.05.

**RESULTS**

**NsPEFs Increased Socs1 and Socs3 but not Socs2 Gene Expressions**

Previous study reported that 20 kV/cm during 80 ns duration at 20 and 30 shots treated into HeLa S3 cell decreased 15% and 30% cell viability and cell growth (41). Our results showed that these numbers of nsPEFs increased socs1 (Figure 1A) and socs3 (Figure 1C) but not socs2 (Figure 1B) gene expressions.

Based on the one way ANOVA, we suggest that nsPEFs significantly increased socs1 and socs3 but not socs2 gene expressions. At 1 hr, 20 and 30 shots nsPEFs increased socs1 gene expression approximately 2.45 and 5.78 folds. Socs1 gene expression increased up to 4.90 and 10.34 folds at 4 hr. NsPEFs also increased socs3 gene expression. At 1 hr, 20 and 30 shots nsPEFs increased socs3 gene expressions approximately 3.19 and 5.42 folds. At 4 hr, 20 and 30 shots nsPEFs increased socs3 gene expression approximately 2.28 and 3.33 folds. NsPEFs did not increase socs2 gene expression.

Effect of nsPEFs on socs1, socs2 and socs3 gen expressions at 1 hr and 4 hr were observed using different analysis respectively using real time PCR and RT-PCR. Based on the simple t-test, the expression of socs1, socs2 and socs3 were significantly different between at 1 hr and 4 hr in 0, 20, and 30 shots nsPEFs respectively socs1 (p<0.000; p<0.014, p<0.010), socs2 (p<0.015; p<0.024; p<0.047), and socs3 (p<0.044; p<0.002; p<0.006).

**Shot Number of NsPEFs Correlated with Socs1 and Socs3 but not Socs2 Gene Expressions**

Based on the correlation-regression analysis, we suggest that different shot number of nsPEFs correlated with socs1 and socs3 but not socs2 gene expressions as we show at the Figure 2.
Gene expression pattern of socs2 in control group and treatment group increased, but these expressions were not significantly different. These data showed that nsPEFs resulted in changes gene expression pattern in socs1 and socs3, but not in socs2.

**DISCUSSION**

**NsPEFs Probably Decrease Proliferation and Induce Apoptosis via Increasing of Socs1 and Socs3 Gene Expressions**

In the cervical cancer, HPV infections cause socs1 and socs3 methylations (36, 37, 39). These methylations implicate to STAT3 and 5 overexpressions (38). Generally, methylation of socs1 and socs3 implicate to high proliferation and low apoptosis in some cancers (36-39). Wherease, the roles of socs2 have not been known in this cancer. Our results show that 20 and 30 shots of 20 kV/cm nsPEFs in 80 ns duration increased socs1 and socs3 but not socs2 gene expressions. From the control group of our results indicate that there were not any changes on socs2 gene expressions.

**Different Pattern of Socs1 and Socs3 Gene Expressions Followed NsPEFs**

Gene expression pattern was determined to know the dynamic of nsPEFs effect on socs1, socs2, and socs3 gene expressions during a certain time (see Figure 3). Gene expression pattern of socs1 due to 20 and 30 shots nsPEFs increased from 1 hr to 4 hrs. In contrary, the control group was decreased during 4 hrs. Unlike socs1, socs3 gene expression initially increased at 1 hr but subsequently decreased after 1 hr up to 4 hr. The pattern of control group of socs3 decreased too, but still lower than treatment group. Gene expression pattern of socs2 in control group and treatment group increased, but these expressions were not significantly different. These data showed that nsPEFs resulted in changes gene expressions pattern in socs1 and socs3, but not in socs2.

**Figure 2. Correlations between shot number of NsPEFs with Socs1, Socs2, and Socs3 gene expressions**

*Note:* The correlation between shot number and socs1 and socs3 gene expressions were indicated with positive correlation, but negative correlation for socs2.

**Figure 3. Profil of gene expression pattern of socs1, socs2, and socs3 responded to 20 and 30 shots nsPEFs from 1 hr to 4 hr**

**Note:**

- ○: Linear (0x)
- ■: Linear (20x)
- ▲: Linear (30x)

**Socs1**

- $0x - y = -0.046x + 1.046$ ($R^2 = 0.997$)
- $20x - y = 0.816x + 1.633$ ($R^2 = 0.977$)
- $30x - y = 1.521x + 4.258$ ($R^2 = 0.937$)

**Socs2**

- $0x - y = 0.401x + 0.481$ ($R^2 = 0.989$)
- $20x - y = 0.345x + 0.654$ ($R^2 = 0.994$)
- $30x - y = 0.450x + 0.150$ ($R^2 = 0.999$)

**Socs3**

- $0x - y = -0.226x + 1.226$ ($R^2 = 1$)
- $20x - y = -0.050x + 2.867$ ($R^2 = 0.926$)
- $30x - y = -0.696x + 6.119$ ($R^2 = 0.963$)
Methylations on promoter of some genes in cervical cancer such as socs1 and socs3 are associated with HPV infections and presence of E6 and E7 oncoproteins (38-39). E6 and E7 increase DNA methyltransferase 1 (DNMT1) that catalyzes attachment of methyl group to cytosine in CpG Island. Binding E7 to pRb (E7/pRb) cause the release of E2F, favoring the expression of DNMT1. Binding of E2F to DNMT1 (E7/DNMT1) induced a conformational change in DNMT1 and exposed its DNA binding site and promoting DNA binding (39).

Difference Gene Expression between Soc1 and Soc3 are Determined by Time of NsPEFs Post Exposure and Activity of Each Gene

Based on gene expression pattern analysis, we know that NsPEFs caused different gene expression pattern between socs1 and socs3 genes. We can see this different expression in Figure 6.

Possibility Mechanism of NsPEFs to Induce Soc1 and Soc3 Gene Expressions

This research did not provide any data to explain the mechanism of nsPEFs to induce socs1 and socs3 gene expressions. The high intensity of nsPEFs enter cell and nucleus and induce some endogen genes (14). Possibly, mechanism of nsPEFs to induce socs1 and socs3 gene expressions is demethylation.

In general, JAK/STAT signal transduction determines all socs genes as a target of this pathway. However, methylation of socs1 and socs3 gene expressions in cervical cancer makes this signal can not raise socs1 and socs3 genes. Therefore, we think that methylation process is more important to determine socs1 and socs3 gene expressions than JAK/STAT signal transductions. Our results show that nsPEFs only induced socsmethylated genes, socs1 and socs3 but not socs2. These results suggest that nsPEFs may induce socs1 and socs3 by demethylation process. We also propose that nsPEFs does not induce socs1 and socs3 by JAK/STAT signal transduction. Our results and hypothesis are describes in Figure 5.

Figure 5. A schematic diagrams of possibility mechanism of NsPEFs to increase socs1 and socs3 gene expressions

Note: NsPEFs probably induce socs1 and socs3 gene expression by demethylation than JAK/STAT signal transduction.

At 1 hr, nsPEFs induced socs1 and socs3 gene expressions. Probably, increasing of these gene expressions are induced by directly effect of nsPEFs on socs1 and socs3 demethylations that involve decreasing of DNMT1 and methyl degradation. However, there are different gene expressions at 4 hrs between socs1 and socs3. Soc1 gene expressions increased from 1 hr up to 4 hrs, but socs3 decreased.

The high expressions of socs3 at 1 hr probably caused negative feedback mechanism on JAK/STAT and decreased the target gene such as c-myc and bcl-xl. From this mechanism, socs3 expression also decreased at 4 hrs but still higher that control (cancer cell). Unlike socs3, socs1 gene expression increased at 4 hrs. The high expression of socs1 gene probably also caused by induction of NFκB.

Figure 6. A schematic diagrams of socs1 and socs3 gene expressions pattern due to nsPEFs exposure.

Note: 1: primary demethylation effect by nsPEFs; 2a: secondary demethylation effect by E7 degradation; 2b: decreasing of demethylation effect; ?: The process between 1 hr to 4 hrs, it may be associated with negative feedback mechanism of SOCS on JAK/STAT
cervical cancer, E6 and E7 induce NFκB signaling pathway and increase expression of the target genes including cyclin, cdk, bcl-xl, and socs1. However, socs1 was methylated in HPV infection (39) and cannot decrease O65 subunit (45). Therefore, proliferation increase and apoptosis decrease. Demethylated socs1 by nsPEFs probably involve in JAK/STAT and NFκB. High expression of NFκB by E6 and E7 may induce socs1 gene expression without methylation at 4 hrs.

SOCS1 has many activities in cervical cancer than SOCS3. SOCS1 is better to decrease proliferation in HeLa S3 than SOCS3 (42). This study also showed that SOCS1 is adaptor of E3 that specific to degrade E7. E7 degradation is important to repress genetic instability in cervical cancer. Low level of E7 is implicated to DNMT1 repression and demethylation some tumor supressor genes such as socs1 and socs3 (39). Low level of E7 also implicated to inhibition E2F and repression of some genes such as c-jun and c-fos (46). SOCS1 can degrade p65 subunit of NFκB and decrease cyclin, cdk, and bcl-xl (45). SOCS1 but not SOCS3 can increase p53 and p21 expressions and implicated to low proliferation and apoptosis induction (47).

According to the discussion, nsPEFs probably directly induce demethylation and activation of socs1 and socs3. However, after activation, the expressions of socs1 and socs3 are determined by activity of each gene in cellular mechanism. From this result we know that the higher expression of socs1 than socs3 probably determine the activity of these gene to decrease proliferation in HeLa S3 cell. This result is suitable with the previous research (42).

**High Shot Number do not always Associate with High Gene Expressions**

Shot number is one of characteristic that also determine the electric fields. Different shot number is proven associated with different cell viability (41) and expression of some gene (13). Our result show that shot number is positively correlates with socs1 and socs3 gene expressions. Higher shot number caused higher socs1 and socs3 gene expression, but not in socs2. We suggest that high shot number do not always associate with high gene expressions.

Finally, we conclude that nsPEFs increase socs2 and socs3 but not socs2 gene expressions. The shot number correlates positively with the socs1 and socs3 gene expressions. High shot number do not always correlate with high gene expression. Effect of nsPEFs on socs1 and socs3 gene expression pattern depends on time of nsPEFs post exposure and type of gene. Because socs1 and socs3 have some roles in tumor suppressor, nsPEFs possibly have potentially as anti cancer.

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