Nanosecond Pulsed Electric Fields (nsPEFs) Induce Socs1 and Socs3 but not Socs2 Gene Expressions in Hela S3 Cells

Nanosecond Pulsed Electric Fields (nsPEFs) Menginduksi Ekspresi Gen Socs1 dan Socs3 namun bukan Socs2 pada Sel HeLa S3

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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

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INTRODUCTION

Nanosecond pulsed electric fields (nsPEFs) are one of 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 Ca \(^+\) \(\text{channels and increases Ca}^{2+}\) 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 Ca \(^{2+}\) influx into mitochondria and induce dissipation of mitochondrial potential (ΔΨm) that implicate to cell viability (7,8). NsPEFs also cause Ca \(^+\) 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), decrease tumor mass and size (12)(20), haveantiangiogenic activity (21,22), and increase the immune system (23).

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, overexpression 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 defective 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 overexpressions are also associated with epigenetic control as well as methylation at promotor 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 investigating socs2 gene expression (40).

The role and molecular mechanisms of nsPEFs in cancer have not been fully elucidated. In this research, we will investigate the effect of nsPEFs on socs1, socs2 and socs3 gene expressions. This research will provide a new possibility role of nsPEFs on cancer therapy.

METHOD

Cell Culture and Sample Preparation

HeLa S3 cells (ATCC) were cultured in α-minimum essential medium (α-MEM)(WAKO) supplemented with 10% fetal bovine serum (FBS) (Equitech Bio), and penicillin/streptomycin (WAKO). Cells were maintained in a humidified atmosphere of 5% CO\(_2\) at 37°C. The cells were counted using cell counter coulter Z.1. The 2x10\(^6\) cell nsPEFs cells were suspended in 400 µl α-MEM-10%FBS lacking antibiotics.

Application of NsPEFs

The cell suspensions (400 µl) were placed in an electroporation cuvette that contained a pair of parallel aluminium electrodes with a 4 mm-gap (#5540, M Laboratory, Institute of Pulse Science, Kumamoto University, Japan). After exposure, the cells were immediately diluted 4-folds α-MEM-10%FBS lacking antibiotics and incubated in 5% CO\(_2\) incubator at 37°C during 1 and 4 hours (hr).

RNA Isolation

Total RNA was isolated from all samples using RNAiso Plus reagent (Takara Bio). Aliquots of the total RNA were subjected to real time PCR SYBR Green and RT-PCR. The total RNA concentration and purity were confirmed using Biophotometer plus (eppendorf).

Real Time PCR Analysis

Quantitative mRNA analysis was conducted by quantative real time PCR using iScript One-Step RT-PCR kit with SYBR Green (Bio-rad) on an MJ Mini Thermal Cycler equipped with a Mini Opticon Real Time PCR system (BioRad). Real time PCR data were calculated by measuring the average cycle threshold (Ct) number for the mRNA of target, which was normalized to the values of the mRNA for glyceraldehydes-3-phosphate dehydrogenase (GAPDH).

Reverse Transcription PCR (RT-PCR)

Semi quantitative mRNA analysis was conducted by RT-PCR using OneStep RT-PCR kit (Qiagen) on RT-PCR system. PCR products were separated by agarose gel electrophoresis and visualized by UV transluminator. Intensity of band was quantified by Imagej software.

 Primer Optimization

The primer pairs for each gene (socs1, socs2 and socs3) that used in this research were optimized based on primer dimmer presence and consistency of melt curve in the real time PCR (Supplementary data). The primer pairthat used as the result from the optimization of the methods as follows: socs1 (32) (10), socs1 (202 bp), F: 5'- A G A C C C C T T C T C A C C T T T -3', R: 5'- CTGACAAGCAGAAAAATAAACG-3'; socs2 (244 bp), F: 5'- GAGATGATCGGGAAGTAGACTG-3'; R: 5'- AGTCGATCATGAAACCACACTGTC-3'; socs3 (107 bp), F: 5'-
TCC CCA GAG GGC TAT A C - 3'’; R: 5’-TCCGACAGAGATGCTG-3’; GAPDH (452 bp), F: 5’-ACCAGATGCATGATCATC-3’, R: 5’-TCCACCCCCCTGCTGGA-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 gene 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.

\[
y = 0.299x + 0.371 \\
R^2 = 0.923
\]

\[
y = 0.146x + 0.627 \\
R^2 = 0.795
\]
treatment group. Gene expression pattern of soscs2 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 soscs1 and soscs3, but not in soscs2.

**DISCUSSION**

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

In the cervical cancer, HPV infections cause soscs1 and soscs3 methylations (36, 37, 39). These methylations implicate to STAT3 and 5 overexpressions (38). Generally, methylation of soscs1 and soscs3 implicate to high proliferation and low apoptosis in some cancers (36-39). Whereas, the roles of soscs2 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 soscs1 and soscs3 but not soscs2 gene expressions. From the control group of our results indicate that there were not any changes on soscs2 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 soscs1, soscs2, and soscs3 gene expressions during a certain time (see Figure 3). Gene expression pattern of soscs1 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 soscs1, soscs3 gene expression initially increased at 1 hr but subsequently decreased after 1 hr up to 4 hr. The pattern of control group of soscs3 decreased too, but still lower than treatment group. Gene expression pattern of soscs2 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 soscs1 and soscs3, but not in soscs2.

Note: The correlation between shot number and soscs1 and soscs3 gene expressions were indicated with positive correlation, but negative correlation for soscs2.

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Socs1, soscs2 and soscs3 are involved in negative feedback mechanism of JAK/STAT signaling pathway (41). By this mechanism, increasing of soscs1, soscs2, soscs3 decrease JAK/STAT target genes that regulate cell proliferation and apoptosis, such as c-myc and bcl-xl (24). According to these theories, we propose that nsPEFs may decrease proliferation and induce apoptosis via increasing of soscs1 and soscs3 gene expressions. Inducing soscs1 and soscs3 gene expressions by nsPEF probably decrease JAK/STAT signal transduction and implicate to decreasing of c-myc and bcl-xl. Decreasing of c-myc (induce proliferation) and anti apoptotic bcl-xl can decrease cell proliferation and induce apoptosis. Increasing of soscs1 by nsPEFs probably causes decreasing of c-myc and bcl-xl directly by E7 degradations. However, we need further investigations to prove this hypothesis. Our results and hypothesis are described on Figure 4.

Figure 3. Profile of gene expression pattern of soscs1, soscs2 and soscs3 responded to 20 and 30 shots nsPEFs from 1 hr to 4 hr

| Socs1        | 0x – y = -0.046x + 1.046 (R² = 0.997) |
|--------------|-------------------------------------|
|              | 20x – y = 0.816x + 1.633 (R² = 0.977) |
|              | 30x – y = 1.521x + 4.258 (R² = 0.937) |
| Socs2        | 0x – y = 0.401x + 0.481 (R² = 0.989) |
|              | 20x – y = 0.345x + 0.654 (R² = 0.994) |
|              | 30x – y = 0.450x + 0.150 (R² = 0.999) |
| Socs3        | 0x – y = -0.226x + 1.226 (R² = 1)     |
|              | 20x – y = -0.050x + 2.867 (R² = 0.926) |
|              | 30x – y = -0.096x + 6.119 (R² = 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).

**Figure 4**. A schematic diagrams of effect of nsPEFs on *socs1*, *socs2* and *socs3* gene expressions in HeLa S3 cells. Note: Blue sign show our result - (△)= gene expressions inductions, (x)= no effect; green sign show our hypothesis – ( / ) = increasing/decreasing of expressions; = gene methylation; ?= unown effect)

### Possibility Mechanism of nsPEFs to Induce Socs1 and Socs3 Gene Expressions

This research did not provide any data to explain the mechanism of nsPEFs to induces *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 *socs* methylated genes, *socs1* and *socs3* but not *socs2*. These results suggest that nsPEFs may induce *socs1* and *socs3* but not *socs2*. These results suggest that nsPEFs only induced *socs* methylated 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.

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*. *Socs1* 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. In
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 methylated 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 suppressor 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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