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Dexamethasone-administrated BALB/c mouse promotes proinflammatory cytokine expression and reduces $CD4^+CD25^+$ regulatory T cells population

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Dexamethasone has been known as an immunosuppressive drug that globally used to ameliorate a variety of inflammation disorders. However, long-term treatment can induce undesirable effects. With this intention, the experiment aimed to generalize and to explore the effect of dexamethasone in physiological regulations including an immune system on the healthy two-weeks-old BALB/c mice. A bioinformatics approach was used to predict molecules interaction and ligand-protein networking. Furthermore, the BD FACS Calibur™ was used to count the population number of regulatory T cells and the expression of TNF-α and IFN-γ which produced by CD4 T cells in the spleen. In addition, the Haematoxylin-Eosin staining was performed to detect necrosis incidents in excretory organs. Presently, based on ligand-protein networking analysis we found that dexamethasone has wide range effects on several metabolisms and pathways. On the other hand, single dose administration of dexamethasone significantly increases IFN-γ pro-inflammatory cytokines expression. On top of that, it induces necrosis in both liver and kidney tissue. Therefore, the study about dexamethasone is still needed in order to evaluate their effects on the other several physiological mechanisms.

Keywords: BALB/c mice, bioinformatics, dexamethasone, inflammation, immune system.

INTRODUCTION

Autoimmune diseases including immune-mediated inflammatory diseases are a cluster of disease followed by over-reactivity of immune responses and high expression of pro-inflammatory cytokines. This unwanted condition promotes inflammation which might act as an inducer for cancer progression and infection (Beyaert et al. 2013). In the western countries, it has reported that approximately 5% to 7% of the population are affected by this kind of diseases (Kuek et al. 2007).

Nowadays, glucocorticoids are commonly used to treat autoimmune diseases due to their rapid and wide spectrum of action on the immune system (Fan and Morand, 2012). In particular, dexamethasone is widely used as an immunosuppressive agent in order to reduce proinflammatory cytokines and T-cells activation (Nakao et al. 2007; Farrell and Kelleher, 2003). However, long-term treatment and dose-dependent toward dexamethasone inflict undesirable side effects, such as; weight-lose, glucose intolerance, skin fragile, muscle breakdown, calcium unbalance, and central nervous system disruption (Ferris and Kahn, 2012).

In our previous studies, 0.5 $mg.kg^{-1}$ BW of

dexamethasone had promoted the population number of erythroid progenitor cells and erythrocytes (Putra et al. 2015a). Furthermore, by performing similar dose, it improved the production of several types of the cells such as hematopoietic stem cell, granulocyte, and B-progenitor cell (Putra et al. 2015b).

Accordingly, this research aimed to generalize and to explore the effect of dexamethasone toward physiological regulations by examining the evidence of certain immune cells population, cytokines production and side effects on excretory organs of BALB/c mouse.

MATERIALS AND METHODS

Prediction of Receptor-Ligand Interaction via In Silico

The ligands 2D structure were obtained from https://pubchem.ncbi.nlm. nih.gov/ (Kim et al. 2016) and were optimized by using Marvin sketch (http://www.chemaxon.com). The ligands consist of cortisol analog (PubChem CID: 5754) and dexamethasone (PubChem CID: 5743). On the other hand, the 3D structure of GR (PDB ID: 4UDC) was obtained from www.rcsb.org (Berman et al. 2000). Ramachandran plot was performed to evaluate the protein quality and property (http://services.mbi.ucla.edu/SAVES/).

Subsequently, GR was docked with each ligand by using Autodock Vina (Trott and Olson, 2009). Ultimately, the results were analyzed and visualized by using Accelrys Discovery Studio Visualizer software.

Drug and Protein-Protein Interaction

STITCH (http://stitch.embl.de/) was used in order to analyze the interaction between protein and drug (Kuhn et al. 2014), whereas protein-protein interaction was analyzed by using STRING (http://string-db.org/) and was clustered based on the similarity of the pathway (Szklarczyk et al. 2015).

Animal Treatment

Two-weeks-old BALB/c mice were used as experimental animals, then properly maintained in the animal facility of Biology Department, Brawijaya University. There were three experimental groups namely control group (C), dexamethasone administration with dose 0.5 $mg.kg^{-1}$ BW (D1), and dexamethasone administration with dose 10 $mg.kg^{-1}$ BW (D2). This experiment was followed by six times replication. The administration was performed by intraperitoneal injection and continued by examining the effect of dexamethasone administration on day-7 after injection. All experimental procedures have been accepted by the Research Ethics Committee of Brawijaya University with ethics number 441-KEP-UB.

Flow Cytometry

The experimental mice were sacrificed, then dissected on day-7 after administrated by dexamethasone. Spleen lysates were homogenized in PBS and were stained with antibodies. Several antibodies were used in this experiment; fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD-4 (clone: GK 1.5), phycoerythrin (PE)-conjugated anti-mouse CD4 (clone GK 1.5), allophycocyanin (APC)-conjugated anti-mouse IFNɣ (PEC45), PE-anti-mouse TNFα (clone: MP6-XT22), and FITC-conjugated anti-mouse CD25 (clone PC61.5). BD FACS Calibur™ was used for flow cytometry assay.

Histological Tissue Preparation

Isolated liver and kidney were washed in PBS solution, then soaked and incubated them into 4% formaldehyde for fixation. Next processes such as paraffin embedding, tissue slicing, and Haematoxylin-Eosin staining were performed by the regular procedure at Brawijaya University, School of Medicine.

Microscopy Observation

Olympus BX51 binocular microscope was used to observe the morphology of necrotic cells. The microscope magnification was 400× for all specimens. The observation was determined by taking five random fields of view from each specimen, then make ratio for the number of necrotic cells and total cells in each field. After that, all data were converted into percentage ratio.

Statistical analysis

The ANOVA and Tukey-HSD test were performed with a significance level (α) of 0.05. The data that used for analysis were a percentage of the relative number of $CD4^+CD25^+$, $CD4^+IFN\gamma^+$, $CD4^+TNF\alpha^+$, and a percentage of necrotic cells in liver and kidney of experimental animals. The analysis was performed by using SPSS 16.0 for Windows.

RESULTS

Determining dexamethasone profile via bioinformatics approach

Data mining, data checking, and data processing were performed to evaluate molecular interaction similarity between GR-cortisol and GR-dexamethasone. Then, Ramachandran plot validation on GR protein was performed in order to visualize and quantify the allowed region of amino acid residues (Figure 1). Henceforward, all validated data including GR and ligands were docked to visualize the molecular interaction between GR and each ligand (Figure 2). The results showed that both cortisol and dexamethasone were in the similar coordinate, therefore the additional amino acid residues and free binding energy could be calculated and compared between GR-cortisol and GR-dexamethasone interaction.

Besides that, we also found dexamethasone can interact with several kinds of proteins especially the protein which are responsible in immune responses pathway (Figure 3). Furthermore, the correlation between a protein with other proteins could be found by extending the protein network. Then, extended networking could be clustered based on which pathways they were involved in.

Immune system responses toward dexamethasone administration

The expression of $CD4^+CD25^+$ Treg cell, $CD4^+IFN\text{-}\gamma^+$, and $CD4^+TNF\text{-}\alpha^+$ T cells was evaluated to know the effect of dexamethasone in term of inflammation activity. Single dose administration of dexamethasone with 0.5 $mg.kg^{-1}$ BW on two-weeks-old BALB/c mice has no effect to suppress both $CD4^+CD25^+$ (Figure 4) and $CD4^+TNF\text{-}\alpha^+$, but they were significantly decreased with 10 $mg.kg^{-1}$ BW of dexamethasone. Interestingly, $CD4^+IFN\text{-}\gamma^+$ were significantly increased after administrated by 0.5 $mg.kg^{-1}$ BW of dexamethasone (Figure 5).

Dexamethasone toxicity in excretory organs

Dexamethasone toxicity evaluation was performed by examining necrosis incidents on the liver and kidney. By performing HE staining methods, our group found the percentage of necrosis in liver tissue were 13.59%, 21.68% and 27.17% for control, dose 1 and dose 2 group respectively, whereas the percentage of necrosis in kidney tissue for every treatment were 3.94% for control, 10.37% for dose 1 and 15.49% for dose 2 group (Figure 6). This result suggested, both low and high dose of dexamethasone induce necrosis in liver and kidney.

DISCUSSION

The roles of dexamethasone in several regulations system

Glucocorticoids exert its effects on wide range metabolisms and pathways. In general, dexamethasone is commonly used to ameliorate inflammatory disorders due to its role in inhibiting proinflammatory cytokines production like IL-2, IL-6, TNF-α, and INF-γ, inducing anti-inflammatory cytokines, and promoting the apoptosis on T-lymphocytes (Ferris and Kahn, 2012; Bosscher et al. 2016). Many reports have illustrated that glucocorticoids have two kinds mechanism of actions (Figure 7). Firstly, glucocorticoid directly binds with the glucocorticoid receptor (GR) on cytoplasm to form activated GR complex. Subsequently, GR complex enters into the nucleus and stimulate glucocorticoid response element (GRE). Secondly, activated GR complex might binds and interferes the action of such transcription factors namely nuclear factor kappa B (NF-κB) or activator protein 1 (AP-1) which have a crucial role in inducing the transcription of proinflammatory cytokines (Bosscher et al. 2016).

In this work, our group examined dexamethasone mechanism of action and its interaction with other proteins via in silico approach. Nowadays, in silico is a powerful technique which is commonly used to determine and to analyze molecules interaction between ligand and protein model. As reported, ligand like chemical compound interacts and changes conformation and structure of the protein which could interfere the potential activities of the protein. As mentioned before, cortisol and dexamethasone were used as ligands for comparing the similarity of interaction between natural and synthetic glucocorticoids to GR (Figure 2). Abduljabbar et al. (2015) have reported that both cortisol as natural glucocorticosteroid and dexamethasone as synthetic glucocorticoid are main ligands for GR. Based on the molecular docking result, our group found amino acid residues interpolation (PRO625 and TYR660) in GR–dexamethasone interaction, therefore dexamethasone has interacted with more amino acid residues than GR-cortisol. At the same circumstance, GR-dexamethasone interaction has better interaction affinity comparing with GR-cortisol interaction because it

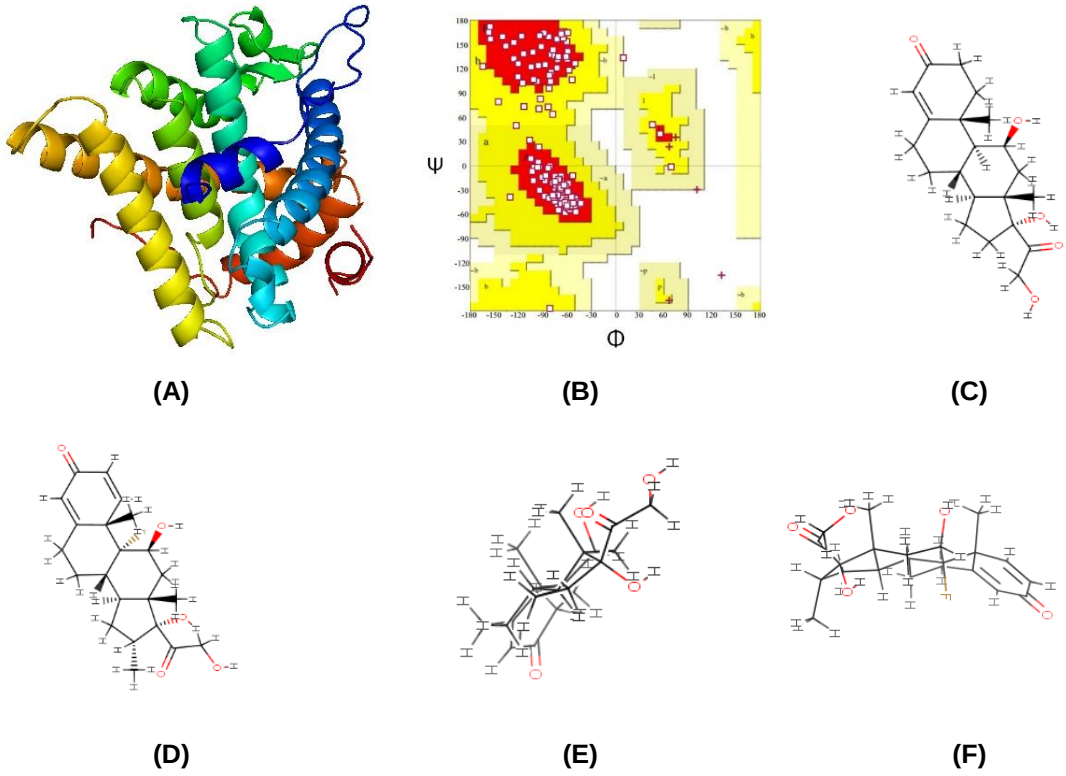

Figure 1. Glucocorticoid receptor and its ligands. (A) Chemical 3D structure of glucocorticoid receptor. (B) Ramachandran plot of glucocorticoid receptor. (C) Chemical 2D structure of cortisol and (D) dexamethasone. (E) Chemical 3D structure of cortisol and (F) dexamethasone.

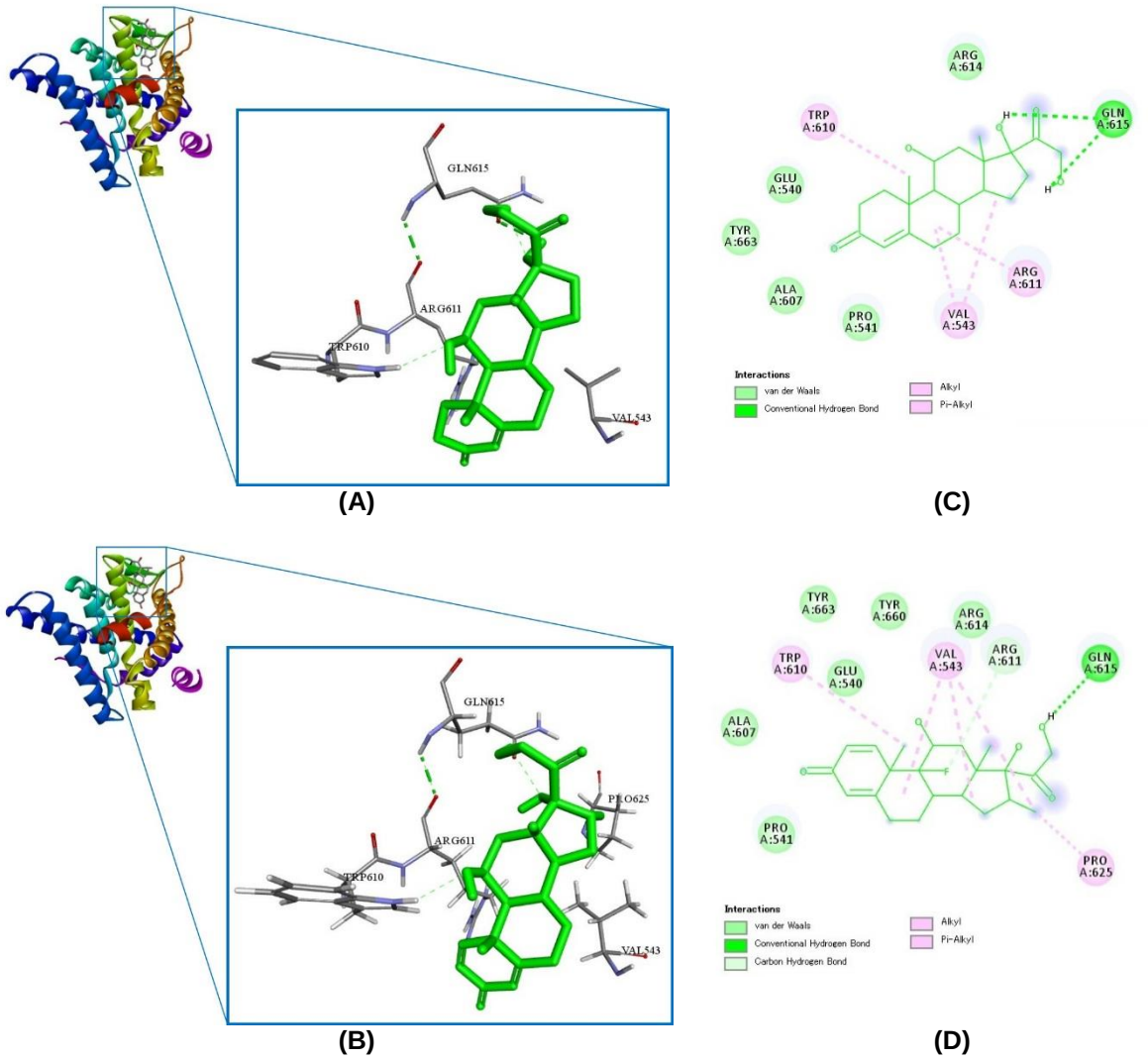

Figure 2. Highlight chemical 3D structure of glucocorticoid receptor and its binding interactions with (A) cortisol (B) dexamethasone. Identically, chemical 2D structure of glucocorticoid receptor and its binding interactions with (C) cortisol (D) dexamethasone.

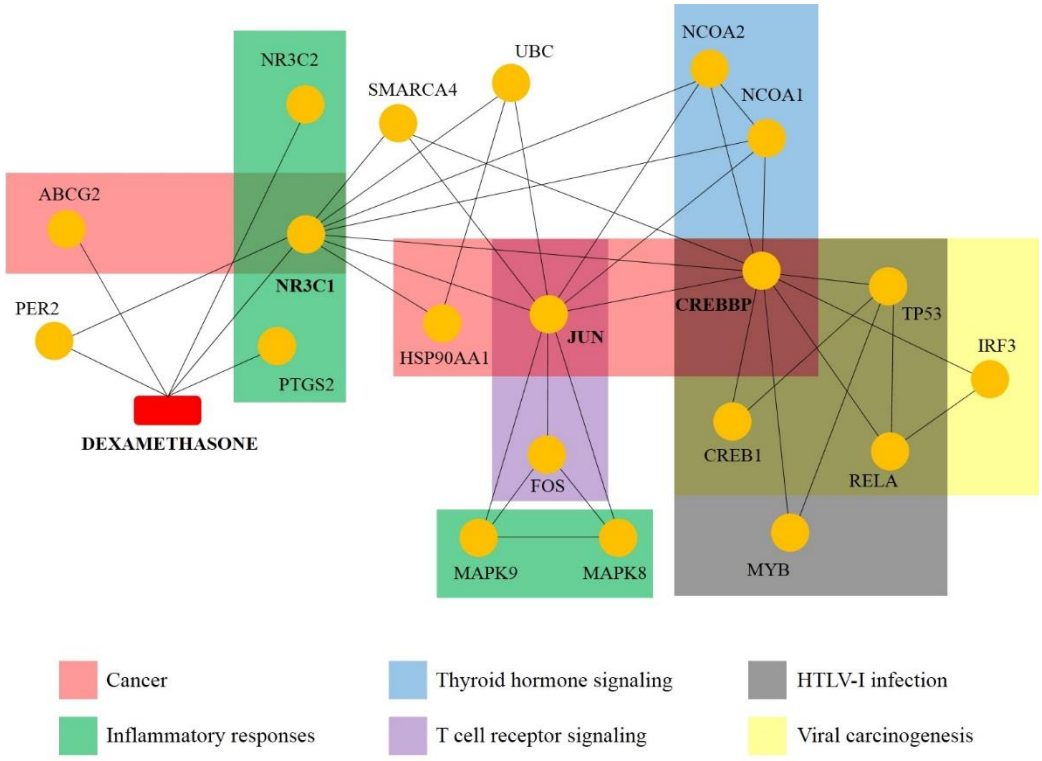

Figure 3. Dexamethasone can interacts with several proteins, further the protein it self has their own interaction to the other proteins which are responsible in certain biological regulation. Data were obtained from http://stitch.embl.de/ and http://string-db.org/.

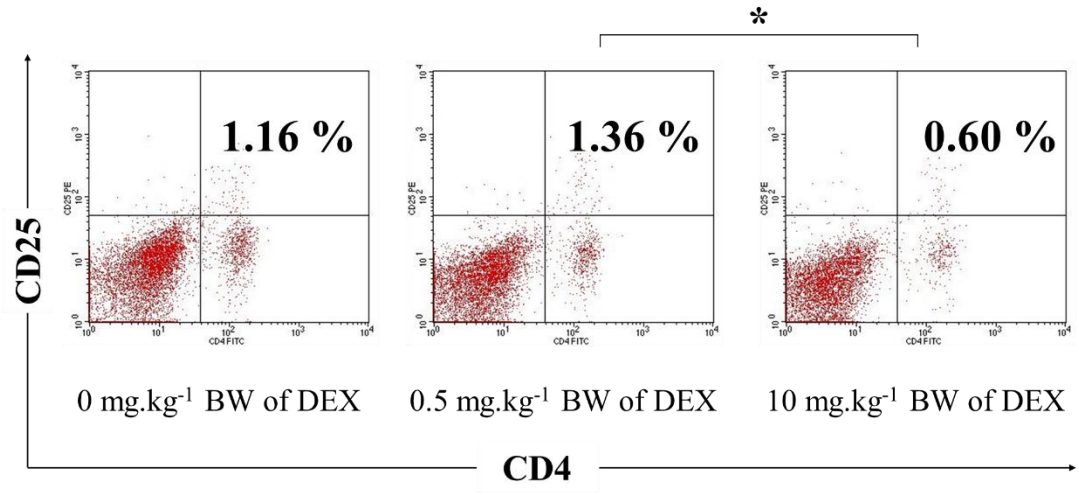

Figure 4. The relative number of $CD4^+CD25^+$ Treg cells were evaluated by FACS Calibur™ on day-7 after administration of dexamethasone. The $CD4^+CD25^+$ Treg cells were significantly promoted by single dose 0.5 $mg.kg^{-1}$ BW of dexamethasone. The data are mean ± SD in each group with p-value<0.05.

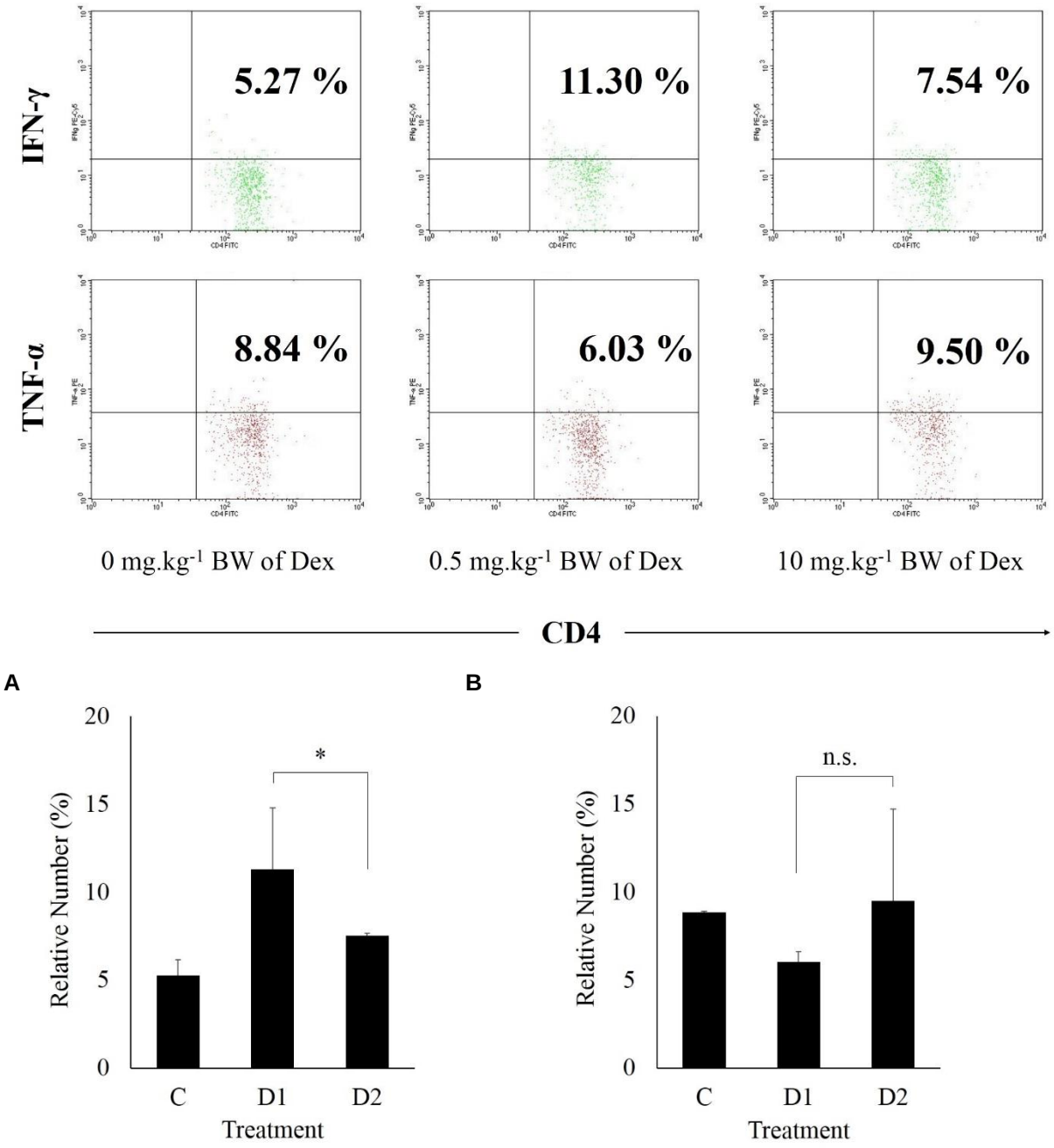

Figure 5. The relative number of $CD4^+IFN\text{-}\gamma^+$ and $CD4^+TNF\text{-}\alpha^+$ cells were evaluated by FACS Calibur™ on day-7 after administration of dexamethasone. (A) The $CD4^+IFN\text{-}\gamma^+$ cells were significantly promoted by single dose 0.5 $mg.kg^{-1}$ BW of dexamethasone, (B) whereas there were no significant difference between the treatment groups in a relative number of $CD4^+TNF\text{-}\alpha^+$ cells. The data are mean ± SD in each group with p-value<0.05.

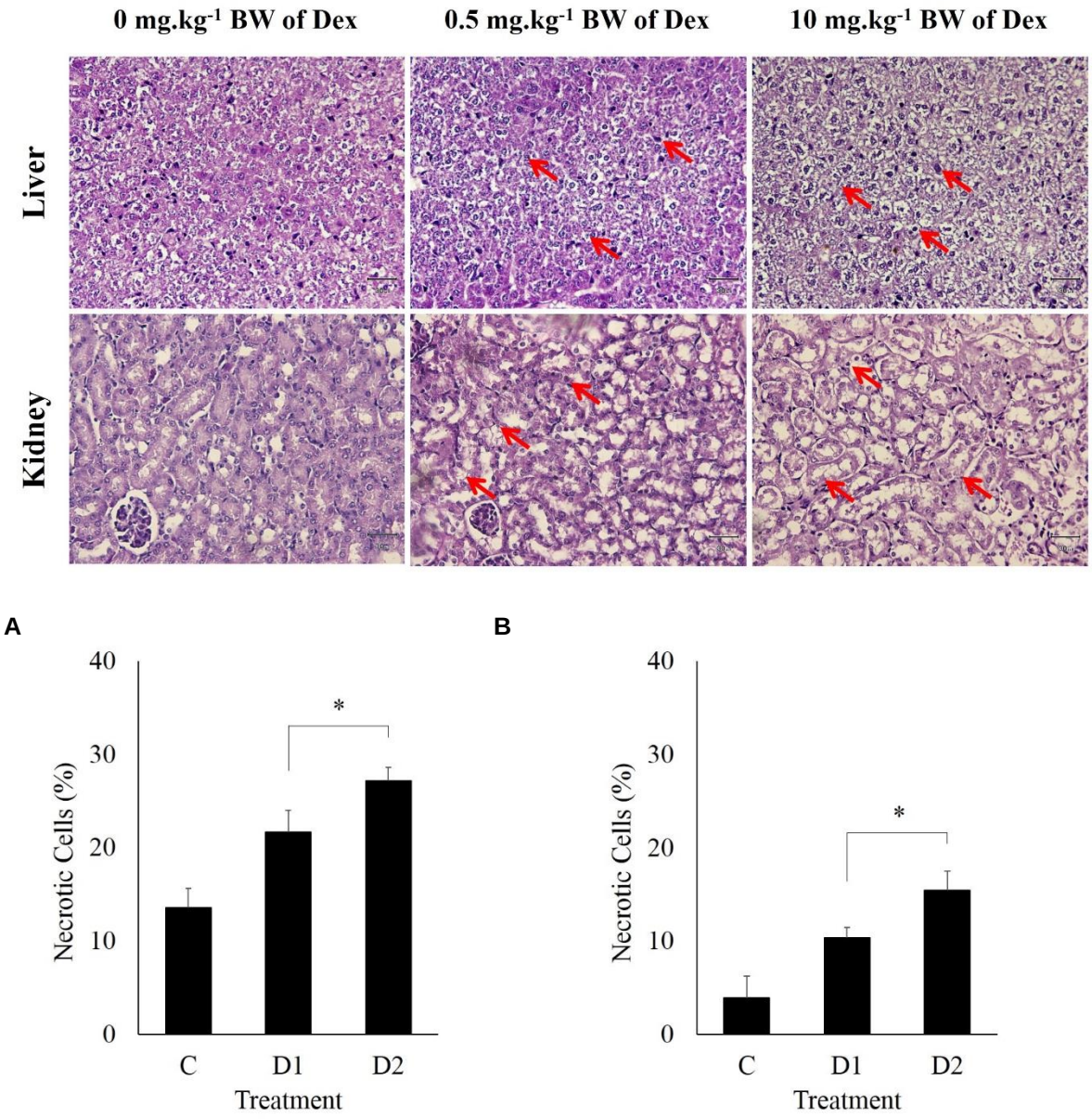

Figure 6. Representative microphotograph of liver and kidney section from experimental mice after administrated by dexamethasone (HE staining, M=400×). Red arrows indicate necrotic cells in both liver and kidney section (↓). The bars are the percentage of necrotic cells in both liver and kidney section of experimental mice. (A) Percentage of necrotic cells in liver section were increased by a single dose of 10 mg.kg^{-1} BW of dexamethasone. (B) Equally important, the percentage of necrotic cells in kidney section also were increased by a single dose of 10 mg.kg^{-1} BW of dexamethasone. The data are mean ± SD in each group with p-value<0.05.

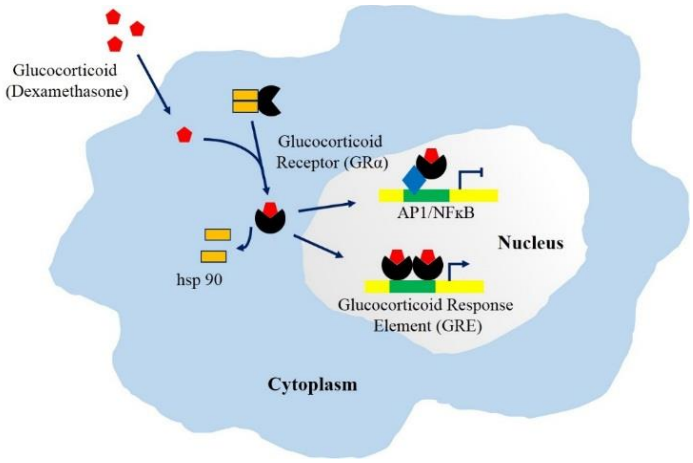

Figure 7. Glucocorticoid's mechanism of action in order to mediates anti-inflammatory responses. There are two common transcriptional mechanisms, namely direct interaction with glucocorticoid response element (GRE) to exert its effect and interfere other transcription factors (AP1/NFκB) to block their effects.

Table 1. Active site residues and binding energy of ligands to glucocorticoid receptor

No	Receptor-Ligand Interaction	Active Site Residues	$\Delta G_{binding}$ (kcal/mol)
1	Glucocorticoid receptor – Cortisol	GLU540, PRO541, VAL543, ALA607, TRP610, ARG611, ARG614, GLN615, TYR663	-6.6
2	Glucocorticoid receptor – Dexamethasone	GLU540, PRO541, VAL543, ALA607, TRP610, ARG611, ARG614, GLN615, PRO625, TYR660, TYR663	-6.8

showed more negative on Gibbs free binding energy value (Table 1). It has been known that in order to modulate and to activate the protein, ligand must possess a high affinity to activated-residues. The molecular interaction affinity is related to the binding energy, the lower binding energy of the complex resulted on the high affinity (Asif et al. 2014; Asthana et al. 2014).

Equally important, protein-protein interaction analysis were used in order to expand the understanding of dexamethasone in several mechanism and regulation. Recently, studies about protein-protein interaction and its network could be such powerful approach to figure out the interaction among proteins which are responsible for certain pathway (Sabetian and Shamsir, 2015). Here, our group performed the interaction analysis by using STITCH and STRING to identify drug-protein interaction and protein-protein interaction. High confidence value (>0.7) based on functional and predicted interaction was used to filter interaction among the proteins. The results showed that dexamethasone has widely interacted with several kinds of proteins, one of them is NRC31 (nuclear receptor subfamily 3, group C, member 1) that involved in inflammatory response pathway. Interestingly, NRC31 is GR-encoded gene which also acts as anti-proliferative and anti-apoptosis (Bosscher et al. 2016). To a greater extent, NRC31 has shared the interaction with several proteins embroiled for various pathways cluster such as; cancer, inflammatory response, thyroid hormone, T cell receptor signaling, Human T-lymphotropic virus 1 (HTLV-1)

infection, and viral carcinogenesis (Figure 3). Supported by several studies, it has been reported that glucocorticoid which acts and exerts its physiological effects via GR is associated with wide range mechanisms in several disorders like inflammatory disorders, autoimmune diseases, cardiovascular diseases and cancers (Abduljabbar et al. 2015; Oakley and Cidlowski, 2013).

Immune system is altered by dexamethasone

Regulatory T Cell (Treg) cell is part of T cell subpopulation. Particularly, Treg cell which is identified by the existence of $CD4^+CD25^+$ has a pivotal role in T-cell development and activation (Rifa'i, 2013). Furthermore, Treg cell activity is correlated with suppressive mechanism against T helper 1 (Th1) cell on the immune system. Many reports have notified the roles of Treg cell, especially on regulating self-tolerance responses against self-antigen on autoimmune diseases (Corthay, 2009). In general, Treg cell mechanism of actions is directly killing cytotoxic cells, block and suppress cytokines production and actively produce anti-inflammatory cytokines (Askenasy et al. 2008).

Currently, the mechanism how dexamethasone affects Treg cell is still unclear. Our group revealed there was no significant differences in population number after administrated by 0.5 $mg.kg^{-1}$ BW of dexamethasone comparing with a non-treatment group. Surprisingly, 10 $mg.kg^{-1}$ BW of dexamethasone reduced the number of $CD4^+CD25^+$ Treg cell population (Figure 4). This result was contradicted with Hu et al. (2012) and Tuckermann et al. (2005), in addition to induces apoptosis mechanism on T cell, dexamethasone definitely promotes Treg cell population for creating the proper condition in term of Th1/Th2 stability.

These unexpected findings also happened on the expression number of IFN-γ on CD4 T cell. Commonly, the proinflammatory cytokines like TNF-α and IFN-γ which also act as antiviral and bacterial immunity should be downregulated after induced by dexamethasone (Keystone and Ware, 2010; Schroder et al. 2003). In the fact, we found the expression number of IFN-γ was increasing and there were no significant changes in TNF-α expression after induced by dexamethasone (Figure 5). We suggested, there were any factors that are responsible for IFN-γ stimulation. Another report already revealed that IL-18 is a factor that can enhance IFN-γ production in immune cells (Yu et al. 2012). However, the existence TNF-α and IFN-γ which are produced by Th1 cells could be considering as inflammation condition (Oliveira et al. 2011). It suggested that single dose dexamethasone administration promotes inflammation in healthy two-weeks-old BALB/c mouse.

High-dose administration of dexamethasone induce several defects in liver and renal

The liver and kidney are part of excretory organs which targeted by side effects of chemical compounds because their role is related for clearing the chemical excess in the body. Furthermore, it has reported that the susceptibility of organ injury caused by several factors such as genetic, chemical compounds, and environmental factors. The drug, including dexamethasone, could be considering as an inducer of apoptosis and necrosis. The mechanism of apoptosis is caused by the intrinsic stress of the cell and sometimes it could be interfered by the immune system, while necrosis caused by metabolite-over reactivity and mitochondrial dysfunction in huge number (Kaplowitz, 2004).

The effect of dexamethasone administration on two-days-old BALB/c mice was obviously detectable by the number of necrosis in both liver and kidney (Figure 6). The result exhibited that low or high dose of dexamethasone were strongly caused tissue injury. Opposite with the research conducted by Eken et al. (2006), it showed that low dose of corticosteroid which had given to bile duct ligation rat model approximately 0.125-0.400 $mg.kg^{-1}$ BW per day could suppress the possibility of liver injury that caused by bile duct obstruction. Dexamethasone has a role to maintenance the stability of anti-oxidant enzymes production like superoxide dismutase (SOD), catalase (CAT) and phospholipid hydroperoxide glutathione peroxidase (GSH-Px) which are responsible for the protection and the prevention the liver from injury. However, another study also has reported that dexamethasone administration with high dose causes serious side effects and growth retardation. As same as liver, the kidney also has a high risk to get a negative effect from the drug. Nephrogenesis is caused by chemicals toxicity that followed by nephron damages which occurred in renal caused by the drug. Another experiment showed that postnatal induced by dexamethasone surely increases kidney damage followed by a low number of the nephron and tubular damage (Schreuder et al. 2011). This result suggests that dexamethasone is dose-dependent manner

toward its effect on tissue

CONCLUSION

Dexamethasone does not only acts as an immunosuppressive agent but based on the bioinformatics prediction also exert their effects in a wide spectrum of the physiological mechanism. Interestingly, single dose administration of dexamethasone with 0.5 $mg.kg^{-1}$ BW in two-weeks-old mice increases IFN-γ pro-inflammatory cytokines produced by T cells. In addition administration with higher dose, 10 $mg.kg^{-1}$ BW, were decreased $CD4^{+}CD25^{+}$ Treg cells population. On top of that, dexamethasone has induced necrosis in both liver and kidney tissue.

CONFLICT OF INTEREST

We found no conflict of interest in this work.

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AUTHOR CONTRIBUTIONS

WEP designed and performed the experiments and also wrote the manuscript. EW, OA, and IDJ performed animal treatments, flow cytometry experiments, tissue collection, and data analysis. AS and MR designed experiments and reviewed the manuscript. All authors read and approved the final version.

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