Effect of Gold Nanoparticle size on TLR2/4 signaling in Hek293 cell line

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การศึกษาผลกระทบของอนุภาคทองคำระดับนาโนเมตรที่ขนาดต่างกันต่อการส่งสัญญาณผ่านโปรตีนตัวรับชนิดเสมือนโทลล์ 2 และ 4 ในเซลล์ ไตของตัวอ่อนมนุษย์

นางสาวชวิกานต์ บุญวงศ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ชวิกานต์ บุญวงศ์ : การศึกษาผลกระทบของอนุภาคทองคำระดับนาโนเมตรที่ขนาดต่างกันต่อการส่งสัญญาณผ่านโปรตีนตัวรับชนิดเสมือนโทลล์ 2 และ 4 ในเซลล์ ไตของตัวอ่อนมนุษย์ (Effect of Gold Nanoparticle size on TLR2/4 signaling in Hek293 cell line) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร. นพ.อมรพันธุ์ เสรีมาศพันธุ์, 4 หน้า.

ในปัจจุบันอนุภาคทองคำระดับนาโนเมตร ถูกนำมาประยุกต์ใช้ในงานต่างๆได้หลากหลายด้าน เช่น ทางด้านงานวิจัย เครื่องสำอาง และ ในทางการแพทย์ เนื่องจากอนุภาคทองคำระดับนาโนเมตรถูกรายงานว่ามีความปลอดภัยต่อสิ่งมีชีวิต และรวมถึงช่วยลดการอักเสบได้ ถึงแม้ว่าจะมีหลายรายงานที่กล่าวถึงประโยชน์ของอนุภาคทองคำระดับนาโนเมตร แต่ก็ยังคงมีการกล่าวถึงความเป็นพิษของอนุภาคทองคำระดับนาโนเมตรเช่นกัน ซึ่งกลไกการทำงานต่างๆที่เกิดขึ้นภายในสิ่งมีชีวิตของอนุภาคทองคำระดับนาโนเมตรนั้นยังคงต้องการศึกษาเพิ่มเติม นอกจากนี้งานวิจัยนี้ยังสนใจ โทลล์ไลค์ รีเซปเตอร์ หรือโปรตีนตัวรับชนิดเสมือนโทลล์ เป็นโปรตีนตัวรับชนิดหนึ่งที่สำคัญในระบบภูมิคุ้มกัน โดยทำหน้าที่จับกับสิ่งแปลกปลอมที่บุกรุกเข้าภายในร่างกาย ทำให้ระบบภูมิคุ้มกันหลั่งสารออกมา เพื่อใช้ในการทำลายสิ่งแปลกปลอมนั้น วัตถุประสงค์ของงานวิจัยนี้เพื่อต้องการศึกษา ผลของอนุภาคทองคำระดับนาโนเมตรต่อการตอบสนองของระบบภูมิคุ้มกันโดยผ่านทาง โปรตีนตัวรับชนิดเสมือนโทลล์ 2 และโปรตีนตัวรับชนิดเสมือนโทลล์ 4 ในเซลล์ไตของตัวอ่อนมนุษย์ ที่มีการเพิ่มการแสดงออกของโปรตีนตัวรับชนิดเสมือนโทลล์ 2 และ 4 โดยอนุภาคทองคำระดับนาโนเมตรที่มีขนาดที่แตกต่างกันได้ถูกนำมาทดสอบในงานวิจัยนี้ จากการศึกษา พบว่าอนุภาคทองคำระดับนาโนเมตรขนาด 10 นาโนเมตรนั้นสามารถกระตุ้นการแสดงออกของโปรตีนตัวรับชนิดเสมือนโทลล์ 2 และ 4 เพิ่มมากขึ้น รวมถึงส่งผลต่อกระบวนการต่างๆหลังกระตุ้นโปรตีนตัวรับชนิดโทลล์ คือ อนุภาคทองคำระดับนาโนเมตรเพิ่มการแสดงออกของนิวเคลียร์ แฟคเตอร์แคปปาบี และยังส่งผลต่อการเพิ่มการแสดงออกของไซโตไคน์ชนิดกระตุ้นให้เกิดการอักเสบอีกด้วย ในทางกลับกันอนุภาคทองคำระดับนาโนเมตรขนาด 20 นาโนเมตร ไม่ส่งผลต่อการเปลี่ยนแปลงระดับการแสดงออกของโปรตีนตัวรับชนิดเสมือนโทลล์ 2 และ 4 แต่พบว่าสามารถช่วยลดการแสดงออกของ นิวเคลียร์ แฟคเตอร์แคปปาบี และไซโตไคน์ชนิดกระตุ้นให้เกิดการอักเสบได้ การศึกษานี้แสดงให้เห็นว่า การตอบสนองในระบบภูมิคุ้มกันโดยผ่านโปรตีนตัวรับชนิดเสมือนโทลล์ ของอนุภาคทองคำระดับนาโนเมตรนั้นขึ้นอยู่กับขนาดของอนุภาคทองคำระดับนาโนเมตร ซึ่งทำให้เรามีความรู้และสามารถนำอนุภาคทองคำระดับนาโนเมตรไปประยุกต์ใช้ในทางการแพทย์เพื่อรักษาโรคที่เกี่ยวกับการอักเสบต่อไป

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CHAWIKAN BOONWONG: Effect of Gold Nanoparticle size on TLR2/4 signaling in Hek293 cell line. ADVISOR: ASST. PROF.AMORNPUN SEREEMASPUN, M.D., Ph.D, 4 pp.

Gold nanoparticles (AuNPs) have tried to apply in many fields such as biological research, cosmetic, and medicine as clinical diagnostics because it has been reported to be safe for various applications and decrease severity of inflammation. Although the several advantages of AuNPs have been reported, but they still have found to cause in many cytotoxicity tests. However, mechanisms inside the cells requiring deeper investigation. Toll-like receptors are one of the key factors that play an important role in the innate immunity, which provide immediate responses to invading pathogens. The main aim of this study was to examine the effect of AuNPs on inflammatory response via TLR2 and TLR4. Using hTLR2 and hTLR4-Hek 293 cell lines, treatment with various size of citrate-stabilized AuNPs for 24 h. In our findings, small size of AuNPs has been shown to be able to up-regulate TLR2/4 gene expression, while 20 nm AuNPs do not change the expression level of TLR2/4 genes. Next, we investigated down-stream of TLRs, NF-kB activation and pro-inflammatory cytokine have been studied. Inductions of NF-kB activity and expression of pro-inflammatory were observed in 10 nm AuNPs treated cells. However, the level of both NF-kB activity and expression of pro-inflammatory is reduced after exposure with 20 nm AuNPs. Ours result conclude that TLR2/4-mediated cytotoxicity of AuNPs by size dependent pattern. These findings suggest that AuNPs may have important guideline the treatment of inflammations application.

Field of Study: Medical Science Student's Signature .....

Academic Year: 2014 Advisor's Signature .....

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CONTENTS

Page

LIST OF FIGURES

Page

LIST OF TABLES

Page

CHAPTER I
BACKGROUND

Currently, there are several interests in using nanotechnology for biomedical applications, such as diagnosis and therapy in many diseases because of unique properties. Nanotechnology is the technology to construct and manipulated of the small item at the atomic or molecular scale that are approximately 1 to 100 nanometers in size (1, 2). Recently, gold nanoparticles (AuNPs) are becoming one of the most interesting nanoparticles that have widely used to apply in the medical field due to their low cytotoxicity, biocompatibility and stability (1, 3). The previous study has been reported that AuNPs can reduce the expression of pro-inflammatory cytokines (1, 4). Thus, AuNPs have been recommended to apply for treatment in many diseases such as rheumatoid arthritis, psoriasis and cancer therapy (4-6)

Despite AuNPs have been expanded in many applications, there have still few reports on their potential toxicity, which has been shown to depend on size, dose, shape, and surface charge of AuNPs (7-9). Previous studies report that 100 nm silver nanoparticles lead to cell toxicity more than 10 nm, but in AuNPs size 5 nm more toxic than 15 nm AuNPs (8, 10). Moreover, AuNPs are able to be increased pro-inflammatory cytokines were reported in different model studies (11, 12). It remains unclear whether the effects of nanoparticles on toxicity or modulate the regulation of pro-inflammatory cytokines and what are the factors of nanoparticles that affects to immunity response.

Toll-like receptors (TLRs) are important members of the first line for defense against invasion of unknown materials. These receptors belong to the pattern recognition receptors (PRRs) family that recognizes pathogen-associated molecular patterns (PAMPs) (13). To date, 13 TLRs have been identified in mammals and can be

divided into two groups that comprising of cell surface TLRs and intracellular organelles TLRs (14, 15). Their activation to TLRs triggers downstream signaling which results in the NF-kB signaling stimulation (16, 17). Activation of the TLRs pathway associated with upregulation of the genes encoding proinflammatory cytokine, thereby initiating an inflammation (18, 19). There are several molecules can stimulate TLRs, in this study, we focused on TLR2 and TLR4. Because of previous studies were shown dose-dependent nanoparticles (titanium dioxide nanoparticles) having the effect on up-regulating the levels of TLR2 and TLR4 (20). Interestingly, previous studies have indicated that AuNPs have the potential to inhibit the expression of TLRs (21, 22). However, cellular interactions between nanoparticles and TLRs have not been adequately evaluated.

Having learned from previous study, we hypothesize these different factors of AuNPs may be affected on TLRs modulation, leading to immunity responses. The objectives of this study were to evaluate whether TLR2 and TLR4 play an important role in cellular response to various sizes of AuNPs. To study the effect of AuNPs exposure in HEK-Blue-hTLR2 cells (hTLR2) and HEK-Blue-hTLR4 cells (hTLR4) (Human Embryonic Kidney 293 cells). Next we aim to study what are the factors of AuNPs affecting the pro-inflammatory gene expression. In this study, *Leptospira interrogans* were used for TLR2 agonists and were selected from the most common serovars in the tropical country. (*Leptospira interrogans* serovar Autumnalis, serovar Bratislava, and serovar pyrogenes) (23-25).

CHAPTER II

LITERATURE REVIEW

Nanotechnology

Physicist Richard Feynman, the father of nanotechnology, who says that "Nanotechnology is science, engineering, and technology conducted at the nanoscale, which is about 1 to 100 nanometers." Nowadays nanotechnology expanding at a rapid rate, and have many applications in industry, computer technology, biology, cosmetic and medicine. In medical, nanotechnology applies to nanomedicine applications such as medical diagnostics (figure 1) (26-28).

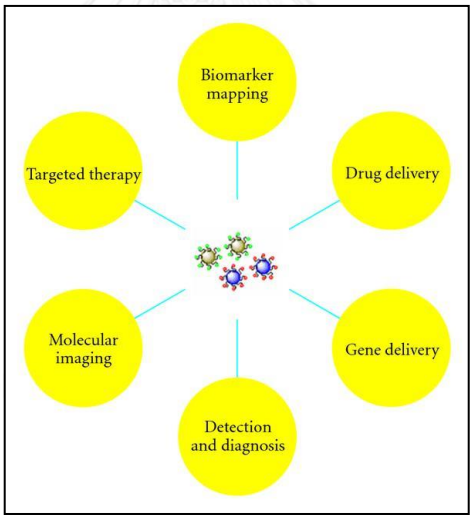


Figure 1. State of the art of multi-directional applications in nanomedicine (Zhe Liu, et al., 2010).

Despite AuNPs have been expanded in many applications, there have still few reports on their potential toxicity, which has been shown to depend on the size of AuNPs (7-9) show in Table 1. And it remains concerned about the immune response that effect by nanoparticles. Because of nanoparticles can enter into the body through various routes and distributes into many organs (figure 2A, B).

Table 1. Shows size dependent AuNPs toxicity

Size of AuNPs	Type exposed organism	Biological effect	Reference
1.4 and 15 nm	Tissue fibroblasts (L929), epithelial cells (HeLa), macrophages (J774A1) and melanoma cells (SK-Mel-28	1.4 nm AuNP was observed as the most toxic responsible for rapid cell death by necrosis as compare to 15 nm which was shown to be non-toxic	Yu Pan et al. (2007)
5 and 15 nm	Mouse fibroblast (Balb/3T3)	Cytotoxicity of AuNPs 5 nm at the exposure time of 72 h was observed, but 15 nm shows no toxicity.	Rosella Coradeghini et al. (2013)
Naked 3 to 100 nm	BALB/C Mice	AuNPs of 8, 12, 17, 37 nm induced fatigue, loss of appetite, change of fur color, and weight loss. Most died within 21 days.	Chen YS et al. (2009)
10, 50, 100 and 250 nm	Male WU Wistar derived rats	10 nm were present in all blood, liver, spleen, kidney, testis, thymus, heart, lung and brain. Larger particles were only detected in blood, liver and spleen	De Jong WH et al. (2008)
86.2 ± 10.8 nm and 11.6 ± 0.9 nm	Zebra fish embryo	The larger AuNPs cause fewer embryos to become dead than the smaller AuNPs	Lauren M. Browning et al. (2013)
2.81, 5.52 and 38.05 nm	J774 A1 murine macrophages	The cytotoxic effect of small size NPs of macrophages was more pronounced than that of the medium or large size of AuNPs	Hung-Jen Yen et al. (2009)

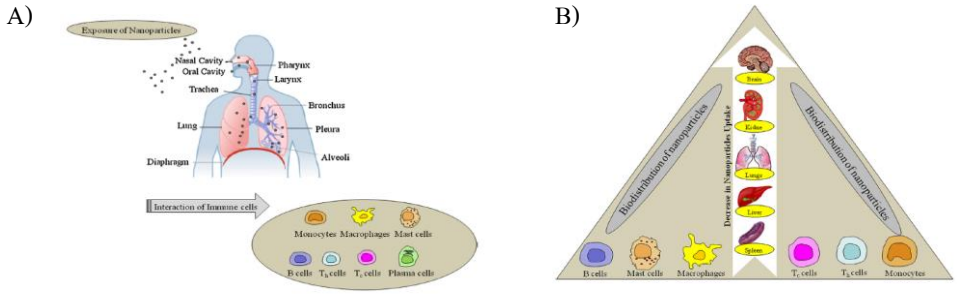


Figure 2. Nanoparticles can enter the body by the various sites and distribution to the various organs (Ruchi Roy, et al., 2014).

After nanoparticles entering the systemic circulation and distributed to the various organs, maybe they have been detected as a non-self by the immune response against nanoparticles. Moreover, the previous study found that when inside the cell, nanoparticles can stimulate effect as the expression of pro-inflammatory cytokines and increased ROS generation (29, 30). Many previous reports showing the association of nanoparticles and proinflammatory cytokines. (Table 2).

Table 2. Shows correlation between nanoparticles and pro-inflammatory cytokines (Modified from Ruchi Roy, et al., 2014).

Nanoparticles	Target cells/organs	Interleukin and TLRs
Cobalt (120 nm)	Human dermal microvascular endothelial cells	↑ IL8
Cobalt (50-200 nm)	PMA-differentiated myelomonocytic U-937 cells	↑ TNF-alpha, reduced IL-1Ra and IL1-beta
AuNPs (2 nm)	Apo E−/− mice	↑ mRNA (Mip-2, Mcp-I, and IL-6)
AuNPs (50 nm)	Bovine retinal pigment epithelial cells	Inhibit VEGF and IL-1beta induced proliferation and migration
Ag (1.5 nm)	PBMC, hMSCs, J774 A1 macrophages	↓ IL-5, INF-gamma, TNF-alpha, IL-1, IL-6, IL-8, IL-11
Ag-tiopronin-capped (5 nm)	Macrophages	Impaired the IL-6 secretion mediated by TLR2, TLR2/6, TLR3 or TLR9 stimulation
Ag (68 nm)	RAW264.7 cells	↑ TNF-alpha in protein and gene levels
Al2O3	U937 & A549 (co-cultured)	↓ Immune response
Fe2O3 (36 nm)	BALB/c mouse (♂)	↑ IL-1beta, IL-6, TNF-alpha, MIP-1alpha mRNA

Gold nanoparticle (AuNPs), one of the most widely used nanomaterials in nanomedicine applications because of their properties such as physical properties increased surface area-to-volume ratio of the structure or chemical properties as electrical conductivity properties, optical properties, and solubility properties. Including able to adapt to many different functions or activities, easy to be synthesized, high stability, controllable morphology, and size dispersion. AuNPs widely used in many nanomedicine applications such as drug and gene delivery, imaging, photodynamic therapy, and tissue engineering (31-34) because of their properties also prove to be nontoxic and biocompatibility (3, 35).

Although, AuNPs be applied in medicine, but the immune system in the human body may be detected AuNPs as foreign materials and leading to an immune response. It is still unclear effect of nanoparticles or AuNPs encounter with the

immune system resulted in immunosuppression or immunostimulation (figure 4). From many previous studies, found AuNPs effect on the immune system that showed both of immunosuppression and immunostimulation (Table 3).

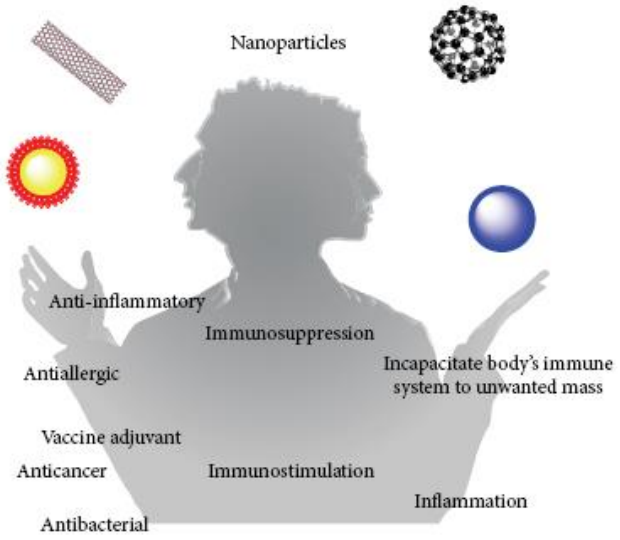


Figure 3. Shows the effect of nanoparticles to the immune system may benefit treatment or may bring harm. (Qing Jiao, et al. 2014)

Table 3. Shows immunomodulating effects of AuNPs used in nanomedicine on the immune system (Modified from Qing Jiao, et al. 2014).

Nanomaterial	Size	Exposure routes/doses	Outcomes	Cytokine/ Chemokines	Animal
PEG coated GNP	13 nm	Intravenously 0, 0.17, 0.85 or 4.26mg/kg	Acute inflammation	MCP-1/CCL-2↑, MIP-1alpha/CCL-3↑, MIP-1beta↑, RANTES/CCL-5↑, IL-1beta↑, IL-6↑, IL-10↑, IL-12beta↑, TNF-alpha↑	BALB/c
GNP functionalized with 2-mercaptoethanesulfonic acid (36) orN,N,N-trimethylammonium-ethanethiol (TMAT)	1.5 nm	Media exposure 0.016–250 ppm	Activate immune response Inflammatory response	Il-5↓, IL-12↓, IL-15↓, IL-18↓	Zebrafish embryos
Citrate-stabilized GNPs	40 nm	Oropharyngeal aspiration 0.8mg/kg	Hypersensitivity	MMP-9↑, MIP-2↑, TNF-alpha↓, IL-6↓	TDI-sensitized mice (BALB/c)
GNP	21 nm	Intraperitoneally injection 7.85 ug/g	Antiflammatory	TNF-alpha mRNA↓, IL-6 mRNA↓	Male C57BL/6
Citrate-stabilized GNPs	5 nm	100 nmolAu/kg	Antiflammatory	IL-1beta↓	IL-1beta model mice (male C57BL/6)

Recently, there have been many research studies the immune response by using AuNPs as a nanomedicine for medical application. In 2005, Ravi Shukla and coworkers studied the biocompatibility of AuNPs on RAW264.7 macrophage cells. The results showed AuNPs do not show cytotoxicity of AuNPs, including decreased ROS generation, and do not induce secretion of TNF-alpha and IL-1beta cytokine. These data suggest that AuNPs suitable candidates nanoparticles for medical application because of AuNPs have safety properties such as non-cytotoxicity, nonimmunogenic, and biocompatibility (3).

In 2007, Chaiau-Yuang Tsaic *et al.* induced arthritis (collagen-induced arthritis-CIA) in rats. In AuNPs treated groups, showed the decrease of macrophage infiltration in synovial tissue by immunohistochemical staining and in the ankle joint of CIA rats, levels of TNF-alpha and IL-1beta were also decreased when compared with the PB-treated and PEG-AuNPs treated groups (37).

In 2012, Marcelo B Dohnert and coworkers induced Achilles tendinitis (tendinous injury model) in male Wistar rats. Rats were divided into three treatment groups (1) iontophoresis + diclofenac diethyl ammonium, (2) iontophoresis + GNP gel, and (3) combination with iontophoresis + diclofenac diethylammonium + GNP gel. The results showed a significant decrease in the levels of IL-1beta and TNF-alpha in the group treated with iontophoresis + diclofenac + GNPs (38).

In 2012, Pereira DV *et al.* studied the effect of AuNPs on endotoxin-induced uveitis in rats. The finding of this study showed LPS administration induced the expression of TNF-alpha in aqueous humor. The LPS-induced elevation of the TNF-alpha levels was inhibited by AuNPs were topically applied to both eyes of rats. Moreover, AuNPs was able to decrease TLR4 and NF-kB levels. This result suggests

that AuNPs decreased LPS-induced uveitis in vivo by reducing the expression levels of TNF-alpha, TLR4, and NF-kB (21).

In 2013, Vadim V. Sumbayev *et al.* revealed AuNPs in a size dependent manner, downregulate IL-1beta induced pro-inflammatory responses. After THP-1 cells were treated with IL-1beta in the presence or absence of 5 nm and 20 nm AuNPs, the expression levels of PI3K, HIF-1alpha, and TNF-alpha were evaluated. The results showed that, 5 nm AuNPs be able to decrease the expression levels of 3 markers more than 20 nm AuNPs. The researchers suggest possible mechanisms of the anti-inflammatory activities by AuNPs is interaction between AuNPs and extracellular IL-1beta, leading to decrease the number of IL-1beta molecule interaction with IL-1beta receptor may result in down-regulation of IL-1beta expression (4).

In 2013, Hui Chen and coworkers investigated the inflammatory effect and distribution of 21 nm spherical AuNPs in mice. The results showed no detectable cell or organ toxicity in mice. They found AuNPs accumulated in abdominal adipose tissue and in the liver. Moreover, TNF-alpha and IL-6 mRNA levels were decreased from 1 h to 72 h after AuNPs injection (1)

The previous reports have been discovered AuNPs can modulate the cytokine expressed in many cell types. In the immune system, TLRs is the first line to detect non-self component invading into the body such as AuNPs. TLRs are up-stream of the NF - kB pathway that regulating cytokine gene expression to response invaders. There are few established about AuNPs and TLRs. Eliza Hutter *et al.* studied the effect of AuNPs with three morphologies, spheres, rods, and urchins, coated with polyethylene glycol (PEG) in microglial cell line. The results showed that urchin GNPs

and rod GNPs can induce TLR2 expression, moreover, only urchin GNPs stimulated IL-1alpha secretion (39).

In 2012, Chiau-Yuang Tsai and coworkers studied the effect of GNP internalization. RAW264.7 cells pretreated with 4 nm GNPs for 24 h then incubated with various TLR ligands for 3 h and measured TNF-alpha production. The CpG-ODN is ligand of TLR9 induced TNF-alpha production while treated in combination with GNPs could inhibit the CpG-ODN induced TNF-alpha, IL-6, and NF-kB activation. They suggest that NF-kB pathway attenuated by GNPs therefor; the cytokine productions were modulated (22).

However, there have been still some studies reported the toxicity of AuNPs may effect on the immune response to stimulating the production and expression of pro-inflammatory cytokines in another model. In 2009, Wan-Seob Cho and coworkers studied acute toxicity of 13 nm-sized PEG-coated AuNPs in BALB/c mice. The results showed cytokine levels of IL-1beta, IL-6, IL-10, and TNF-alpha were significantly secreted at 30 min and 4 h after treatment showed elevated of IL-12beta in a dose-dependent manner (11).

In 2012, Min Wei *et al.* have shown that in RAW264.7 cell exposed by both 15 and 30 nm CpG-AuNPs could stimulate the secretion of TNF-alpha and IL-6 levels in a dose-dependent manner when compared group treated AuNPs alone or CpG Oligonucleotide alone. Suggests CpG-AuNPs as an efficient stimulator that induced production of the pro-inflammatory cytokines in this cell (12).

In 2013, Haseeb A. Khan *et al.* studied the effect of 10 and 50 nm AuNPs on expression of IL-1beta, IL-6 and TNF-alpha in the liver and kidney rats. The results showed, in rat liver, both of 10 and 50 nm AuNPs elicited the expression of IL-1beta, IL-6, and TNF-alpha mRNA at first-day post injection. In rat kidney, 10 nm AuNPs do

not induce the expression all of 3 cytokines, but 50 nm AuNPs increased the expression of IL-6 and TNF-alpha on first-day (40). This finding in this report indicates that, 50 nm AuNPs induced more severe inflammatory response compared with in 10 nm AuNPs, in accordance with the study by Monita Sharma and colleagues in year 2013, showed the effect of 10 nm AuNPs can induce NF-kB activation in the highest dose of AuNPs (5 ug/ml) at 24 and 48 h (30). Many reports showed both of immunosuppression and immunostimulation of AuNPs, these results which have also been shown to depend on size, shape, and surface chemistry of the AuNPs.

Pro-inflammatory cytokines by immune response

Toll-like receptors (TLRs) are major members of the innate immunity that are likely to be the first route to encounter non-self materials such as bacterial infections, and thus play an important role in combating pathogen. Nowadays, 13 TLRs have been identified and discovered contain several leucine-rich repeats. Moreover, TLRs can be divided into two groups are cell surface TLRs and intracellular organelles TLRs. Several molecules can stimulate TLRs, including lipopolysaccharide (LPS) from gram-negative bacteria as a trigger or bacterial lipoproteins and peptidoglycan of gram-positive bacteria. Their activation triggers as a signalling which results in the NF-kB signalling pathway, being downstream of TLRs-mediated signalling and lead to initiating an inflammatory response such as upregulation of the genes that encoding proinflammatory cytokines (14, 15, 17, 18). The previous reported in 2012 was demonstrated that TLR2 and TLR4 are the receptors for *Leptospira interrogans* secretes many hemolysins as inducer through TLR2-and TLR4-dependent JNK and NF-kB pathways (41).

In 2014, Yijie GUO and coworkers stimulated pig fibroblasts by LPS from *Escherichia coli* (E-LPS) and *Leptospira interrogans* serovar Hebdomadis (L-LPS). They

found that L-LPS can induce mRNA expression of TLR2 but does not induce the level of TLR4 mRNA. In addition, E-LPS showing up-regulations the expression of TLR2 and TLR4 mRNA. Moreover, L-LPS can induce the expression of IL-6 and IL-8 mRNA after stimulation in pig fibroblast for 6 h (42). Also, it is was already known that component of *Leptospira* species can activate the immune system through TLR2 more than TLR4, while LPS of gram-negative bacteria can induce proinflammatory cytokine through TLR4.

When the body infected by pathogen, viruses, bacteria or foreign materials, and the immune system can detect and get them out of the body. The mechanisms of immune system are produced and secreted cytokines to response the invading foreign materials. The previously reported in 2006, Vernel-Pauillac and coworkers studied the expression levels of cytokine mRNA in hamsters after infected with *Leptospira interrogans.* The results showed that the increase in the expression of TNF-alpha during 10-18 h post infection and remains to be determined until 24 h (43).

In 2012, Huan Wang and coworkers observed the main pro-inflammatory cytokine expression of IL-1beta, IL-6 and TNF-alpha were found in the sera of leptospirosis patients and in the mice induced with leptospires (41).

In 2012, Sonia Lacroix-Lamande *et al.* studied the response of inflammation in bone marrow-derived macrophages (BMDMs) after stimulated for 24 h with live and heat-killed *Leptospira interrogans* infection. The results showed the dose-dependent secretion of IL-1beta, when stimulated with live higher than heat-killed *Leptospira interrogans* but the secretion level of IL-6, was equivalent when stimulated with live or heat-killed bacteria (44).

When foreign factors enter into the body, such as bacteria or virus infection, the immune system response to the toxins that may contribute to systemic

inflammatory leading to drop in blood pressure, preventing the blood delivery by blocking oxygen and nutrients to the vital organs. These responses are called septic shock. Septic shock can affect any part of the body can lead to respiratory, heart or organ failure, and may result in death.

In 1997, Martin and coworkers studied the expression patterns of TNF-alpha and IL-6 in septic shock and multiple trauma patients. These clinical results showed that is associated with systemic inflammatory response. They found high concentration levels of TNF-alpha and IL-6 in septic shock patients, but in trauma patients showed high concentration levels of IL-6, and showed normal concentration levels of TNF-alpha (45).

CHAPTER III
MATERIALS AND METHODS

Synthesis of gold nanoparticles

The AuNPs were prepared in Milli-Q water, followed the standard citrate-reduction protocol. Add Chloroauric solution (HAuCl4.3H2O) 500 µL and Milli-Q water 24.5 ml to 250 ml conical flask for heating and stirred vigorously. When Chloroauric solution boiled, 980 µL of 38.8 mM sodium citrate was quickly added. Heating continued for 15 minutes and the AuNPs was cooled at room temperature. The gold nanoparticles solution was stored at 4°C.

Dynamic light scattering and zeta-potential measurement

For determining the average size of particles was using Dynamic light scattering (DLS). For analysis of particle surface charges were measured by zeta-potential, and the laser Doppler electrophoresis technique was applied based on the Henry equation. Both techniques were performed on a Malvern Zetasizer Nano Series (Malvern Instruments, England).

Transmission electron microscopy

Characterize gold nanoparticles by TEM (Hitachi, Japan) operated at a voltage of 100 kV.

Cell culture

HEK-Blue-hTLR2, HEK-Blue-hTLR4, HEK-Blue-Null1 and HEK-Blue-Null2 cells using in this study were performed by the manufacturer (Catalog code. hkb-htlr2, hkb-htlr4, hkb-null1 and hkb-null2, Invivogen, USA). Cells derived from laboratory of Associate Professor Pattama Ekpo, Ph.D, Department of Immunology, Faculty of Medicine Siriraj Hospital, Mahidol University. These cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Sigma, USA) supplemented with 10% heat fetal bovine serum (FBS) (Gibco, USA), 50 U/ml penicillin, 50 µg/ml streptomycin (Gibco, USA), and 100 µg/ml Normocin (Invivogen, USA). All cells were cultured at 37°C under 5% CO_2 atmosphere (Esco, Singapore). Maintain HEK-Blue-hTLR2 and HEK-Blue-hTLR4 cells in Growth Medium supplemented with 1X HEK-Blue Selection (Invivogen, USA). Maintain HEK-Blue-Null1 and HEK-Blue-Null2 cells in Growth Medium supplemented with 100 µg/ml of Zeocin (Invivogen, USA) (product description from InvivoGen, USA).

Leptospires culture

Leptospira interrogans serovars Bratislava, Autumnalis, Pyrogenes, and *Leptospira biflexa* serovar Patoc were obtained from Department of Immunology, Faculty of Medicine Chulalongkorn Hospital, Chulalongkorn University.

Cell viability assay

HEK-Blue-hTLR2, HEK-Blue-hTLR4, HEK-Blue-Null1 and HEK-Blue-Null2 cells were seeded into 96-well plates at a density of 5×10^3 cells/well in 45 µL of culture media, and incubated at 37°C under 5% CO_2 atmosphere for 12 h. The cells were treated with various concentrations of 10 nm AuNPs (10, 30, and 50 ppm) and 20 nm AuNPs (10, 20, 50 and 80 ppm). Pathogenic *Leptospira interrogans* 3 serovars,

including: *Leptospira interrogans* serovar Bratislava, *Leptospira interrogans* serovar Autumnalis, *Leptospira interrogans* serovar Pyrogenes and nonpathogenic *Leptospira biflexa* serovar Patoc. Treated leptospires at a multiplicity of infection (MOI) of 100 (100 leptospires per 1 host cell) and AuNPs treated in combination with each leptospires in 45 µL. Plates were incubated for 24 h. Following incubation, 10 µL PrestoBlue™ reagent (Invitrogen, USA) was added to each well, and incubated for 30 min. Fluorescence was measured using a microplate reader at 560 and 590 nm (Thermo, Varioskan Flash, England).

Cell morphology

HEK-Blue-hTLR2, HEK-Blue-hTLR4, HEK-Blue-Null1 and HEK-Blue-Null2 cells were seeded into 24-well plates at a density of 5 x 10^4 cells/well in 500 µL of culture media, and incubated at 37°C under 5% CO_2 atmosphere for 12 h. The cells were treated with appropriate concentrations of AuNPs, 4 serovars of leptospires and AuNPs treated in combination with leptospires in 500 µL and incubated for 24 h. Cell morphology of the cells was studied using a phase contrast inverted microscope (Nikon, Eclipse TS 100, Japan).

ROS generation

HEK-Blue-hTLR2, HEK-Blue-hTLR4, HEK-Blue-Null1 and HEK-Blue-Null2 cells were seeded into 96-black well plates at a density of 5 x 10^3 cells/well in 100 µL of culture media, and incubated at 37°C under 5% CO_2 atmosphere for 12 h. The cells were treated with 10 µM H_2O_2 as a positive control, vitamin C as an antioxidant known to quench ROS, various concentrations of AuNPs, 4 serovars of leptospires and AuNPs treated in combination with leptospires in 200 µL and incubated for 24 h. The cells were washed with phosphate buffer saline (PBS) after that the cells were

incubated with 0.1 μM of 2′, 7′-dichlofluorescein-diacetate (H_2DCF-DA) (Molecular probes, USA) at a volume of 100 μL/well for 30 mins. Cells were washed with PBS again and 200 μL of PBS was added to each well. Fluorescence was measured using a microplate reader at excitation and emission wavelengths of 485 and 528 nm.

TLRs activation by detection and quantification of alkaline phosphatase

HEK-Blue-hTLR2, HEK-Blue-hTLR4, HEK-Blue-Null1 and HEK-Blue-Null2 cells were used to study secreted embryonic alkaline phosphatase (SEAP). HEK-Blue-hTLR2 and HEK-Blue-hTLR4 cells are designed for studying the stimulation of human TLR2 (hTLR2) and human TLR4 (hTLR4), respectively, by monitoring the activation of NF-kB (product description from InvivoGen, USA). The cells, which do not express hTLR2 is HEK-Blue-Null1 cells as a control cell line of HEK-BLUE-hTLR2 and cells which do not express hTLR4 is HEK-Blue-Null2 cells as a control cell line of HEK-BLUE-hTLR4. The levels of SEAP were determined by QUANTI-Blue, a detection medium that turns to purple/blue in the presence of the SEAP. The levels of SEAP determine the level of TLRs activation. Cells were seeded into 96-well plates at a density of 5 x 10^4 cells/well in 150 μL of culture media, and incubated at 37°C under 5% CO_2 atmosphere for 12 h. The cells were treated 100 μL with appropriate concentration of AuNPs, 4 serovars of leptospires and AuNPs treated in combination with leptospires. Plates were incubated at 37°C in a CO_2 incubator for 24 h. Following incubation, 20 μL of induced cell supernatant was added to 180 μL of QUANTI-Blue™ (Invivogen, USA) and incubated at 37°C in a CO_2 incubator for 1-3 h. Measured the SEAP levels using a microplate reader at 620-650 nm.

RNA extraction and quantitative real-time PCR

HEK-Blue-hTLR2 and HEK-Blue-hTLR4 cells were seeded into 24-well plates at a density of 10^5 cells/well in 500 µL of culture media, and incubated at 37°C under 5% CO_2 atmosphere for 12 h. The cells were treated with appropriate concentration of AuNPs, 4 serovars of leptospires and AuNPs treated in combination with leptospires in 500 µL and incubated for 24 h. The total RNA was isolated from cells using TRIzol reagent (Invitrogen). One microgram of total RNA was used for cDNA synthesis with a First strand cDNA synthesis kit (Roche®) according to the manufacturer's instructions. Real-time PCR system using the Express SYBR GreenER qPCR Supermix Universal (Invitrogen). The cDNA as a template for measurement IL-1beta, IL-6, TNF-alpha, TLR2 and TLR4 gene expression. Primer sequences are listed in Table 3. Quantitative Real-time PCR performed using StepOnePlus Real-Time PCR System (ABI Applied Biosystems). The C_T (threshold cycle) values obtained for target gene were normalized to the endogenous GAPDH level (housekeeping gene) and relative to the normalized calibrator. PCR conditions for each primer couple were as follows: pre-PCR heat step 50°C for 2 min, 95°C for 2 min is required to activate the enzyme in real-time kits, pre-denaturation step at 95°C/10 min, followed by 40 cycles of denaturation at 95°C/15 sec, annealing temperature shown in table 3 at 1 min and extension at 72°C/3 min.

Table 4. Sequence of primers used in Quantitative Real-time PCR.

Genes	Primers	**Sequence 5' – 3'**	**Annealing temp.**	**Reference**
IL-1beta	Forward Reverse	AATCTTCATTGCTCAAGTGT TCAGCTTCAAAGAACAAGTC	63°C	Qiuwang Zhang, *et al.* (2013)
IL-6	Forward Reverse	TGCAGAAAAAGGCAAA CAACAACAATCTGAGGTG	63°C	Adam Steensberg, *et al*. (2002)
TNF-alpha	Forward Reverse	CCAGGCAGTCAGATCA TGGGAGTAGATGAGGTACAG	64°C	Qiuwang Zhang, *et al*. (2013)
TLR2	Forward Reverse	GGCCAGCAAATTACCTGTGTG AGGCGGACATCCTGAACCT	60°C	Nathalie Satta, *et al.* (2011)
TLR4	Forward Reverse	CAGAGTTTCCTGCAATGGATCA GCTTATCTGAAGGTGTTGCACAT	60°C	Nathalie Satta, *et al.* (2011)
GAPDH	Forward Reverse	CAGGGGCCATCCACAGTCTTC CATCACCATCTTCCAGGAGCG	59°C	Ming Cui, *et al.* (2014)

Statistical analysis

The result from triplicates of the sample was expressed as mean ± SD. Statistical significance was analyzed by using one-way ANOVA (Analysis of variance), followed by the Tukey's multiple comparison tests. Statistical significance was defined as *the P - value* ≤ 0.05 in comparison to control.

CHAPTER IV
RESULTS AND DISCUSSION

RESULTS

Characterization of gold nanoparticles (AuNPs)

Gold nanoparticles size was measured by using a UV-VIS spectrophotometer, Zetasizer and transmission electron microscope (TEM). The UV-Vis spectrum of AuNPs size 10 and 20 nm revealed a single peak between 500 and 550 nm (figure 4A,4B). Average size of AuNPs was measured by TEM and results showed that AuNPs have spherical morphology and measured size approximately 10 and 20 nm, respectively (figure 4C,4D).

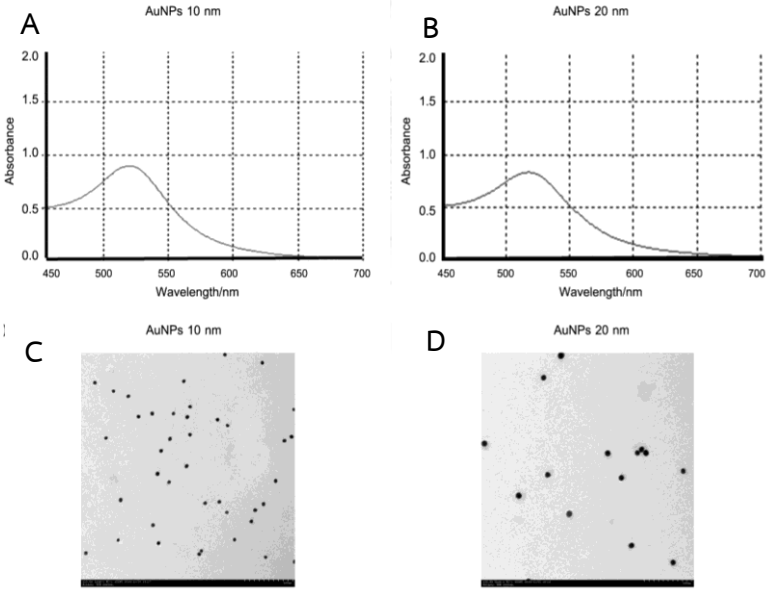


Figure 4. Characterization of AuNPs synthesis citrate-stabilized. A) UV-Vis spectra of 10 nm of AuNPs. B) UV-Vis spectra of 20 nm of AuNPs C) and D) TEM image of monodisperse citrate-stabilized 10 nm and 20 nm. The sample was prepared by drying placed in the aqueous solution of nanoparticles in the coated copper grids.

Furthermore, stability and surface charge of nanoparticles are represented strongly cationic or anionic, the zeta potential should be greater than +30 mV or less than -30 mV, respectively (ref-zeta potential and measurement). The zeta-potential result showed a high negative charge both sizes of AuNPs (Table 5).

Table 5. Show Zeta potential measurements to characterize the surface AuNPs in different size.

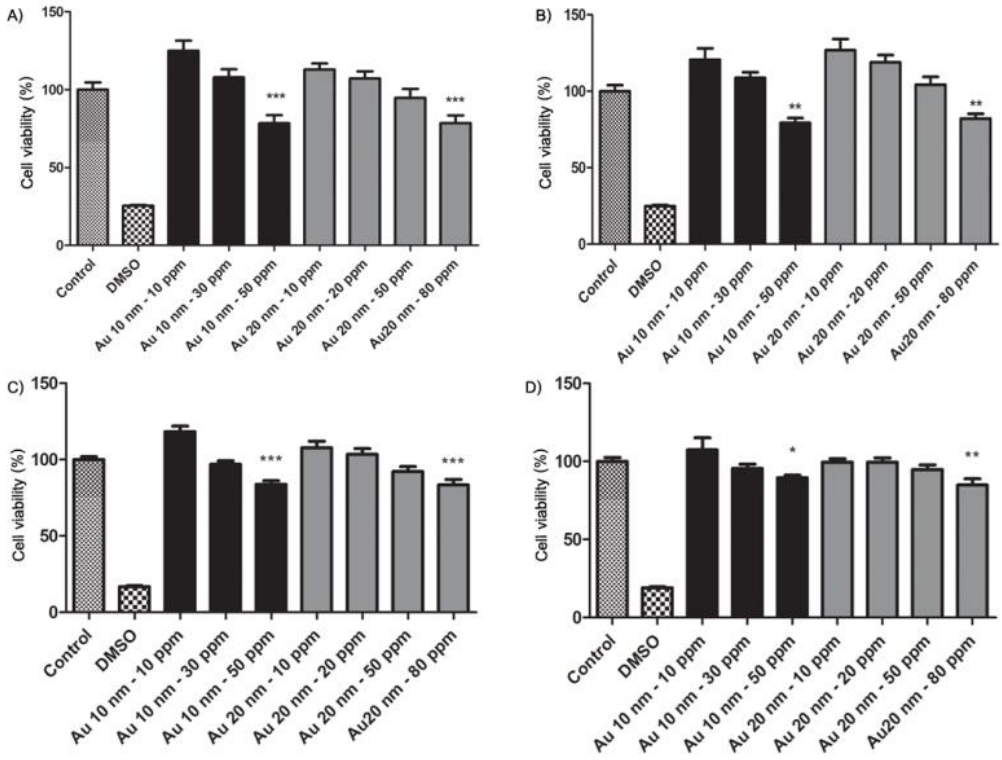
Nanoparticles	Zeta Potential
Gold nanoparticle 10 nm	-37.3 mV
Gold nanoparticle 20 nm	-31.3 mV

Cellular toxicity studies

Cell morphology and viability assay

To show no harmful the effect of AuNPs, leptospires and AuNPs with leptospires on cell viability of HEK-Blue-hTLR2, HEK-Blue-hTLR4, HEK-Blue-Null1, and HEK-Blue-Null2 cells. First, we checking concentrations of AuNPs and various serovars of leptospires are appropriate for treatment. The first group of the cells was treated with AuNPs at various sizes and concentrations (10, 30, 50 ppm of AuNPs size 10 nm and 10, 20, 50, 80 ppm of AuNPs size 20 nm) for 24 h. The second group, the cells was treated with various serovars of leptospires at multiplicity of infection (MOI) = 100: 1 for 24 h. Cell viability was evaluated by the activity of mitochondrial

dehydrogenase using a resazurin-based technique. The result showed that exposure to increasing of AuNPs resulted in significant reduction in cell viability. Further studies of the effect of AuNPs that no toxicity on cell viability were carried out using 30 ppm of 10 nm AuNPs and 50 ppm of 20 nm AuNPs for the next experiment (figure 5A-D). Including, the results suggest that each serovars of leptospires has no obvious toxicity to these cells. (figure 5E-H)



Including, the results suggest that each serovars of leptospires has no obvious toxicity to these cells. (figure 5E-H)

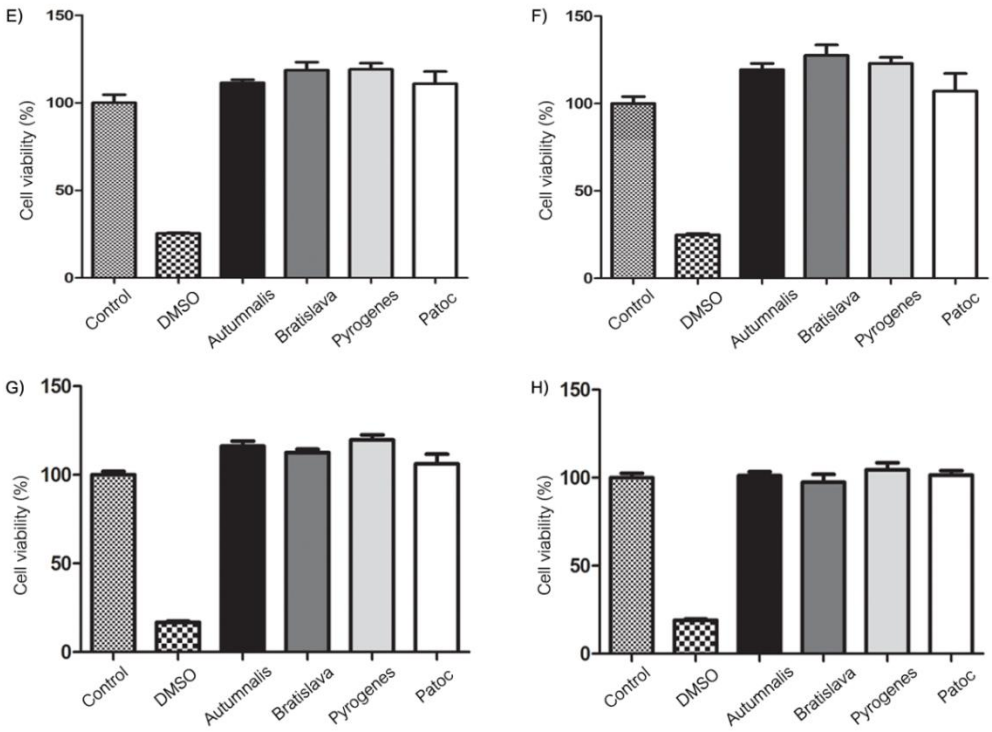


Figure 5. Effect of various sizes and concentrations of AuNPs on cell viability (A-D). Percentage of cell viability in HEK-Blue-hTLR2 (A), HEK-Blue-hTLR4 (B), HEK-Blue-Null1 (C) and HEK-Blue-Null2 (D) cells after incubated with AuNPs 24 h. Effect of various serovars of leptospires on cell viability. Percentage of cell viability in HEK-Blue-hTLR2 (E), HEK-Blue-hTLR4 (F), HEK-Blue-Null1 (G) and HEK-Blue-Null2 (H) cells after incubated with AuNPs 24 h. Data were shown as a percentage of the control group. Values are mean ± SD (n=6). Significance represents the *p<0.05, **p<0.01 and ***p<0.001 versus control group.

Next, cells were treated with appropriate concentrations of AuNPs and various serovars of leptospires. The result showed that percentage of viable cells did not different significantly between control group and other treated group after 24 h (figure 6).

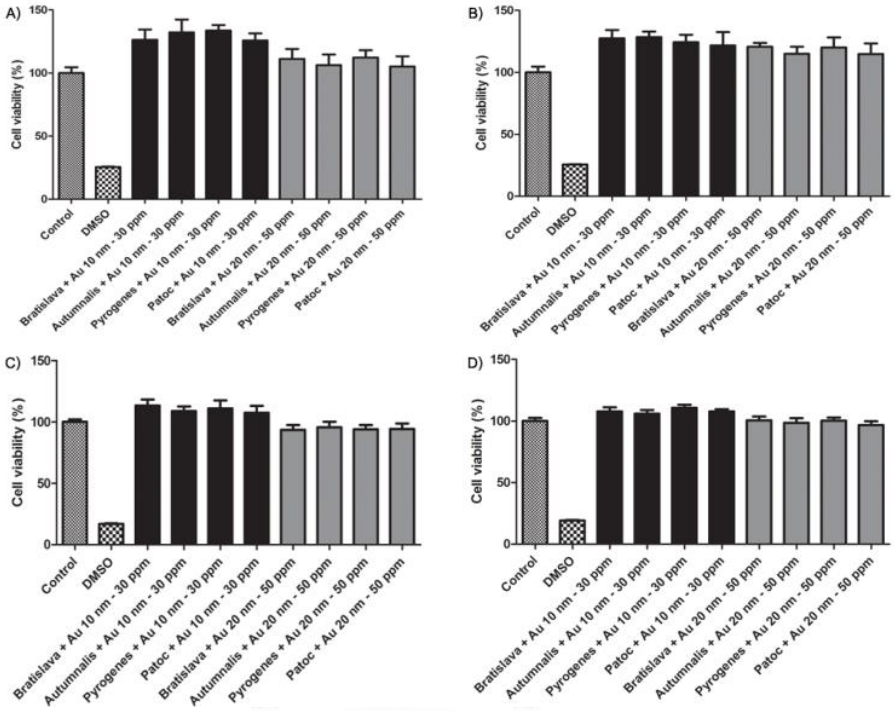


Figure 6. The effect of AuNPs treated in combination with different serovars of leptospires on cell viability**.** Percentage of cell viability in HEK-Blue-hTLR2 (A), HEK-Blue-hTLR4 (B), HEK-Blue-Null1 (C) and HEK-Blue-Null2 (D) cells after incubated with AuNPs and different serovar of leptospires 24 h. Data were shown as a percentage of the control group. Values are mean ± SD (n=6). Significance represents the *p<0.05, **p<0.01 and ***p<0.001 versus control group.

In addition, there was no difference in cell morphology after treated with AuNPs, leptospires and AuNPs with leptospires when compared with the control group (untreated) in all of the cell line (figure 7-10). These data confirm that treatment of various serovars of leptospires with appropriate concentrations of AuNPs did not affect cell viability.

A) Morphology of HEK-Blue-hTLR2 cells after treatment

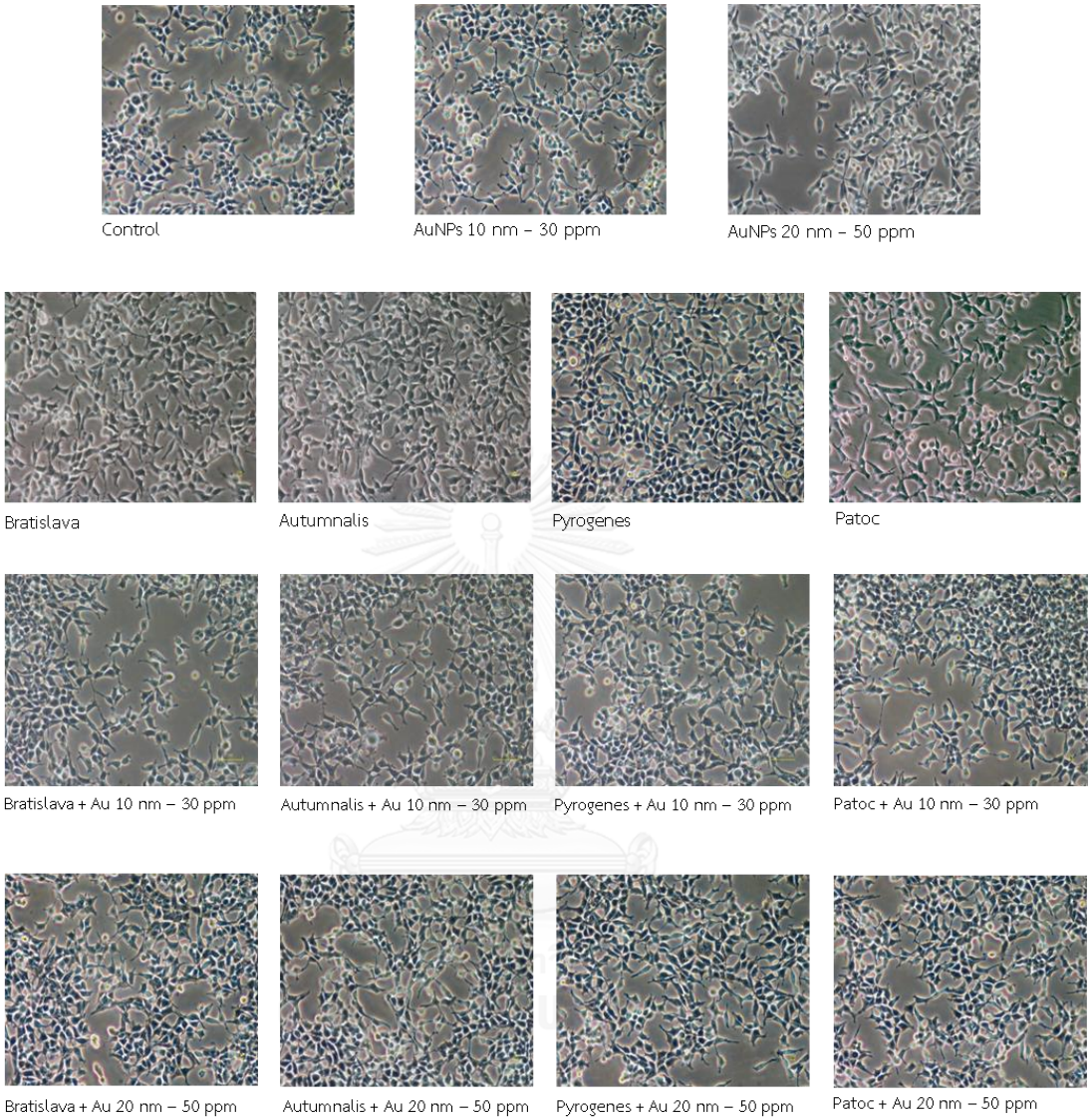


Figure 7. The effect of AuNPs treated in combination with different serovars of leptospires on cell morphology. Morphological changed in HEK-Blue-hTLR2 cells after stimulated for 24 h observation by phase contrast microscopy.

B) Morphology of HEK-Blue-hTLR4 cells after treatment

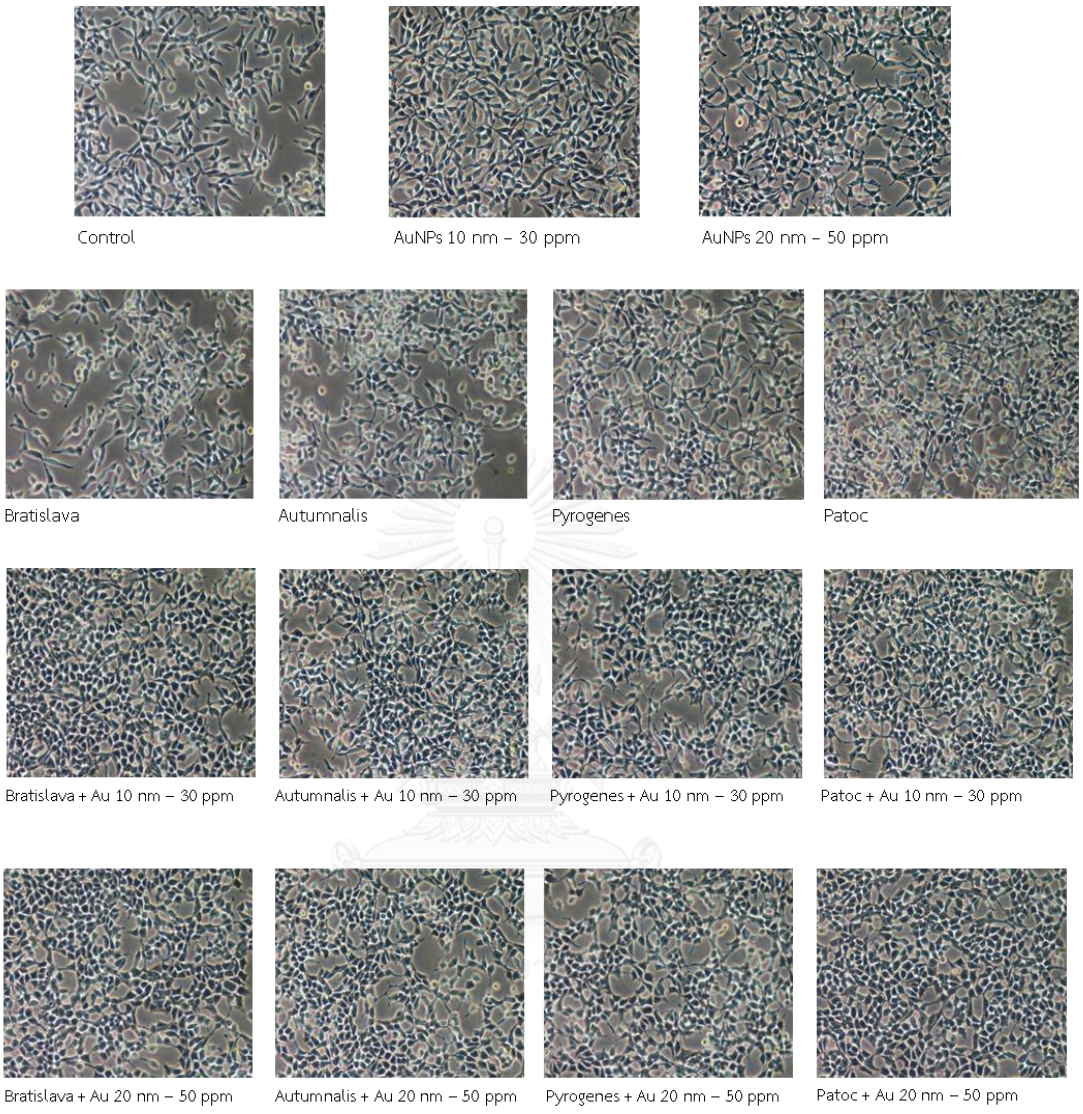


Figure 8. The effect of AuNPs treated in combination with different serovars of leptospires on cell morphology. Morphological changed in HEK-Blue-hTLR4 cells after stimulated for 24 h observation by phase contrast microscopy.

C) Morphology of HEK-Blue-Null1 cells after treatment

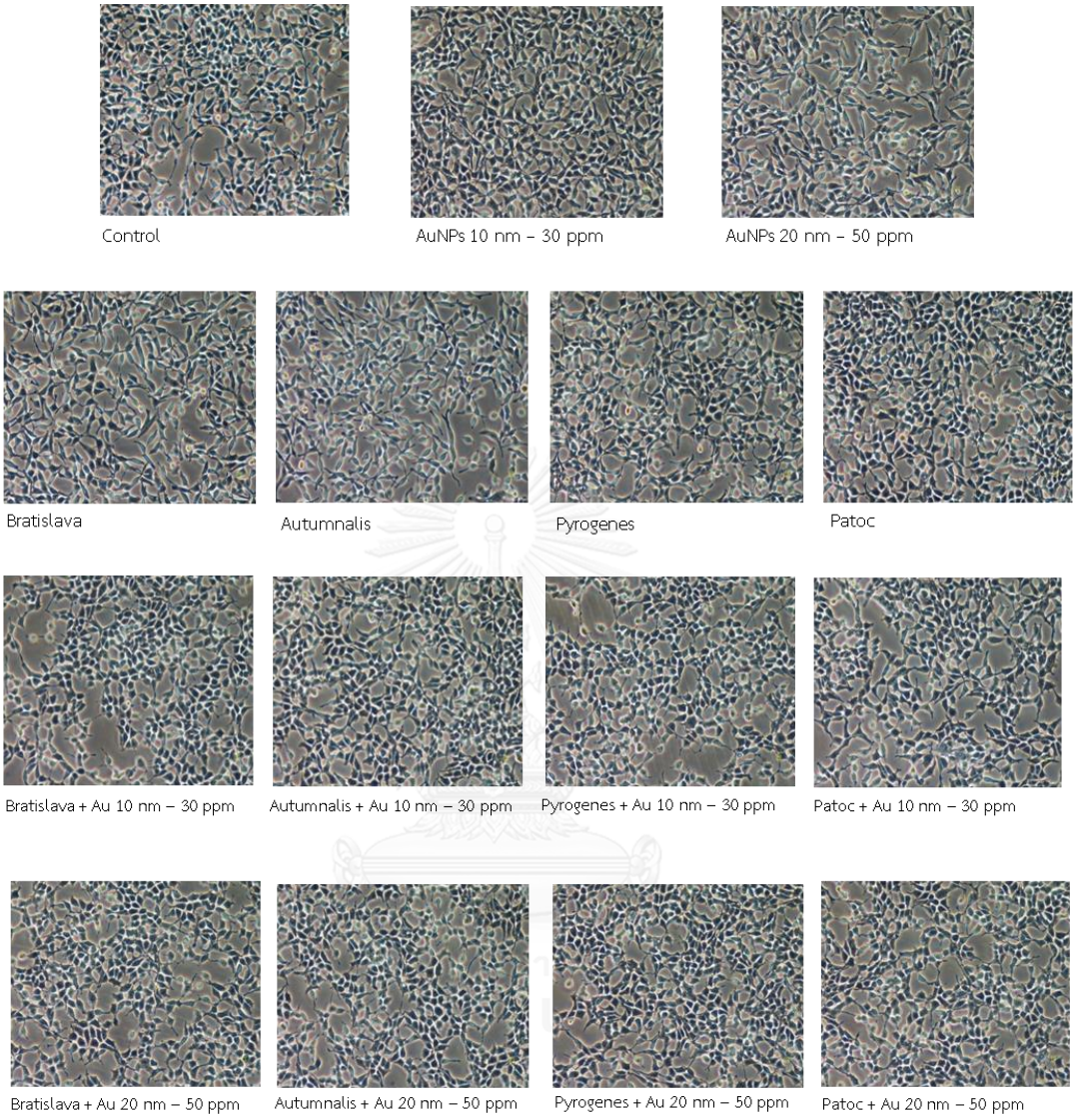


Figure 9. The effect of AuNPs treated in combination with different serovars of leptospires on cell morphology. Morphological changed in HEK-Blue-Null1 cells after stimulated for 24 h observation by phase contrast microscopy.

D) Morphology of HEK-Blue-Null2 cells after treatment

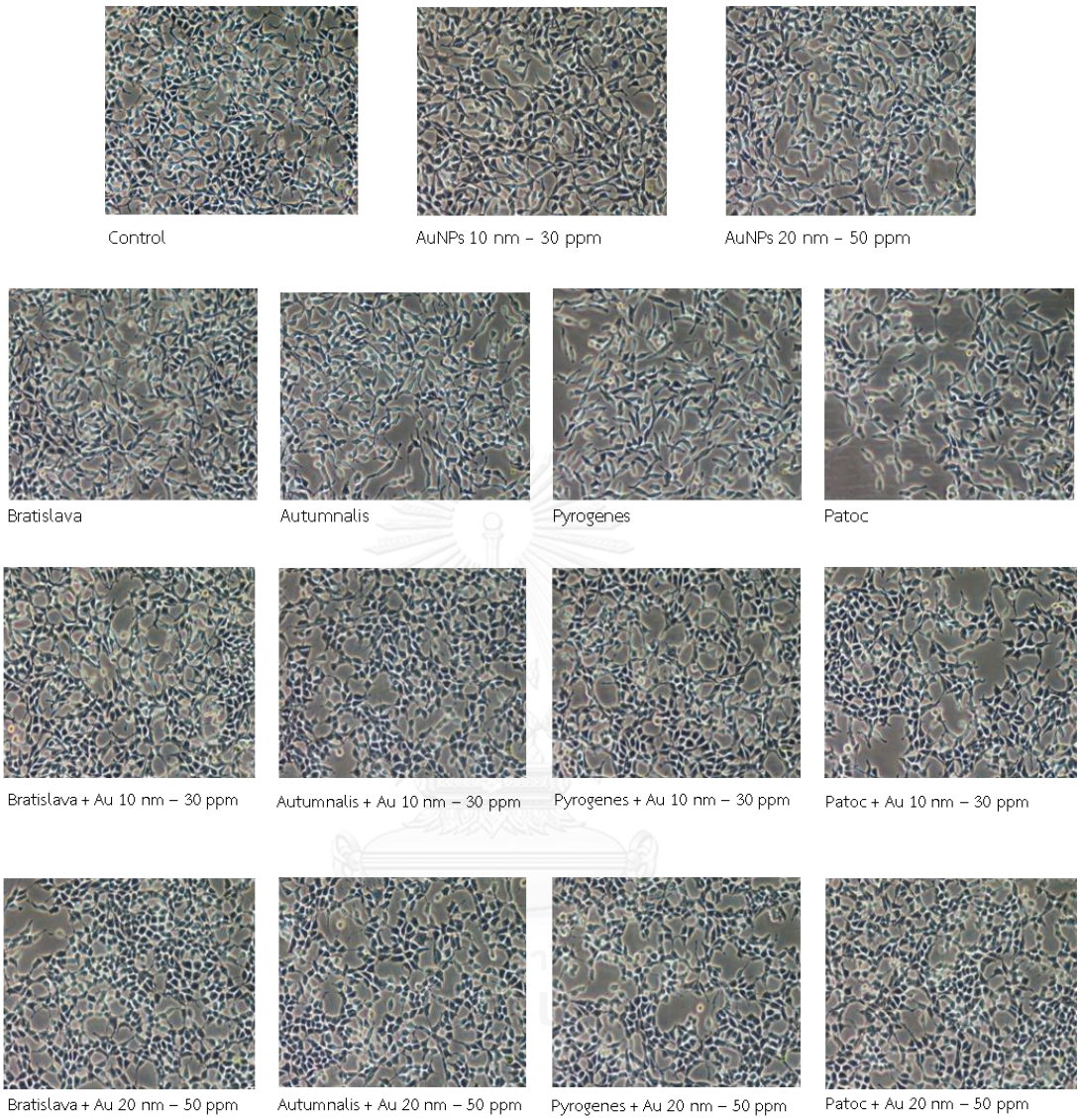


Figure 10. The effect of AuNPs treated in combination with different serovars of leptospires on cell morphology**.** Morphological changed in HEK-Blue-Null2 cells after stimulated for 24 h observation by phase contrast microscopy.

ROS generation

To studies the effect of AuNPs and leptospires on reactive oxygen species generation following treatment in HEK-Blue-hTLR2, HEK-Blue-hTLR4, HEK-Blue-Null1, and HEK-Blue-Null2 cells. The evaluation was performed using the DCFH-DA technique each from 1 h to 24 h. First, we evaluated by varying the concentration of AuNPs for detect ROS generation. Cells were treated with AuNPs at various sizes and concentrations (10, 20, 30, 50, 80 ppm of AuNPs size 10 nm and 10, 20, 30, 50, 100 ppm of AuNPs size 20 nm). The results showed that in AuNPs size 10 nm significantly increases in ROS generation at 80 ppm treatment (figure 11) but in AuNPs size 20 nm treatment showed no ROS generation (figure 12) when compared with the control group.

ROS generation after treated with AuNPs 10 nm

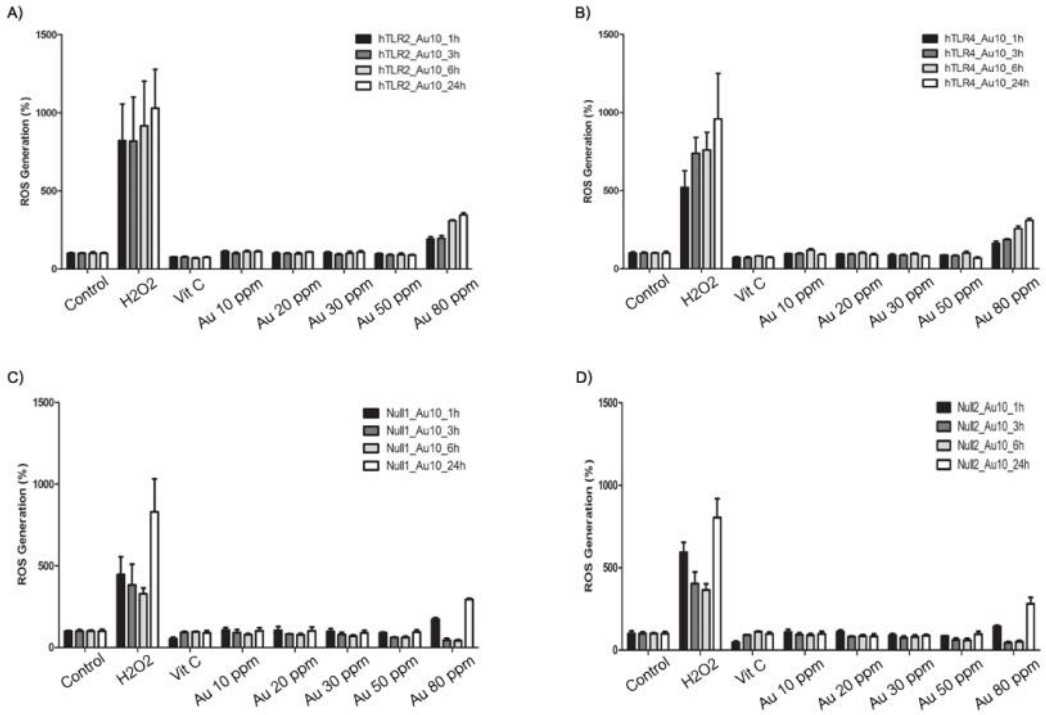


Figure 11. Effect of AuNPs size 10 nm on ROS generation. Percentage of ROS generation in HEK-Blue-hTLR2 (A), HEK-Blue-hTLR4 (B), HEK-Blue-Null1 (C), and HEK-Blue-Null2 (D) cells after incubated with AuNPs at 1, 3, 6 and 24 h. ROS generation was detected with DCFH-DA assay and fluorescence intensity were calculated compared with the control group. Values are mean ± SD (n=6). Significance represents the *p<0.05, **p<0.01 and ***p<0.001 versus control group.

ROS generation after treated with AuNPs 20 nm

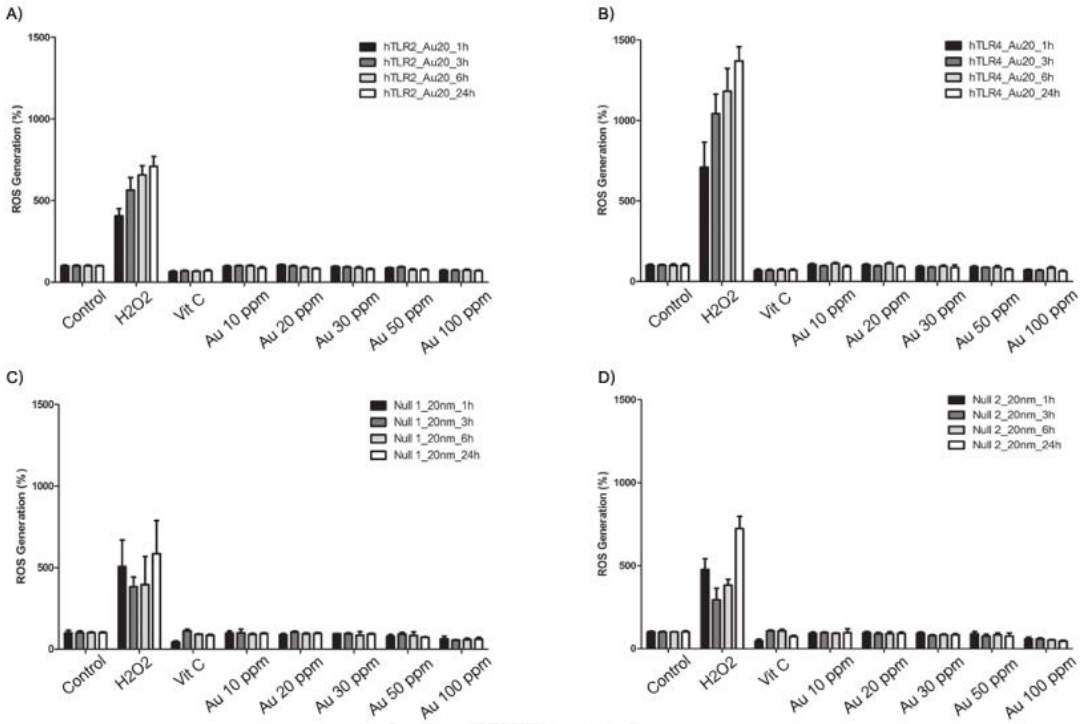


Figure 12. Effect of AuNPs size 20 nm on ROS generation. Percentage of ROS generation in HEK-Blue-hTLR2 (A), HEK-Blue-hTLR4 (B), HEK-Blue-Null1 (C), and HEK-Blue-Null2 (D) cells after incubated with AuNPs at 1, 3, 6 and 24 h. ROS generation was detected with DCFH-DA assay and fluorescence intensity was calculated compared with control group. Values are mean ± SD (n=6). Significance represents the *p<0.05, **p<0.01 and ***p<0.001 versus control group.

Next, to reveal the effect of AuNPs and leptospires on ROS generation. Cells were treated with AuNPs and various serovars of leptospires for 24 h. The appropriate concentration of AuNPs and leptospires following the cell viability test. The result showed that percentage of ROS generation did not different significantly between control group and another group after treated with both sizes of AuNPs while H_2O_2 significantly higher ROS levels as a positive control (figure 13,14).

ROS generation after treated with AuNPs 10 nm in combination with leptospires

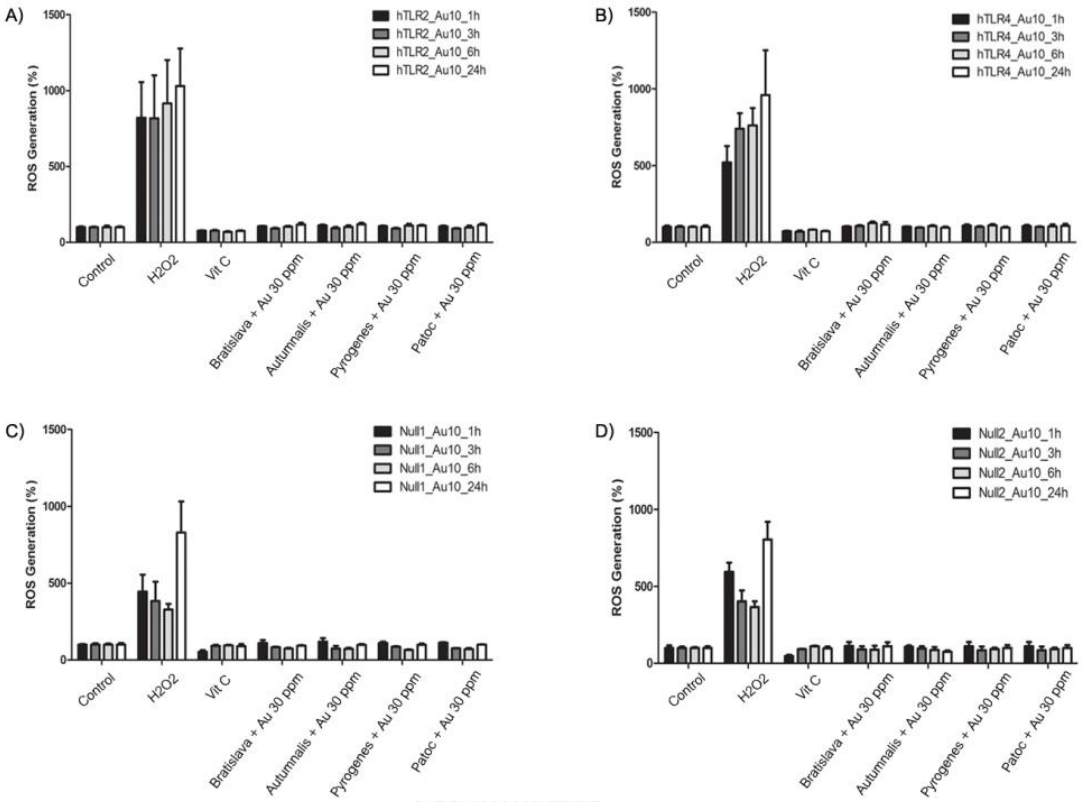


Figure 13. Effect of AuNPs size 10 nm treated in combination with different serovars of leptospires on ROS generation**.** Percentage of ROS generation in HEK-Blue-hTLR2 (A), HEK-Blue-hTLR4 (B), HEK-Blue-Null1 (C) and HEK-Blue-Null2 (4) cells after incubated with AuNPs and different serovars of leptospires 1, 3, 6 and 24 h. ROS generation was detected with DCFH-DA assay and fluorescence intensity were calculated compared with the control group. Values are mean ± SD (n=6). Significance represents the *p<0.05, **p<0.01 and ***p<0.001 versus control group.

ROS generation after treated with AuNPs 20 nm in combination with leptospires

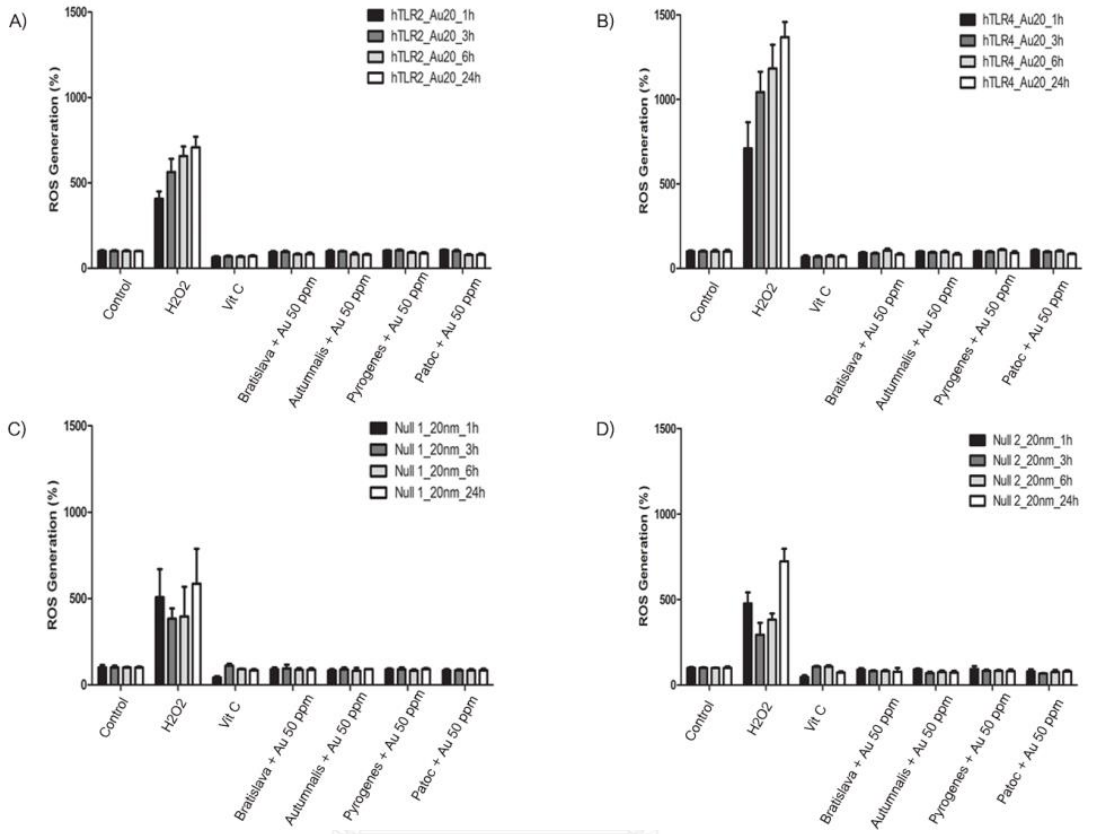


Figure 14. Effect of AuNPs size 20 nm treated in combination with different serovars of leptospires on ROS generation. Percentage of ROS generation in HEK-Blue-hTLR2 (A), HEK-Blue-hTLR4 (B), HEK-Blue-Null1 (C) and HEK-Blue-Null2 (D) cells after incubated with AuNPs and different serovars of leptospires 1, 3, 6 and 24 h. ROS generation was detected with DCFH-DA assay and fluorescence intensity were calculated compared with the control group. Values are mean ± SD (n=6). Significance represents the *p<0.05, **p<0.01 and ***p<0.001 versus control group.

Human TLR2 and TLR4 stimulation by monitoring the activation of NF-kB

HEK-Blue-hTLR2 and HEK-Blue-hTLR4 Cells were designed for studying the stimulation of human TLR2 (hTLR2) and human TLR4 (hTLR4), respectively by monitoring the activation of NF-kB. HEK-Blue-hTLR2 or HEK-Blue-hTLR4 Cells were obtained by co-transfection of the hTLR2 or hTLR4 and SEAP (secreted embryonic alkaline phosphatase) reporter genes into HEK293 cells. Stimulation with a TLR2 ligand or TLR4 ligand activates NF-kB which induces the production of SEAP release into the supernatant. HEK-Blue-Null1 (Null1) and HEK-Blue-Null2 (Null2) cells express the SEAP reporter gene, but no express hTLR2 and hTLR4. These cell lines are the parental cell lines of HEK-Blue hTLR2 and HEK-Blue-hTLR4, respectively (Product descriptions, InvivoGen).

To characterize whether the AuNPs, leptospires and AuNPs in combination with leptospires regarding their biological function, their ability to activate the NF-kB signaling through TLR2 and TLR4 pathway were evaluated on hTLR2 and hTLR4 cells. HEK-Blue-hTLR2, HEK-Blue-hTLR4, HEK-Blue-Null1 and HEK-Blue-Null2 cells were incubated with AuNPs, leptospires and AuNPs in combination with leptospires for 24 h and SEAP in the supernatant were collected and determined with QUANTI-Blue™ detection medium were measured using microplate reader OD at 650 nm. The response ratios of NF-kB in each HEK-Blue-hTLR were determined by subtraction and comparison with their negative controls. And the response ratios of each HEK-Blue-hTLR were compared with their negative cells. The result indicated in HEK-Blue-hTLR2 cell, AuNPs 10 nm showed to be significantly stimulated SEAP secretion, but AuNPs 20 nm did not induce SEAP secretion in this cell. Moreover, when incubated with leptospires alone the results showed that each serovar of leptospires induced HEK-Blue-hTLR2 cells to stimulate SEAP secretion. AuNPs 10 nm, treated in combination with each serovar of leptospires showed to increase higher SEAP secretion than leptospires treatment group. In contrast, AuNPs 20 nm, treated in

combination with each serovars of leptospires showed to suppress SEAP secretion in supernatant compared with the leptospires treatment group (figure 15A, C). In HEK-Blue-Null1 and HEK-Blue-Null2 cells, showed no produced SEAP secretion in all treatment.

In HEK-Blue-hTLR4 cells, AuNPs 10 nm showed to be significantly stimulated SEAP secretion but AuNPs 20 nm did not induce SEAP secretion, these result similarly in HEK-Blue-hTLR2 cells. In contrast with the result of HEK-Blue-hTLR2 when stimulated with each serovar of leptospires in hTLR4 cells did not induce NF-kB assay. In the other group, cells were incubated with AuNPs 10 nm, treated in combination with each serovars of leptospires, the result showed increased SEAP secretion more than leptospires treatment group. In the last group, AuNPs 20 nm, treated in combination with each serovar of leptospires, result showed no change SEAP secretion compared with the leptospires treatment group (figure 15B, D).

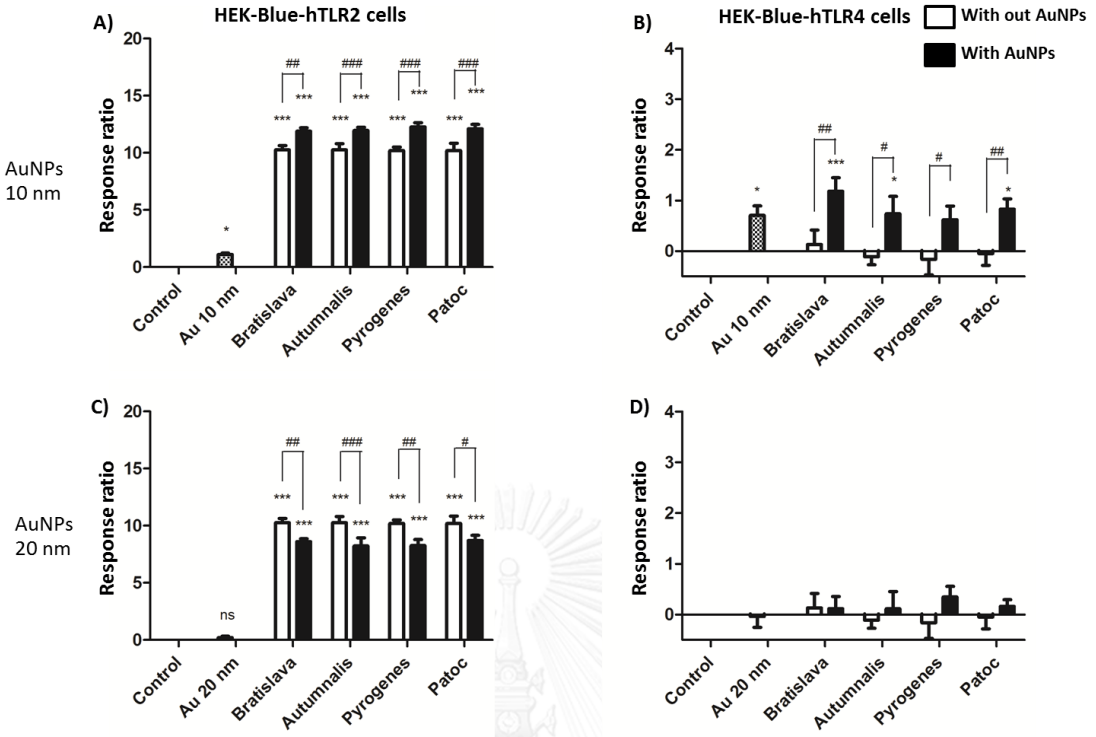


Figure 15. Effect of size of gold nanoparticles and leptospires on NF-kB response in HEK-Blue-hTLR2 and HEK-Blue-hTLR4 cells. Cells were incubated with different serovars of *Leptospira* and 2 size of AuNPs. (A, B) 10 nm AuNPs exposure on HEK-Blue-hTLR2 and HEK-Blue-hTLR4 cells. (C, D) 20 nm AuNPs exposure on HEK-Blue-hTLR2 and HEK-Blue-hTLR4 cells. The levels of NF-kB-induced SEAP were determined by reading the OD at 655 nm. Data were shown as a percentage of the control group. Values are mean ± SD (n=6). Significance represents the *p<0.05, **p<0.01 and ***p<0.001 versus control group.

Finding indicated that AuNPs 10 nm, leptospires and AuNPs 10 nm in combination with each serovar of leptospires activated TLR2, as indicated by the increased secretion of SEAP. While AuNPs 20 nm in combination with each serovar of leptospires suppressed SEAP secretion in hTLR2 cells. In hTLR4 cells activation was mediated by AuNPs 10 nm, which still induced SEAP secretion, including with AuNPs 10 nm in combination with each serovar of leptospires. However, leptospires and AuNPs 20 nm in combination with each serovar of leptospires group were not able to induce TLR4 signaling.

Pro-inflammatory mRNA expression

During the TLRs stimulation by monitoring the activation of NF-kB study indicated that AuNPs, leptospires and AuNPs treated in combination with leptospires induced through TLR2 and TLR4 then released secreted embryonic alkaline phosphatase (SEAP) in HEK-Blue-hTLR2 and HEK-Blue-hTLR4 cells. The important roles of NF-kB pathway in regulating the immune response of the body in the expression of pro-inflammatory genes such as cytokines for eliminating foreign materials from the body. When microbial molecules or foreign materials into the body may trigger TLRs and induce NF-kB pathway lead to an inflammatory response that regulates expression of pro-inflammatory mediators.

In the immune modulation study, pro-inflammatory cytokines were minimally expressed in normal stage, whereas they were all markedly higher expressed after foreign materials administration. Pro-inflammatory cytokine interested in this study such as TNF-alpha, IL-1beta, and IL-6 mRNA expression were evaluated. To determine whether AuNPs was able to modulate the expression of pro-inflammatory cytokine mRNA after treated with AuNPs, leptospires and AuNPs combination with leptospires in HEK-Blue-hTLR2 and HEK-Blue-hTLR4 cell incubated for 24 h. The results showed that in hTLR2 cells, treated with AuNPs size 10 nm can induce TNF-alpha, IL-1beta, and IL-6 mRNA expression was significantly increased when compared to the control (untreated) group but AuNPs size 20 nm show no induced the expression of all cytokine mRNA. In the second group, when treated the cells with varies serovars of leptospires, we found that each serovar of leptospires in this study can induce TNF-alpha, IL-1beta, and IL-6 mRNA up-regulates expression that similar results of induced with AuNPs 10 nm. Further group, HEK-Blue-hTLR2 cells were treated with AuNPs 10 nm in combination with each serovar of leptospires can induce TNF-alpha, IL-1beta, and IL-6 mRNA expression significantly higher than

treated with leptospires alone. In the last group, HEK-Blue-hTLR2 cells were treated with AuNPs 20 nm in combination with each serovars of leptospires, the results showed that AuNPs 20 nm significantly suppressed the expression of TNF-alpha, IL-1beta, and IL-6 mRNA when compare treated with leptospires alone group in HEK-Blue-hTLR2 cells (figure 16 A-C).

In HEK-Blue-hTLR4 cells, treated with AuNPs size 10 nm alone can induce TNF-alpha, IL-1beta and IL-6 mRNA expression was significantly increased when compared to the control (untreated) group, but AuNPs size 20 nm alone no induced the expression of 3 cytokine mRNA these results similar with hTLR2 cells. However, in second group, the expression of TNF-alpha, IL-1beta and IL-6 mRNA were unchanged when following incubation with varies serovars of leptospires, the results inferred that leptospires no induced up-regulates expression of 3 cytokines in HEK-Blue-hTLR4 cells. Moreover, when treated the HEK-Blue-hTLR4 cells with AuNPs 10 nm in combination with each serovar of leptospires, we found the expression of TNF-alpha, IL-1beta and IL-6 mRNA significantly higher than treated with leptospires alone. In the last group of HEK-Blue-hTLR4 cell were treated with AuNPs 20 nm in combination with each serovar of leptospires, the results showed that the expression levels of 3 cytokines were unchanged when compared to leptospires alone treatment group. From the results, we conclude that in hTLR4 cells, AuNPs 10 nm induced up-regulates expression of pro-inflammatory genes such as TNF-alpha, IL-1beta, and IL-6 (figure 16 D-F).

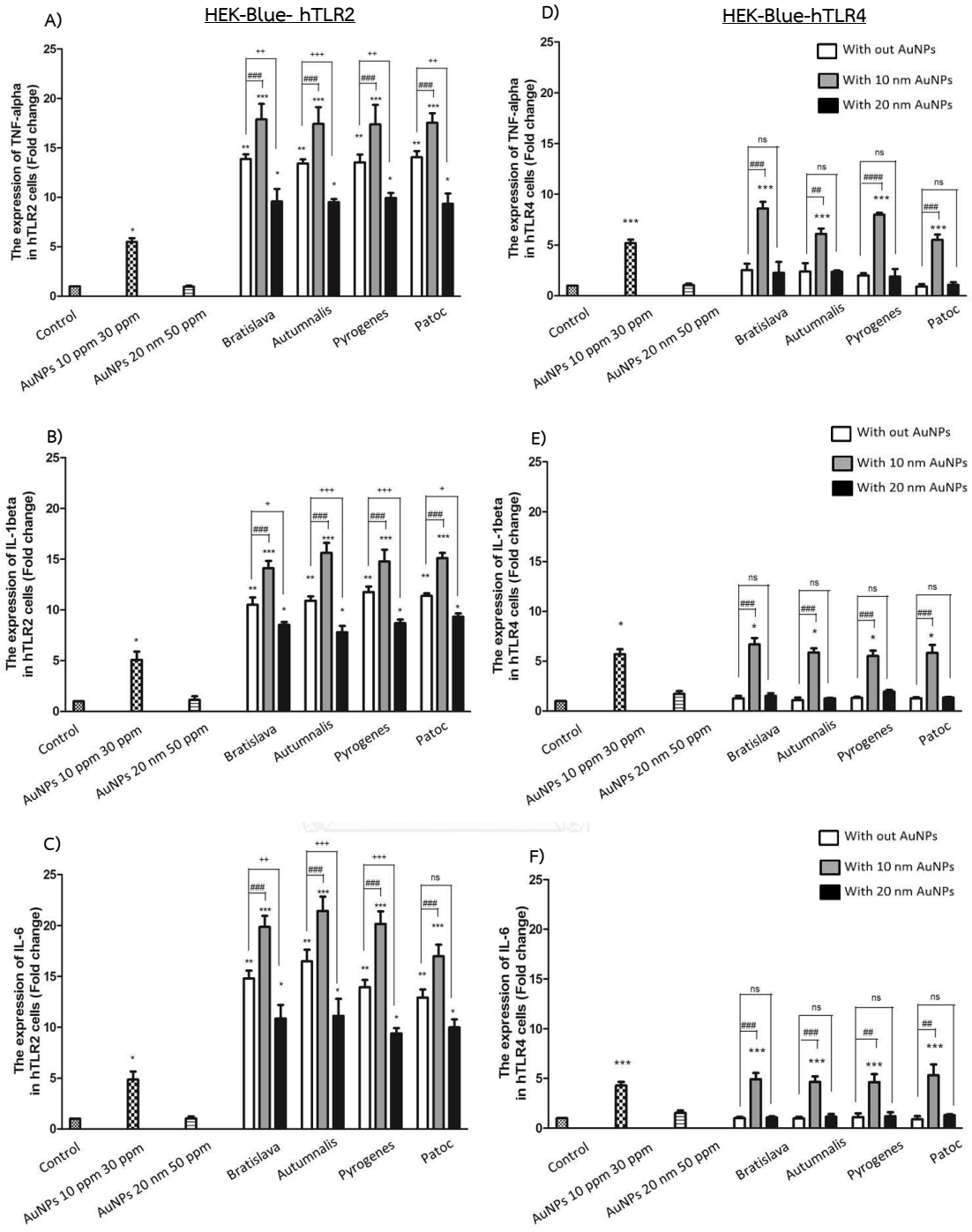


Figure 16. The effect of AuNPs on leptospires-induced pro-inflammatory cytokine mRNA expression as determined by real-time PCR and analyzed by normalized with housekeeping gene. Pro-inflammatory cytokine expression such as TNF-alpha, IL-1beta and IL-6 were following 24 h treatment with AuNPs, leptospires and AuNPs combination with leptospires were evaluated in HEK-Blue-hTLR2 cells (A-C) and HEK-Blue-hTLR4 cells (D-F).

TLR2 and TLR4 mRNA expression response in mediated by AuNPs

To establish the effect of AuNPs on the TLR2 and TLR4 mRNA expression after incubated the cells by varies size of AuNPs in combination with leptospires. From the cytokine mRNA expression results showed that AuNPs 20 nm can suppress TNF-alpha, IL-1beta and IL-6 mRNA expression when incubated the cells in combination with leptospires. But not be clear, how the AuNPs that effect on the modulation of pro-inflammatory cytokine mRNA expression. To directly test whether the activation of AuNPs effect on TLR2 and TLR4 alteration in HEK-Blue-hTLR2 and HEK-Blue-hTLR4 cells, respectively because TLRs are upstream of the NF-kB pathway that be related to produced pro-inflammatory cytokine. The results showed that in HEK-Blue-hTLR2 cells, TLR2 mRNA most highly expressed in treated with AuNPs 10 nm in combination with each serovar of leptospires but no changed when treated with AuNPs 20 nm in combination with leptospires. Accordingly, with HEK-Blue-hTLR4 cells, AuNPs 10 nm in combination with each serovar of leptospires showed to increase TLR4 expression, but AuNPs 20 nm in combination with each serovar of leptospires showed no changes the expression of TLR4 when compared with leptospires treatment alone (figure 17 A-B).

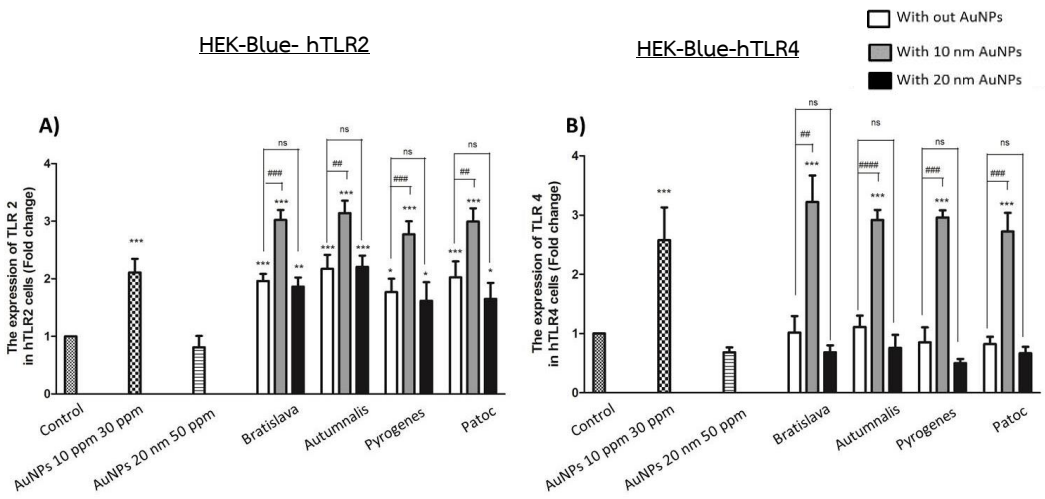


Figure 17. The effect of AuNPs on leptospires-induced TLR2 and TLR4 mRNA expression in HEK-Blue-hTLR2 and HEK-Blue-hTLR4 cells, respectively, as determined by real-time PCR and analyzed by normalized with housekeeping gene. TLR2 and TLR4 expression were following 24 h treatment with AuNPs, leptospires and AuNPs combination with leptospires were evaluated in HEK-Blue-hTLR2 cells (A) and HEK-Blue-hTLR4 cells (B).

Discussion

In this study, we found that the body has been invaded by the unknown substance, the first line of immunity for against invaders is toll-like receptors (TLRs). Our finding showed that the fate of AuNPs response to inflammation is dependent on size of AuNPs. In this study HEK-293 cell stably transfected with human TLR2 (HEK-Blue-hTLR2) and TLR4 (HEK-Blue-hTLR4) was used in all experiments. The smallest 10 nm AuNPs were found to have cellular response TLRs dependent on both of hTLR2 and hTLR4 cells. In addition, TLRs activation showed 10 nm AuNPs stimulate high levels of SEAP, we assume that 10 nm AuNPs can induce NF-kB pathway through TLRs. Moreover, cytokine results confirm TLRs activation assay results that downstream of the NF-kB transcription factors are many cytokines. One group of interesting pro-inflammatory cytokine genes, mainly in bacterial infection, including IL-1beta, IL-6, and TNF-alpha was showed increasing the expression by 10 nm AuNPs, whereas 20 nm AuNPs showed no changes in expression of these cytokines.

To confirm that the observed change induced by AuNPs and leptospires was not related to cell damage, cell viability assay, and morphological change were analyzed. Cell viability was studied to demonstrate the cytotoxicity of AuNPs and leptospires. Our data clearly showed cell viability decreases after cell exposure to 10 nm AuNPs at 50 ppm and 20 nm AuNPs at 80 ppm. Therefore, in our experiment, we used 10 nm at 30 ppm and size 20 at 50 ppm of AuNPs that does not toxic to cells. In this context, Rosella Coradeghini *et al.* (2013) showed cytotoxicity of AuNPs 5 nm at the exposure time of 72 h and show no cytotoxicity was observed in Balb/3T3 cells of AuNPs 15 nm (8). After we got the appropriate concentrations of AuNPs that no cytotoxicity, then we evaluate the cytotoxicity of AuNPs when treated in combination with leptospires at MOIs of 100. Our results showed the percentage of viable cells was not significantly different between treated and untreated groups. This work in accordance with previous studies reported that J774A.1 cells (mouse macrophage cell line) infected with *Leptospira interrogans* at MOIs of 100 for 8 h showed reductions in cell viability when compared with uninfected group. However, HUVEC infected by *L. interrogans* at MOIs of 100 shows no loss of cell viability after

12 h incubation time. Thus, macrophages (J774A.1) were more sensitive to apoptosis than endothelial cells (HUVEC) due to *L. interrogans* (46). In addition, we found that when treated AuNPs in combination with leptospires, the morphological quality of cells was not changed. These data confirm the cell viability results. Moreover, it is very possible that AuNPs and leptospires did not affect cell viability in terms of size, shape, conformation, and number of cells. It is postulated AuNPs showed good biocompatibility activity in *in vitro* (3, 22). The ROS generation is a key mediator of cellular inflammation. Macrophages produce excessive oxidative stress and secrete pro-inflammatory cytokine for eliminating pathogens or invaders (47). Therefore, ROS generation was studied to evaluate cell stress from AuNPs and leptospires. Our results showed that no induced ROS generation in all of the cells exposure with both sizes of AuNPs. Moreover, in the treatment group, at appropriate concentration of both sizes of AuNPs treated in combination with leptospires show no induces ROS generation in all these cells. These results inconsistent with the previous study, they revealed 15 nm AuNPs showed no induction of oxidative stress in the triple cell co-culture (A549-alveolar epithelial cells, human blood monocyte-derived macrophages, and dendritic cells) (48). On the contrary, previous observations Vecchio G, et *al.* showed high levels of ROS in the small size of AuNPs (5 nm), but the larger sizes of AuNPs showed a decrease of the ROS levels in *the Drosophila melanogaster model* (49). In addition, the previous study indicated that rat Kupffer cells produce ROS production after infected with alive *L. interrogans* (50). These data suggest that the AuNPs, leptospires and AuNPs with leptospires do not affect cellular toxicity based on the exposed cell type specific or different model. Thus, we conclude that ROS generation independent inflammatory response of these cells.

Toll-like receptors (TLRs) act as pattern recognition receptors in host animals or humans stimulated by invading pathogens such as bacteria or virus. TLRs play an important role as initiating an inflammation of the body to against the foreigner. In the present study, HEK-293 cell stably transfected with human TLR2 (HEK-Blue-hTLR2) and TLR4 (HEK-Blue-hTLR4) with a vector expressing secreted embryonic alkaline phosphatase (SEAP) reporter gene were used as a model study to identify the TLRs responsible for the activation of AuNPs and leptospires. Therefore,

activations of TLRs will show in quantity of SEAP in the supernatant of cell that related to the levels of NF-kB stimulation. Our results show that AuNPs 10 nm at 30 ppm stimulates SEAP secretion, but AuNPs 20 nm at 50 ppm did not induce SEAP secretion in HEK-Blue-hTLR2 and HEK-Blue-hTLR4 cells. Our data consistent with previous study by Monita Sharma *et al.* which revealed that AuNPs 10 nm activate the NF-kB pathway in a B-lymphocyte cells at 24 and 48 h (30). Furthermore, the level of SEAP secretion in the group treated with AuNPs in combination with leptospires was determined. We hypothesized that AuNPs can suppress the stimulation through TLR2/4 and reduce induction of NF-kB result in reducing SEAP secretion in HEK-Blue-hTLR2 cells stimulated by leptospires and AuNPs. The results showed that in HEK-Blue-hTLR2 and HEK-Blue-hTLR4 cells were treated with smaller size of AuNPs in combination with leptospires induced higher levels of SEAP secretion. In contrast, larger size of AuNPs showed the suppress SEAP secretion. This finding established that the induction of TLRs depends on the size of AuNPs. 10 nm AuNPs activation of NF-kB by TLRs dependent via TLR2 and TLR4 lead to inflammation in the body, but 20 nm AuNPs inductions SEAP as a TLRs independent.

TLRs play important roles in the activation of both innate and adaptive immune systems. When TLRs as up-stream were triggered leading to activation NF-kB pathway. We focus on down-stream of NF-kB pathway which is the result of inflammatory response that regulates expression of pro-inflammatory mediators. In the immune modulation study, TNF-alpha, IL-1beta, and IL-6 mRNA, these are major cytokines involved in inflammation and infection responses were used to study. The results showed consistent in the previous TLRs activation results, AuNPs 10 nm induces the expression of pro-inflammatory cytokine in HEK-Blue-hTLR2 and HEK-Blue-hTLR4 cells. Our result in accordance with the previous report that the 13 nm sized PEG-coated AuNPs were seen to induce acute liver inflammation by the increase inflammatory cytokine (11). In contrast, Haseeb A. Khan *et al.* studied the effect of AuNPs size 10 and 50 nm on expression of pro-inflammatory cytokine gene in the liver and kidney of rat. The results showed that AuNPs size 50 nm highly increases the level of pro-inflammatory expression more than AuNPs size 10 nm (40).

In the group of cells treated with AuNPs 10 nm in combination with leptospires showed up-regulate expression of these cytokines. But AuNPs 20 nm in combination with leptospires show suppresses the expression of these pro-inflammatory mRNA in HEK-Blue-hTLR2 cells. According to many researches reported that AuNPs size approximately 20 – 35 nm show decreased the expression of proinflammatory cytokine levels (1, 21, 37, 38, 51). In fact, we believe that synergistic or inhibition effect on the inflammatory response dependent on size of AuNPs. To study how the AuNPs effect on the modulation of proinflammatory cytokine mRNA expression, the expression of TLRs were evaluated. Our results showed that AuNPs in small size induced the expression of TLR2 and TLR4 mRNA in HEK-Blue-hTLR2 and HEK-Blue-hTLR4 cells, respectively. Accordingly, the high level expression of TLR2/4 mRNA was observed in hTLR2/4 cells treated with AuNPs size 10 nm in combination with leptospires. However, the treatment group with AuNPs size 20 nm in combination with leptospires showed no changes the expression of TLR2 and TLR4 in HEK-Blue-hTLR2 and HEK-Blue-hTLR4 cells, respectively. From the results, we hypothesize that size dependent of AuNPs have an effect on TLRs level and lead to the modulation of pro-inflammatory cytokine mRNA expression in HEK-Blue-hTLR2 and HEK-Blue-hTLR4 cells. According to previous research reported the inflammation-dependent on up-regulation of TLR2 and TLR4 expression during intestinal inflammation (36). The previous study about the effect of AuNPs on endotoxin-induced uveitis in rats by David Valter Pereira *et al.* revealed that 30 nm AuNPs decreases TNF-alpha, NF-kB and TLR4 levels. The researcher summarized that AuNPs affect to the down-regulations of the TLR4-NF-kB pathway (21).

In this work, leptospires were selected as a model for study size-dependent cytotoxicity. We used pathogenic *Leptospira interrogans* serovars Bratislava, Autumnalis, Pyrogenes and non-pathogenic *Leptospira biflexa* serovar Patoc as a stimulator for inducing immune system via TLR2. These serovars are common in tropical regions. Our data showed different *Leptospira* species are cause same results of inflammation between pathogenic *Leptospira* species and non-pathogenic

Leptospira species. It can conclude that the inflammation of leptospires independent on serovars of *Leptospira*.

Although AuNPs has the advantage of being nontoxic, there have been still some limitations in the preclinical research that should be addressed. The responsiveness of humoral immunity for AuNPs and leptospires still unclear and should be assessed in more details. Further studies should also be performed to examine the protein that produces by these pro-inflammatory mRNA genes after treatment. Moreover, should be to study in *in vivo* model for applying in the treatment of leptospirosis. In this study, we use only citrate stabilizer and two sizes of AuNPs for evaluating the effect of AuNPs on inflammation. However, other physical of AuNPs as size, shape or other chemical stabilizer that may effect on the inflammation did not reveal in this work. Including, other TLRs and down-stream of TLRs should be in the future work.

CHAPTER V
CONCLUSION

In conclusion, we report the effect of AuNPs response on the immune system in term of TLRs, NF-kB, and down-stream is cytokine production. Our findings, using *in vitro* TLR2/4 overexpressed models, suggest that TLR2/4-mediated response of gold nanoparticles by size dependent pattern. Size 10 nm of AuNPs has been shown to be able to up-regulate TLR2/4 gene expression, while 20 nm AuNPs do not change the expression level of TLR2/4 genes. NF-kB is one of the major pathways that lead to AuNPs-induced cytotoxicity such as regulation of proinflammatory gene expression. 10 nm AuNPs can induce NF-kB assay and results in up-regulates the expression of proinflammatory genes. On the other hand, 20 nm AuNPs have ability to down-regulation of both NF-kB assay and proinflammatory genes. Moreover, we found that TLR2/4-mediated cytotoxicity by AuNPs is ROS-independent. The results of this work described here have expanded the current basic knowledge of cellular responses to AuNPs for keeping away from the hazard or reduce its harmful effects. Furthermore, to guide the treatment of infectious diseases, such as leptospirosis diseases. In addition to this information, we can choose appropriate physical of nanoparticles to apply for drug delivery application.

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APPENDIX

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
AuNPs	Gold nanoparticles
cDNA	Complementary DNA
CO_2	Carbon dioxide
C_T	Threshold cycle
DLS	Dynamic light scattering
DMEM	Dulbecco's Modified Eagle Medium
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GNPs	Gold nanoparticles
H_2DCF-DA	2', 7'-dichlofluorescein-diacetate
$HAuCl_4.3H_2O$	Chloroauric solution
HIF-1alpha	Hypoxia-inducible factor 1-alpha
hTLR2	Human toll-like receptor 2 cell line
hTLR4	Human toll-like receptor 4 cell line
IL-1beta	Interleukin-1beta
IL-6	Interleukin-6
IL-10	Interleukin-10
IL-11	Interleukin-11
IL-12beta	Interleukin-12beta
IL-13	Interleukin-13
IL-17	Interleukin-17
LPS	Lipopolysaccharide
MCP-1	Monocyte chemotactic protein 1
MOI	Multiplicity of infection
NF-kB	Nuclear factor kappa B

OMPs	Outer membrane proteins
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositol 3-kinase
RAW264.7	Mouse leukaemic monocyte macrophage cell line
SEAP	Secreted Embryonic Alkaline Phosphatase
ROS	Reactive oxygen species
TEM	Transmission electron microscopy
TLR2	Toll-like receptor 2
TLR4	Toll-like receptor 4
TNF-alpha	Tumor necrosis factor-alpha

EQUIPMENT AND CHEMICALS

1. Autoclave (Hirayama, Japan)
2. Biohazard Laminar Flow (Gibco, USA)
3. CO_2 incubator (Esco, Singapore)
4. Fluorescent microscope (Nikon, Japan)
5. Laboratory balance (Denver instrument, Germany)
6. Malvern Zetasizer Nano Series (Malvern Instrument, England)
7. Microcentrifuge (Hettich, Germany)
8. pH meter (Denver instrument, Germany)
9. Phase contrast inverted microscope (Nikon, Japan)
10. Shaker incubator (Heidolph, Germany)
11. Transmission Electron Microscope (Hitachi, Japan)
12. Varioskan Flash microplate reader (Thermo, England)
13. Vortex mixer (Scientific industries, USA)
14. StepOnePlus Real-Time PCR System (ABI Applied Biosystems)
15. Water bath (Memmert, Germany)
16. 24-well plate (Corning, USA)
17. 96-well plate (Corning, USA)
18. Cell culture flask (SPL, Korea)
19. Centrifuge tube 1.5 mL (Corning, USA)
20. Centrifuge tube 15 mL (Corning, USA)
21. Centrifuge tube 50 mL (Corning, USA)
22. Filter Tip (Corning, USA)
23. Dulbecco's Modified Eagle's Medium (Sigma, USA)

24. Fetal Bovine Serum (Gibco, USA)
25. Penicillin/Streptomycin (Gibco, USA)
26. Normocin (Invivogen, USA)
27. Zeocin (Invivogen, USA)
28. Hek-Blue selection (Invivogen, USA)
29. LPS (Sigma, USA)
30. Gold nanoparticles 10 nm (Sigma, USA)
31. H_2DCFDA (Invitrogen, USA)
32. QUANTI-Blue (Invivogen, USA)
33. TRIzol reagent (Invitrogen, USA)
34. First strand cDNA synthesis kit (Roche®) (Thermoscientific, USA)
35. PrestoBlue™ Cell viability Reagent (Invitrogen, USA)
36. SYBR GreenER qPCR Supermix Universal (Invitrogen, USA)

CHEMICAL PREPARATIONS

1. Phosphate buffer saline

KCl	0.2	g
KH_2PO_4	0.2	g
NaCl	8.0	g
Na_2HPO_4	1.15	g

Mix all of chemical component and add DI water to 1,000 mL, then adjust pH to 7.4 with HCl

2. Dulbecco's Modified Eagle's Medium (DMEM)
    1) Dissolve 13.4 g of DMEM with 800 mL DI water
    2) Add 3.7 g of Na_2HCO_3
    3) Adjust pH to 7.2 with HCl
    4) Add DI water to 1,000 mL
    5) Filtrate by 0.2 μM filter and keep as a stock medium
    6) For working medium preparation, add 100 mL of heat Fetal Bovine Serum,
       5 mL of antibiotic (Pen-Strep), and 1 mL of normocin into 900 mL of stock medium.

- Working media for hTLR2 and hTLR4 cells add 2 ml of 1X Hek-Blue selection into

  500 mL of media.

- Working media for Null1 and Null2 cells add 0.5 mL of zeocin into 500 mL of media.

Cell Viability Assay Protocol (PrestoBlue™, Invitrogen, USA, Catalog number A13261)

1. Cell culture

Materials

1. 96-well plate
2. Cell Culture Media
3. Micropipetters
4. CO_2 incubator

Method

1. Cells are seed in 96-well plates at a density of 1-10 X 10^3 cells/well in 45 µL.
2. Incubate at 37°C and 5% CO_2 for 12 h.

2. Cell viability assay

Materials

1. Unknown sample for toxicity test
2. DMSO (positive control)

Method

1. Add 45 µL of culture medium for negative control.
2. Add 45 µL of DMSO for positive control.
3. Treat with 45 µL of unknown samples.
4. Incubate at 37°C and 5% CO_2 for 24 or 48 h.
5. Add 10 µL PrestoBlue™ reagents and incubate for 30 min.
6. Measure fluorescent product by using a microplate reader at 560 and 590 nm.

Reference

- Product Information Sheet : PrestoBlue™ Cell Viability Reagent Protocol from Invitrogen™

Reactive Oxygen Species (ROS) Generation Protocol (H_2DCFDA, Invitrogen, USA, Catalog number D399)

1. Cell culture

Materials

1. 96-black well plate
2. Cell Culture Media
3. Micropipetters
4. CO_2 incubator

Method

1. Cells are seed in 96-black well plate at a density of 1-10 X 10^3 cells/ well in 100 µL.
2. Incubate at 37°C and 5% CO_2 for 12 h.

2. DCFH-DA assay

Materials

1. Unknown sample for ROS generation test
2. H_2O_2 (positive control)
3. Vitamin C (negative control)
4. Phosphate Buffer Saline (PBS)

Method

1. Wash the cells 2 times with PBS
2. Add 100 µL of 0.1M H_2DCFDA
3. Incubate at 37°C and 5% CO_2 for 30 min.
4. Wash 2 times with PBS
5. Add 100 µL of culture medium for control.
6. Add 100 µL of 0.5% H_2O_2 for positive control.
7. Add 100 µL of 5 mg/mL vitamin C for negative control.

8. Treat with 100 µL of unknown sampless.
9. Measure fluorescence excitation and emission at 485 and 528 respectively by using microplate reader.

Reference

- Product Information Sheet : H_2DCFDA from Invitrogen™

QUANTI-Blue detection Protocol (QUANTI-Blue, Invivogen, USA, Catalog code rep-qb1)

1. Cell culture

Materials

1. 96- well plate
2. Cell Culture Media
3. Micropipetters
4. CO_2 incubator

Method

1. Cells are seed in 96-well plate at a density of 2 X 10^4 cells/ well in 150 µL.
2. Incubate at 37°C and 5% CO_2 for 12 h.

2. Detection and quantification of alkaline phosphatase assay

Materials

1. Unknown sample for SEAP test
2. QUANTI-Blue medium(Invivogen, USA)

Method

1. Add 100 µL of culture medium for negative control.
2. Treat with 100 µL of unknown samples.
3. Incubate at 37°C and 5% CO_2 for 24 h.
4. Add 180 µL of QUANTI-Blue™ per well to new 96-well plates.
5. Add 20 µL of induced cells supernatant to QUANTI-Blue medium.
6. Incubate the plate at 37°C in a CO_2 incubator for 1-3 h.
7. Measured SEAP levels using a microplate reader at 650 nm.

Reference

- Product Information Sheet : QUANTI-Blue from Invivogen™

VITA

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