Chapter 1.1: Introduction

Human beings use electricity at their disposal for convenience and improvement in life. Possible undesirable effects of electric and magnetic field exposures have brought up great concerns [1-4]. Most of the epidemiologic research focused on exposure to low frequency electromagnetic fields or radiofrequency fields; however there are only a few epidemiologic studies specifically designed to study potential health hazards of static field exposure. It is of general concern that the present knowledge regarding the health concerns of static electromagnetic fields (static EMF) is lagging behind medical development such as magnetic resonance imaging (MRI). New studies are needed in order to fill the gaps in knowledge and provide assurance that novel medical technology will not cause any unwanted health hazards [5].

There have been few studies on the effects of static EMF at the cellular level [6]. static EMF alone does not have a lethal effect on the basic properties of cell growth and survival under normal culture conditions [7, 8]. Most studies also suggest that static EMF failed to affect proliferation of the cells [9] or to influence cell cycle. However, disturbance in gene expression have been reported. In HeLaS3 cells, static EMF enhanced *c-fos* expression as examined by Northern blot. Morphological analyses indicate that static EMF induce modifications of cell shape, cell surface and cytoskeleton [10-17]. static EMF may also modulated apoptosis through influence on cytoplasmic calcium ion concentration [11].

In multi-cellular organism minor effects of static EMF at the cellular level might accumulate and end up with distinct symptoms. Since *Caenorhabditis elegans* provides a rich resource in the genetic and molecular investigation with advantages of being a multi-cellular organism, it might provide insights into the gaps in knowledge of possible health hazards. For *C. elegans*, short term treatment of static EMF induced fluctuation of heat shock protein gene expression [18, 19]. But in general, low genotoxicity was observed. The current study is based on the hypothesis that static EMF might induce abnormality in the nematode and this abnormality might be correlated to molecular pathways such as apoptosis.

Chapter 1.2: Materials and Methods

1.2.1. Strains and chemicals.

Strains of C. elegans used in this research including wild type (N2), ced-3, ced-4, ced-6, ced-9, and cbp-1 were obtained from the Caenorhabditis Genetics Center (CGC), University of Minnesota, St Paul. Populations of C. elegans exist primarily as hermaphrodites. Nematodes were propagated at 20 °C on nematode growth-medium (NGM) plates (Brenner 1974) with Escherichia coli strain OP50 as a food source. All culture media and related chemicals including Bacto agar, Bacto tryptone, and yeast extract were purchased from Gibco Co. Other chemicals of analytical grade or higher were purchased from Sigma or Merck. Nd-Fe-B magnets were purchased from Taiwan Magnetic Corp. Ltd. The magnetic field strength of the device was measured at The National Measurement Laboratory, Taiwan.

1.2.2. Mobility assay.

The static EMF device was composed of two Nd-Fe-B permanent magnets sandwiching one Petri dish at the center. The field intensity of the static EMF device was measured and varied from 0 to 200 mT by adjusting the distance between magnets (Fig.1.). Synchronized wild-type nematodes (N2) from L1 to L4 staged larvae were treated by 200 mT static EMF for 3 days and measured for possible reduction in movement. To effectively capture the

locomotion behavior of a freely moving worm, a grid paper (5mm per grid) was placed underneath the Petri dish and the movement of worms was recorded through optical microscope equipped with a CCD camera. The moving speed was obtained by analyzing the recorded video. Number of sine wave per minute (sw/min) propagated along the anterior/posterior axis in the dorsal/ventral plane was also analyzed. The measurement was performed on 30 worms. And the result was expressed as mean ± SD (standard deviation). An adult nematode travels at a speed of approximately 8 mm/min with mobility of 12 sw/min. All experiment were performed at 20℃.

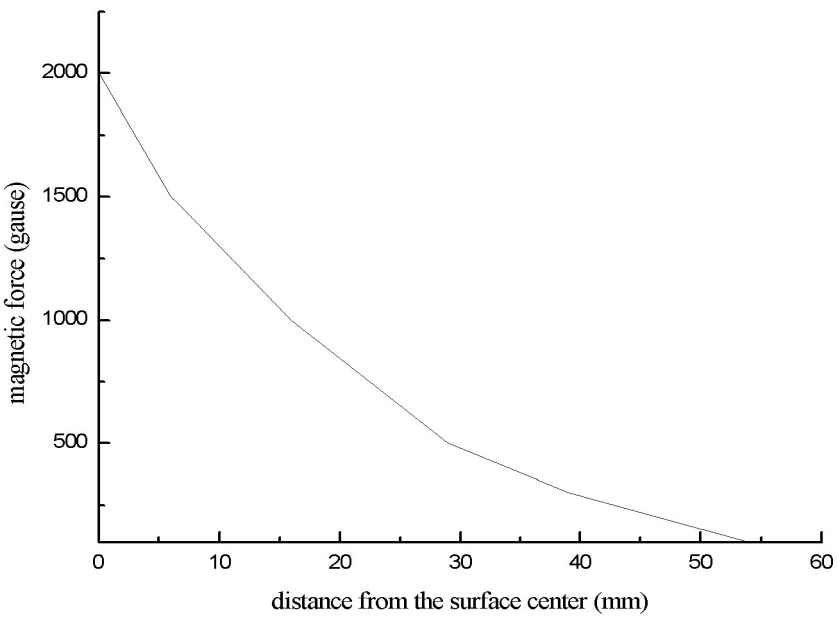

Fig. 1. Magnetic field strength at the center of SEMF settings used in the current study. Distance depicts the distance in mm between two permanent magnets. Field intensity was represented by mT.

1.2.3. Quantitative real time RT-PCR.

Total RNA was extracted from 100 worms using TRI-reagent (Talron Biotech) according to the manufacturer's specifications. Worms were picked and washed three times with M9 and collected in a 2ml eppendorf tube. The pellet was dissolved in 1ml Tri-regent and disrupted by homogenizer on ice, followed by chloroform extraction and isopropanol precipitation. The crude RNA extract was immediately purified by RNeasy Mini Kit (Qiagen) to remove impurities and unwanted organics. Purified RNA was resuspended in DEPC water and quantified by OD260. OD260 to OD280 ratio usually exceeded 2.0 at this stage. For cDNA synthesis, 1μg total RNA was annealed with 1 μg oligo-dT, followed by reverse transcription using SuperScript® III Reverse Transcriptase (Invitrogen) in a total volume of 50 μl. Between 0.2 and 0.5 μl of the reverse transcription reactions were used for quantitative real time PCR using the SYBR Green I on an iCycler iQ5 (Bio-Rad Laboratories). Cycling conditions were: 1× [5 min 95°C] and 50× [20 s 95°C, 20 s 60°C, 40 s 72°C] fluorescence was measured after each 72°C step. Expression levels were obtained as threshold cycles (Ct) determined by the equipped software of iCycler iQ Detection System.

Relative transcript quantities were calculated by the ΔΔCt method using ribosomal protein L18 and L21 as reference genes amplified from the same cDNA sample. ΔCt is the difference in threshold cycles of the sample mRNAs relative to ribosomal protein L18 or L21 mRNA. ΔΔCt is the difference between ΔCt of normal control and ΔCt of static EMF-treated sample.

Fold change in mRNA expression was expressed as 2ΔΔCt. Results are expressed as mean ± SD of 6 experiments.

1.2.4. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay [20].

TUNEL assay was performed using DeadEnd™ Fluorometric TUNEL System (promega) according the manufacturer's specification with minor modification. Worms were washed there times with M9 and collected in a 2 ml eppendorf tube. Worms were fixed in 4% formaldehyde for 15 minutes at room temperature followed by PBS wash for three times. Worms were further incubated in 100ul of a 20ug/ml Proteinase K solution for 10 minutes at RT, washed in PBS for 5 minutes, fixed in 4% formaldehyde for 5 minutes at RT, washed in PBS for 5 minutes, and incubated in 100ul Equilibrate buffer for 10 minutes at RT. The fixed worms were resuspended in 100 µl TdT reaction mix (90ul equilibrate buffer, 10ul nucletide mix, and 2rTdT enzyme) and incubated at 37 °C for 1 hour in a humidified dark chamber, followed by 2X SSC wash for once and PBS wash for three times. The fluorescence image was obtained using Lica confocal microscope DMI4000 at 488 nm.

1.2.5. Immunostaining.

Immunostaining was performed based on the procedure described previously with minor

modification [21]. Worms were washed there times with M9 then fixed by 4% formaldehyde for 10 minutes at RT. Cell wall was disrupted by adding 0.1% Triton X-100 diluted in 0.1% BSA for 30 minutes at RT. After PBS washing worms were incubated with goat anti-CED-3 IgG diluted 1:200 in 0.1% BSA overnight at 4 °C. The worms were washed three times with PBS and incubated with fluorescein isothiocyanate (FITC)-conjugated donkey anti-goat IgG diluted 1:1000 in 0.1% BSA for 1 hour at RT, followed by PBS wash for three times. Imaging was performed with Leica confocal microscope. With excitation wavelength set at 488 nm and emission set at 530 nm.

Chapter 1.3: Result and Discussion

1.3.1. Static EMF flux density stronger than 150 mT reduces the mobility of nematode.

The crawling action of nematodes was measured by moving speed (mm/min) and the mobility was characterized by number of sine waves generated per minute (sw/min). Synchronized wild-type nematodes (N2) from L1 to L4 staged larvae were treated by 200 mT static EMF for 3 days and measured for possible reduction in movement (Fig. 2.).

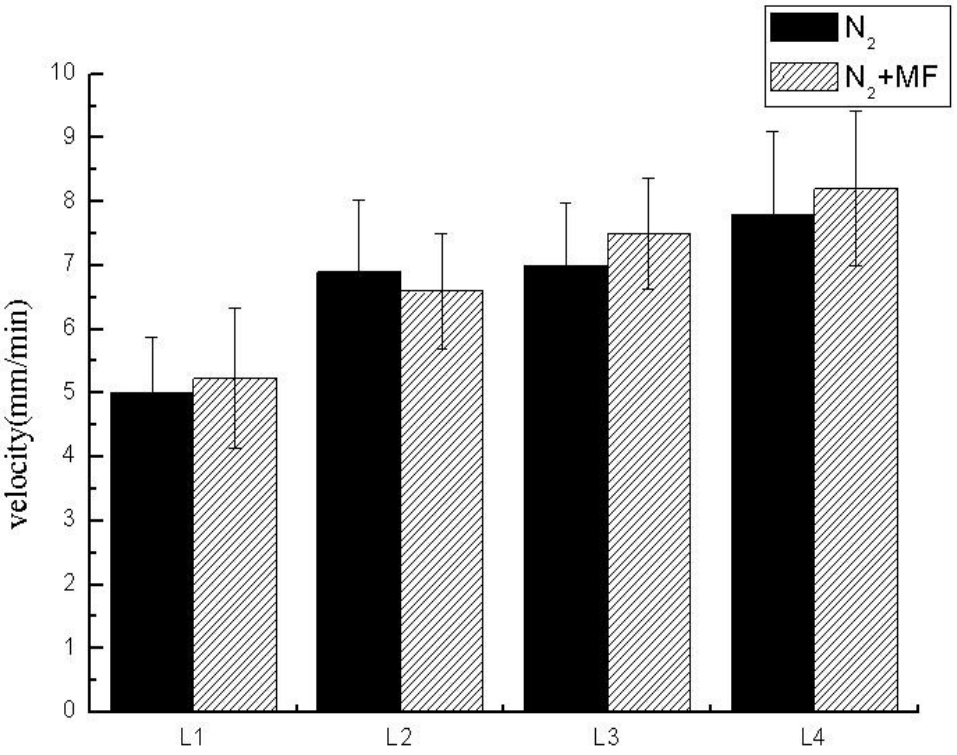

Fig. 2. Crawling speed for larvae of various stages treated with static magnetic field. Nematodes at designated stage were treated by 200 mT for 4 hours before mobility

measurement. The crawling speed is measured in the absence (black bars) or in the presence (shaded bars) of magnetic fields.

Nematodes did not show difference in moving speed probably due to the insufficient time of treatment. Time course experiment was then performed to synchronized adult N2 nematodes under static EMF varied from 0 to 200 mT (Fig. 3.). At 20℃, adult N2 nematodes move approximately at 8 mm/min with mobility of 12 sw/min. Extended static EMF treatment affected crawling action of worms. The reduction in moving speed and mobility was dosage-dependent and also time-dependent. Flux density of static EMF under 50 mT has no significant effect. Significant reduction in mobility occurred when the flux density higher than 150 mT for treatment longer than 4 days. Moving speed reduced to 1.5 mm/min under our extreme condition (200 mT for 8 days, Fig. 4.).

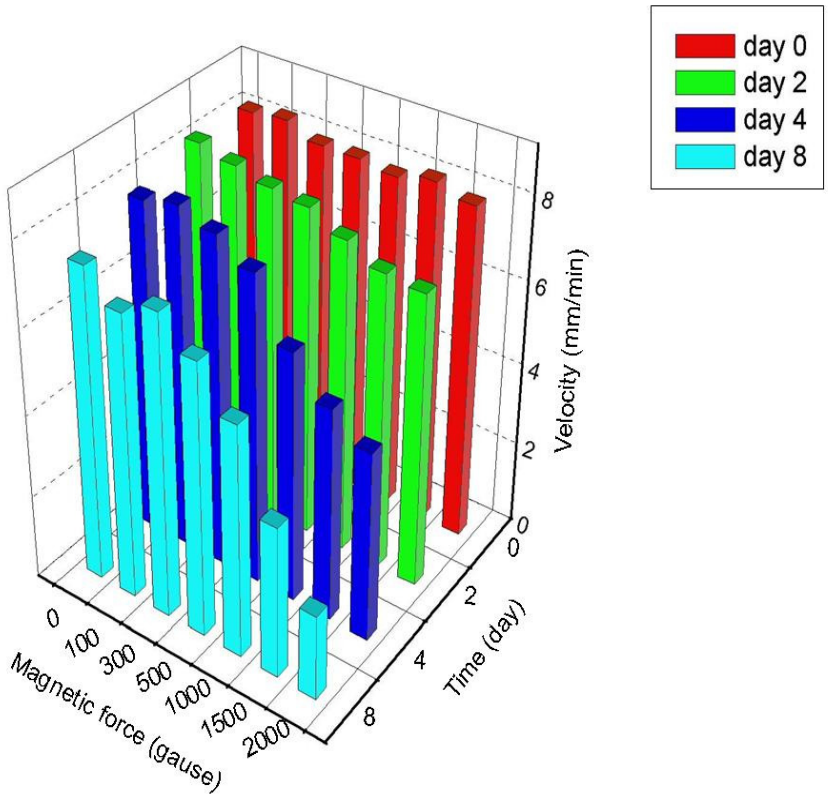

Fig. 3. Surveillance for intensity and duration of static EMF on the crawling speed of adult nematodes. Adult worms are grown under the static EMF from 0 to 200 mT and for 0 to 8 days. The crawling speed is measured in the absence of static EMF and under 20℃. The result is the averaged value from 30 worms.

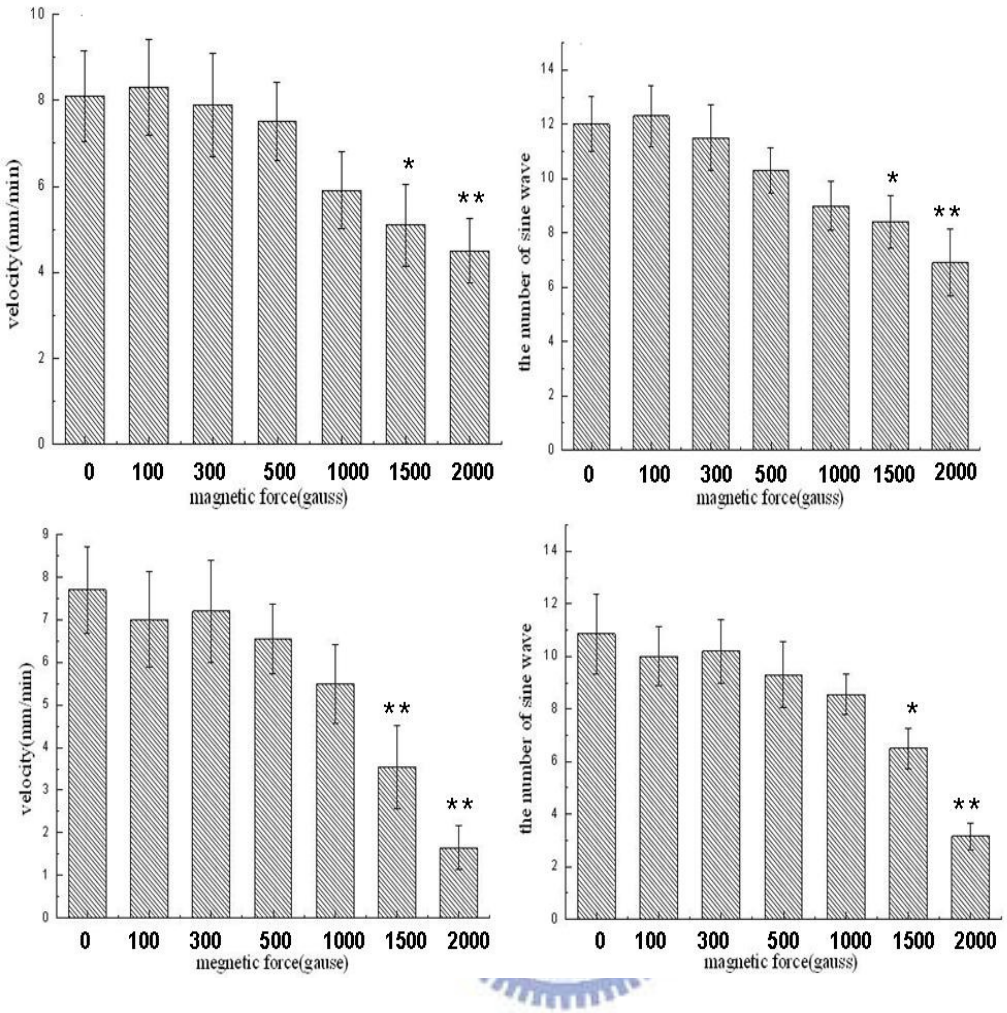

Fig. 4.Crawling speed and mobility of nematodes tread by static EMF. The upper panel shows the effects of a four-day static EMF treatment in crawling speed(left) and mobility(right). The lower panel shows the effects of an eight-day static EMF treatment in crawling speed(left) and mobility(right). All experiment were performed under 20℃. The result is expressed as mean ± SD from 30 worms. Student's *t*-test was performed comparing the crawling speed of static EMF-treated group with untreated control. (*) depicts $p < 0.05$ while (**) depicts $p < 0.01$.

1.3.2. Apoptotic genes were differentially expressed in the presence of static EMF.

Reduced moving speed of nematodes indicated possible damage to the cellular function related to movement such as muscle cells or neuronal cells. It is likely that gene expression of those cells was altered by static EMF treatment. To screen for genes differentially expressed under static EMF treatment, quantitative real time RT-PCR was performed. For the preliminary screening 120 genes were selected from cancer genes, apoptosis, development, stress response, and metabolism (genes and primer sequences were listed in Table 1 of complimentary information) that several pathway may affected by static EMF. Among them, 26 genes consistently showed differential expression (Table 1). These genes are associated with apoptosis (*ced-2, ced-3, ced 6, ced-8*), cancers genes (*abl-1, cbp-1*), stress response (*hsp 16, hsp70, hsp90*), and aging (*age-1*). Of most interests is the enhanced effect on genes associated with apoptosis. Apparently apoptotic pathway could play a role in the static EMF-induced mobility reduction of nematode.

Table 1 List of differentially expressed genes derived from quantitative RT-PCR

Symbol[a]	**Annotation**	**ΔΔCt**[b]	**SD**[c]	**Fold**[d]
Abl-1	Related to oncogene abl	5.56	0.17	47.18
Alx-1	Alix (apoptosis-linked gene 2 interacting protein x) homolog	-1.32	0.26	0.40
Bir-1	Bir (baculovirus inhibitory repeat) family	2.43	0.51	5.39

Ced-3	Cell death abnormality	4.16	1.00	17.88
Ced-2	Cell death abnormality	2.71	0.36	6.54
Ced-6	Cell death abnormality	-0.71	0.22	0.61
Ced-8	Cell death abnormality	4.27	1.64	19.29
Che-13	Abnormal chemotaxis	-0.641	0.09	0.64
Mel-26	Maternal effect lethal	3.43	0.88	10.78
T27F7.2	Shc-2 - (shc (src homology domain c-terminal) adaptor homolog)	2.05	0.68	4.14
Tir-1	Tir (toll and interleukin 1 receptor) domain protein	4.83	0.50	28.44
Nft-1	Nitfhit family	1.06	0.56	2.08
Par-4	Abnormal embryonic partitioning of cytoplasm	4.63	0.42	24.76
Bub-1	Yeast bub homolog	5.98	0.34	63.12
Daf-18	Abnormal dauer formation	2.35	0.46	5.10
Cbp-1	Cbp/p300 homolog	3.23	0.88	9.38
Dic-1	Human dice1 (deleted in cancer) homolog	5.5	0.43	45.25
Hoe-1	Homolog of elac2 (cancer susceptibility locus)	-1.35	0.29	0.39
Cyp-44A1	Cytochrome p450 family	-1.82	0.53	0.28
Sod-2	Sod (superoxide dismutase)	2.14	1.22	4.41
Hsp 16	Heat shock protein	2.67	0.78	6.36
Hsp 70	Heat shock protein	2.94	0.22	7.67
Hsp 90	Heat shock protein	3.68	0.57	12.82
Act-1	Actin	4.85	0.65	28.84
Age-1	Ageing alteration	1.86	0.98	3.63
Dif-2	Differentiation abnormal	5.62	1.24	49.18

[a]This list contains 26 genes which are selected from real time PCR of 120 genes. The selected genes are consistently differentially expressed with *p*-value less than 0.05 in the Student's *t*-test.

[b]ΔΔCt: Relative transcript quantities were calculated by the ΔΔCt method using ribosomal protein L18 and L21 as reference genes amplified from the same sample. ΔCt is the difference

in threshold cycles of the sample mRNAs relative to ribosomal protein L18 or L21 mRNA. ΔΔCt is the difference between ΔCt of normal control and ΔCt of treated sample. The values are averaged from 6 sets of independent experiments.

[c]SD: Standard deviations are calculated from results of 6 experiments.

[d]Fold: Fold change in gene expression was calculated by $2^{\Delta\Delta Ct}$.

1.3.3. Biochemical evidence indicated increase of apoptotic activity under the influence of static EMF.

The abnormality of apoptosis was further verified by functional assays. One of the characteristics of apoptosis is the degradation of DNA after the activation of Ca/Mg dependent endonucleases. This DNA cleavage leads to strand breaks within the DNA. The TUNEL method detects the broken ends of DNA and identifies apoptotic cells in situ by using terminal deoxynucleotidyl transferase (TdT). Fluorescent TUNEL staining for the static EMF-treated *C. elegans* was performed (Fig. 5.). Cytochalasin D was administered as positive control of apoptosis. Cytochalasin D inhibits actin polymerization and induces depolymerization of actin filaments formed during platelet shape change [22]. Cytochalasin-D treatment triggers premature apoptosis of insect ovarian follicle and nurse cells [23]. Cytochalasin D-treated nematode showed a global TUNEL staining for the whole body. static EMF-treatment, on the other hand, induced localized apoptotic reaction. Fluorescence was

distributed along both sides of intestinal adducts of nematode which is likely the muscle portion of the worms.

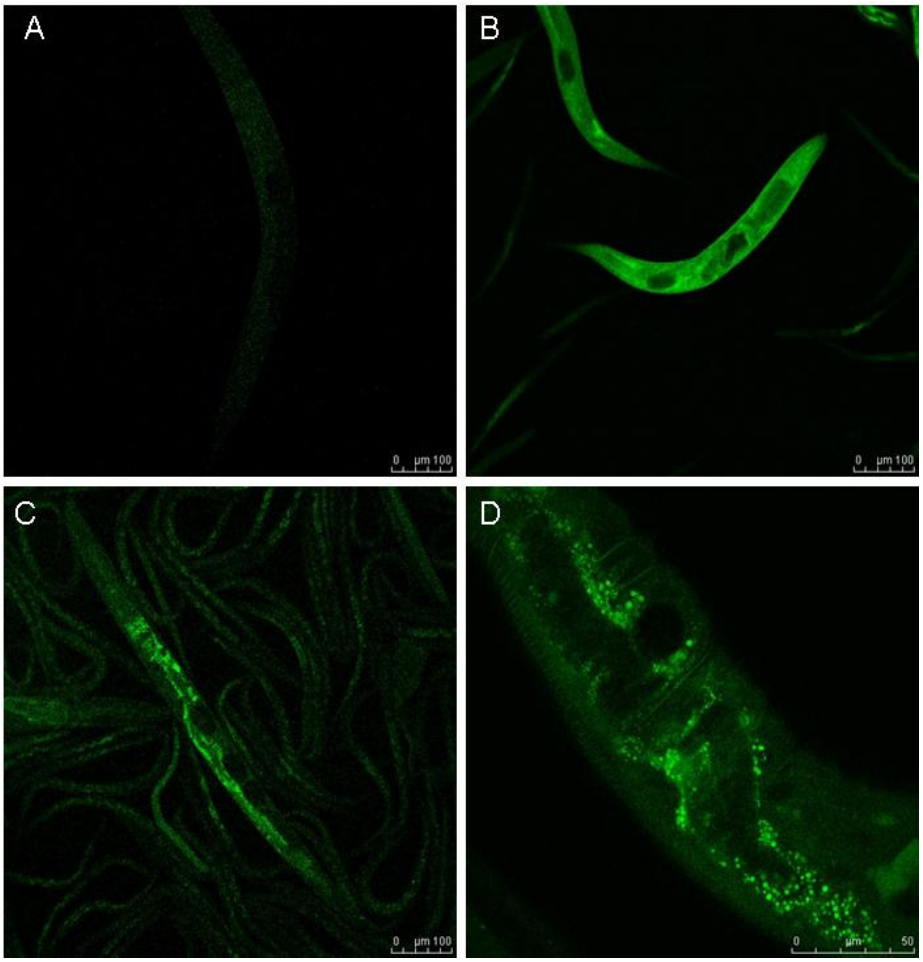

Fig. 5. Fluorescence TUNEL assay on *C. elegans* under the influence of static EMF. (A) Worms without any treatment serve as negative control. (B) Worms are treated with cytochalasin D (0.5 µg/ml) to induce apoptosis and served as positive control. (C) Worms are treated by 200 mT static EMF for 4 days. (D) Worms same as (C) but in a larger magnification to show the localization of fluorescence.

Immunostaining using antibody against gene product of ced-3 was also performed (Fig. 6.). *Ced-3* encodes gene product which plays essential role in apoptosis [24]. Positive staining for the static EMF-treated nematode confirmed the occurrence of apoptosis. Of most interest is that the staining was also localized at the muscle portion of nematodes. Both TUNEL staining and immunostaining are consistent with the hypothesis that static EMF induced apoptosis at muscle cells of nematodes thus affected the mobility of the worms.

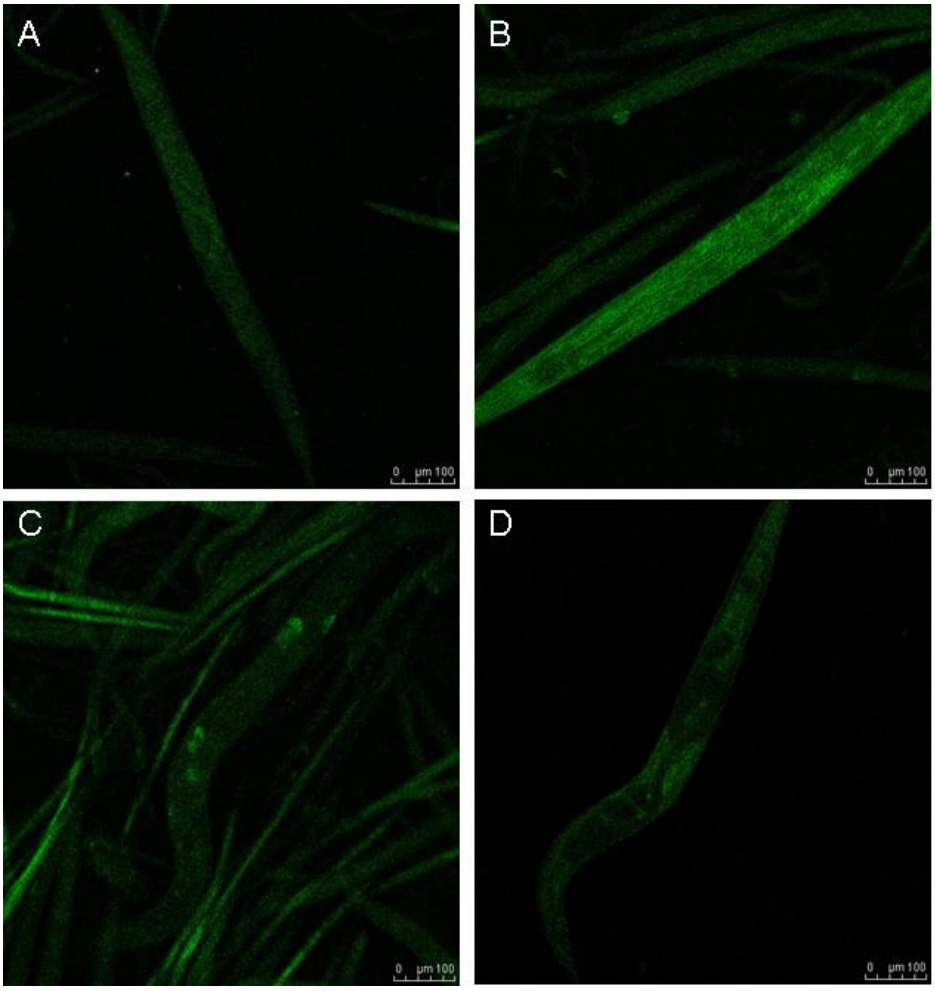

Fig.6. Immunostaining of static EMF-treated nematodes. Immunostaining using antibody against Ced-3 is performed to nematodes. (A) Worms without any treatment serve as negative

control. (B) Worms are treated with cytochalasin D (0.5 μg/ml) to induce apoptosis and served as positive control. (C) Worms are treated by 200 mT static EMF for 4 days. (D) Worms same as (C) but in a larger magnification to show the localization of fluorescence

1.3.4. The major apoptotic pathway mediates static EMF-induced mobility reduction.

Although apoptosis was involved in the mobility reduction of static EMF-treated nematode, it is yet to be demonstrated if apoptosis plays a central role of this mobility reduction. We took the advantage of the mutant nematodes that carry mutation at genes involved in the apoptotic pathways. Mutant strains of *ced-3*, *ced-4*, *ced-6*, *ced-9*, and *cbp-1* were tested for their resistance to static EMF. The *ced-3*, *ced-4*, and *ced-9* encode gene products belongs to major apoptotic pathway. *Ced-6* does not participate in apoptosis directly; however *ced-6* is involved in the pathways to regulate cell corpse engulfment. *Cbp-1* encodes a homolog of the mammalian transcriptional cofactors CBP and p300 that have been shown to possess histone acetyltransferase activity, and which, when mutated, lead to Rubinstein-Taybi syndrome and colorectal cancer [25].

Mobility of these mutant nematodes was measured after the treatment of 200mT static EMF for 4 days (Fig. 7.). For wild-type nematode, static EMF-treatment significantly reduced 41 % of the moving speed, from 8 mm/min to 5 mm/min. Mutant nematodes that are directly involved in the main apoptotic pathway, i.e. *ced-3*, *ced-4*, and *ced-9*, were insensitive to static EMF treatment. On the other hand, mobility of *ced-6* and *cbp-1* mutants was significantly reduced 40 % and 45% respectively by static EMFtreatment. Apparently, the major apoptosis pathway is associated with the static EMF-induced mobility reduction of nematodes (Fig. 8.).

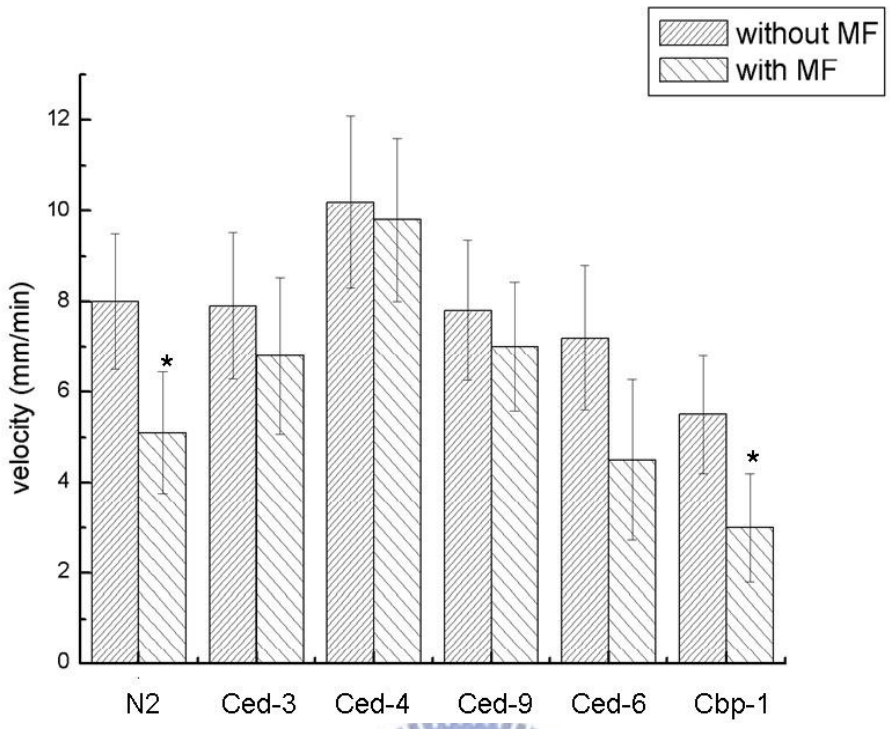

Fig. 7. Effect of static EMF on the mobility of wild-type nematode and mutant strains. N2 and mutant nematodes (*ced-3, ced-4, ced-6, ced-9*, and *cbp-1*) are treated with 200 mT static EMF for 4 days. Mobility assay is performed. The black bars depict crawling speed of worms without static EMF treatment. The shaded bars depict crawling speed of worms with static EMF treatment. All experiment were performed under 20℃. The result is expressed as mean ± SD from 30 worms. Student's *t*-test was performed comparing the crawling speed of static EMF-treated group with its original strain. (*) depicts $p < 0.05$.

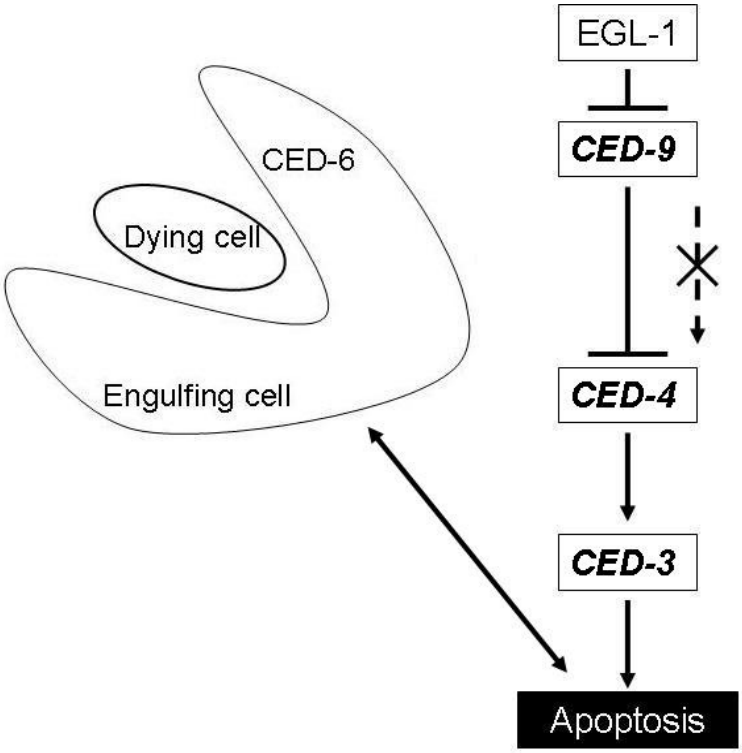

Fig. 8. Apoptosis pathways involved in the static EMF-induced reduction of mobility. Our results show that static EMF globally alters expression of apoptosis-related genes. The occurrence of apoptosis defects the moving ability of nematodes. Mutations at locus among major apoptosis pathways abolish the ability of static EMF to reduce the mobility of nematodes.

We have demonstrated that apoptosis was directly involved in the mobility reduction of static EMF-treated nematode. Immunostainig using antibody against CED-3 and fluorescence TUNEL staining indicated that apoptosis occurred at the muscle cells of the worms. However, it remains to be proven that besides mobility static EMF might be capable of inducing other

abnormalities such as the incidence of cancer.

Chapter 1.4: Conclusion

Here we show that application of static electromagnetic field to nematode at the flux density above 150 mT and for duration longer than 4 days significantly reduced the moving speed of *C. elegans*. By applying real time RT-PCR we were able to identify 26 genes whose expression was affected by static EMF-treatment. These genes are associated with apoptosis, cancer, stress response, and aging. TUNEL assay and immunostaining of caspase 3 validated the occurrence of apoptosis in the worm body and located the apoptotic activity to muscle cells. Genetic analysis using mutant strains indicated that apoptotic pathway mediates the static EMF-induced mobility reduction. Magnetic flux density, when given sufficient intensity and duration, induces apoptosis and causes damaging effect to nematodes.

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Chapter 2.1: Introduction

Human beings use electricity at their disposal for convenience and improvement in life. Possible undesirable effects of electric and magnetic field exposures have brought up great concerns [1-4]. Most of the epidemiologic research focused on exposure to low frequency electromagnetic fields or radiofrequency fields; however there are only a few epidemiologic studies specifically designed to study potential health hazards of static field exposure. It is of general concern that the present knowledge regarding the health concerns of static electromagnetic fields (we call SEMF in this paper) is lagging behind medical development such as magnetic resonance imaging (MRI). New studies are needed in order to fill the gaps in knowledge and provide assurance that novel medical technology will not cause any unwanted health hazards [5].

There have been few studies on the effects of static EMF at the cellular level[6]. Static EMF alone does not have a lethal effect on the basic properties of cell growth and survival under normal culture conditions [7, 8]. Most studies also suggest that SEMF failed to affect proliferation of the cells [9] or to influence cell cycle. Morphological analyses indicate that static EMF induce modifications of cell shape, cell surface and cytoskeleton [10-17]. SEMF may also modulated apoptosis through influence on cytoplasmic calcium ion concentration [15]. EMF induced double stranded DNA break in rat brain cells. SEMF is also associated

with cancer risk. It is likely that SEMF may affect the longetivity of life [18, 19].

The median life spans ranging from 11.8 days [20] to 20 days [21], when grown on agar plates at 20℃ with *Escherichia coli*. Populations of *C. elegans* exist primarily as hermaphrodites. Hundreds of progeny are generated per individual during its short lifetime. The newly born progeny mature within 24 hours and soon are indistinguishable from their parents. So we have to transferred nematodes to fresh plates during the egg laying everyday [22]. The short lifespan of *C. elegans* makes it attractive for the development of whole-organism compound screening [23].

In multi-cellular organism minor effects of static SEMF at the cellular level might accumulate and end up with distinct symptoms. Since *Caenorhabditis elegans* provides a rich resource in the genetic and molecular investigation with advantages of being a multi-cellular organism, it might provide insights into the gaps in knowledge of possible health hazards. For *C. elegans*, short term treatment of static EMF induced fluctuation of heat shock protein gene expression [24, 25]. But in general, low genotoxicity was observed. The current study is based on the hypothesis that Static electric magnetic field, when given sufficient intensity, induces life cycle reduction and causes aging to nematodes.

Chapter 2.1: Materials and Methods

2.2.1. Strains and chemicals

Strains of *C. elegans* used in this research including wild type (N2), *lim-7*、*lin-14*、*lin-41*、*clk-1*、*age-1* and *unc-3* were obtained from the Caenorhabditis Genetics Center (CGC), University of Minnesota, St Paul. Populations of *C. elegans* exist primarily as hermaphrodites. Nematodes were propagated at 20 °C on nematode growth-medium (NGM) plates (Brenner, 1974) with *Escherichia coli* strain OP50 as a food source. All culture media and related chemicals including Bacto agar, Bacto tryptone, and yeast extract were purchased from Gibco Co. Other chemicals of analytical grade or higher were purchased from Sigma or Merck. Nd-Fe-B magnets were purchased from Taiwan Magnetic Corp. Ltd. The magnetic field strength of the device was measured at The National Measurement Laboratory, Taiwan.

2.2.2. Isolation of Nematode Developmental Stages

We used body length to define each stage of nemaodes. We defined 250um as L1, 370um as L2, 500um as L3, 630um as L4, 920um as young adult and 1130 as adult. To effectly capture the locomotion behavior of a freely moving worm, a grid paper (5mm per grid) was placed underneath the petri dish and the movement of worms was recorded through optical by analyzing the recorded video. And the result was expressed as mean ± SD (standard deviation).

All experiment were performed under 20℃.

2.2.3. Quantitative real time RT-PCR

Total RNA was extracted from 100 worms using TRI-reagent (Talron Biotech) according to the manufacturer's specifications. Worms were picked and washed three times with M9 and collected in a 2ml eppendorf tube. The pellet was dissolved in 1ml Tri-regent and disrupted by homogenizer on ice, followed by chloroform extraction and isopropanol precipitation. The crude RNA extract was immediately purified by RNeasy Mini Kit (Qiagen) to remove impurities and unwanted organics. Purified RNA was resuspended in DEPC water and quantified by OD_{260}. OD_{260} to OD_{280} ratio usually exceeded 2.0 at this stage. For cDNA synthesis, 1μg total RNA was annealed with 1 μg oligo-dT, followed by reverse transcription using SuperScript® III Reverse Transcriptase (Invitrogen) in a total volume of 50 μl. Between 0.2 and 0.5 μl of the reverse transcription reactions were used for quantitative real time PCR using the SYBR Green I on an iCycler iQ5 (Bio-Rad Laboratories). Cycling conditions were: 1× [5 min 95°C] and 50× [20 s 95°C, 20 s 60°C, 40 s 72°C] fluorescence was measured after each 72°C step. Expression levels were obtained as threshold cycles (Ct) determined by the equipped software of iCycler iQ Detection System. Because There is no *let-7* gene sequence provided by the wormbase, and the *lin-41* didn't react for quantitative real time PCR, so the data of *let-7* and *lin-41* didn't showed in the result.

Relative transcript quantities were calculated by the ΔΔCt method using ribosomal protein L18 and L21 as reference genes amplified from the same cDNA sample. ΔCt is the difference in threshold cycles of the sample mRNAs relative to ribosomal protein L18 or L21 mRNA. ΔΔCt is the difference between ΔCt of normal control and ΔCt of static EMF-treated sample. Fold change in mRNA expression was expressed as $2^{\Delta\Delta Ct}$. Results are expressed as mean ± SD of 6 experiments.

2.2.4. Lifespan spay assay

Synchronized wild-type nematodes were putted on the growth-medium (NGM) plates with *Escherichia coli* strain OP50 as a food source. On the next day, sixty L1 larvae nematodes were picked to there new NGM plate, twenty namatodes in each plates. And we defined this day as first day. Two groups were set up in the same condition. One was with SEMF, and the other was without SEMF. The nematodes were picked to a new NGM plate with *Escherichia coli* strain OP50 as a food source every day, and recorded the alive nematodes to calculate the percentage of living nematodes. The result was expressed as mean ± SD (standard deviation). All experiment were performed under 20℃.

Chapter 2.3: Results and Discussion

2.3.1. SEMF at 200 mT shortens lifespan of C. elegans

The SEMF device was composed of two Nd-Fe-B permanent magnets sandwiching one Petri dish at the center. The field intensity of the SMF device at the center was measured and varied from 0 to 200 mT by adjusting the distance between magnets (Fig.1.).

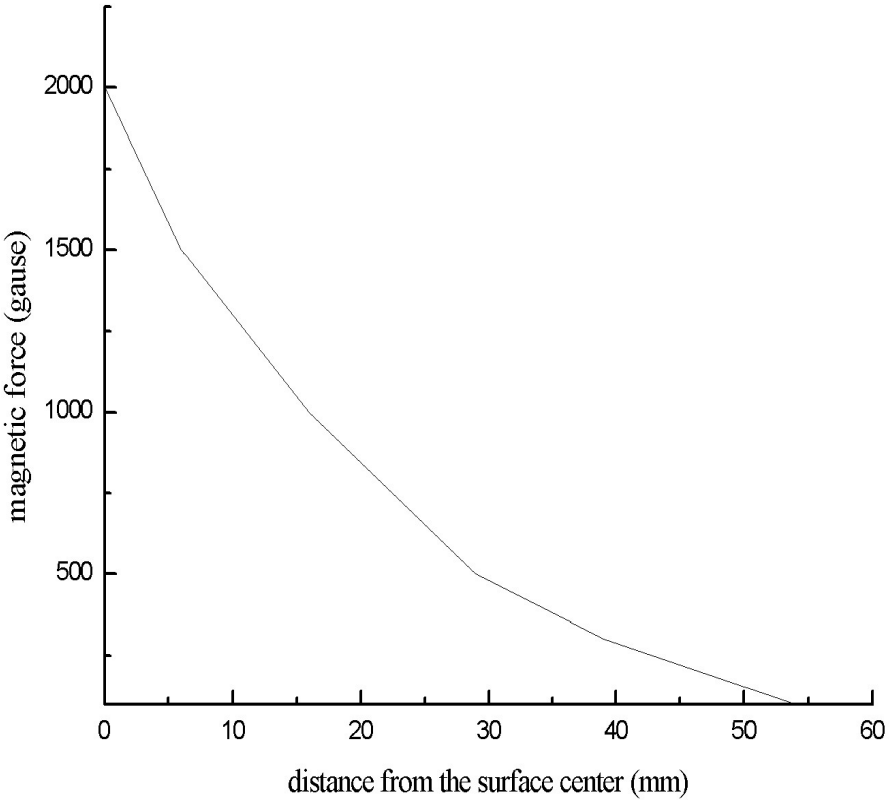

Fig. 1. Magnetic field strength at the center of SEMF settings used in the current study. Distance depicts the distance in mm between two permanent magnets. Field intensity was represented by mT.

To investigate possible effect of SEMF on the lifespan of *C. elegans*, synchronized nematodes were grown under 200 mT SEMF at 20°C. Lifespan assay was performed and the result was compared with untreated group (Fig. 2). Medium lifespan of *C. elegans* is 16 days for untreated group and is reduced to 13 days upon the treatment of 200 mT SEMF. The normal lifespan for *C. elegans* is 31 days at 20°C. SEMF treatment shortened lifespan to 24 days. There is a reduction of 23% normal lifespan upon the treatment of 200 mT SEMF.

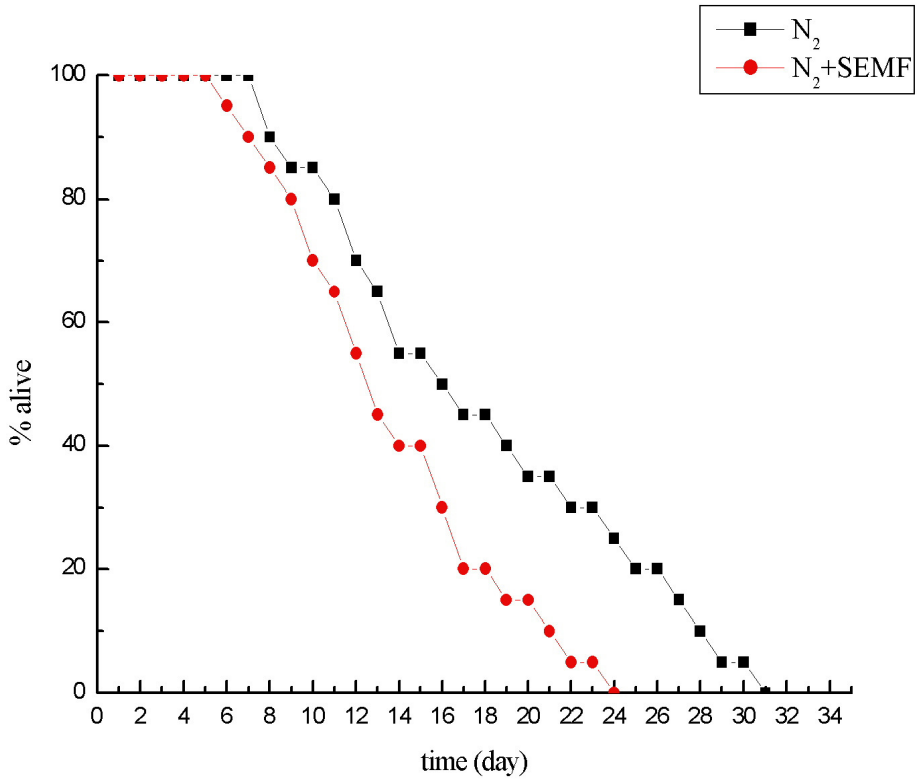

Fig. 2. The effect of SEMF on the lifespan of wild-type nematodes. Sixty wild-type nematodes were transferred onto NGM plates with *Escherichia coli* strain OP50 as a food source. Lifespan is measured at 20℃. Medium lifespan of wild-type nematode is 16 days for

untreated group and is reduced to 13 days upon the treatment of 200 mT SEMF. The normal lifespan for wild-type nematode is 31 days at 20 °C. SEMF treatment shortens lifespan to 24 days.

2.3.2. SEMF stronger than 150 mT accelerated the development time of nematode

It is possible that the lifespan of nematodes is shortened through the interference with time required in the early stages of nematode development. The life cycle of nematodes was measured from egg to adult stage (development time). Synchronized wild-type nematodes (N2) were treated by SEMF from 0 to 200mT for 3 days. Total development time from egg to adult staged larvae was measured (Fig. 3A). At 20℃, the complete life cycle of wild-type nematodes is 57.5 hours. There is a trend of reduction when the intensity of SEMF increases. A reduction of 22% was observed for 200 mT treatment compared to untreated group.

Because there is a distinct difference of appearance at each stage of nematode during development, it is possible to measure the duration of each stage from egg to adult. No significant difference was observed for all groups from egg to L2 stage (Fig.3B). Significant difference in development time occurred from L2 to L3. There is reduction of 18% for 150 mT treatment and 23% for 200 mT treatment for the development time from L2 stage to L3 stage, respectively (Fig.3C). SEMF treatment seems to accelerate development of nematodes.

For later stages, tendency of reduction in development time was observed. Although SEMF did not induce statistically significant difference from L3 to L4 stage, there is a 19% reduction of the development time from L4 to young adult and 23% reduction from young adult to adult under 200mT SEMF treatment (Fig.3E, 3F).

SEMF accelerated the development of nematode and shortened the development time from L2 to adult. The result is reflected by the reduction of lifespan under static EMF treatment.

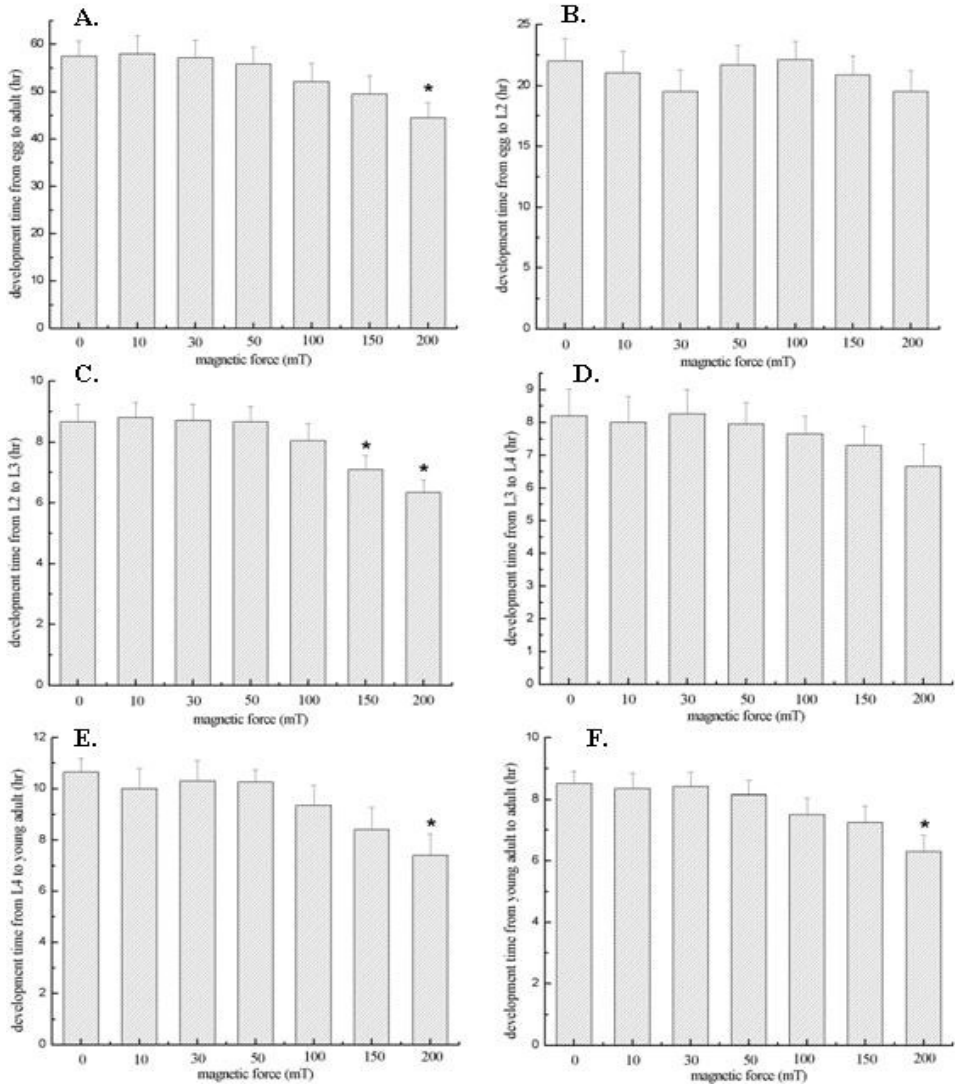

Fig. 3. The effect of SEMF on the life cycle of wild-type nematodes. Synchronized wild-type namatodes are transferred onto NGM plates with *Escherichia coli* strain OP50 as a food source. Larvae worms are grown under the SEMF from 0 to 200 mT, and the development time of each stage are measured at 20℃. (A) The total development time of wild-type nematodes, and the life cycle is significantly reduced 23% at 200mT SEMF. (B) No significant reduction is observed for the development time from egg to L2 under SEMF. (C) The development time from L2 to L3. The life cycle is significantly reduced 18% and 23% at 150mT and 200mT SEMF. (D) The development time from L3 to L4. There is no significant reduction under the SEMF from 0 to 200 mT. (E) The development time from L4 to young adult. The life cycle is significantly reduced 19% at 200mT SEMF. (F) The development time from young adult to adult. The life cycle is significantly reduced 23% at 200mT SEMF.

2.3.3. Genes associated with development and aging were differentially expressed by the treatment of SEMF

Reduced life cycle of nematodes indicated that pathways associated with development and aging were altered by SEMF treatment. To verify differential gene of associated pathways under SEMF treatment, quantitative real time RT-PCR was performed. For the preliminary screening 7 genes were selected and the primer pairs were designed based on the sequences provide in GenBank (Table 1). These genes are associated with cell development (*lin-4, lin-14,*

lin-41); growth rate (*clk-1*); growth of somatic gonadal sheath cells from the L4 stage to adulthood (*lim-7*), regulation of development and differentiation of B lymphocytes, adipocytes, and nerve cells (*unc-3*); and aging (*age-1*);. Real time RT-PCR showed consistently differential expression for *clk-1*, *lim-7*, *unc-3*, and *age-1* (Fig. 4). Expression levels increased 3.3 folds, 10.2 folds, 2.5 folds, and 7.5 folds for *clk-1*, *lim-7*, *unc-3*, and *age-1* respectively. Apparently pathways associated with development and aging play a role in the SEMF–induced reduction of lifespan and development time.

Table 1 Primer sequences for genes of development and aging

Symbol	Annotation	Primer sequence (5' →3')
Lin-4	abnormal cell LINeage	F[a]: gtgccagcctcacggaaagg R[b]: gggaggagtagctgaaggag
Lin-14	abnormal cell LINeage	F: aaccagcatcgccgacattac R: ggagtggtggagctgtttcaac
Lin-41	abnormal cell LINeage	F: tcccgcaagactcctttcgg R: gcgtcggagacaggtacatc
Lim-7	LIM domain family	F: accaccgatggcagtttgtgc R: caggcaacacacgcaaagcag
Clk-1	CLocK (biological timing) abnormality	F: aggtgcaatggcttgtacaattgc R: tccatcgtgttctactccagtatc
Unc-3	UNCoordinated	F: gatgtgccgagtgcttctcac R: gcatcggtgcgcttagttctc
Age-1	AGEing alteration	F: agagctccacggcactttcc R: ctcagcttggcagccttgac

[a] F elicits the sequence of forward primer.

[b] R elicits the sequence of reverse primer.

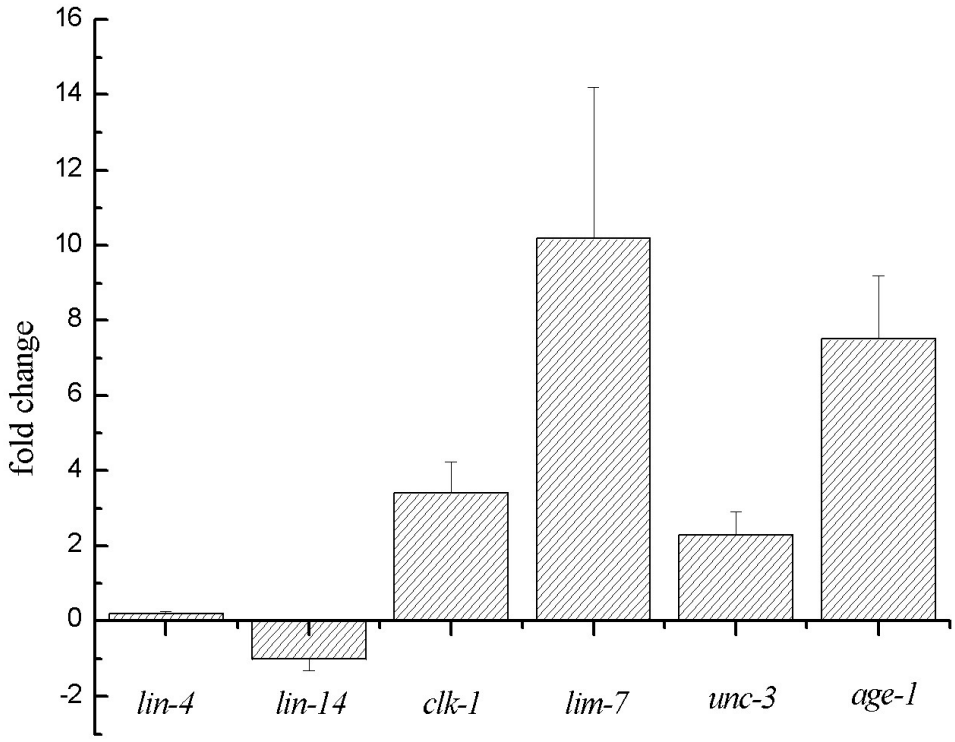

Fig. 4. Quantitative real time RT-PCR for genes associated with development and aging pathways under SEMF treatment. Expression levels increase 3.3 folds, 10.2 folds, 2.5 folds, and 7.5 folds for *clk-1*, *lim-7*, *unc-3*, and *age-1* respectively. The fold changes are calculated from the difference in cycle numbers of real time RT-PCR.

2.3.4. Mutant nematodes of development and aging are resistant to SEMF-induced life cycle reduction

Although the expression levels of genes associated with development and aging was altered upon the treatment of SEMF, it is yet to be demonstrated if the inactivation of these genes might cause resistance of nematodes against SEMF treatment. We took the advantage of

the mutant nematodes that carry mutation at genes involved in the development and aging pathways. Mutant strains of *let-7*, *lin-14*, *lin-41*, *clk-1*, *lim-7*, *unc-3*, and *age-1*were scored for total development time with or without SEMF treatment (Fig. 5.). For wild-type nematode, static EMF-treatment significantly reduced 22 % of the development time, from 58 hours to 45hours. Life cycle of *lim-7* , *lin-14*, and *lin-41* was significantly reduced 20%、23% and 21% by SEMF treatment, respectively. Life cycles of *let-7*, *unc-3*, *clk-1*, and *age-1* mutants were not affected by SEMF treatment. *Let-7* is directly involved in the transition from late larval to adult cell fates. Apparently, pathways associated with development and aging mediated SEMF-induced life cycle reduction in nematodes.

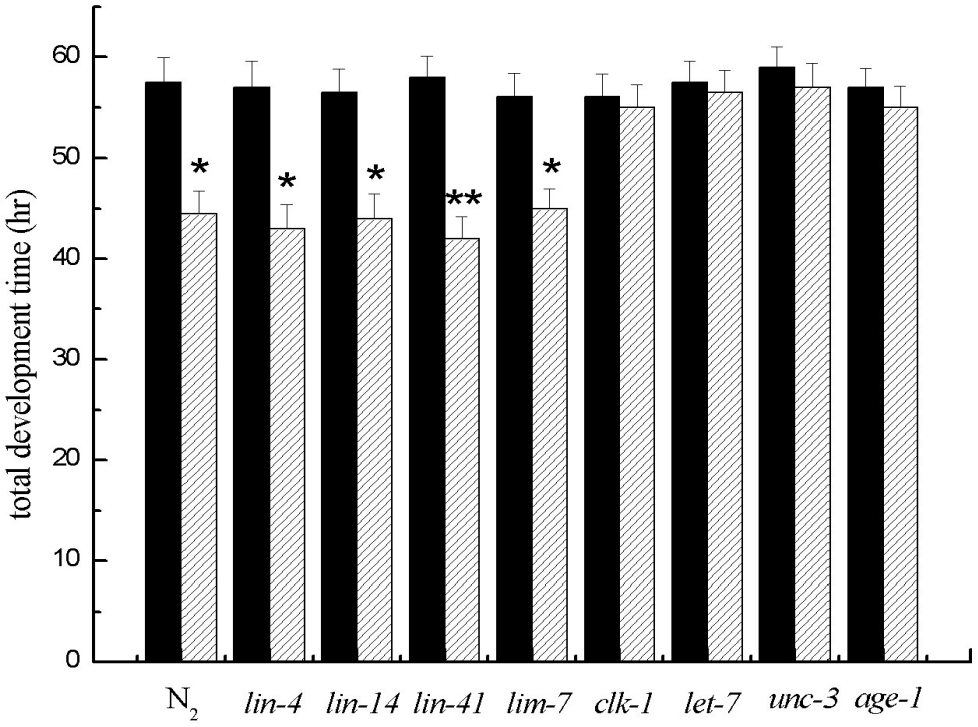

Fig. 5. The effect of SEMF on the life cycle of mutant nematodes. Synchronized wild-type namatodes are transferred onto NGM plates with Escherichia coli strain OP50 as a

food source. Larvae worms are grown under the SEMF from 0 to 200 mT, and the development time of each stage are measured at 20℃. For wild-type nematode, SEMF-treatment significantly reduces 22 % of the development time, from 58 hours to 45hours. Life cycles of *let-7*, *unc-3*, *clk-1*, and *age-1* mutants are not affected by SEMF treatment. Life cycle of *lim-7* , *lin-14*, and *lin-41* is significantly reduced 20%、23% and 21% by SEMF treatment, respectively.

2.3.5 Mutant nematodes of development and aging are resistant to SEMF-induced life span reduction

To investigate possible effect of static EMF on the lifespan of *C. elegans*, synchronized wild-type and mutant nematodes were grown under 200 mT SEMF at 20°C. Lifespan assay was performed to N2, *let-7*, *unc-3*, and *age-1* with or without SEMF treatment (Fig. 6). Medium lifespan of wild-type nematode is 16 days for untreated group and is reduced to 13 days upon the treatment of 200 mT SEMF. The normal lifespan for wild-type nematode is 31 days at 20 °C. SEMF treatment shortened lifespan to 24 days. Lifespan of *let-7*, *unc-3*, and *age-1* showed only minimal reduction by SEMF treatment: from 30 days to 29 days, from 34 days to 33 and from 33 days to 32 days, for *let-7*, *unc-3*, and *age-1* respectively. In summary, *let-7*, *unc-3*, and *age-1* mutants were not affected by SEMF treatment. The pathways associated with these genes, that is, development and aging are associated with

SEMF-induced reduction in development time and lifespan.

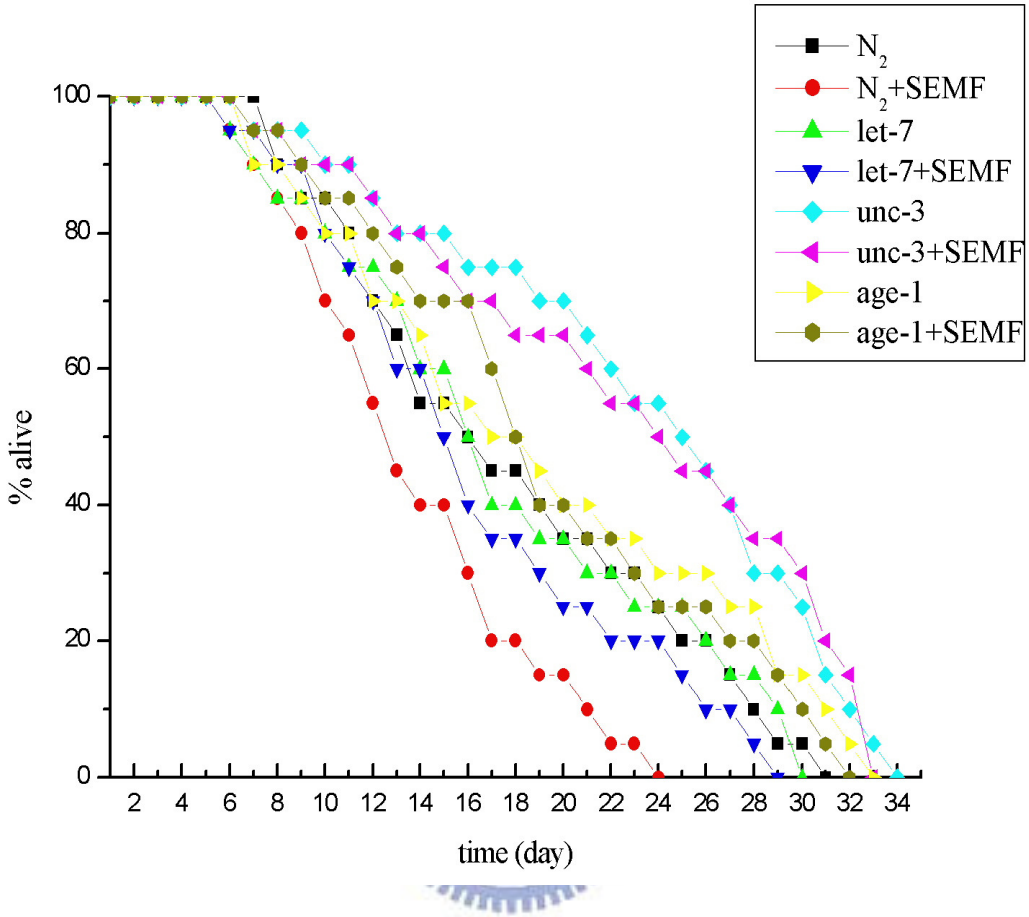

Fig. 6. The effect of SEMF on the lifespan of wild-type nematodes. Each experiment contains sixty nematodes. The lifespan is measured at 20℃. Medium lifespan of wild-type nematode is 16 days for untreated group and is reduced to 13 days upon the treatment of 200 mT SEMF. The normal lifespan for wild-type nematode is 31 days at 20 °C. SEMF treatment shortens lifespan to 24 days. Lifespan of *let-7*, *unc-3*, and *age-1* shows only minimal reduction by SEMF treatment: from 30 days to 29 days, from 34 days to 33 and from 33 days to 32 days, for *let-7*, *unc-3*, and *age-1* respectively. The difference is within experimental error.

Genetic analyses indicated that 200 mT SEMF treatment induced over expression of genes associated with aging and development. There is a significant reduction of lifecycle time from L2 stage to adult nematode. The overall effect is the acceleration of development and reduction of lifespan.

Chapter 2.4: Conclusion

Here we show that application of SEMF to nematode at the strength above 150 mT significantly reduced the life cycle of *C. elegans*. SEMF accelerated nematode development from L2 to adult stages. The effect may be as enhanced as 23% from L2 to L3 stage. By applying real time RT-PCR we identified differentially expressed genes under SMF-treatment (*clk-1*, *lim-7*, *unc-3*, and *age-1*). These genes are associated with development and aging. Genetic analysis using mutant strains indicated that development and aging pathways were involved in the SMF-induced development time and lifespan reduction. Static electric magnetic field, when given sufficient intensity, induces life cycle reduction and causes aging to nematodes.

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