어류 내분비계장애의 분자생물학적 지표를 이용한 환경위해성평가기법

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발표내용

1. 내분비계장애물질 시험법 연구
2. 분자생물학적(난막전구체, Choriogenin) 지표 연구
3. 분자생물학적 지표를 이용한 환경위해성평가

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1. 내분비계장애물질 시험법연구

- 국제적으로 합의된 시험법이 없으면 내분비계장애물질을 규정할 수 없음

- 시험법이 없으면 위해성평가의 첫 단계인 유해성 확인(Hazard Identification)이 불가능

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내분비계장애물질의 정의(?)

- 체내 항상성유지 및 발생에 관여하는 체내 호르몬의 생산, 분비, 이동, 대사, 결합, 작용 및 배설을 방해하는 외인성물질(Kavlock *et al*, 1996)

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OECD 환경국 화학물질위원회

- 내분비계장애물질에 대한 특별계획 수립(1996, 11)
  - 주요내용
    1. EDC평가관련 국제적 정보제공
    2. EDC를 확인할 수 있는 시험법의 제개정
    3. EDC위해성평가방법의 조화

EDTA Task Force 설립 (1997, 12)

- 주요활동목표
  1. EDC검색을 위한 시험방법개발 우선순위결정
  2. EDC시험법 개발전략의 국제적 조화
  3. 포유류/비포유류를 이용한 시험법개발 계획수립

Validation Management Group(VMG) 설립

- VMG for mammalian tests (1998)
  1. Uterotrophic assay
  2. Hershberger assay
  3. Enhanced TG407 (28d repeated dose toxicity)

- VMG for ecotoxicity tests (2000)
  1. Test protocols for fish
  2. Test protocols for birds
  3. Test protocols for amphibians
  4. Test protocols for invertebrates

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EDF전문가자문그룹 운영 (1998 - 현재)

- OECD/National TG 검토 및 EDF 제/개정분야 도출
- EDF시험법 국가간 검증사업 추진방안 도출
- EDF1
  - 1998년 10월 영국 런던, 11개국 28인 참석
  - 내분비계장애물질 검색 시험법 제정 및 추진계획마련
  - TIER 1, TIER2, TIER3 시험법 제안
- EDF2
  - 2000년 3월 일본 동경, 13개국 38인 참석
  - Proposal for EDs fish assay
  - Working Group for drafting fish protocols

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OECD 어류시험법제정 방안(EDF2결과)

- Screening Tier
1. Fish, prolonged toxicity test (enhanced TG204)
2. Fish, juvenile growth test (enhanced TG215)

- Higher Testing Tier
1. Developmental Early life-stage test(enhanced TG210)
2. Reproduction test
3. Full life-cycle test

* core endpoints : gross morphology, biomarker(vitellogenin), gonadal histology

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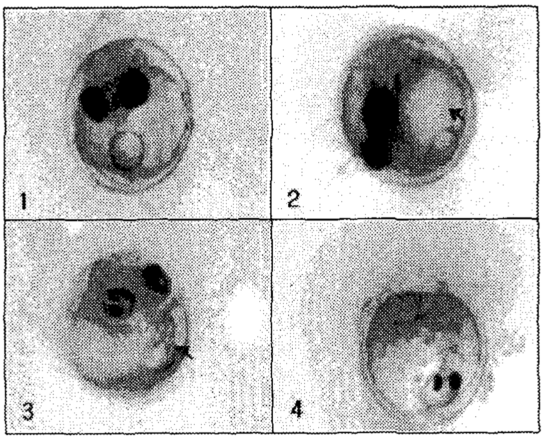
Normal and Abnormal Embryos



1. Normal	2. Reduced blood circulation
3. Hemorrhage	4. Dwarf and reduced eyeball

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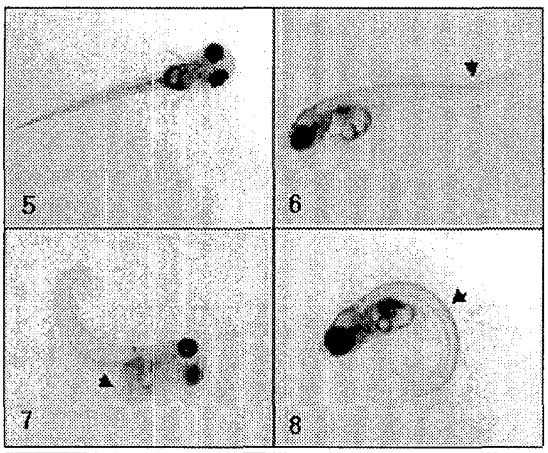
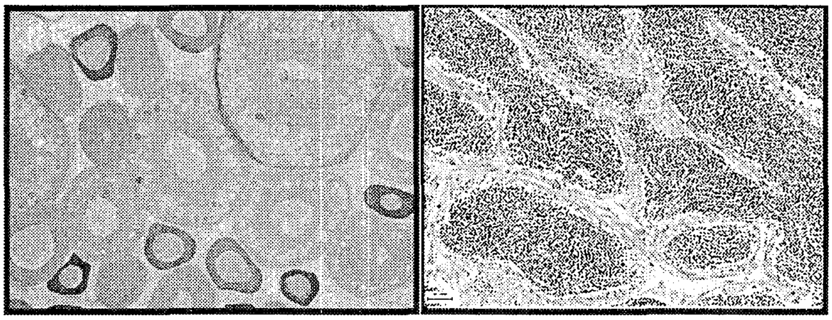
Deformed Hatched Larvae



5. Normal	6. Distortion of tail
7. Visceral edema	8. Distortion of spinal cord

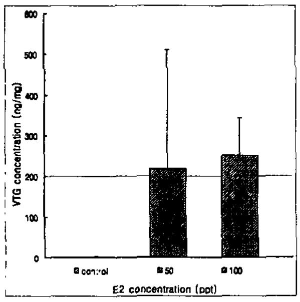
Histological observation

Ovary and Testis of Fish

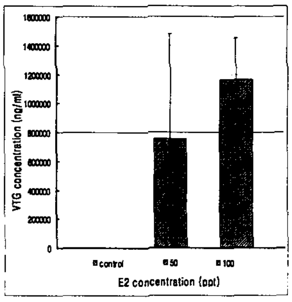


- Different developmental stages of oocytes were observed in control ovary (left)
- Typical histology of testis (right)

어류 간 및 혈액에서의 비텔로제닌 측정



간에서의 VTG 정량



혈액에서의 VTG 정량

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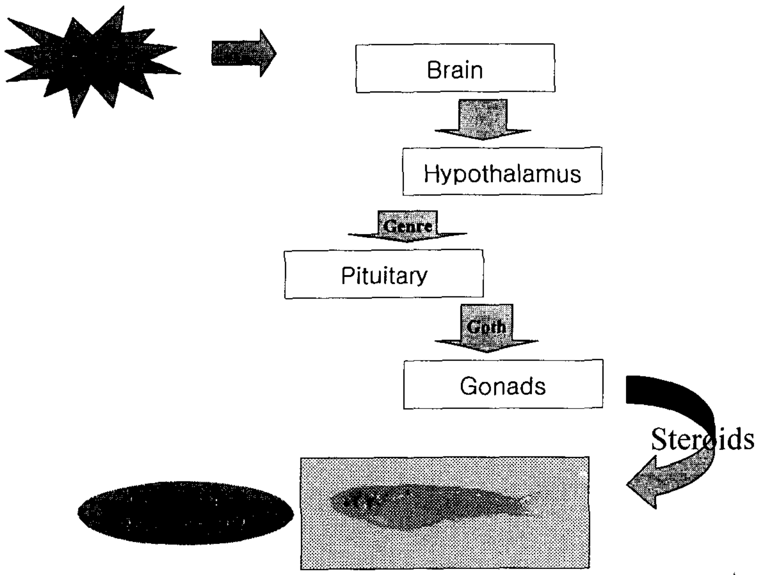
어류 생식능력의 조절



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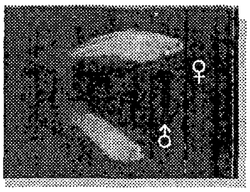
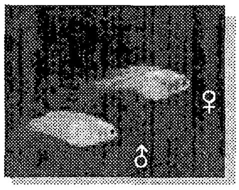
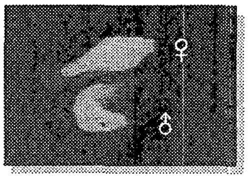
Mating behavior in male toward the female



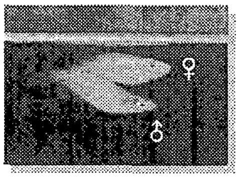
(a) Normal swimming



(b) Following



(c) Courtship dance



(d) Crossing

This mating behavior between normal medaka male and female was recorded by video camera for 1 hour

2. 분자생물학적 지표연구

- 내분비계장애물질에 의해 나타나는 보다 민감한 영향을 확인하기 위해서는 분자수준의 변화를 측정할 수 있는 분자생물학적 지표 연구가 필요

- 저용량에서 나타나는 영향을 평가할 수 있으므로 보다 엄격한(Conservative) 위해성평가가 가능

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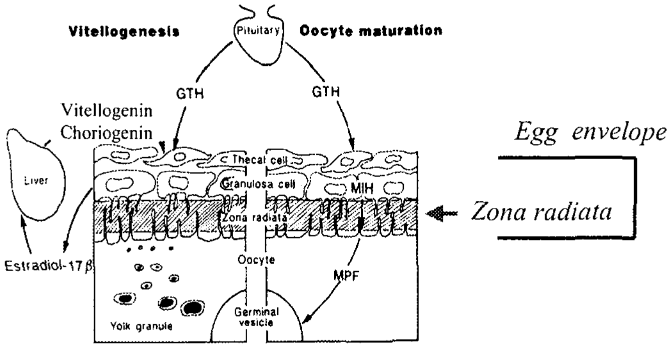
난막전구체(Choriogenin)

Choriogenin is a precursor of the zona radiata protein which is the major glycoprotein of egg envelope in fish



Synthesis of Choriogenin / ZR proteins / ZP proteins

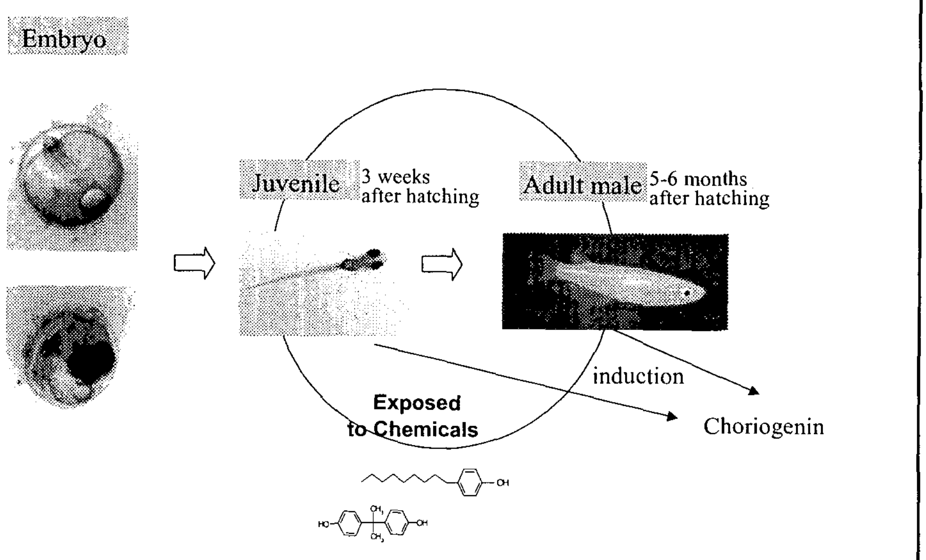
□ **In Liver** : Winterflounder, Medaka, Rainbow trout, Turbot, Cod, Atlantic salmon, Long-spined bullhead, pollack, Sea scorpion, Coalfish, Eelpout, Perch, Tilapia, and Plaice

□ **In Ovary** : Carp, Goldfish, Seahorse, Pipefish, and Eel

Choriogenin of Medaka, *Oryzias latipes*

- **Zona radiata protein consists of the glycoprotein subunits ZI-1, ZI-2, and ZI-3 in medaka**
- **ZI-1, ZI-2, and ZI-3 are formed from two groups of precursor proteins named as *Choriogenin H*(high Mw) and *Choriogenin L* (low Mw), respectively**

Zona radiata proteins	Precursors
ZI-1 : 76,000Da ZI-2 : 74,000Da	*Choriogenin H*
ZI-3 : 49,000Da	*Choriogenin L*



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Choriogenin cDNA & Primers Selection

reported by *Murata et al(1995)*

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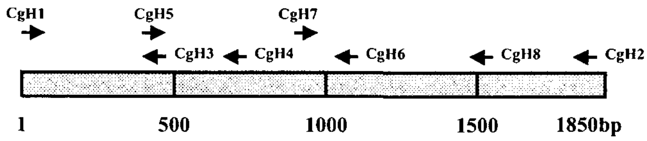

reported by *Murata et al(1997)*

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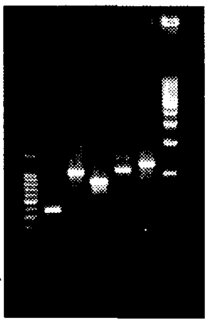
RT-PCR Products of Choriogenin L mRNA

1000bp →
500bp →



M 1 2 3 4 5 M

Primers	cDNA products size
1 : CgL1-3	380bp
2 : CgL1-5	1050bp
3 : CgL4-5	860bp
4 : CgL4-2	1150bp
5 : CgL1-2	1340bp

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Choriogenin L mRNA Expression in E2-treated males

M : Maker
1 : Untreatd
2 : E2 0.1mg/L
3 : E2 0.2mg/L
4 : E2 0.4mg/L
5 : E2 0.8mg/L
6 : Female

← 860bp
← β-actin (540bp)

M 1 2 3 4 5 6

RT- PCR was performed using CgL4 and CgL5 primers for 25 Cycles

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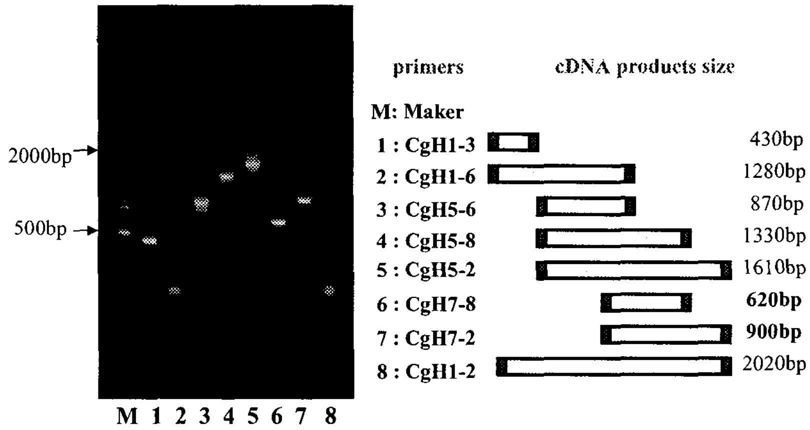
RT-PCR Products of Choriogenin H mRNA



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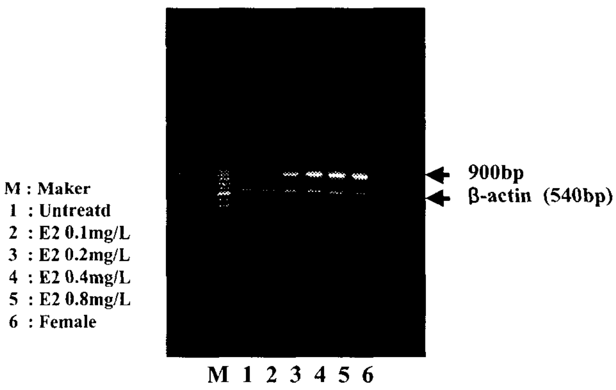
Choriogenin H mRNA Expression in E2-treated males



RT- PCR was performed using CgH7 and CgH2 primers for 25 Cycles

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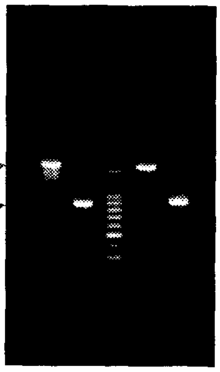
PCR Products of Genomic DNA and cDNA

ChgL genomic DNA
(ChgL4&5)
1700bp

ChgL cDNA 860bp
(ChgL4&5)



ChgH genomic DNA
(ChgH7&2)
1600bp

900bp *ChgH* cDNA
(ChgH7&2)

PCR products were obtained from genomic DNA(lane1,2) and cDNA (lane 4,5)
Lane 3 is molecualr marker

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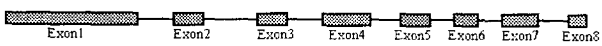
Sequence analysis of choriogenin gene of Medaka(*Oryzias latipes*) and mRNA expression

Choriogenin H

2645bp

Choriogenin L



2145bp

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Choriogenin H cDNA sequence and deduced amnino acid sequence
(ORF: 1998 bp, 666 amno acids)

```
ACC ATG GCA AGG CAC TGG AGT ATC ACG GTT TTT TCC GCA CTA GCT CTG CTA TGT TCT TTC  60
    M   A   R   H   W   S   I   T   V   F   S   A   L   A   L   L   C   S   F
CTG GGG ACC GAA GTG GAC GCT CAA AAA GGC AAC CCT CAA GAC CCT AAG GTT CCA TAC CCT 120
L   G   T   E   V   D   A   Q   K   G   N   P   Q   D   P   K   V   P   Y   P
CCA TAC TAT CCA CAG CCG AAG CCT CAG GAC CCT CAA CAC GTT TCG CCG CCT TAC TAC CCA 180
P   Y   Y   P   Q   P   K   P   Q   D   P   Q   H   V   S   P   P   Y   Y   P
GGG AAG CCA CAG AAC CCT CCT CAG AAG CCT TCG AAT CCT CAG TAT CCT TCG TAT CCT CAA 240
G   K   P   Q   N   P   P   Q   K   P   S   N   P   Q   Y   P   S   Y   P   Q
ACT CCT CAG AAT CCT CAG GTT CCT CAA AAT CCT CAG GTT CCT CAG AAT CCC CAG TAT CCT 300
T   P   Q   N   P   Q   V   P   Q   N   P   Q   V   P   Q   N   P   Q   Y   P
TCG TAT CCT CAG AAT CCT TCG TAT CCT CAG AAT CCT TCG TAT CCT CAG TAT CCT TCA AAT 360
S   Y   P   Q   N   P   S   Y   P   Q   N   P   S   Y   P   Q   Y   P   S   N
CCT CCG ACT TCT CAG AAT CCT TCG TAT CCT CAG AAT CCT AAG CTG TTT CAA GAT GGG AAG 420
P   P   T   S   Q   N   P   S   Y   P   Q   N   P   K   L   F   Q   D   G   K
CCT AGC AAT CCT CAG CAG CCC CAG GTT CCC CAG TAC CCA TCA AAG CCT CAG CCC CCT CAG 480
P   S   N   P   Q   Q   P   Q   V   P   Q   Y   P   S   K   P   Q   P   P   Q
AAT CCT CAG GTC CCC CAG TAC CCA TCA AAG CCT CAG CCC CCT CAG AAT CCT CAG GTC CCC 540
N   P   Q   V   P   Q   Y   P   S   K   P   Q   P   P   Q   N   P   Q   V   P
CAG TAC CCA TCA AAG CCT CAG CCC CCT CAG AAT CCT CAG GTC CCC CAG TAC CCA TCA AAG 600
Q   Y   P   S   K   P   Q   P   P   Q   N   P   Q   V   P   Q   Y   P   S   K
CCT CAG CCC CCT CAG AAT CCT CAG GTC CCC CAG TAC CCA TCA AAG CCT CAG CCC CCT CAG 660
P   Q   P   P   Q   N   P   Q   V   P   Q   Y   P   S   K   P   Q   P   P   Q
AAT CCT CAG GTC CCC CAG TAC CCA TCA AAG CCT CAG CCC CCT CAG AAT CCT CAG GTC CCC 720
N   P   Q   V   P   Q   Y   P   S   K   P   Q   P   P   Q   N   P   Q   V   P
CAG TAC CCA TCA AAG CCT CAG CCC CCT CAG AAT CCT CAG GTC CCC CAG TAC CCA TCA AAG 780
Q   Y   P   S   K   P   Q   P   P   Q   N   P   Q   V   P   Q   Y   P   S   K
CCT CAG AAC CCC CAG TAC CCA TCA AAG CCT CAG TAC CCC CAG GCC CCT CAG CAG CCT CAG 840
P   Q   N   P   Q   Y   P   S   K   P   Q   Y   P   Q   A   P   Q   Q   P   Q
AAC CCC CAG TAC CCA TCA AAG CCT CAA GAT CCA GGC AAG AAC CCA AAT ACA CCT CCC ATT 900
N   P   Q   Y   P   S   K   P   Q   D   P   G   K   N   P   N   T   P   P   I
GGT CCT CCC CCT CCC AAG AGC TGT GAA GTG CCC CGC GAT GTG AGA GTC CCC TGT GGA GTT 960
G   P   P   P   P   K   S   C   E   V   P   R   D   V   R   V   P   C   G   V
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Choriogenin H cDNA sequence and deduced amnino acid sequence
(ORF: 1998 bp, 666 amno acids)

```
TAC TTT GGA ACA GGA GCA ACC GTT CAG TGC ACC AAG GAT GGA CAC TTC ATC GTT GTG GTG 1080
Y   F   G   T   G   A   T   V   Q   C   T   K   D   G   H   F   I   V   V   V
GCC AAG GAT GTC ACC TTA CCT CAC ATT GAC CTG GAA ACT ATT TCC CTA TTG GGC CAG GGT 1140
A   K   D   V   T   L   P   H   I   D   L   E   T   I   S   L   L   G   Q   G
CAA GAC TGT GGA CCT GCT GAC TCT AAT TCA GCT TTC GCC ATC TAC TAC TTT CCC GTC ACT 1200
Q   D   C   G   P   A   D   S   N   S   A   F   A   I   Y   Y   F   P   V   T
TAT TGC GGC ACT GTT GTC ATG GAA GAG CCT GGG GTT ATA GTC TAT GAA AAC CGG ATG ACA 1260
Y   C   G   T   V   V   M   E   E   P   G   V   I   V   Y   E   N   R   M   T
TCC TCT TAT GAA GTG GGA GTT GGA CCG CTT GGA GCT ATT ACC AGA GAC AGT TCC TTT GAG 1320
S   S   Y   E   V   G   V   G   P   L   G   A   I   T   R   D   S   S   F   E
CTG CTC TTC CAG TGC AGA TAC CGT GCA ACA TCT GTT GAA ACT CTG GTC GTG GAA GTG CAG 1380
L   L   F   Q   C   R   Y   R   A   T   S   V   E   T   L   V   V   E   V   Q
CCA CCG GAT AGT CCT CTT TCC ATT GCT GAG CTT GGA CCC CTC AAT GTG TAC TTG CAA ATT 1440
P   P   D   S   P   L   S   I   A   E   L   G   P   L   N   V   Y   L   Q   I
GCC AAT GGG CAG TGT CAA ACA AAG GGT TGC GAC GAA GCG GCG GCA GCC TAC ACC TCT TTC 1500
A   N   G   Q   C   Q   T   K   G   C   D   E   A   A   A   A   Y   T   S   F
TAC ACG GAT GCT GAC TAT CCT GTG ACA AAG GTA CTG AGA GAT CCT GTC TAC GTG GAC GTA 1560
Y   T   D   A   D   Y   P   V   T   K   V   L   R   D   P   V   Y   V   D   V
CAA ATC CTT GGC AGA ACG GAT CCA AAT CTG GTT CTG ACT CTT GGA CGC TGT TGG GCA ACC 1620
Q   I   L   G   R   T   D   P   N   L   V   L   T   L   G   R   C   W   A   T
ACA AGC CCC AAT GCT TTC AGC CTG CCC CAG TGG GAC ATT TTG ATT GAC GGG TGT CCA TAT 1680
T   S   P   N   A   F   S   L   P   Q   W   D   I   L   I   D   G   C   P   Y
GCA GAT GAT CGT TAC CTG TCC GCG TTG GTC CCA ATT GAT CAT TCC TCT GGT CTG CCA TTC 1740
A   D   D   R   Y   L   S   A   L   V   P   I   D   H   S   S   G   L   P   F
CCA ACT CAT CAC AGT CGC TTC CTG TTC AAG ATG TTT ACT TTT GTT GAT CCT CAT TCA ATG 1800
P   T   H   H   S   R   F   L   F   K   M   F   T   F   V   D   P   H   S   M
GAA CCA CTA AGG GAA AAG GTG TAT ATT CAC TGT AGC ACA GCT GCA TGC GTT CCA GGA CAA 1860
E   P   L   R   E   K   V   Y   I   H   C   S   T   A   A   C   V   P   G   Q
GGA GTC AGC TGT GAA CCC TCA TGC AGC AGA AGA AAA GGT AGA GAT ACT GAA GCT GTG GCC 1920
G   V   S   C   E   P   S   C   S   R   R   K   G   R   D   T   E   A   V   A
ATT AGA ACG GAT GAA AGA AGG GTT GTG GTA TCG TCT GGA GAA GTG CTC ATG CTC GCC GCC 1980
I   R   T   D   E   R   R   V   V   V   S   S   G   E   V   L   M   L   A   A
GCT GAC GAA CCA TCT GAA CAG TGA ACC GAC AGA AGC TCC AGT TTT CAG AAA AAA TAA CAT 2040
A   D   E   P   S   E   Q   stop
TTT GAA AAT CTG TTT CAT CAT GGG TCA AAA TTA AAT GCA TAA AAT GAA AAA AAA AAA AAA 2100
AAA AAA AAA                                                                     2109
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Choriogenin H 염기서열 특성

반복염기서열

5'-ccc cag tac cca tca aag cct cag
ccc cct cag aat cct cag gtc -3'

반복아미노산서열

PQYPSKPQPPQNPQV (15 amino acids)

반복회수

7 complete repeats of 45bp

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어종별 반복서열의 특성

어 종	반 복 서 열	반복수	
Medaka	PQYPSKQPPQNPQV (15)	7	Complete
Carp	QQTSQQFQPQKPV (13)	14	Complete
Goldfish	QQLTNQQFPLQKPV (14)	14	Incomplete
Winter flounder	PQQPQQPQQPQQPKY(15)	4	Complete

* 반복서열은 수정란의 **hardness**와 상관성이 있음

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Choriogenin H gDNA sequence (underlined : introns)

```
atggcaaggcactggagtatcacggtttttccgcactagctctgctatgttctttcctggggaccgaagtggacgctca   80
aaaaggcaaccctcaagaccctaaggttccataccctccatactatccacagccgaagcctcaggaccctcaacacgttt  160
cgccgccttactacccaggaagccacagaaccctcctcagaagccttcgaatcctcagtatccttcgtatcctcaaact  240
cctcagaatcctcaggttcctcaaaatcctcaggttcctcagaatccccagtatccttcgtatcctcagaatccttcgta  320
atcctcagaatccttcgtatcctcagtatccttcaaatcctccgacttctcagaatccttcgtatcctcagaatcctaag  400
ctgtttcagatgggaagcctagcaatcctcagcagccccaggttccccagtacccatcaaagcctcagcccctcagaat  480
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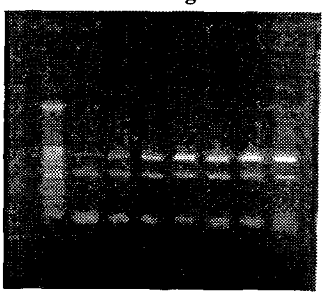
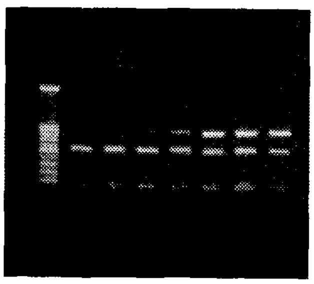
17α-ethynylestradiol 용량-반응 평가

Choriogenin L



860 (CgL)
540bp (-actin)

Choriogenin H



900bp(CgH)
540bp (-actin)

M : Markers 1 : Untreatd 2 : EE2 10ug/L 3 : EE2 20ug/L 4 : EE2 40ug/L
5 : EE2 80ug/L 6 : EE2 160ug/L 7 : Female exposure duration : 7days

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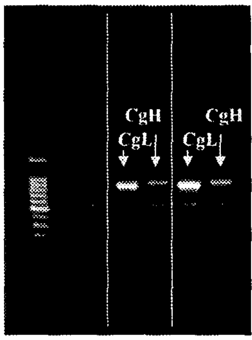
Choriogenin L is more Sensitive than Choriogenin H

M : Markers
1 : CgL (untreated)
2 : CgH (untreated)
3 : CgL (E2 200ug/L)
4 : CgH (E2 200ug/L)
5 : CgL (E2 800ug/L)
6 : CgH (E2 800ug/L)



900bp
860bp
β-actin (540bp)

RT-PCR was performed with CgL3/4 or CgH7/2 for 25 cycles, *exposure duration : 96hrs*

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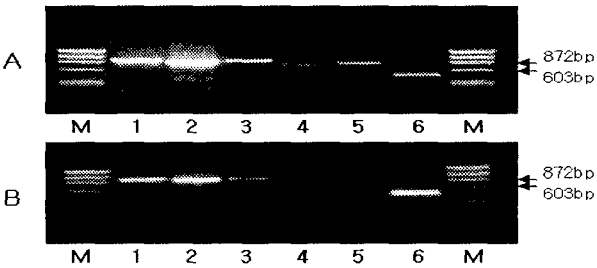
Sensitivities of Choriogenin L, H, Vitellogenin I, II and Estrogen receptor



RT-PCR of choriogenin, vitellogenin and estrogen receptor in adult male medaka (A) and juvenile medaka (B) exposed to 100㎍/L of 17 -ethinylestradiol. RT-PCR was performed for 30 cycles (M: *size marker* X173-HaeIII, 1: choriogenin H, 2: choriogenin L, 3: vitellogenin I, 4: vitellogenin II, 5: estrogen receptor, 6: -actin)

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3. 분자생물학적 지표를 이용한 환경위해성평가

- 내분비계장애물질에 의해 영향을 받지 않을 농도와 현재의 오염농도를 비교하여 위해도를 결정

환경위해성평가방법

Risk = PEC/PNEC

- PEC : Predicted Exposure Concentration
- PNEC : Predicted No-Effect Concentration
- Risk value 가 1보다 클 경우 위해판정

PEC

평가대상물질의 환경중 노출농도는 사용량, 용도, 배출, 분포, 분해, 물리화학적 성질 등으로 부터 예측하거나 환경매체중 농도를 실측

$$PEC = I(1-R)/D$$

I ; Influent Conc, R; Removal Percentage, D; Dilution Factor

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PNEC

환경중 서식하는 생물체가 악영향을 받지 않을 수준의 농도로서 화학물질 최대무영향농도값에 안전계수를 고려하여 산출된 값

$$PNEC = NOEC/AF$$

* 내분비계장애물질 환경위해성평가를 위해서는 내분비계장애를 평가할 수 있는 적정 시험법(또는 Biomarker)에 의한 독성 값이 필요
* Assessment Factor에 대한 합의가 필요

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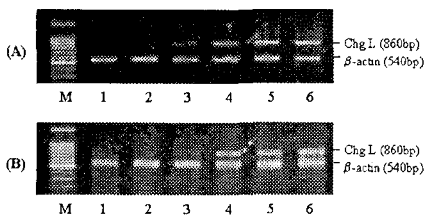
Proposed assessment Factor for Application to Aquatic Toxicity Data
For Estimating a PNEC

Available information applied	Assessment factor applied to the lowest value (modification not included)		
	(a)OECD Workshop	(b)EU Technical Guidance Document	(c)ECETOC proposal
One acute $L(E)C_{50}$ for acute toxicity from one trophic level	1000	-	
At least one acute ***$L(E)C_{50}$ from each of three trophic levels of the base-set(fish, *Daphnia* and algae)	100	1000	200
One chronic NOEC(either fish or *Daphnia*)	-	100	-
Two chronic NOECs from species representing two trophic levels(fish and/or *Daphnia* and/or algae)	-	50	5
Chronic NOECs from at least three species(normally fish, *Daphnia* and algae) representing three trophic levels	10	10	
Field data or model ecosystems	-	case- by-case	1

*** in the EU Technical Guidance Document, "short-term toxicity"and "long-term toxicity" are used instead of "acute toxicity" and "chronic toxicity"

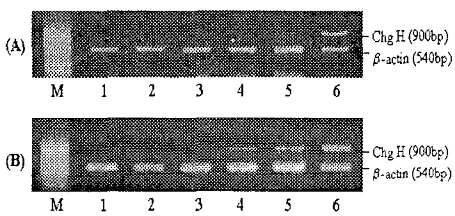
노닐페놀의 PNEC

Choriogenin L



Choriogenin L gene expression in the liver of male medaka exposed to biphenol A (A) and nonylphenol (B), respectively. Amplified cDNA fragments of choriogenin L and -actin were produced by RT-PCR performed for 25cycles. M: markers, 1: control, 2: 5μg/L, 3: 50μg/L, 4: 100μg/L, 5: 200 μg/L, 6: 500μg/L

Choriogenin H



Choriogenin H gene expression in the liver of male medaka exposed to biphenol A (A) and nonylphenol (B), respectively. Amplified cDNA fragments of choriogenin H and -actin were produced by RT-PCR performed for 25cycles. M: markers, 1: control, 2: 5μg/L, 3: 50μg/L, 4: 100μg/L, 5: 200 μg/L, 6: 500μg/L

PNEC

○ endpoints

- molecular biomarker (choriogenin L) induction

○ NOEC : 5 ug/L

○ Assessment factor : 10 (*)

○ Nonylphenol PNEC = 0.5 ug/L

* molecular biomarker를 end point할 경우 AF에 대한 합의과정이 필요

노닐페놀의 PEC

수계별 Nonylphenol 검출농도			
수 계	농도(ug/L)	수 계	농도(ug/L)
W1	0.0768	W23	0.1849
W2	0.4477	W24	0.2310
W3	0.7702	W25	0.2757
W4	0.8719	W26	0.5479
W5	0.1671	W27	0.4148
W6	0.3455	W28	0.3510
W7	0.2521	W29	0.1490
W8	5.8830	W30	0.1442
W9	2.4861	W31	0.1465
W10	0.1037	W32	0.1540
W11	0.2505	W33	0.1627
W12	0.0399	W34	0.1172
W13	0.2573	W35	0.2361
W14	0.1042	W36	0.5075
W15	0.2245	W37	0.1976
W16	0.1675	W38	0.2051
W17	0.4801	W39	0.1806
W18	0.1538	W40	0.1369
W19	0.2146	W41	0.1296
W20	0.3401	W42	0.5057
W21	0.2588	W43	0.1821
W22	0.1390		

노닐페놀의 PEC

Percentile	95	90	75	50	Arithmetic mean
PEC (ug/L)	1.4369	0.5924	0.3496	0.2146	0.4464

노닐페놀의 위해도

PCT	95	90	75	50	Arithmetic mean
R i s k	> 1	> 1	< 1	< 1	< 1

노닐페놀의 지점별 위해도

노닐페놀의 수계 지점별 환경위해도			
지점	위해도	지점	위해도
W1	<1	W23	<1
W2	<1	W24	<1
W3	1.54	W25	<1
W4	1.74	W26	1.10
W5	<1	W27	<1
W6	<1	W28	<1
W7	<1	W29	<1
W8	11.77	W30	<1
W9	4.97	W31	<1
W10	<1	W32	<1
W11	<1	W33	<1
W12	<1	W34	<1
W13	<1	W35	<1
W14	<1	W36	1.01
W15	<1	W37	<1
W16	<1	W38	<1
W17	<1	W39	<1
W18	<1	W40	<1
W19	<1	W41	<1
W20	<1	W42	1.01
W21	<1	W43	1.01
W22	<1		

종 결

1. 분자생물학적 지표를 이용한 환경위해성평가는 보다 엄격한 관리기준을 마련하는 데 이용될 수 있음

2. 특정 화학물질그룹 또는 질환에 특이적인 생체지표를 발견하고 그 측정법에 대한 표준화가 필요

3. Toxicogenomic 및 Proteomics 분야는 새로운 생체지표를 발견하고 정량화하는 최신 기법으로서 향후 환경오염물질의 위해성평가에 필수적인 수단으로 정립될 것으로 판단됨