Antioxidant effect of Estrogen on Bovine aortic endothelial cells

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- Background
- Objectives
- Materials & Methods
- Results & Discussion
- Limitation
- Conclusion

폐경이행기(Menopausal transition)

- Declining estrogen levels

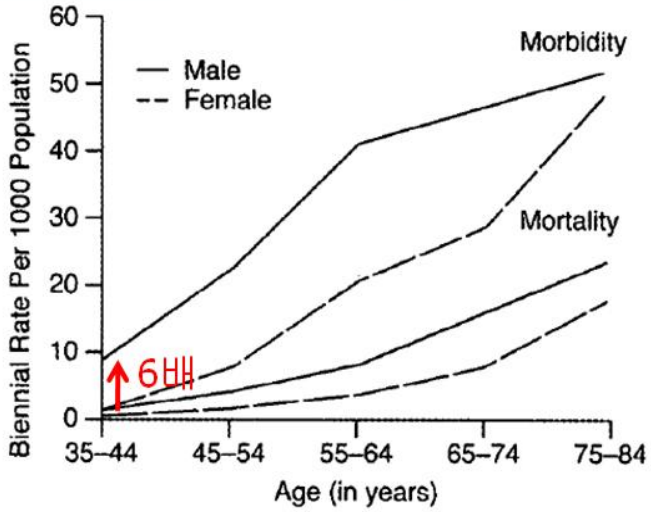
  -> multifarious metabolic and physiologic changes

  ->strongly associated with increased incidence of hypertension and susceptibility to CVD

Menopause and CVD

Framingham Cohort 1971–1974

Incidence of coronary heart disease morbidity and mortality



Age (y)	Average annual rate per 1 000							
	All CV events[a]		CHD		Stroke		CHF	
	Men	Women	Men	Women	Men	Women	Men	Women
35–64	18	9	14	6	3	2	3	2
65–94	43	30	27	17	12	11	11	9
Risk ratio[b]	2.4	3.3	1.9	2.8	4.0	5.5	3.7	4.5

a Also includes peripheral vascular disease.

b (65-94)/(35-64).

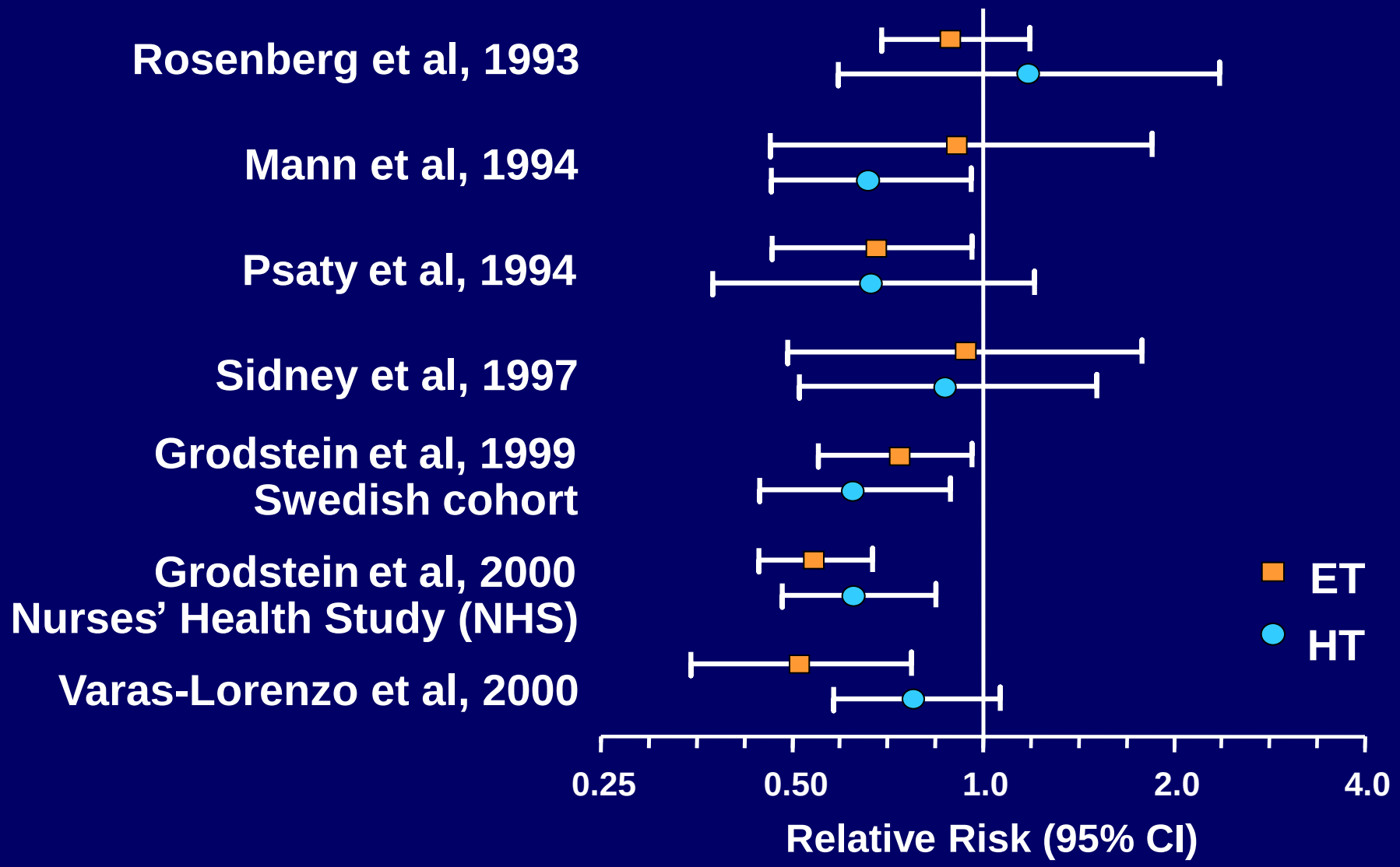
CHD = coronary heart disease; **CHF** = congestive heart failure; **CV** = cardiovascular.

Am Heart J. 1986.111:383-390 ; Kannel WB, Prev Cardiol 1998

Menopause and CVD

- Mainly due to Estrogen deficiency
  - Change in body composition
    : android type fat distribution
  - Metabolic syndrome
  - More atherogenic lipoprotein profile
  - Impaired fibrinolytic potential

Observational Studies of CVD Risk: ET Compared With HT



Women's Health Initiative (WHI)
Study Outline

end in March 2005 F/U : 8.5 yrs

AGE 50–79 (mean 63)

CEE(0.625r

<Outcomes>

- **Primary outcome**

  Coronary heart diseases (CHD)

  non-fatal myocardial infarction and CHD death

- **Secondary outcome**

  Stroke, Venous Thromboembolism,

  Colorectal Cancer, Osteoporosis-related fractures,

  all-cause Mortality

-**Primary adverse outcome**

ive breast cancer

stopped in Feb. 2004 F/U : 6.8 yrs

stopped in May, 2002 F/U : 5.2 yrs

WHI clinical outcome

Event	Relative Risk CEE/MPA vs. placebo (Hazard ratio)	Relative Risk CEE vs. placebo (Hazard ratio)
CHD event	1.29 (1.02–1.63)*	0.91 (0.75–1.12)
Nonfatal MI	1.32 (1.02–1.72)*	0.94 (0.65–1.36)
CHD death	1.18 (0.70–1.97)	0.84 (0.70–1.12)
Invasive breast cancer	1.26 (1.00–1.59)	0.77 (0.59–1.01)
Stroke	1.41 (1.07–1.85)*	1.39 (1.10–1.77)*
Pulmonary embolism	2.13 (1.39–3.25)*	1.34 (0.87–2.06)
Colorectal cancer	0.63 (0.43–0.92)*	1.03 (0.75–1.55)
Endometrial cancer	0.83 (0.47–1.47)	–
Hip fracture	0.66 (0.45–0.98)*	0.61 (0.41–0.91)*
Vertebral fracture	0.66 (0.44–0.98)*	0.62 (0.42–0.93)*
Death due to causes other than events above Global index	0.92 (0.74–1.14)	1.01 (0.91–1.12)

*statistical significance

Estrogen as antioxidant

- Antioxidant protection of LDL by physiological concentrations of 17 beta-estradiol.

  Shwaery GT et al. *Circulation. 1997 Mar 18;95(6):1378-85.*

- The inhibition of low-density lipoprotein oxidation by 17-beta estradiol. *Rifici VA et al.* *Metabolism. 1992 Oct;41(10):1110-4.*

- Effects of oral estrogen on aortic ROS-generating and -scavenging enzymes and atherosclerosis in apoE-deficient mice. *Wing LY et al.* *Exp Biol Med (Maywood). 2009 Sep;234(9):1037-46. Epub 2009 Jun 22.*

  Long-term oral estrogen treatment reduces ROS levels and atherosclerosis progression in apoE(-/-) mice. Oral estrogen alters ROS-generating and -scavenging enzyme expression, suggesting that anti-oxidative actions in the vessel wall contribute to atheroprotective effects of estrogen.

Objectives

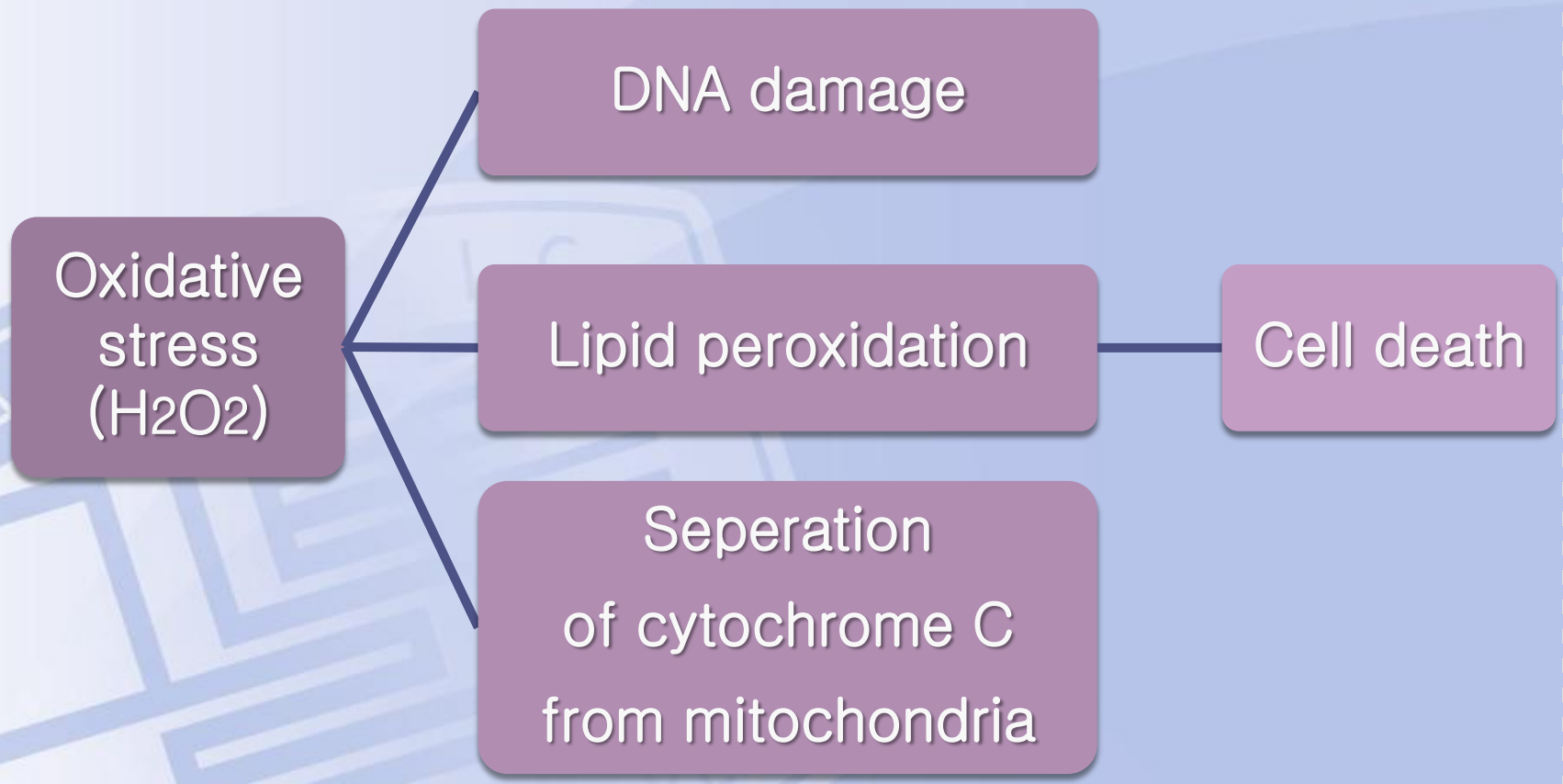
- 대동맥 혈관 내피세포에

  Hydrogen peroxide (H_2O_2)를 이용한

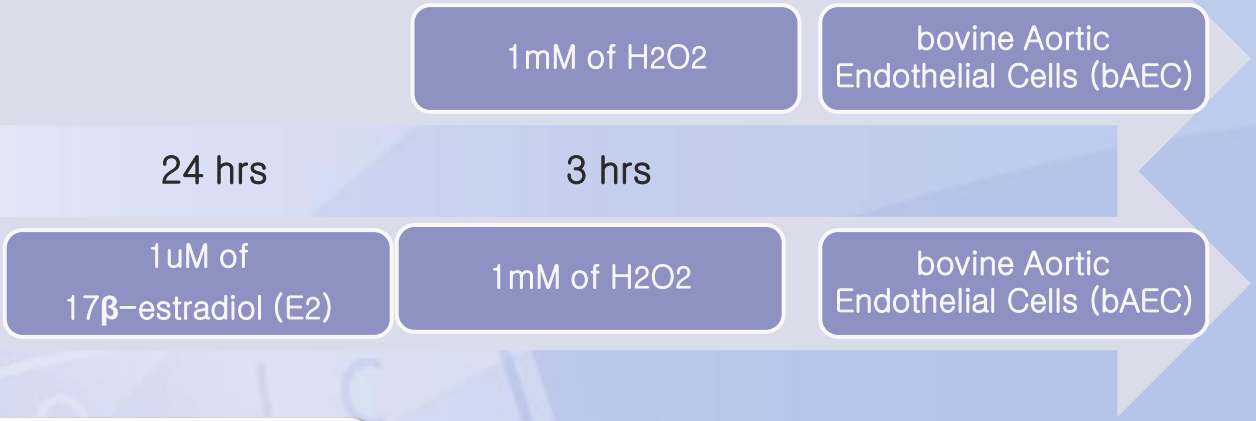
  직접적인 산화자극에 대한

  Estrogen의 작용을 알아보고

  그 작용 기전에 대하여 알아보는데 목적이 있다.


Oxidative stress (H_2O_2)
DNA damage
Lipid peroxidation
Seperation of cytochrome C from mitochondria
Cell death

Materials & Methods



Cell survival	• MTT assay
Reactive oxidative stress	• 2,7-dichlorofluorescin diacetate (DCF-DA)
Cellular apoptosis	• Hoechst 33342 staining • Fluorescence activated cell sorter (FACS)
Intracellular mechanism	• Western blotting for phospho-p38, p38, and Bcl-2

Materials & Methods (contin.)

1.세포배양 및 전처치

소혈관 내피세포주인 bAEC(bovine Aortic Endothelial Cell), DMEM (Dulbecco's Modified Eagle Medium, Gibco/BRL, MD, U.S.A.)

대조군은 산화자극을 가하기 위하여 30%의 H_2O_2(Sigma Chemicals, St. Louis, MO, U.S.A.)를 단독처리 하였으며, 에스트로겐 처리군은 1 μM의 17β-estradiol(E_2) (Sigma Chemicals, St. Louis, MO, U.S.A.)을 산화자극을 가하기 24시간 전에 투여하여 전배양

2. 산화자극과 MTT분석

96 well plate에 1 x 10^4 /㎖ 개의 bAEC 세포를 배양시켜 대조군은 30%의 H_2O_2 (Sigma Chemicals)를 인산완충용액(PBS)에 500 μM 로 희석하여 15, 30, 60분 동안 산화자극을 가하였으며, 에스트로겐 처리군은 1 μM의 17β-estradiol(E_2)을 24시간 전에 처리하여 전배양 후 15, 30, 60분동안 산화자극을 가했다.

세포의 산화제에 대한 감수성 평가는 tetrazolium based colorimetric assay 방법을 변형한 MTT[3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyl-tetrazolium bromide] (Colorimetric assay kit, Chemicon Inc. CA, U.S.A.) 측정방식을 이용하여 450 ㎚에서 흡광도를 측정했다.

Materials & Methods (contin.)

3. 세포 내 reactive oxygen species (ROS) 측정

6 well tissue culture dish에 5 x 10^4/㎖ 개의 bAEC 세포를 배양하고 1 mM 농도의 H_2O_2에 3시간 노출시켰다. 에스트로겐 처리군은 1 μM의 17β-estradiol을 산화 자극을 가하기 24시간 전에 투여하여 전배양 한 후에 같은 방법으로 산화자극.

이후에 PBS 완충용액으로 2회 세척, 30 μM의 2,7-dichlorofluo- rescin diacetate (DCF-DA (Sigma Chemicals, St. Louis, MO, U.S. A.)를 첨가하여 1시간 동안 CO_2를 5% 함유한 37℃ incubation - 세척 후 flow cytometer (FACScan, Becton-Dickinson, Mountain View, CA, U.S.A.)로 분석.

4. 세포자멸사의 형태학적 관찰

4 well chamber slide에 1 x 10^4/㎖ 개의 bAEC 세포를 배양하고 1 mM 농도의 H_2O_2에 3시간 노출. 에스트로겐 처리군은 방법 2와 같이 전처치 후 역시 H_2O_2에 3시간 노출. 인산염완충식염수로 2회 세척하고 3.7% formaldehyde로 세포를 고정시키고, 다시 인산염완충식 염수로 3회 씻어낸 후 10 ㎍/㎖의 Hoechst 33342 (Sigma Chemicals, St. Louis, MO, U.S.A.)를 첨가하여 1시간동안 실온의 암실에서 반응 시키고 세척 후 형광현미경으로 관찰했다.

Materials & Methods (contin.)

5. Western blotting

- 2 x 10^5/㎖ 개의 bAEC 세포를 100 ㎟ dish에 배양시켜 30%의 H_2O_2를 인산완충 용액 (PBS)에 1 mM로 희석하여 3시간 동안 산화자극을 가했다. 에스트로겐 처리군은 역시 위와 동일한 방법으로 전 처치 후 3시간 동안 산화자극을 가했다.
- phospho-p38의 활성 억제를 확인하고자 대조군과 에스트로겐 처리 군에서 SB203580(Sigma Chemicals)를 20 μM 농도로 1시간 전처치한 후 산화 자극을 가하였다.
- 일차항체는 phospho-p38, p38 MAP kinase (Thr180/Tyr182) (Cell signaling Technology, Beverly, MA, U.S.A.), Bcl-2 (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) 항체

Materials & Methods (contin.)

6. 형광표지세포분리기(fluorescence activated cell sorter, FACS)를 이용한 세포자멸사 분석

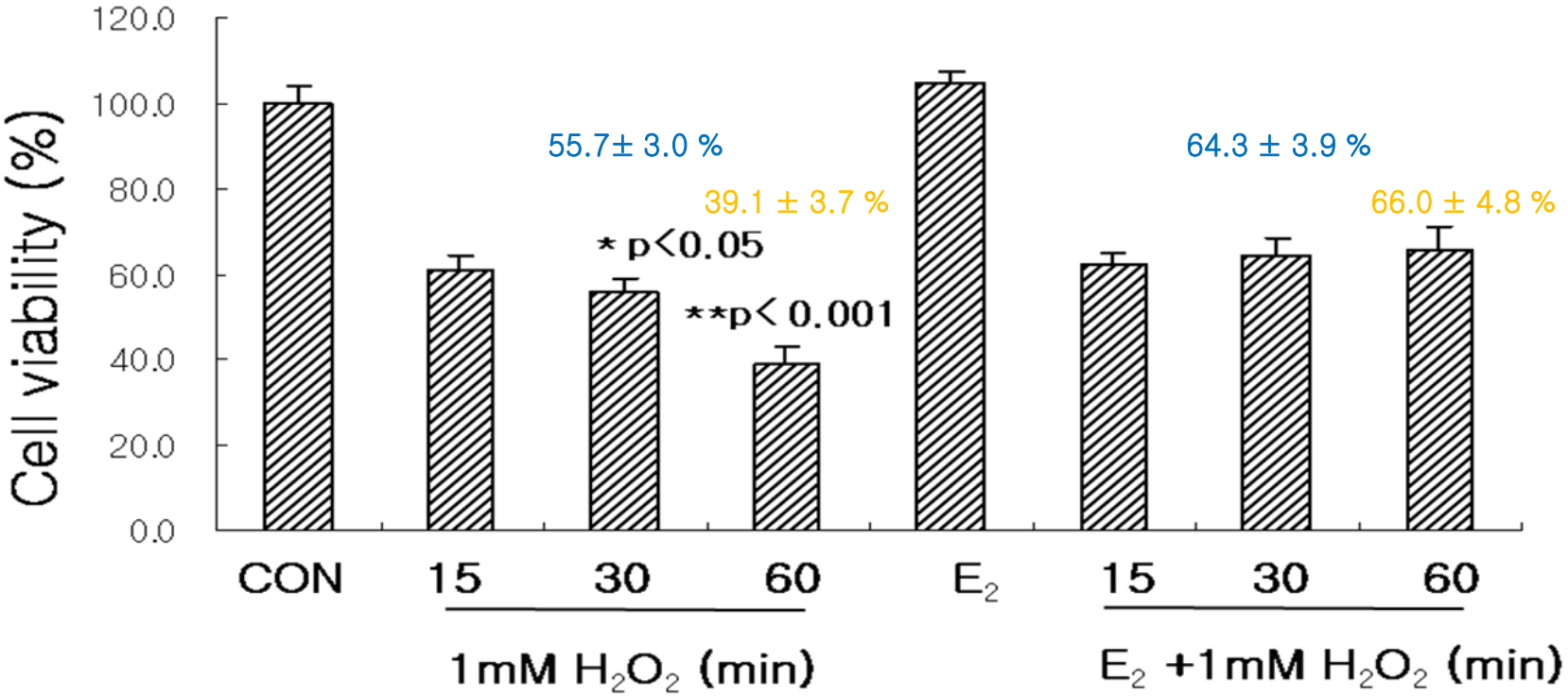
인산염완충식염수로 세척한 후 100 ㎕의 Annexin V binding buffer(140 mM NaCl, 10 mM Hepes, pH 7.4, 25 mM $CaCl_2$)로 재부유시켰다. 5 ㎕의 Annexin V-FITC conjugate와 5 ㎕의 propidium iodide (PI)를 넣어 암실에서 15분간 반응시켰다. 400 ㎕의 Annexin V binding buffer를 재첨가하여 FACScan (Becton-Dickinson)으로 분석.

7. 통계분석 (Statistical analysis)

같은 실험을 3회에 걸쳐서 반복하여 결과를 얻으며, 모든 자료는 평균 ± 표준 편차로 표시하고, 각 군들 사이의 비교는 SPSS (SPSS Inc, Chicago, U.S.A)를 사용하며 t-test로 분석. 통계학적 유의 수준은 $P < 0.05$로 정의.

Results
– cell survival by MTT assay

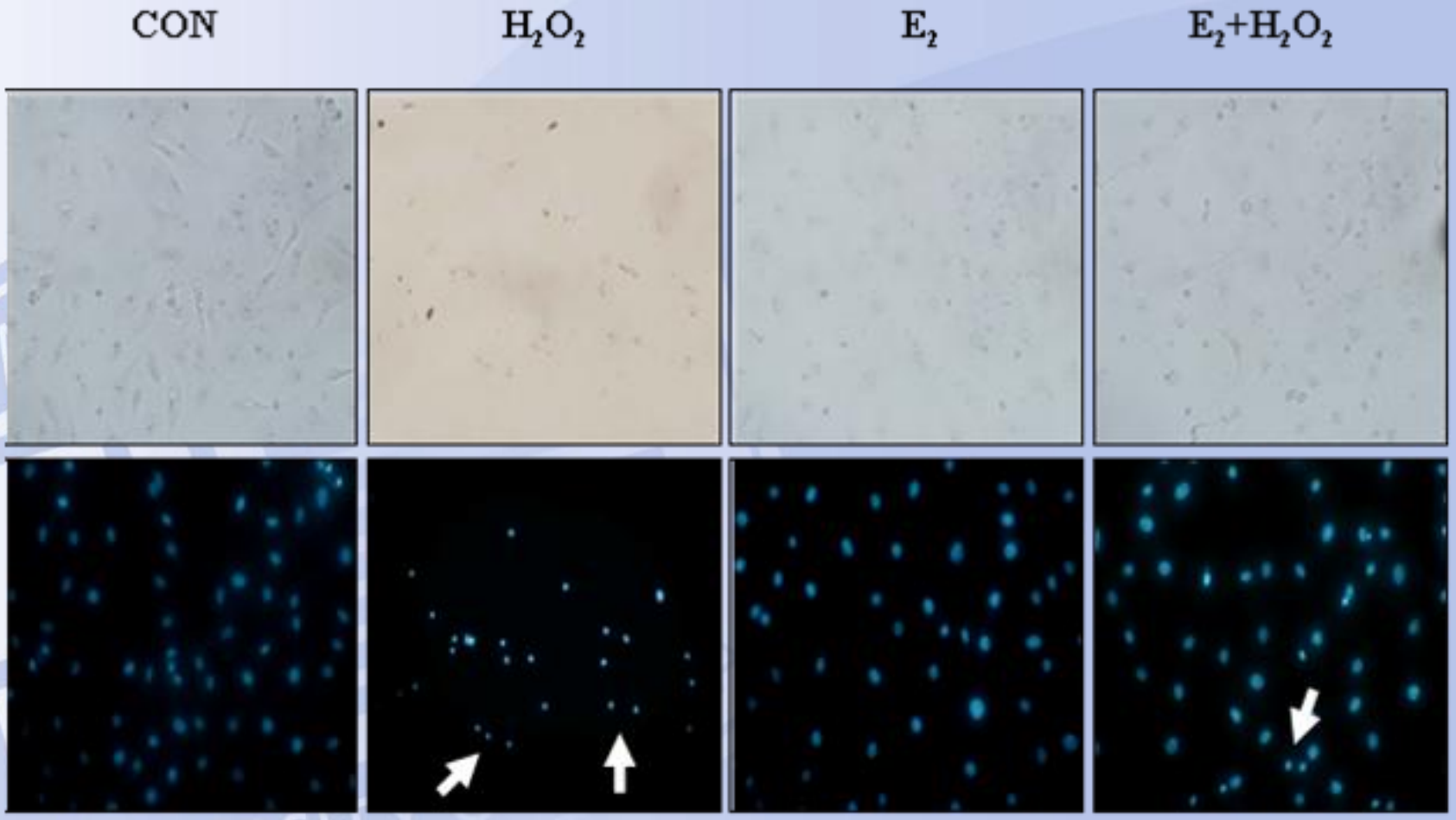
17β-estradiol inhibited H_2O_2 induced bAEC death.



Results

– cellular apoptosis by Hoechst 33342 staining

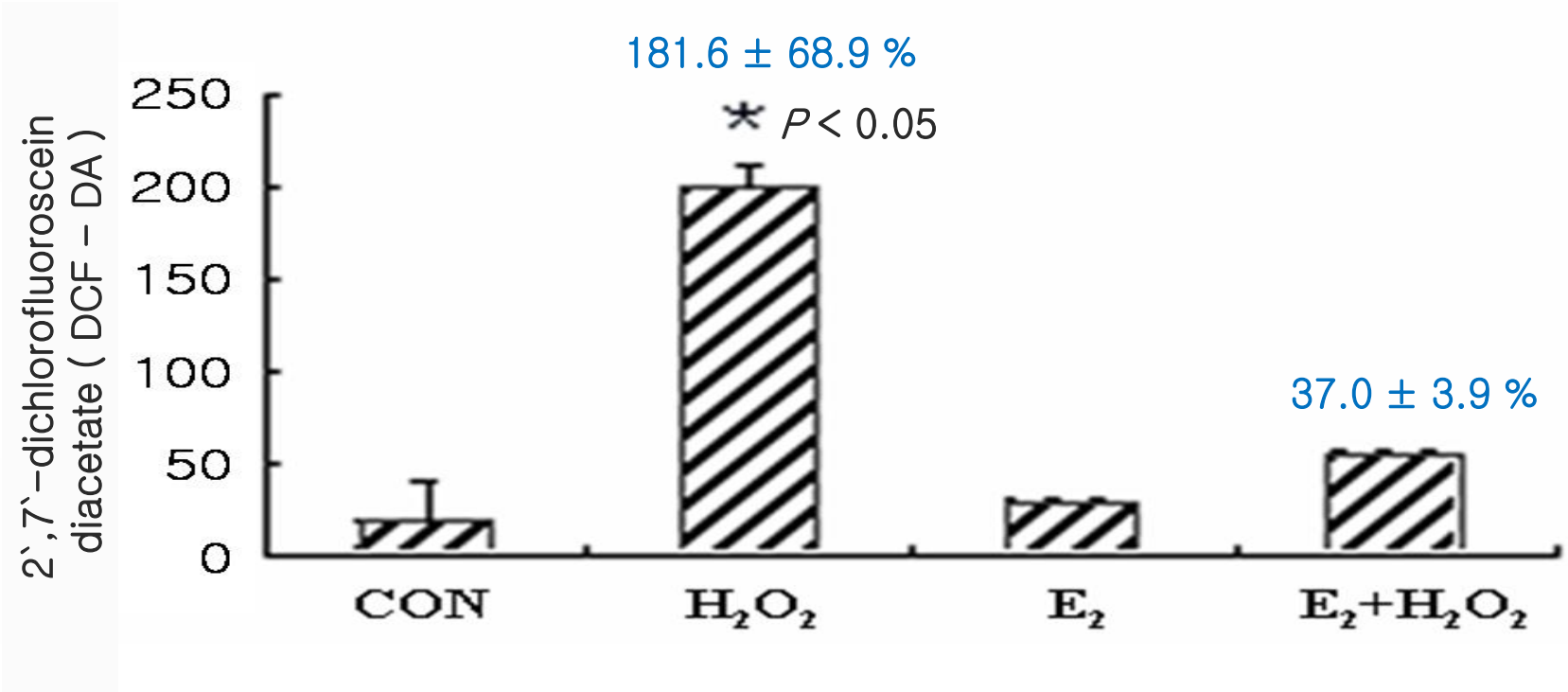
17β-estradiol reduced apoptotic bodies and nuclear chromatin condensation.



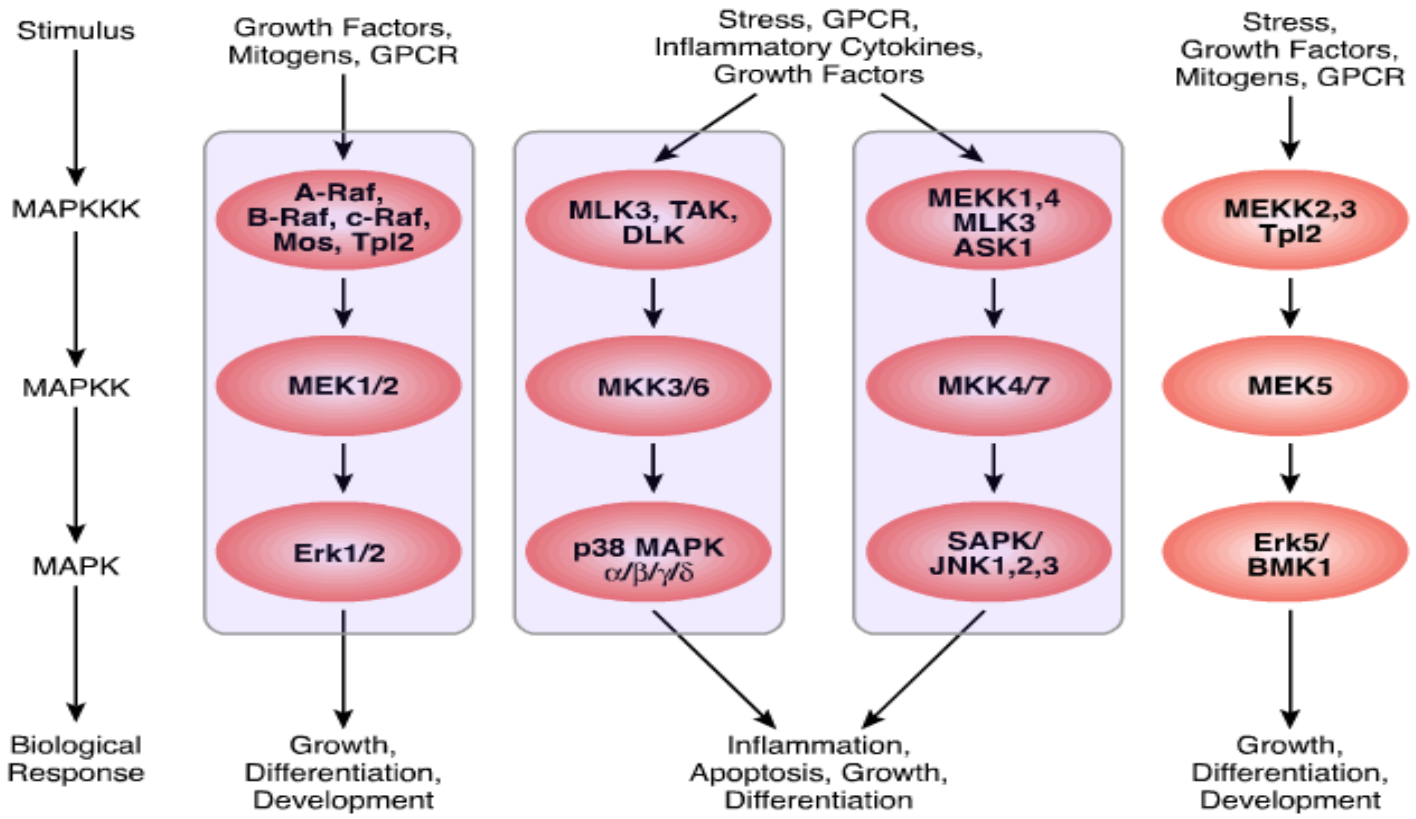
Results

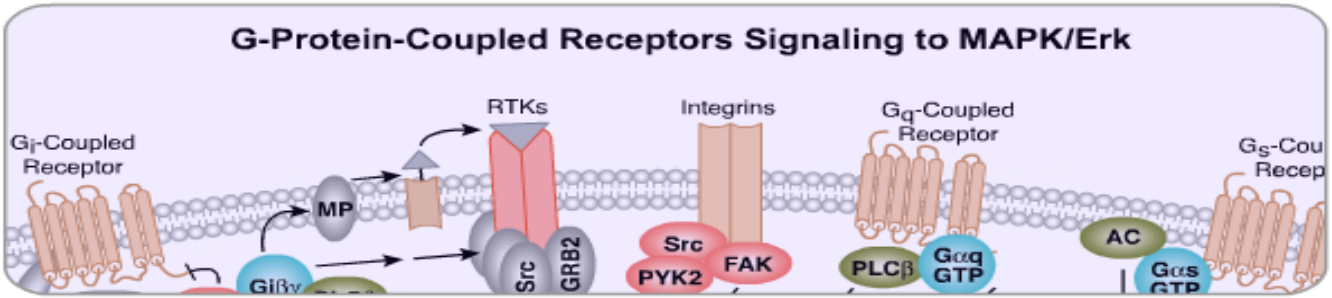
– Reactive oxygen species by DCF-DA

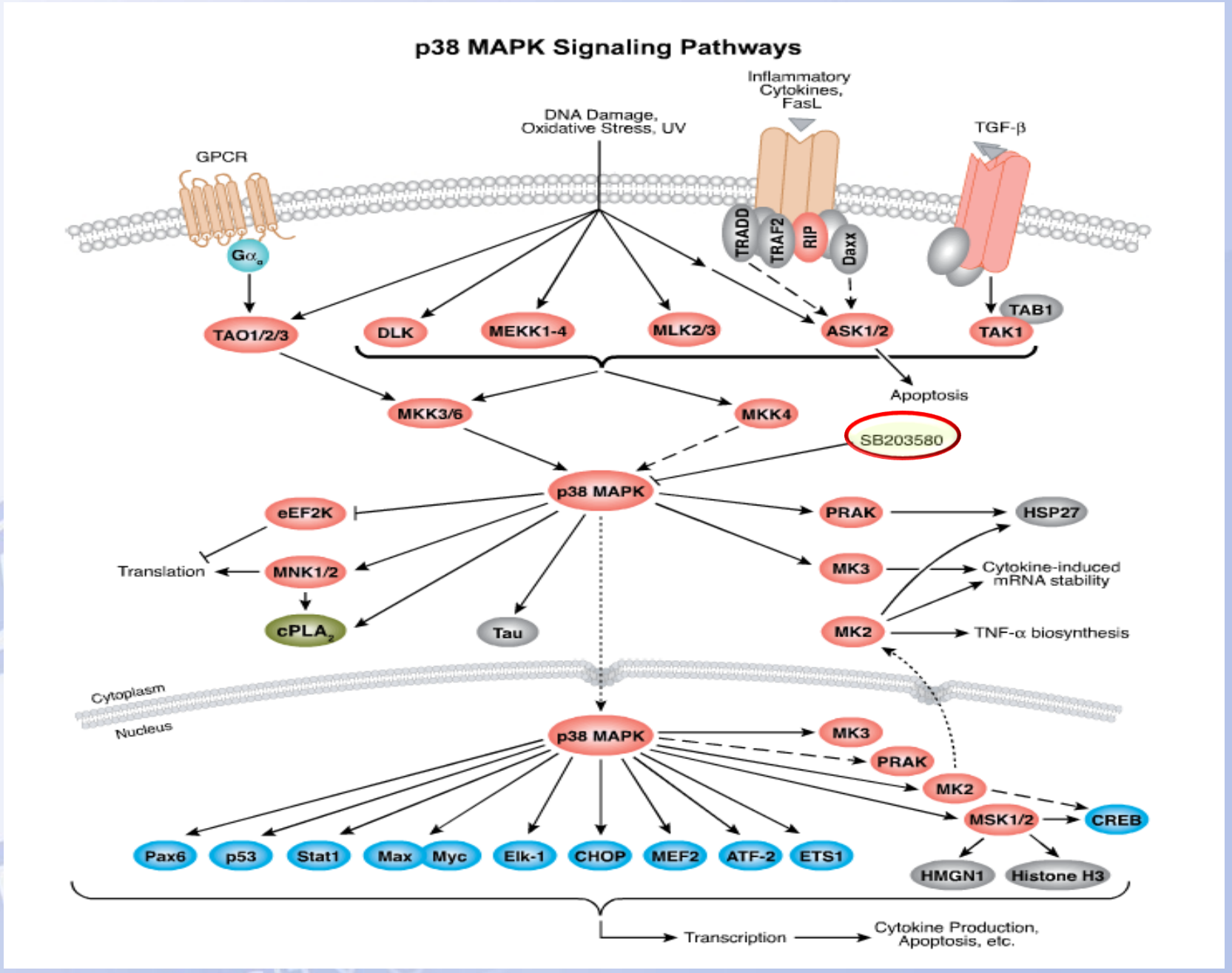
17β-estradiol inhibited intracellular ROS production.



Mitogen-Activated Protein Kinase Cascades

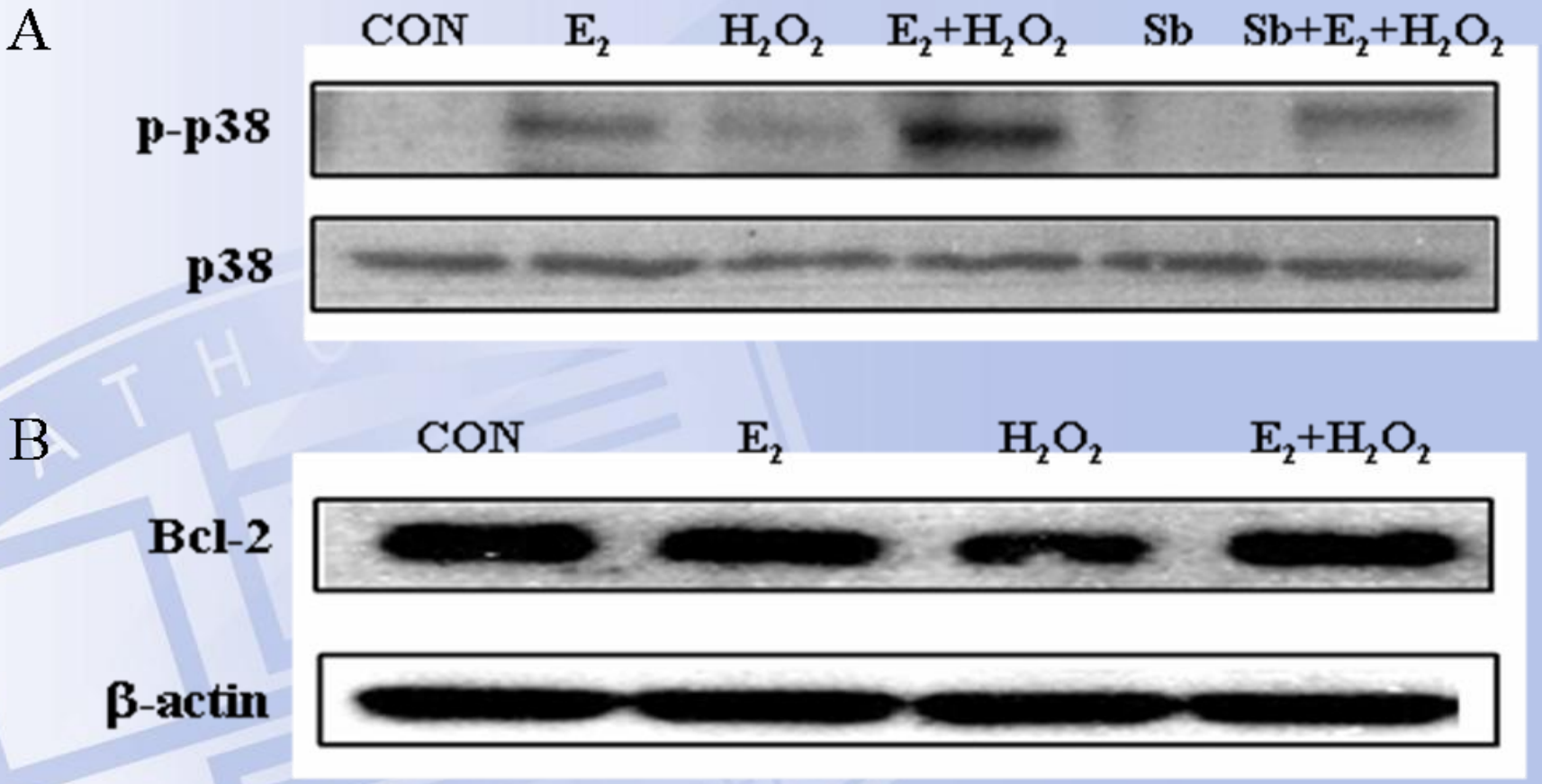





p38 MAPK Signaling Pathways
Inflammatory Cytokines, FasL
DNA Damage, Oxidative Stress, UV
TGF-β
GPCR
Gα
TRADD
TRAF2
RIP
Daxx
TAB1
TAO1/2/3
DLK
MEKK1-4
MLK2/3
ASK1/2
TAK1
Apoptosis
MKK3/6
MKK4
SB203580
p38 MAPK
eEF2K
Translation
MNK1/2
cPLA2
Tau
PRAK
HSP27
MK3
Cytokine-induced mRNA stability
MK2
TNF-α biosynthesis
Cytoplasm
Nucleus
p38 MAPK
MK3
PRAK
MK2
MSK1/2
CREB
Pax6
p53
Stat1
Max
Myc
Elk-1
CHOP
MEF2
ATF-2
ETS1
HMGN1
Histone H3
Transcription
Cytokine Production, Apoptosis, etc.

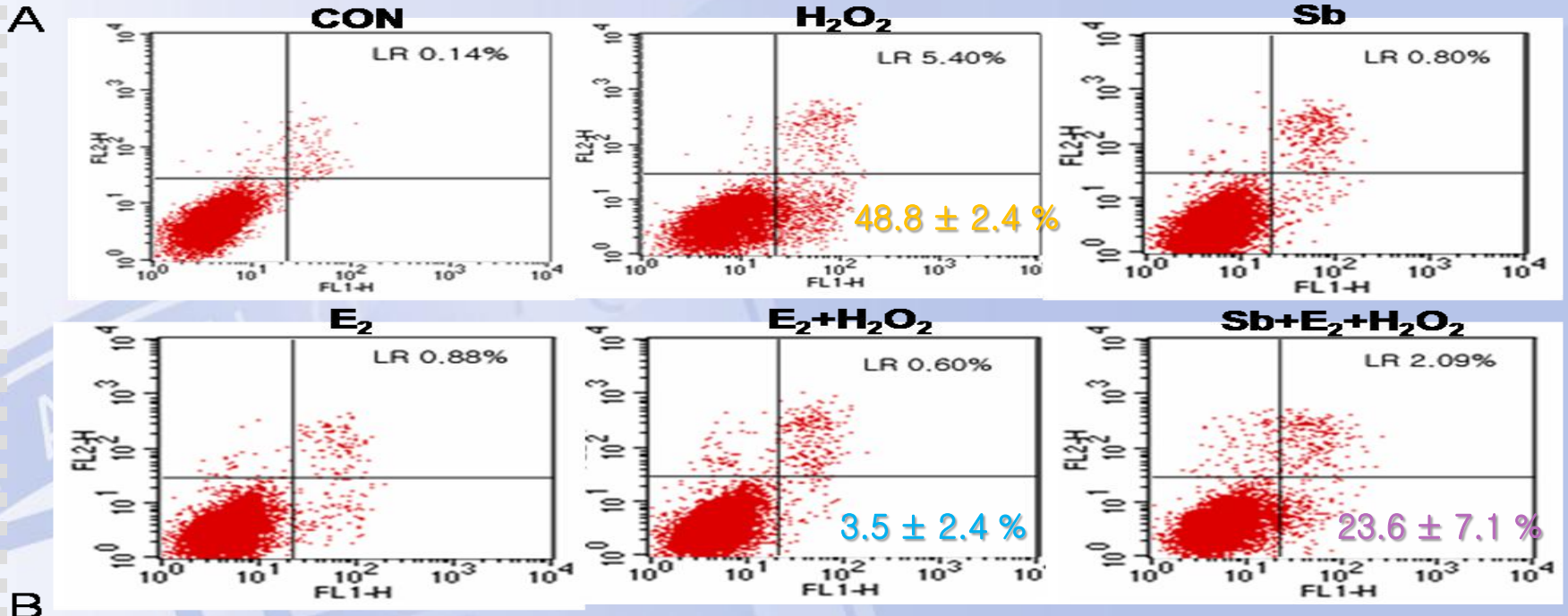
Results

– intracellular mechanism of apoptosis after oxidative stress by western blotting for phospho-p38, p38, and Bcl-2

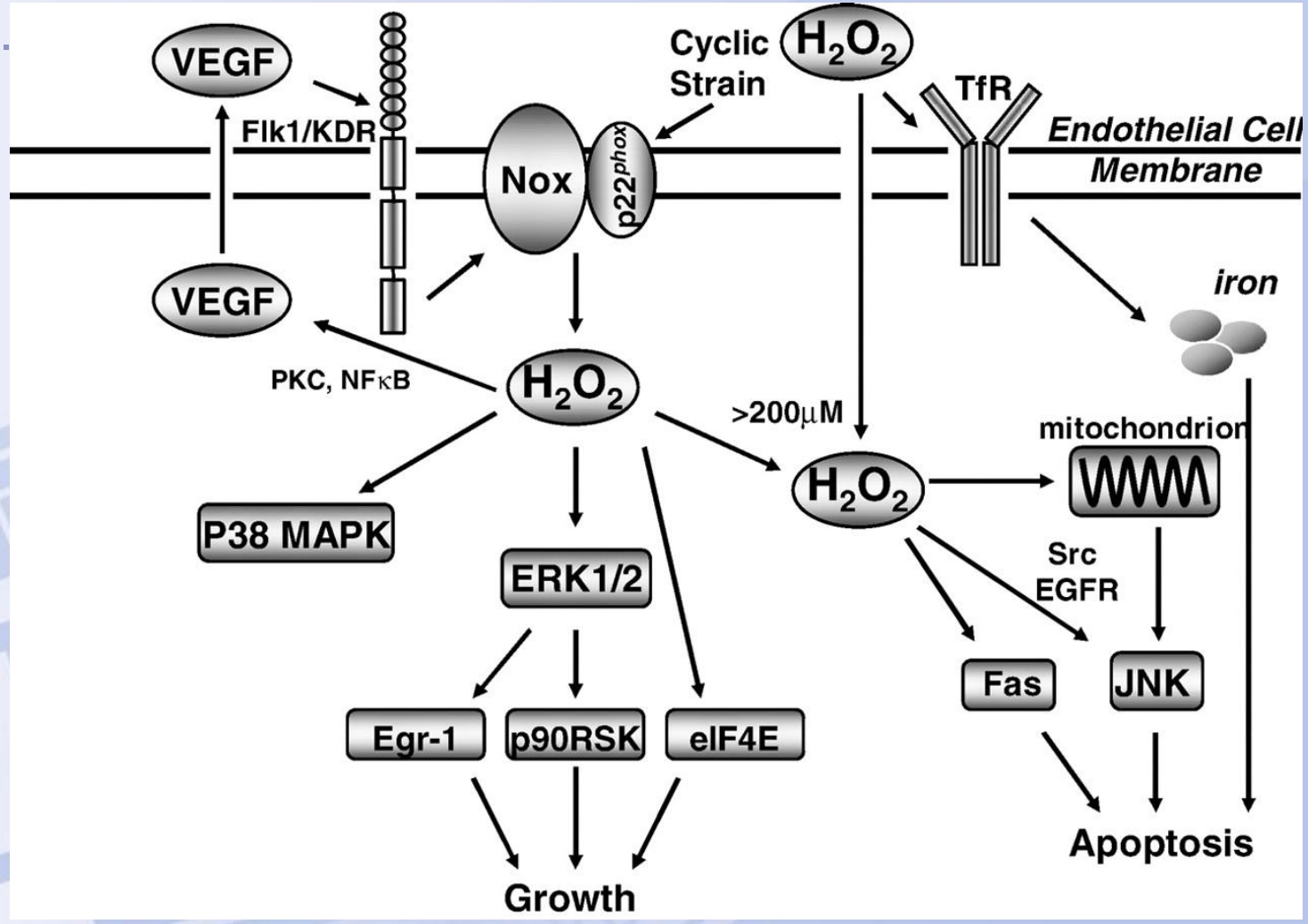


Results

– cellular apoptosis by FACS with Annexin V and propidium iodide (PI).



Signaling events mediating hydrogen peroxide modulation of endothelial cell growth and apoptosis.



Cai H Cardiovasc Res 2005;68:26-36

Limitations

- Bovine aortic endothelial cells
- In vitro experiment

Conclusion

- 소의 대동맥내피세포에서 E2의 전처치는
  H_2O_2에 대한 직접적인 산화자극에 대해서
  혈관내피세포를 보호하는 작용을 하였다.
- E2는 세포 내의 Reactive oxygen species를 줄이고,
  antiapoptotic effect를 보였다.
- 이러한 보호작용에 대한 기전으로는
  세포 내 p38 MAP Kinase를 활성화시키는 것으로 생각되며,
  향후 이러한 기전에 대한 연구가 더 필요하다.
- 따라서, 여성 호르몬 치료가 폐경 여성에서
  산화자극에 대하여 cardiovascular integrity 유지에 중요한
  역할을 할 것이라는 과학적 근거로 제시될 수 있겠다.

Thank you
for your attention !!