

Effects of Cigarette Smoke Condensation under different smoking regimens on human oral epidermoid carcinoma cell

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- **Experimental:** Design, CSCs, Cell Culture and Assay Test.
- **Results and Discussion:** Cytotoxicity, Emissions and Gene Expression.
- **Conclusions**

Background



FCTC:

- Provisions for testing and regulating cigarette emissions.
- Current Smoking Method: ISO Machine Smoking Regime (ISO).
- Alternative Method: Health Canada Intense Smoking Regime (HCI).



Exposure to Human:

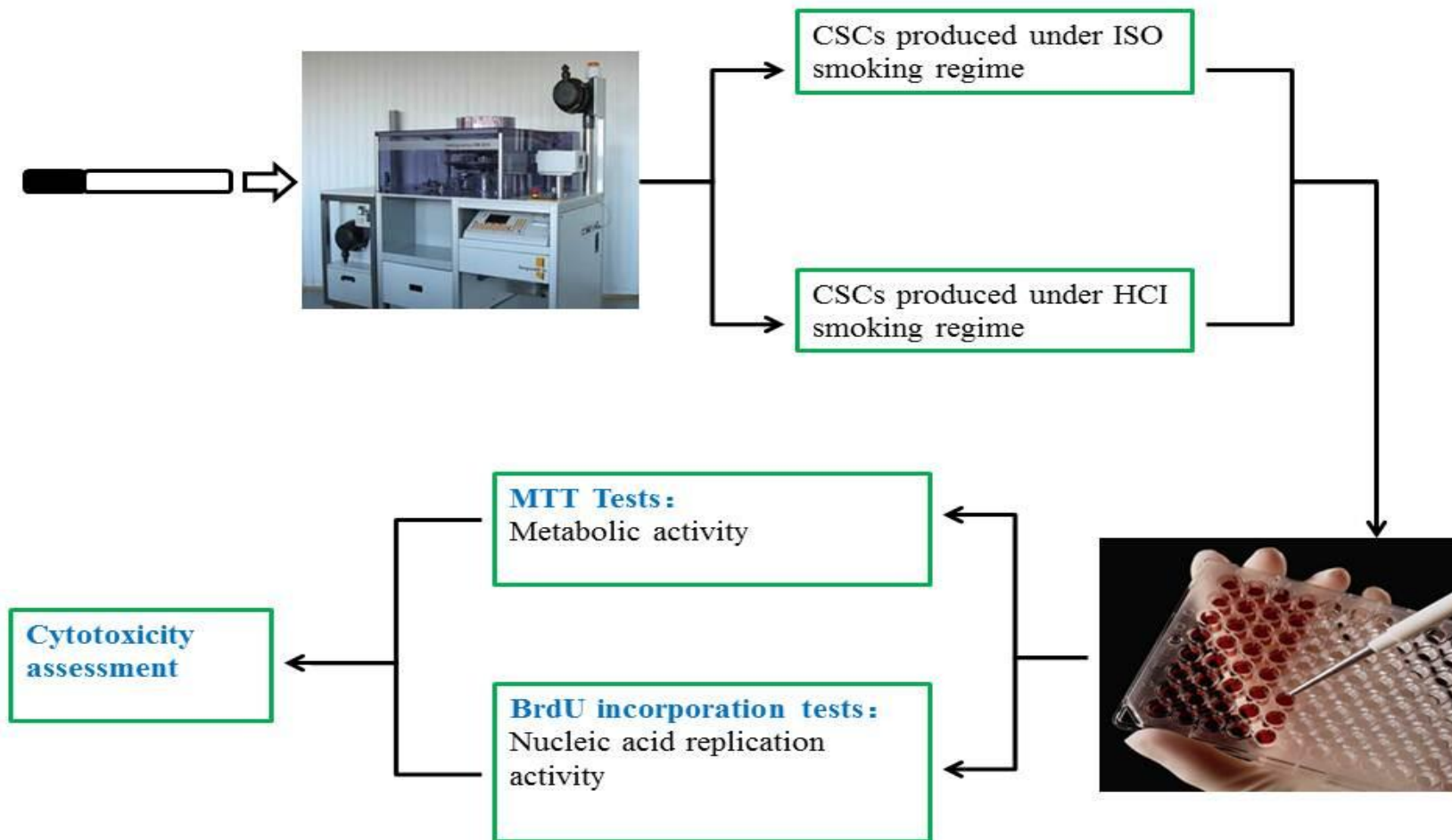
- Tobacco use is a major etiological factor for oral cancer.
- The smoke emissions is different between the two regimes.
- The exposure to human may also different between two regimes.



Toxicology Studies:

- Toxicology assessment *in vitro* using cigarette smoke condensation
- Human Clinical Studies using the biomarker
- Genotoxicity is another way to evaluate the toxicological effects.

Experimental-Design

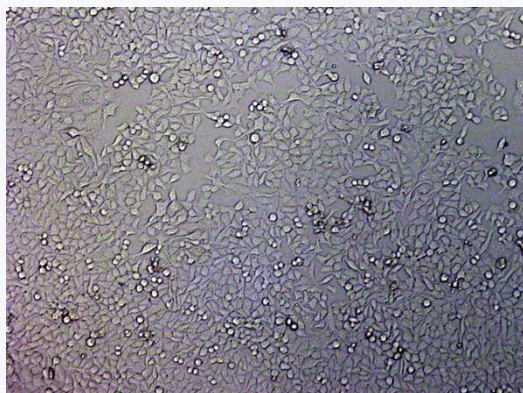


Experimental-CSCs preparation

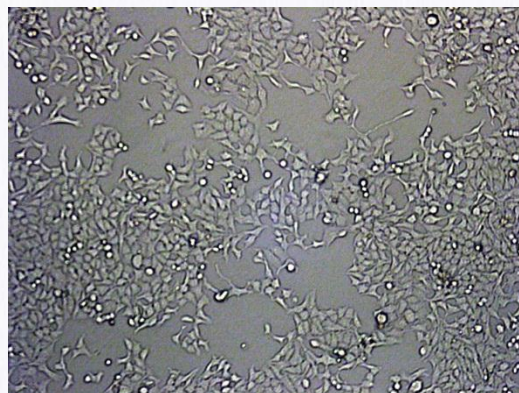
Reference Cigarette	3R4F
Smoking machine	Borgwalt RM-20H
Smoking regime 1	ISO smoking (35/60/2 without blocking of filter ventilation)
Smoking regime 2	HCI smoking (55/30/2 with complete blocking of filter ventilation)
CSCs	Prepared by dissolving the collected smoke particulates in the dimethyl sulfoxide (DMSO).
Store	Filtered through sterile cheesecloth and stored frozen at -80°C

Experimental-Cell culture

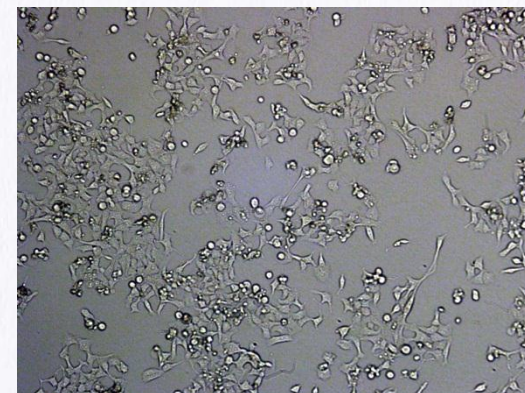
Cells line	Oral epidermoid carcinoma cell line KB
Culture	a-MEM complete culture plus 10% fetal bovine serum
Condition	5% carbon dioxide and 95% air at 37°C
Cell passage	Seeded into 96-well culture plates at 4×10^4 cells per well and used between passages three and seven.
Exposure	The dose of the total particulate matter exposed to the cells range from 0 to 300 mg/L



Control



Low Exposure



High Exposure

Experimental-Assay Test

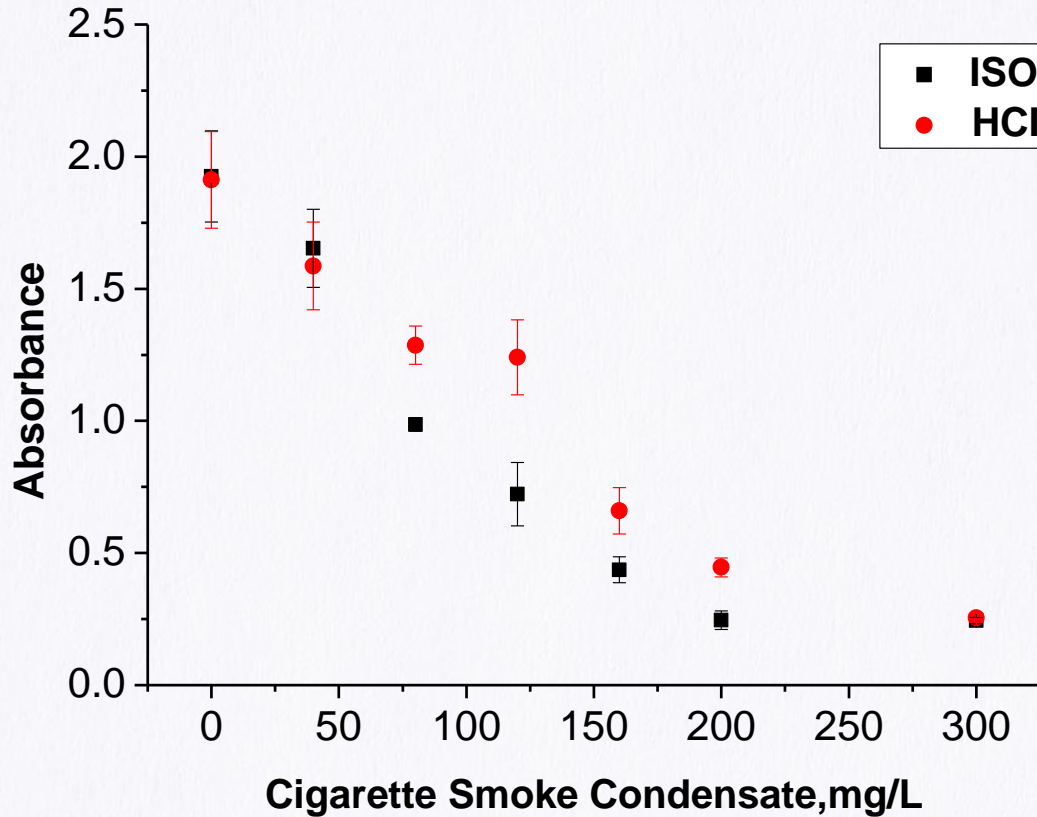
The metabolic activity was assessed by MTT, ROS and protein phosphorylation. MTT was investigated for mitochondrion activity, and ROS was for reactive oxygen radical concentration evaluation, and protein phosphorylation was also investigated using γ H2A.X.

Effects on metabolic activity	MTT	Mitochondrion activity
	Reactive oxygen species assay	Reactive oxygen radical
	Protein phosphorylation	γ H2A.X (phospho S139)

The effects on the nucleic acid expression was assessed by BrdU incorporation assay, micro-RNA expression, m-RNA expression

Effects on nucleic acid expression	BrdU incorporation assay	Nucleic acid replication activity
	micro-RNA expression	mi-RNA
	m-RNA expression	m-RNA

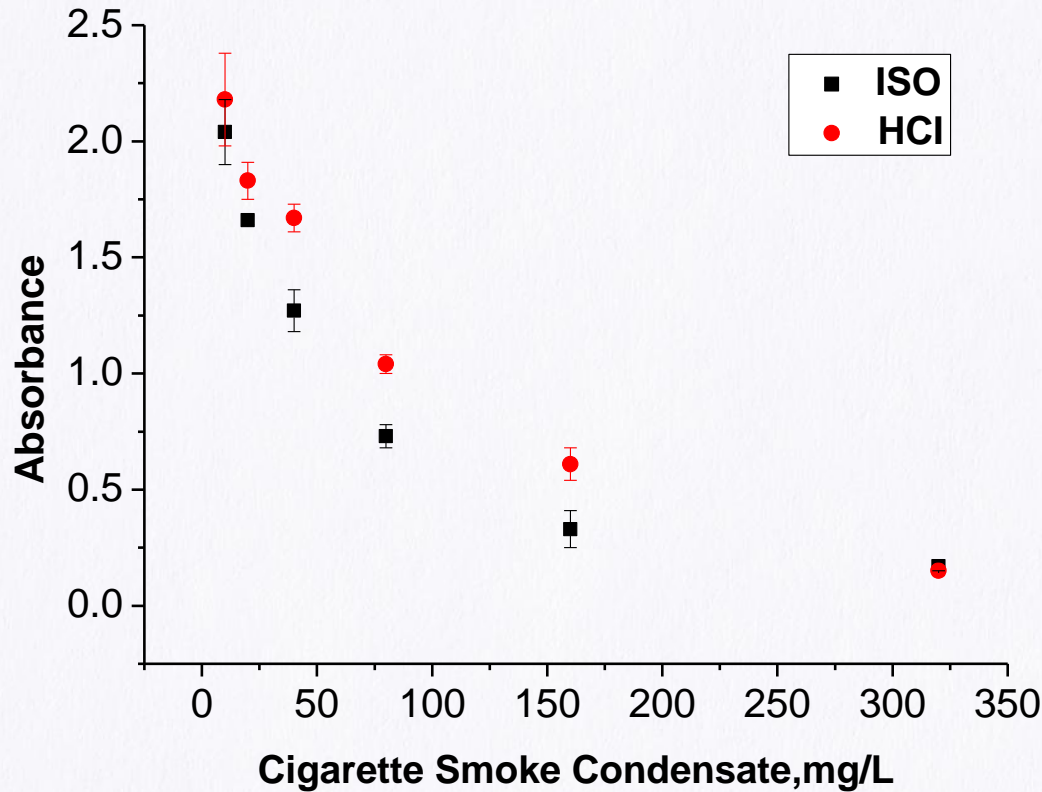
Results and Discussion



MTT Assay

- MTT assay was performed to evaluate the effects on the metabolic activity.
- The result show that the CSCs prepared under ISO regime have higher inhibit effects than HCI regime, with the EC50 range from 92 (ISO) to 123 (HCI) mg/L for MTT test.

Results and Discussion



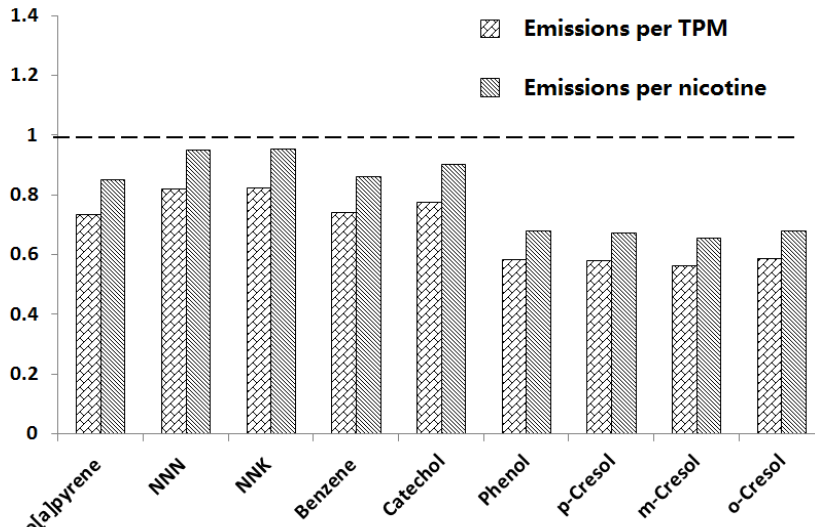
BrdU incorporation assay

- Similar with MTT assay's result, ISO regime show a higher inhibit effects than HCl
- The nucleic acid replication activity was inhibited more significant than metabolic activity, with the EC50 range from 68 (ISO) to 81 (HCl) mg/L for BrdU incorporation assay, and 92 (ISO) to 123 (HCl) μ g/mL for MTT test.

Results and Discussion

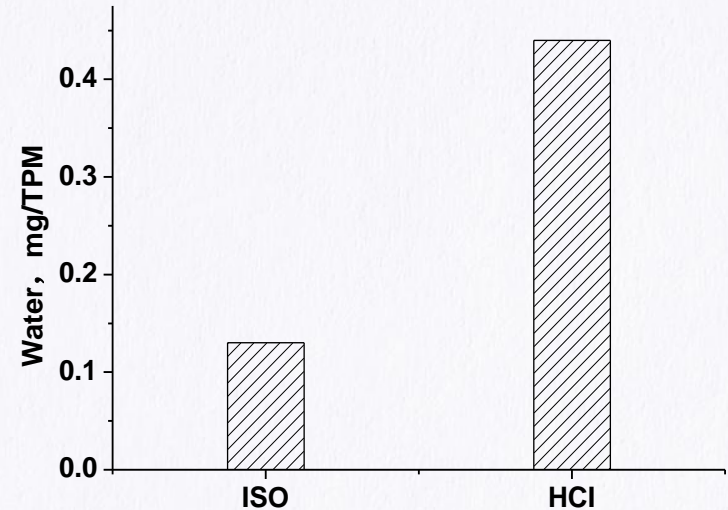
	Emissions		Emissions per TPM		Emissions per Nicotine	
	ISO	HCl	ISO	HCl	ISO	HCl
TPM	9.0 mg	38.6 mg	1	1		
Nicotine	0.72 mg	1.96 mg			1	1
	Emissions (ng)		Emissions per TPM (ng/mg)		Emissions per Nicotine (ng/mg)	
Benzo[a]pyrene	6.8	15.7	0.76	0.41	9.44	8.01
NNN	121	293	13.44	7.59	168.06	149.49
NNK	95	247	10.56	6.40	131.94	126.02
	Emissions (μg)		Emissions per TPM (μg/mg)		Emissions per Nicotine (μg/mg)	
Hydroquinone	32.8	89.6	3.64	2.32	45.56	45.71
Catechol	36.8	90.2	4.09	2.34	51.11	46.02
Phenol	7.1	13.1	0.79	0.34	9.86	6.68
p-Cresol	2.3	4.2	0.26	0.11	3.19	2.14
m-Cresol	1.8	3.2	0.20	0.08	2.50	1.63
o-Cresol	4.6	8.5	0.51	0.22	6.39	4.34

Results and Discussion



The ratio of the emissions under HCl and ISO (HCl/ISO)

Further study on the release of harmful components in mainstream smoke showed that the toxicant output on per unit of TPM and Nicotine for HCl regime is lower than ISO regime for reference cigarette.

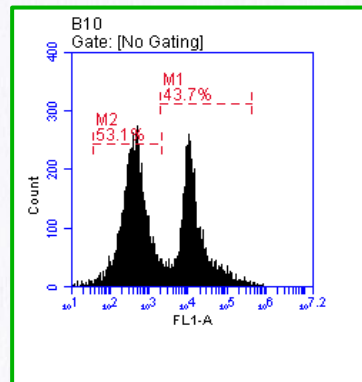
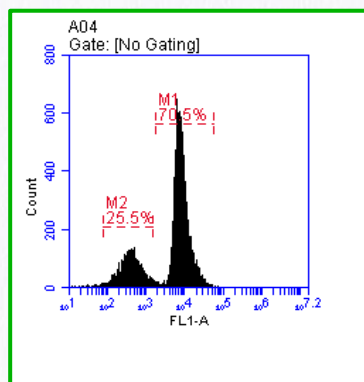
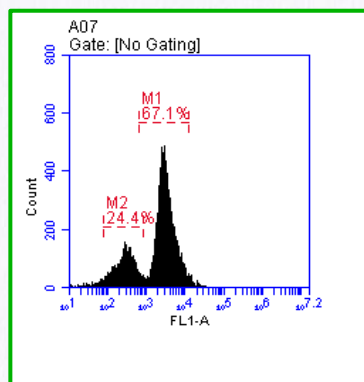
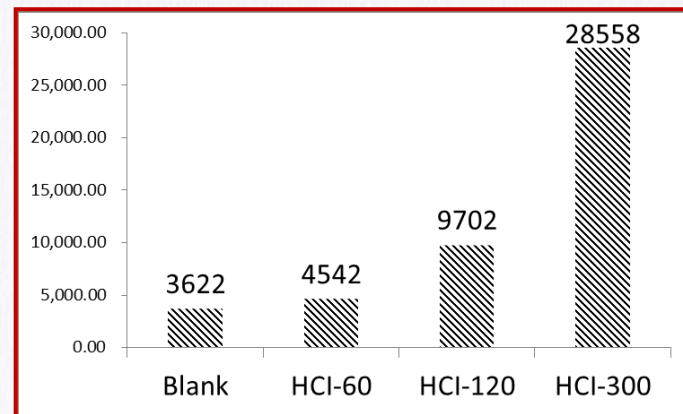
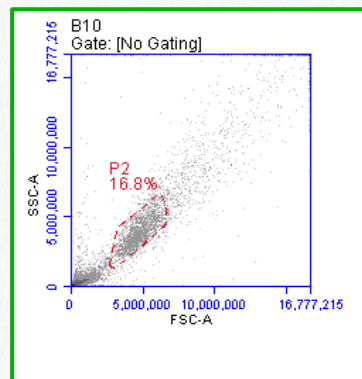
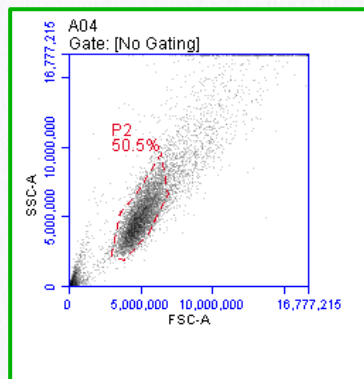
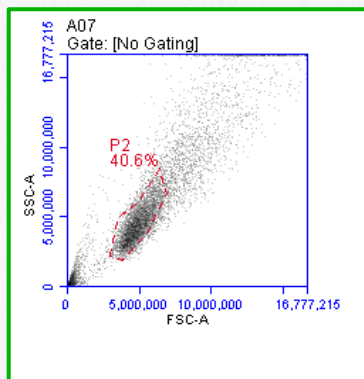


Water content under ISO and HCl

The CSCs under HCl regime have higher water content than ISO.

These two factors might contribute to the decrease of the cytotoxicity for human oral cancer cell under HCl regime than ISO regime.

Results and Discussion-Further Studies



□ As the exposure concentration rise from 0 to 300 mg/L, a huge number of cells was came into apoptosis.

□ The level of protein phosphorylation was also increased, with the concentration of the gamma H2A.X (S139) increase from 3662 (blank) to 28558 (300 mg/L) under HCl regime.

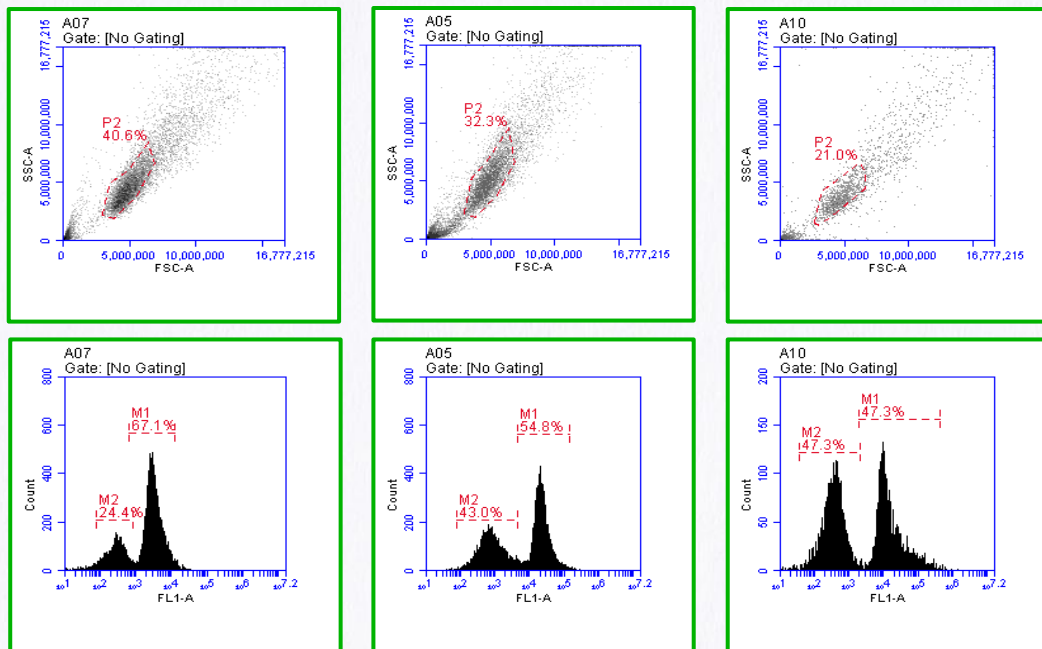
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CSCs Exposure:
120 mg/L

CSCs Exposure:
300 mg/L

Flow Cytometry Test under HCl

Results and Discussion-Further Studies



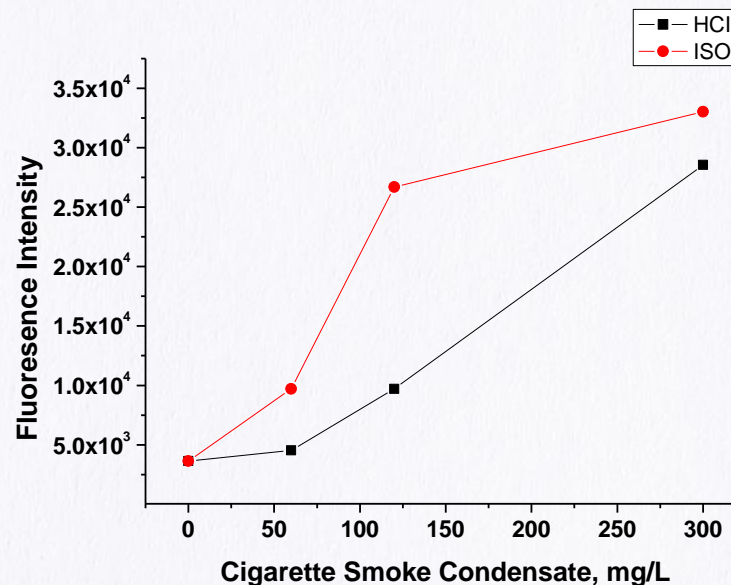
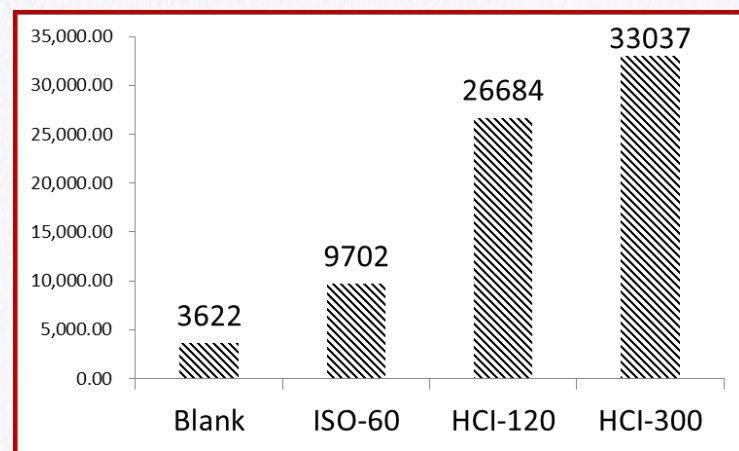
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CSCs Exposure:
120 mg/L

CSCs Exposure:
300 mg/L

Flow Cytometry Test under ISO regime

- Under the ISO regime the trends of the apoptosis and protein phosphorylation was similar with HCl.
- ISO regime show a higher phosphorylation level than HCl regime similar with the result of the MTT and BrdU incorporation assay.



Results and Discussion-Further Studies

Transcriptome Sequencing

Down or up-regulated ≥ 2 -fold, and FDR (false discovery rate) ≤ 0.05

Sample-vs-sample	Ups	Down
ISO60-vs-KB	903	811
ISO120-vs-KB	1533	1083
HCI60-vs-KB	1481	748
HCI120-vs-KB	2061	1048

- (1) NM_001270507: TNF
- (2) NM_000600: Interleukin-6 (IL-6)
- (3) NM_020327: Activin receptor type-1B
- (4) NM_001259 and NM_001145306: Cyclin-dependent kinase 6 (CDK6)
- (5) NM_001168236 and 001136000: Abelson Tyrosine protein Kinase 2
- (6) NM_001199462: Programmed cell death protein 2
- (7) NM_133330 and NM_133331: Histone-Lysine N-methyltransferase NSD 2

Results and Discussion-Further Studies

Micro-RNA Sequencing

Down or up-regulated ≥ 2 -fold, and FDR (false discovery rate) ≤ 0.05

Sample-vs-sample	Ups	Down
ISO60-vs-KB	18	7
ISO120-vs-KB	51	20
HCI60-vs-KB	54	72
HCI120-vs-KB	105	57

Different expressed micro-RNA	HCI-60	Control	Predict Target m-RNA	HCI-60	Control
all_hsa-miR-125a-5p	25939	10093	NM_001270507	0.001	1846.27
all_hsa-let-7a-5p	197644	55725	NM_000600	10	1499.01
all_hsa-miR-210-3p	587	2549	NM_020327	126.46	0.001

- (1) NM_001270507: TNF
- (2) NM_000600: Interleukin-6 (IL-6)
- (3) NM_020327: Activin receptor type-1B

Conclusions

Conclusion1:

The oral cancer cell vitro proliferation can be inhibited after exposed to CSCs, and exposure to the CSCs have affects on metabolic activity and nucleic acid replication activity of the cells.

Conclusion2:

Both for MTT tests and BrdU incorporation tests, the cytotoxicity is decreased from ISO regimen to HCI regimen. BrdU incorporation assay show that the nucleic acid replication activity was inhibited more significant than metabolic activity.

Conclusion3:

The different release of harmful components and water content might contribute to the decrease of the cytotoxicity for human oral cancer cell under HCI regimen than ISO regimen.

Conclusion4:

As the exposure concentration rise from 0 to 300 mg/L, the level of protein phosphorylation was also increased, ISO regime show a higher phosphorylation level than HCI regime.

Conclusion5:

The exposure to the CSCs, the gene expression of the oral cell was down or up-regulated, specially, some cancer, inflammation and apoptosis related genes were differently expressed.

Thank you for you attention~