# Liposomal formulation,characterization, and therapeutic efficacy

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# Introduction

- The clinical utility of most conventional chemotherapeutics is limited either by the inability to deliver therapeutic drug concentrations to the target tissues or by severe and harmful toxic effects on normal organs and tissues.

# Pharmacological Basis for Liposome Delivery

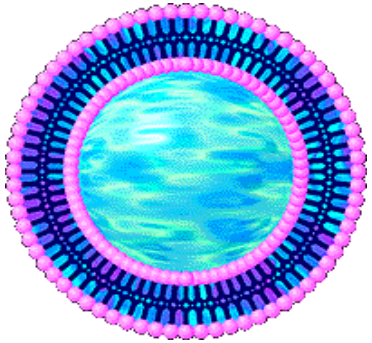
- Slow Release: reduced peak levels of free drug and prolonged tumor exposure
- Change in Biodistribution: avoiding drug deposition in certain tissues will reduce tissue-specific toxicities
- Tumor Targeting: passive accumulation by enhanced permeability and retention (EPR) effect

# Liposomes

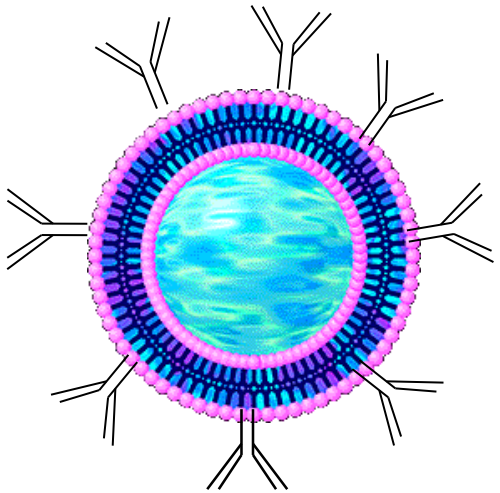
- Described by Bangham in 1965
- Study of membranes
- Spherical vesicles with a phospholipid bilayer

# Application

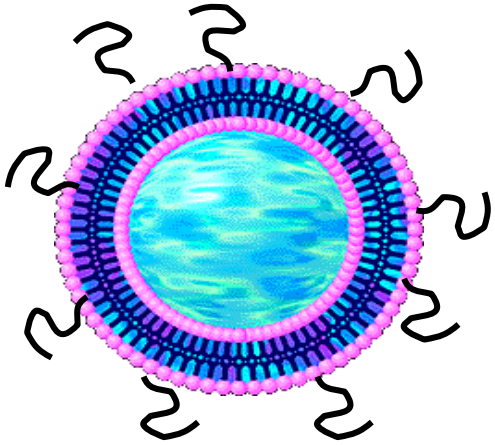
## Traditional



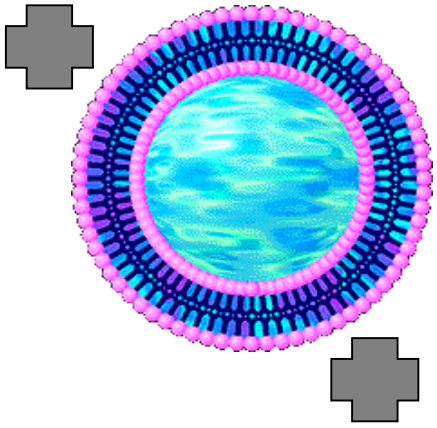
## Immunoliposome



## Long circulating

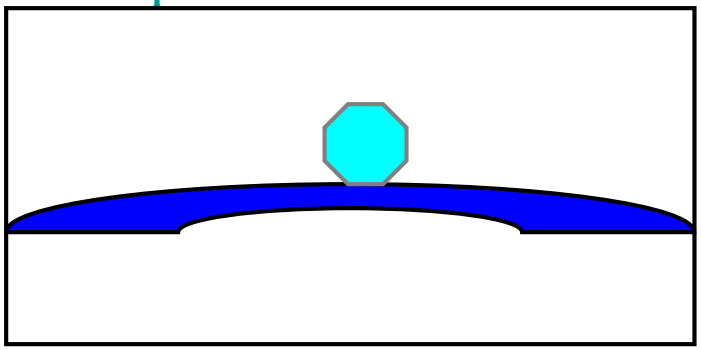


## Cationic

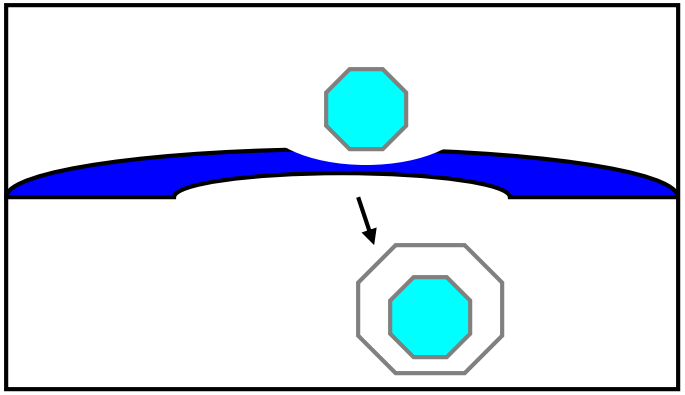


# Interaction of Liposome with Cell

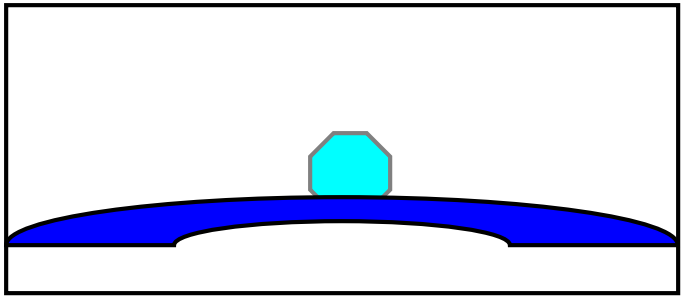
Adsorption



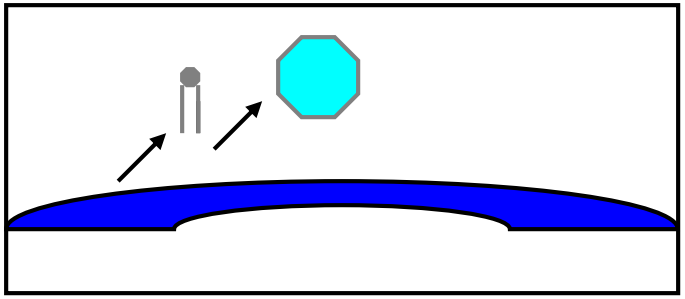
Endocytosis



Fusion



Lipid transfer



# Liposomal formulation

Liposomes can prepared by the modified thin-film hydration method (Tonnesen et al., 1994).

Dry lipid film

Extruction to form 100nm nanoparticles

# Liposomal formulation

- Bangham method, the detergent depletion method, the ether/ethanol injection method, the reverse phase evaporation and the emulsion method, have been reported for preparing liposome with high entrapment efficiency, narrow particle size distribution and long term stability.
- Recently, some alternative methods including dense gas and supercritical fluid techniques have been introduced for liposome preparation without using any organic solvent.

- Liposomes were first discovered by Bangham in 1965 and the first liposomal pharmaceutical product, Doxil, received FDA approval in 1995 for the treatment of chemotherapy refractory AIDS-related Kaposi’s sarcoma.

- Currently, there are about 12 liposome-based drugs approved for clinical use and more are in various stages of clinical trials.

# Table 1 Liposome based drugs in market

| Product Name | Route of injection | Drug | Lipid composition | Approved indication |
|---|---|---|---|---|
| Ambisome | Intravenous | Amphotericin B | HSPC, DSPG, cholesterol and amphoteracin B ( 2:0.8:1:0.4) | Sever fungal infections |
| Abelcet | Intravenous | Amphotericin B | DMPC and DMPG (7:3) | Sever fungal infections |
| Amphotec | Intravenous | Amphotericin B | cholesteryl sulfate | Sever fungal infections |
| DaunoXome | Intravenous | Daunorubicin | DSPC and cholesterol (2:1) | Blood tumors |
| Doxil | Intravenous | Doxorubicin | HSPC, cholesterol and PEG 2000-DSPE (56:39:5) | Kaposi's sarcoma, Ovarian/Breast Cancer |
| Lipodox | Intravenous | Doxorubicin | DSPC, cholesterol and PEG 2000-DSPE (56:39:5) | Kaposi's sarcoma, Ovarian/Breast Cancer |
| Myocet | Intravenous | Doxorubicin | EPC and cholesterol (55:45) | Combination therapy with cyclophosphamide in metastatic breast cancer |
| Visudyne | Intravenous | Verteporfin | EPG and DMPC (3:5) | Age-related molecular degerneration, , pathologic myopia, ocular histoplasmosis |
| Depocyt | Spinal | Cytarabine | Cholesterol, Triolein, DOPC and DPPG (11:1:7:1) | neoplastic meningitis and lymphomatous meningitis |
| DepoDur | Epidural | Morphine sulfate | Cholesterol, Triolein, DOPC and DPPG (11:1:7:1) | Pain management |
| Epaxal | intramuscular | Inactivated hepatitis A virus (strain RG-SB) | DOPC and DOPE | Hepatitis A |
| Inflexal V | intramuscular | Inactivated hemaglutinine of Influenza virus strains A and B | DOPC and DOPE | Influenza |

# Table 2 Liposome based drugs in clinical trials

| Product Name | Route of injection | Drug | Lipid composition | Approved indication | Trial phase |
|---|---|---|---|---|---|
| LEP-ETU (Powder/12 months) | Intravenous | Paclitaxel | DOPC, cholesterol and cardiolipin (90:5:5 molar ratio) | ovarian, breast and lung cancers | Phase I/II |
| LEM-ETU | Intravenous | Mitoxantrone | DOPC, cholesterol and cardiolipin in 90:5:5 molar ratio | Leukemia, breast, stomach, liver, ovarian cancers | Phase I |
| EndoTAG-1 (Powder/ 24 months) | Intravenous | Paclitaxel | DOTAP, DOPC and paclitaxel (50:47:3 molar ratio) | Anti-angiogenic properties, breast cancer, pancreatic caner | Phase II |
| Arikace | portable aerosol delivery | Amikacin | DPPC and cholesterol | Lung infection | Phase III |
| Marqibo | Intravenous | Vincristine | cholesterol and egg sphingomyelin (45:55 molar ratio) | Metastatic malignant uveal melanoma | Phase III |
| ThermoDox | Intravenous | Doxorubicin | DPPC, MSPC and PEG 2000-DSPE (90:10:4 molar ratio) | non-resectable hepatocellular carcinoma | Phase III |
| Atragen | Intravenous | Tretinoin | DMPC and soybean oil | acute promyelocytic leukemia, hormone-refractory prostate cancer | Phase II |
| T4N5 liposome lotion | Topical | Bacteriophage T4 endonuclease 5 | unknown | xeroderma pigmentosum. | Phase III |

# Table 2 Liposome based drugs in clinical trials

| Product Name | Route of injection | Drug | Lipid composition | Approved indication | Trial phase |
|---|---|---|---|---|---|
| Liposomal Grb-2 | Intravenous | Grb2 antisense oligodeoxynucleotide | unknown | Acute myeloid leukemia, chronic myelogenous leukemia, Acute lymphoblastic Leukemia | Phase I |
| Nyotran | Intravenous | Nystatin | DMPC, DMPG and cholesterol | systemic fungal infections | Phase I/II |
| LE-SN38 | Intravenous | SN-38, the active metabolite of irinotecan | DOPC, cholesterol and cardiolipin | metastatic colorectal cancer | Phase I/II |
| Aroplatin | Intrapleural | Cisplatin Analog (L-NDDP) | DMPC and DMPG | metastatic colorectal carcinoma | Phase II |
| Liprostin | Intravenous | Prostaglandin E1 | unknown | Peripheral Vascular Disease | Phase II/III |
| Stimuvax | subcutaneous | BLP25 lipopeptide (MUC1-targeted peptide) | monophosphoryl lipid A, cholesterol, DMPG and DPPC | Cancer vaccine for multiple myeloma developed encephalitis | Phase III |
| SPI-077 | Intravenous | Cisplatin | SHPC, cholesterol and DSPE-PEG | Head and neck cancer, Lung cancer | Phase I/II |
| Lipoplatin (suspension /36 months) | Intravenous | Cisplatin | SPC, DPPG, cholesterol and mPEG 2000-DSPE | Pancreatic cancer, head and neck cancer, mesothelioma, breast and gastric cancer, and non-squamous non-small-cell lung cancer | Phase III |

# Table 2 Liposome based drugs in clinical trials

| Product Name | Route of injection | Drug | Lipid composition | Approved indication | Trial phase |
|---|---|---|---|---|---|
| S-CKD602 | Intravenous | Camptothecin analog | DPSC, and DSPE-PEG ( 95:5 molar ratio) | Recurrent or progressive carcinoma of the uterine cervix | Phase I/II |
| OSI-211 | Intravenous | Lurtotecan | HSPC and cholesterol (2:1 molar ratio) | Ovarian cancer, head and neck cancer | Phase II |
| INX-0125 | Intravenous | Vinorelbine | cholesterol and egg sphingomyelin (45:55 molar ratio) | Advanced solid tumors | Phase I |
| INX-0076 | Intravenous | Topotecan | cholesterol and egg sphingomyelin (45:55 molar ratio) | Advanced solid tumors | Phase I |
| Liposome-Annamycin (powder) | Intravenous | Annamycin | DSPC, DSPG and Tween | Acute lymphocytic leukemia | Phase I/II |

- Most liposomal drug formulations, such as Doxil and Myocet, are approved for intravenous application.
- Other administration routes such as intramuscular delivery have been approved for delivery of surface antigens derived from the hepatitis A or influenza virus (Epaxal and Inflexal V).
- Oral delivery has been examined however this is more troublesome due to the potential for liposome breakdown following exposure to bile salts.

# Storage of Liposomes: lyophilization

- Liposomes dispersed in aqueous solution generally face physical and chemical instabilities after long term storage.
- Hydrolysis and oxidation of phospholipids and liposome aggregation are the common cause of liposome instabilities.
- According to the literature, many methods have been investigated for the stabilization of liposomes, such as lyophilization, freezing and spraying drying.
- In commercial liposome-based drugs, AmBisome, Amphotec, Myocet, Visudyne and LEP-ETU are all lyophilized products.

# Storage of Liposomes: lyophilization

- In general, freeze-drying increases the shelf-life of liposomal formulations and preserves it in dried form as a lyophilized cake to be reconstituted with water for injection prior to administration.
- Furthermore, cryoprotectants need to be added to maintain particle size distribution of liposomes after freeze-drying- rehydration cycle.
- Various types and concentrations of sugars have been investigated for their ability to protect liposomes against fusion and leakage during lyophilization processes.

# Storage of Liposomes: lyophilization

- In commercial liposome lyophilized products, lactose was used as a cryoprotectant in the formulation of Amphotec, Myocet and Visudyne and sucrose was added in the formulation of Ambisome and LEP-ETU to increase liposome stability during lyophilization.
- Interestingly, these commercial lyophilized products showed similar shelf-life in comparison with other liposome products (eg: suspension and emulsions) and hence lyophilization may not have the expected effect on liposome stability.

# Storage of Liposomes: lyophilization

- Clemons et al. compared the potency and therapeutic efficacy among the different lipid-based formulations of amphotericin B (Amphotec, AmBisome and Abelcet) for the treatment of systemic and meningeal cryptococcal disease.
- Their work indicated that the therapeutic efficacy of Amphotec and AmBisome was superior to that of Abelcet by up to 10-fold in survival and in clearing infection from all organs.
- In these three commercially available lipid-based formulations of amphotericin B, Amphotec and AmBisome are both lyophilized products and Abelcet is formulated as a suspension form. Therefore, lyophilization may not extend the shelf-life of products but may increase therapeutic efficacy *in vivo*.

# Storage of Liposomes: lyophilization

- We also investigated the stability of the siRNA-loaded liposomes in suspension and lyophilized powder form up to 1 month post manufacture.
- Following formulation, the siRNA-loaded liposomes were stored at either 4°C or room temperature. The particle size and zeta potential of siRNA-loaded liposomes remained unchanged for both storage conditions.

| | | Storage condition | | | |
|---|---|---|---|---|---|
| | | 4°C | | Room temperature | |
| | Freshly prepared | 2 weeks | 4 weeks | 2 weeks | 4 weeks |
| (A) HFDM method | | | | | |
| Particle size (nm) | 189.5±3.76 | 193.7±12.5 | 199.8±10.7 | 187.6±15.8 | 192.0±3.63 |
| Zeta potential (mV) | 49.8±4.04 | 48.1±3.84 | 49.1±3.20 | 52.7±2.57 | 52.7±0.95 |
| Entrapment % | 94.7±0.61 | 88.1±2.04 | 84.4±4.00 | 88.8±1.34 | 84.2±4.56 |
| (B) Post-insertion (PI) method | | | | | |
| Particle size (nm) | 163.7±4.99 | 174.5±2.84 | 172.6±8.47 | 172.1±1.97 | 171.0±1.28 |
| Zeta potential (mV) | 43.6±5.18 | 43.9±9.21 | 47.5±7.62 | 53.2±3.87 | 51.3±1.36 |
| Entrapment % | 89.9±2.66 | 87.4±0.76 | 82.7±2.96 | 86.4±2.60 | 83.1±1.00 |

# Storage of Liposomes: lyophilization

- However, siRNA entrapment efficiencies were observed to have decreased slightly after 1 month storage for both of suspension (90→83%) and lyophilized powder (94→84%) forms.
- Surprisingly, the gene-silencing efficiency of siRNA-loaded liposomes in aqueous solution showed 80% reduction following 1-month of storage at either 4°C or room temperature.

| | | Storage | |
|---|---|---|---|
| | | 4°C | |
| | Freshly prepared | 2 weeks | 4 weeks |
| (A) HFDM method | | | |
| Particle size (nm) | 189.5±3.76 | 193.7±12.5 | 199.8± 10.7 |
| Zeta potential (mV) | 49.8±4.04 | 48.1±3.84 | 49.1±3.20 |
| Entrapment % | 94.7±0.61 | 88.1±2.04 | 84.4±4.00 |
| (B) Post-insertion (PI) method | | | |
| Particle size (nm) | 163.7±4.99 | 174.5±2.84 | 172.6±8.47 |
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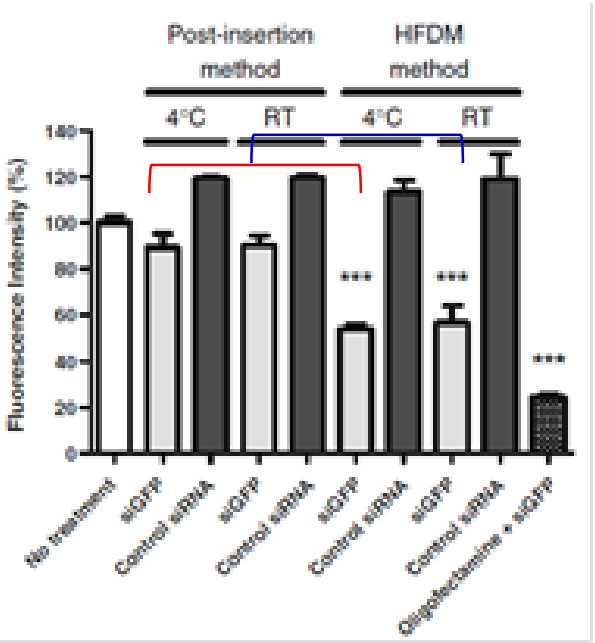
| Entrapment % | 89.9±2.66 | 87.4±0.76 | 82.7±2.96 |



# Storage of Liposomes: lyophilization

- Although therapeutic efficiency of liposome-based drugs may vary depending on the choice of lipids, the preparation technique, the physico-chemical characteristics of the bioactive materials, and the overall charge of the liposome, lyophilisation is useful for the long term storage of liposome-based drugs.

# Clinical studies of liposomal based anti-cancer drugs: Doxorubicin

- Liposome delivery systems offer the potential to enhance the therapeutic index of anticancer drugs, either by increasing the drug concentration in tumor cells or by decreasing the exposure in normal host tissues.
- Doxorubicin is an anthracycline widely used to treat solid and hematological tumors, but its major drawback is its related cardiotoxicity.
- In cardiotoxicity, positively charged doxorubicin's affinity for negatively charged cardiolipin, a lipid abundant in heart tissue, is thought to be involved in drug localization in the heart tissue.
- Therefore, doxorubicin-loaded liposomes were developed to combat aggressive tumors, like breast and ovary metastatic cancers and Kaposi’s sarcoma.

# Clinical studies of liposomal based anti-cancer drugs: Doxorubicin

- Myocet and Doxil were the first approved liposome-based drugs for cancer treatment. Both products contain doxorubicin but are different particularly in the presence of poly(ethylene glycol) (PEG) coating.
- In pharmacokinetic studies of doxorubicin-loaded liposomes, free doxorubicin had an elimination half-life time of 0.2 hours and an AUC of 4 μg h $ml^{-1}$ in patients as compared with 2.5 hour and 45 μg h $ml^{-1}$ for Myocet and with 55 hours and 900 μg h $ml^{-1}$ for Doxil, respectively.

# Clinical studies of liposomal based anti-cancer drugs: Doxorubicin

- The particle size of Myocet is about 190 nm and Doxil is about 100 nm.
- Both liposome products have longer circulating half-life in blood as compared with free drug but Doxil displays greatly longer circulation time in blood than Myocet.
- Generally, the blood circulation time of liposomes ($T_{1/2}$) increases with decreasing size, negative charge density and fluidity in the bilayer or PEG surface coating.

# Clinical studies of liposomal based anti-cancer drugs: Doxorubicin

- In phase III head to head comparison of free doxorubicin vs Myocet in patients with metastatic breast cancer, similar results were presented in first year survival rate (64 vs 69%) and progression-free survival (3.8 vs 4.3 months) but Myocet had low incidence of cardiac events (13 vs 29%), mucositis/stomatitis (8.6% vs 11.9%) and Nausea/vomiting (12.3% vs 20.3%).
- Therefore, Myocet tends to reduce drug-related toxicity (eg: cardiotoxicity) rather than to enhance antitumor efficacy.
- Similar to Myocet, Doxil had a better safety profile including the reduction of cardiotoxicity (3.9 vs 18.8%), neutropenia (4 vs 10%), vomiting (19 vs 31%) and alopecia (20 vs 66%) in phase III trial of metastatic breast cancer whereas its progression-free survival times (6.9 vs 7.8 months) and overall survival times (21 vs 22 months) demonstrated equivalent efficacy to conventional doxorubicin.

# Clinical studies of liposomal based anti-cancer drugs: Doxorubicin

- Lipo-dox is the second generation of PEGylated liposomal doxorubicin, composed of distearoyl phosphatidycholine (DSPC) and cholesterol with surface coating with PEG.
- DSPC, which has two completely saturated fatty acids, has high phase transition temperature (Tm), 55°C, and good compatibility with Cholesterol.
- Normally, lipid bilayer has two thermodynamic phases: gel phase or liquid-crystal phase. At temperature < Tm, the lipid membrane is in the gel phase which is relatively rigid and tight because the lipid molecules have lower energy of random motion and the hydrocarbon chains are fully extended and closely packed.
- In phase I clinical study, Lipo-dox has achieved the most prolonged circulation half-life (65 hours).

# Clinical studies of liposomal based anti-cancer drugs: Doxorubicin

- Moreover, stomatitis became the new dose-limiting toxicity of PEGylated liposomal doxorubicin.
- For Lipo-dox, stomatitis appeared at doses of 30mg/$m^2$ and reached dose limit at 50mg/$m^2$. In contrast, Doxil reached dose limit at 80mg/$m^2$ and hence Lipo-dox had higher incidence of severe stomatitis than Doxil.
- In comparison with Myocet (the non PEGylated form of liposomal doxorubicin), Doxil and Lipodox (both PEGylated forms of liposomal doxorubicin) both showed significant incidence of stomatitis and this is mainly due to the long circulation properties of PEGylated liposomes.

# Clinical studies of liposomal based anti-cancer drugs: Doxorubicin

- The new generation of doxorubicin-loaded liposomes is thermosensitive liposomes (TSL) which release their encapsulated drugs in regions where local tissue temperatures are elevated.
- Compared with non-TSLs that remain stable and do not release drug in the physiologic temperature range, TSLs undergo a gel-to-liquid crystalline phase change when heated that renders the liposomes more permeable, releasing their encapsulated drugs.
- ThermoDox®, a proprietary TSL encapsulation of doxorubicin, recently is in phase III clinical trials for the treatment of hepatocellular carcinoma.

# Clinical studies of liposomal based anti-cancer drugs: Doxorubicin

- In the design of TSL, it is necessary to choose a phospholipid that has a gel-to-liquid crystalline phase transition temperature (Tc) in the temperature range of clinically attainable local hyperthermia (41- 42$^{o}$C).
- The mechanism behind TSL is the temperature induced membrane instability at the Tc of the used lipids. Dipalmitoylphosphatidylcholine (DPPC) with a Tc=41.5$^{o}$C, is an ideal lipid according to temperature triggered technology.
- For liposomes composed of DPPC alone, the rate of release and the amount released are relatively small.

# Clinical studies of liposomal based anti-cancer drugs: Doxorubicin

- Dromi et al. compared the accumulation of doxorubicin in mice tumors among free doxorubicin, Doxil and ThermoDox.
- Results showed that over time, doxorubicin gradually increased in tumors when both Doxil and ThermoDox were used but not with free doxorubicin.
- At 24 hours after administration, doxorubicin concentrations in tumors were found to be significantly higher with Doxil than ThermoDox.

**Clinical studies of liposomal based anti-cancer drugs: paclitaxel**

- In a Phase II trial of patients with pancreatic adenocarcinoma who were not candidates for surgery, Median survival in patients who received gemcitabine alone was 7.2 months, whereas it was up to 9.4 months in those who received combination treatment of EndoTAG-1 plus gemcitabine.
- The 12-month survival rates in patients given the two higher doses of EndoTAG-1 (22 and 44 mg/$m^2$ plus gemcitabine) were 36% and 33%, respectively, compared with 17.5% in those given gemcitabine alone.
- Combination treatment with EndoTAG-1 plus gemcitabine was well tolerated and led to substantially prolonged survival rates compared to standard therapy in this phase II trial.

# Research Topic 1

Influence of liposomal compositions on the balance between adipogenesis and osteogenesis in bone regeneration

Chang SF, Yeh CC, Chen PJ, Chang HI.* Molecules. 2018 Jan 2;23(1). pii: E95.

# Bone

- Bone is a dynamic tissue that is constantly remodeled.
- The process carried out by:

  Osteoclasts are bone-resorbing cells.

  **Osteoblasts** are bone-forming cells that synthesize bone matrix, regulate mineralization and finally differentiate into osteocytes or bone lining cells.

# Osteogenesis and adipogenesis

- Both osteoblasts and adipocytes are derived from a common multipotent mesenchymal stem cell (MSCs).
- Presence of fat may increase adipocyte proliferation, differentiation and fat accumulation while inhibiting osteoblast differentiation and bone formation.

- Previous studies have demonstrated that the disruption of the balance between osteogenesis and adipogenesis of MSCs leads to bone diseases such as osteoarthritis and osteoporosis (J. Dragojevic et al., 2011).

# Osteoarthritis (OA)

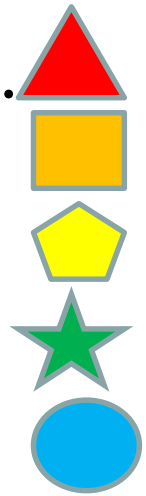
- It is a progressive degenerative joint disease, characterized by the breakdown of joint cartilage.
- Cause: muscle weakness, overweight/obesity, joint injury, aging
- Symptoms: **joint pain**, stiffness, swelling, bone deforming
- Treatment: 1) lifestyle modifications 2) medication 3) physical therapy 4) surgery

# Liposome

- An artificially-prepared spherical vesicle composed of a lamellar phase phospholipid bilayer.
- Application: nutrients and pharmaceutical drugs

# Aim

1.

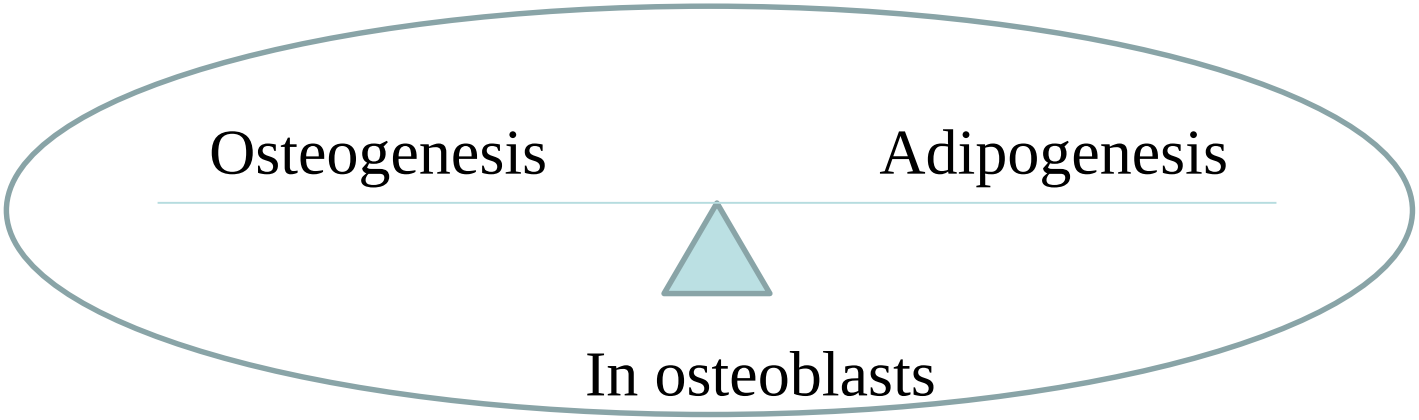


Phosphatidylcholine
DOPE
Cholesterol
DC-Cholesterol (+)
DOTAP (+)
Liposomes


Cell viability
Differentiation
Mineralization
Inflammation

?

2.

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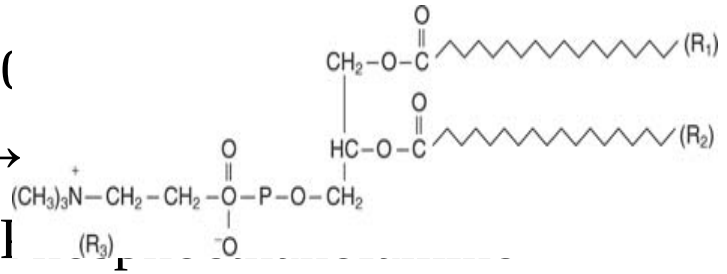
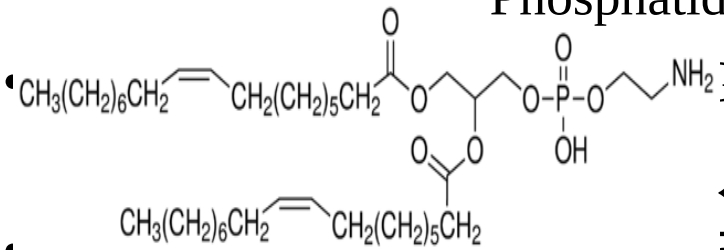
# Lipids

- Phosphatidylcholine - Phospholipon 9(

Phosphatidylcholine →

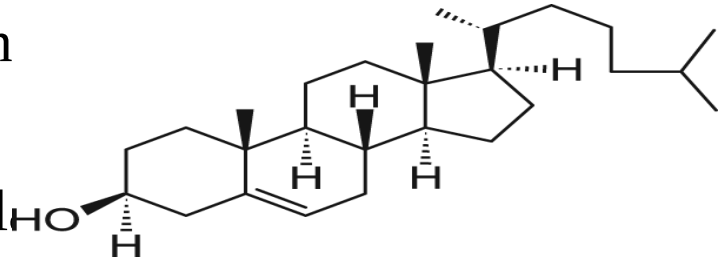


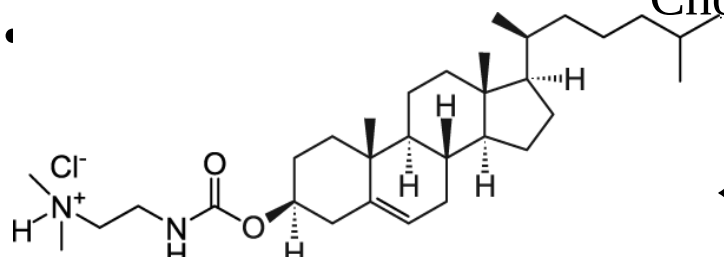


←DOPE

- Cholesterol – 3β-Hydroxy-5-cholesten

Cholesterol →

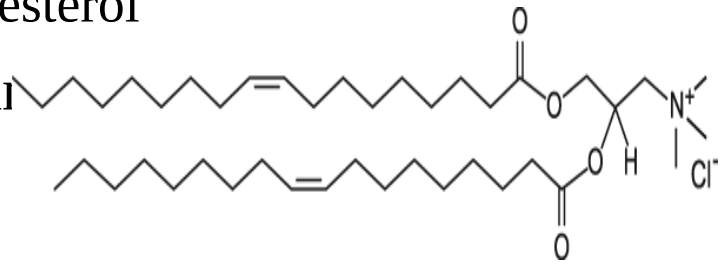




←DC-Cholesterol

- DOTAP - 1 ,2- Dioleoyl-3- trimethylar Salt)

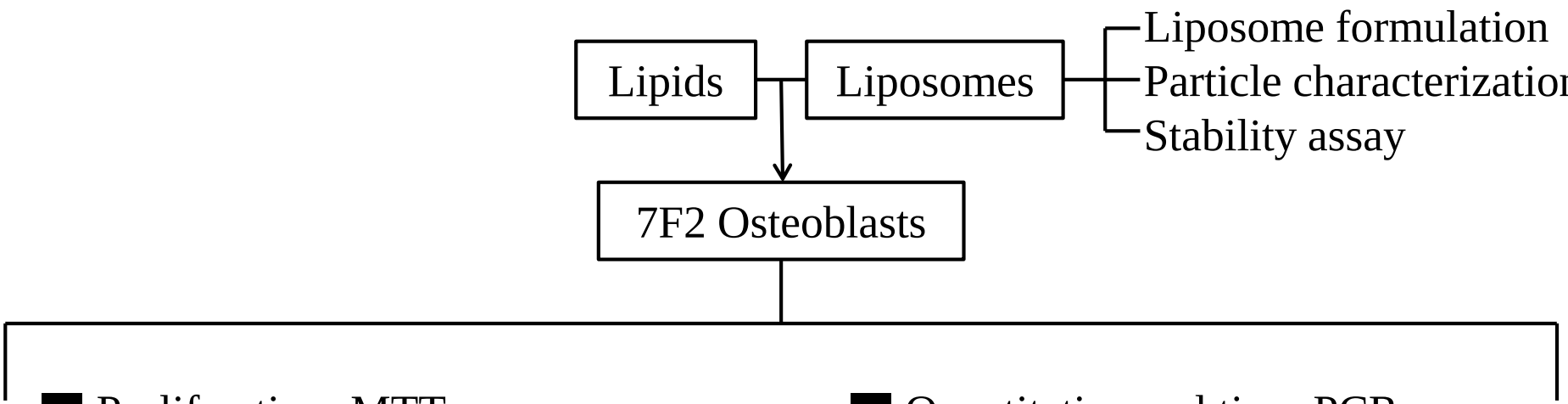
DOTAP →



# Comparison of lipids

| Lipids | PC | DOPE | Chol | DC-Chol | DOTAP |
|---|---|---|---|---|---|
| Chemical Formula | $C_{42}H_{80}NO_8P$ | $C_{41}H_{78}NO_8P$ | $C_{27}H_{46}O$ | $C_{32}H_{57}N_2O_2Cl$ | $C_{42}H_{80}NO_4Cl$ |
| Molecular weight | 758.07 | 744.03 | 386.654 | 537.260 | 698.542 |
| Physical Form | Granular (pale yellow to yellow) | powder (white) | powder (white) | powder (white) | powder (white) |
| Electric charge | neutral | neutral | neutral | cationic | cationic |

# Method




- Proliferation- MTT assay
- Differentiation- Alkaline phosphatase activity
- Mineralization- Alizarin Red S stain
- Adipogenesis- Oil Red O stain
- Quantitative real time-PCR
  Osteogenesis-related gene analysis
  Adipogenesis-related gene analysis
  Inflammation-related gene analysis
- Statistical analysis

Scheme 1. Experimental design

# Liposomal formulation

Group1: Phosphotidylcholine only
Group2: Phosphotidylcholine : DOTAP
Group3: Cholesterol : DOTAP
Group4: DC-Cholesterol : DOPE
at the ratio of 1:1

+

Dissolved in the mixture of ethanol and chloroform

↓

Rotary evaporation & Vacuum drying

↓

Rehydration with PBS

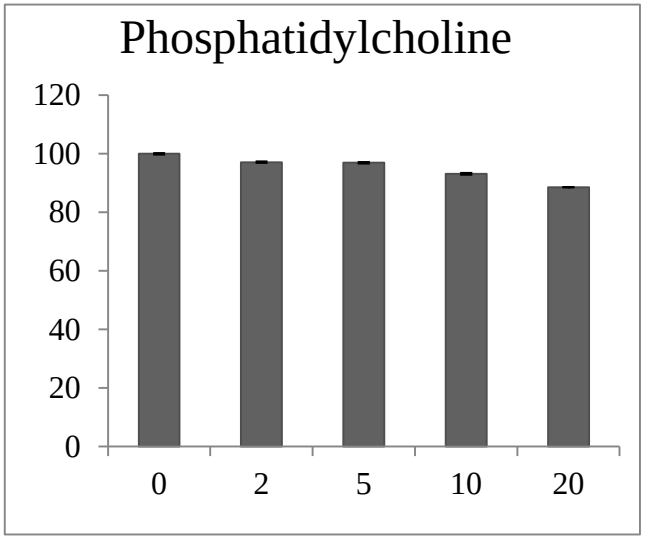
↓

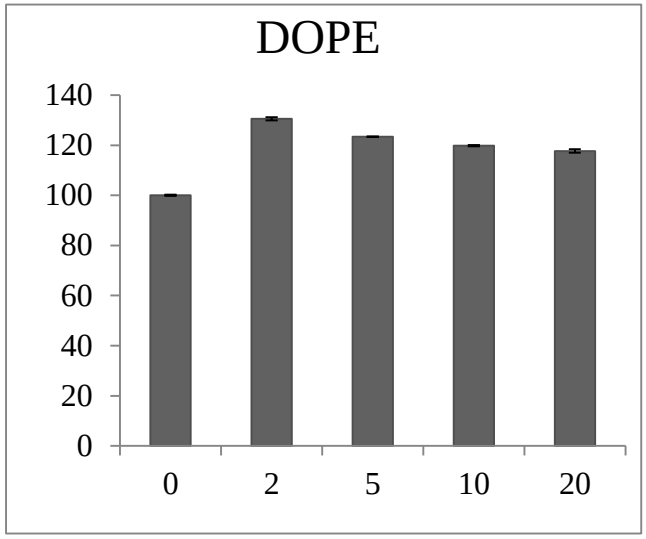
Through extruder

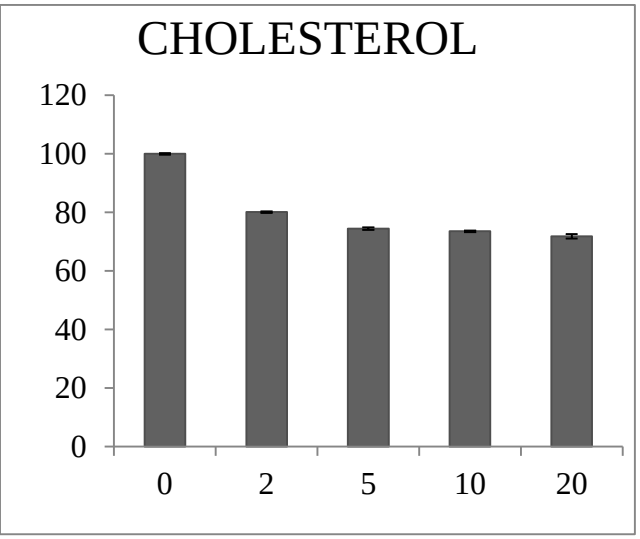
# RESULTS

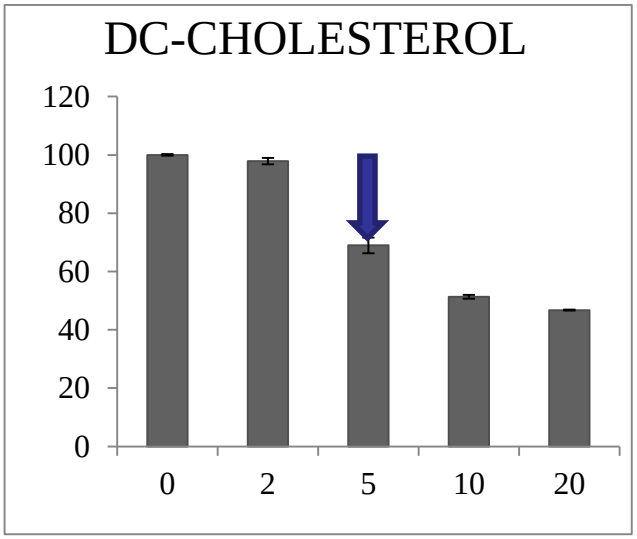
# 3.1A. Dose-dependent effect of lipids on cell viability of 7F2 osteoblasts.

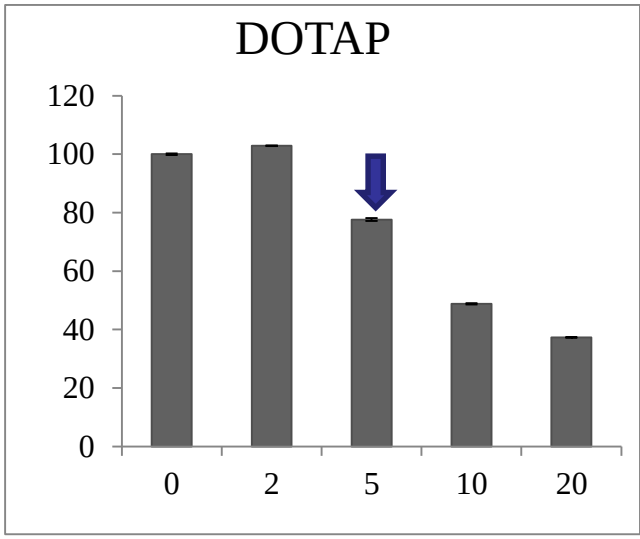
(Unit: μg/ml)



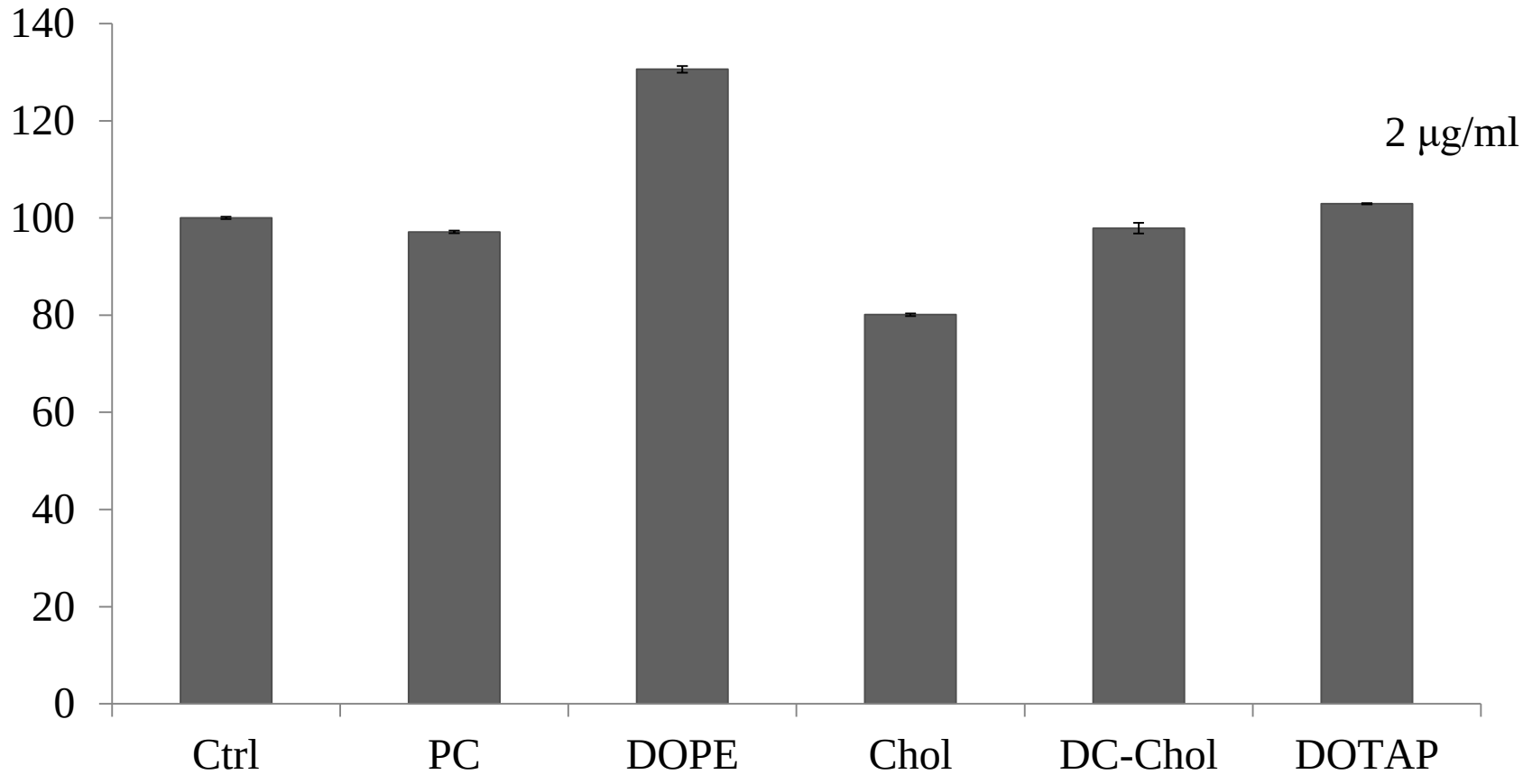



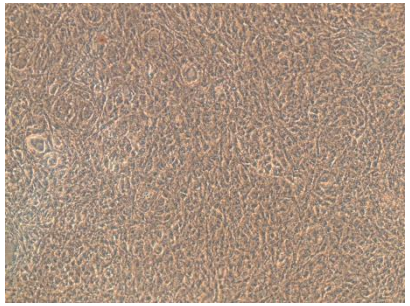



# 3.1B. The influence of lipids on cell viability of 7F2 osteoblasts.



# Histochemical staining of lipid accumulation by Oil Red O.



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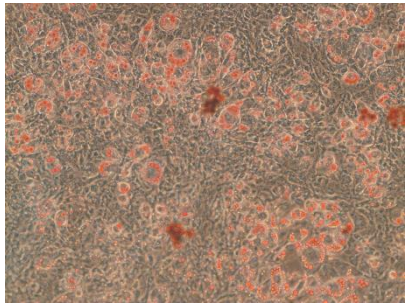
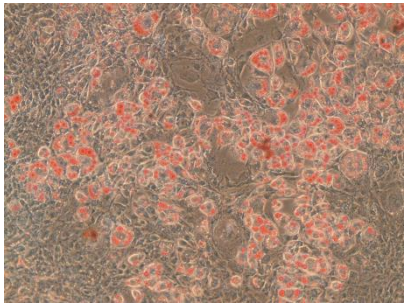
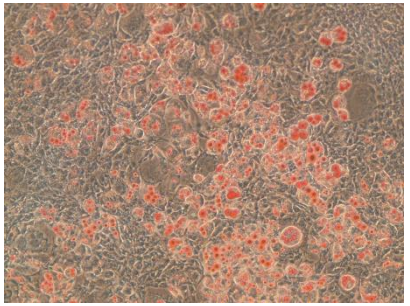


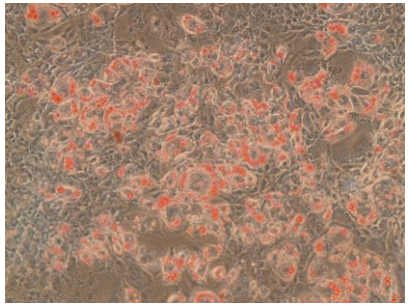
ADM



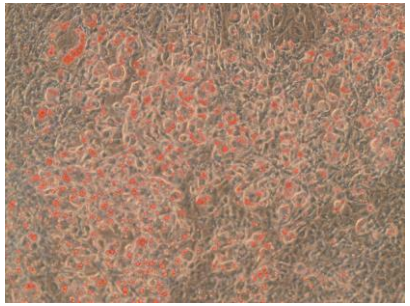
EtOH



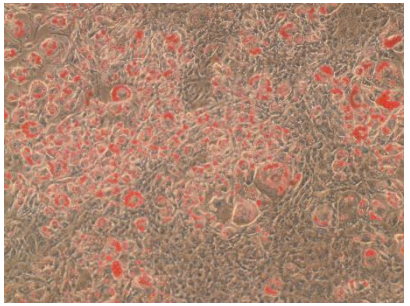
PC



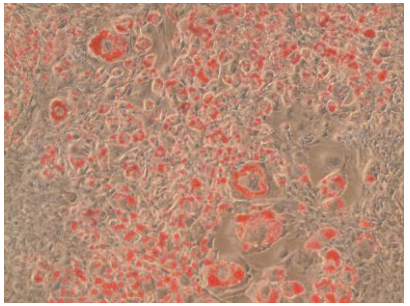
DOPE



Chol

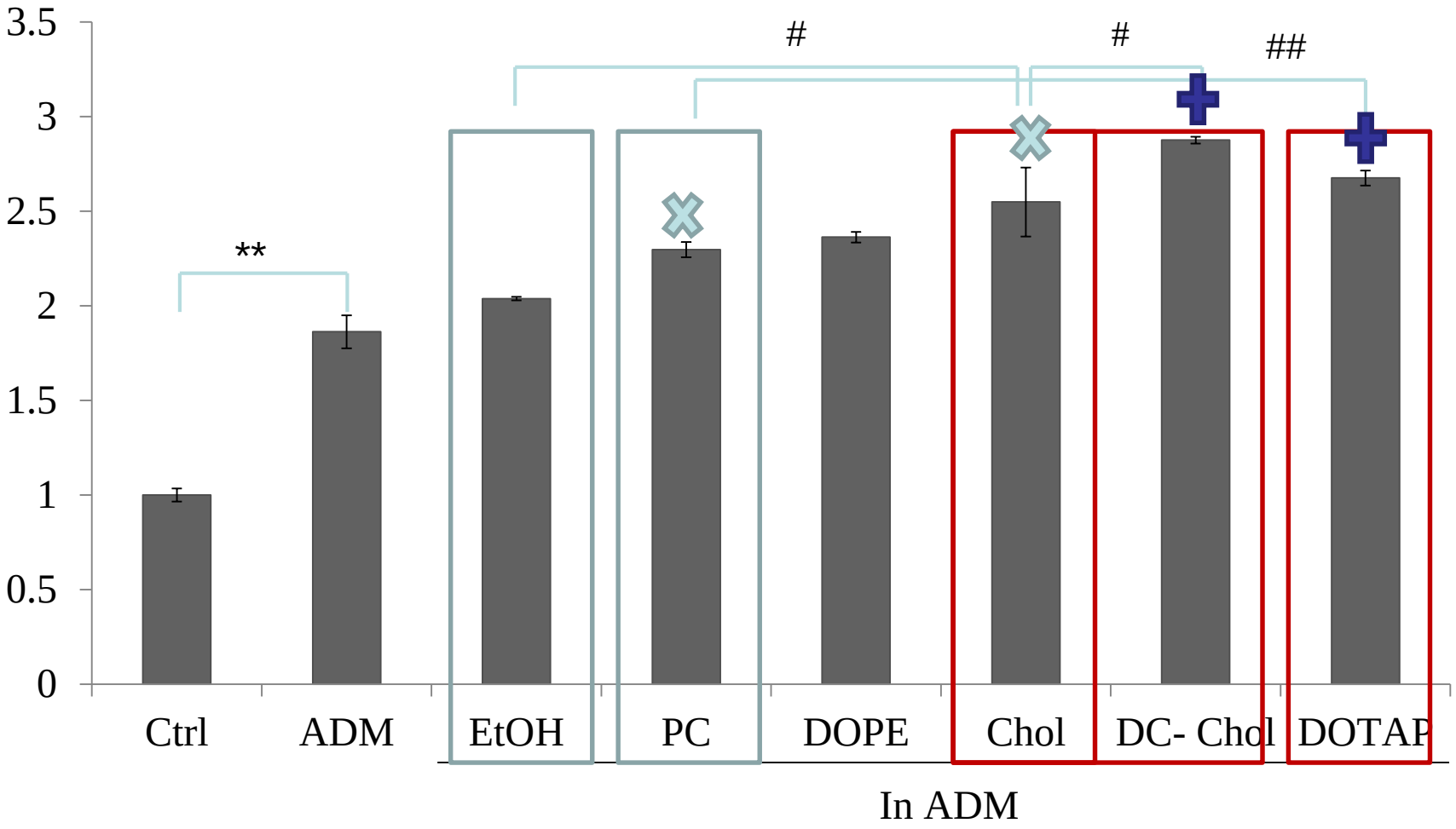


DOTAP

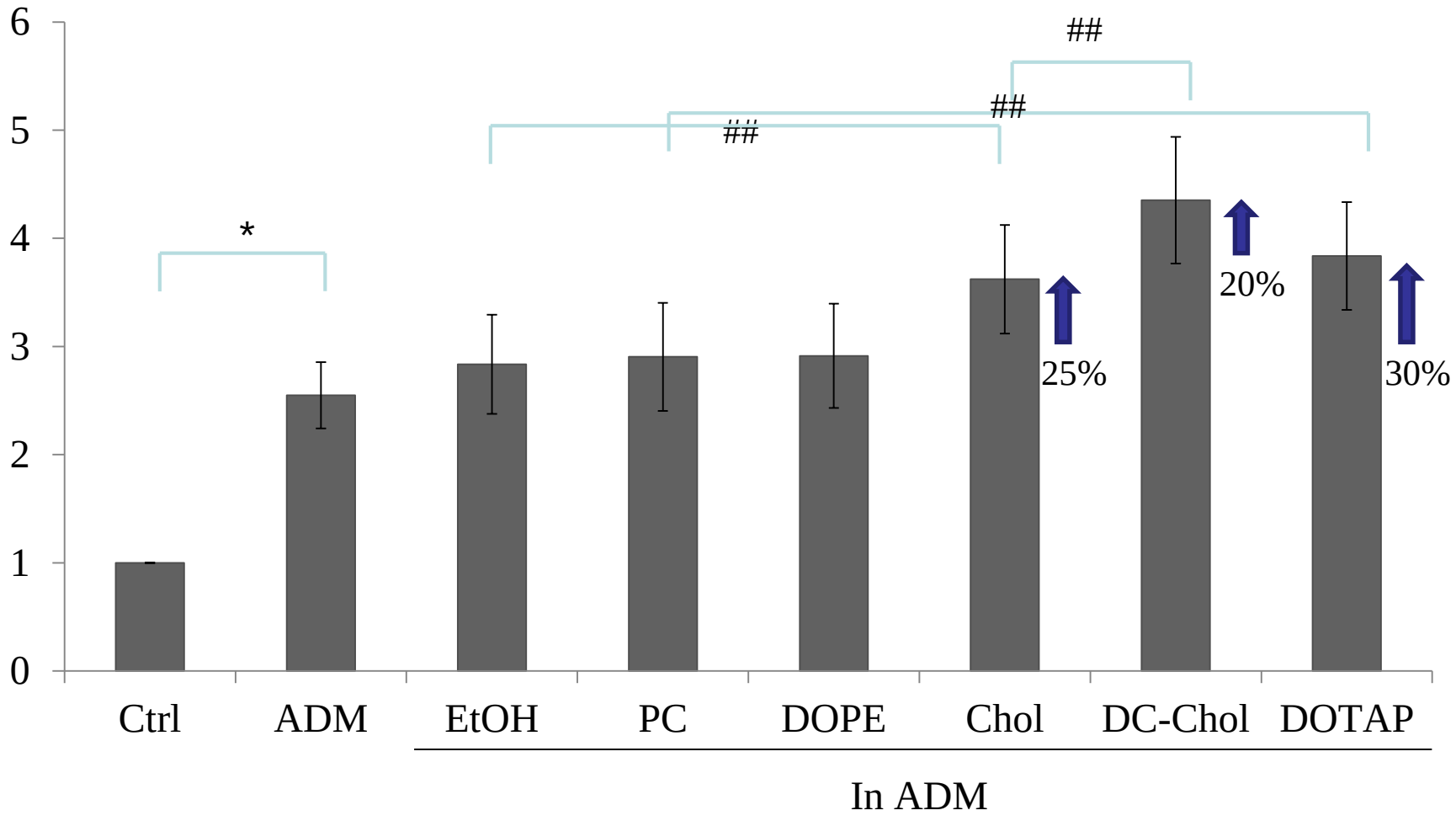


DC-Chol

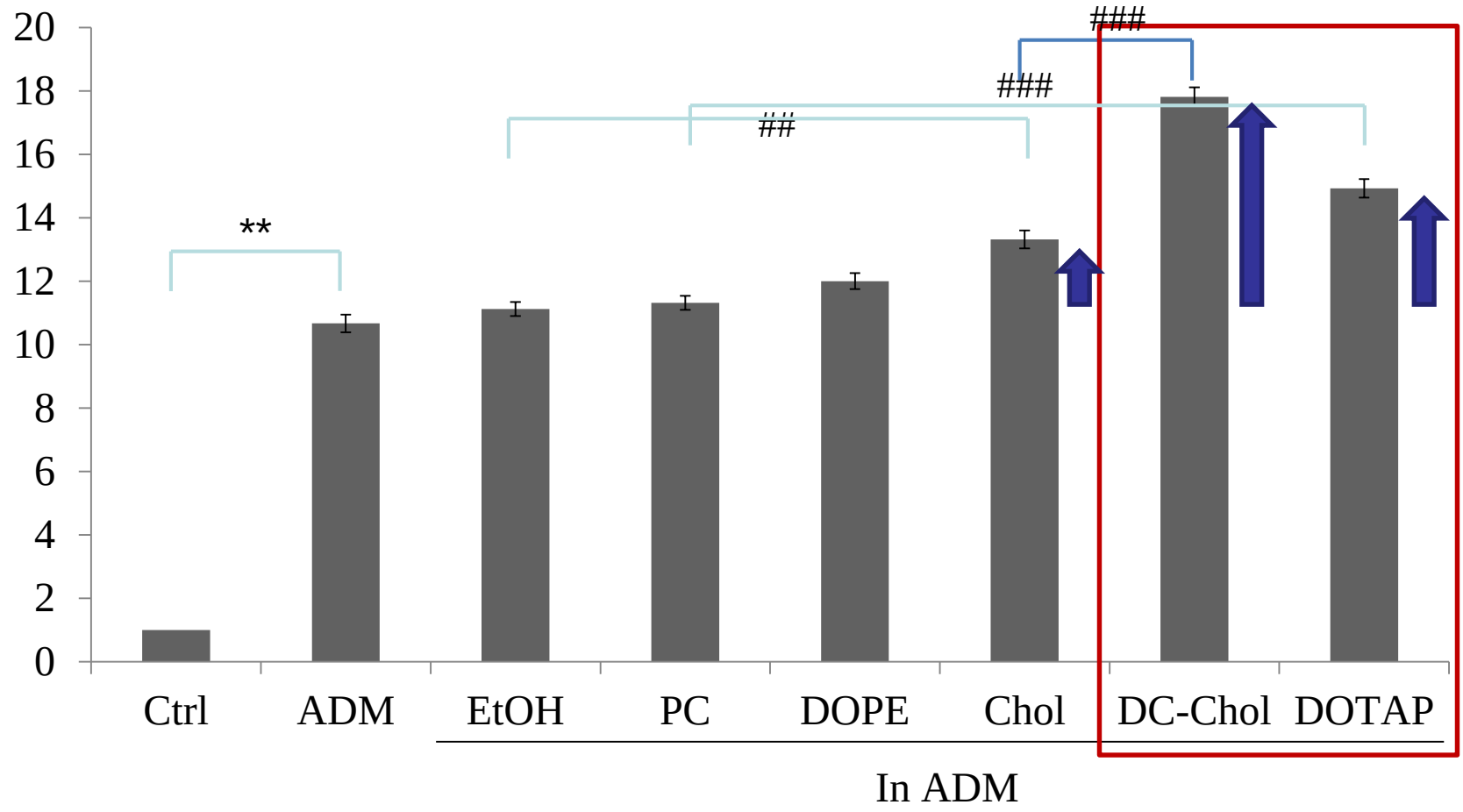
# The quantification of lipid accumulation



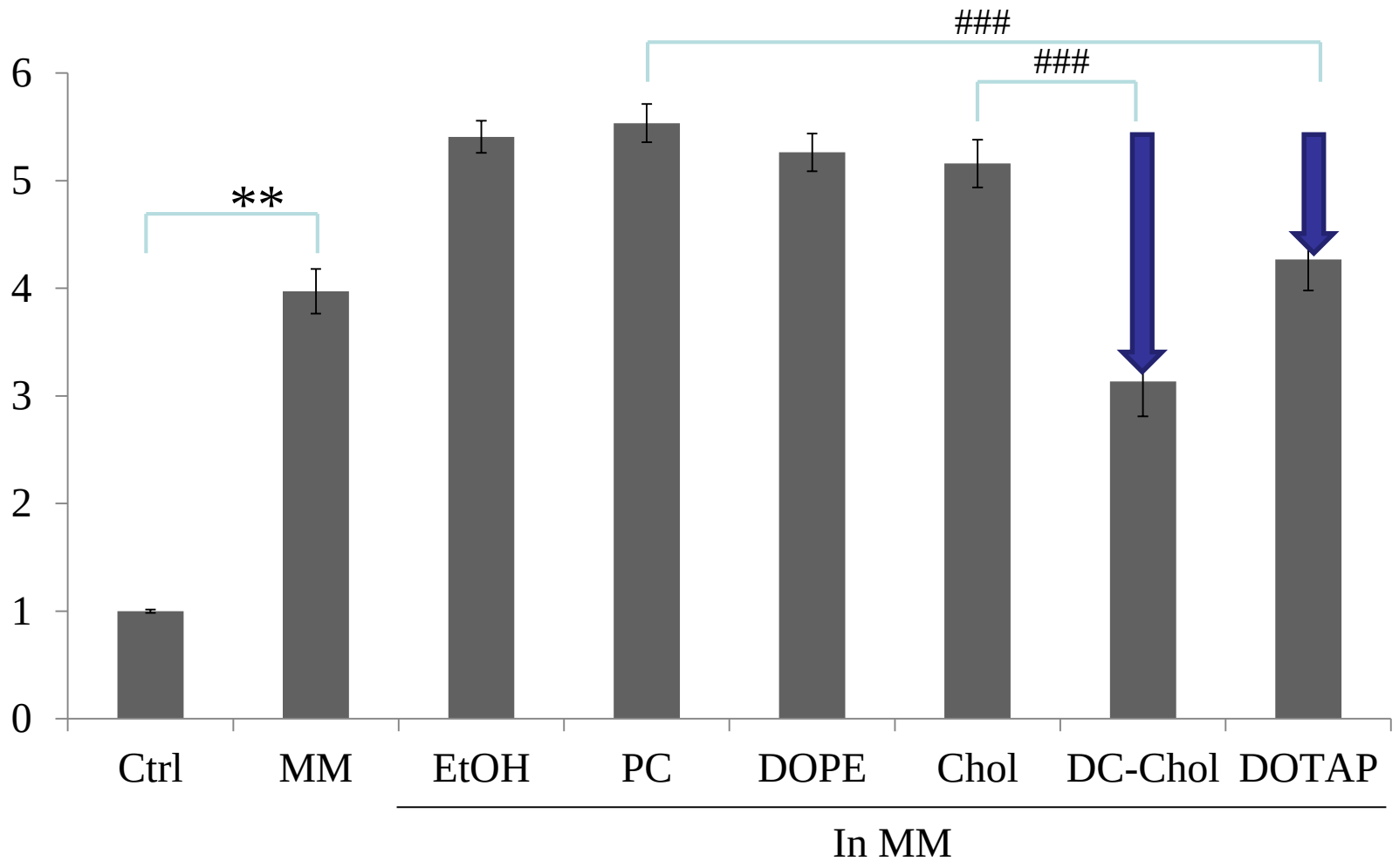
# The effect of lipids on the expression of fatty acid synthase (FAS).



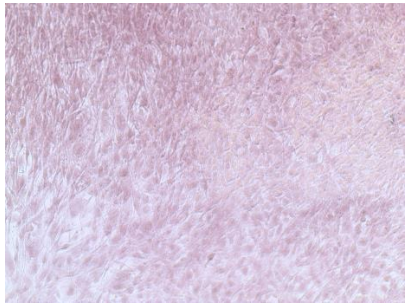
# The effect of lipids on the expression of fatty acid binding protein 4 (FABP4).



# The activity of alkaline phosphatase (ALP) in 7F2 osteoblasts.



# Histochemical staining of calcium deposition by Alizarin Red S.



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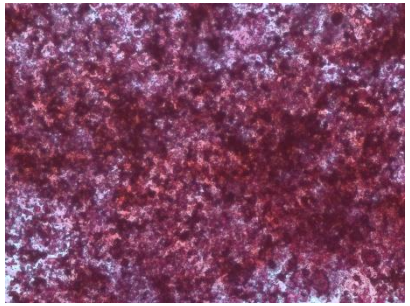
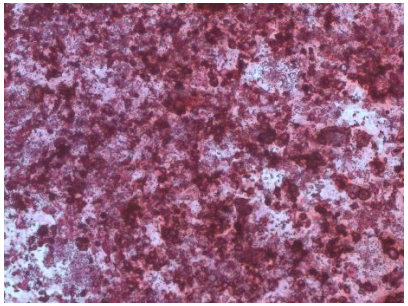
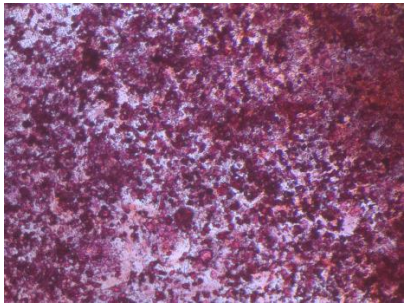


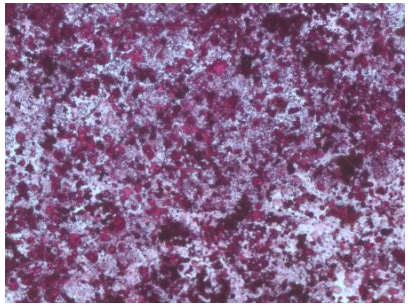
MM



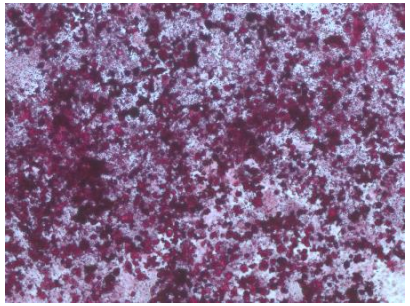
EtOH



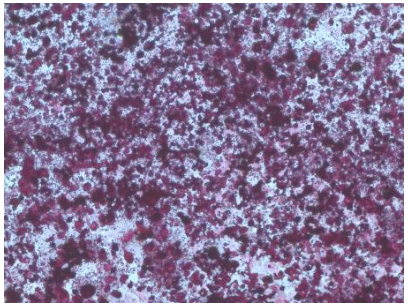
PC



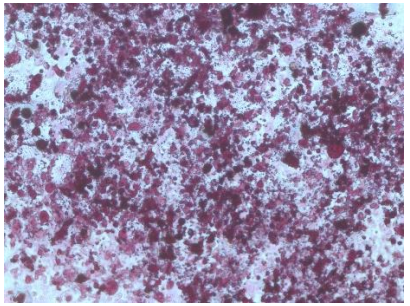
DOPE



Chol

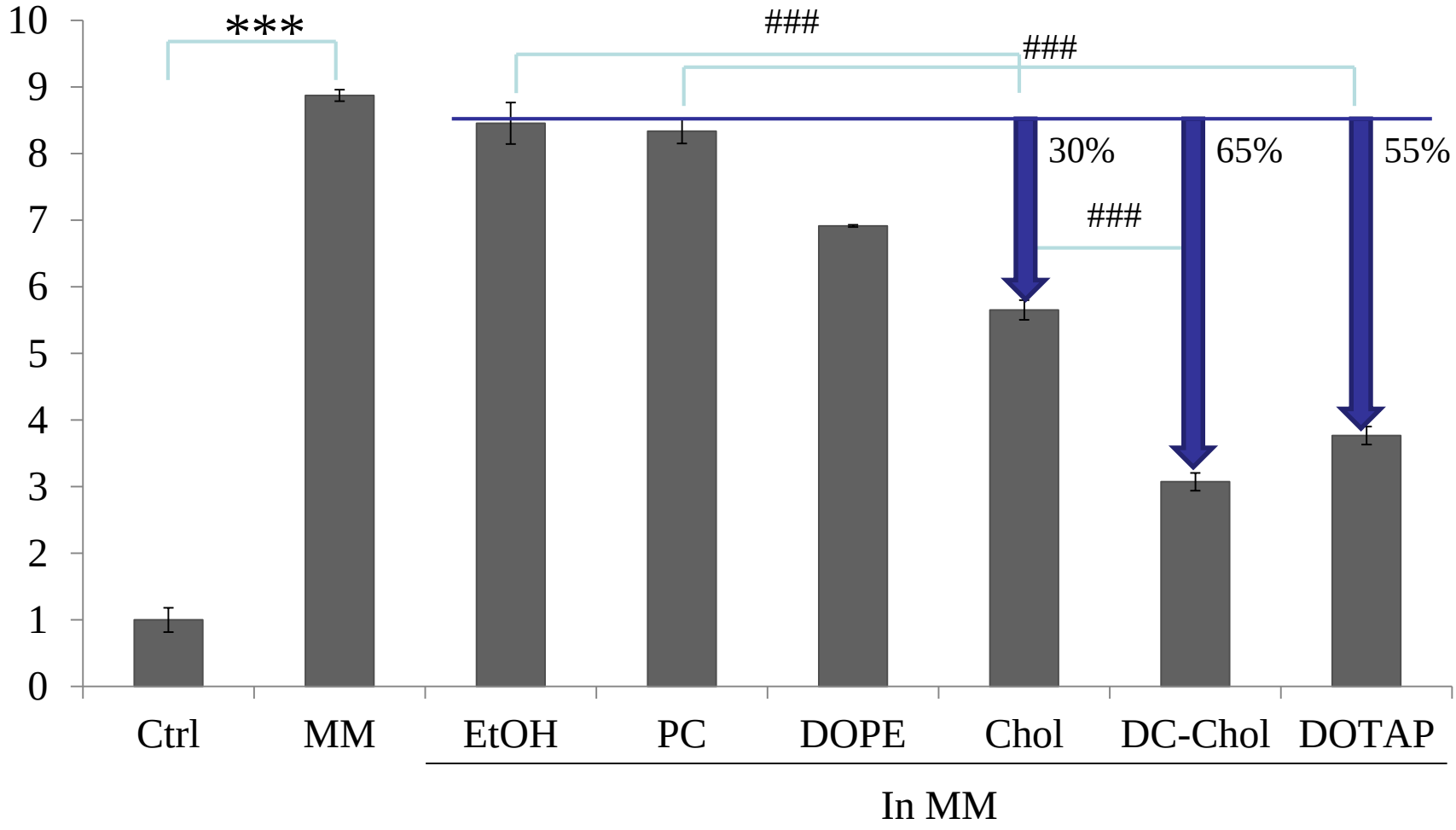


DOTAP

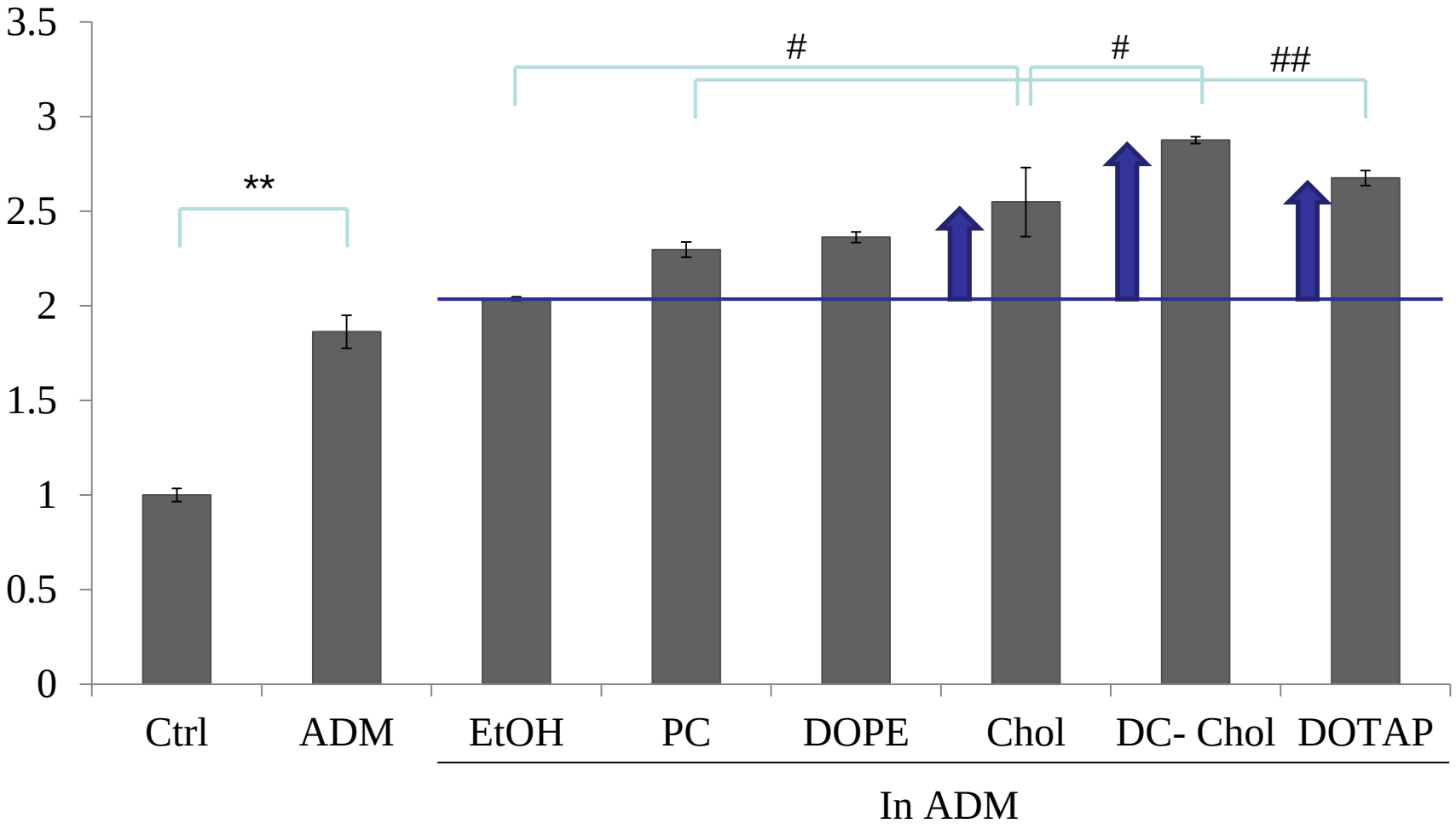


DC-Chol

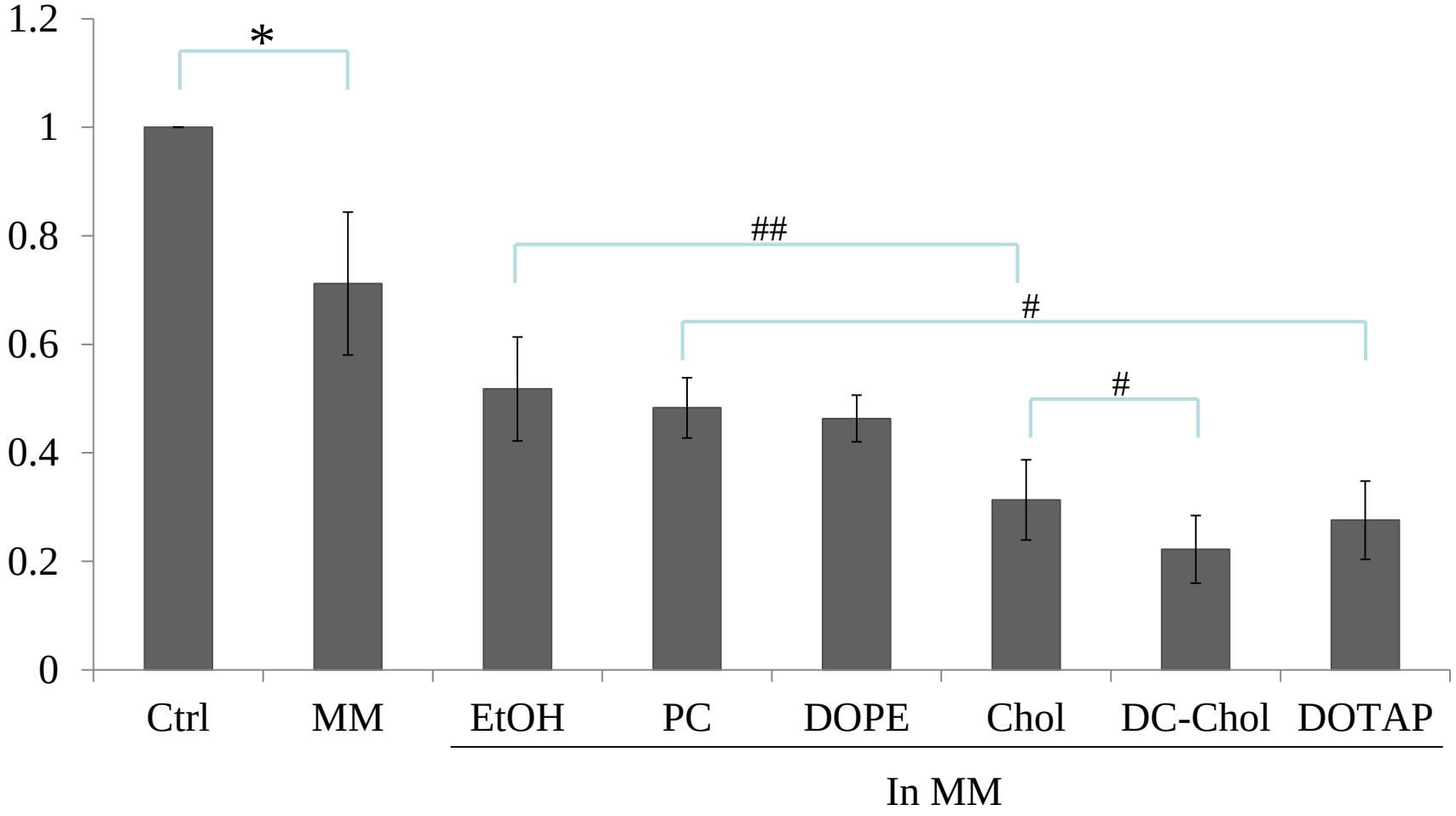
# The quantification of osteoblast mineralization.



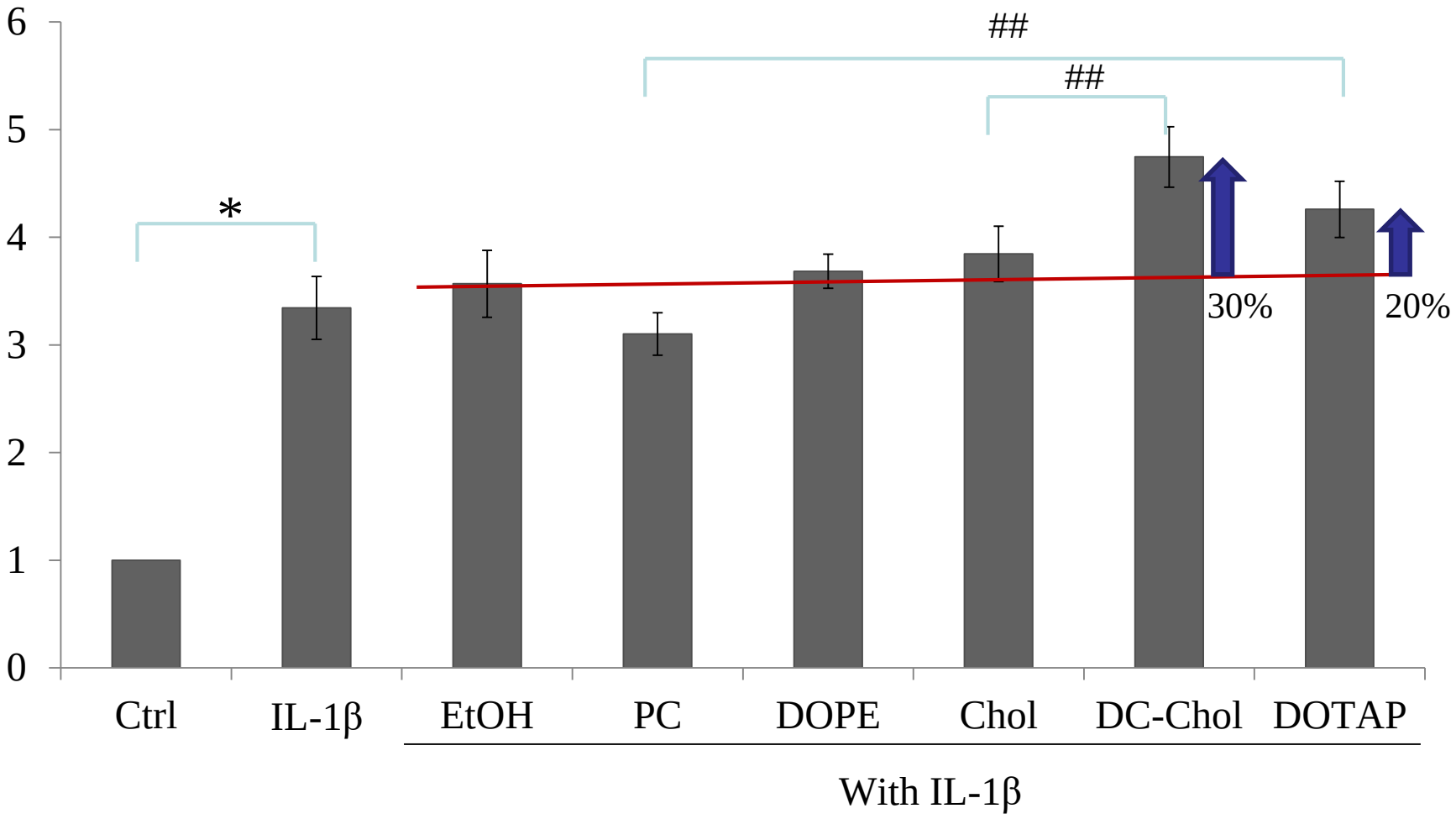
# The quantification of lipid accumulation.



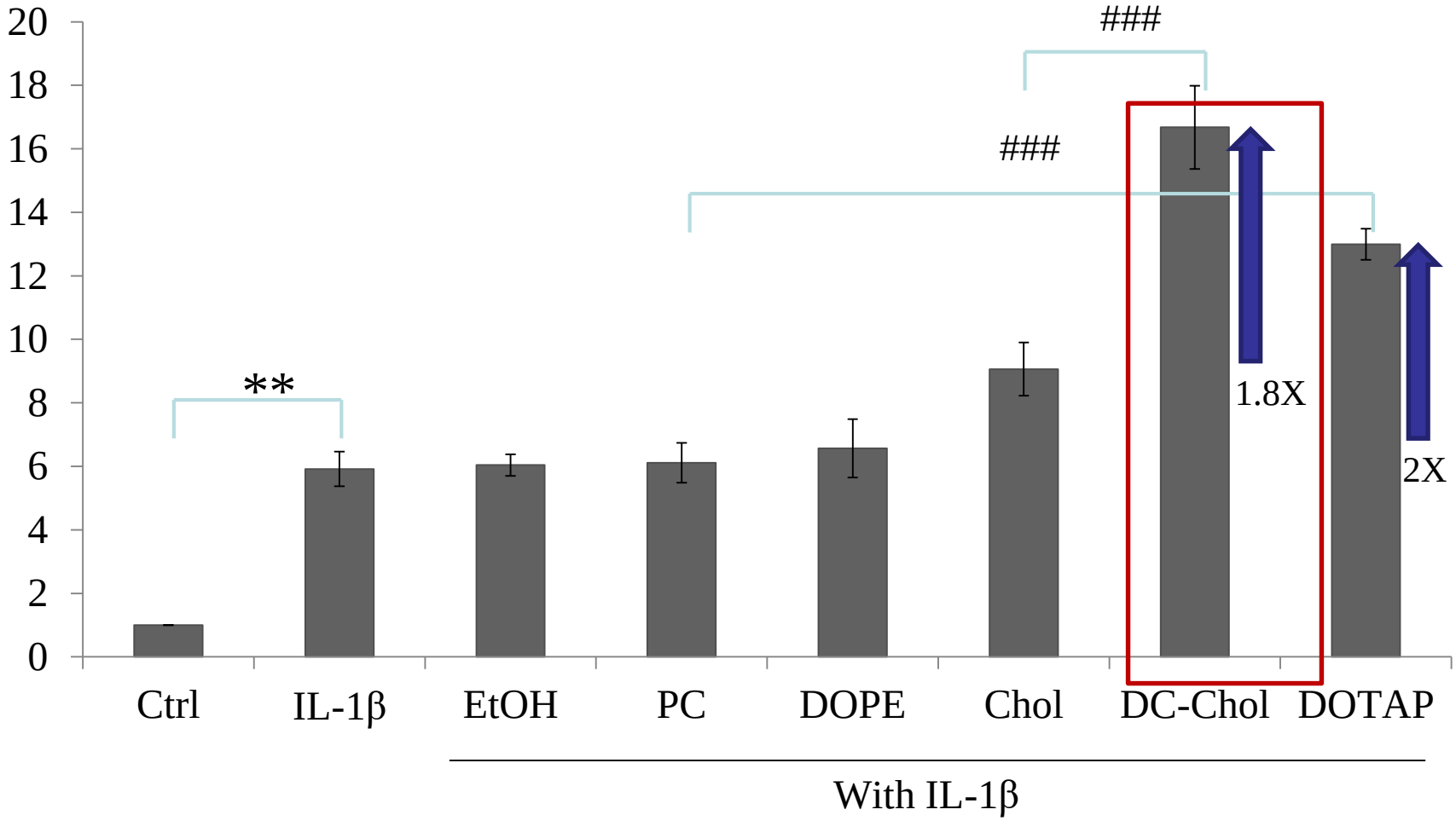
# The influence of lipid content on the OPG/RANKL ratio of 7F2 osteoblasts.



# The effect of lipid content on the expression of COX-2 of 7F2 osteoblasts in the of 10ng/ml IL-1β stimulation.



# The effect of lipid content on the expression of MMP-3 of 7F2 osteoblasts in the of 10ng/ml IL-1β stimulation.



# Summary 1

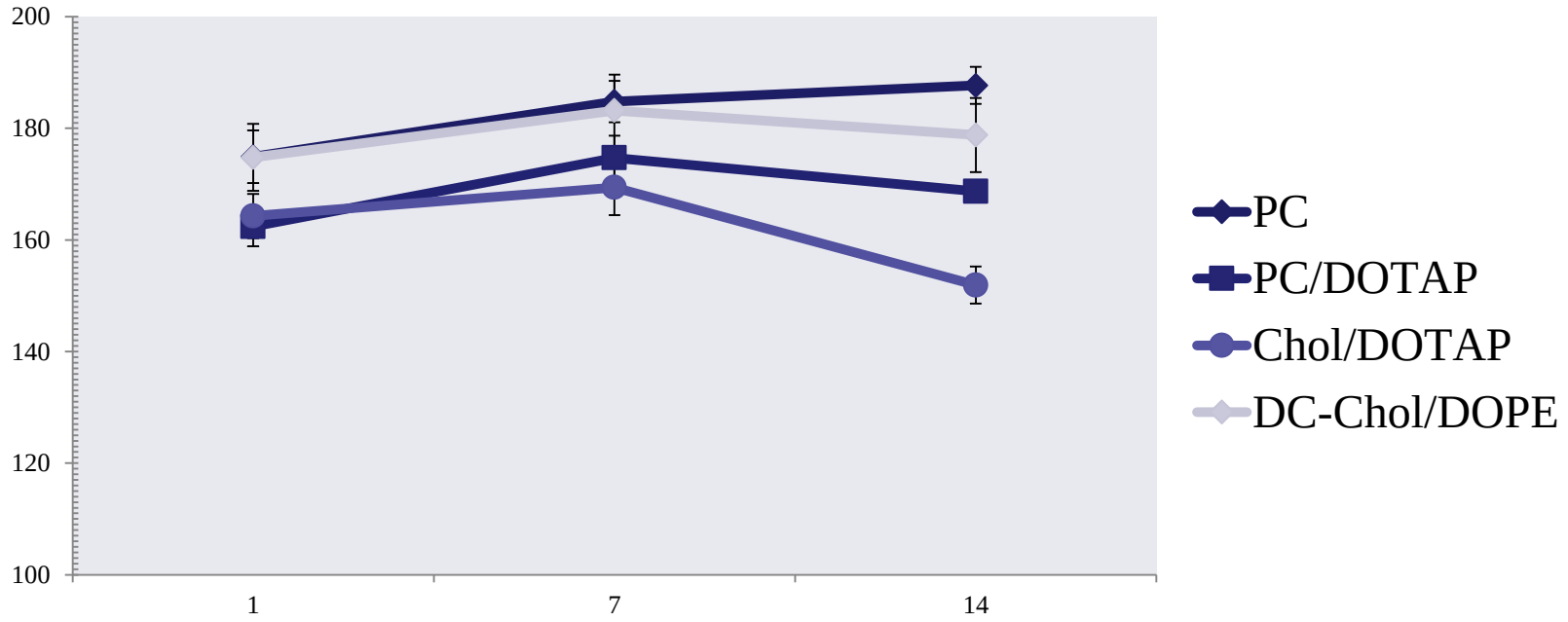
- Neutral lipids：PC、DOPE、Cholesterol
- Cationic lipids (+)：DC-Cholesterol、DOTAP

| | Cytotoxicity | Inflammation | Adipogenic differentiation | Inhibition of osteoclasto-genesis | Inhibition of osteoblast |
|---|---|---|---|---|---|
| Neutral lipids | L | L | L | H | L |
| Cationic lipids (+) | H | H | H | L | H |

Osteogenesis Adipogenesis

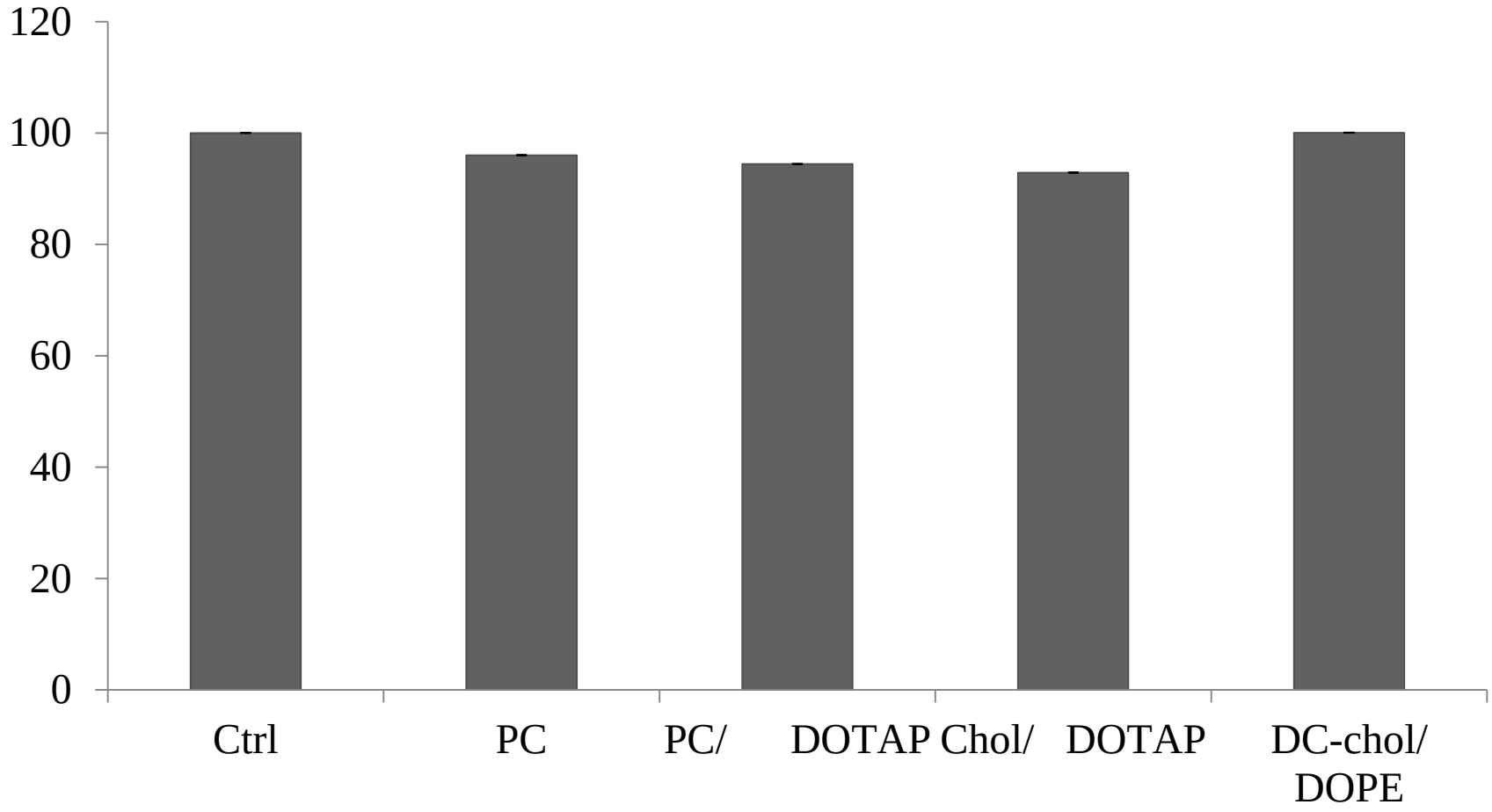
PC <<< DOPE <<< Cholesterol >>> DOTAP >>> DC-Cholesterol

# The particle size and stability of liposomes prepared with various lipid compositions at 4°C for 14 days.

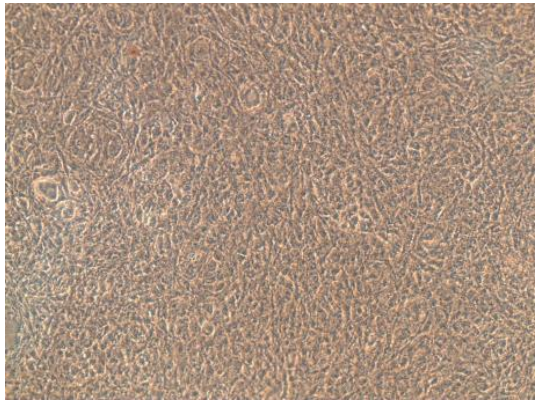



| Unit：nm | PC (4 | PC<br>DOTAP (2 | Cholesterol<br>DOTAP (1 | DC- Cholesterol<br>DOPE (3 |
|---|---|---|---|---|
| 1 day | 174.9 | 162.4 | 164.3 | 174.8 |
| 7 day | 184.8 | 174.7 | 169.4 | 183.2 |
| 14 day | 187.7 | 168.7 | 151.9 | 178.8 |

# The survival rate of 7F2 osteoblasts after 24 hour treatment with 2.5 μg/ml liposome samples.



# Histochemical staining of lipid accumulation.



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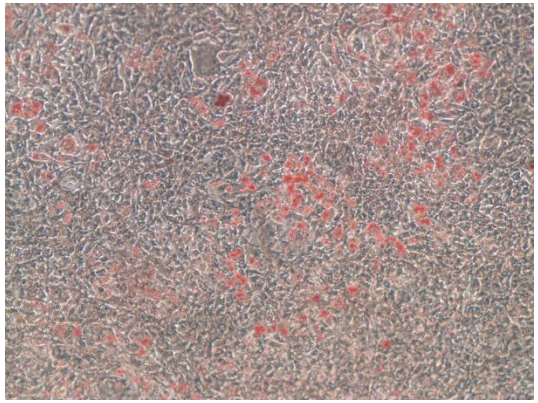
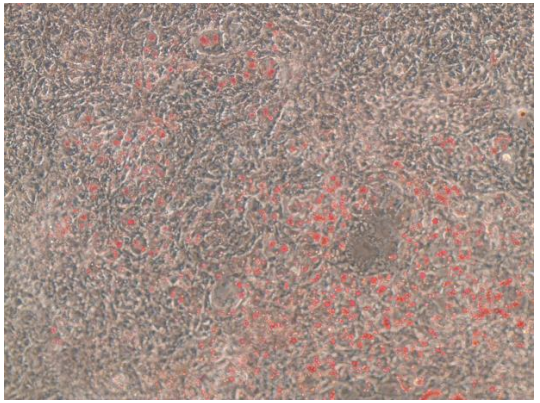
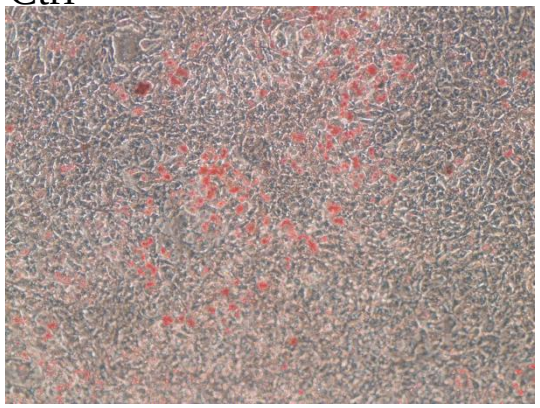


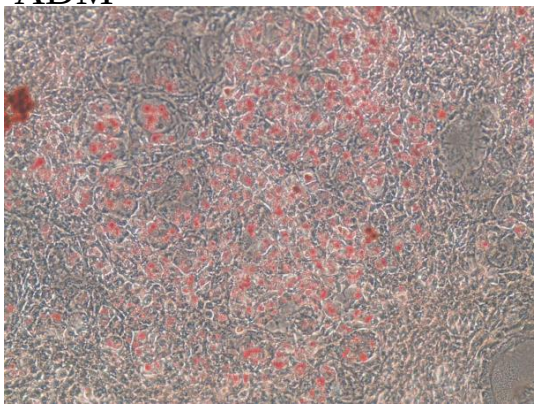
ADM



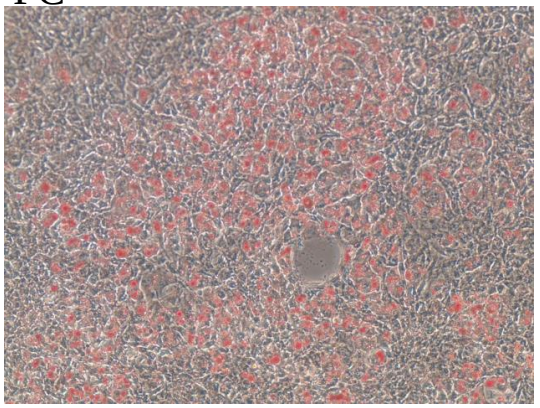
PC



PC/DOTAP

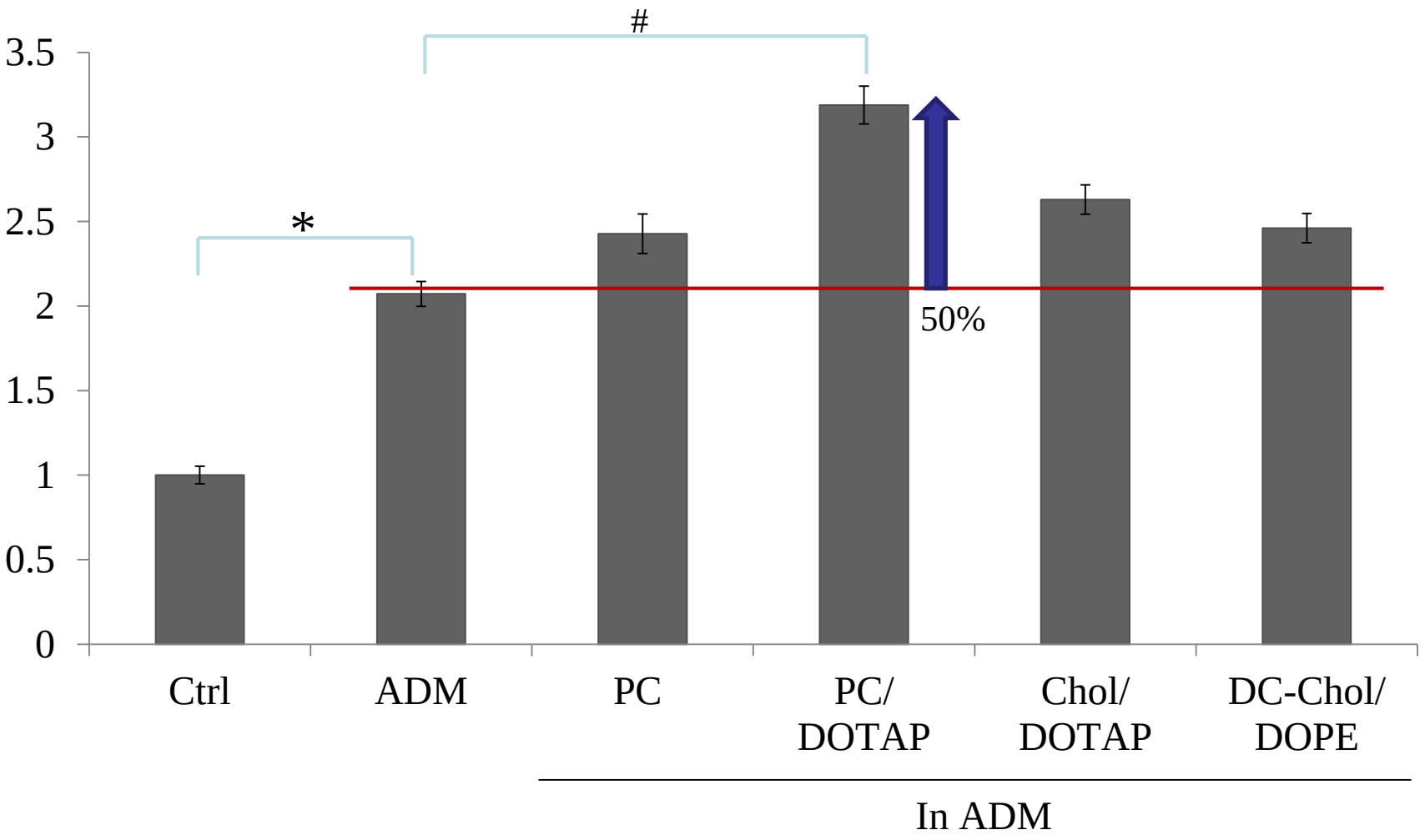


Chol/DOTAP

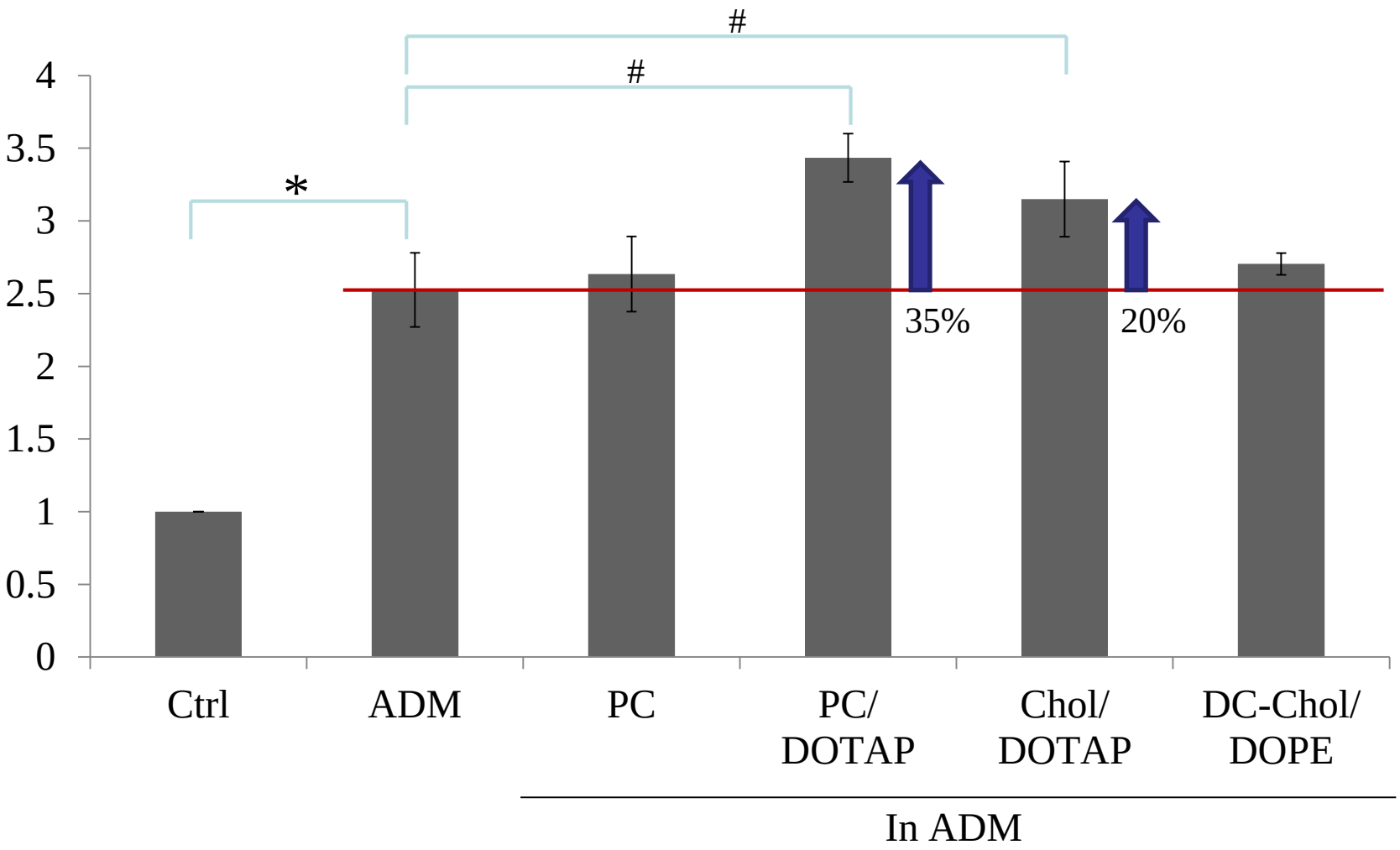


DC-Chol/DOPE

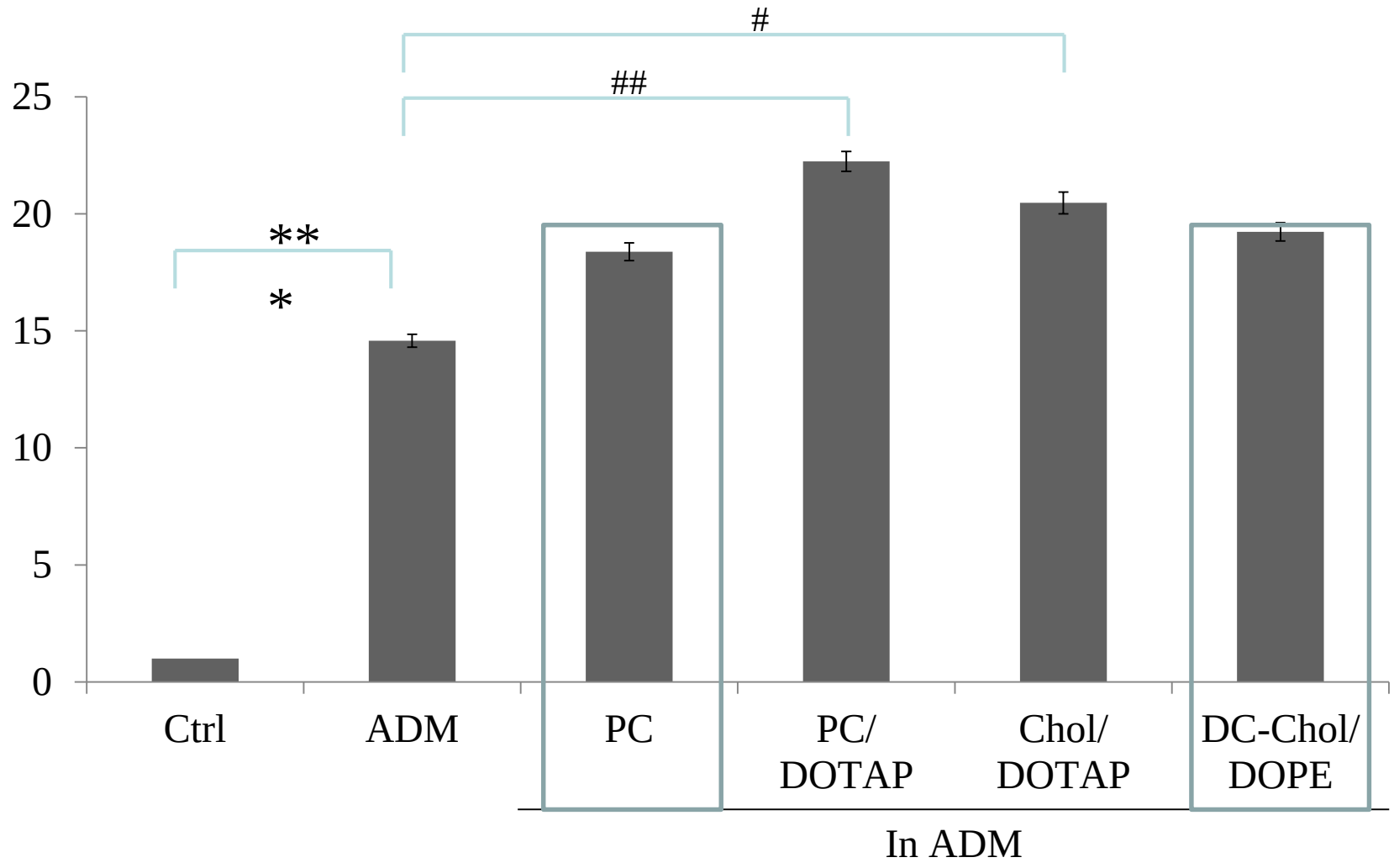
# The quantification of lipid accumulation.



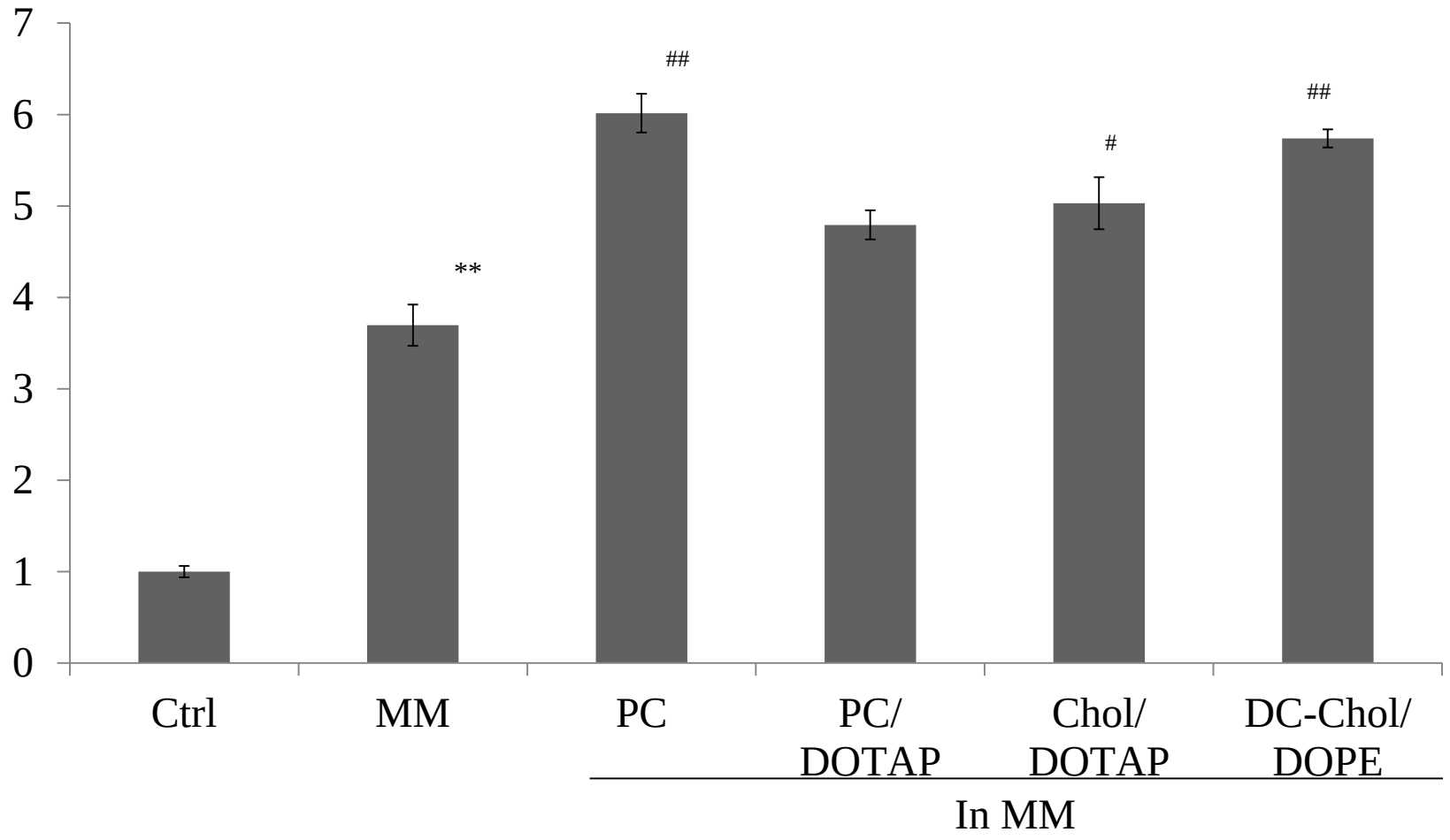
# The influence of liposomes on adipogenic gene expression of FAS.



# The influence of liposomes on adipogenic gene expression of FABP4.

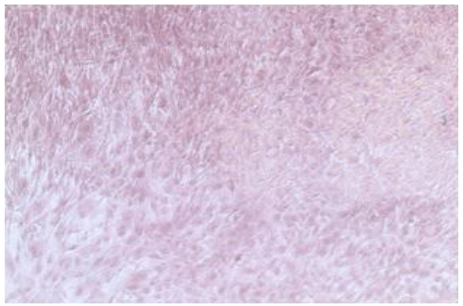


# The activity of alkaline phosphatase (ALP) in 7F2 osteoblasts.



# Histochemical staining of calcium deposition by Alizarin Red S.

B.



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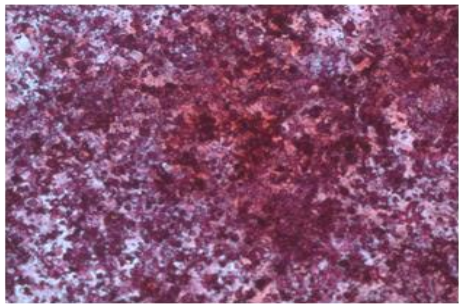
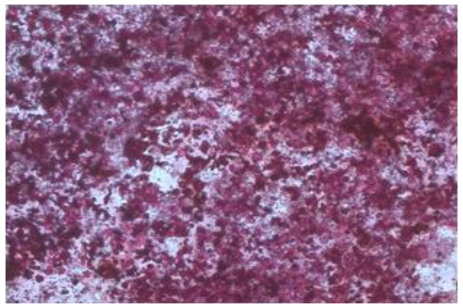
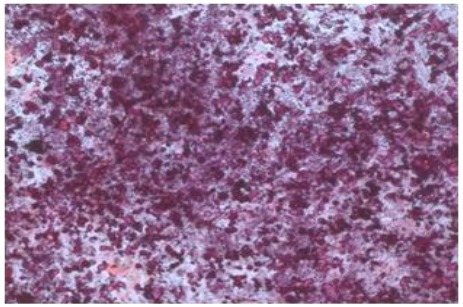


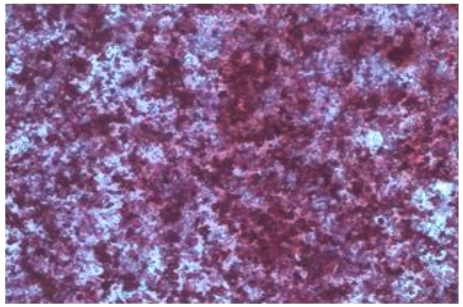
MM



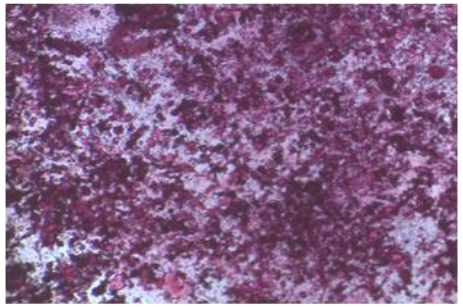
Liposome PC



PC/DOTAP

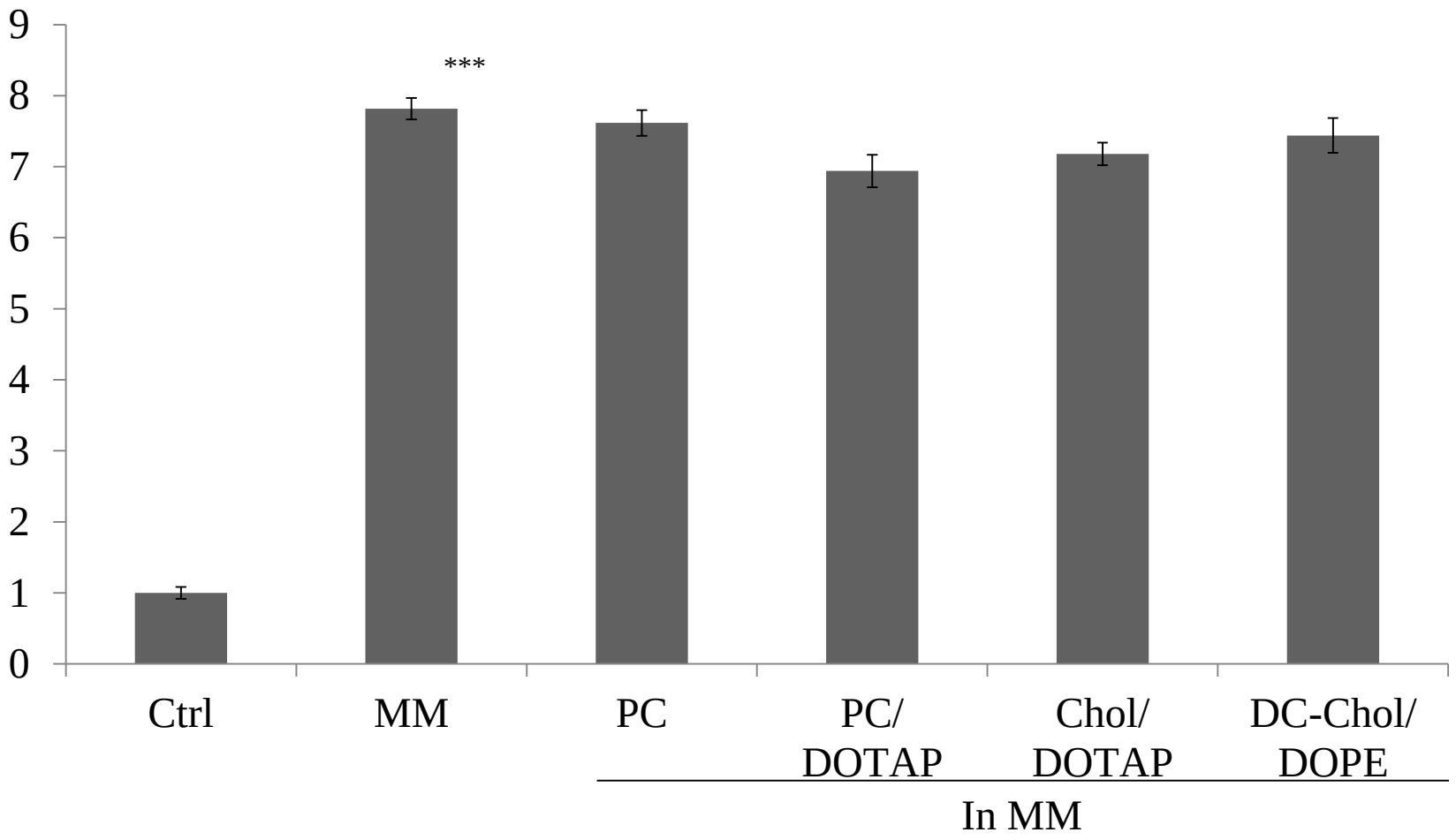


Chol/DOTAP

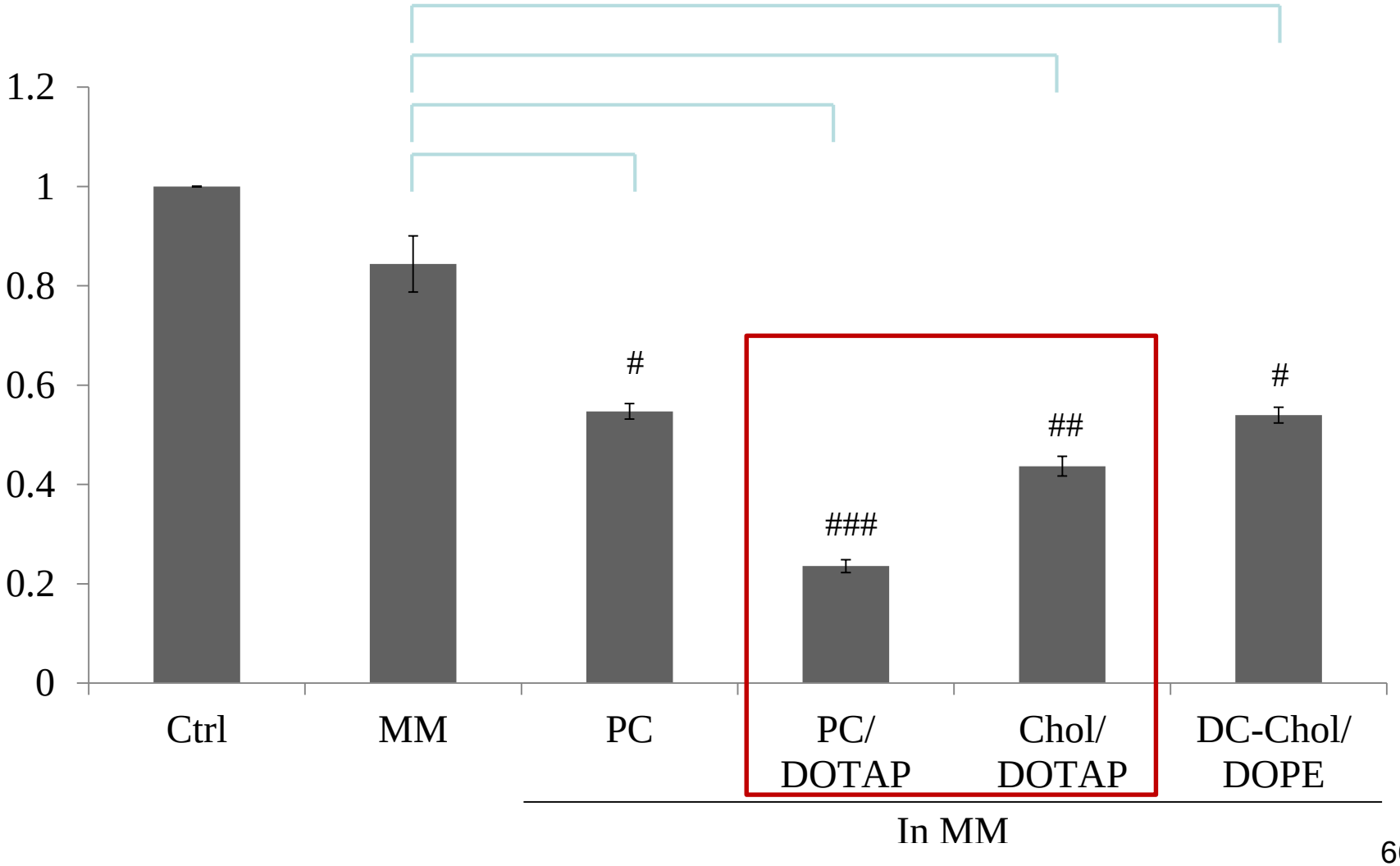


DC-Chol/DOPE

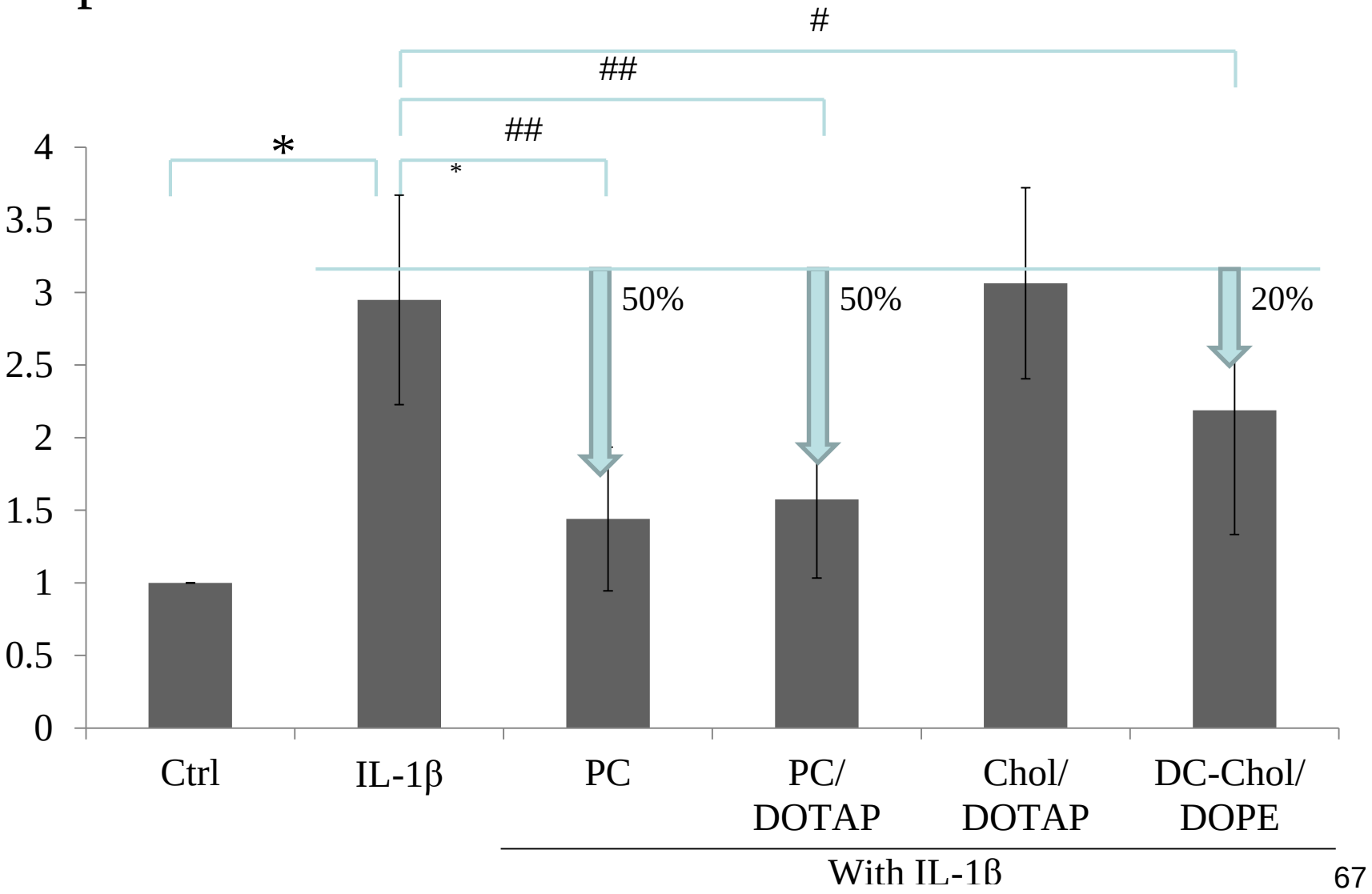
# The quantification of osteoblast mineralization.



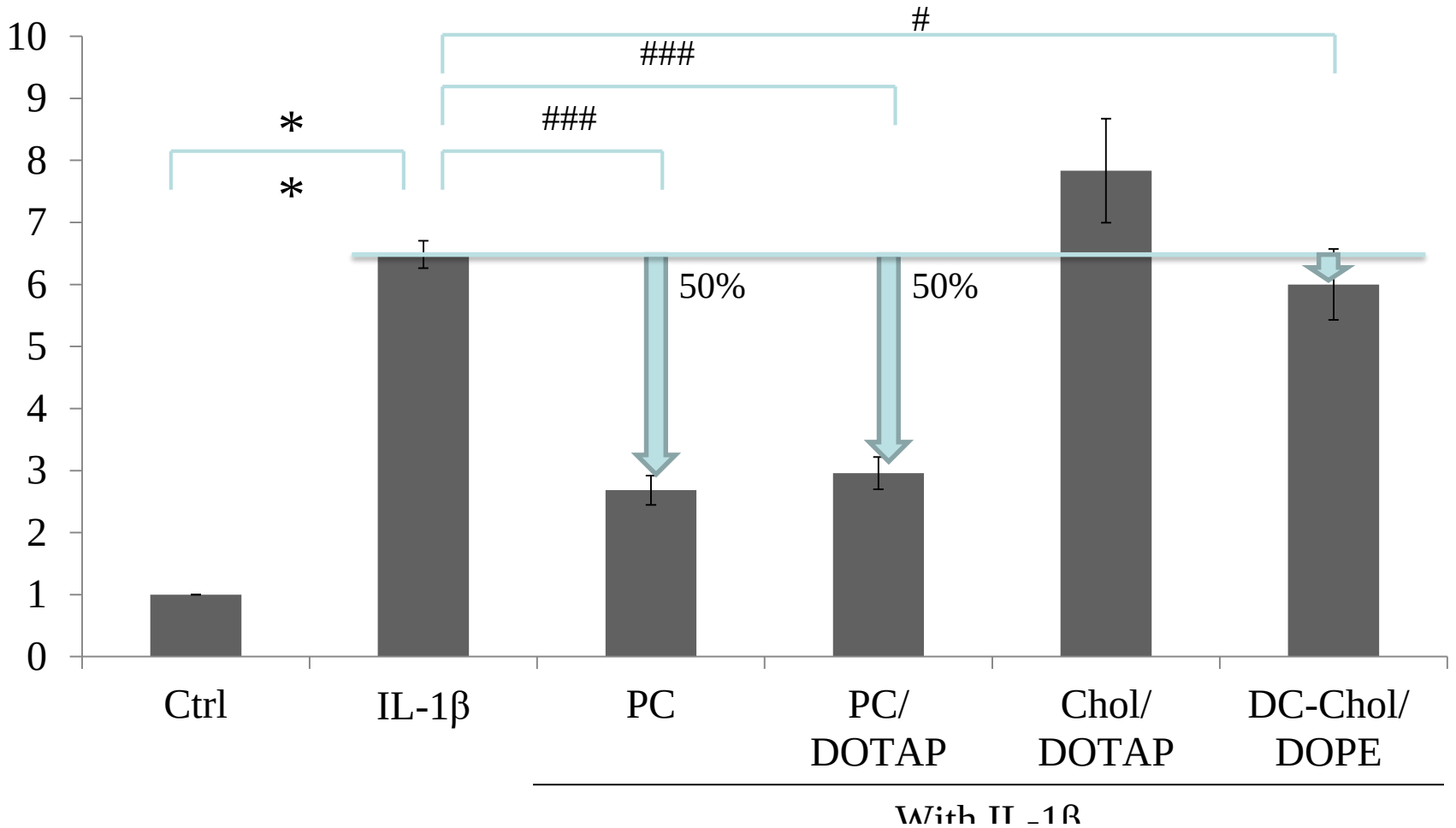
# 3.7A. The effect of liposome compositions on the OPG/RANKL ratio of 7F2 osteoblasts.



# The effect of liposome compositions on the gene expression of COX-2 of 7F2 osteoblasts.



# The effect of liposome compositions on the gene expression of MMP-3 of 7F2 osteoblasts.



# Summary 2

1. Liposomes are stable in 7 days.

2. Liposomes made by
   Cholesterol or
   DC- Cholesterol

   ⬇ Lipid accumulation

3. Liposomes prepared with
   PC only, PC and DOTAP,
   DC-Cholesterol and DOPE

   ⬇ COX-2 expression
   MMP-3 expression

# Conclusion

1. Osteogenesis  Adiopogenesis

   adverse relationship

2. Comparison : neutral and cationic(+) lipids

   Neutral — Cytotoxicity — Cationic(+)

   Inflammatory response

   Adipogenic differentiation

   Inhibition of osteogenesis

3. Liposomes made by lipids also minimized the reverse effects on osteoblast differentiation, mineralization, and inflammation.

4. PC could potentially be applied for the formulation of liposomes to be the treatment of osteoarthritis.

# Research Topic 2

## Curcumin and bis-demethoxycurcumin liposomes applied in bone treatment

Yeh CC, Su YH, Lin YJ, Chen PJ, Shi CS, Chen CN, Chang HI*. Drug Des Devel Ther. 2015 Apr 20;9:2285-300.

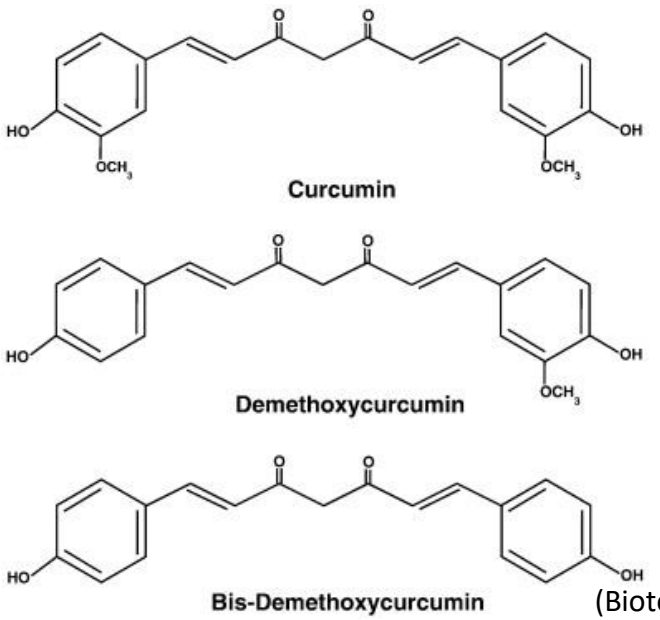
# *Curcuma longa* (turmeric)

- Curcumin is a non-water-soluble polyphenol that can be derived from *C. longa* by ethanol extraction.

(J. Wickenberg et al., 2010 )

- Curcuminoids, comprising curcumin, demethoxycurcumin, and bis-demethoxycurcumin.

(C. Capini et al., 2009)





(Biotechnology Advances, 2012)

# Curcumin

- Curcumin derived from turmeric (Curcuma longa) has been shown to suppress proliferation of cultured osteoblasts.

(Dorai and Aggarwal, 2004; Belakavadi and Salimath, 2005; Karunagaran et al., 2005)

- Curcumin  Inhibits Receptor Activator of NF-B Ligand-Induced NF-B Activation in Osteoclast Precursors and Suppresses Osteoclastogenesis.

(Alok C. Bharti , 2004)

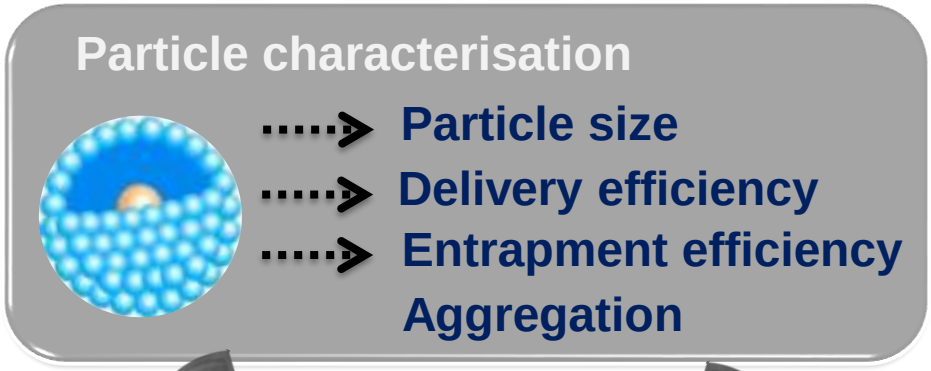
- Curcumin can prevent different cancers, decrease blood cholesterol levels, suppress psoriasis, prevent Alzheimer0s disease and improve the symptoms of osteoarthritis.

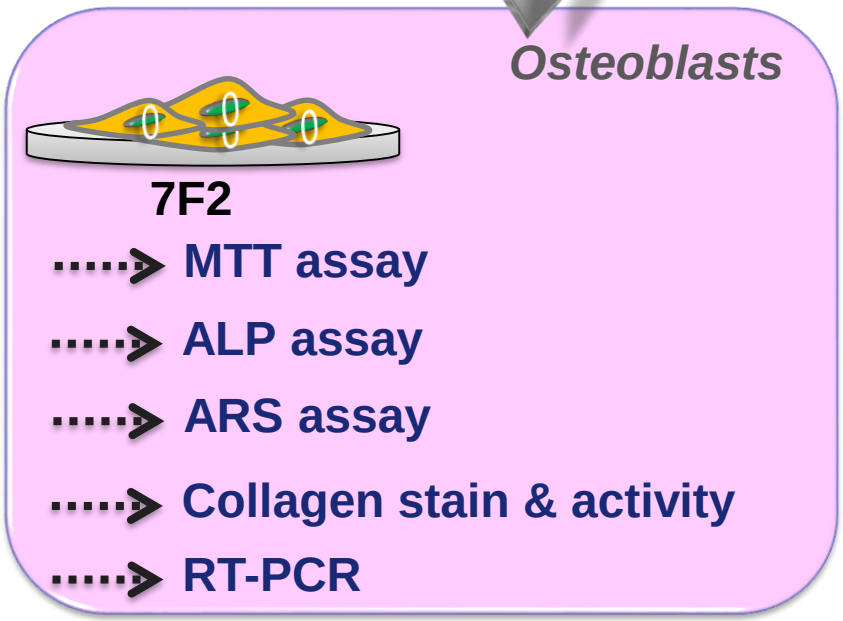
(Aggarwal et al., 2003)

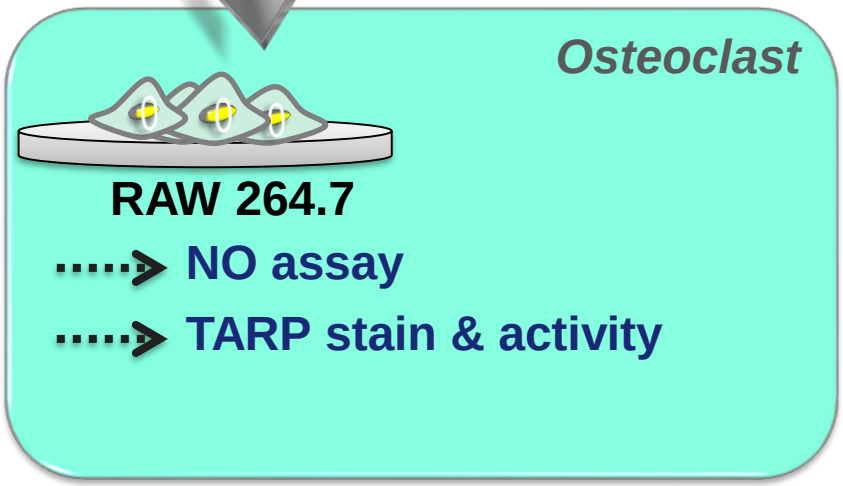
# Aim of study

- To increase its solubility and bioavailability, attempts have been made through encapsulation in liposomes.
- The formation of liposome–curcumin complexes also leads to an increase in the stability of curcumin *in vitro* to treat bone cells.

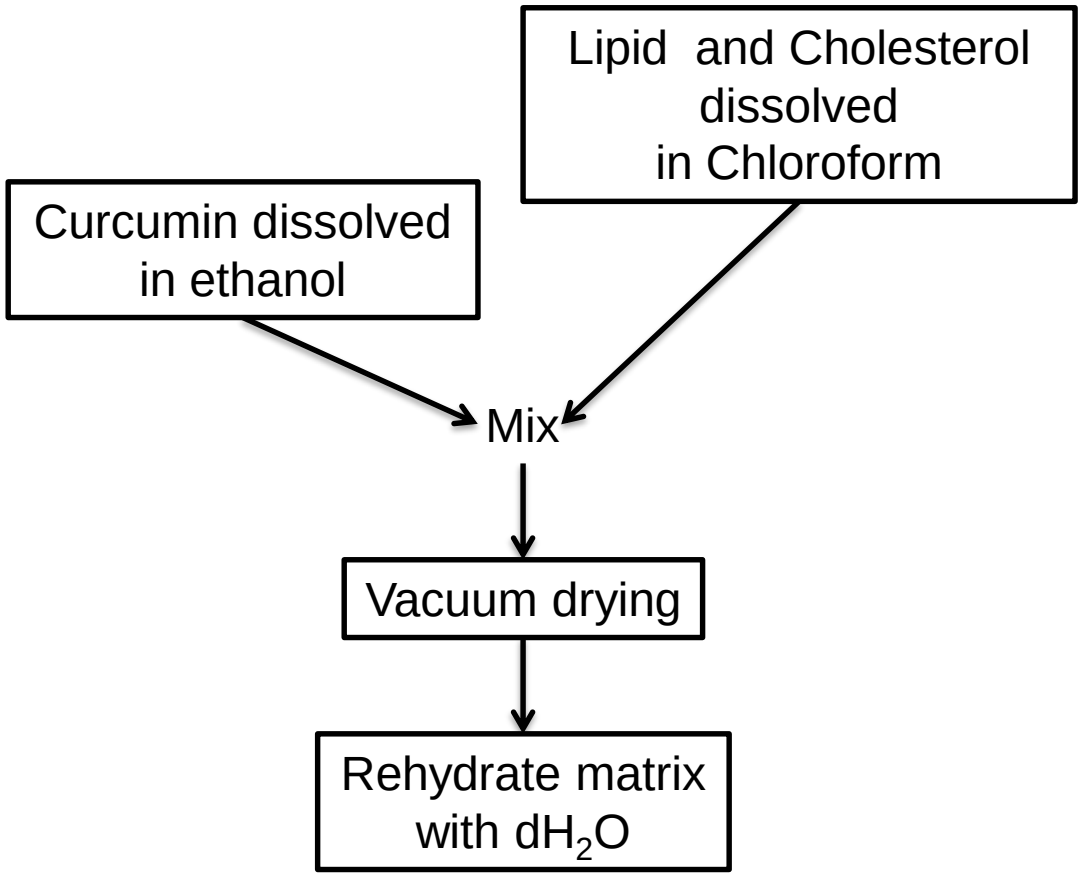
# The structure of experiment



# Liposome Formulations



(A)

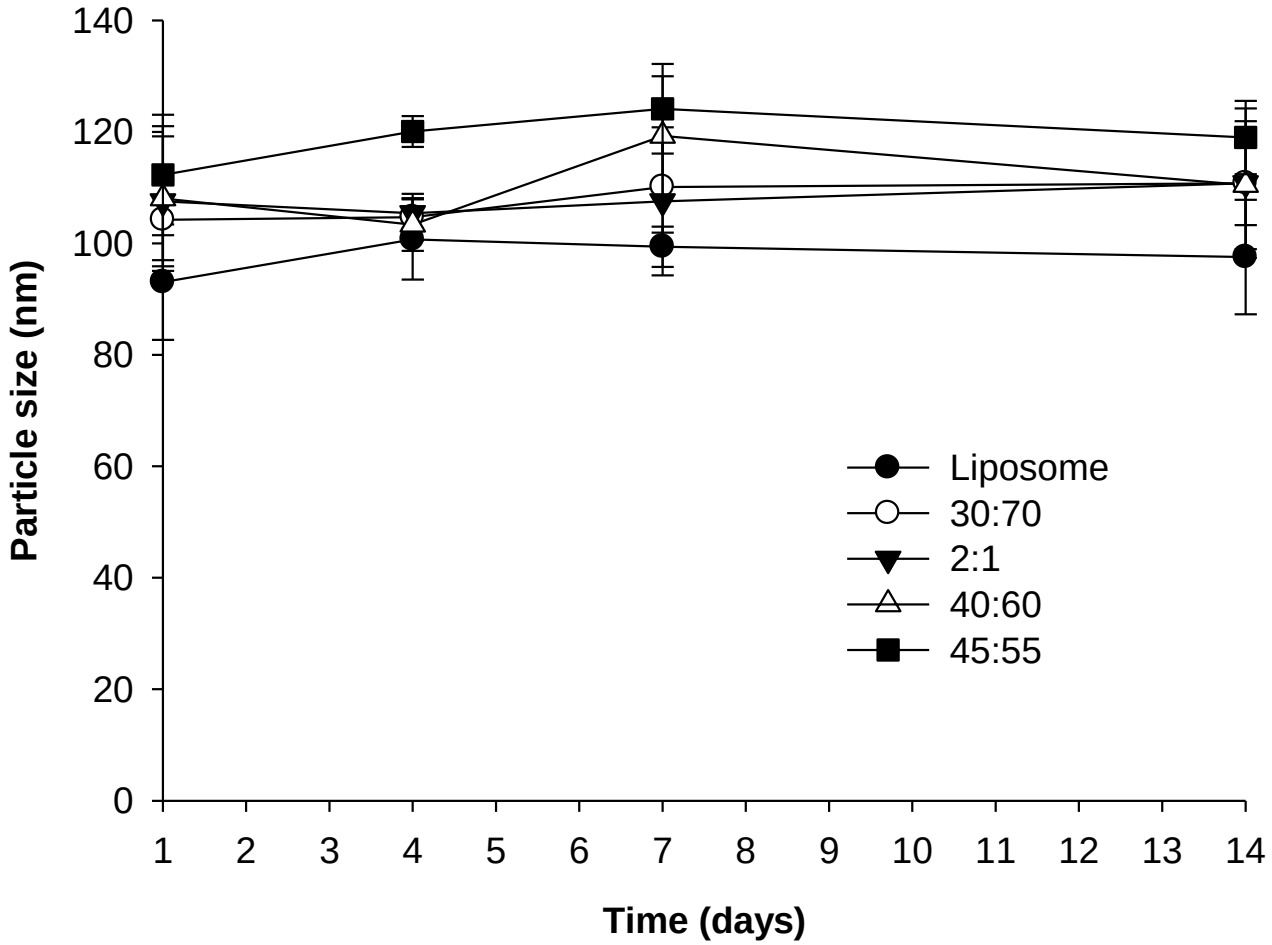



Fig 1. Particle size of liposome formulations. (A) The stability of liposomes prepared with different compositions of SPC and cholesterol in 1, 4, 7, and 14 days incubation

(B)

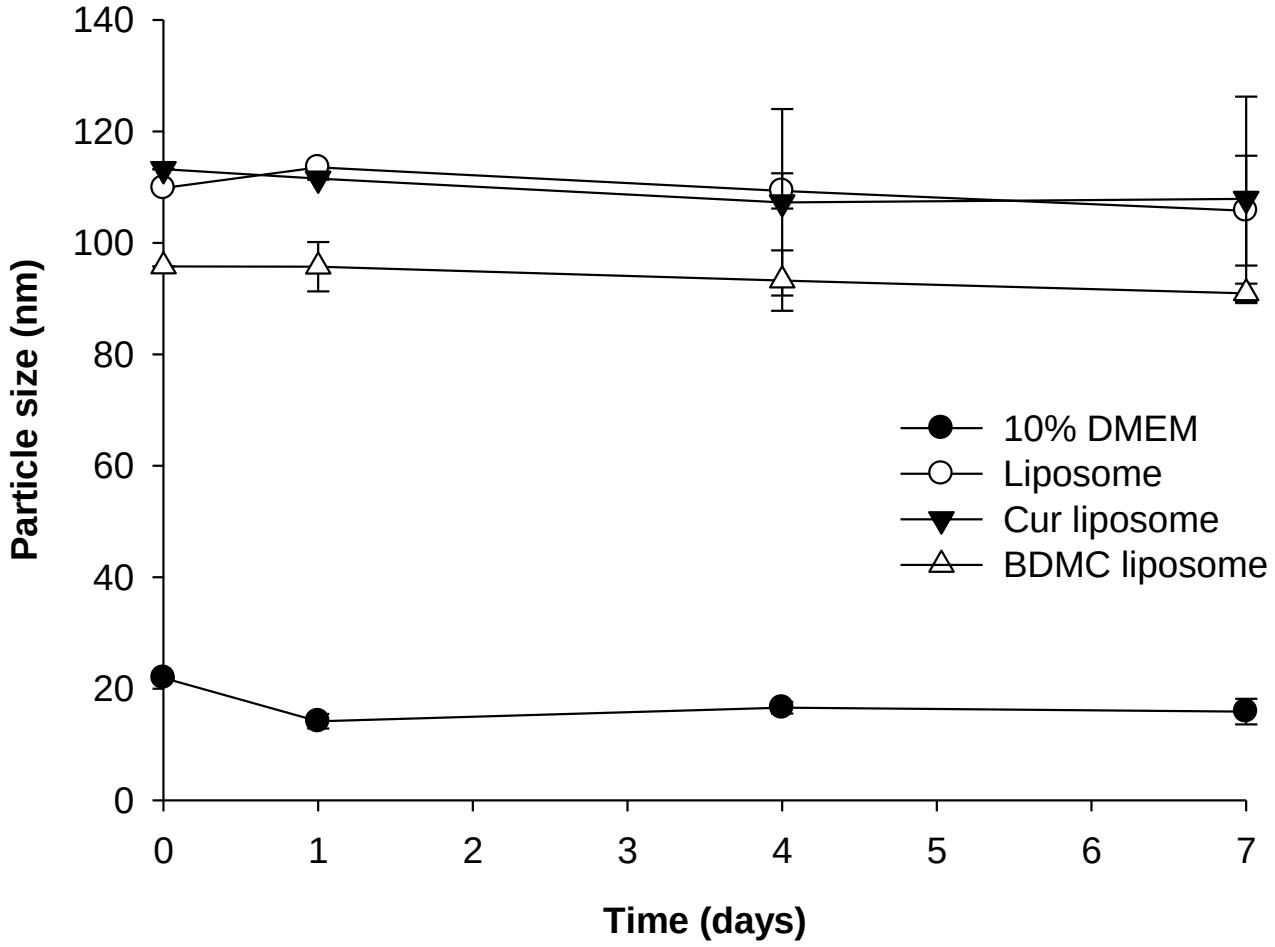


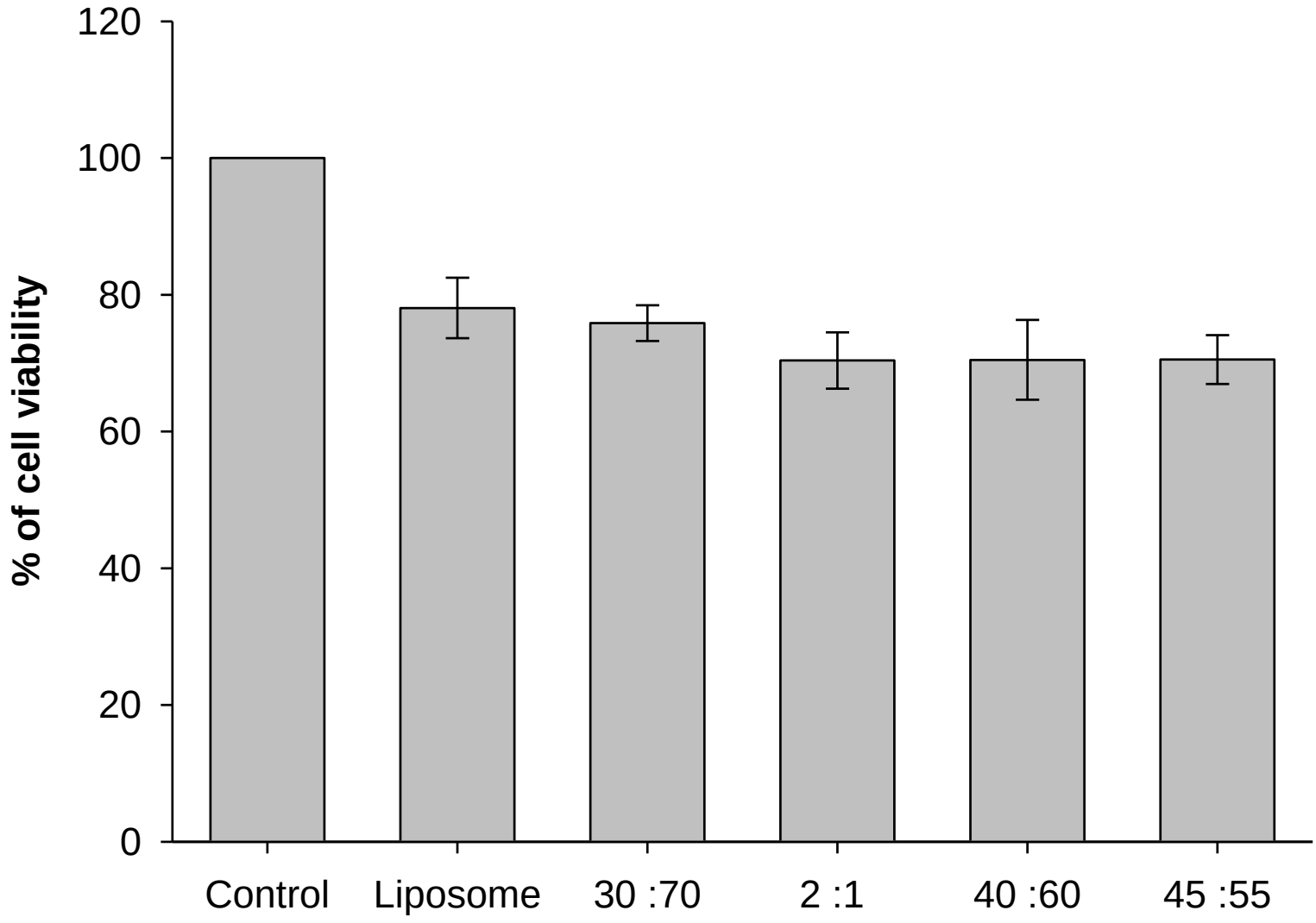

Fig 1. Particle size of liposome formulations. (B) The stability of empty liposomes and Cur-loaded liposomes in DMEM with 10% FBS in 1, 4, and 7 days incubation.

# Encapsulation parameters of the liposomal formulations

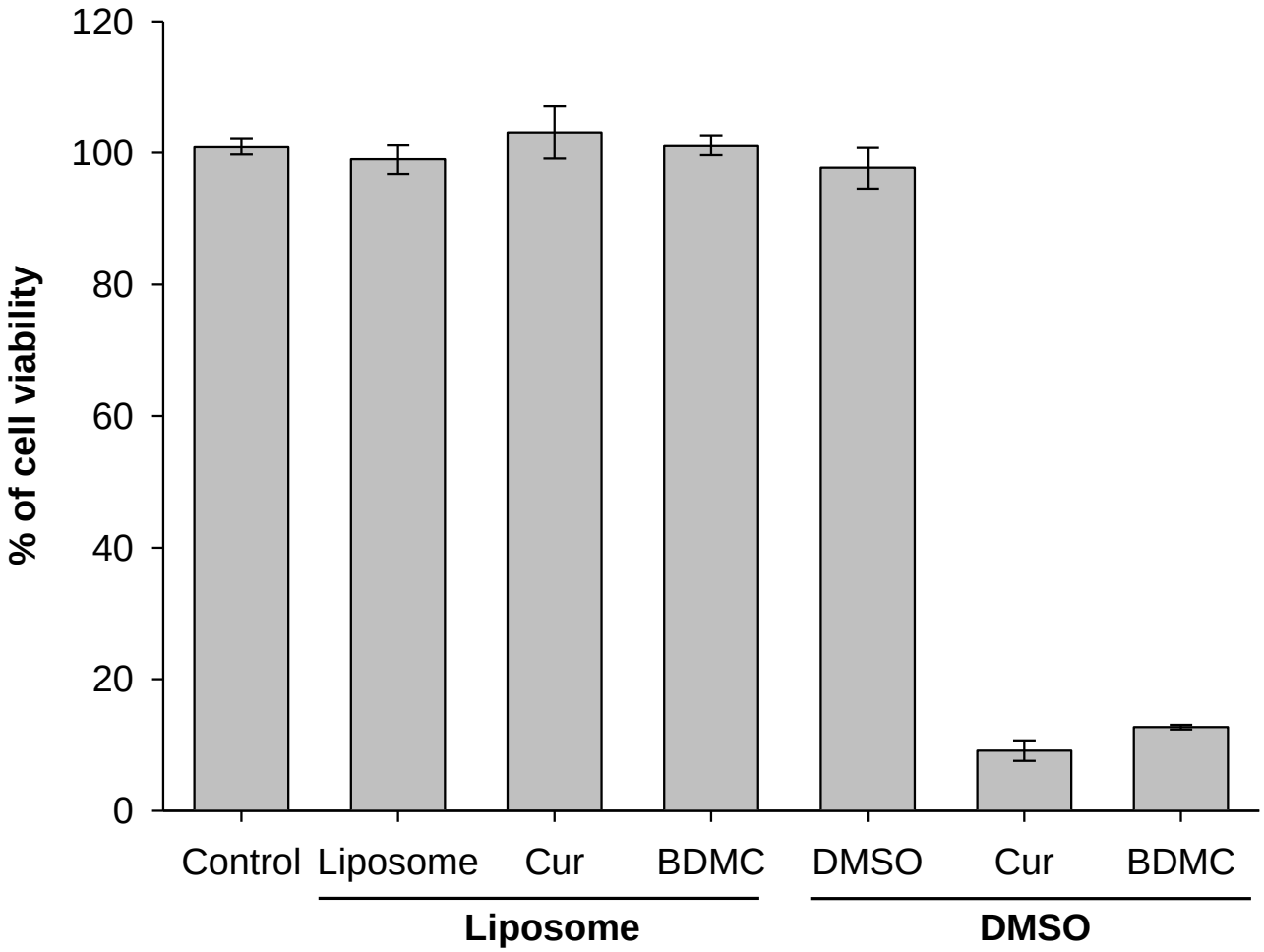
| Drug | Particle size (nm) | | Entrapment (%) | |
|---|---|---|---|---|
| | No extruder | Extruder | No extruder | Extruder |
| Empty | 959.6± 204.3 | 99.7± 5 | - | - |
| Curcumin (Cur) | 1110.0±149.0 | 110.0±4.8 | 92.5±4.2 | 69.5±1.4 |
| Bis-demethoxycurcumin (BDMC) | 1097.9±135.4 | 110.8±8.5 | 89.4-±0.7 | 71.4±7.4 |

Values represent the mean ± SD for at least three experiments.

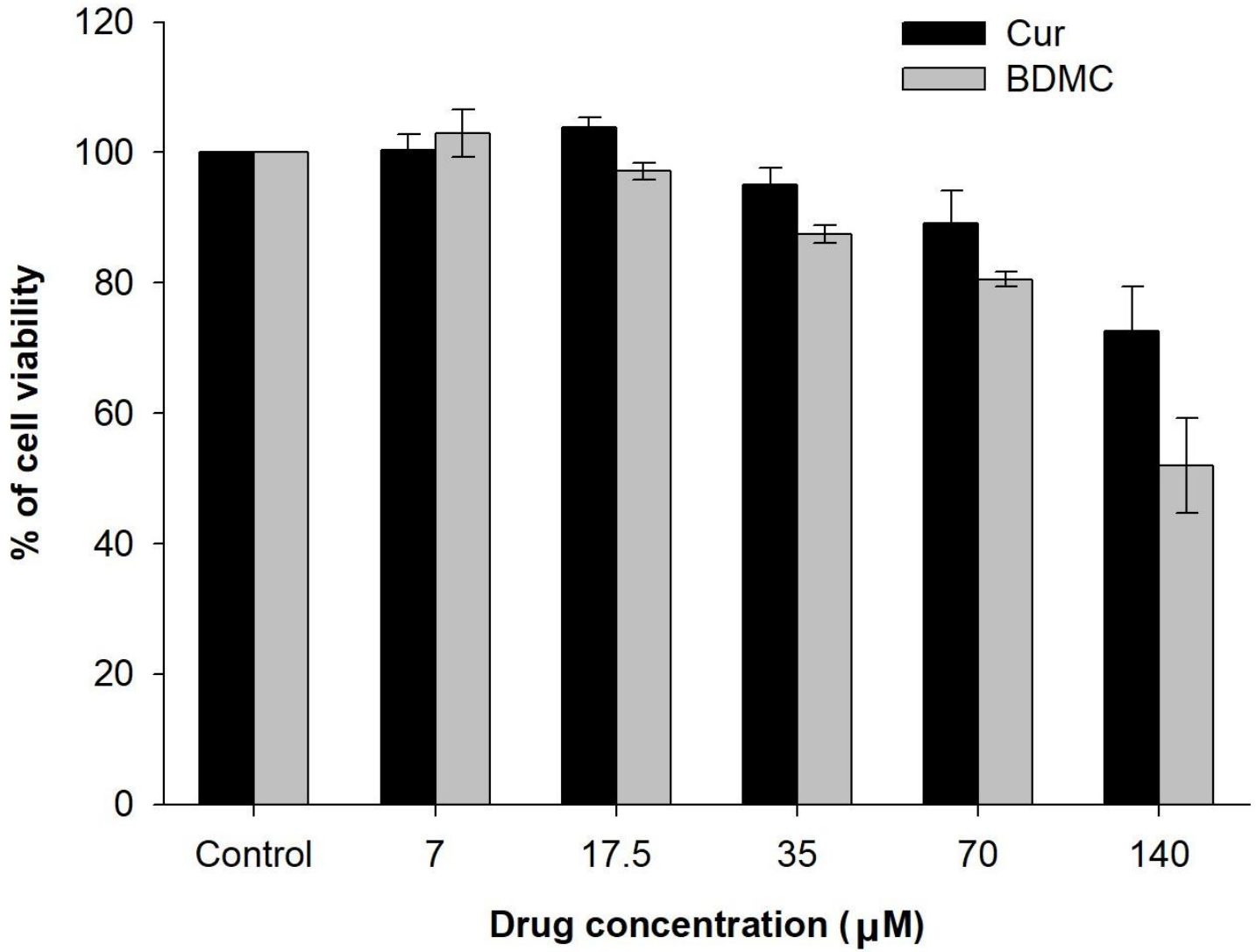
# Liposomal compositions on cell viability



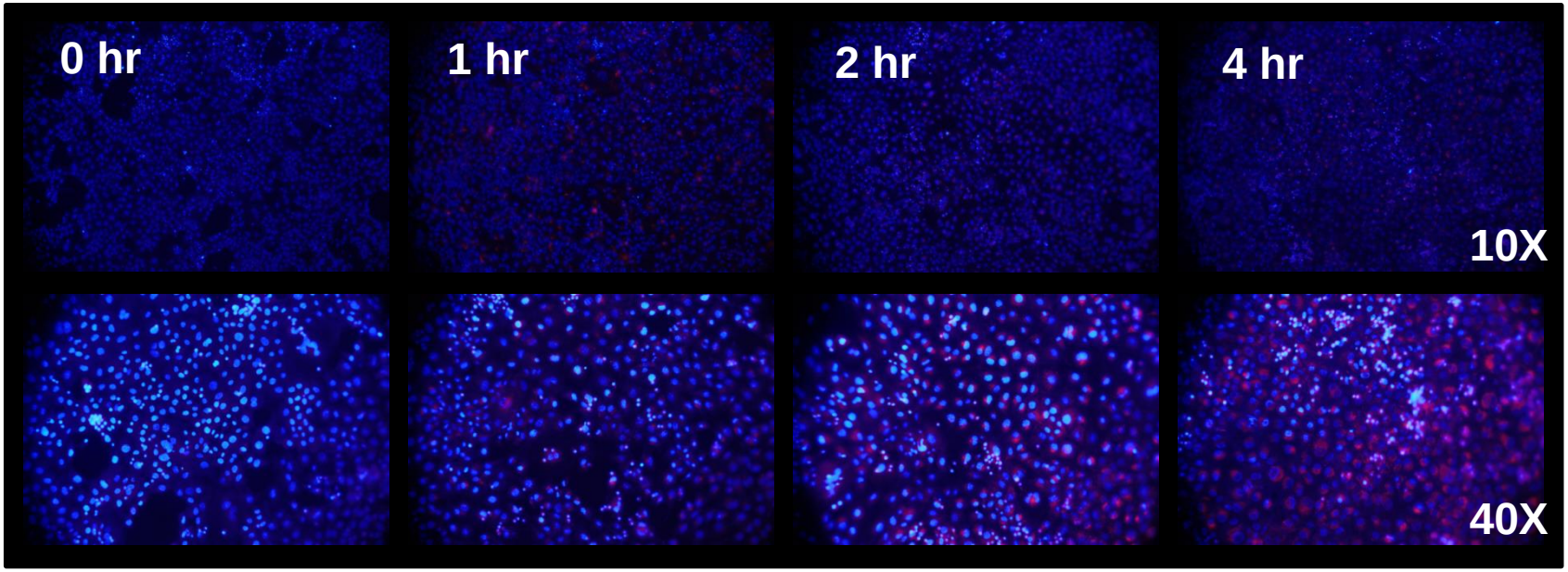
# Cur- and BDMC- loaded Liposomes on cell viability

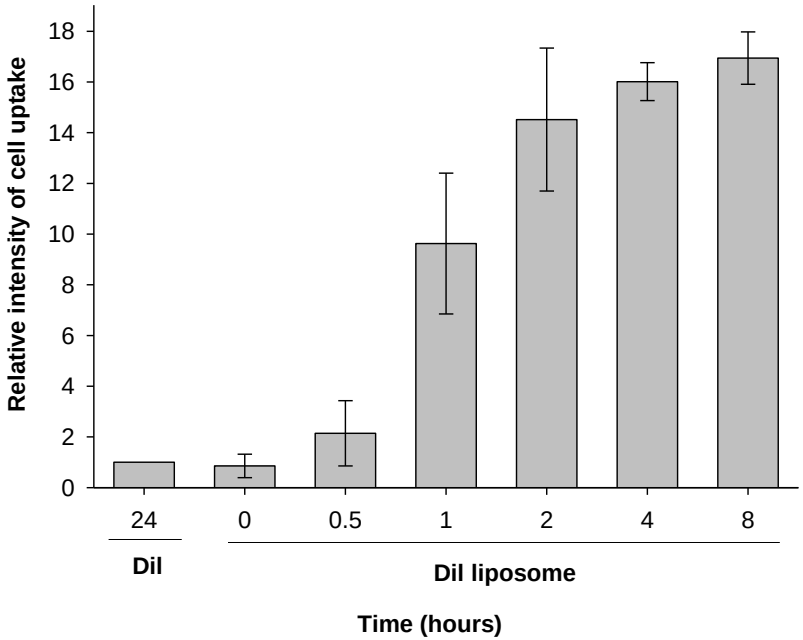


# The effect of drug concentrations for Cur- and BDMC- loaded Liposomes on cell viability



# Cell uptake of liposomes on 7F2 osteoblasts.

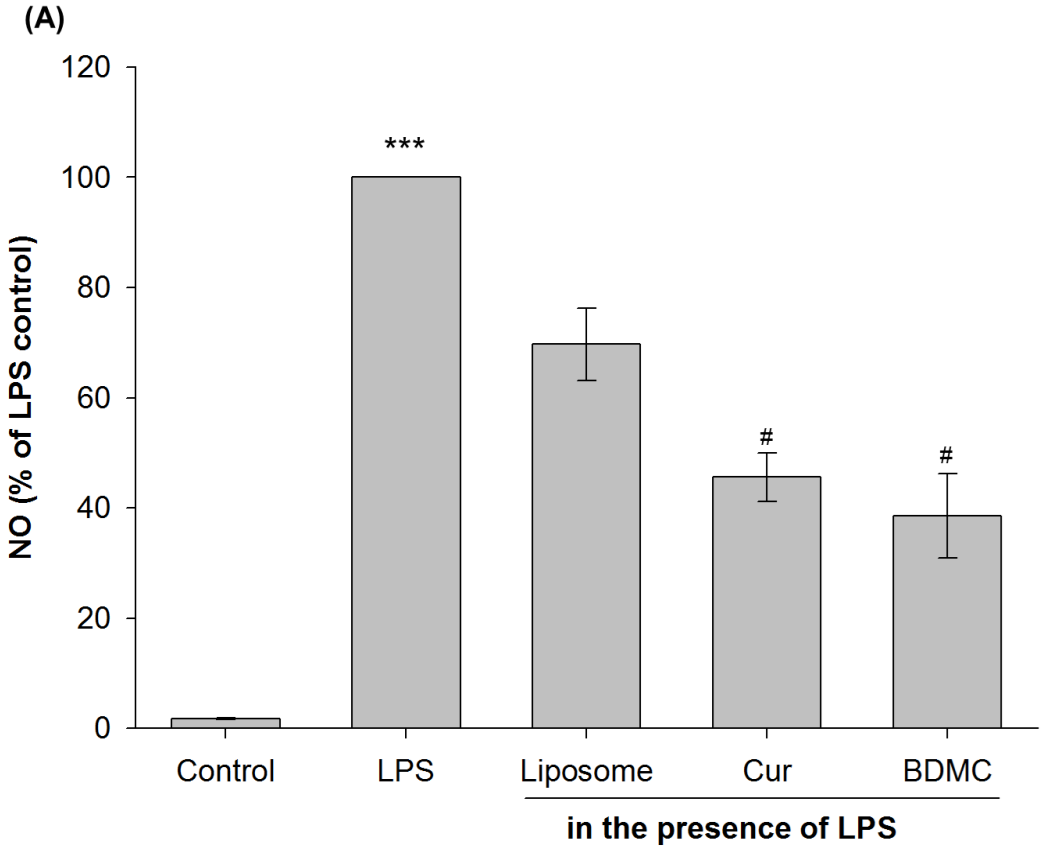


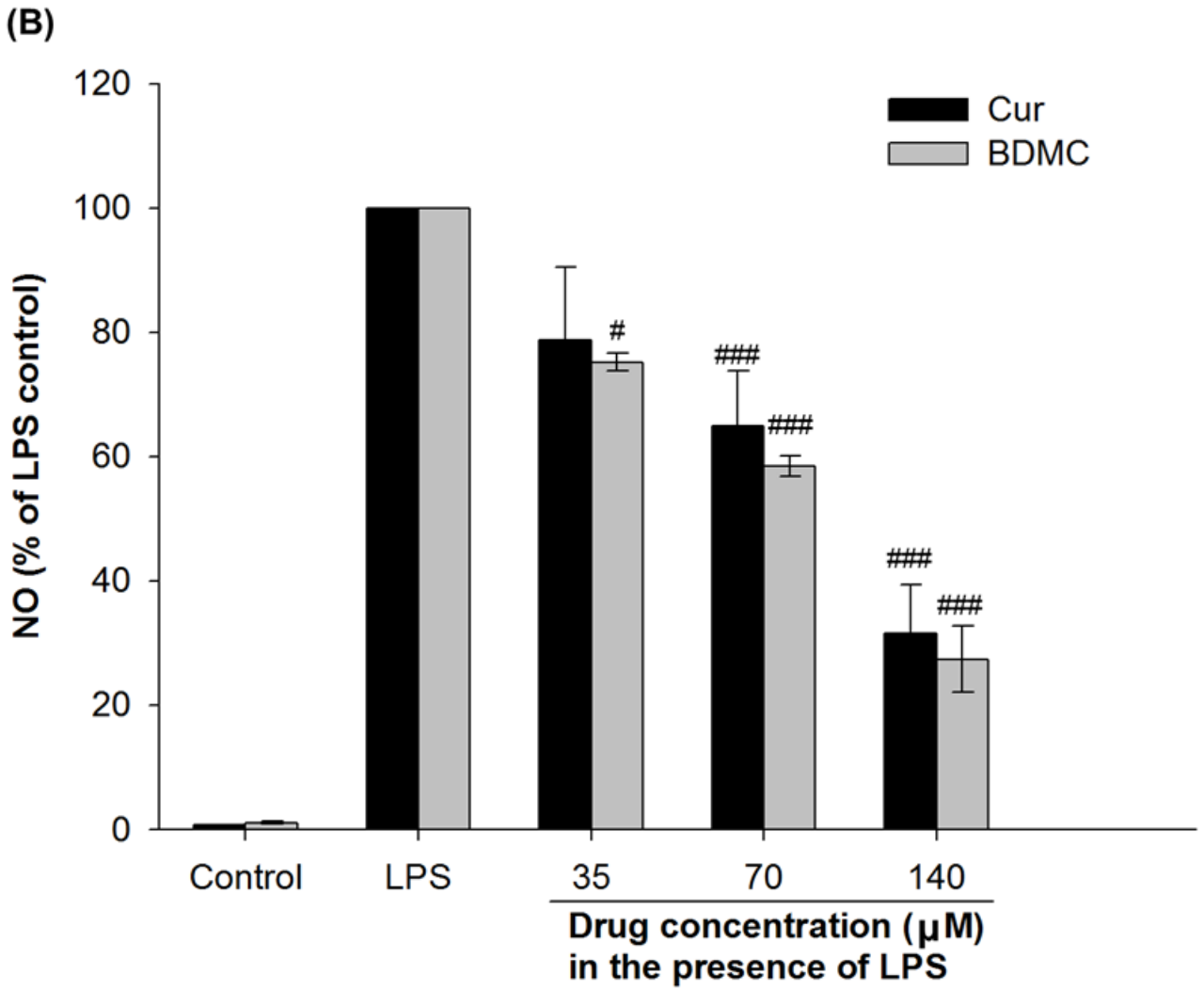
# Summary 3

- There are about 70% entrapment efficiency of curcumin in liposomes and particle sizes of liposomes are stable.
- Using liposomes to encapsulate curcumin could reduce cell cytotoxicity and increase cellular uptake.

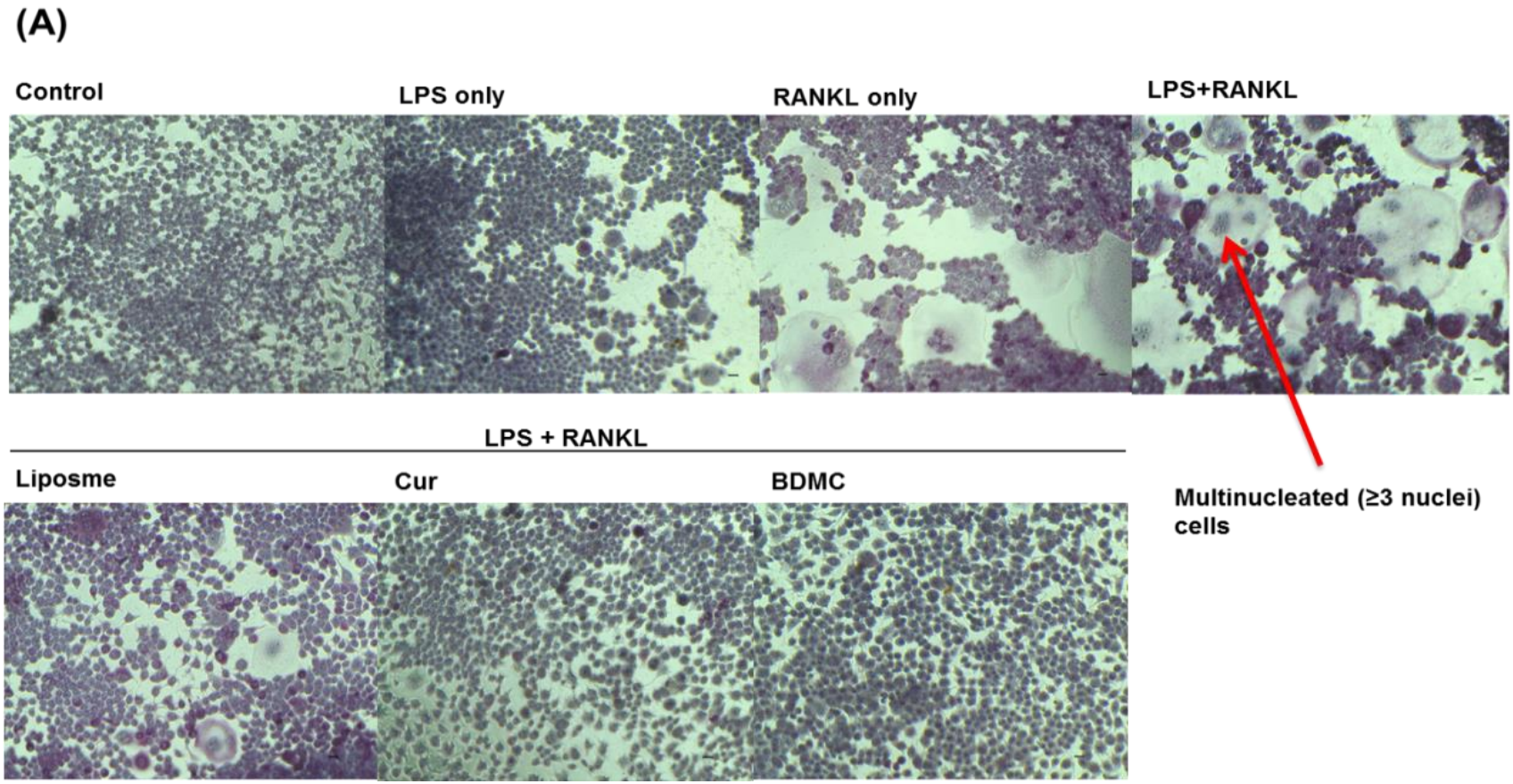
Effect of Curcuminoid-loaded liposomes on NO production in LPS (500ng/ml) induced RAW264.7 macrophages for 24 hours. Curcuminoids-loaded liposomes and lipsomes on nitric oxide (NO) production.



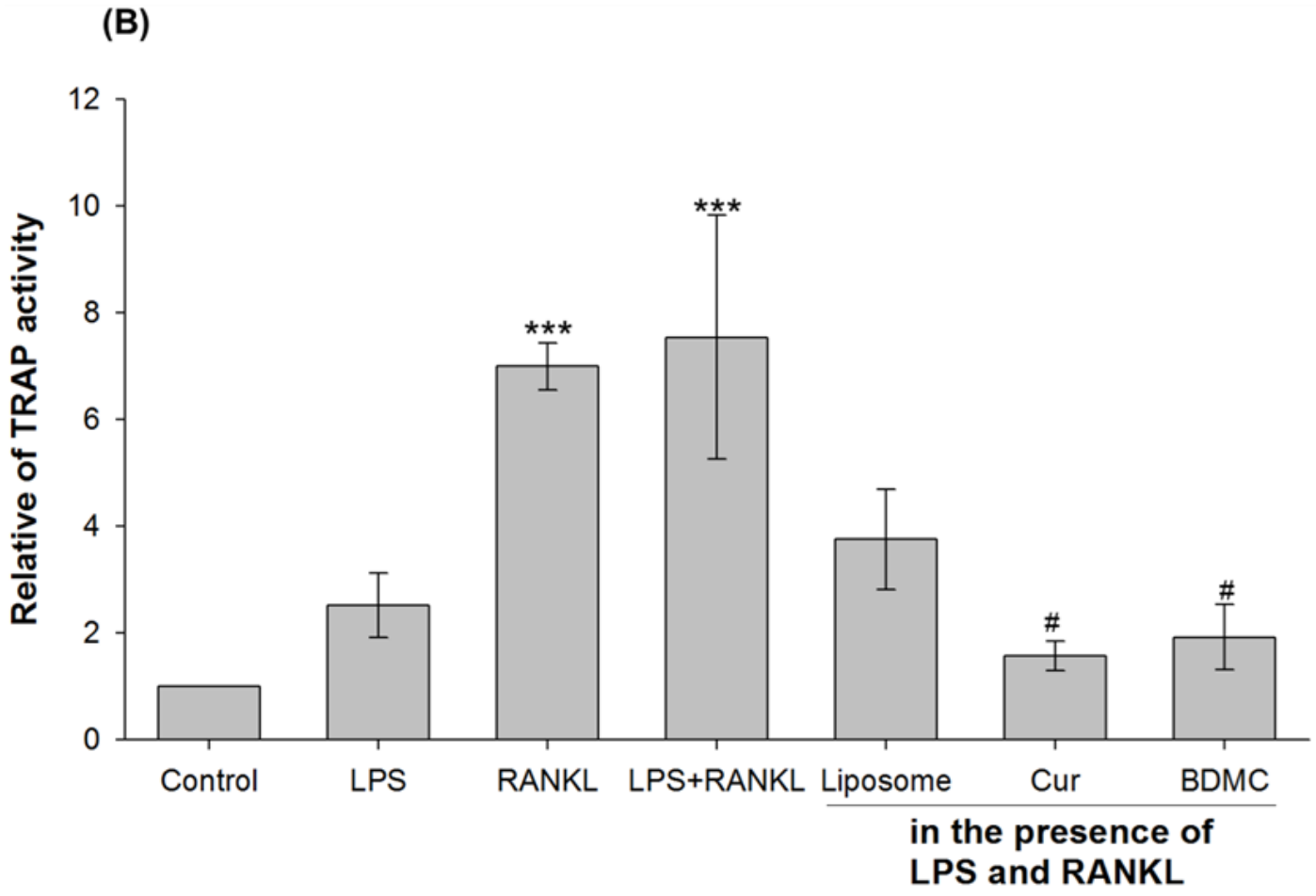
Effect of Curcuminoid-loaded liposomes on NO production in LPS (500ng/ml) induced RAW264.7 macrophages for 24 hours. RAW264.7 macrophages were incubated with different concentrations of Cur liposomes and BDMC liposomes.



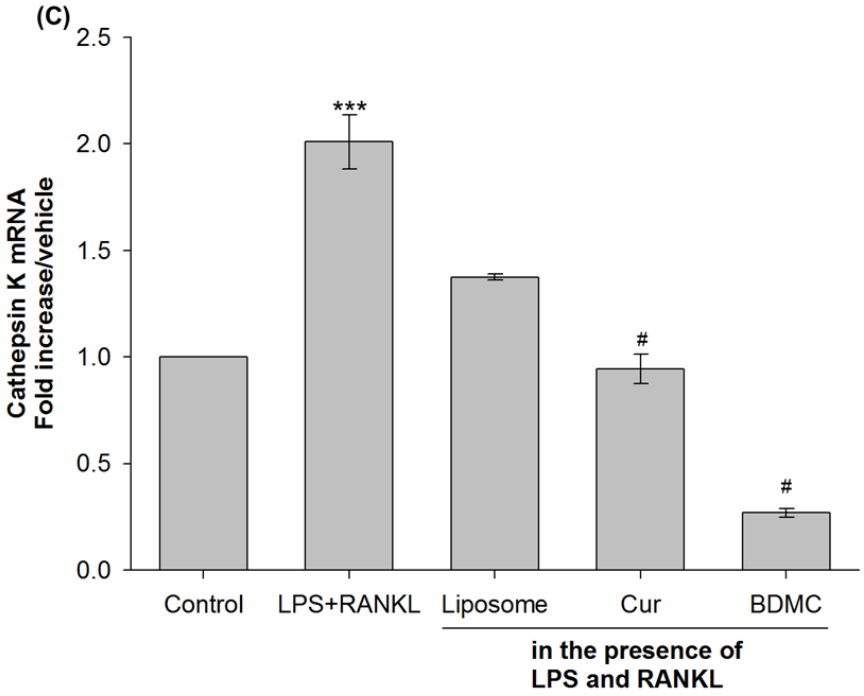
The effect of curcuminoid-loaded liposomes on tartrate-resistant acid phosphatase (TRAP) staining of RAW264.7 macrophage.

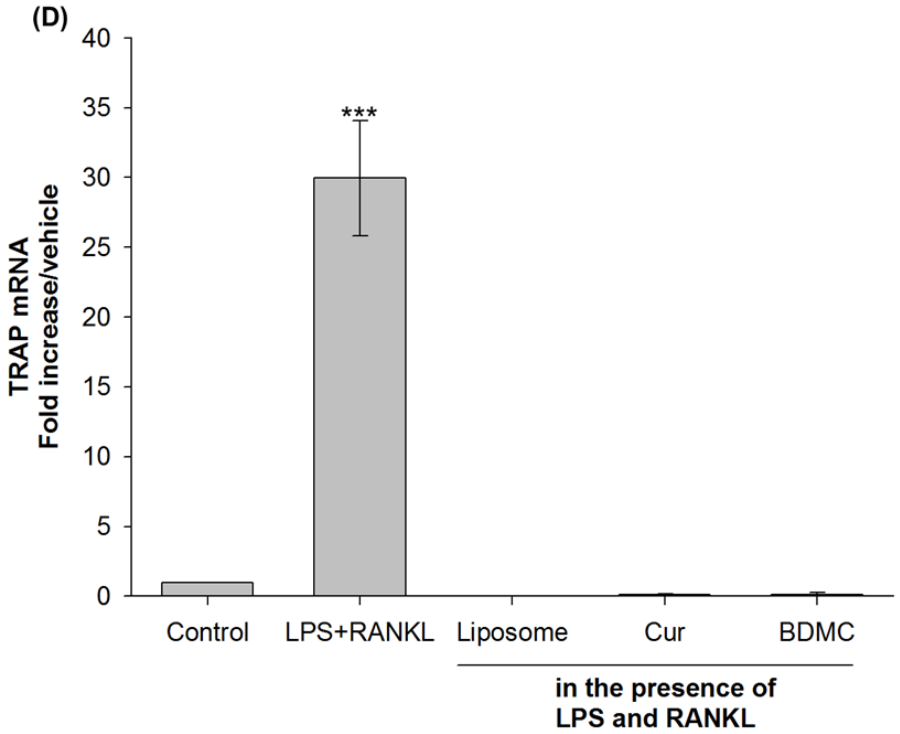


# Curcuminoid-loaded liposomes inhibit TRAP activity on LPS and RANKL-induced RAW264.7 macrophages.



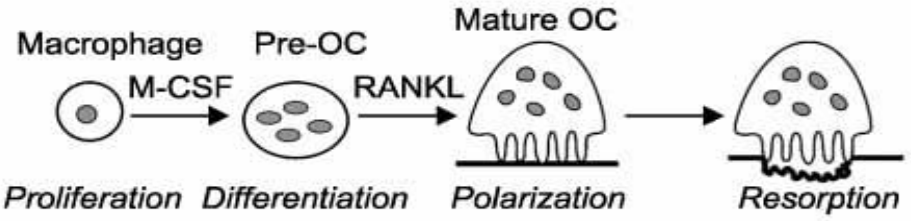
# LPS and RANKL-induced cathepsin K and TARP mRNA expression and cur liposomes and bis liposomes inhibits it in RT-PCR.



# Summary 4

- Curcuminoid-loaded liposomes can suppress the osteoclastogenic activity and down-regulate the gene expression of TARP and Cathepsin K in RAW 264.7 macrophages.

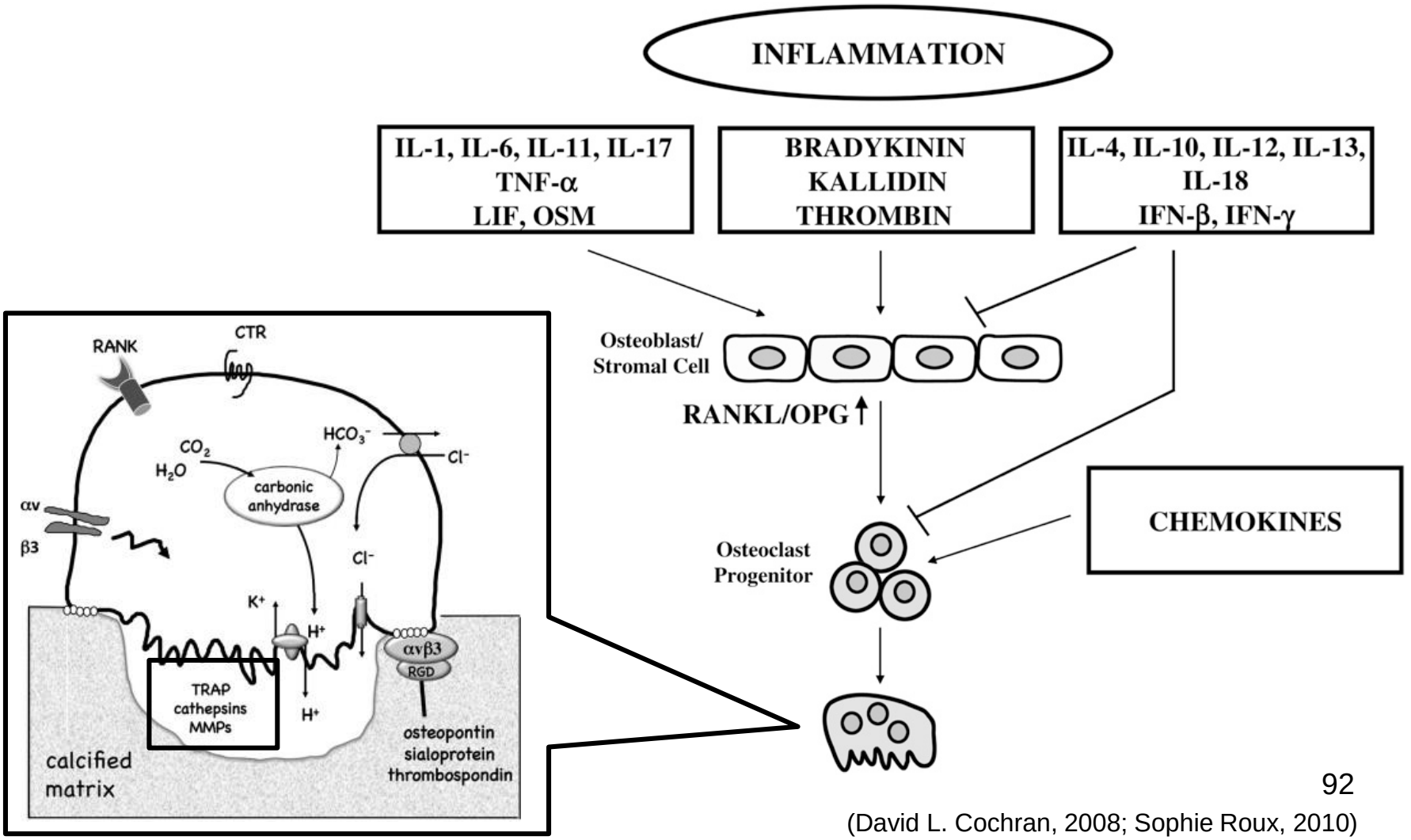



(Shin-Yoon Kim et al., 2009)

# OSTEOBLASTS

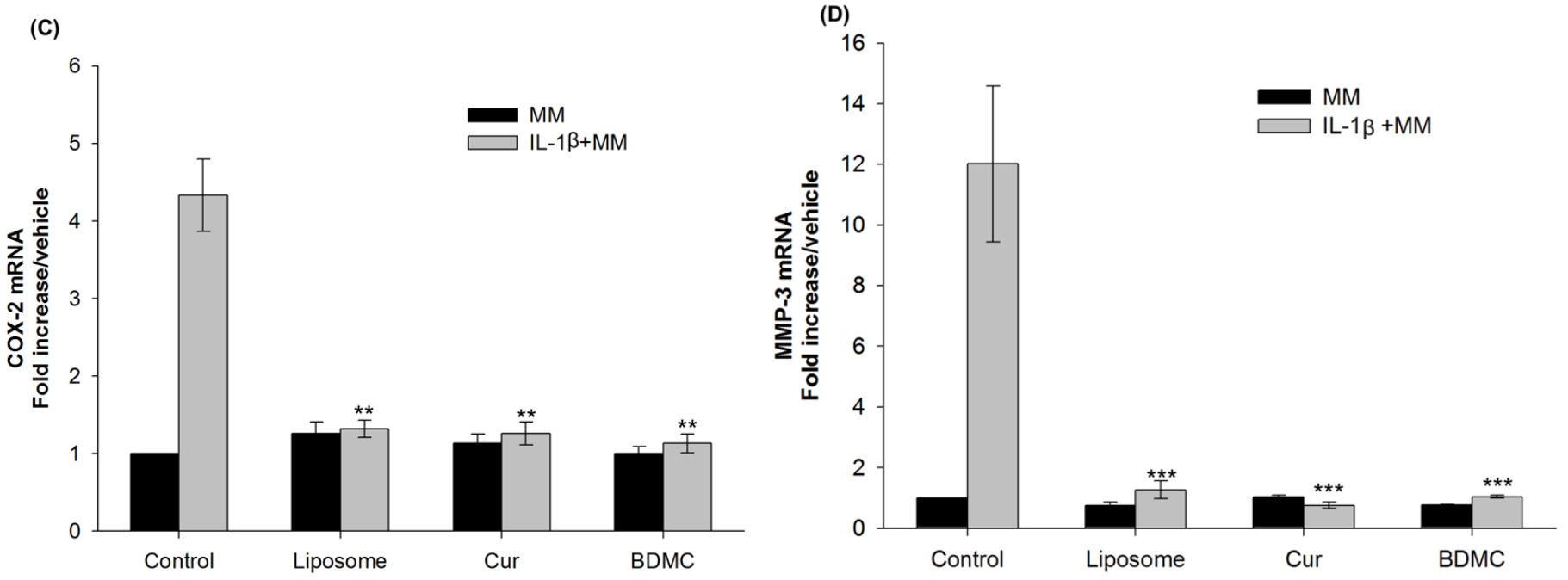
**The effect of curcuminoid-loaded liposomes on 7F2 osteoblasts**

# Inflammation and Bone Loss

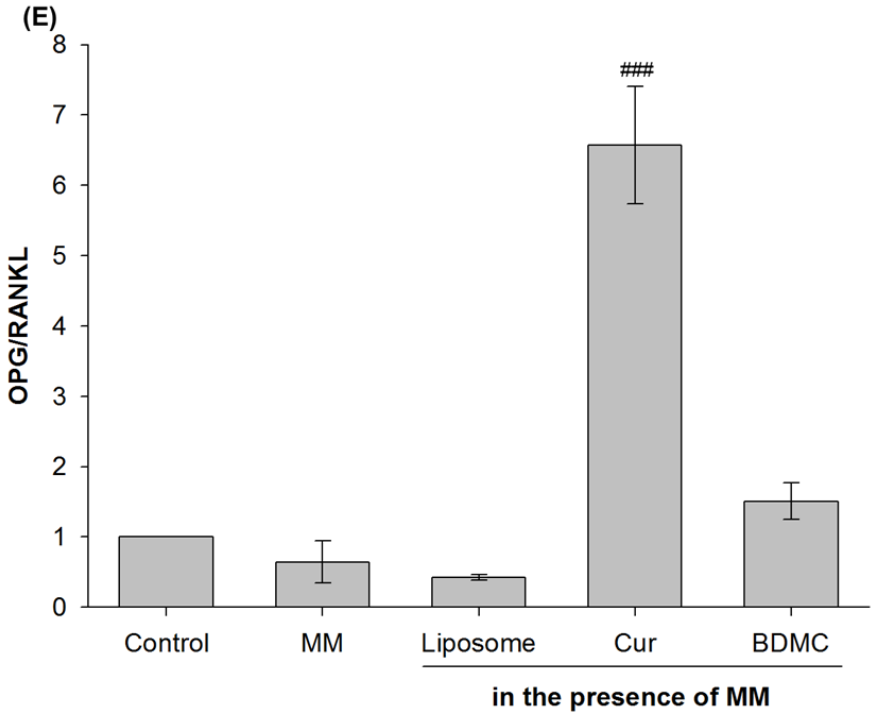


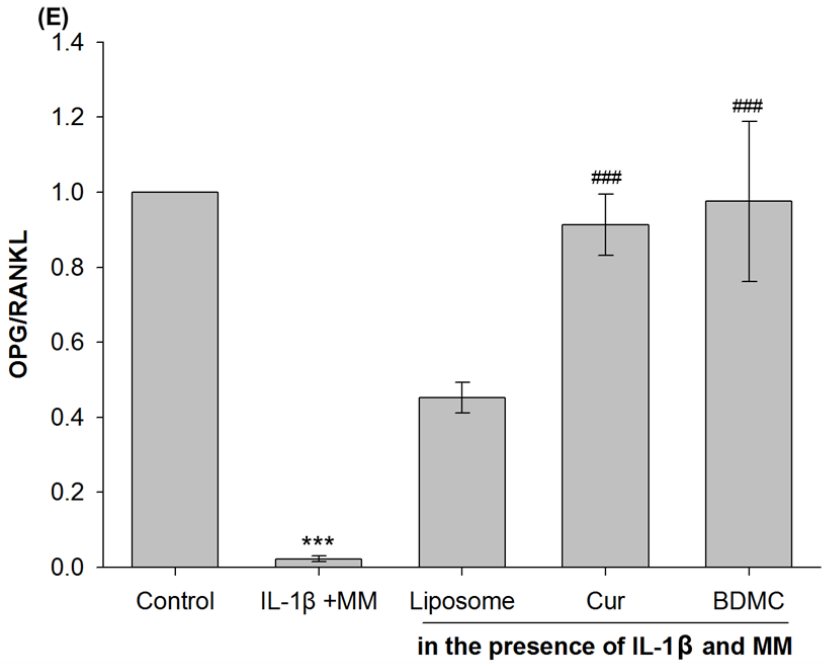



(David L. Cochran, 2008; Sophie Roux, 2010)




The effect of Cur/BDMC-loaded liposomes on the expression of pro-inflammatory mediators (cyclooxygenase 2; COX-2) and metalloproteinase-3 (MMP-3) of 7F2 osteoblasts in the presence or absence of 10ng/ml IL-1β stimulation.7F2 osteoblasts were treated with 50 μg/ml ascorbic acid and 10 mM βGP to induce osteoblast differentiation and mineralization (MM) and inflamed by 10ng/ml IL-1β.



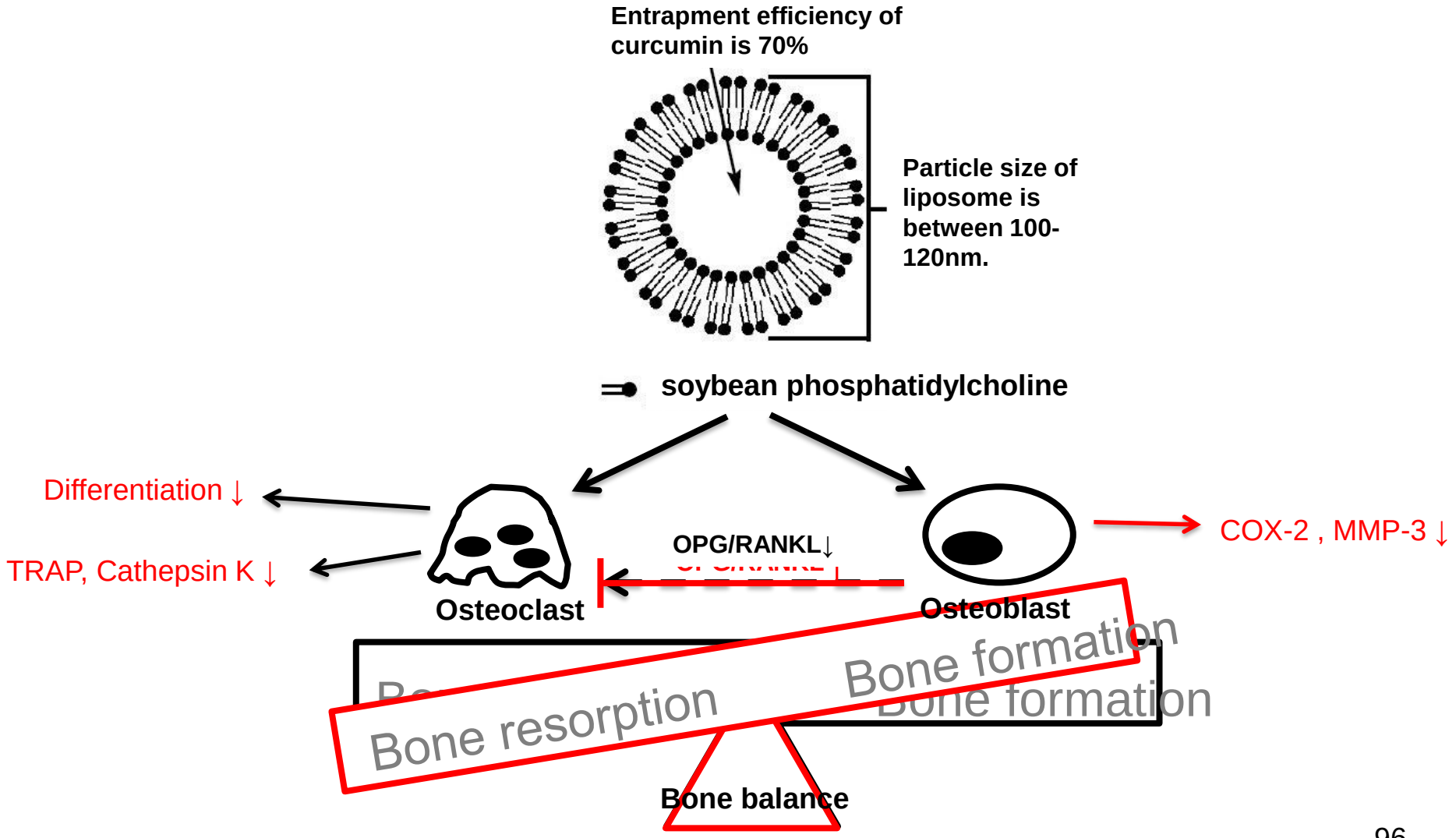




Effects of Cur/BDMC-loaded liposomes on OPG/RANKL ratio of 7F2 osteoblasts in the absence or presence of IL-1β and MM.

# Summary 5

- Curcuminoid-loaded liposomes can successfully decrease COX-2 and MMP-3 expression and show high OPG/RANKL ratio to prevent osteoclastogenesis.

# Conclusion


Entrapment efficiency of curcumin is 70%
Particle size of liposome is between 100-120nm.
soybean phosphatidylcholine
Differentiation ↓
TRAP, Cathepsin K ↓
OPG/RANKL↓
COX-2 , MMP-3 ↓
Osteoclast
Osteoblast
Bone resorption
Bone formation
Bone balance

Thank you for your attention