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EXPERIMENTAL RESULTS OF COLORECTAL CANCER CHEMOPREVENTION BY CURCUMINOIDS LOADED NANO-CARRIER DRUG DELIVERY SYSTEM INCREASED *IN VITRO* BIOCOMPATIBILITY

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Colorectal cancer (CRC) has the second highest cancer mortality rate in Taiwan. Curcumin, a major ingredient in the popular spice - curry, is derived from the rhizome of curcuma longa. Curcumin derivatives, curcuminoids, have shown anti-cancer activity and apoptosis induction in a variety of cancer cells. We developed novel poloxamer 188 based oil-in-water (o/w) nanoemulsions (NE) containing curcuminoids for drug delivery systems (DDS) to enhance the localized delivery of curcuminoids to colorectal cancer. The size of curcuminoids loaded NEs (F1 to F4) was found to be less than 100 nm, and F2 and F3 could further increase the rectal bioavailability of curcuminoids via low mucosa permeation and high accumulation. The growth rate of 5 primary colon cancer cells was inhibited by F2 and F3. Our research demonstrates a curcuminoids loaded poloxamer 188-based nanoemulsion could be a potential formulation for the development of a colorectal DDS in colorectal cancer therapy.

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*Keywords***:** Nanoemulsion, Curcuminoids, Colorectal cancer**.**

1. Introduction

Interest in this herb has grown in recent years based on its putative beneficial pharmacological effects including antioxidant, anti-inflammatory, and cancer chemopreventive actions [1-3].Turmeric (Curcumin) is a spice and a coloring agent derived from the root of the plant (*Curcuma Longa*) which is present in curry, and it is used extensively in Asian countries, also in traditional herb medicine [4-5].The low incidence of colon cancer in Asian countries could be related to low meat intake, but also to the regular use of curcumin in the diet [3].In animal experiments, curcumin has a profound effect on colon carcinogenesis or the multiplicity of colon adenomas [6].While commercial curcumin products contain a mixture of Curcumin, the primary ingredient, with minor components of demethoxycurcumin (DMC) and bisdemethoxycurcumin (bDMC), it has been found impossible to quantify these individual ingredients of curcuminoids by using the spectrophotometric method. Literature search has discovered the recent use of liquid chromatography-mass spectrophotometry for the detection of curcumin [1, 7-9]. In rats, absorption of curcuminoids from the intestine was reported to be about 60% [10]. As the colon is exposed to

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both curcumin and its metabolites, it is a likely target for the anti-carcinogenic activity of these compounds. Moreover, the fact that humans were able to consume up to 8 grams of curcumin per day without toxic effects makes curcumin a very interesting chemopreventive agent [11-12].Curcuminoids have been found to be poorly soluble in water and the maximum solubility in aqueous systems (pH 5.0) was reported to be as low as 11 ng/mL [13].The limited solubility of curcuminoids, as well as extensive systemic metabolism, could be responsible for the low bioavailability of curcuminoids after oral delivery [14]. In addition, curcuminoids in solution state could be sensitive to UV light, so that marked photochemical degradation could occur under UV exposure [15].This elimination, however, is avoidable and the bioavailability of curcuminoids (curcumin, demethoxycurcumin and bisdemethoxydrocurcumin) could be significantly improved if the drug were to be administered intrarectally [16-17], but this leads to difficulty in its handling for clinical use.

In order to enhance the availability and distribution of anti-tumor agents such as curcuminoids to tumor sites, we have proposed that nanotechnology-based drug delivery systems could provide a unique strategic advantage, as nanoemulsions (NE) have been used to enhance bioavailability of drugs. There has been increased interest during recent years in the use of nano- or microemulsions that could modify drug permeation [18]. NE is one of the most promising techniques for transmucosal delivery of drugs and present many advantages such as higher storage stability, lower preparation cost, good production feasibility, thermodynamic stability, and absence of organic solvents [19]. NE are thermodynamically stable transparent or translucent dispersions of oil and water stabilized by an interfacial film of surfactant usually in combination with surfactant having a droplet size less than 100 nm [20]. Aqueous phase titration or spontaneous emulsification methods have been successfully investigated for the preparation of oil-in-water (o/w) NE of many lipophilic drugs in many articles [21]. Curcuminoids-loaded NE suppositories would be expected to be more acceptable to patients and to cause less irritation to rectal mucosa compared with conventional suppositories [22].

In order to clarify this aim, nanoemulsions containing curcuminoids were prepared and used to treat five colorectal cancer cell lines in the present study. Relationships between the bioavailability of curcuminoids and physicochemical or biological characteristics were investigated.

2 Experimental

2.1. Materials

Commercial curcuminoids (>95% pure curcumin), which was selected as a model for drug and Poloxamer 188 (Pluronic F68), was purchased from Sigma (USA) and was used as surfactant. Double distilled water was prepared in-house and was used to prepare the aqueous solutions. All products were used as received. All other chemicals were of reagent grade. The formulation of curcuminoids-poloxamer 188 oil-in-water (O/W) nanoemulsions (NE) is listed in Table 1.

Table 1. Formulation of curcuminoids loaded nanoemulsion. The curcuminoids dissolved in the EtOH (oil phase) and the size, polydispersity index (PI) and Zeta potential were measured using Zetasizer Nano-ZS. All data were reported as the Means ±SEM of at least three separate experiments.

Formulation	Curcuminoids (mg/ml)	Oil phase (%, v/v)	Water phase (%, v/v)	Surfactant (%, v/v)	Size (nm)	Polydispersity index (PI)	Zeta potential (mV)
F1	-	25.4	59.68	14.92	75.43±1.97	0.32±0.02	-19.13±1.17
F2	0.25	25.4	59.68	14.92	87.54±1.86	0.35±0.03	-23.12±1.43
F3	0.25	25.4	55.95	18.65	76.34±1.47	0.34±0.02	-20.04±1.35
F4	0.25	25.4	52.22	22.38	71.26±1.94	0.32±0.04	-17.54±1.27

Surfactant: poloxamer 188
(Mean±SEM, N=4)

2.2. Human primary colorectal cancer cell culture

The specimens of human colon cancer were obtained from a total of five colon patients suffering from adenocarcinoma (CP-1, -2, -3 and -5) or tubulovillous adenoma (CP-4) on the colon (2 cases), sigmoid colon (2 cases) or descending colon (one case). These patients consisted of three men (age: 54-82 years) and two women (age: 62-72 years), who were receiving treatment at the Kaohsiung Medical University Hospital (KMUH). The specimens were taken during the operation for colon cancer removal in the period of 1/13/04 to 5/10/05. Specimens were removed from only the typical and clinically clear-cut (Grade II) cases. Prior written informed consent was obtained from the patients and all procedures used had been reviewed and approved by the IRB at Kaohsiung Medical University (KMU) Hospital in adherence to the Helsinki Principles. The five primary cell lines of colon cancer cells were derived, as a gift, from the cell bank maintained in the MedicoGenomics Research Center at KMU [1].The cells were grown at 37℃ in Dulbecco's Modified Eagle Medium (GibcoBRL) supplemented with 10% (v/v) Fetal Bovine Serum (HyClone) and a combination of antibiotics (penicillin, 200 unit/ml, and streptomycin, 200 g/ml) (HyClone) under an atmosphere of CO_2/air (5%) for this series of studies.

2.3. Cell proliferation assay

The cells were seeded into 96-well culture plates at 5000 cells/well. The cells were treated with F2 and F3 (the formulations containing various concentrations of curcuminoids) for 1 to 3 days, and then treated with MTS (3-(4,5-dimethyl-thiazol-2-yl)-5-(3-carboxymeth-oxy-phenyl)-2-(4-sulfo-phenyl)-2H-tetrazolium) for 4 hrs. The absorbance was determined by the Powerwave XS reader (Bio-Tek) at 490nm. Each assay was carried out in triplicate and the results were expressed as the mean+/-SEM. Cell proliferation was expressed as the percentage of the assay data determined for the control group.

2.4. Preparation of curcuminoids loaded nanoemulsion

From each phase diagram constructed, different formulae were selected from nanoemulsion regions for incorporation of curcuminoids into the oil phase. 25.4% (v/v) of curcuminoids was dissolved in the oil phase (EtOH) of all selected nanoemulsion formulations. Then, surfactant was added in the oil phase of the drug and stirred for 10 min. The water phase (distilled water) was added slowly with continuous stirring. Selected formulations were subjected to different dispersion stability tests. (Data not shown).

2.5. Characterization of curcuminoids loaded nanoemulsions

Droplet size distribution and zeta potential of the curcuminoids loaded nanoemulsion was determined by photon correlation spectroscopy (PCS) using a Zetasizer Nano-ZS (Malvern Instruments, UK). Particle solution (1.0 ml) was pipetted into a cuvette. Data were collected at room temperature (23-25℃). Zeta potentials of curcuminoids loaded nanoemulsion were also analyzed by Zetasizer Nano-ZS (Malvern Instruments, UK). Each assay was carried out at least in triplicate and the results were expressed as the mean+/-SEM.

2.6. In vitro permeation and mucosal accumulation studies

Permeation and mucosal accumulation studies of curcuminoids loaded nanoemulsion in pig mucosa was monitored by the Villa-Chien method [23]. One ml of the nanoemulsion or 1mg of curcuminoids was placed in a pig mucosal with Villa-Chien permeation cells. The cells were then immersed in the dissolution medium (i.e., 5 ml of phosphate buffer, pH 6.8) and the medium was then stirred at 100 rpm by the paddle. The dissolution medium was kept at 37°C during the experiment. At time intervals for 0 to 24 hours, 5 µl aliquots were collected and assayed for curcumin by high-performance liquid chromatography (HPLC) [1].

2.7. Preparation of tissue lysates

Frozen tissues were minced and transferred into a volume of ice-cold lysis buffer equivalent to tissue weights (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM $Na_4O_7P_2$, 1 mM β-glycerol-phosphate, 1 mM Na_3VO_4, 1µg/mL leupeptin, supplemented with 1 mM PMSF). Minced tissue fragments were sonicated on ice using a Vibra Cell Model VC 375 ultrasonic processor (Sonics & Materials, Inc., Danbury, CT, USA) for 14 s with a 14-s break between sonications; total of 4 min at a 40% duty cycle. Homogenates were centrifuged at 14,000×*g* for 10 min at 4°C. Supernatants were aliquoted and stored at −80°C after the total protein content was determined using the micro-BCA assay (Pierce Biotechnology, Rockford, IL).

2.8. Recovery of curcuminoids from mucosa

Tissue lysate (500 µL) was first acidified with 6N HCl (1:1 (v/v)) and vortexed for 30 seconds. Following addition of the extracting buffer (500 µl) prepared above, samples were each vortexed again and then shaken in the Orbited shaker (at 100 rpm) for 15min. After centrifugation at 18000rpm for 20min, the upper organic layer was filtered through a membrane filter (0.22 µm) and then transferred to a clean injection sample vial (about 100 µL) for quantitative analysis by the HPLC method [1].

2.9. Permeation data analysis

Cumulative amounts of curcuminoids that permeated through the pig mucosa (µg/cm2) was plotted as a function of time (t) for each formulation. Rate of drug permeation at steady state (Jss) was determined from the slope of the linear portion of graph plotted between cumulative drug permeated and time. Permeability (P^b) was calculated by dividing Jss with initial concentration of drug in the donor cell [23].

2.10. Statistical evaluation

All data were reported as the mean±SEM of at least three separate experiments. Statistical analysis was conducted using a one-way ANOVA test, with the significant differences determined at the level of $P<0.05$.

3. Results

3.1. Effects of poloxamer 188 on the size, polydispersity index (PI) and zeta potential of curcuminoids loaded nanoemulsion

The objective of the present study was to determine whether poloxamer 188-based nanoemulsion for curcuminoids had any effect on colorectal cancer chemoprevention. Particular size of bare poloxamer 188 nanoemulsion and curcuminoids loaded nanoemulsions were measured (Table 1). The average size of bare poloxamer 188 nanoemulsion and curcuminoids loaded nanoemulsions were 75.43+/-1.97 nm (F1), 87.54+/-1.86 nm (F2), 76.34+/-1.47 nm (F3) and 71.26+/-1.94 nm (F4) respectively. The size of curcuminoids loaded (F2) was enlarged 1.16 fold than bare poloxamer 188 nanoemulsion. Emendation of the surfactant percentage of curcuminoids loaded nanoemulsions could reduce the effect (F3 and F4).

In this study, bare poloxamer 188 nanoemulsion and curcuminoids loaded nanoemulsion procedures are as described in the Methods section. Surface electrical potentials (Zeta potential) for formulations (F1 to F4) are shown in Table 1. The average Zeta potential of bare poloxamer 188 nanoemulsion and curcuminoids loaded nanoemulsions were -19.13+/-1.17 mV (F1), -23.12+/-1.43 mV (F2), -20.04+/-1.35 mV (F3) and -17.54+/-1.29 mV (F4) respectively.

3.2. In vitro mucosa permeation studies

In vitro mucosa permeation studies were performed to compare the permeation of curcuminoids from 4 different nanoemulsion formulations (F1 to F4) and aqueous solution of curcuminoids (curcuminoids free form), all having the same quantity (0.25 mg/ml) of curcuminoids as shown in Table 2. In vitro mucosa permeation profile of F2 and F3 nanoemulsions was significantly lower compared with control (aqueous solution of curcuminoids free form) in 24 hours as shown in Figure 1 (* $P < 0.05$). The in vitro mucosa permeation profile of F4 was significantly different from other nanoemulsion formulations F2 and F3 (# $P < 0.05$). The droplet size of F4 was lowest of other formulations. The significant difference in curcuminoids permeation between nanoemulsion formulations and aqueous solution of curcuminoids free forms could be due to the mean size of internal phase droplets, which was different in the nanoemulsions.

*Table 2. Effect of Steady-state mucosa flux and permeability on the nanoemulsions. Data represents the Mean±SEM of at least three separate determinations. Statistical analysis was using t-test, with the significant differences determined at the level of *P<0.05 versus control group (Curcuminoids; Cur), # P<0.05 versus other formulation groups (F2 and F3).*

Formulation	Curcuminoids (mg/ml)	Steady-state mucosa flux (Jss)[a]	Permeability $x10^{-3}$ (P)[b]
F1	-	ND	ND
F2	0.25	0.53±0.22 *	2.13±0.86 *
F3	0.25	0.75±0.32 *	2.99±1.36 *
F4	0.25	1.40±0.39 #	5.61±1.55 #
Cur	0.25	1.69±0.30	3.69±1.27

Cur: Curcuminoids free form
ND: non detectable

(Mean±SEM, N=3)
Jss[a] (μg cm-2h-1)
P[b] (cm h-1)

3.3. Permeation data analysis

The graph between cumulative curcuminoids permeation and time was plotted for each

nanoemulsion formulation (Figure. 1). Permeability parameters like Jss and P^b were significantly decreased in nanoemulsions (F2:Jss 0.52+/-0.22, P^b 2.13+/-0.86 and F3:Jss 0.72+/-0.32, P^b 2.99+/-1.30) (Mean+/-SEM, *n*=3) as compared to the aqueous solution of curcuminoids (Jss 1.69+/-0.30, P^b 6.69+/-1.27) (* $P < 0.05$) as shown in Table 2. Aqueous solution of curcuminoids was used as control formulation for the determination of permeability parameters. The permeability parameters were significant for formulation of F4 as compared to other formulations (# $P < 0.05$). The values of Jss and P^b for formulation F4 were found to be 1.40±0.39μg/cm^2/h and 5.61 ±1.55 cm^2/h respectively (Table 2). The enhanced permeability parameters of F4 could be due to the presence of permeation enhancers such as poloxamer 188 [24].

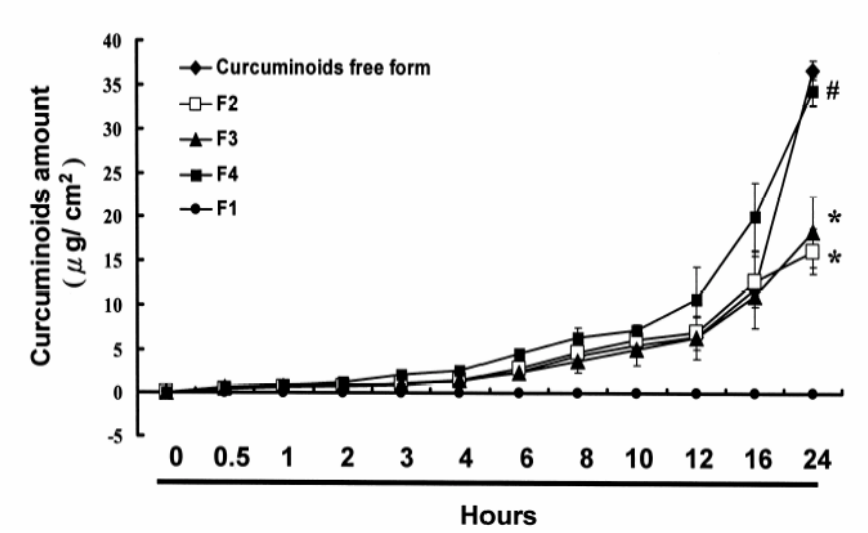

*Fig. 1. In vitro mucosa permeation. The permeation of curcuminoids from 4 different nanoemulsion formulations (F1 to F4) and aqueous solution of curcuminoids (curcuminoid free form). Statistical analysis was using t-test, with the significant differences determined at the level of *P<0.05 versus control group (Curcuminoids; Cur), # P<0.05 versus other formulation groups (F2 and F3).*

3.4. Non-cytotoxicity of bare poloxamer 188 nanoemulsion in colorectal cancer cell

The cytotoxicity of bare poloxamer 188 nanoemulsion in colorectal cancer cell lines (CP1 to 5) has been studied. While the cells were treated with formulation one (F1) for 24 to 72 hours, the survival was measured by MTS method. The results summarized in Figures 3 and 4 show that colorectal cancer cell line survival rates treated with bare poloxamer 188 nanoemulsion (Curcuminoids 0 μM) Poloxamer 188 from 24 to 72 hours was 105.61+/-4.26% to 95.40+/-3.31% (versus control, n=6). Statistical analysis of the result indicated that the bare poloxamer 188 nanoemulsion was shown to have no evidence of crisis or injury.

3.5. Effect of curcuminoids loaded nanoemulsions on the release of curcuminoids from nanoemulsions to mucosa.

The release of curcuminoids into mucosa from various formulations (curcuminoids only, F1 to F4) is shown in Fig. 2. Curcuminoids release from curcuminoids loaded nanoemulsions and accumulation in mucosa was generally elevated (Fig. 2.) (vs. 0 hours, * $P < 0.05$). The curcuminoids accumulation in mucosa had positive correlation with treatment time. Aqueous solution of curcuminoids was used as control formulation for the determination of mucosal accumulation parameters. The accumulation was significant for formulation F2 (29.92+/-3.59 μg/cm^2) and F3 (28.29+/-1.08 μg/cm^2) as compared to other formulations in 24 hours (# $P < 0.05$). The curcuminoids loaded nanoemulsion formulations (F2 and F3) could further increase the rectal

bioavailability of curcuminoids.

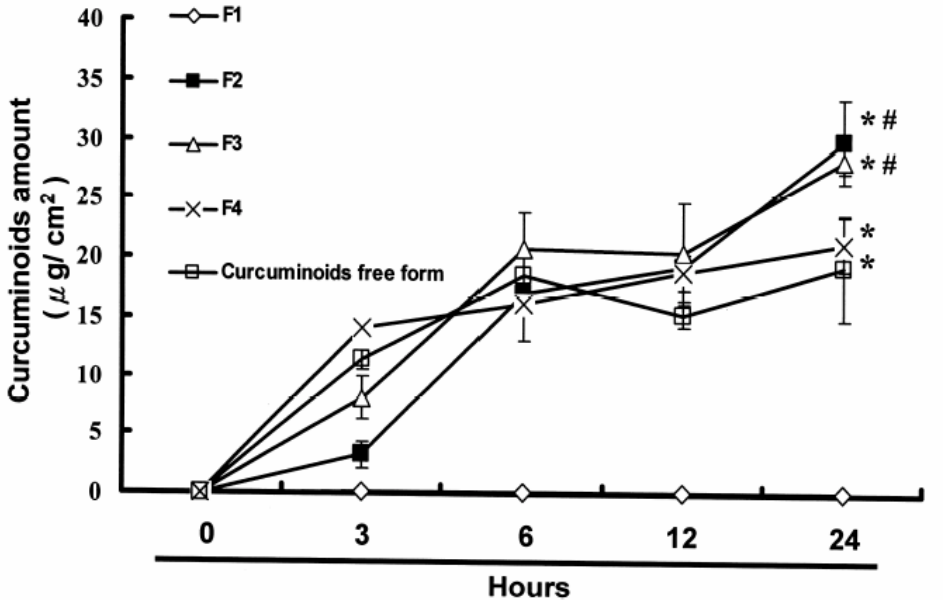

*Fig. 2. In vitro release and accumulation in mucosa. The accumulation of curcuminoids from 4 different nanoemulsion formulations (F1 to F4) and aqueous solution of curcuminoids (curcuminoid free form). Data represents the Mean±SEM of at least three separate determinations. Statistical analysis was using t-test, with the significant differences determined at the level of *P<0.05 versus control group (F1), # P<0.05 versus other formulation groups (F2 and F3).*

3.6. Chemoprevention of curcuminoids loaded nanoemulsions in human colorectal cancer cells

We hypothesized that curcuminoids loaded nanoemulsions could mediate the survival of primary colon cancer cells and thus inhibit their proliferation. To explore this anti-tumor activity of curcuminoids loaded nanoemulsions against primary colon cancer cells, we initiated an in vitro study and exposed the primary colon cancer cells to two formulations of curcuminoids loaded nanoemulsions (F2 and F3) for 24, 48 and 72 hours (Figures 3 and 4). The survival and proliferation of cancer cells were then assessed respectively by MTS assays. The results summarized in Figures 3 and 4 indicate that the survival and proliferation of the primary colon cancer cells both decrease as the doses of curcuminoids loaded into formulation in the cell culture increase, which show a dose- dependent reduction. Taken together, the observations imply that the curcuminoids loaded nanoemulsions have significantly elevated the death of all five primary colon cancer cell lines.

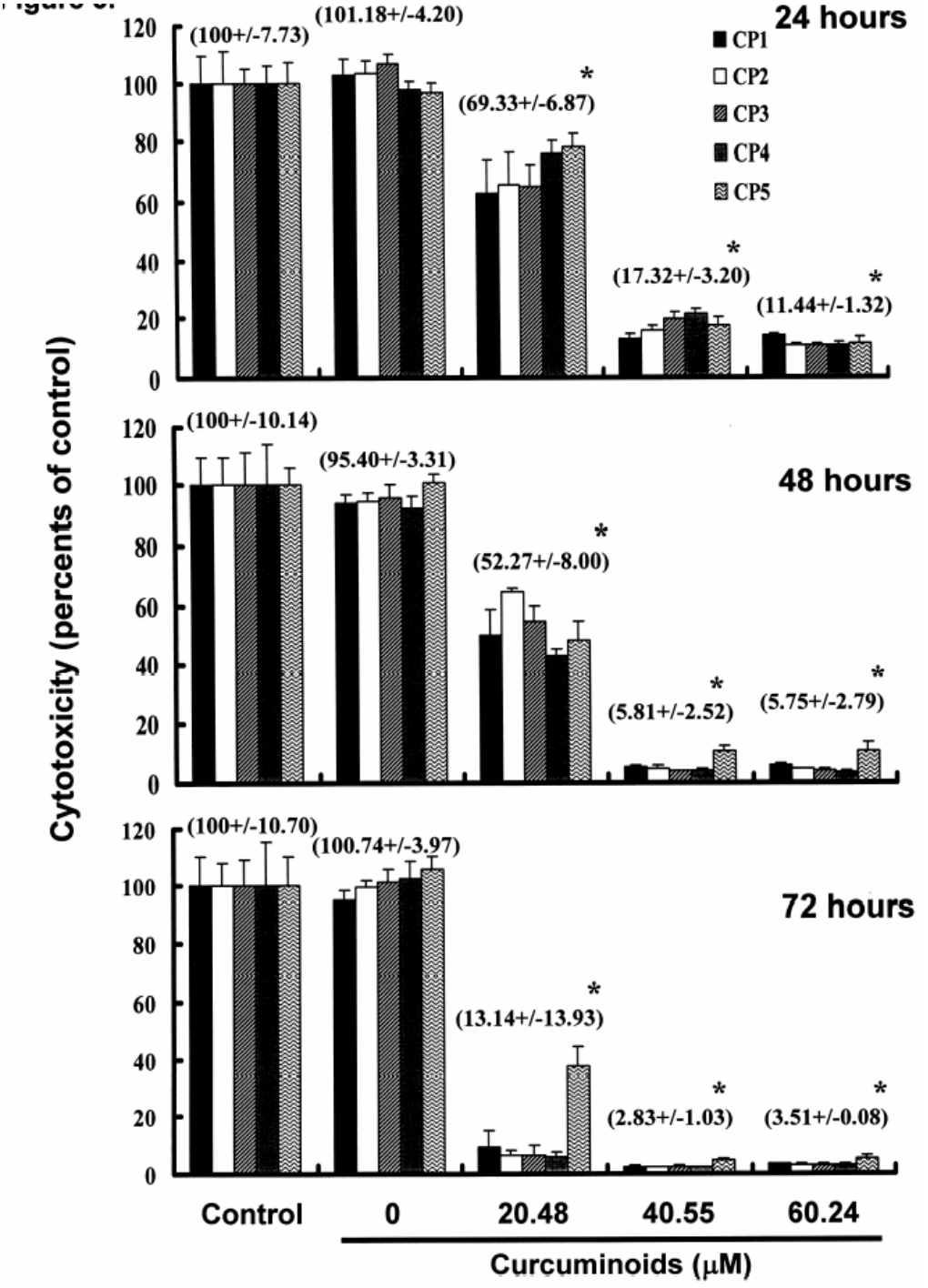

*Fig. 3. Curcuminoids loaded nanoemulsion F2 mediated the survival of CRC cancer cell lines (CP1 to CP5) and thus inhibited their proliferation. In vitro study was initiated by treating each of the CRC cell lines to the F2 (with curcuminoids 0, 20.48, 40.55 and 60.24 μM) for 24, 48 and 72 hours. The survival of these treated cancer cell was then measured by MTS method. Results were expressed as a percentage of control, which was considered as 100%. All data were reported as the Mean±SEM of at least three separate experiments. Statistical analysis was using t-test, with the significant differences determined at the level of *P<0.05 versus curcuminoids 0 μM group.*

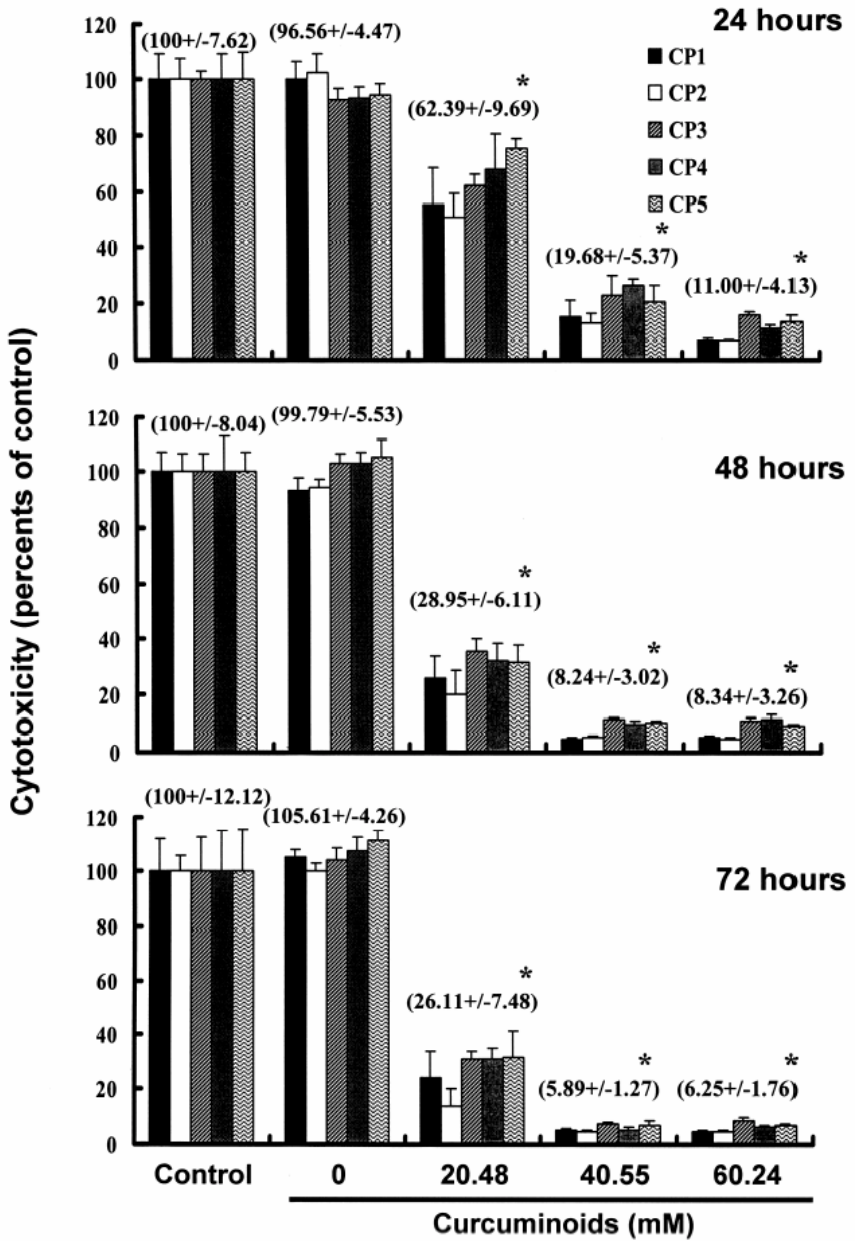

*Fig. 4. Curcuminoids loaded nanoemulsion F3 mediated the survival of CRC cancer cell lines. Results were expressed as a percentage of control, which was considered as 100%. All data were reported as the Mean±SEM of at least three separate experiments. Statistical analysis was using t-test, with the significant differences determined at the level of * $P<0.05$ versus curcuminoids 0 μM group.*

4. Discussion

Curcuminoids were found to produce, in a number of cell types, an anti-cancer activity and induce apoptosis [1-2].Our results collected in this series of studies with the cell lines of colorectal cancer cells (isolated from the colorectal cancer patients) have provided experimental evidence to indicate that curcumin could irreversibly induce the apoptosis of CRC cell lines [1].The bioavailability of curcuminoids, which was only 60% after oral administration [10], could be elevated through rectal administration [25].

The important criterion for selection of components is their pharmaceutical acceptability. It has been demonstrated that only very specific pharmaceutical excipient combinations lead to

efficient nanoemulsion formulations [26-27]. The solubility of the drug in oils is most important, as the ability of the nanoemulsion to maintain the drug in solubilized form is greatly influenced by the solubility of the drug in the oil phase. Among the oils, the solubility of curcuminoids was found to be highest in DMSO than EtOH (data not shown), but the DMSO has more toxicity to cells. Thus, EtOH was selected as the oil phase for the development of the formulations in this study.

If the surfactant is contributing to drug solubilization, the chosen one must be able to lower the interfacial tension to a very small value to aid the dispersion process during the preparation of the nanoemulsion, provide a flexible film that can readily deform around droplets, and be of the appropriate lipophilic character to provide the correct curvature at the interfacial region for the desired nanoemulsion type (ie, oil/water, water/oil, or bicontinuous) [28].

The droplet size analysis of the selected formulations showed that the size increased with the combination of curcuminoids in the formulation F2, but not in F3 and F4 (Table 1). This may have been due to the increase in the surfactant concentration from 14.92% to 22.38% (v/v), but the F4 has smallest droplet size and also worst mucosal accumulation. The results summarized in Figures 3 and 4 indicate that the survival of the primary colon cancer cells both decrease as the dose of curcuminoids loaded into nanoemulsion in the five CRC cell lines is increased, which show a dose- dependent reduction. The IC_{50} of F3 and F4 to the five CRC cell lines were 24.66 +/-1.30 μM (9.08+/-0.48 μg/ml) and 24.02+/-1.76 μM (8.85+/-0.65 μg/ml) in 24 hours treatment. Simultaneously, curcuminoids loaded nanoemulsions also induced apoptosis in the five CRC cell lines (data not shown). Therefore, it can be concluded that, for drugs that are sensitive to an extensive first-pass effect, rectal administration in the form of curcuminoids loaded nanoemulsion represents a promising approach for increasing the bioavailability of a drug (Figure 5).

5. Conclusions

Poloxamer 188 was the most effective nanoemulsion polymer for increasing the rectal bioavailability of curcuminoids. Among the curcuminoid release and mucosal accumulation parameters examined, Poloxamer 188 nanoemulsion containing curcuminoids appears to be one of the preferred formulations for these drugs.

Acknowledgments

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