CHAPTER IV
RESULTS

4.1. Reviewed tapioca derivative properties (Data from Starch Unit, Kasetsart University)

Tapioca derivative product was provided from Ban Pong Industrial Company. This polysaccharide polymers was prepared by the hydrolysis of tapioca starch, fractionated for specific profile of molecular weight range. The molecules are not the same size (polydispersity polymer) with a range of molecular weights distribution. Optimal molecular weight profile was defined. Its molecular weight molecules were similar to commercial corn derivative-based peritoneal dialysis fluid.

The molecular weight properties of tapioca derivative were reviewed. Approximately 90% of molecule is linked by α-(1-4) and less than 10% bonded by α-(1-6) glucosidic linkage (Table 4.1.1a). The α-(1-4) glycosidic bonds (dextrin molecule) can be catalyzed by enzymes, amylases, maltase in tissue in to maltose and eventually glucose. While, α-(1-6) glucosidic (dextran molecule) is more resistant to the normal body carbohydrases, so the polymer is expected much more slowly hydrolyzed.

Fig. 4.1A showed chromatogram from size exclusion chromatogramphy (SEC) displaying the molecules according to their size. The larger molecules do not have access to the pores and therefore pass sown the column earlier, while the smaller are retained by the porous structure of the column. The overlaid chromatograms of polydispersed molecular weights distribution patterns of tapioca derivative compared to corn derivative (peaks 1, 2 and 3). However, CPDF had slightly higher ratio of dextrose equibalent (DE) distribution of peak 2 than in TPDF

These results obtained from HPLC were consistent with those obtained from enzymatic method (Table 4.1A). It demonstrated also the similar characteristics of molecular weight distribution. These results indicated that the molecular weight distribution and degree of polymerization of tapioca derivative and corn derivative was similar (Table 4.1B). However, the percentage of a smaller molecule of tapioca is slightly higher than in corn derivatives, which presented by having higher reflective index (RI) response, as showed in Fig. 4.1A.

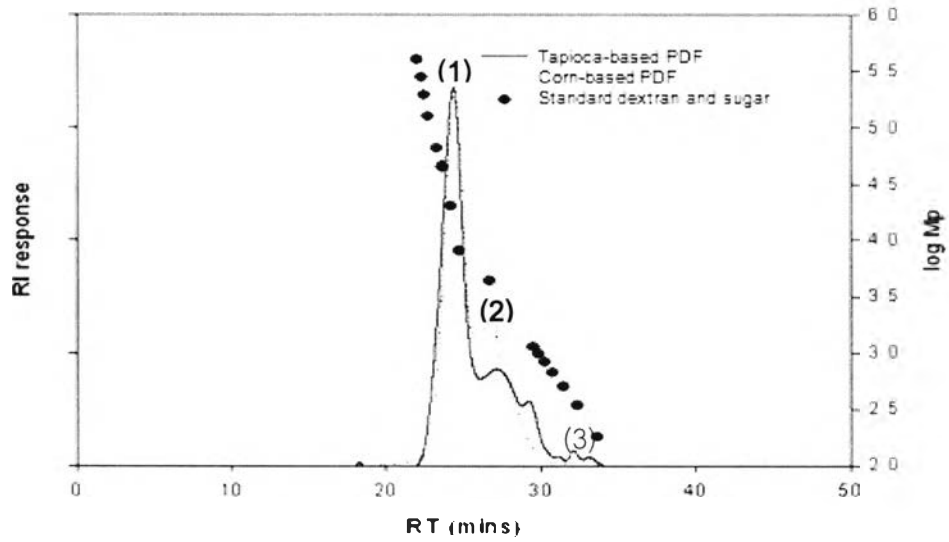

Figure 4.1A: Chromatographic analysis on molecular weight distribution using Ultrahydrogel 250 and mobile phase 0.02M phosphate buffer in 250 mM NaCl.

Table 4.1A summarizes the results of branching percentage, number average molecular weight (Mn), weight average molecular weight (Mw), and average degree of polymerization (DP) compared between the two tapioca and corn derivative polymers.

Table 4.1A: Structural properties of glucose polymer derivative powders

Parameters	Analysis	Tapioca	Corn
	Methods	derivative	derivative
%Branching	β-amylolysis limit	7.5-7.7	5.7-6.3
	By enzymatic & colorimetric method*		<10
	Proton Nuclear Magnetic	7.2-7.8	5.86
	Resonance (^{1}H NMR)		6
MWw	Gel Permeation Chromatography	12,000	13,000-19,000
MWn		6,200	5,000-6,500
% Polydispersity		2.90	2.4-3.0

* Enzymes pullulanase is specific to small polymer molecule such as maltose, while isoamylase is specific to maltotriose and maltotretabose. Using isoamylase and

pullulanase enzymes to digest α-1,6 glucosidic linkages and polysaccharide was obtained in chain length distribution with different specificity digestion.

The physiochemical properties of glucose polymer property are dependent on their molecular distribution and oligosaccharide profiles. Whether, this difference property reflects on different reaction of water transportation have been also investigated and that will be discussed in later part.

Table 4.1B showed the molecular weight characteristics of TPDF compared to CPDF. Lot 1 was the first production of tapioca derivative form. This had Mw, Mn and polydisperse polymer higher than corn derivative. However, after it has been development so far, the latest production lot 4 showed more similarity molecular weight distribution, Mw, Mn and polydispersibility to corn derivative starches, but lower percentages of higher Mw molecule (DP13-24 and DP≥37) 40.81 and 3.71 in tapioca compared to 44.78 and 4.03 in corn derivatives, respectively. The higher percentage of Mw of molecule (DP6-12 and DP25-36) was 37.54 and 17.94 compared to 36.61 and 14.58 observed in tapioca compared to corn derivative respectively.

Table 4.1B: the molecular weight property of tapioca derivative compared to cornstarch derivatives.

Sample		% Molecular weight distribution				Mw* (Dalton)	Mn**	Polydis-persibility
		DP6-12	DP13-24	DP25-36	DP≥37			
Tapioca	Lot1	37.71	40.71	17.48	4.10	16429±362	4782±254	3.44±0.15
	Lot2	40.02	40.38	16.73	2.88	10711±586	3672±94	2.92±0.21
	Lot3	39.87	40.89	16.43	2.88	10787±356	3656±127	2.95±0.17
	Lot4	37.54	40.81	17.94	3.71	13832±95	4951±252	2.80±0.15
Cornstarch		36.61	44.78	14.58	4.03	11322±415	4389±215	2.58±0.13

*Average molecular weight by weight (Mw) by size exclusion chromatography

**Average molecular weight by number (Mn) by size exclusion chromatography

Tapioca derivative-based peritoneal dialysis fluid (TPDF) preparation at CU

The composition of PDF was compared; the electrolyte formulas of TPDF were composed similar to CPDF and GPDF, only sodium chloride, which had 2 mg higher than in CPDF. The osmolality's TPDF were increased, the more tapioca derivative concentration and the higher osmolarity was observed. At percentage of interested 7.5%TPDF, it had similar osmolarity value to 7.5% CPDF, while the pH of TPDF had more physiological equivalent to 6.6 compared to CPDF of pH 5.27(Table 4.1C). Autoclaving is the acceptable sterilization method that has been used in industrial sterilization process including peritoneal dialysis preparation. The result from this study indicated that if tapioca derivative is less than 10%, autoclave condition at 121°C for 20 minutes did not have any significant effects on osmolarity and pH or physical characteristics change compared to filtration.

Table 4.1C: Compositions of peritoneal dialysis fluids

	TPDF				7.5% CPDF	1.5% GPDF
	5%	7.5%	10%	15%		
Glucose (g/dL)	-				-	1.5
Corn derivative (g/dL)					7.5	-
Tapioca derivative (g/dL)	5	7.5	10	15	-	
Sodium Chloride; NaCl (mg)	540	540	540	540	538	538
Sodium Lactate; $C_3H_5NaO_3$ (mg)	448	448	448	448	448	448
Calcium Chloride; $CaCl_2$ (mg)	25.7	25.7	25.7	25.7	25.7	26.7
Magnesium Chloride; $MgCl_2$ (mg)	5.08	5.08	5.08	5.08	5.08	5.08
Osmolarity (Osm/Kg)	275	295	304	339	284	291
pH (pH4=4.0, pH7=6.98)	6.72	6.60	6.70	6.51	5.27	5.13

Fig. 4.1B presented the osmolarities of TPDF compared to commercial PDFs. The osmolarities were not significant changed between varies concentration of TPDF compared to 7.5%CPDF and 1.5%GPDF, but it was significantly different compared to 4.25% GPDF ($p<0.05$)

(A)

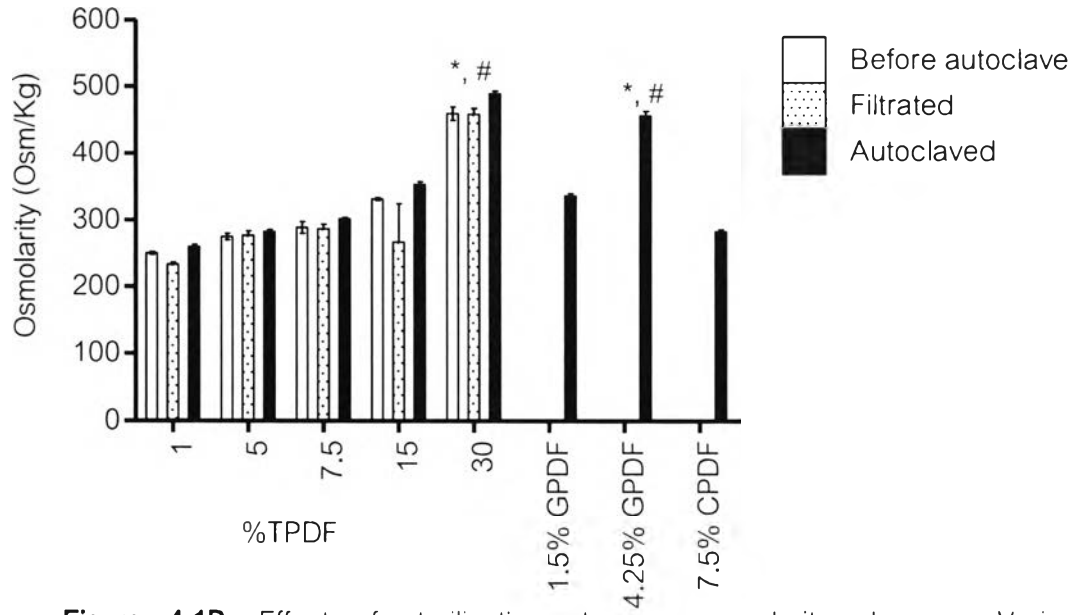

Figure 4.1B: Effect of sterilization step on osmolarity changes. Varies concentration of TPDFs 1, 5, 7.5, 15% showed not statistic significantly difference (Paired *t*-test) of by either autoclaved (black bars) or filtrated (dot bars) compared to before (white bars), but the osmolarity increased significantly if they were compared (unpaired t-test) to both 30%TPDF ($^{*}p<0.05$) and compared to 4.25%GPDF ($^{\#}p<0.05$)

Thirdly, pH changes were examined before and after sterilizations. Fig. 4.1C showed that, the pH decrease significantly if increase the tapioca derivative percentage by dose dependent manner in all three conditions (before, after filtrate or after autoclaved) ($p<0.05$). Sterilization by autoclave made pH significant trend of decline than before and filtration states ($p<0.05$), while filtration was declined but it was not difference compared to before sterilization step.

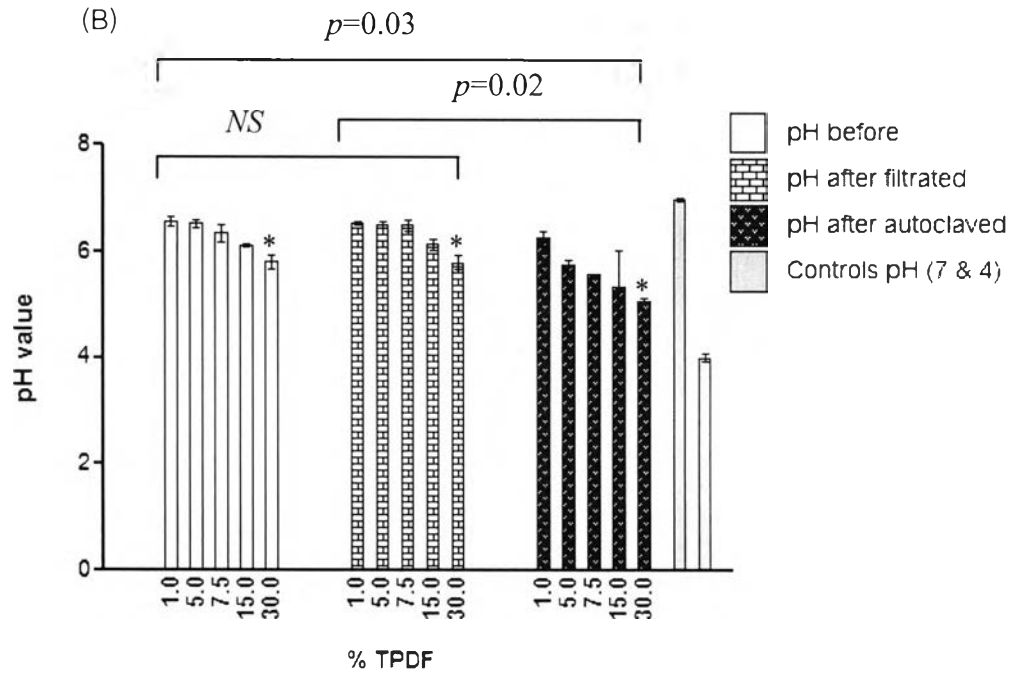

Figure 4.1C: Effect of sterilization process on pH of TPDF changes. There was not statistic significantly difference by paired *t*-test between before (white bars) and after filtration (stratified bars) and after autoclaved (black-dot bars), except autoclaved of 30%TPDF, its pH was significantly lower (unpaired *t*-test) compared to other control pH 7.0 (*p<0.05).

Glucose degradation product generation after preparation

After TPDF were prepared at KUBMED laboratory, the GDPs (Hydroxymethyl-furaldehyde (5-HMF), Glyoxal (GO), Methylglyoxal (MGO) and 3-deoxyglucosone (3-DG) were quantified at Kasetsart Universtiy. The variation of HPLC analysis was less than 10% (data not showed).

Table 4.1D showed the analysis of Methylglyoxal (MGO) and 3-deoxyglucosone (3-DG) concentration. The level were higher detected in 4.25%GPDF> 1.5%GPDF> polyglucose based PDFs, while Glyoxal (GO) was higher observed in polyglucose TPDF than in CPDF if stored TPDF in higher temperature, but of that GO level was lower detected than in 1.5%GPDF.

Table 4.1D: Levels of Glyoxal (GO), Methylglyoxal (MGO), 3-deoxyglucosone (3-DG) in glucose and glucose polymers based PDFs

(Data analyzed from Starch Unit, Kasetsart University)

lot		GDPs (μmol/L)			
		5-HMF	GO	MGO	3-DG
4.25% GPDF	Storage at RT	22.84±0.07	47.3±5.70	6.20±0.00	211.70±13.10
1.5% GPDF		15.49±0.08	43.8±0.00	3.00±0.00	83.10±2.40
7.5% CPDF		2.61±0.01	15.9±1.40	1.60±0.00	8.30±1.30
7.5%TPDF (baseline)		2.28±0.01	N/A	3.4±0.1	21.1±2.1
7.5% TPDF (6 Months) Storages	4°C	2.20±0.02	N/A	4.00±0.30	21.13±2.19
	RT	2.74±0.05	13.30±2.80	2.80±0.20	19.13±1.63
	37 °C	5.69±0.01[a,b,c,d]	36.90±3.90[e,f,g]	5.60±0.50	14.00±0.94

[a, b, c] Significantly two fold higher than storage at baseline and RT, 7.5%CPDF, respectively ($p<0.05$)

[d] Significantly lower than either 1.5%GPDF and 4.25%GPDF ($p<0.05$)

[e, f, g] Significantly two fold higher than storage at RT, 7.5%CPDF, and either 1.5% or 4.25%GPDF, respectively ($p<0.05$)

4.2 Cytotoxicity and toxicity testing

4.2.1 Cytotoxicity testing

80-90% confluent monolayers of Human peritoneal mesothelial cells (HPMC) was harvested within 6 days after the cells were propagated (Fig. 3.2.1, upper row). Then the cells were sub-cultured and the second or third passage was obtained used for cytotoxicity testing (Fig. 4.2.1, lower row).

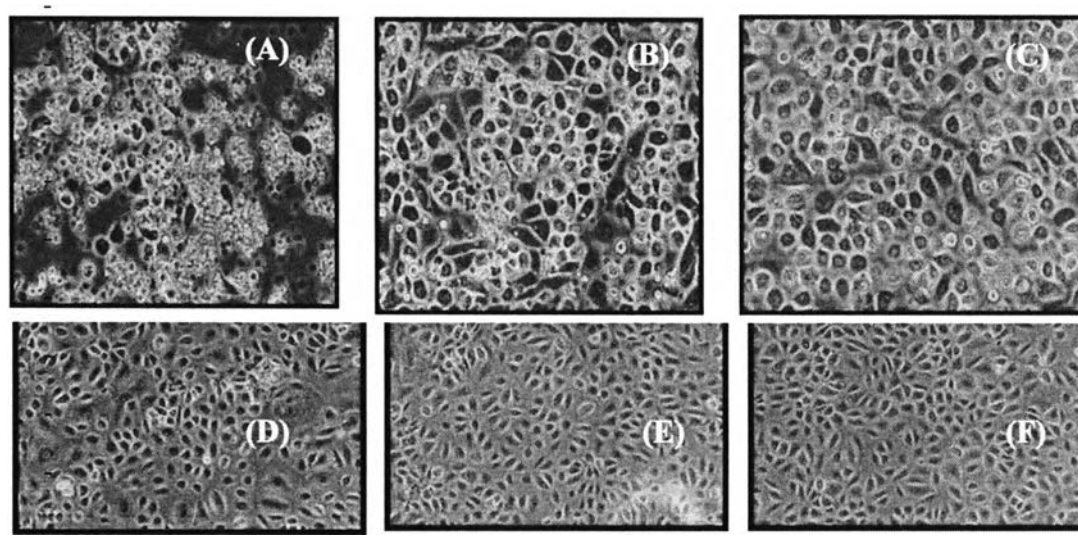

Figure 4.2.1: HPMC cultures presents in days 2nd, 4th, 6th (A, B, C) and passages 1st, 2nd, 3th (D, E, F), respectively.

Human mesothelial morphology change studies

The cell morphology remained normal during the experiment. Although treated with high concentration of 15%TPDF, it had no significant effect on the cell morphology changes when compared to positive control. Slightly morphological changes were observed in cells which were exposed to conventional even 1.5%GPDF. The changes were reduced if the cells were incubated with either 7.5% polyglucose TPDF or CPDF (Fig. 4.2.1A).

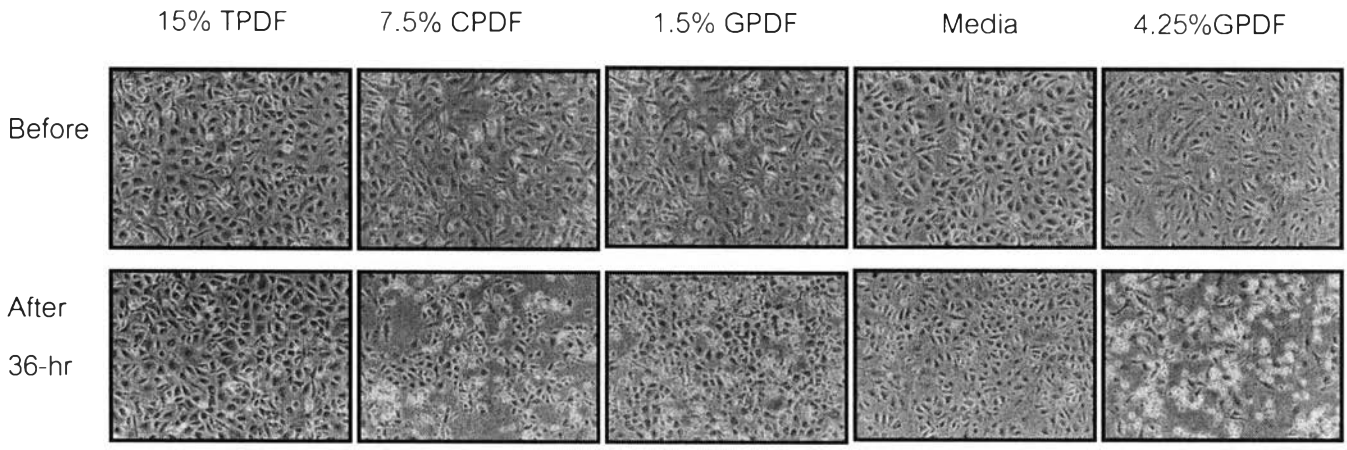

Figure 4.2.1A: PDFs induced mesothelial cell morphological changes. Normal morphology of the cells before the treatment was in upper panel and after the incubation with PDF for 36 hours was in lower panel. 15%TPDF treatment group showed little morphology changed comparing to GPDF. % morphology changes were graded as described in Table 4.2.1A.

Mophology changes were grading, polyglucose preserved better cell morphology compared to especially 4.25%GPDF, even 15% TPDF treated cells showed less morphology change compared to high glucose based PDF and controls. The rate of change were graded as presented in Table 4.2.1A.

Table 4.2.1A: Evaluation of HPMC morphology changes ater 36 hours treatment

Solution	% Morphology changes	% Death cells
15% TPDF	10-20	15
7.5% CPDF	15-20	14
1.5% GPDF	20-30	28
Media control	<10	<10
Positive control	>80	>80

Determination of HPMCs injury

Exposure of HPMC to adjusted pH7.4 PDFs for 6 hours; cell injury was assessed by lactate dehydrogenase (LDH) release. It was found that negative control (mesothelial cell monolayers exposed to HAM-F12 4% v/v albumin) was LDH released 21%. As expected, A significant reduction of cell viability was observed in the group which treated with 4.25% GPDF by increased of % LDH released up to 71% (p<0.01). Cell treatment with 1.5%GDPF and polyglucose groups showed similar levels of LDH release and the cell viability was more preserved significantly compared to 4.25%GPDF as presented in Fig 4.2.1B.

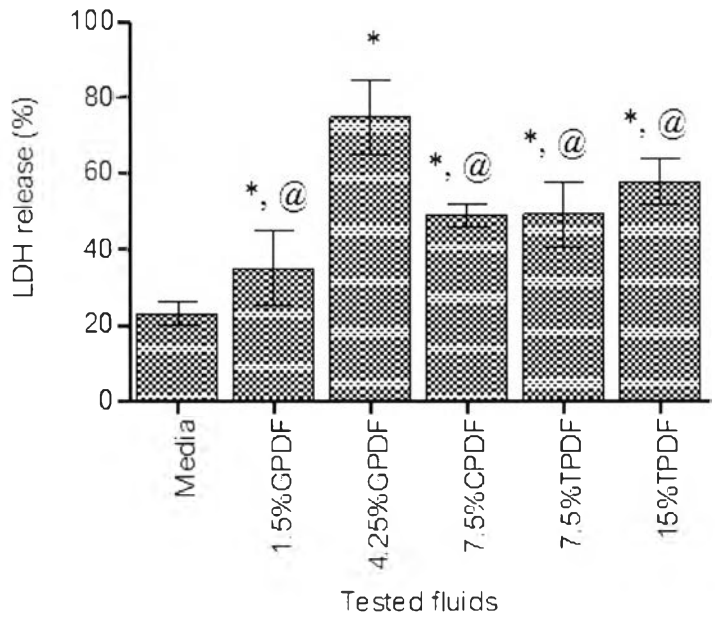

Figure 4.2.1B: Effect of peritoneal dialysis fluids on lactate dehydrogenase (LDH) released by human peritoneal mesothelial cells (HPMCs). After serum depletion, HPMCs were incubated with various experimental conditions and medium control solution (as negative control; NC), incubated up to 18 hours, the supernatants and pellets were collected to detect LDH release. All groups were compared using one-way ANOVA (Bonferroni Test), *p < 0.01 vs control, @p<0.05 vs 4.25%GPDF

HPMC cell death

Pararell with morphology change and cell injury evaluation, apoptotic cells were assessed by Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) assay. The cells were counterstained by propidium iodide (PI), accordingly. dUTP incorporated to DNA nuclear fragmentation of apoptotic cells. 7.5% polyglucose TPDF reduced significantly the number of HPMCs undergoing apoptosis compared to 4.25%GPDF (18% vs 29%; $p<0.05$), and similar effects as 1.5%GPDF and CPDF, respectively as presented in Fig. 4.2.1C.

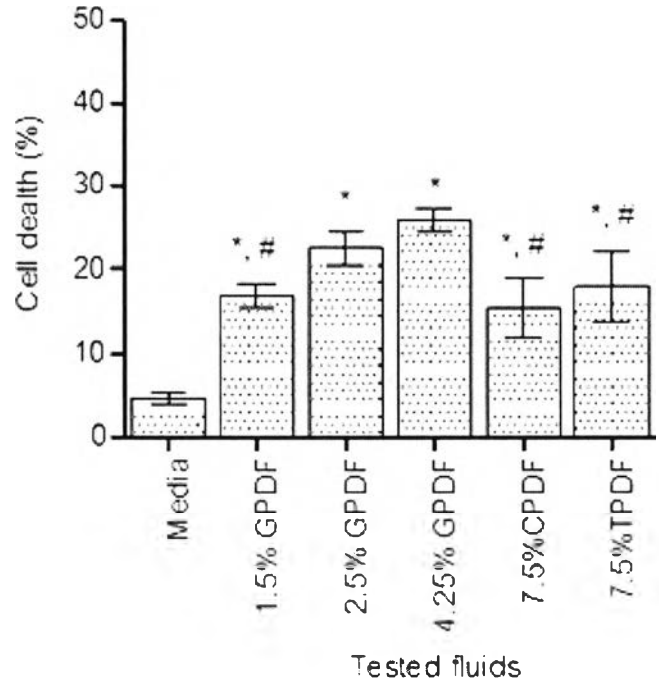

Figure 4.2.1C: Effect of PDFs on mesothelial cells death by TUNEL/PI Assay. PDFs induced HPMC death at 18-hour counted by Flow Cytometer. Using one-way ANOVA (Bonferroni Test), % cell death of all groups were higher compared to medium control ($^{*}p < 0.01$) and significant lower if compared to 4.25% ($^{\#}p<0.05$).

Fibroblast 3T3cell line cytotoxicity testing

Cell injury by LDH release

After PDFs treatment, LDH released was quantified. There was no evidence of significant cell toxicity difference between polyglucose TPDF and CPDF and 1.5%GDPF as assessed by LDH release during 60 minutes with 'recovery' phases of 24 hours. A significant difference was found when TPDF compared to high glucose based PDF (Fig. 4.2.1D)

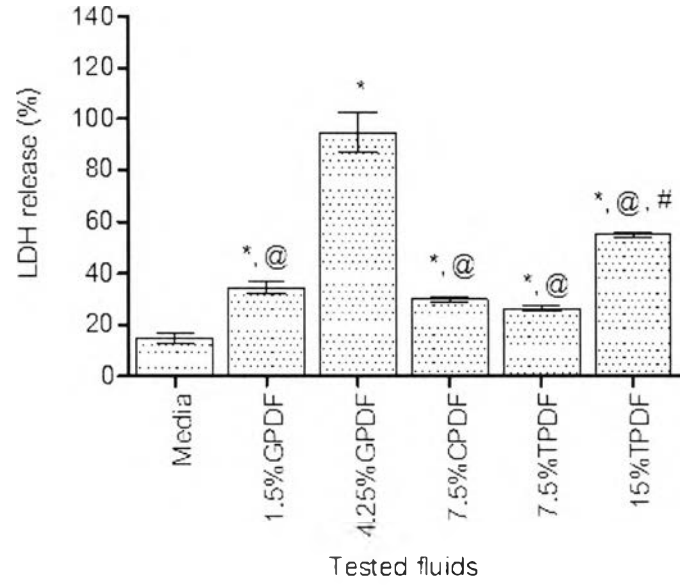

Figure 4.2.1D: Effect of PDFs on lactate dehydrogenase (LDH) released by 3T3 fibroblast cell line. After depleted serum, cells were incubated for 60 minutes with PDFs adjusted pH and medium controls. LDH levels observed in 7.5% polyglucose groups were not significantly different using one-way ANOVA (Bonferroni Test). LDH levels of all groups were significant higher compared to medium control ($^{*}p < 0.01$), but significant lower compared to 4.25%GPDF ($^{@}p<0.01$). 1.5%GPDF, 7.5%CPDF, TPDFs ($^{\#}p< 0.05$), respectively.

3T3 cell viability

The cell viability was not statistically different among pH adjusted group. However, if pH was not adjusted, the cell viability was significantly reduced, especially in 4.25%GPDF group from 78% to 42% ($p<0.05$). TPDF did not show different effect on cell viability between adjusts and none adjusted pH (Fig. 4.2.1E). This may indicate that the glucose polymer TPDF alone did not effect to the cell line.

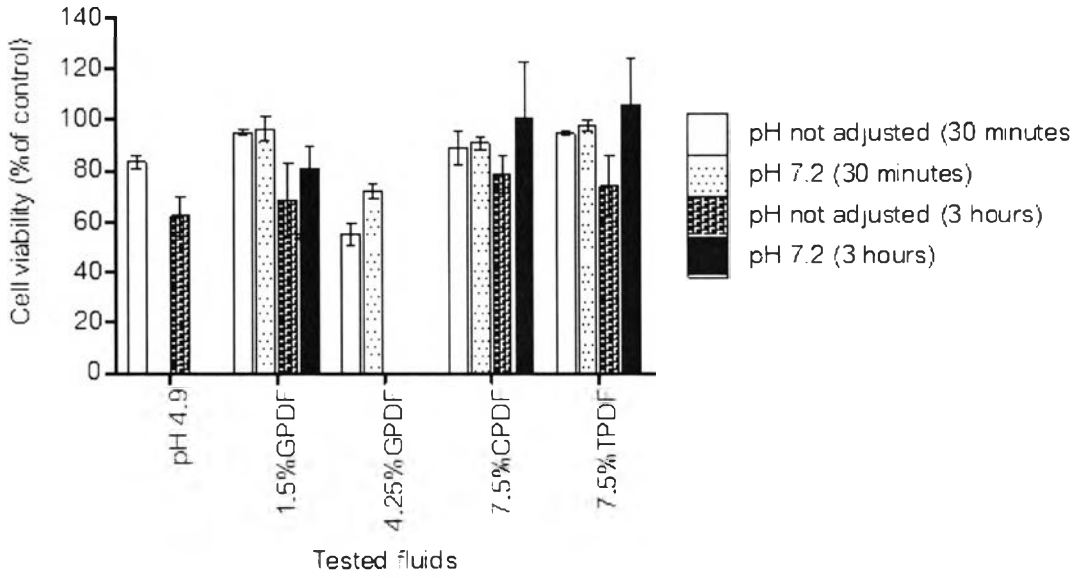

Figure 4.2.1E: Effect of PDFs with and without adjusted pH on 3T3 cell viability by MTT assay. The cells exposure to either pH adjusted and not adjusted PDFs for 30 minutes and 3 hours with recovered in culture medium for 15 hours.(*p<0.05)

Effects of tapioca derivative on 3T3 cell viability

3T3 cells line were pre-exposed to pH adjusted PDF for 24 hours and then allowed to recover in control medium for 15 hours, after which viability was assessed with the MTT test. The results were derived from triplicate experiments. pH adjusted and not adjusted of 30% tapioca derivative in media represented high percentage of cell viability calculated from media control (Fig. 4.2.1F).

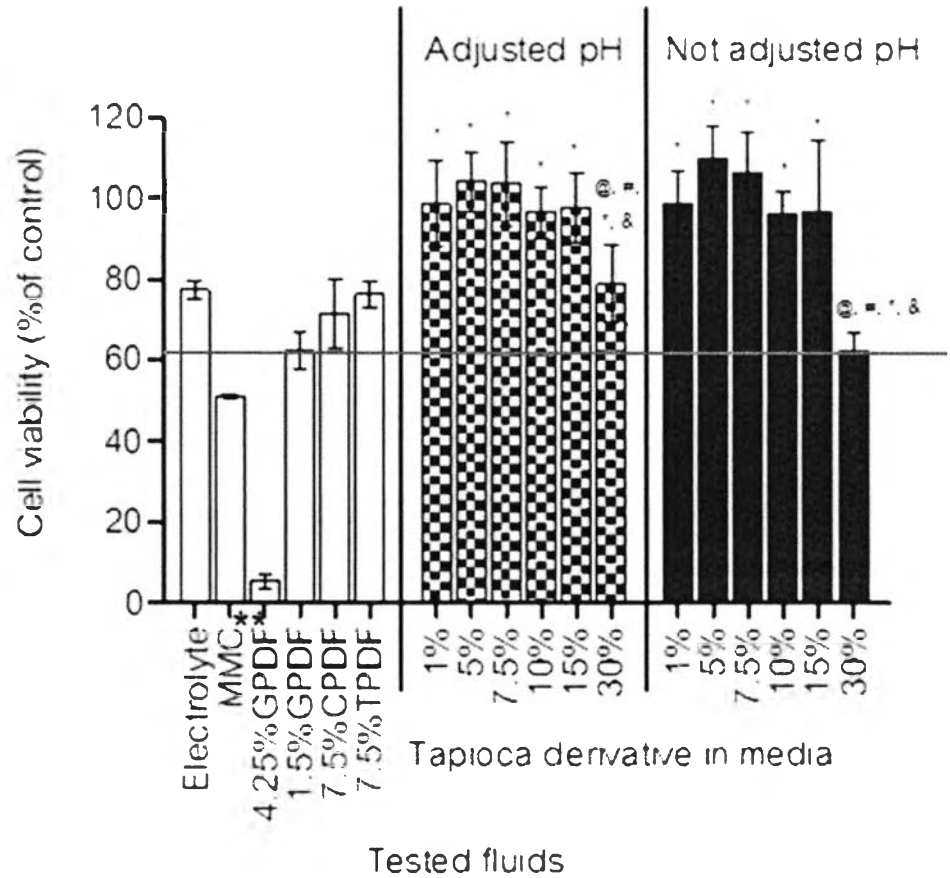

Figure 4.2.1F: Effects of tapioca derivative on 3T3 cell viability by MTT assay. Using one-way ANOVA unpaired t-test, each tapioca derivative in media preserved cell viability higher than media with electrolytes (*$p<0.05$), except 30% tapioca derivative in media showed a statistic significantly reduction of cell viability when compared to each tapioca derivative concentration ($^{\#}p<0.05$). However, it presented better effect on cell viability when compared to 4.25%GPDF ($^{@}p<0.01$) and similar effect to 1.5%GPDF ($^{\&}p<0.05$).

Effects of PDFs on 3T3 cell proliferation

The effects of diluted and undiluted PDFs on 3T3 cell proliferation were tested. The cells exposure to pH adjusted for 24 hours and recovered in culture medium for 15 hours. The cell viability was not statistically different among pH adjusted group; this indicated that the glucose polymer TPDF did not effect to the cell viability. If PDF was undiluted, the more effect on the cell viability was found (Fig. 4.2.1G).

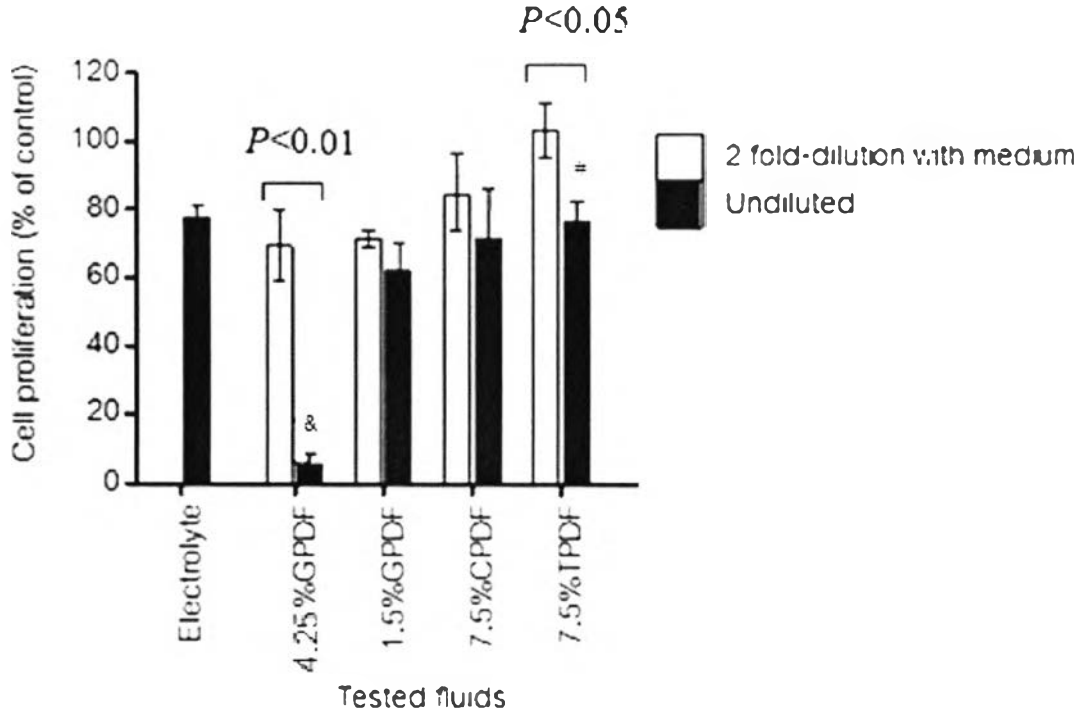

Figure 4.2.1G: Effects of PDFs on 3T3 cells proliferation by MTT Assay.

The 3T3 cells were treated with 100 ul of solutions for 24 hours and recovered for 15 hours. The average growth percent values over all assays were compared. Statistical comparisons were performed using one way ANOVA unpaired t-test. When compared between diluted and undiluted PDFs effect on cell proliferation, the difference was found in undiluted 4.25% GDFP ($^{\&}p<0.01$). Undiluted 7.5%TPDF reduced cell proliferation compared to the diluted two fold TPDF. However, the undiluted TPDF showed similar effect when compared to 7.5%CPDF, 1.5%GPDF and electrolyte in medium control, and better preserved when compared to 4.25%GPDF ($^{\#}p<0.01$).

Peripheral blood mononuclear cells (PBMC) cytotoxicity testing

Effects of PDFs on PBMC cell death

% cell death were not different when compare between TPDF, CPDF and 1.5% GPDF at 6 hours and the percentage of cell intact was decreased at time 24 hours but not statistically different among all PDFs groups. Except 4.25%GPDF in both conditions 6 and 24 hours and 1.5%GPDF at 24 hours. This indicated that all PDFs used in this study have not different biocompatibility effects on PBMC accordance with %cell death as showen in Fig. 4.2.1H.

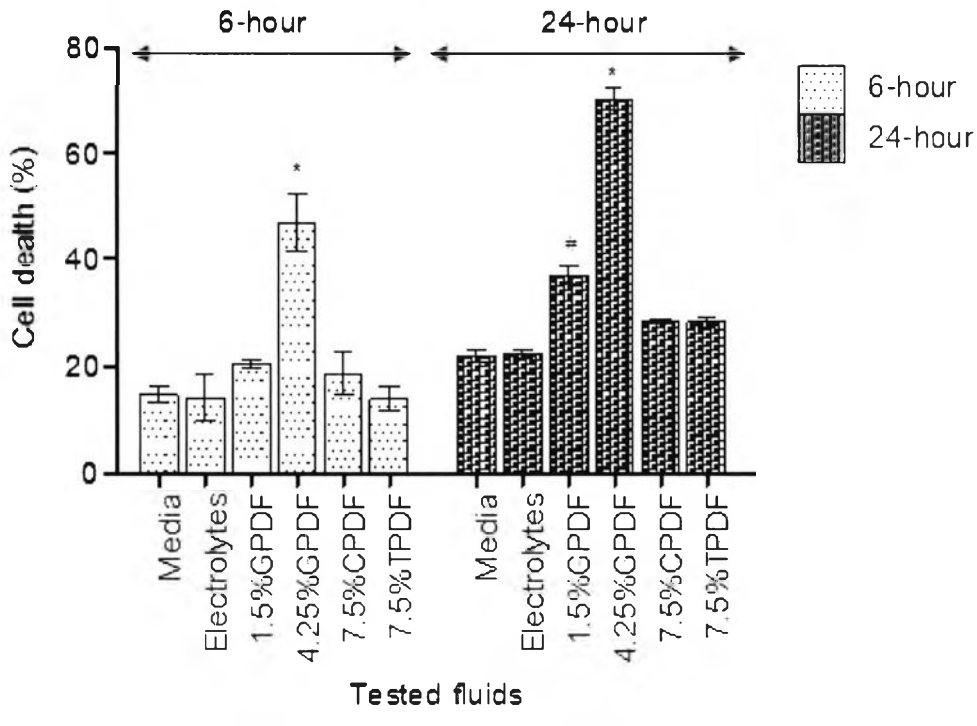

Figure 4.2.1H: Percentage of PBMC death by FITC-Annexin V/PI staining/ flow cytometry. Using One way ANOVA analysis (unpaired *t*-test), %cell death of 4.25%GPDF was significant increased comparing to all groups in both conditions at 6 hours and at 24 hours (*$p<0.01$), 1.5%GPDF was significant increased of cell death when compared to either media or medial with electrolytes at 24 hours experiments (#$p<0.01$).

Effects of PDFs on PBMC cell viability

As well as % cell viability were not different when compare between TPDF, CPDF and 1.5% GPDF at 6 hours and the percentage was slightly decreased when treated time up to 24 hours but not statistically different. This indicated that all TPDFs used in this study did not significant effect on PBMC viability (Fig. 4.2.1l).

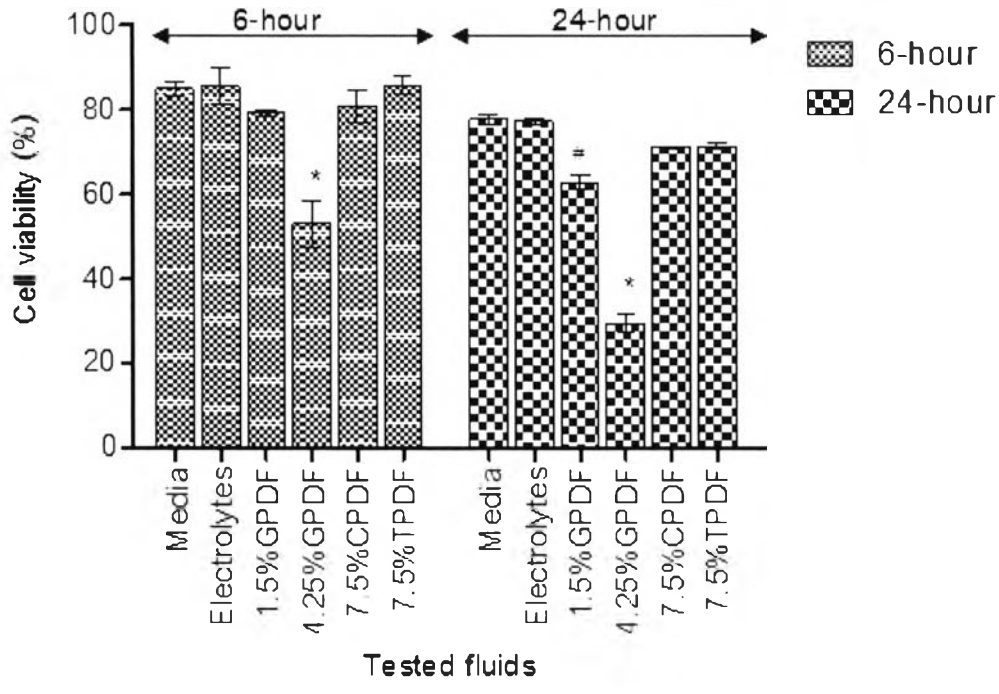

Figure 4.2.1l: Percentage of PBMC viability by FITC-Annexin V/PI staining/ flow cytometry. Simultaneously, %cell viability was reduced among cells treated with 4.25% GPDF in both 6 and 24 hours ($^{*}p<0.01$) and 1.5%GDPF treated group at 24 hours ($^{\#}p<0.05$)

Effects of PDFs on PBMC proliferation

All group showed not different effect on the proliferation at 1 hour growth, except the hypertonic 4.25%GDPF which had the most adverse effect on PBMC proliferation at 1 hour. The cell proliferation was statistic significantly reduction among the TPDFs and 4.25%GPDF compared to either medium or electrolyte controls at 48 hours (Fig. 4.2.1J).

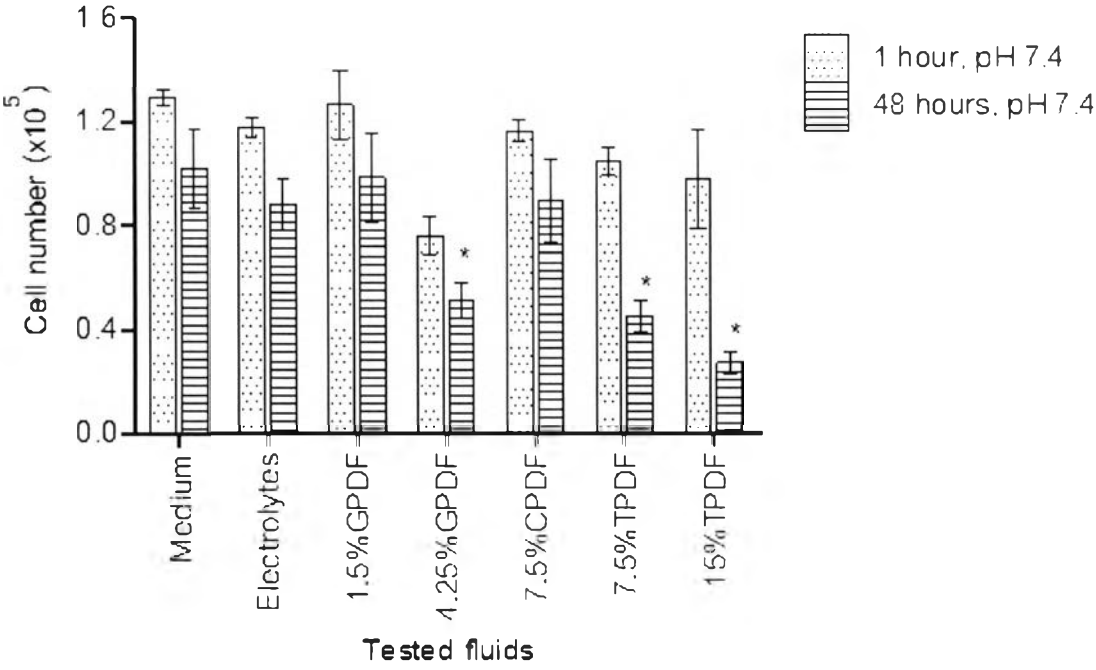

Figure 4.2.1J: Effect of PDFs on PBMC proliferation. PBCM were pretreated to tested fluids for 60 minutes, and followed up again at 48 hours. (N=4 for each group). There was statistical significance versus medium control at the corresponding time point (*$p<0.05$).

4.3 Toxicity testing in animal

The useful results of biocompatibility testing for new PD solutions, besides the involving of *in vitro* peritoneal cells cytotoxicity testing such as in human peritoneal mesothelial cells fibroblast and immune cells, it can be better addressed using *in vivo* animal toxicity testing models to permit the analysis of biocompatibility under conditions that allow insight physiology and dynamic pathologic changes that more closely mimic into the clinical situation.

4.3.1 Acute Intravenous (I.V) Toxicity Test in Mice

Acute 14-day intravenous toxicity test was studied in mice injected with 15% TPDF (dose 5 ml/Kg) compared with control group injected with 0.9% normal saline solution (NSS). Test results, including clinical signs, mortality and necropsy finding, are evaluated. There was no mortality and no clinical signs were noted. No abnormalities of necropsy finding were detected. Results details are in Table 4.3.1A.

Table 4.3.1A: Acute Intravenous Toxicity Test in Mice.

Animal No:	Sex	Mortality	Clinical signs	Necropsy findings
1	15% TPDF	Male 0/4	NAD	NAD
2	15% TPDF		NAD	NAD
3	15% TPDF		NAD	NAD
4	15% TPDF		NAD	NAD
5	15% TPDF	Female 0/4	NAD	NAD
6	15% TPDF		NAD	NAD
7	15% TPDF		NAD	NAD
8	15% TPDF		NAD	NAD
9	0.9% NSS	Female 0/1	NAD	NAD
10	0.9% NSS	Male 0/2	NAD	NAD
11	0.9% NSS		NAD	NAD

NAD= no abnormalities detected

Body weight monitoring

There were no differences in the weekly mean body weight between controls and the animal treated with the 15%tapioca derivative based with electrolytes (TPDF) in both female and male groups, except on day7 and day14 male group had increased of body weight with 6.45% body weight changes (Table 1.3.1B). However, body weight gains of male were acceptable since here were no significant differences in both male and female compared to control group (Fig. 4.3.1A)

Table 4.3.1B: Mean of body weight and its %change (acute toxicity testing, I.V)

Monitoring time point	Mean of body weight (mg)		% Body weight changes*	
	Male (n=4)	Female (n=4)	Male (n=2)	Female (n=1)
7 days before	30.05	30.10	0.33	0.66
day0	30.25	30.11	-1.65	0.58
day7	38.75	30.28	6.45	1.49
day14	38.75	30.10	6.45	0.66

*%Difference= 100x(test-control)/average) versus average

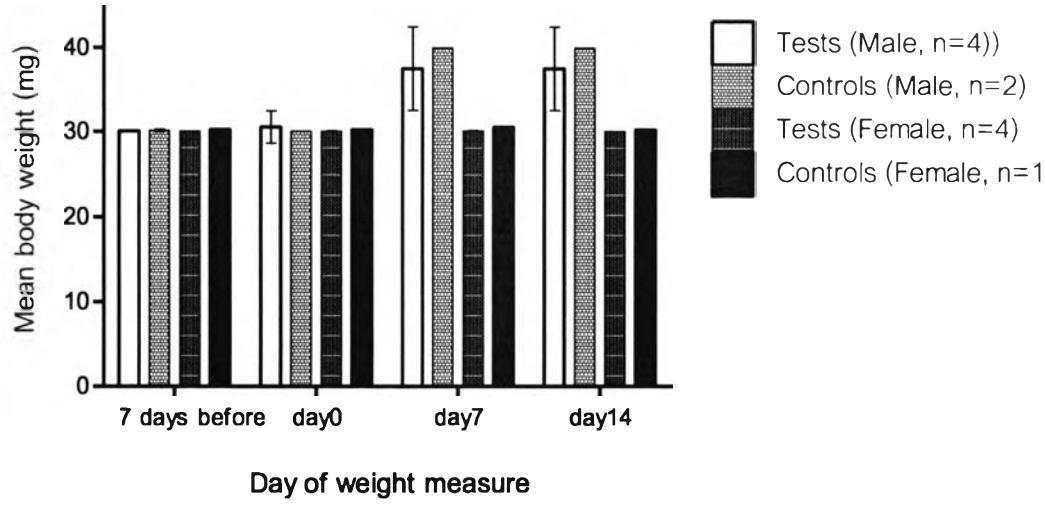

Figure 4.3.1A: Body weight monitoring by group/sex among acute intravenous toxicity (Limit) 14-days testing in mice. One way ANOVA (Bonferroni's multiple comparison test), there was not statistical significant differences between test and control groups in both male and female.

Organ weight monitoring

Table 4.3.1C showed the results of organ weight per body weight. There were no significant differences those comparisons of organs liver, spleen, heart, pancreas, kidneys between the controls and the tested animals injected with 15%TPDF (Fig.4.3.1B).

Table 4.3.1C: Organ weight per body weight monitoring (Acute Toxicity Test in Mice, I.V)

I.V Dose	Test (n=8) ($X10^{-3}$)	Control (n=3) ($X10^{-3}$)	Unpaired *t*-test (Mann Whitney test)
Liver	52.55±3.12	64.67±1.51	Not significance
Spleen	4.68±0.52	3.95±1.01	Not significance
Heart	5.39±0.60	7.26±1.32	Not significance
Pancreas	4.08±0.62	6.86±2.40	Not significance
Lt Kidney	7.37±0.53	5.40±1.38	Not significance
Rt. Kidney	7.11±0.61	5.32±1.58	Not significance

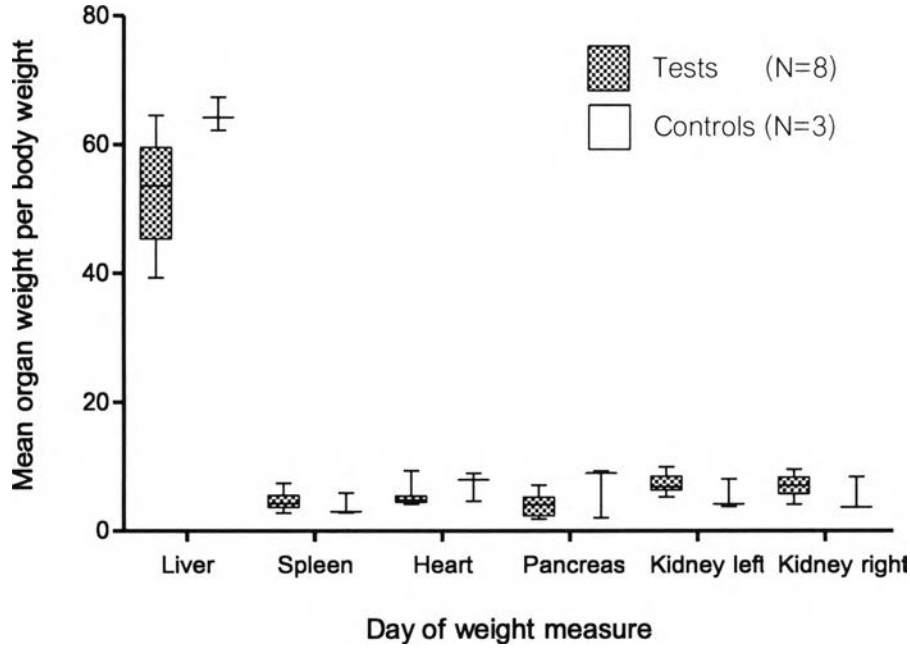

Figure 4.3.1B: Organs weight per brain weight monitoring (Limit) test in 14-day. The differences were within ranged of mean (min, max) by box plot analysis.

4.3.2 Acute Intraperitoneal (I.P) Toxicity Test in Mice

Acute 14-day intraperitoneal toxicity test was studied in mice injected with 15% TPDF (dose 10 ml/Kg) compared with control group injected with 0.9% NSS.

Test results, including clinical signs, mortality and necropsy finding, are evaluated. There was no mortality and no clinical signs were noted. No abnormalities were detected at necropsy. Results details are in Table 4.3.2A.

Table 4.3.2A: Acute Intraperitoneal Toxicity Test in Mice.

Animal No:	Sex	Mortality	Clinical signs	Necropsy findings
12	15%TPDF	Male 0/5	NAD	NAD
13	15%TPDF		NAD	NAD
14	15%TPDF		NAD	NAD
15	15%TPDF		NAD	NAD
16	15%TPDF		NAD	NAD
17	15%TPDF	Female 0/5	NAD	NAD
18	15%TPDF		NAD	NAD
19	15%TPDF		NAD	NAD
20	15%TPDF		NAD	NAD
21	15%TPDF		NAD	NAD
22	0.9%NSS	Male 0/5	NAD	NAD
23	0.9%NSS		NAD	NAD
24	0.9%NSS		NAD	NAD
25	0.9%NSS		NAD	NAD
26	0.9%NSS		NAD	NAD
27	0.9%NSS	Female 0/5	NAD	NAD
28	0.9%NSS		NAD	NAD
29	0.9%NSS		NAD	NAD
30	0.9%NSS		NAD	NAD
31	0.9%NSS		NAD	NAD

NAD= No abnormalities detected; TPDF= tapioca derivative based PDF

Body weight monitoring

Body weight gains/loss was comparable with control group in both male and females. There were no significant different of the weekly mean body weight and the change was acceptable compared with control group in both male and female during acute intraperitoneal toxicity testing. Details results are showed in Fig. 4.3.2A.

Table 4.3.2B: Mean of body weight and its %change (acute toxicity testing, I.P)

Monitoring time point	Mean of body weight (mg)		% Body weight changes*	
	Male (n=5)	Female (n=5)	Male (n=5)	Female (n=5)
7 days before	36.46	1.71	29.24	-2.63
day0	38.13	1.66	31.06	-2.60
day7	37.16	1.32	29.80	-4.25
day14	37.39	-0.71	31.02	-3.09

*%Difference= 100x(test-control)/average) versus average

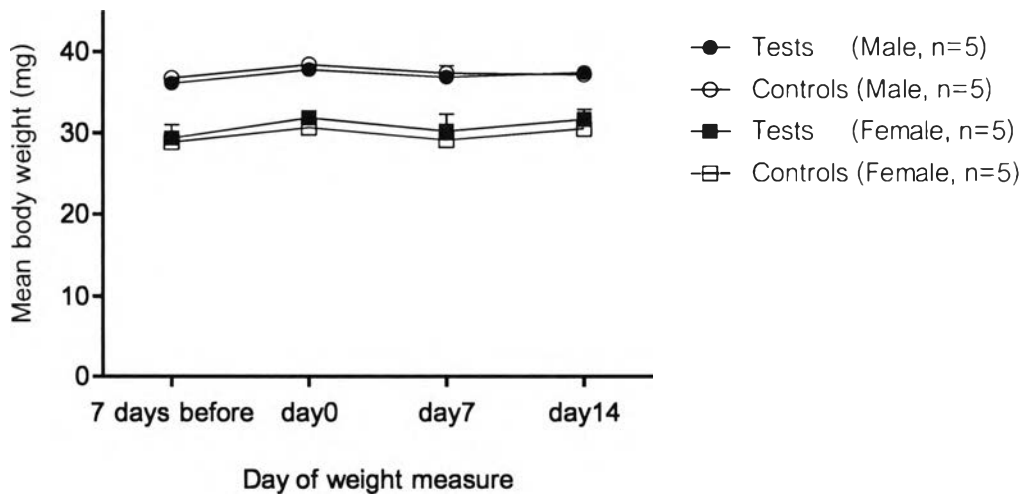

Figure 4.3.2A: Body weight monitoring by group/sex among acute intraperitoneal toxicity (Limit) 14-days in mice.

Organ weight monitoring

There were no differences in mean organs weight per body weight between control and the animal intraperitoneal injection with the 15%TPDF in both male and female groups (Table 4.3.2C) and (Fig. 4.3.2B).

Table 4.3.2C: Organ weight per body weight monitoring (Toxicity Test in Mice, I.P)

I.V Dose		Test (n=10) ($X10^{-3}$)	Control (n=10) ($X10^{-3}$)	Unpaired *t*-test (Mann Whitney test)
Liver	Male	63.1±1.15	64.91±3.11	Not significance
	Female	60.03±2.71	49.34±3.21	Not significance
Spleen	Male	3.54±0.34	64.91±3.11	Not significance
	Female	4.58±0.14	7.03±0.55	Not significance
Heart	Male	5.16±0.28	5.80±0.41	Not significance
	Female	5.64±0.17	5.32±0.50	Not significance
Pancreas	Male	10.6±1.69	7.40±1.11	Not significance
	Female	7.64±0.52	4.50±0.71	Not significance
Lt Kidney	Male	9.68±0.34	10.19±0.29	Not significance
	Female	7.89±0.36	6.00±0.38	Not significance
Rt. Kidney	Male	10.18±0.36	10.43±0.36	Not significance
	Female	8.32±0.12	6.12±0.24	Not significance

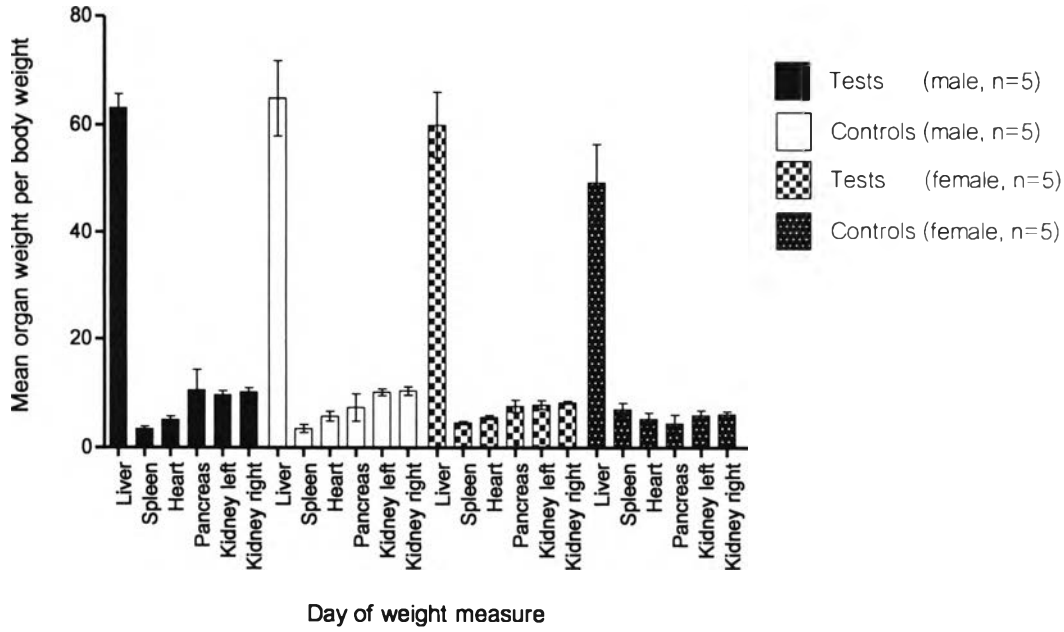

Figure 4.3.2B: Organs weight monitoring during acute intraperitoneal toxicity test in Mice divided by group and sex. Each organ did not difference between test and control using unpaired t-test.

The presence of abnormality was examined and scored (Table 4.3.2D). The four sites of the peritoneal were sampling. The abscess formation in liver, abdominal wall, bowel and peritoneum were examined. The standard scoring protocol was used (see annex). The photos of histology examination were presented (as shown in Fig. 4.3.2: C-F). The scoring was conducted as fashion of a blinded labeling sample. Mean abnormal grading in each organ were summarized between the test and control groups (Table 4.3.2E).

Table 4.3.2D: Pathology finding* (Acute I.V & I.P Toxicity Test in Mice)

Mice	Liver	Spleen	Heart	Pancreases	Ab. wall	Lymph	Diaphragms
1T	0.5	0	0.5	0	N/A	0	N/A
2T	0.5	0	0.5	0	N/A	0	N/A
3T	0	0	0	0	N/A	0	N/A
4T	0	0	0.5	0	N/A	0	N/A
5T	0.5	0	0.5	0	N/A	0	N/A
6T	0	0	0	0	N/A	0	N/A
7T	0.5	0	0	0	N/A	0	N/A
8T	0.5	0	0	0	N/A	N/A	N/A
9C	0.5	0	0	0	N/A	0	N/A
10C	0.5	0	0	0	N/A	0	N/A
11C	0.5	0	0	0	N/A	0	N/A
12T	0	0	0	0	0	N/A	0
13T	0.5	0	0.5	0	1	0	1
14T	0.5	0	0	0	0	N/A	2
15T	0.5	0	0	0	0	0	0
16T	0.5	0	0	0	0	0	0
17T	0.5	0	0	0	0.5	0	0
18T	0.5	0	0	0	0.5	0	0
19T	0	0	0	0	0.5	1	0
20T	0	0	0	0	2	1	0
21T	0.5	0	0	0	1.5	1	0
22N	0.5	0	0.5	0	1.5	N/A	0
23C	0.5	0	0	0	-	0	0
24C	0.5	0	0	0	0	0	0
25C	0.5	0	0	0	-	0	0
26C	0.5	0	0	0	1	N/A	0
27C	0.5	0	0	0	0	0	0
28C	0	0	0	0	0.5	1	1.5
29C	0.5	0	0	0	0	Recut	Recut
30C	0	0	0	0	0.5	0.5	0

Mice	Liver	Spleen	Heart	Pancreases	Ab. wall	Lymph	Diaphragms
31C	0.5	0	0	0	0	N/A	0

0= no abnormality observed

1= focal congestion 1+

1T= Tested mice number 1 injected with 15%TPDF with electrolytes solution

10C= Controlled mice number 10 injected with 0.9% normal saline solution

*Clinical pathologist examined the slides (blind samples) and peer reviewed with experienced medical technologist.

Table 4.3.2E: Summary of abnormality findings in pathology examination

Group	Liver N=28	Spleen N=31	Heart N=31	Pancreases N=31	Ab. wall N=18	Mes. Lym N=18	Diaph. N=19
Control	11/12	0/13	1/13	0/13	4/8	2/6	1/9
Test	10/16	0/18	5/18	0/18	6/10	3/12	2/10
Unpaired *t*-test	ns	ns	ns	ns	ns	ns	ns

ns= not significance

Note: The most abnormality grading: focal congestion in liver, inflammation and fat necrosis in pancreases, sinus cell dialate/hyperplasia in mesenteric lymph node and focal fibrosis in abdominal wall.

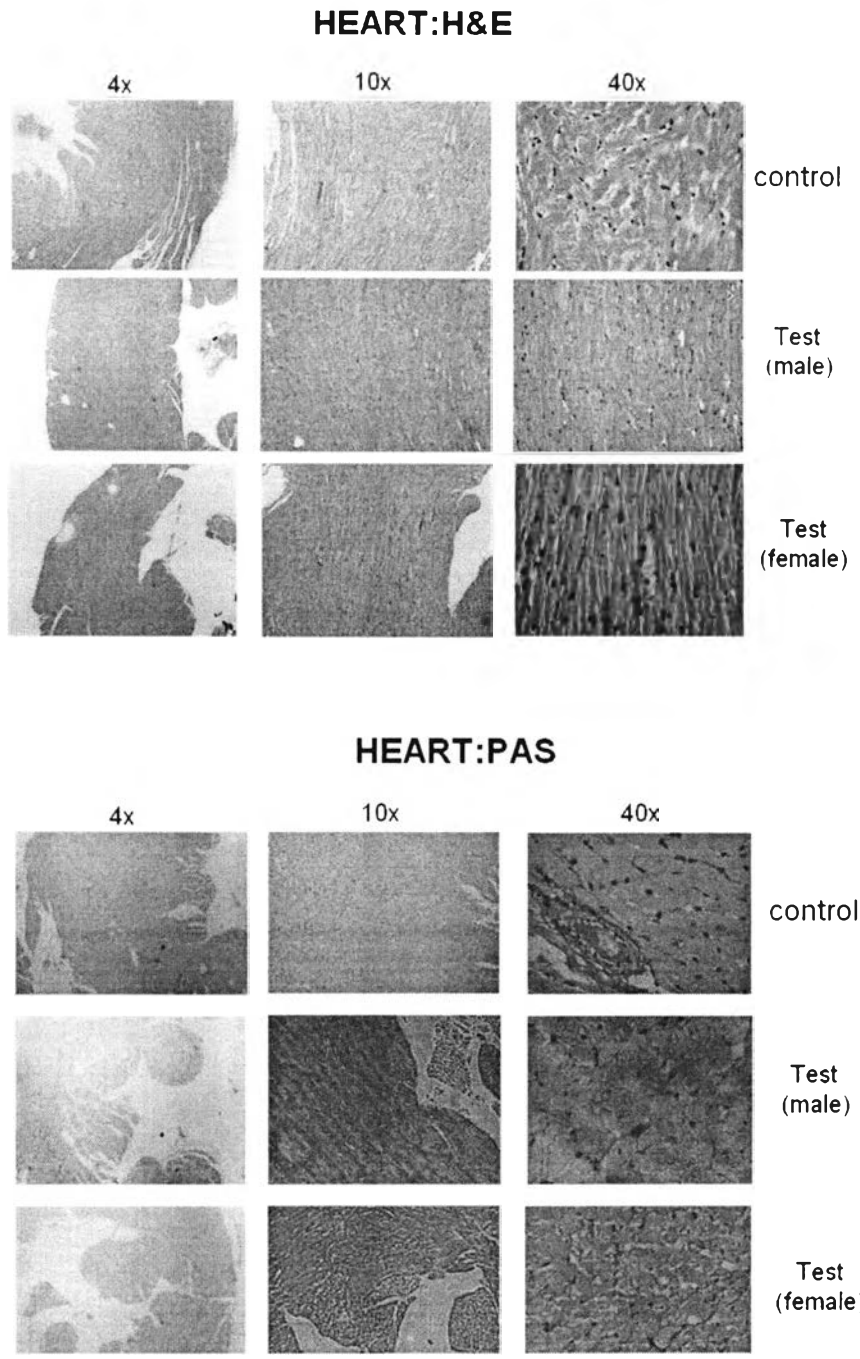

Figure 4.3.2C: Light micrograph of heart sections with H&E and PAS staining. Tissue 2x2 cm in size was collect from same position of different part of organs. Then it was immediately fixed in neutral pH-buffered 4%formaline solution. After, samples were dehydrated, embedded in paraffin and 5 um sections were obtained for histological

staining and analysis. Tests and controls showed not significant in morphology and structure changes.

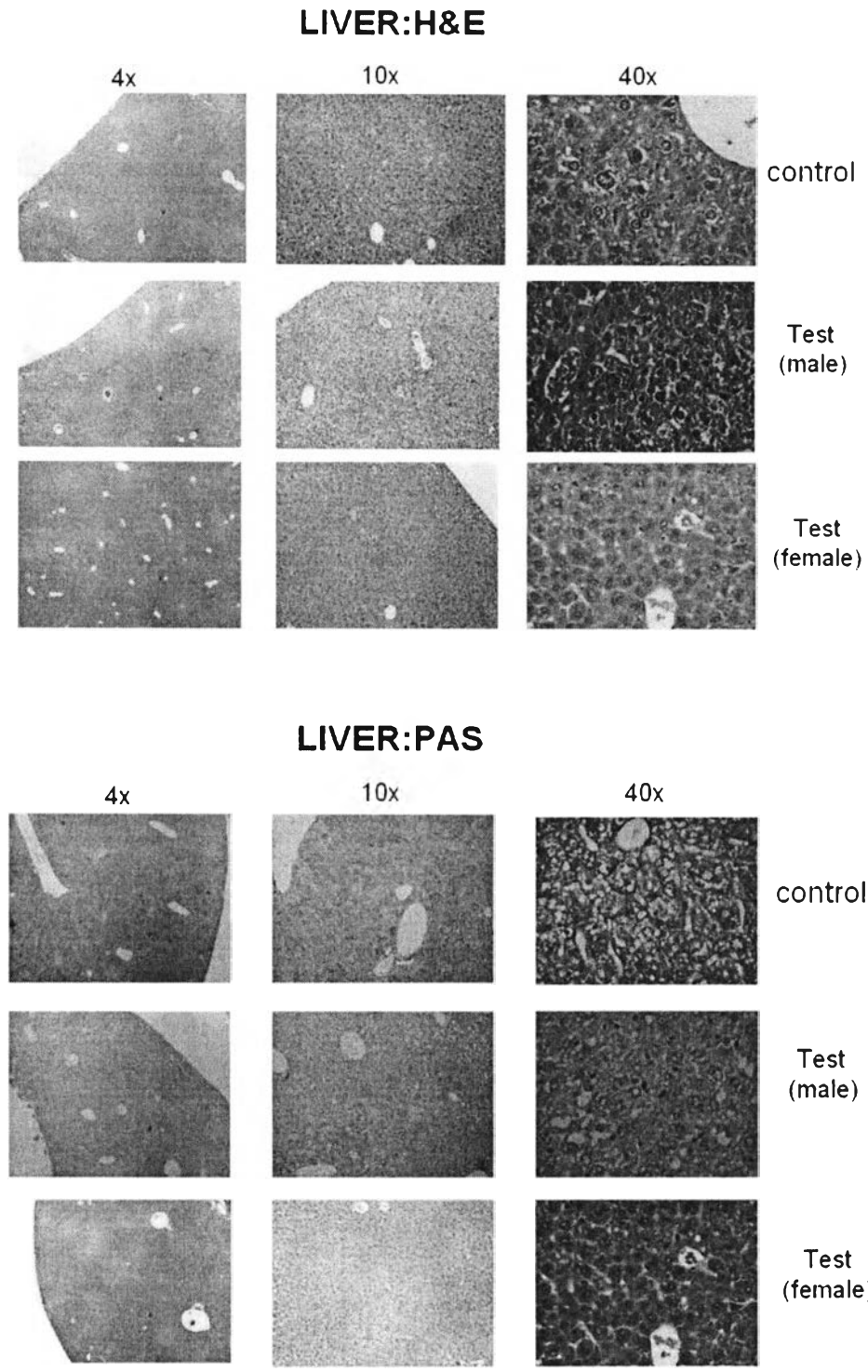

Figure 4.3.2D: Light micrograph of liver sections with H&E and PAS staining

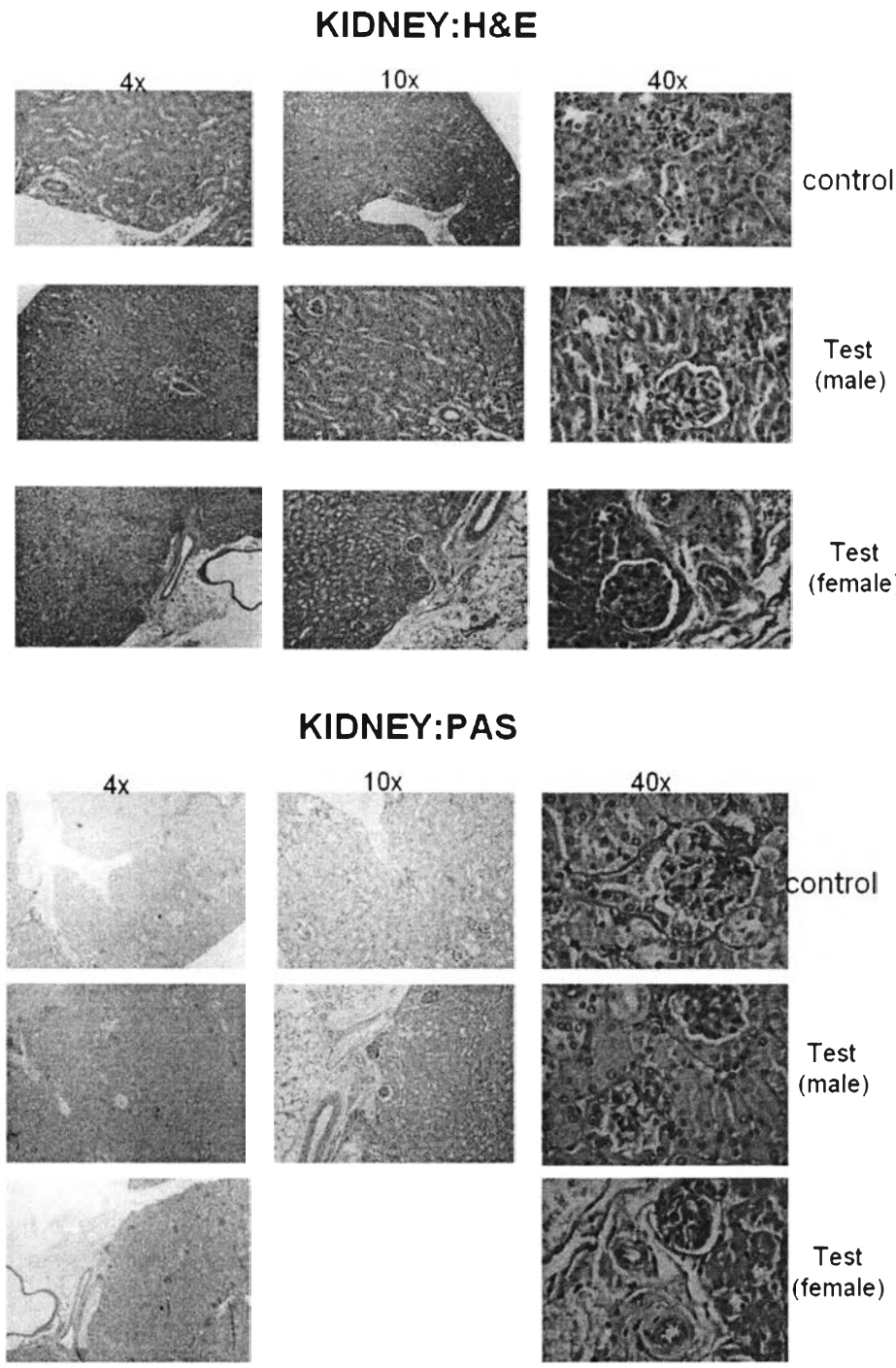

Figure 4.3.2E: Light micrograph of kidney sections with H&E and PAS staining.

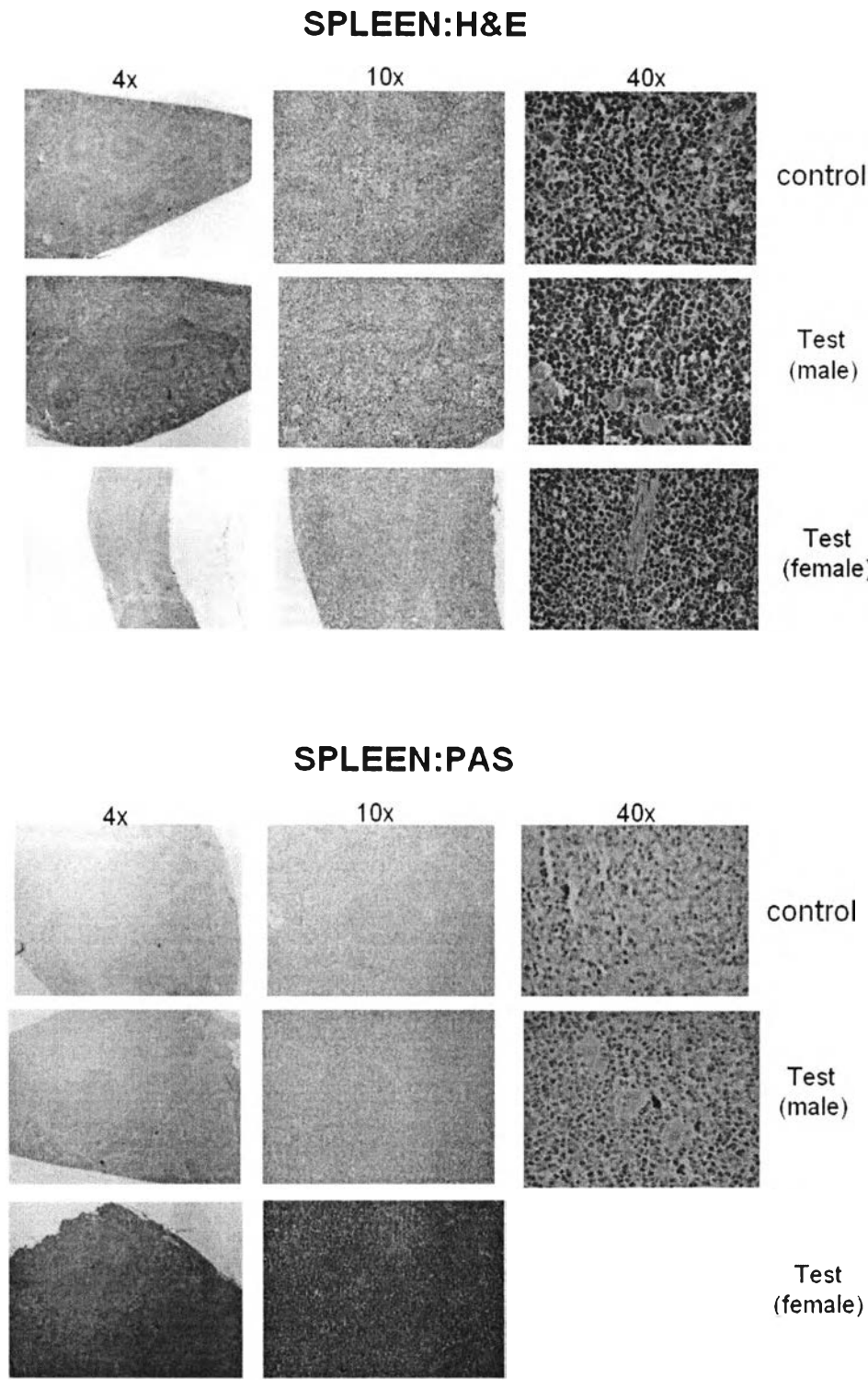

Figure 4.3.2F: Light micrograph of spleen sections with H&E and PAS staining.

4.4. Effectiveness of TPDF and its mechanism

4.4.1 Efficacy of TPDF on water osmosis compared to GPDF and CPDF

Fig. 4.4.1A shows the percentage of mass changes induced by glucose and polyglucose based PDFs through dialysis tubing with the molecular weight cut-off 10 kDa in a water and plasma container as a function of dwell time.

In cellophane bags in water, the mass increase of TPDF cellophane bags with MWCO equals to 10 kDa was 58% in water and 38% in plasma containers. This result indicated the greater effectiveness of TPDF to sustain water osmosis longer than GPDF and similar effect to induce water transport as CPDF (Fig. 4.4.1A). This result was confirmed with computer simulation analysis. The good agreement was obtained (Fig. 4.4.1B)

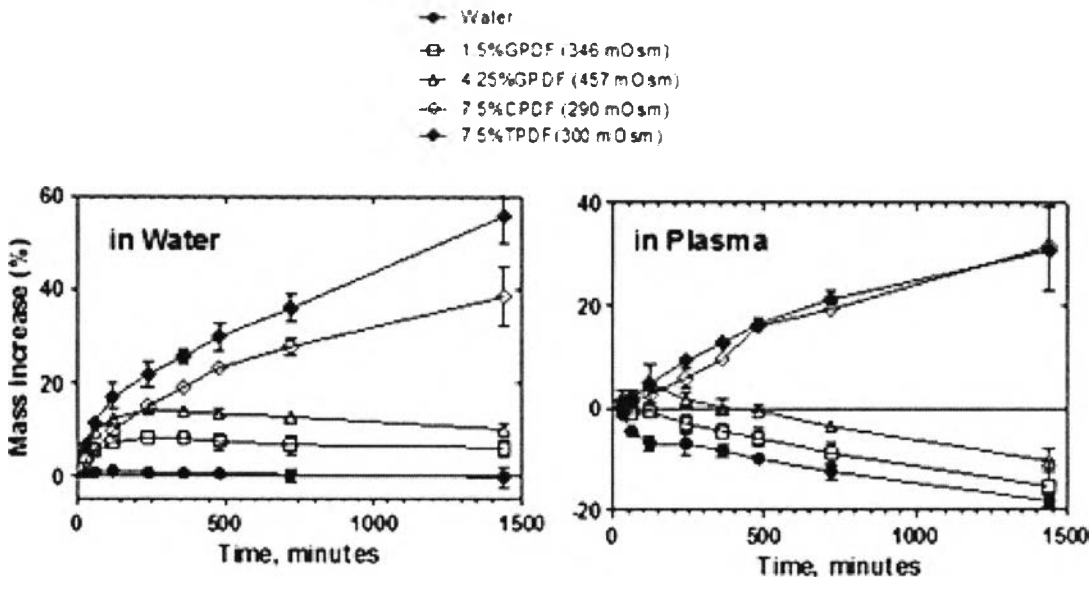

Figure 4.4.1A: Water osmosis in water and plasma studies

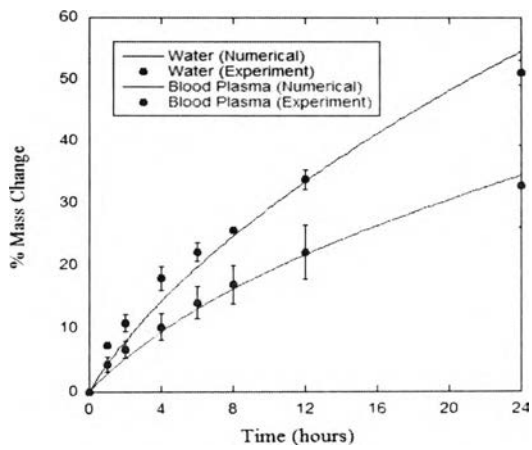

Figure 4.4.1B: Water osmosis by mass changes between experiments compared to computer simulation

4.4.2 Mechanism of polyglucose base as peritoneal dialysis fluid

The effect of small molecules on water transportation using CPDF as a model

Using CPDF as a model, we eliminated the crystalloid effect from electrolytes to see the efficacy of corn derivative molecules alone to induce water osmosis.

Fig. 4.4.2A showed that no difference was noted in effect between desalted and non-desalted CPDFs in 10 kDa cellophane bags placed in a water container. Desalted CPDF induced more water osmosis in the smaller pore size 3.5 kDa, but the difference was not statistically significant. This result indicated without crystalloid effect from electrolyte component, polyglucose alone can induce water osmosis.

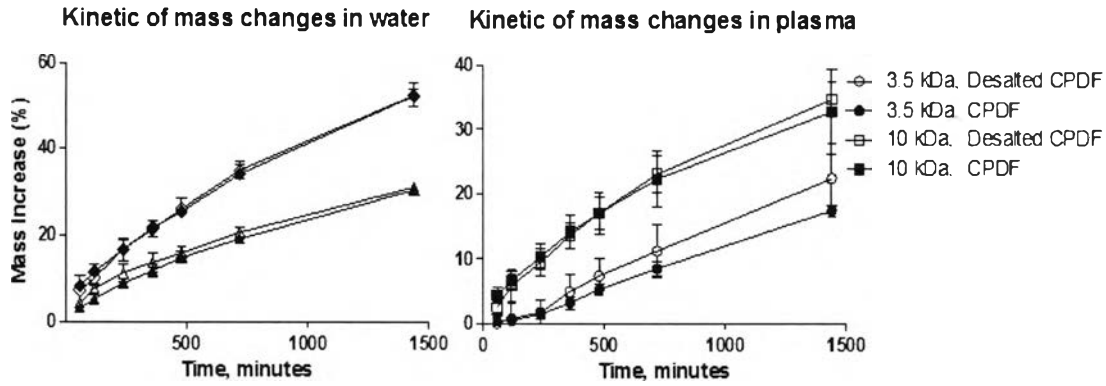

Figure 4.4.2A: Effects of polyglucose molecules of CPDF induced water osmosis.

We found that CPDF can induce water osmosis through both from diffusion of small molecule and from convection of large molecules. The small molecule effect on water osmosis into cellophane bags placed in water was stronger than the cellophane bags placed in plasma. Reduction of the effective membrane pore size increased the small molecule effect.

It was confirmed that small molecule of polyglucose can diffuse out of the bags as an example shown the distribution of eluted polyglucose molecule concentration from the HPLC analysis (Fig. 4.4.2B).

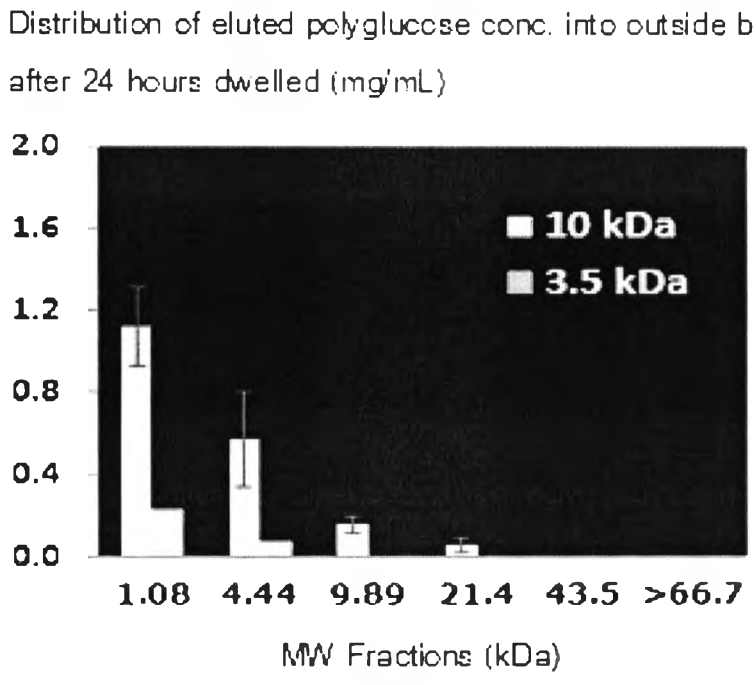

Figure 4.4.2B: The HPLC chromatogram of diffused CPDF into outside bag, showing a pattern of concentration of each molecular weight fraction found in water outside the bags. As expected, small solutes (crystalloid properties) can diffuse out.

With cellophane bags in beakers containing water, the solid lines with the symbols are from the experiment. As shown in the Fig. 4.4.2C, the mass increase of cellophane bags with MWCO equal to 10 kDa at 68% (solid line, left) is larger than that of cellophane bags with MWCO equal to 3.5 kDa at 40% (solid line, right) and the dashed lines were obtained from the computed theory. They agree quite well with experimental results, with small errors.

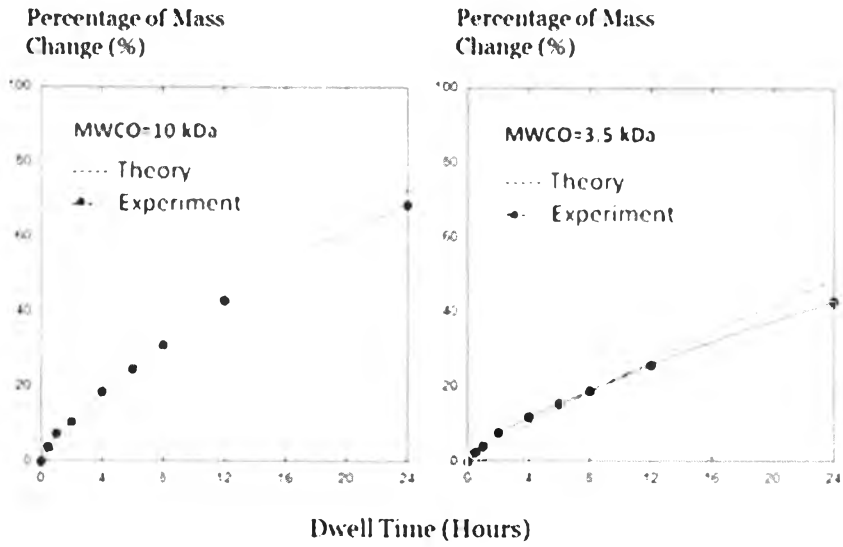

Figure 4.4.2C: Experiment compared to simulated theory

What if the theory without small fraction in water container

Then we did the same simulation but without the fraction of glucose polymers with MW <1.08 kDa. It is shown as the lower dashed line in the Fig. 4.4.2D. For cellophane bags with MWCO 10 kDa without crystalloids, the mass change is reduced to about 49%. On the other hand, cellophane bags with MWCO 3.5 kDa, have a larger reduction of mass change of about 71% without the small molecules. The smaller the effective pore size, the larger the "crystalloid" effect becomes.

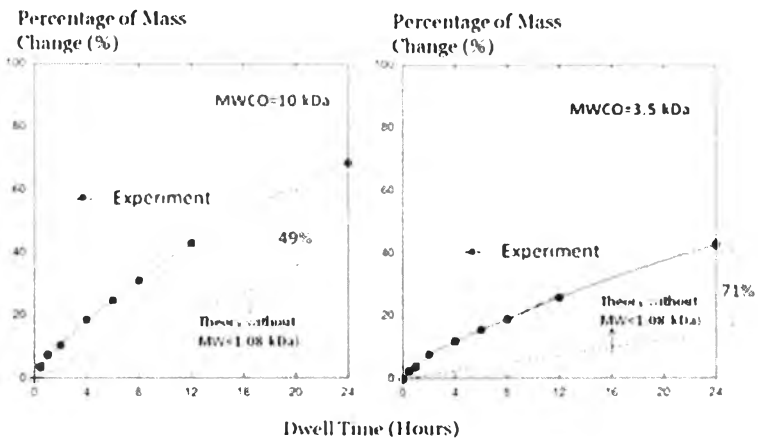

Figure 4.4.2D: Computer simulation without small MW<1.08 kDa compared to experiment study in water. Induce increased %mass change using MWCO 3.5 kDa (right) greater than in 10 kDa (left).

Reduction percentage of mass change of the cellophane bag placed in water after 24 hours due to the absence of the molecular weight fraction displayed in the horizontal axis. The calculations were done with membrane MWCO = 10 kDa (blue) and MWCO = 3.5 kDa (red). This result confirmed that the small molecular weight fractions played more effective role when MWCO barrier was reduced (red bar) in both condition as shown in Fig. 4.4.2E of water (upper) and plasma (lower).

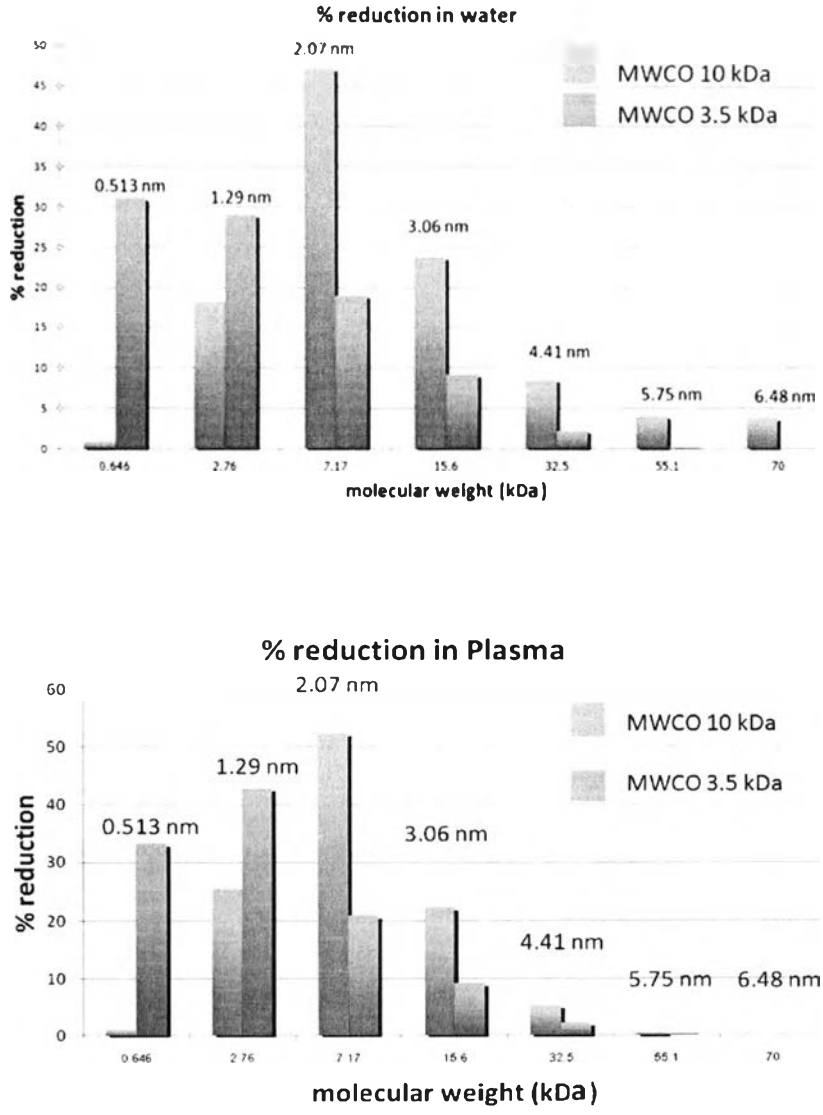

Figure 4.4.2E: Effect of each fraction on %water reduction (in Water, upper) and (in Plasma, lower)