AIM OF THE WORK

The aim of the present study was to compare vitamin K level in exclusively breast fed infants versus formula fed infants aged 2 month ±1 week.

PATIENTS AND METHODS

Study Setting

The study was conducted in Alexandria University Children's Hospital's outpatient clinics.

Study Population and sampling

From children attending outpatient clinics of Alexandria University Children's Hospital, 45 breast fed and 45 formula fed infants were selected after fulfilling the following criteria:

Inclusion criteria

- Home delivery.
- Gestational age between 37 and 42 weeks.
- Birth weight ≥ 2500 g.
- Uncomplicated postnatal period. (No asphyxia, sepsis nor DIC)
- Exclusive breastfeeding or formula feeding.
- Age 2 month ± 1 week.

Exclusion criteria

- Previous blood transfusion, NICU admission or jaundice.
- History of operations.
- History of medications (anticoagulants, antibiotics to the infant or the mother).
- History of vitamin k supplementation.

Data collection methods

All studied infants were subjected to the following:

A. Thorough history taking stressing on:

- Personal data: age, sex, residence.
- Perinatal history: Gestational age, vitamin K supplementation, NICU admission.
- Nutritional history: breastfeeding, formula feeding.
- Drug history: antibiotic therapy**.**

B. Physical examination:

Physical examination of infants to exclude:

- Jaundice,
- Ecchymotic patches,
- Bleeding,
- Organomegaly,
- Ascites,
- Eodema and/or thrombosis.

C. Laboratory investigations:

Venous blood samples were drawn to measure vitamin K level (ELISA), Prothrombin Time (PT) and Prothrombin Activity (PA).

1. Measurement of vitamin K:

Principle of Test

The kit is for the quantitative level of VK1 in the sample, adopt purified human VK1 to coat microtiter plate, make solid-phase antibody, then add samples or standards to wells with a labeled antibody specific to VK1, then add labeled HRP to the well. After washing completely, add TMB substrate solution, TMB substrate becomes blue color in wells that contains antibody - antigen - enzyme-antibody complex, reaction is terminated by the addition of a stop solution and the color change is measured at a wavelength of 450 nm. The concentration of VK1 in the samples is then determined by comparing the O.D. of the samples to the standard curve. [78]

Washing method

- **Manually washing method:** shake away the remained liquid in the enzyme plates; place some bibulous papers on the test-bed, and flap the plates on the upside down strongly. Inject at least 0.35ml after-dilution washing solution into the well, and marinate 1~2 minutes. Repeat this process according to your requirements.
- **Automatic washing method:** if there is automatic washing machine, it should only be used in the test when you are quite familiar with its function and performance.

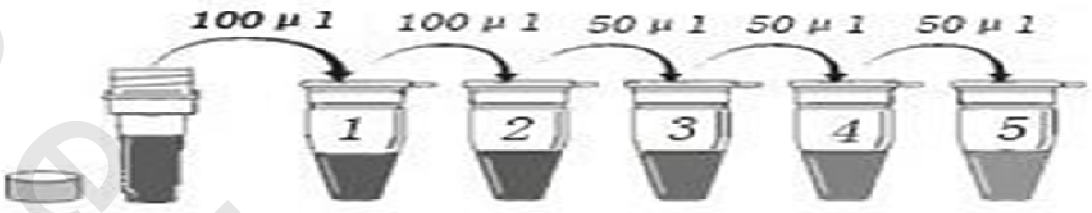
Sample Preparation

1. Serum-coagulation at room temperature for 10-20 min, centrifuge at the speed of 2000-3000 rpm for 20-min. Remove supernatant, if precipitation appeared, centrifuge again.
2. Plasma-use suited EDTA or citrate plasma as an anticoagulant, centrifuge at the speed of 2000-3000 rpm for 20-min. Remove supernatant, if precipitation appeared, centrifuge again.
3. Cell culture supernatant-Detect secretory components, remove particulates by centrifugation for 20-min at the speed of 2000-3000 rpm. Remove supernatant detect the composition of cells, dilute cell suspension with PBS (PH7.2-7.4) to make cell concentration 1 million / ml, repeated freeze-thaw cycles, damage cells and release intracellular components, centrifugation 20-min at the speed of 2000-3000 rpm. Remove supernatant, if precipitation appeared, centrifugal again.
4. Tissue homogenates-after cutting samples, check the weight, pipette PBS (PH7.2-7.4), frozen with liquid nitrogen, maintain samples at 2-8℃ after melting. Pipette PBS (PH7.4), homogenized by hand or Grinders, centrifugation 20-min at the speed of 2000-3000 rpm. Remove supernatant.
5. Extract as soon as possible after samples collection, and should be tested as soon as possible after the extraction. If not, samples can be kept in -20℃.Avoid repeated freeze-thaw cycles.
6. Don't detect the samples which contain NaN3, because NaN3 inhibits HRP activity.

Assay procedure

Step 1: Standard: Bring all reagents to room temperature.

Dilute the standard Pipette 50µl standard dilution in each tube. Pipette 100µl standard (135pg/ml) in the first tube. And take out 100µl from the first tube into the second. Pipette 50µl from the second tube to the third tube and produce dilution series as below. Repeat each of the concentration to get the mean value of each well.



Tube	0	1	2	3	4	5
pg/ml	135	90	60	30	15	7.5

Fig.(5): Dilution series

Step 2: Prepare sample: Set blank wells separately (blank comparison wells don't add sample and HRP-Conjugate reagent, other each step operation is same). Pipette Sample dilution 40µl to testing sample well, then add testing sample 10µl (sample final dilution is 5-fold), Pipette sample to wells, don't touch the well wall as far as possible, and mix gently.

Step 3: Incubate: Cover with the adhesive strip provided, incubate for 30 min at 37°C.

Step 4: Configurate liquid: Dilute wash solution 30-fold (or 20-fold) with distilled water.

Step 5: Washing: Uncover the adhesive strip, discard liquid, Pipette washing buffer to every well, still for 30s then drain, repeat 5 times.

Step 6: Add enzyme: Pipette HRP-Conjugate reagent 50µl to each well, except blank well.

Step 7: Incubate: Operation with 3.

Step 8: Washing: Operation with 5.

Step 9: Color: Pipette Chromogen Solution A 50ul and Chromogen Solution B to each well, avoid the light preservation for 15 min at 37°C.

Step 10: Stop the reaction: Pipette Stop Solution 50µl to each well, Stop the reaction (the blue change to yellow).

Step 11: Calculate: take blank well as zero, Read absorbance at 450nm after Pipetteing Stop Solution within 15min.

Calculation of result

Take the standard concentration as the horizontal, the OD value for the vertical ,draw the standard curve on graph paper, Find out the corresponding concentration according to the sample OD value by the Sample curve, multiplied by the dilution multiple, or calculate the straight line regression equation of the standard curve with the standard concentration and the OD value, with the sample OD value in the equation, calculate the sample concentration, multiplied by the dilution factor, the result is the sample actual concentration.

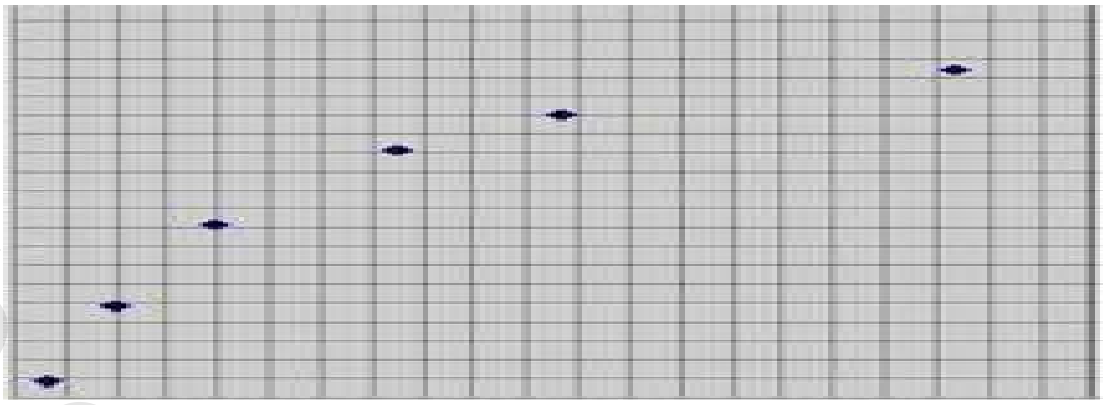


Fig. (6): Graphical Representation

Description

1. The standard curve is drawn under ideal conditions, and is just for reference rather than the actual standard curve diagram of the kit.
2. It is advisable to establish proper assay data and standard curve according to respective laboratory conditions.

Reference range: The serum level of vitamin K1 ranges from 200 to 1000 ng/l in healthy people consuming adequate quantities of vitamin K1 (50 to 150 µg/day).[79]

Prothrombin time and activity

Thromborel® S Reagent is used for the determination of the prothrombin time (PT) and for the determination of the activity of coagulation factors II, V, VII and X. [80]

Summary and Explanation

The prothrombin time measured with Thromborel® S Reagent is a rapid, sensitive screening test for coagulation disorders in the domain of the extrinsic system (Factors II, V, VII and X).[80, 81]

Principle of the Method

The coagulation process is triggered by incubation of plasma with the optimal amount of thromboplastin and calcium. The time to formation of a fibrin clot is then measured. [82]

Equipment

Thromborel® S Reagent can be used manually or on automated coagulation analyzers. Siemens Healthcare Diagnostics provides Reference Guides (Application Sheets) for several coagulation analyzers.

Specimen Collection and Preparation

Carefully mix 1 part sodium citrate solution (0.11mol/L) with 9 parts venous blood, avoiding the formation of foam.

Centrifuge the blood specimen at 1500 x g for no less than 15 minutes at room temperature. Store in an unopened tube at room temperature. Plasma should be tested within 24 hours of blood collection.

Procedure

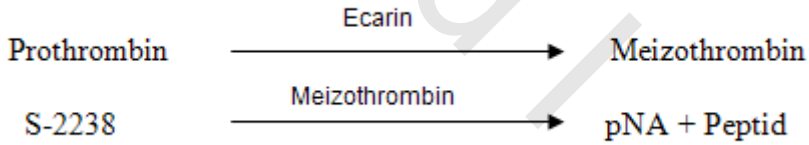
Pipette into a test tube prewarmed to +37 °C
Citrated plasma 100 µL
Incubate for 1 minute at +37 °C
Thromborel® S Reagent (warmed to +37 °C) 200 µL
On addition of Thromborel® S Reagent start stop-watch or timer on the coagulation analyzer and determine the coagulation time.

Results

The results can be reported in seconds and should always be interpreted in conjunction with the patient's medical history, clinical presentation and other findings.
Reference Range: (sec): 11-15 (12-16). [83]

- **Prothrombin activity:**

Prothrombin is activated to meizothrombin by the snake venom enzyme Ecarin from Echis Carinatus. After a certain incubation time, the amount of meizothrombin formed is measured with the thrombin selective substrate S-2238, which also is cleaved by meizothrombin. The absorbance recorded at 405 nm is proportional to the prothrombin activity in the sample. [84]



Reference Range: (%): 75 – 130% (mean 102% ± 2 SD). [85]

Statistical analysis data

Comparison between study groups according to demographic data, maternal vitamin K supplementation during pregnancy and after labor was done using chi square (or Fisher exact test when indicated). Comparison of these variables between groups of different vitamin K levels in cases was also done using chi square or student (t) tests.

Comparison of lab investigation parameters was done using t test (or Mann Whitney U test for none normally distributed parameters). Significance level was set at 5% level. Statistical analysis was done using SPSS version 17.0. [86]

RESULTS

Table (3): Demographic data of the studied infants

	Breast feeding (n = 45)		Formula feeding (n = 45)		Test of sig.	p
	No.	**%**	**No.**	**%**		
Sex						
Male	18	40.0	26	57.8	χ^2=2.846	0.092
Female	27	60.0	19	42.2		
Age (Days)						
Min. – Max.	53.0 – 67.0		55.0 – 65.0			
Mean ± SD.	62.02 ± 6.31		60.36 ± 6.06		t= 1.278	0.204
Median	60.0		60.0			
Birth weight (kg)						
Min. – Max.	2.55 – 3.80		2.60 – 3.80			
Mean ± SD.	2.87 ± 0.33		2.98 ± 0.32		t= -1.649	0.103
Median	2.75		2.85			
Gestational age (weeks)						
Min. – Max.	37.0 – 40.0		37.0 – 40.0			
Mean ± SD.	38.04 ± 0.74		38.31 ± 0.67		t= -1.798	0.076
Median	38.0		38.0			

χ^2: Chi square test
t: Student t-test

Table (3), figure (7,8,9 and 10) demonstrates some demographic data of studied infants; it shows no statistical significance regarding sex, age, birth weight and gestational age of both groups.

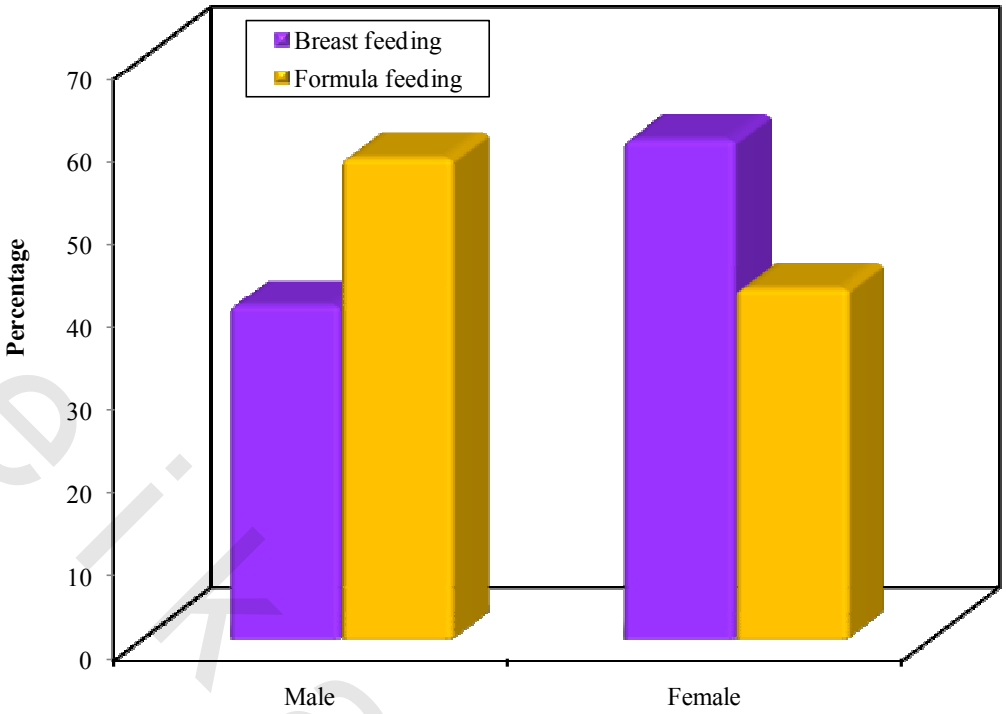


Fig. (7): Sex of the studied infants

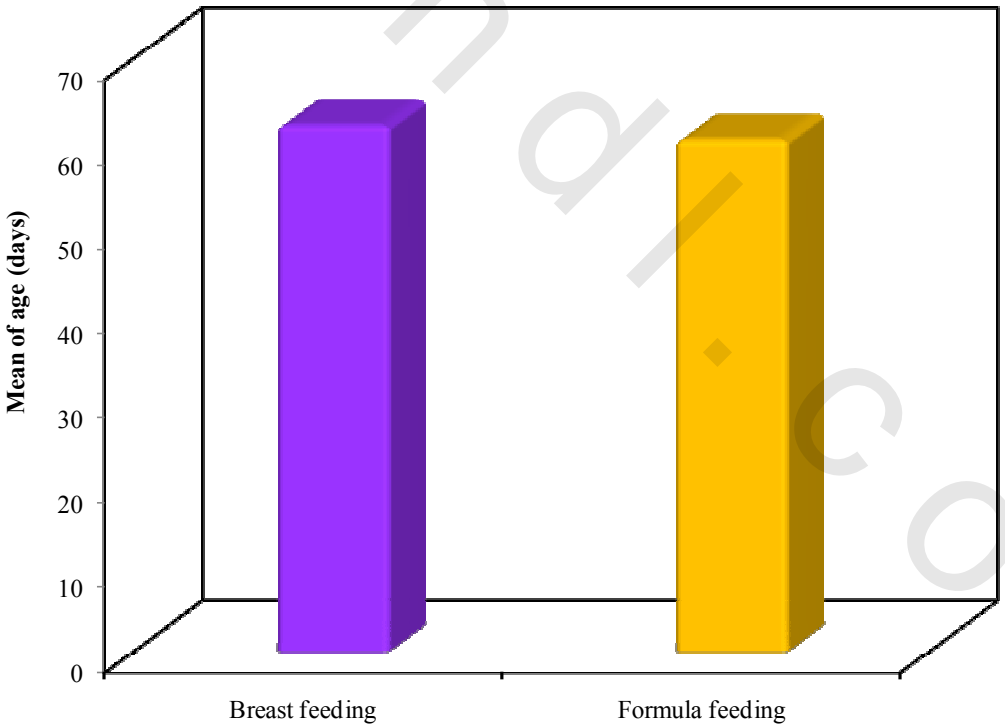


Fig. (8): Age of the studied groups

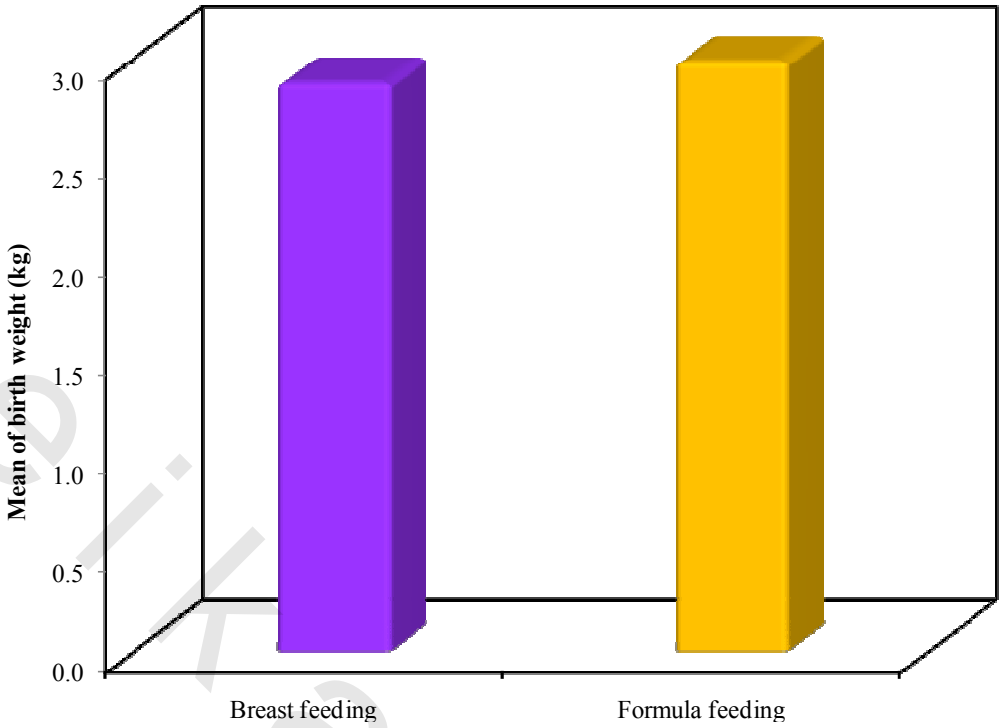


Fig. (9): Birth weight of studied infants

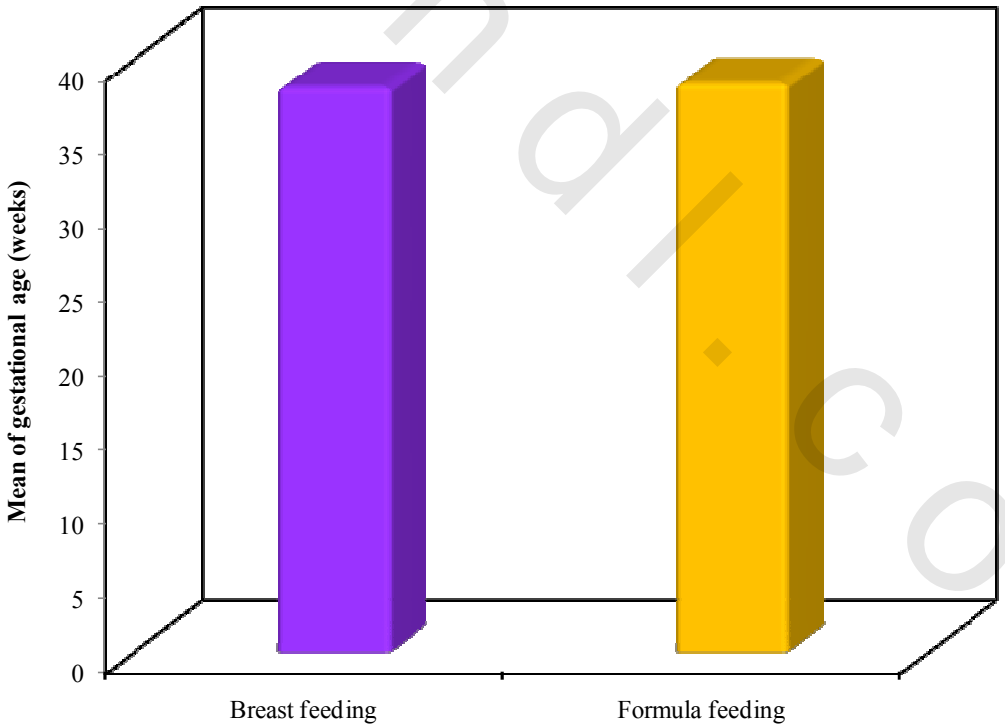


Fig. (10): Gestational age of studied infants

Table (4): Comparison between the studied groups according to ProthrmbinTime

	Breast feeding (n = 45)	Formula feeding (n = 45)	t	P
PT(in sec)				
Min. – Max.	11.0 – 14.0	11.20 – 14.0		
Mean ± SD.	12.28 ± 0.72	12.47 ± 0.82	1.150	0.253
Median	12.20	12.50		

t: Student t-test

Table (4) and figure (11) shows that the mean prothrombin time was 12.28± 0.72 seconds among breast fed children while it was 12.47± 0.82 seconds among formula fed children. Statistically, no significant difference was found between the two groups. (P= 0.253)

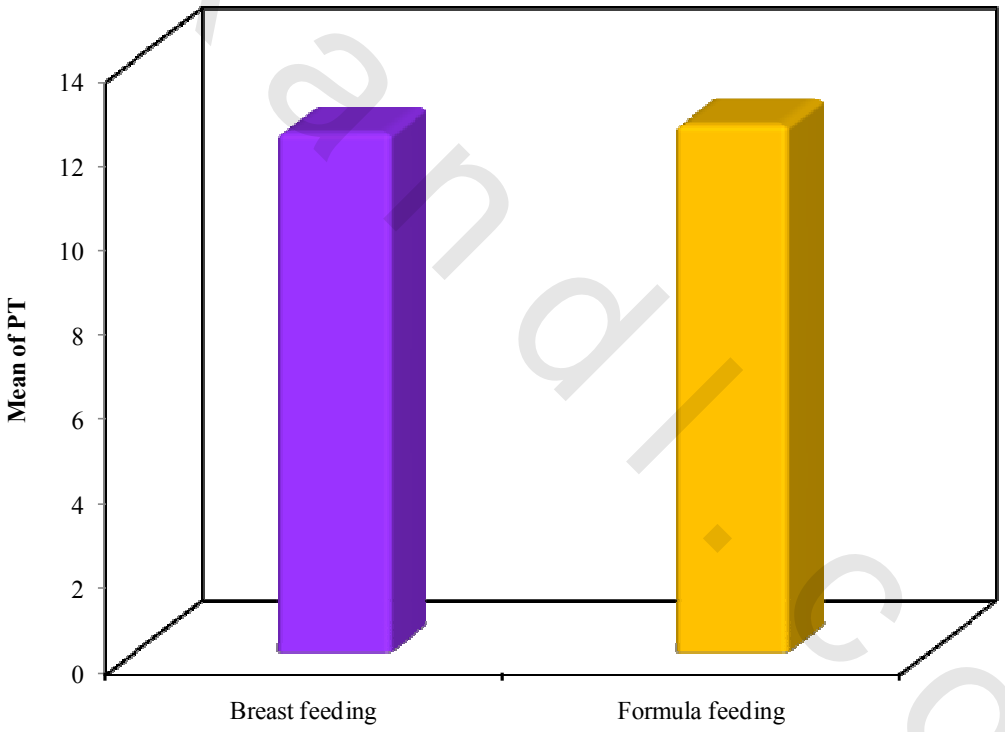


Fig. (11): Prothrombin time of studied infants

Table (5): Comparison between the studied groups according to ProthrombinAct

	Breast feeding (n = 45)	Formula feeding (n = 45)	T	P
PA (%)				
Min. – Max.	70.0 – 100.0	70.0– 100.0		
Mean ± SD.	90.16 ± 5.81	87.88 ± 13.04	1.070	0.288
Median	90.0	90.0		

t: Student t-test

Table (5) figure (12) shows that the mean prothrombin activity among breast fed children was 90.16 % ± 5.81 and 87.88 % ± 13.04 among formula fed ones. Statistically, no significant difference was found between the two groups (P=0.288).

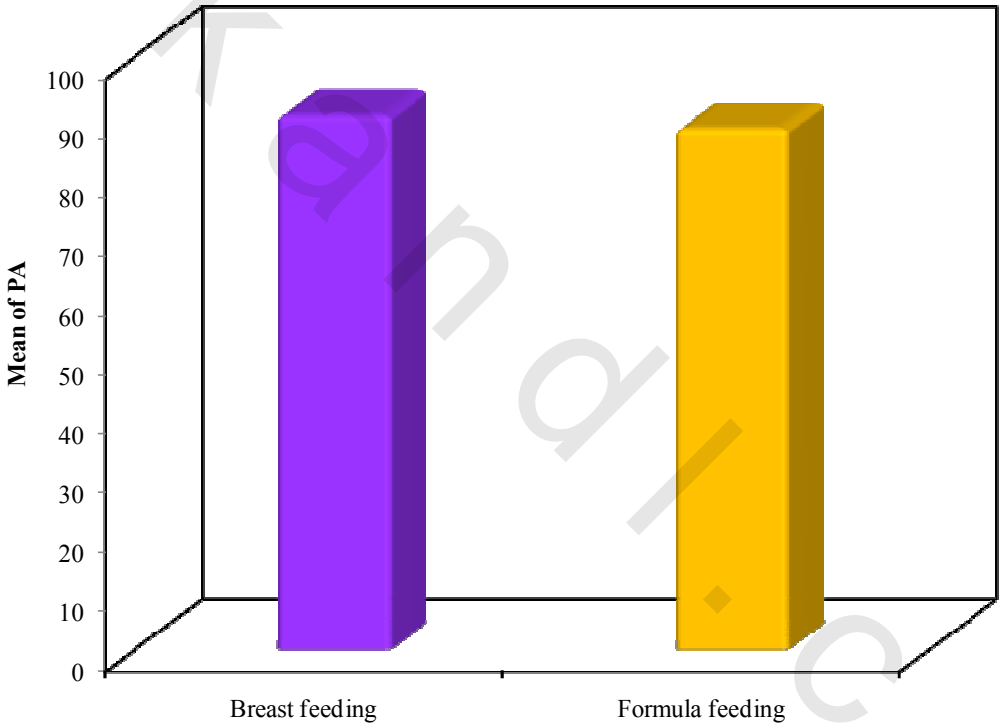


Fig. (12): Prothrombin activity among studied groups

Table (6a): Mean serum vitamin k level among studied groups

	Breast feeding (n = 45)	Formula feeding (n = 45)	Z	P
VitaminK level ng/l				
Min. – Max.	59.92 – 408.0	79.36 – 414.30		
Mean ± SD.	125.62 ± 69.63	134.29 ± 59.27	1.852	0.064
Median	92.87	110.64		

Z: Z for Mann Whitney test normal level (200-1000 ng/l)

Table (6a) figure (13) demonstrates serum level of vitamin k ranged between 59.92 - 408 ng/L with mean of 125.62±69.63 ng/l among breast fed infants compared to a range of 79.36-414.30 ng/L and a mean of 134.29±59.27 ng/l among formula fed infants. This difference is of no statistical significance (P=0.064).

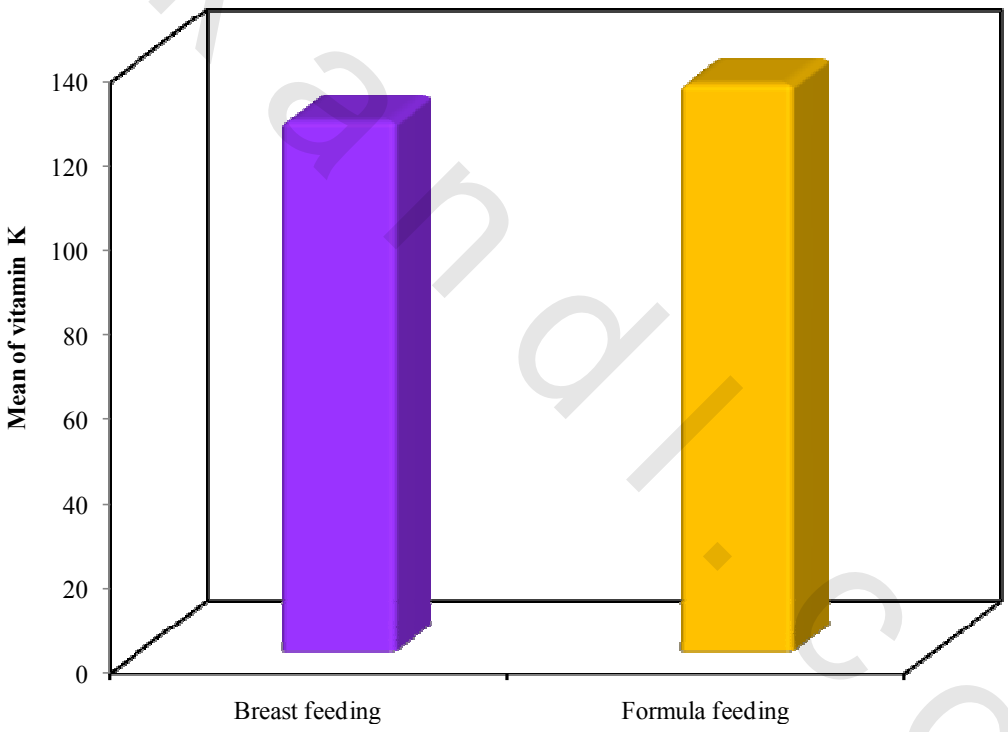


Fig. (13): Mean serum vitamin k level among breast fed and formula fed infants

Table (6b): Mean serum vitamin k level among studied groups

	Breast feeding (n = 45)		Formula feeding (n = 45)		χ^2	^{FE}p
	No.	%	No.	%		
Vitamin K level ng/l						
<200	40	88.9	42	93.3	0.549	0.714
≥200	5	11.1	3	6.7		

χ^2: Chi square test
FE: Fisher Exact test

Table (6b) demonstrates serum level of vitamin k among studied infants where vitamin k levels were below 200 ng/l in 88.9% of breast fed and 93% of formula fed ones. No statistical significance was found (P=0.714).

Table (7): Comparison between the studied groups according to maternal demographic data

	Breast feeding (n = 45)		Formula feeding (n = 45)		Test of sig.	P
	No.	%	No.	%		
Maternal age						
`21-30(years)	29	64.4	37	82.2	$\chi^2 = 3.636$	0.057
`>30(years)	16	35.6	8	17.8		
Min. – Max.	21.0 – 35.0		21.0 – 33.0		t = 1.586	0.116
Mean ± SD.	27.93 ± 4.38		26.60 ± 3.55			
Median	29.0		26.0			
Previous pregnancy						
No	23	51.1	22	48.9	$\chi^2 = 0.044$	0.833
Yes	22	48.9	23	51.1		
Employed						
No	26	57.8	25	55.6	$\chi^2 = 0.045$	0.832
Yes	19	42.2	20	44.4		
Attended breast feeding education						
No	23	51.1	20	44.4	$\chi^2 = 0.401$	0.527
Yes	22	48.9	25	55.6		
Social state						
High	19	42.2	22	48.9	$\chi^2 = 0.403$	0.525
Low	26	57.8	23	51.1		
Vit supplement during pregnancy						
No	26	57.8	26	57.8	$\chi^2 = 0.000$	1.000
Yes	19	42.2	19	42.2		
Vit supplement during Lactation						
No	23	51.1	25	55.6	$\chi^2 = 0.179$	0.673
Yes	22	48.9	20	44.4		

t: Student t-test χ^2: Chi square test

Table (7) and figure (14, 15 and 16) demonstrates comparison between the studied infants according to maternal demographic data regarding maternal age, previous pregnancy, employed, attending breast feeding health education, social state, vitamin k supplementation during pregnancy and lactation. It shows no significant difference between both groups.

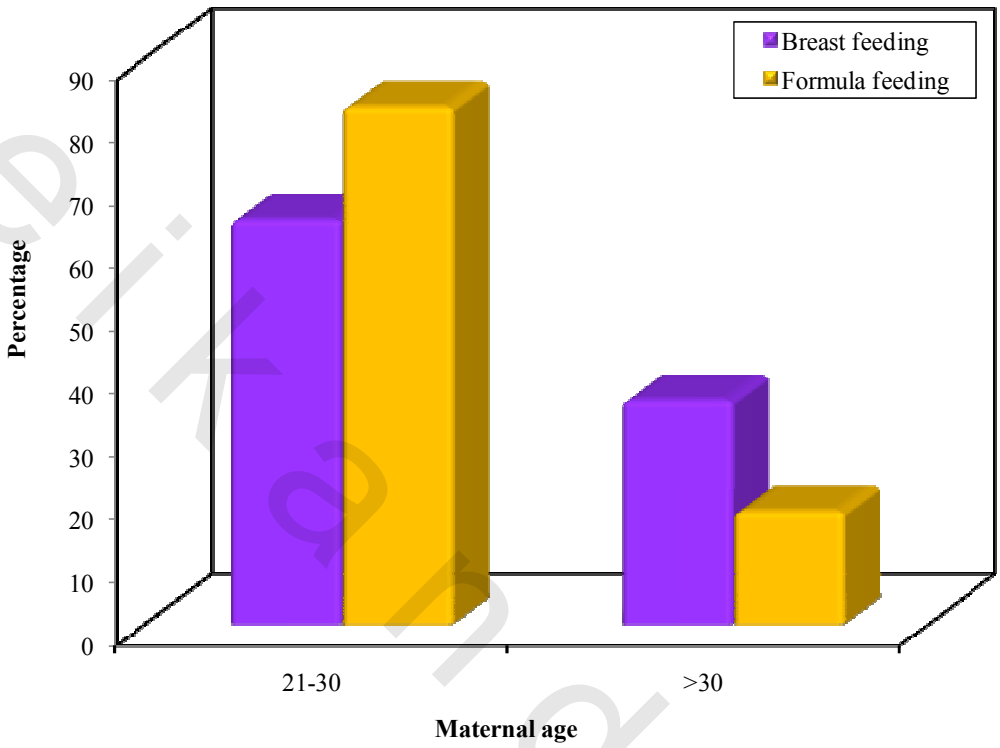


Fig. (14): Maternal age of the studied mothers among both groups

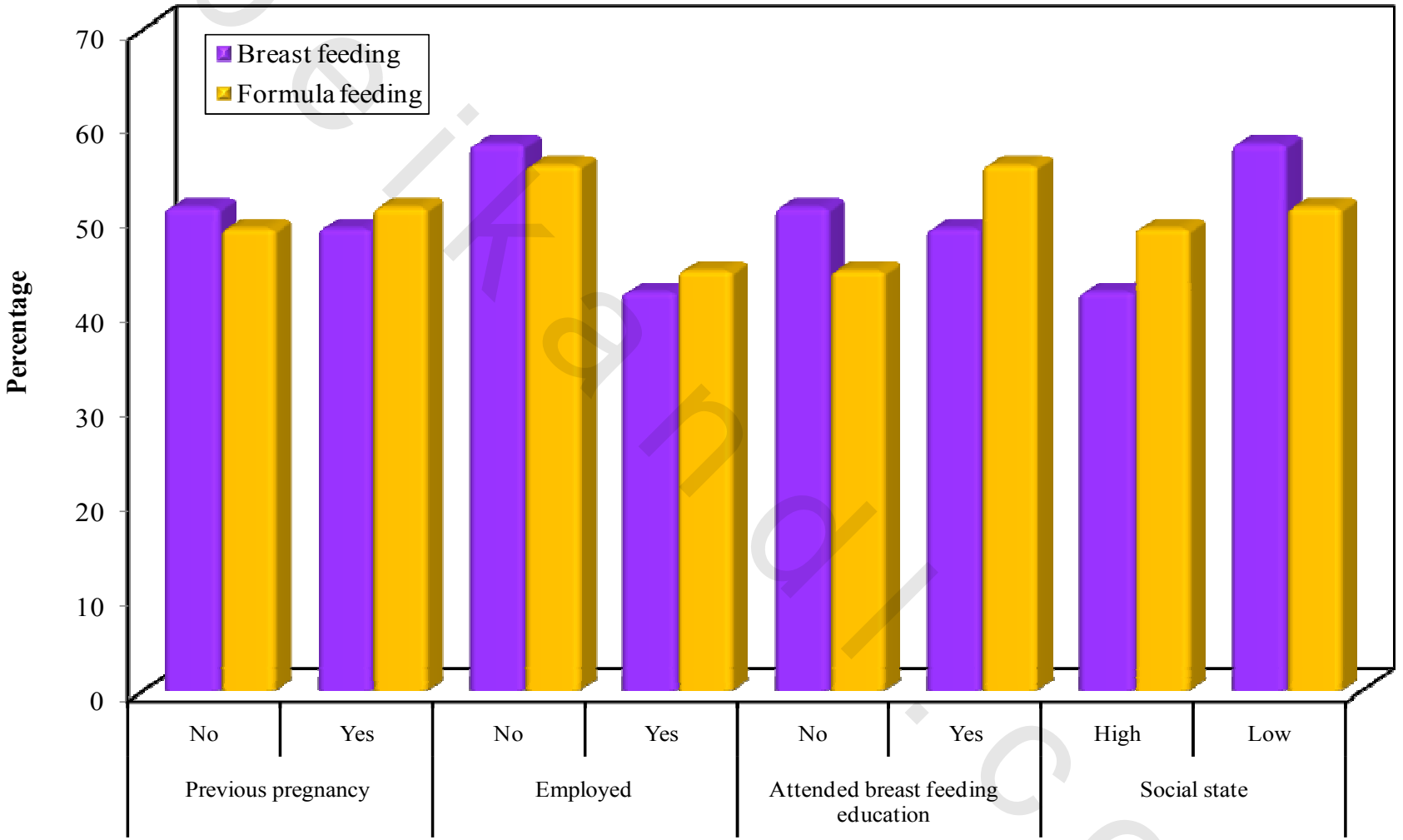


Fig. (15): Data of studied mothers

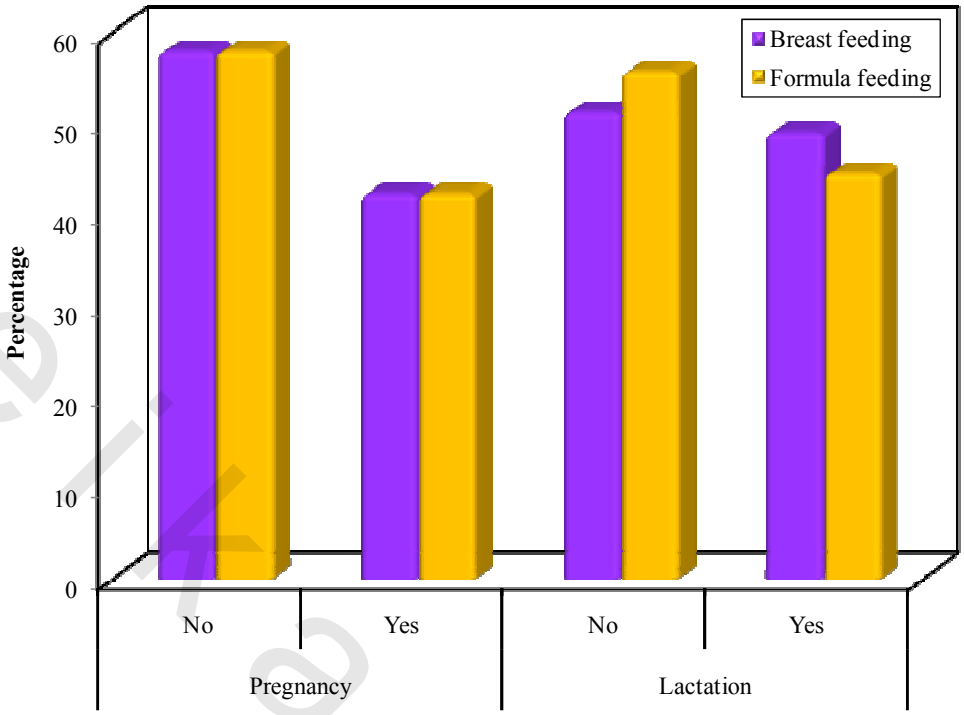


Fig. (16): Maternal vitamin k supplementation during pregnancy and lactation

Table (8): Relation between vitamin K and sex of the infants

	Sex		Z	p
	Male	**Female**		
Vitamin K level ng/l in Breast fed infants	**(n = 18)**	**(n = 27)**		
Min. – Max.	68.90-230.0	59.92-408.0		
Mean ± SD.	116.35±44.55	131.80±82.50	0.093	0.926
Median	109.43	92.87		
Vitamin K level ng/l in Formula fed infants	**(n = 26)**	**(n = 19)**		
Min. – Max.	79.36 – 155.25	88.39 – 414.30		
Mean ± SD.	104.77 ± 17.24	174.69 ± 72.07	4.251*	<0.001*
Median	99.33	161.39		

Z: Z for Mann Whitney test
*: Statistically significant at $p \leq 0.05$

Table (8), figure (17) among breastfed infants, the mean serum vitamin K level was higher among females in comparison to that among males. No statistical significance was seen (P=0.926). This was also seen among formula fed infants but this difference was statistically significant (P<0.001).

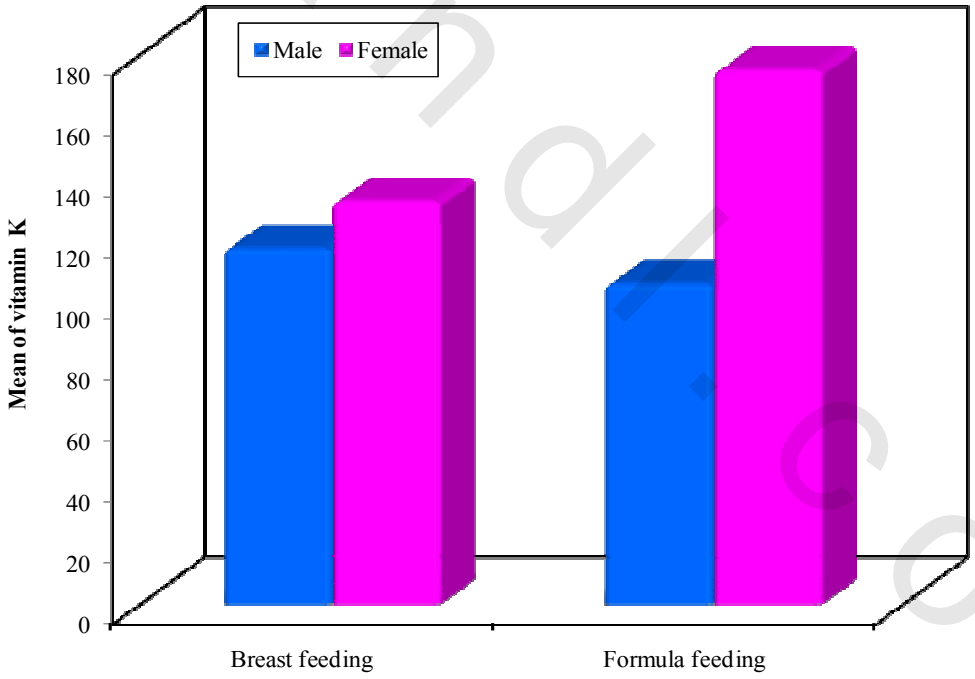


Fig. (17): Relation between sex of studied infants and mean serum vitamin k level

Table (9a): Relation between vitamin K level in breast fed infants and maternal vitamin k supplementation during lactation

	Maternal Vit K suppl during Lactation		Z	p
	No (n = 23)	Yes (n = 22)		
Vitamin K level(ng/l) in Breast fed infants				
Min. – Max.	59.92 – 92.87	123.33-408.0		
Mean ± SD.	76.54±10.07	176.93±68.23	5.745*	<0.001*
Median	70.55	152.69		

Z: Z for Mann Whitney test
*: Statistically significant at $p \leq 0.05$

Table (9a), figure (18) shows that maternal vitamin K supplementation during lactation reflects significantly on vitamin K level in breastfed infant. Infants of lactating mothers who received vitamin K show higher levels of mean vitamin K than those of lactating mothers who didn't receive vitamin K (176.93 ± 68.23 vs. 76.54 ± 10.07ng/l). This difference shows statistical significance (P<0.001).

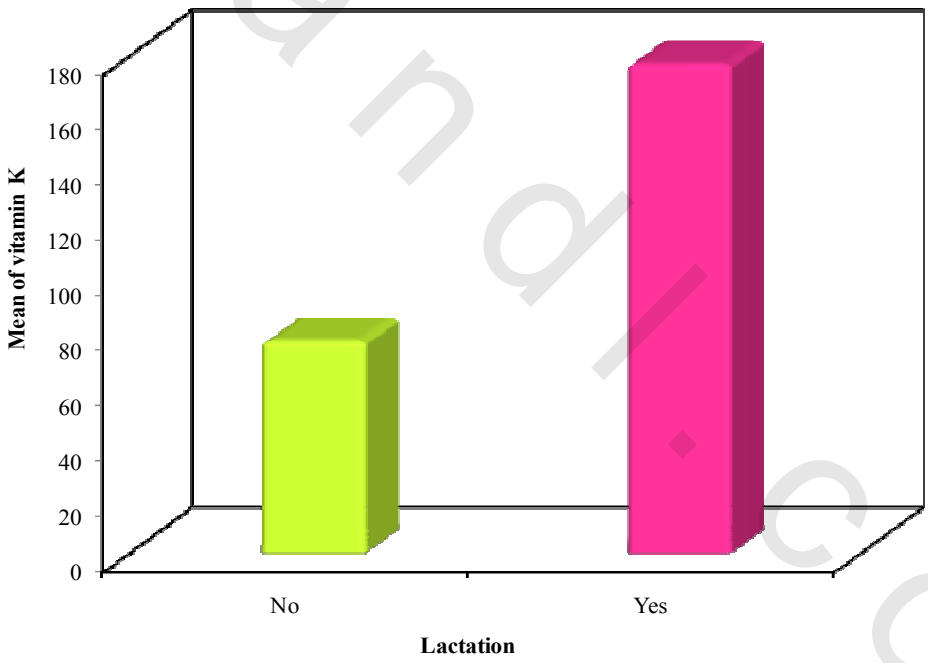


Fig. (18): Maternal vitamin k supplementation during lactation and mean serum vitamin k level among breast fed group.

Table (9b): Relation between vitamin K level in breast fed infants and maternal vitamin k supplementation during lactation

	Maternal Vit K suppl during Lactation				χ^2	^{FE}p
	No (n = 23)		Yes (n = 22)			
	No.	%	No.	%		
VitaminK level ng/l						
<200	23	100.0	17	77.3	5.881*	0.022*
≥200	0	0.0	5	22.7		

χ^2: Chi square test
FE: Fisher Exact test

Table (9b), shows that 100% of breast fed infants had vitamin k levels below 200 ng/l if their mothers did not receive vitamin K supplementation during lactation while 77.3% were below 200ng/l in those whose mothers received vitamin K supp in lactation. This difference is of statistical significance (P<0.022).

Table (10): Relation between sex and PT and PA in breast fed group

	Sex		t	p
	Male (n = 18)	Female (n = 27)		
PT(in sec)				
Min. – Max.	11.0 – 13.80	11.20 – 14.0		
Mean ± SD.	12.24 ± 0.77	12.31 ± 0.69	0.302	0.764
Median	12.30	12.20		
PA (in %)				
Min. – Max.	80.0 – 100.0	70.0 – 100.0		
Mean ± SD.	90.56 ± 5.60	89.89 ± 6.04	0.373	0.711
Median	90.0	90.0		

t: Student t-test

Table (10), figure (19 and 20) shows that the mean prothrombin time was 12.24± 0.77 seconds among male breast fed infants while it was 12.31± 0.69 seconds among female breast fed infants. Also the mean Prothrombin activity among male breast fed infants was 90.56 % ± 5.60 and 89.89 % ± 6.04 among female breast fed ones. Statistically, no significant differences were shown between the two groups (P=0.764, P=0.711 respectively).

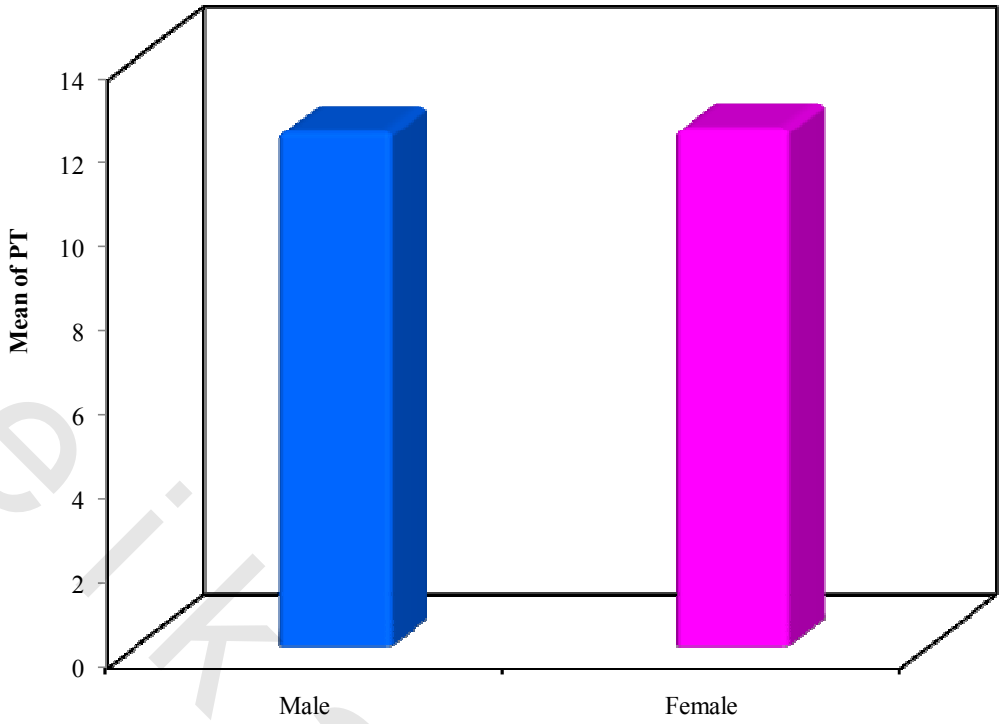


Fig. (19): Relation between sex and prothrombin time among breast fed group

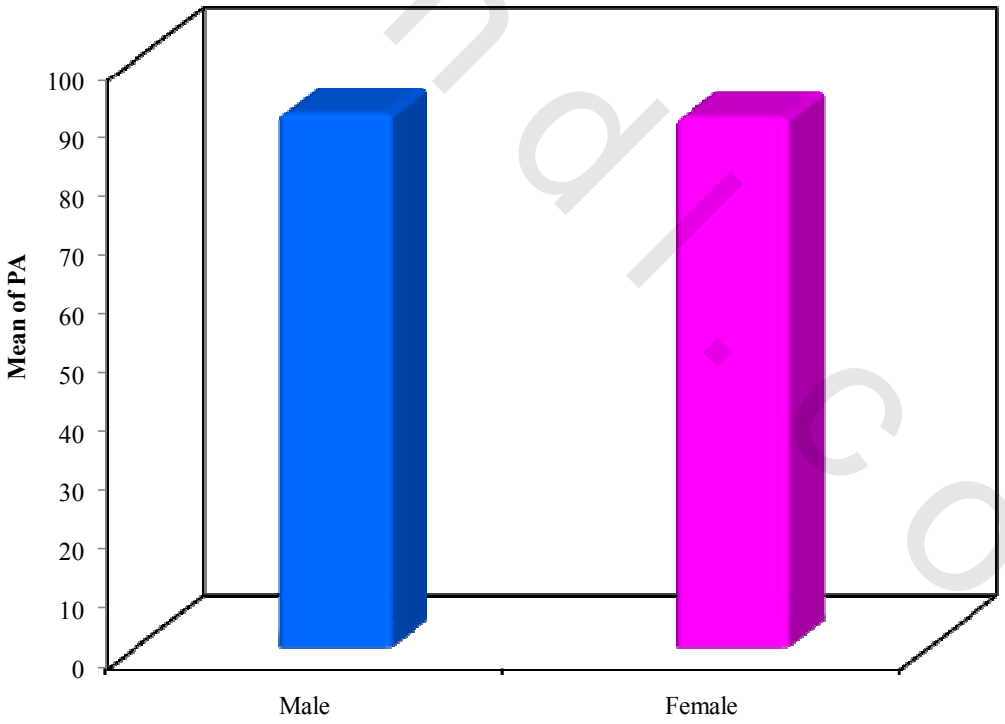


Fig. (20): Relation between sex and prothrombin activity among breast fed group

Table (11): Relation between sex and PT and PA in formula fed group

	Sex		t	P
	Male (n = 26)	**Female (n = 19)**		
PT(in sec)				
Min. – Max.	11.20 – 13.80	11.50 – 14.0		
Mean ± SD.	12.32 ± 0.82	12.67 ± 0.80	1.435	0.159
Median	12.20	12.60		
PA(in %)				
Min. – Max.	11.60 – 100.0	80.0 – 100.0		
Mean ± SD.	87.56 ± 16.57	88.32 ± 5.81	0.190	0.851
Median	90.0	90.0		

t: Student t-test

Table (11), figure (21 and 22) shows that the mean prothrombin time was 12.32± 0.82 seconds among male formula fed infants while it was 12.67± 0.80 seconds among female formula fed infants. Also the mean Prothrombin activity among male formula fed infants was 87.56% ± 16.57 and 88.32% ± 5.81 among female formula fed ones. Statistically, no significant differences were shown between the two groups (P=0.159, P=0.851 respectively).

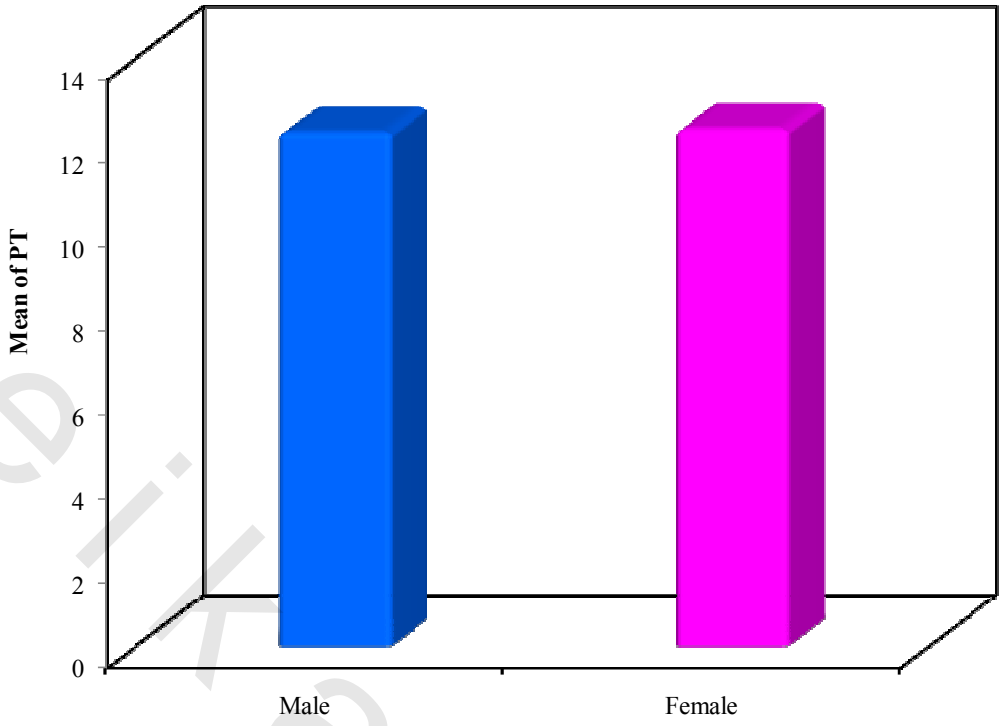


Fig. (21): **Relation between sex and prothrombin time among formula fed group**

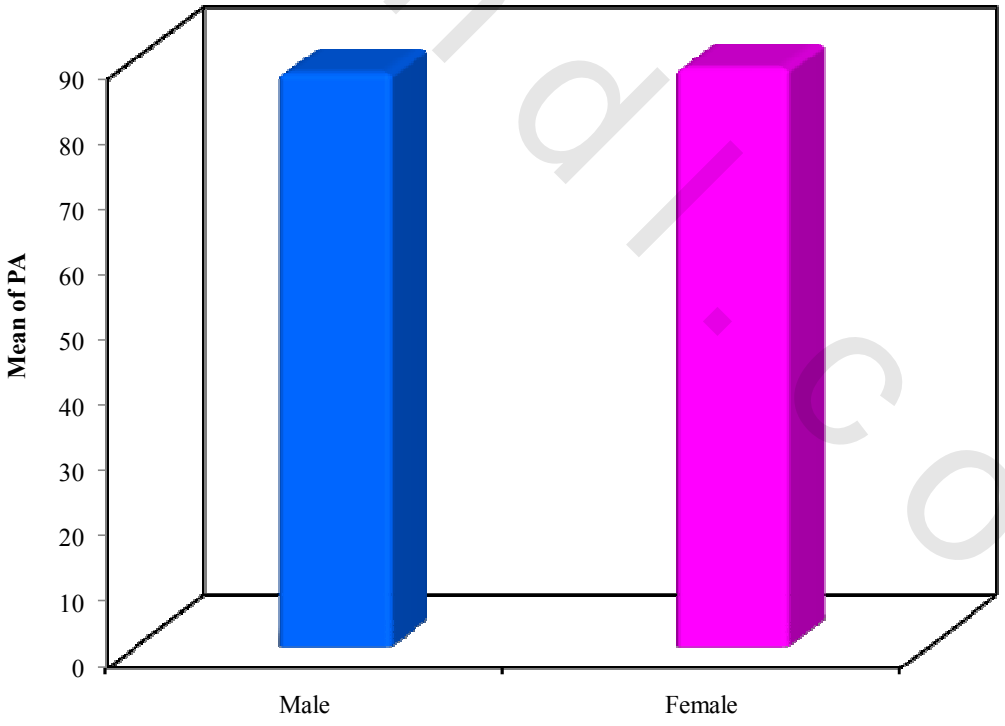


Fig. (22): Relation between sex and prothrombin activity among formula fed group

Table (12): Relation between maternal vitamin k supplementation during lactation and PT and PA in breast fed infants

	Maternal vit k supp during Lactation		t	P
	No (n = 23)	Yes (n = 22)		
PT(in sec)				
Min. – Max.	11.0 – 14.0	11.20 - 13.0		
Mean ± SD.	12.37 ± 0.83	12.19 ± 0.58	0.852	0.399
Median	12.20	12.0		
PA(in %)				
Min. – Max.	70.0 - 100.0	80.0 – 100.0		
Mean ± SD.	89.78 ± 6.79	90.55 ± 4.71	0.436	0.665
Median	90.0	90.0		

t: Student t-test

Table (12), figure (23 and 24) shows that the mean prothrombin time was 12.19 ± 0.58 seconds among breast fed infants whose mothers were taking vitamin k supplementation during lactation while it was 12.37± 0.83 seconds among breast fed infants whose mothers were not. Also the mean prothrombin activity was 90.55% ±4.71% among breast fed infants whose mothers were taking vitamin k supplementation during lactation and 89.78% ±6.79 among those who were not. Statistically, no significant differences were shown between the two groups (P=0.399, P=0.665 respectively).

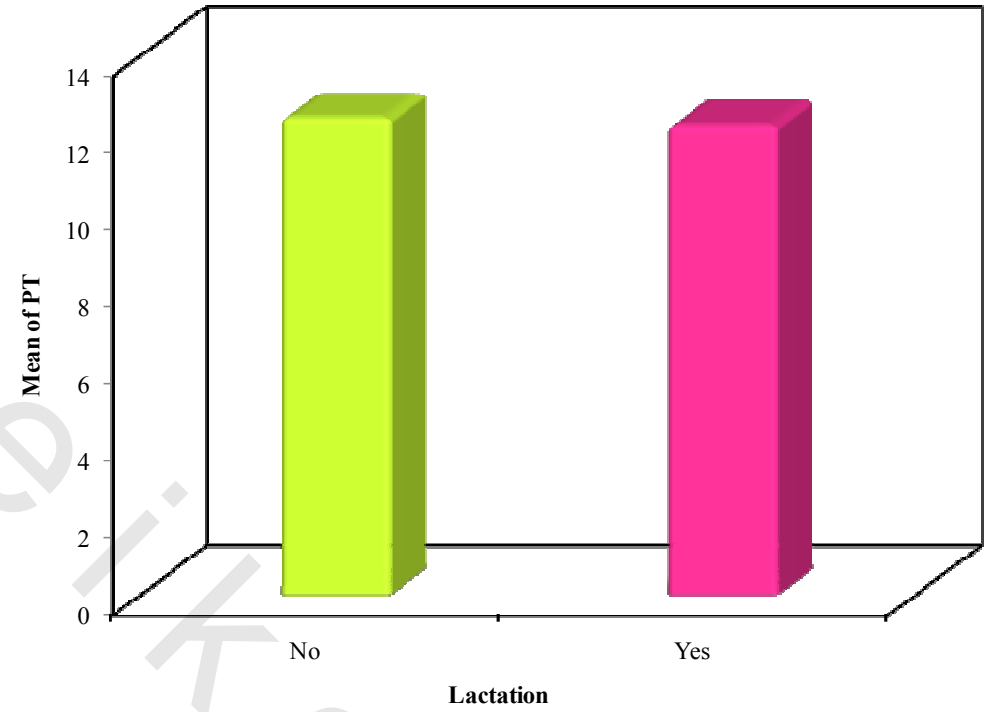


Fig. (23): Maternal vitamin k supplementation during lactation and PT of breast fed infants

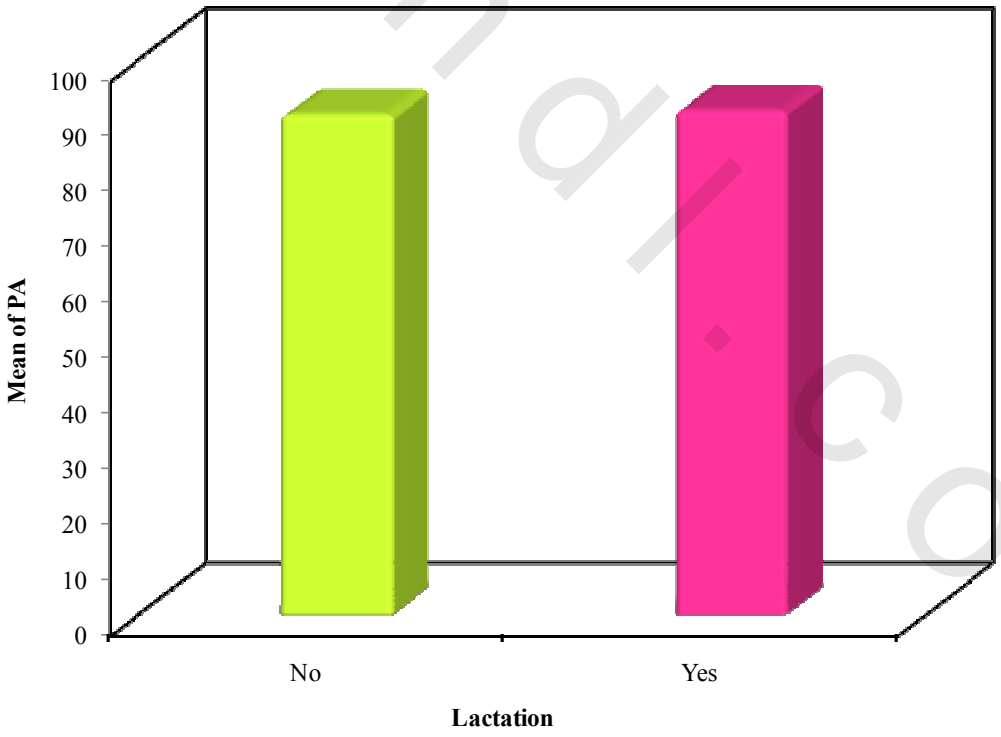


Fig. (24): Maternal vitamin k supplementation during lactation and PA of breast fed infants

Table (13): Correlation between vitamin K and different parameters in each studied group

	Vitamin K			
	Breast feeding		Formula feeding	
	r_s	p	r_s	p
Age (Days)	-0.051	0.741	-0.120	0.432
Birth weight (kg)	0.450*	0.002*	0.485*	0.001*
Gestational age (weeks)	0.197	0.195	0.074	0.628
PT(sec)	-0.076	0.619	0.161	0.292
PA(%)	0.051	0.738	-0.161	0.290
Maternal age (years)	0.556*	<0.001*	0.342*	0.021*

r_s: Spearman coefficient
*: Statistically significant at $p \leq 0.05$

Table (13) studies the relationship between vitamin k level of both breast fed and formula fed infants and different parameters in each studied groups, it demonstrates positive correlations (r=0.103) between levels of serum vitamin K of breastfed infants and both birth weight (r= 0.450) and maternal age (r= 0.556). These correlations are statistically significant (P=0.002 and P<0.001 respectively).

Among formula fed infants, positive correlations are found between serum vitamin K level and both birth weight (r=0.485) and maternal age (r=0.342). These correlations are of statistical significance (P=0.001 and P=0.021 respectively).

Table (14): Multiple stepwise linear regression for factors affecting Vitamin K level in breast fed group

	B	SE	t	p
Vit k supplementation during Lactation	63.106	17.112	3.688*	0.001
Birth weight	99.329	20.012	4.963*	<0.001
Maternal Age	0.972	1.859	0.523	0.604
$R = 0.842$, $R^2 = 0.710$, $F = 33.389^*$, $P < 0.001$				

Table (14) shows that the maternal vitamin k supplementation during lactation and birth weight of the infants greatly influence vitamin k levels among the breast fed infants.

Table (15): Multiple stepwise linear regression for factors affecting Vitamin K level in formula fed group

	B	SE	t	p
Sex	46.154	15.424	2.992*	0.005
Birth weight	61.184	25.810	2.371*	0.023
Maternal Age	2.953	2.010	1.469	0.150
$R = 0.699$, $R^2 = 0.489$, $F = 13.068^*$, $P < 0.001$				

Table (15) shows that sex and birth weight of the infants greatly influence vitamin k levels among the formula fed infants.