

## Effect of punicalagin and human chorionic gonadotropin on body weight and reproductive traits in maiden rabbit does.



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### ABSTRACT

Little is known about the effect of punicalagin extracted from pomegranate or in combination with human chorionic gonadotropin on enhancing the productive and reproductive performance in rabbits that gestate for the first time. The impact of punicalagin alone or in combination with human chorionic gonadotropin on maiden doe body weight, progesterone concentrations, conception rate, gestation rate, litter size, and kit weight was investigated in this study. A completely randomized and balanced experimental design was used to allocate 21 naturally mated maiden does at 6 months of age into the following three treatment groups of 7 does each: Control - intramuscular injection with sterilized water only; Treatment 1 - pre-mating intramuscular injection with punicalagin (100 µg/doe) twice a week and once 3 days post-mating; and Treatment 2 - pre-mating intramuscular injection with punicalagin twice a week and human chorionic gonadotropin (25 IU/doe) once 3 days post-mating. Progesterone was assayed from blood samples taken from the ear marginal vein at mating, post-mating, gestation, and post-kindling phases when all does were weighed. Results indicated significantly positive impacts of punicalagin alone or in combination with human chorionic gonadotropin on all doe reproductive traits, birth, and weaning weights of kits. The study clearly demonstrated an improvement in doe fertility, reproductive performance, and kit survival to weaning. In conclusion, PL and PL+HCG improved maternal body weights and their offspring as well as pregnancy outcomes of young rabbits particularly in the 2nd pregnancy, hence findings of this study could be recommended for improving reproductive health and fertility in maiden rabbit does.

### 1. Introduction

Antioxidants and natural progesterone (P4) in adequate concentrations are needed for reproductive health and sustenance of pregnancy to term in rabbit does that gestate for the first time. Reactive oxygen species (ROS) such as metallic cations, amine oxidase, visible light, and oxygen consumption may originate directly from the embryos or their surrounding environment. Antioxidants play an important role in either preventing free radicals from being formed or removing them before they damage vital cell components. Punicalagin (PL) extracted from pomegranate (*Punica granatum*) is very rich in polyphenols and flavonoids (Seeram et al., 2008) which may maintain reproductive health and survivability of kits. In their first parity, the does may not have the capacity to produce adequate P4 because of inadequate corpus luteum. Corpus luteum is a temporary endocrine gland formed in the ovary from a ruptured Graffian follicle after ovulation. It secretes progesterone responsible for the sustenance of pregnancy. If no pregnancy

the corpus luteum (CL) will degenerate near the end of the estrous cycle. Barnes (2000); Mann and Lamming (1999) reported that rising P4 concentration during the first week of pregnancy resulted in a decrease in embryo mortality. For the maintenance of pregnancy, natural P4 is more preferable than its synthetic form. It is known that human chorionic gonadotropin (HCG) after binding to LH receptors, may provoke an extra P4 synthesis from the accessory CL by increasing interferon-tau (INF-t) secretion (Mann, 2002) which is responsible for extending the CL lifespan (Spencer & Bazer, 1996). Stevenson et al. (2007) confirmed that HCG caused extra natural P4 synthesis from accessory luteal cells after binding to LH receptors. ROS usually accumulate in the reproductive tract and initiate lipid peroxidation (Mishra & Acharya, 2004), which distorts key steps relevant to oocyte maturation and embryo growth. ROS also promotes embryo fragmentation and DNA damage, hence the need for cells to be constantly protected from these harmful ROS.

It has previously been suggested that using pomegranate extract

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could protect embryos from ROS and increase the number of live mice offspring (Kishore, Sudhakar & Parthasarathy, 2009). Little is known about the effect of PL on pregnancy outcomes in rabbits that gestate for the first time, because the first pregnancy may result in either early embryonic loss or weak litters. About 50% of embryonic losses in rabbits occur pre-implantation (Santacreu, Moce, Climent & Blasco, 2005), due to asynchronization between embryonic growth and uterus condition (Chang, 1969; Geisert & Schmitt, 2002). Based on the fact that PL is a potent antioxidant and resistant to ROS and HCG as a promoter of the CL for producing more natural P4, the goal of this study was to investigate the effects of intramuscular injections of PL and PL + HCG on maternal body weight (M BW), conception rate (CR), gestation length (GL), kindling rate (KR), total litter size (TLS), live litter size (LLS), kit weight (KW) and concentrations of P4 in the first two pregnancies of young rabbit does.

## 2. Materials and methods

### 2.1. Animals

The experiment was carried out from March to July 2018 in the experimental unit of the Poultry Production Farm, Faculty of Agriculture, Assiut University, Assiut (Upper Egypt) Twenty-one healthy maiden white new Zealand (WNZ) rabbit does (6 months of age with an average body weight (3.15 ± 0.21 kg) 3. ± 0.21 kg) were randomly allocated into 3 treatment groups of 7 does each as follows: Group I, each doe was injected IM with 100 µg PL twice a week pre-mating and once 3 days post-mating. Group II, each doe was injected IM with 100 µg PL twice a week pre-mating plus once 3 days post-mating and a single dose of 25 IU HCG 3 days post-mating. The choice of 3 days post-mating was made because HCG receptors are more active within this interval. Group III, each doe was injected IM with sterilized water and considered as the control for the two treated groups. All does were weighed using a digital balance in the intervals mentioned above during 1st and 2nd pregnancy. Before starting the experiment, all animals were vaccinated with Formalized Polyvalent Rabbit Pasteurellosis Vaccine (Vet. Ser. and Vac. Res. Inst. Cairo, Egypt) against bacterial septicemia and viral hemorrhagic diseases. All animals received adequate care and all aspects of animal welfare commensurate with international standards of good practice were observed during the study.

### 2.2. Feeding

All does rabbit does were fed a ration containing ingredients and chemical composition as in Table 1. Each doe was fed *ad libitum* on this commercial diet throughout the experiment. Water was available

**Table. 1**  
Represents the ingredients and chemical composition of ration NRC, 1977.

Ingredients	Chemical composition
Yellow corn (15 %)	Crude protein (18.25 %)
Barely (15 %)	Crud fat (3.29 %) 2700 Kcal/Kg of ME (NRC, 1977)
Soybean meal (20 %)	Crude fiber (14.40 %)
Wheat bran (17 %)	
Alfalfa hay (25 %)	
Wheat straw (3.0 %)	
di-calcium phosphate	5 %
Limestone	
Digestive enzymes	
sodium chloride	
Antitoxins	
Anticoccidia	
Lysine	
Methionine	
Dry yeast	

through automatic drinkers attached to the galvanized wire cages.

### 2.3. Housing

All animals were individually housed in galvanized wire batteries (60 × 50 × 35 cm)(60 × 50 × 35 cm) located in 3 naturally ventilated and lighted (13 h light: 11 h dark) rooms. The dimensions of each room were 5.3 × 3.6 × 2.8 m. 5.3 × 3.6 × 2.8 m. The average ambient temperature and relative humidity ranged from 26.01–31.21 oC and 36.29 - 48.59% respectively, about 5 days pre-kindling wooden nest boxes (containing straw or hay) with dimension 40 cm (length) x 26 cm (width) x 27 cm (height) attached to the dam's cages). The bedding was replaced daily by a new one to avoid any contamination from urine or faecal material. A quick mammary structure examination of the dam to confirm lactation status was conducted. Kits were taken out of nest box and weighed before being removed at weaning (4 weeks). From birth to weaning, all kits remained with their dams to suckle without any treatment. All does rabbit in their 1st parity were naturally re-mated after 10 days of kindling without any additional treatments. This part was conducted to confirm the results of treatments obtained from the first pregnancy has no negative impact on 2nd pregnancy outcomes.

### 2.4. Mating

Does were naturally mated by fertile bucks with high-quality semen, good physical conformation, and vigor. Each buck was mated to an equal number of control and treated does. After 10 days of the first kindling, the does were naturally mated by the same bucks used in the 1st pregnancy. CR was determined by palpation after 10 days of mating in both 1st and 2nd pregnancies.

### 2.5. Blood collection

Blood samples collected at mating, 3 days post-mating, each week during pregnancy and immediately post-kindling at 1st and 2nd pregnancies were withdrawn from the lateral ear vein by using a 21 gauge needle. Blood samples were stored overnight in a refrigerator and later centrifuged at 2000 x g for 20 min and the sera were frozen at -20 °C until ready for hormonal assay.

### 2.6. Progesterone (P4) assay

P4 concentrations were assayed using enzyme immunoassay (ELISA) kits (Cal biotech Inc., 1935 Cordel CT., El Cajon, CA 92020 USA). The method was based on the principle of competitive binding between P4 in the test specimen and enzyme conjugate for a constant

amount of anti-progesterone antibody with an assay range of 0–60 ng/ml for P4. A standard curve was prepared relating color intensity at 450 nm to the concentration of P4.

### 2.7. Statistical analysis

Excel spreadsheet was used for data entry and revision. Continuous variables were presented as means ( ± standard errors). All calculations were performed by using Statistical Package for Social Science (SPSS), version 22 (SPSS Inc., Chicago, IL, USA). The normality of the quantitative variables was tested with the Shapiro-Wilk test and all variables were not normally distributed. So, the Mann-Whitney test was used to compare quantitative variables between two groups and the Kruskal Wallis Test for more than two groups. Significance was considered when the P-value was ≤ 0.05. No interaction has been done in the statistical analysis, simply because of dissimilarity between the two pregnancies, where the animals in the first pregnancy were treated by PL and HCG + PL, while the animals in the second pregnancy were not treated by any treatment. Thus, the variation between the two parities prevented the interaction. The data were analyzed by least-square multivariable rather than non-parametric analysis between different treatments of each period because the parametric analysis can assess group means, while the non-parametric analysis assesses group medians. The mathematical model used in the study is as follows:

$$Y_{ij} = \mu + T_i + E_{ij}$$

Where:

Y<sub>ij</sub> = Observation, μ = General mean, T<sub>i</sub> = Effect of treatments and E<sub>ij</sub> = Errors.

### 3. Results

As shown in Table 2, M BW was significantly influenced by treatment. For instance, except for 3 days post-mating interval in the first

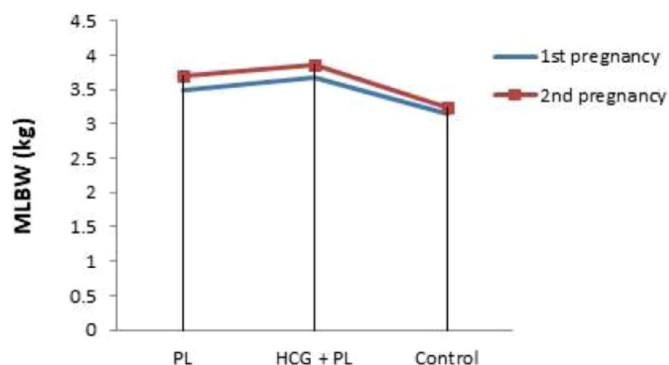


Fig. 1. Overall mean MLBW during 1st and 2nd pregnancy in the treated does compared to the control.

pregnancy, M BW was significantly greater in the combined treatment than the control. The overall mean MLBW was greater than PL and control groups by 0.18 and 0.52 kg respectively, during 1st pregnancy, while during the 2nd Pregnancy, the increases in M BW were 0.15 and 0.62 kg in the combined treatment group compared to PL and control, respectively (Fig. 1). Moreover, PL and PL + HCG achieved weight gain (+ 0.22 and + 0.19 kg in the 2nd pregnancy) as compared with the controls (+ 0.09 kg). Fig. 2

Although CR was not significantly different between the studied groups in the 1st pregnancy, it was significantly higher in PL and HCG + PL groups (100%) than controls (85.71%) in the 2nd pregnancy (one rabbit was aborted) as shown in Table 2.

Results indicated that GL was not significantly different among the groups in the 1st pregnancy, while it was significantly shorter by 1.69 days in PL group than the control in the 2nd pregnancy (Table 2). This means that PL may require some while to display its impact on GL. Its impact on GL in the 2nd pregnancy could have improved the vital components of the oviduct and uterus which supports the early

Table. 2

Mean ± SE of MLBW, CR, GL, KR, TLS, LLS and KW throughout the different periods of the study at 1st and 2nd pregnancy of young NZW rabbit does.

Periods and traits	Pregnancy	PL	HCG + PL	Control	P-value	I vs. II	I vs. III	II vs. III	Total
<b>MLBW (kg) at</b>									
1st mating	I	3.19 ± 0.08	3.40 ± 0.06	2.95 ± 0.06	0.05	0.025*	0.002*	0.007*	
3 days post-mating		3.29 ± 0.09	3.60 ± 0.04	3.10 ± 0.04	0.025*	0.096	0.002*	0.006*	
1st week		3.60 ± 0.09	3.73 ± 0.09	3.18 ± 0.05	0.406	0.004*	0.002*	0.002*	
2nd week		3.75 ± 0.08	3.83 ± 0.11	3.29 ± 0.05	0.609	0.003*	0.002*	0.002*	
3rd week		3.85 ± 0.10	3.95 ± 0.11	3.39 ± 0.05	0.482	0.002*	0.002*	0.001*	
1st kindling		3.29 ± 0.11	3.52 ± 0.11	2.99 ± 0.01	0.180	0.004*	0.003*	0.001*	
CR%		100	100	100					
GL (days)		32.00 ± 0.00	32.14 ± 0.26	32.29 ± 0.57	0.533	0.602	0.789	0.823	
KR%		100	100	85.71					
TLS		7.71 ± 0.47	8.14 ± 0.80	7.43 ± 0.57	0.474	0.740	0.365	0.755	
LLS at birth	7.71 ± 0.47	8.14 ± 0.80	5.86 ± 0.40	0.474	0.269	0.015*	0.331		
LLS at weaning	5.86 ± 1.01	6.43 ± 0.75	3.14 ± 0.86	0.845	0.042*	0.020*	0.014*		
KW at birth (g)	59.11 ± 3.85	51.70 ± 3.96	30.83 ± 2.17	0.142	0.017*	0.017*	0.011*		
Kw at weaning (g)	621.18 ± 55.70	587.03 ± 43.95	390.53 ± 51.74	0.749	0.019*	0.007*	0.005*		
<b>MLBW (kg) at</b>									
2nd mating	II	3.88 ± 0.11	4.00 ± 0.10	3.38 ± 0.06	0.406	0.007*	0.003*	0.002*	
3 days post-mating		3.90 ± 0.11	4.07 ± 0.11	3.45 ± 0.08	0.338	0.010*	0.003*	0.004*	
1st week		3.77 ± 0.10	3.98 ± 0.12	3.36 ± 0.06	0.225	0.015*	0.003*	0.003*	
2nd week		3.75 ± 0.12	4.01 ± 0.13	3.32 ± 0.07	0.180	0.022*	0.003*	0.001*	
3rd week		3.67 ± 0.10	3.81 ± 0.10	3.10 ± 0.06	0.522	0.007*	0.003*	0.002*	
2nd kindling		3.30 ± 0.11	3.31 ± 0.10	2.80 ± 0.04	0.848	0.004*	0.004*	0.008*	
CR%		100	100	85.71					
GL (days)		31.71 ± 0.36	32.43 ± 0.43	33.40 ± 0.60	0.219	0.037*	0.213	0.110	
KR%		100	100	85.71					
TLS		6.43 ± 1.09	6.43 ± 0.48	4.57 ± 0.53	0.515	0.367	0.014*	0.354	
LLS at birth	6.43 ± 1.09	6.43 ± 0.48	2.29 ± 0.75	0.515	0.006*	0.040*	0.070		
LLS at weaning	4.57 ± 0.48	4.71 ± 0.42	0.57 ± 0.43	0.691	0.002*	0.002*	0.002*		
KW at birth (g)	59.00 ± 3.83	51.08 ± 4.21	34.09 ± 3.51	0.110	0.014*	0.059	0.022*		
KW at weaning (g)	381.93 ± 26.26	335.61 ± 29.97	209.50 ± 4.50	0.141	0.040*	0.040*	0.068		

\*Significance at 0.05.

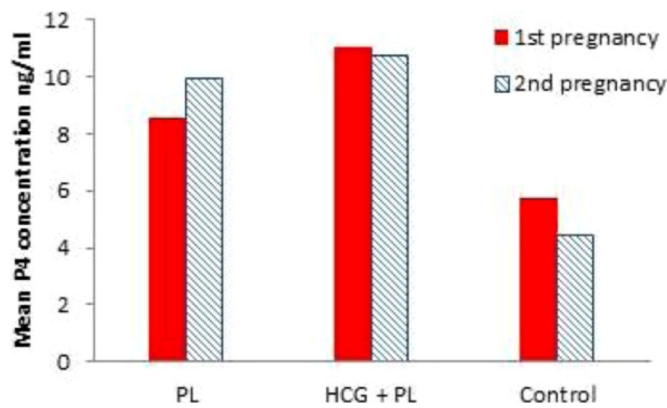


Fig. 2. Overall mean P4 concentration in the does treated by PL and PL + HCG in the first and second pregnancy compared to the control.

implantation.

Table 2, showed that KR was not significantly influenced by the treatments in the 1st pregnancy, while in the 2nd pregnancy it was significantly higher in the treated does than the controls.

Data in Table 2, showed that TLS at birth were not influenced by any treatment in the 1st pregnancy. While LLS at birth was significantly greater by 2.28 kits in the combination group (HCG + PL) than the controls. In the 2nd pregnancy, TLS at birth were ( $P < 0.05$ ) greater in HCG + PL group (1.86 kits) than the control, while LLS at birth was greater by 4.14 kits in both PL and HCG + PL groups than the controls. At weaning LLS were ( $P < 0.05$ ) greater by 2.72 and 3.29 kits in both PL and HCG + PL groups than the controls in the 1st pregnancy, while in the 2nd pregnancy, LLS were ( $P < 0.05$ ) greater by averages 4.0 kits and 4.14 kits in PL and HCG + PL groups than the controls. This means that the treatment with PL and HCG + PL maintained the fetal stability because of copiousness of PL polyphenols and adequacy of natural P4 motivated by HCG.

Data in Table 2, showed also that KWs at birth were ( $P < 0.05$ ) greater by 28.28 g and 20.87 g in PL and PL + HCG groups than the controls in the 1st pregnancy, while in 2nd pregnancy, KWs were ( $P < 0.05$ ) greater by 24.91 g in PL group than the control. At weaning the KWs produced from the mothers treated with PL and HCG + PL in the 1st pregnancy was significantly greater than the controls by 230.65 g and 196.5 g, respectively. In the 2nd pregnancy, KWs were also ( $P < 0.05$ ) greater in PL and HCG + PL groups by 172.43 g and 126.11 g, respectively than the controls.

Results in Table 3, showed that P4 concentrations at mating were significantly greater in the PL group than the control in the two pregnancies (2.3 ng/ml blood serum at 1st pregnancy vs. 1.75 ng/ml blood

serum at 2nd pregnancy). The table indicated also that the first remarkable increase of P4 was at 3 days post-mating in the two pregnancies, where P4 values of the PL group were significantly higher than the controls in the two pregnancies, while the P4 concentration of the HCG + PL group was significantly greater than the control in the 2nd pregnancy and insignificant in the 1st pregnancy. All animals in the experiment displayed their maximum levels of P4 in the 2nd week of the two pregnancies, where the P4 concentrations were significantly higher in combination group by 7.88 ng/ml at 1st pregnancy and 13.18 ng/ml at 2nd pregnancy than the controls, while P4 concentrations in PL group were significantly higher by 11.38 ng/ml in the 2nd week of the 2nd pregnancy than the controls (Table 3). The overall mean of P4 concentrations throughout the study was greater in the treated animals than the controls in the two pregnancies. Otherwise, P4 concentrations dropped suddenly to  $\leq 2.2$  ng/ml blood serum) in all animals after their 1st and 2nd kindling directly. No significant differences for the dropped P4 concentration post-1st kindling were present between the studied groups, while the significance was found between the treated animals and the controls post-2nd kindling. Furthermore, percentages of dropping the P4 concentrations post-kindling (calculated from the maximum level of P4 in the 2nd week of pregnancy to the minimum level post-kindling) were 89.63% (PL), 93.58% (HCG + PL) and 89.66% (control) in the 1st kindling. While in the 2nd kindling, they were 99.35% (PL), 94.10% (HCG + PL), and 74.39% (control).

Treatment with PL achieved true increase (+1.4 ng/ml) for P4 in the 2nd pregnancy, while the combination (- 0.30 ng/ml) and controls (- 1.26 ng/ml) did not achieve that.

#### 4. Discussion

Protecting tissue proteins from ROS damage is of importance in maintaining body health. Herein, PL and PL + HCG combinations led to an increase in M BW at mating and during pregnancy. This increase could be attributed to potent PL polyphenols which protected lipids and amino acids in the uterus from ROS damage, hence foetal growth improved. Kits born from dams treated with PL and PL + HCG combination were heavier at birth and weaning, consistent with the findings of Vazquez-Gomez et al. (2017) who reported that piglets produced from dams treated with hydroxytyrosol (a polyphenol present in olive) improved pre-and-post-natal development of offspring with mean birth weights higher than in the control treatment. This indicates that PL and its combination with HCG may convey to the uterus optimal supply of nutrients which results in maximum embryo development. Previous studies showed that HCG regulated the foetal growth through trophoblastic differentiation, placental growth, uterine angiogenesis and vasculogenesis (Cole, 2012), and stimulated the production of

Table. 3

Mean  $\pm$  SE P4 concentrations throughout the different periods of the first two pregnancies of maiden NZW rabbit does.

Periods of study	Pregnancy	PL	HCG + PL	Control	P-value I vs. II	P-value II vs. III	P-value III vs. Total
1st Mating	I	3.30 $\pm$ 0.72	1.38 $\pm$ 0.19	1.00 $\pm$ 0.00	0.043*	0.003*	0.008*
3 days post-mating		10.36 $\pm$ 0.79	10.02 $\pm$ 2.19	6.01 $\pm$ 1.43	0.848	0.013*	0.160
1st week		13.11 $\pm$ 0.43	17.72 $\pm$ 1.30	7.09 $\pm$ 0.82	0.009*	0.002*	0.000*
2nd week		17.74 $\pm$ 1.59	22.29 $\pm$ 1.25	14.41 $\pm$ 1.82	0.064	0.180	0.009*
3rd week		5.32 $\pm$ 0.48	13.49 $\pm$ 1.59	4.34 $\pm$ 0.35	0.003*	0.180	0.002*
1st kindling		1.84 $\pm$ 0.17	1.43 $\pm$ 0.07	1.49 $\pm$ 0.10	0.110	0.110	0.949
Overall mean		8.55 $\pm$ 0.70	11.06 $\pm$ 1.10	5.72 $\pm$ 0.75			
2nd Mating	II	2.75 $\pm$ 0.62	1.17 $\pm$ 0.11	1.00 $\pm$ 0.00	0.033*	0.005*	0.041*
3 day post-mating		13.87 $\pm$ 0.73	13.00 $\pm$ 0.90	4.53 $\pm$ 0.48	0.338	0.003*	0.000*
1st week		13.68 $\pm$ 0.25	17.66 $\pm$ 1.37	6.27 $\pm$ 0.53	0.025*	0.003*	0.000*
2nd week		20.05 $\pm$ 0.55	21.85 $\pm$ 0.86	8.67 $\pm$ 1.30	0.225	0.003*	0.001*
3rd week		9.20 $\pm$ 0.68	9.61 $\pm$ 0.75	4.05 $\pm$ 0.14	0.565	0.003*	0.000*
2nd post-kindling		0.13 $\pm$ 0.00	1.29 $\pm$ 0.25	2.22 $\pm$ 0.27	0.003*	0.001*	0.046*
		9.95 $\pm$ 0.46	10.76 $\pm$ 0.71	4.46 $\pm$ 0.45			

\*Significance at 0.05.

endocrine gland-derived vascular endothelial growth factor involved in placental development (Brouillet, Hoffmann, Feige, & Alfai, 2012). Barker, Lampl, Roseboom and Winder (2012) reported that intrauterine conditions were completely dependent on placental function that nourishes fetuses, hence a suboptimal intrauterine environment may obstruct foetal growth and lower birth weight (Albu, Anca, Horhoianu and Horhoianu (2014).

CR was higher in the treated groups than in the controls, particularly during the 2nd pregnancy. This observation may be attributed to the positive effects of HCG on CL development for either producing more natural P4 or due to PL acting as a potential antioxidant for providing optimal uterine milieu for embryonic growth or both. Santos, Thatcher, Pool & Overton, 2001 (2001) indicated that pregnancy rate increased after treating cows with HCG on day 6 after oestrus, while the administration HCG on day 5 of the oestrous cycle induced additional CL formation (86.2%), elevated P4 levels and increased CR in cows. CR decreased by 12.4% with a 1 ng P4/ml decrease during the luteal phase of oestrous cycle preceding the first service, where the delay in the normal rise of natural P4 between days 4–5 post-mating reduced pregnancy and CR (Shams-Esfanabadi & Shirazi, 2006). It could be concluded that both PL as potential antioxidant against ROS and HCG + PL improved CR in the first two consecutive pregnancies.

GL during the 2nd pregnancy was shorter in PL group than the controls. This means that PL could extend its positive impact to the 2nd parity. However, data are scarce on the relationship between PL and GL in small or large animals. The decrease in GL may be due to PL polyphenols which protect the zygote from ROS and accelerate the implantation process in the uterine wall. Jenes and Harper, (1984) indicated that the synthesis of prostaglandins involved in embryo implantation, resulted in ROS production, and through the natural antioxidants, protected the cellular components from ROS (Su et al., 2007). To maintain KR in rabbit does, natural P4 must be adequate.

The current results show that KR in all treatments was greater than in the controls, particularly during the 2nd pregnancy. This may be due to the antioxidant activity of PL which protects the embryo from ROS damage, and HCG which differentiates trophoblast to produce more natural P4 in the early stages of pregnancy. In rabbits, HCG blocked apoptosis in the CL cultured *in vitro*, suggesting that this had an important role in the maintenance of luteal cells in humans (Dharmarajan, Goodman, Tilly & Tilly, 1994). Treatment with PL resulted in KR increases, this observation suggests that PL reduced ROS and enhanced the total antioxidant capacity of the placenta, these findings are in agreement with the report of Wang, Huang, Yang & Yang, (2018), where the punicalagin enhanced placental nitric oxide levels and reduced oxidative stress in pregnant rats. Similarly, PL improved cardiovascular activity, attenuated hypoxia-induced apoptosis, reduced inflammation and restored endothelial-cell function *in vitro* (BenSaad, Kim, Quah, Kim & Shahimi, 2017). Celia, Cullere, Gerencsér & Matics, (2015) showed that KR in does rabbit received 300 mg/kg of digestarom (product consisting of herbs, extracts, and essential oils) in the 1st kindling was higher than that reported in another study considering Pannon White rabbits (Szendrő Jováncai Theau-Clément, Radnai, 1999). In comparison to the other domestic animals, the administration of HCG increased P4 secretions and prevented premature luteolysis in super-ovulated (Shabankareh, Seyedhashemi, Torki, Kelidari & Abdolmohammadi, 2012). Induction of accessory CL for increasing natural P4 in the plasma (Thatcher, Moreira, Pncarci, Bartolome & Santos, 2002) or administration of P4 (Lopez-Gatius, Santolaria, Yaniz & Hunter, 2004) was investigated and both studies reported reduction in early embryonic mortality. Mann and Lamming (1999) indicated that P4 supplementation in the first week of pregnancy resulted in an overall increase in pregnancy rate. One previous study showed that treatment with polyphenols had a positive impact on reproductive health and pregnancy (Coan, Ferguson-Smith & Burton, 2004). Also, polyphenol supplementation improved the placental activity against oxidative stress (Chen, Tuuli, Longtine, & Shin,

2012) and increased plasma antioxidant capacity (Ly et al., 2015).

The present study indicate that LLS at birth at 1st pregnancy were greater in combination than the controls while at 2nd pregnancy, TLS was greater than the control and the LLS were greater in both PL and combination than the controls. The overall mean P4 concentrations were also greater in these groups than the controls. The mechanism by which PL increased LS is unknown, but it is thought that PL injected during the 1st pregnancy may extend its positive impact to the 2nd pregnancy by improving the dam's uterine conditions responsible for increasing embryonic survival as a result of improved maternal carbohydrate status and energy availability during pregnancy (provide supporting reference here (Reed, 1995)). Kulkarni, Mahal, Kapoor and Aradhya (2007) indicated that antioxidants had positive effects against oxidative damage to lipids, amino acids and guanosine. Besides, fecundity increased by increasing blood flow in the uterus. Furthermore, fertility of rabbits increased after injection with PMSG plus HCG or GnRH (Castellini, Canal, Boiti & Battaglini, 1991). At weaning, the present study shows that LLS at 1st and 2nd pregnancies were greater in PL and combination groups than the controls, thus indicating that PL as a potent antioxidant against ROS, and a single dose of HCG as a supporter of foetal growth via elevated P4, played their vital roles in reducing mortality rate at weaning. Huxley and Neil (2003) found that with anti-oxidative activity, there was a decrease in various diseases and mortality, in addition to blastocyst development support in the mouse (Wang et al., 2002). Sheffield (1991) suggested that the increase in lactation performance when caffeine was administered to mice may be due to an increase in mammary developmental capacity during pregnancy.

The current results reveal that KWs at birth and weaning in the first pregnancy were greater in PL and combination groups than the controls, while in the 2nd pregnancy KWs were greater in combination than control. These results are consistent with the findings of Vazquez-Gomez et al. (2017) showing that piglets produced from dams treated with hydroxytyrosol had improved pre- and early post-natal development with higher birth weights than the control. Monsefi, Parvin and Talaei-Khozani (2012) showed that the length of the femur increased in embryos after treatment of mice with 3.3 ml pomegranate juice/kg between days 8 and 18 of pregnancy in addition to its positive impact on bone cells. Furthermore, hCG/LH receptors were found in the foetal organs (Rao, 2001) which in turn play a major role in foetal growth. However, the increase in KW at weaning may be attributed to the positive impact of PL on maternal mammary glands responsible for producing more milk or that the milk produced from those dams contained anti-microbial and antioxidant factors which protected kits from coliform infections. A previous study (Rosenblat & Aviram, 2006) showed that PL and ellagitannins had antimicrobial, antioxidant, anti-inflammatory, antimutagenic, and immune modulatory properties both *in vivo* and *in vitro*. Rehfeldt and Kuhn (2006) indicated that the high birth weight in piglets had better post-natal growth performance. Similarly, piglets treated with hydroxytyrosol were significantly heavier than the controls at 15 and 25 days of age (Vazquez-Gomez et al., 2017). Adequate antioxidant capacity in pregnant women led to alleviation of intra uterine growth restriction from oxidative stress (Jauniaux & Burton, 2016). Injected HCG with PL may also have a positive impact on pre-natal fetal growth which could be reflected in post-natal development, this is in agreement with the report of Cole (2012). Hence, the integration between HCG and PL in the current results supported pregnant does and their offspring during the 1st and 2nd pregnancies. Adequate P4 is necessary for the maintenance of pregnancy in rabbit does (Salem & Gomaa, 2014). The present study indicates that PL and HCG + PL improved P4 concentrations during pregnancy. These results are consistent with the findings of Packova, Carbonell-Barrachina & Kolesarova, (2015), where the P4 released from rabbit ovarian fragments was significantly increased at 100 µg.ml – 1 PL and E2 secretion decreased at 10 µg.ml – 1 PL. This means that PL as potential antioxidant either supported granulosa cells of oocytes to

produce steroid hormones or protected the placenta from ROS damage, while HCG increased the natural P4 through its positive impact on CL development. Huxley and Neil (2003) showed that antioxidant compounds enhanced fertility and activated liver enzymes with low rates of mortality and various diseases. Polyphenol supplementation increased plasma antioxidant capacity (Ly Christina et al., 2015) and improved antioxidant enzymes against placental oxidative stress (Chen et al., 2012).

The current results show that the drop in P4 during the 1st and 2nd kindlings dropped to  $\leq 2.0$  ng/ml in all animals. This is normal because reducing P4 at the end of pregnancy stimulates the onset of parturition and leads to a resumption of myometrial activity to exert contractile effects on the foetus (Csapo, 1969). Hilliard, Spies and Sawyer (1968) showed that the causal factor(s) responsible for P4 drop at the end of gestation in rabbit does could be attributed to a reduction in CL activity and cholesterol turnover. Keikkala et al. (2013) and Rabie and Magann (2014) reported that low HCG concentrations in the first trimester were linked to pregnancy loss, preeclampsia, and lower birth weight. In contrast, the increased P4 concentration observed in the PL group particularly in the 2nd pregnancy increased P4 concentration in the 2nd pregnancy, this observation suggests that PL protected placenta function and foetal development. Chen, Longtine and Nelson (2013) reported that oxidative stress associated with placental dysfunction and lower pregnancy outcomes was reduced with using punicalagin. Furthermore, PL polyphenols were responsible for hormonal regulation, anti-inflammation and antidiabetic protection (Zarfeshany, Asgary & Zarfeshany, Asgary & Javanmard, 2014) and foetal development (Ly Christina et al., 2015). So, it is concluded that PL and HCG + PL improved pregnancy outcomes in the first two pregnancies of maiden rabbit does.

## 5. Conclusion

This study hypothesised that punicalagin (a potential antioxidant against ROS) and its combination with HCG (a vital source of P4 from luteal cells) will improve reproductive and pregnancy outcomes in maiden does. P4 concentrations during 1st and 2nd pregnancies were improved by both treatments before, after mating and throughout pregnancy. In conclusion, PL and PL+HCG improved body weights of dams and their offspring as well as pregnancy outcomes of young rabbits particularly in the 2nd pregnancy, hence the tested hypothesis in this study should be accepted and its findings could be recommended for improving reproductive health and fertility in maiden does.

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## Ethical statement

All animals received high quality care (housing, lighting, ventilation, cleaning, cages, syringes, gloves and treatment) under authors supervision and well-trained technicians, Faculty of Agriculture, Assiut University. During the experimental procedure, the rabbits were protected from specific pathogens (bacterial septicemia and viral hemorrhagic diseases). Further, the other environmental conditions (chemical hazards, radiation, heat, noise, odors and pains) that affect the welfare of animals were avoided in the experiment. Authors must ensure our manuscript has been carried out in accordance with ethics publishing and ethics guidelines. Declaration of Competing Interest No one of the authors cooperated in this paper entitled "Effect of combination punicalagin and single dose of HCG on live body weight, some reproductive traits and progesterone concentration in the first two pregnancies of virgin rabbit does" has a financial or personal relationship with other people or organizations that could affect contents

of this article.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.vas.2020.100140](https://doi.org/10.1016/j.vas.2020.100140).

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