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EFFECT OF DIFFERENT HORMONAL REGIMES ON FERTILITY IMPROVEMENT OF ARTIFICIALLY INSEMINATED SUB-FERTILE NEW ZEALAND DOE RABBITS

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ABSTRACT
Forty eight sub-fertile doe rabbits with no conception rate, repeated mating and low receptivity to male during natural mating were used in this experiment. The females were allocated into three experimental groups for different treatments for improving their reproductive performance; namely; the first group was treated with pregnant mare serum gonadotropin PMSG, while the second group was treated by $PGF_{2\alpha}$ + PMSG. The third group received (5ml/doe/day) of sunflower oil (SF) for 5 days, followed by PMSG injection. After 54 h from the PMSG injection in all treatments, all does were artificially inseminated with diluted raw semen followed by Gn-RH dose for induction of ovulation. The results showed that (PMSG) treated group demonstrated the highest ($P<0.05$) receptivity (69.57%), followed by the ($PGF_{2\alpha}$ + PMSG) treatment (40.00%). Both treatments were significantly higher than using (SF + PMSG) with only (10%) observed receptivity. Mathematically, the overall mean for the effect of different treatments regardless of the vulva colour showed superiority of the (PMSG) treatment (47.91%), as compared to the ($PGF_{2\alpha}$ + PMSG) treatment receptivity (31.25%). In the same frame, mathematically the overall indication of red coloured vulva gave a high indication of receptivity of New Zealand doe rabbits (66.66%), compared to the white or pink colour of the vulva, showing receptivity percentage (31.25%) and (20.83), respectively **(Table 3)**. Both (PMSG) and ($PGF_{2\alpha}$ + PMSG) groups showed a significant superiority for conception rate (73.91% and 66.67%, respectively) compared to the (SF + PMSG) treatment with only (10%) conception. The same aforementioned effect trend of the treatments was observed for litter size after parturition, being (5.08±0.71, 4.47±0.88 and 1.4±1.07) for (PMSG), ($PGF_{2\alpha}$ + PMSG) and (SF + PMSG) treatments, respectively. In conclusion, both (PMSG) and ($PGF_{2\alpha}$ + PMSG) treatments promote the sexual receptivity to sub-fertile doe rabbits, specially does with low receptivity and repeated refusal of natural mating. It could be recommended that, an appropriate hormonal treatment and application of AI using fresh diluted semen can be a solution for fertility improvement and increasing fertility potential of sub-fertile doe rabbits under semi-arid conditions which may affect the normal reproductive behavior and the reproductive efficiency of rabbit herds.

KEYWORDS: Artificial insemination, Doe rabbits, Sunflowers, Synchronization, Litter size, Semen, Conception rate.

1. INTRODUCTION
Artificial insemination (AI) has become a routine practice in rabbit production (Alvariño, 2000). The technique offers significant benefits, including genetic selection, prolonged fertility even during unfavorable times of the year, cycle based production, and more efficient breeding programs. Although this reproductive assisted technique totally suits the new applied production systems in developing countries with the aim of reducing cost and increasing meat production (Abdel-Azeem, 2010), although (AI) as a modern reproductive technology has recently started to be used in rabbit farms in Egypt but still with limited application.

Under arid or semi-arid conditions, animals' fertility may have been reduced due to the surrounding environmental conditions. Marai and Rashwan (2004) reported that the doe that was capable to produce 10 litters a year may give only 4 to 5 litters in hot climate. In addition, the intensive breeding system for rabbit production requires insemination of does during nursing period (0 d – 11 d) post-partum, which may lead to a decrease in the reproductive efficiency due to the contrast of the lactating period with the reproductive functions (Theau-Clément and Roustan, 1992). Several studies have investigated the use of different hormonal regimes to enhance the reproductive efficiency in rabbit does (Dimitrova *et al*., 2009; Castellini, *et al*., 2003; Theau-Clement, *et al*., 2000). The use of pregnant mare serum

gonadotropins (PMSG) is now largely used in the synchronization of rabbit does for stimulating the ovarian dynamics and promoting of the follicular growth (Vanna, *et al*., 1996; Theau-Clément and Lebas 1996). Also, many reports have concluded that using prostaglandin ($PGF_{2\alpha}$) lead to a significant improvement in the reproductive performance and the receptivity of rabbit does (Boiti, *et al*., 2006; Theau-Clement, *et al*., 2008). Prostaglandins injection could have a very rapid significant effect on steroid synthesis in the lutein cell, whereas, normal luteolysis would seem to cause more gradual regression of the gland. This may have some relevance in explaining the mating acceptance post-injection of $PGF_{2\alpha}$ (Abdel-Azeem, 2010; Gogol, 2009). As rabbits are induced animals, thus, ovulation depends on neurohormonal reflex throughout natural mating, but when using AI, the ovulation may require a hormonal treatment such as Gn-RH or hCG injection for induction of ovulation confirmation (El-Ratel, *et al*., 2017; Khalifa, *et al*., 2000). Nevertheless, Castellini, (1996) recommended limiting use of exogenous products such as (hormones, antibiotics…) because of their harmful effects on animal and human health, suggesting the replacement of such materials with natural methods that perform the same purpose. The natural methods include farm management, animal manipulation, feeding programs, photoperiod and buck effects (Boiti, 1998). As well as using natural feed additives for the enhancement of productivity and reproductive efficiency of the herds.

Fats which contain poly unsaturated fatty acids PUSFA such as sunflower or omega 3 can enhance ovarian follicles by increasing precursors for the synthesis of reproductive hormones such as estradiol 17- ß E_2 and prostaglandins PG (Abayasekara and Wathes, 1999; Petit, *et al*., 2004). Oil of sunflower is a good source for (PUFA). Recently in sheep, when supplemented their feeding diet to be rich in palmitic and oleic fatty acids, increased effect on the reproductive performance of animals was observed, this positive improvement included numbers and diameters of follicles, conception rate and twinning rate% (Abd El-Hamid, *et al*., 2016), similar results were reported by Bilby, *et al*., (2006) in dairy cows fed on a diet enriched with PUFA compared to the cows fed on a diet enriched with monounsaturated fatty acids. Recently, in sows, supplementation of minimum dietary intake of 10 g/d of α-linolenic acid, or 125 g/d of linoleic acid led to an improved reproductive efficiency, the observed improvement included rapid return to estrus, high maintenance of pregnancy and improved subsequent litter size (Rosero, *et al*., 2016).

The aim of the present study is to determine the best hormonal protocol to improve the reproductive efficiency including (conception rate and litter size) and the ability of replacing the hormonal injection to sub-fertile rabbit does by oral administration with PUSFA such as sunflower oil to improve low fertility of rabbit does by the aid of artificial insemination application under semi-arid condition.

2. MATERIALS AND METHODS

2.1. Location and climatic data

This study was carried out in Maryout Research Station that belongs to Desert Research Center (DRC), 34 km South West of Alexandria (Lat: 31.0^0 17.748" Long:$29^0$47' 22.2504"). Meteorological data of relative humidity (RH, %), air temperature (AT, ◦C), were obtained from the Egyptian Meteorological Authority (EMA) for the located region during the experiment period with mean values of average ambient temperature (28.32°C) and relative humidity (72.15%, RH) and temperature humidity index THI (26.95%). Temperature-Humidity Index (THI) was calculated according to the equation [THI = db ^oC – [(0.31 – 0.31 × RH) × (db ^oC – 14.4)] proposed by Marai *et al*., (2002).

2.2. Animals and managements

Forty-eight New Zealand White does, 24-30 months old, with an average body weight of 3.27±0.1 kg were used in this experiment, all doe rabbits used in this study were excluded from the main herd with a history of non-conception rate, repeated and/or refuse of natural mating with males. Each rabbit was individually housed in a standard dimension wired metallic cage throughout the experimental period from November to December, 2016. All animals were fed a commercial pelleted concentrate mixture diet (*ad lib*). Analysis of basal contents of the pelleted concentrate with digestible energy of 2630 k cal/ kg. are shown in Table (1).

2.3. Hormonal treatments and experimental groups

Rabbit does were allocated into three groups, the first group (n= 23) was treated by 20 µg of pregnant mare serum gonadotropin PMSG IM (Gonaser, Laboratories HIPRA, S. A.-Avoda. Laselva, 135 17170 Amer (GIRONA), Spain.). While the second group (n= 15) was treated by 62.5 µg $PGF_{2\alpha}$ IM (Estrumate, 250µg Cloprostenol/ml, Schering-Plough Animal Health, Germany) + PMSG 20 µg. The third group (n=10), received (5ml/doe/day) of sunflower oil (Arma Company, Al-Asher Ramadan city, Egypt) for 5 days the chemical composition of sunflowers oil is presented in Figure (1), followed by PMSG (20 µg). After 54 h, all does were artificially inseminated with diluted raw semen followed by IM injection of 25 µg/ dose of Gn-RH (Cystorelin, Ceva Sante` Animal -10, av.de la Ballastie`re-33500 Libourne - France) for confirming induction of ovulation. Experimental groups with different hormonal regimes and experimental design are shown in Figure (2).

Sexual receptivity detection based on morphological examination of the valve during to the different treatments was recorded according to (Quintela *et al.*, 2001). The conception rate percentage was calculated 10 days after insemination via abdominal palpation, as well as recorded litter size percentage after parturition.

2.4. Semen collection

Semen ejaculates (n=40) were collected, evaluated and diluted from New Zealand mature bucks (n=10) the day assigned for artificial insemination of doe rabbits using an artificial vagina made of a plastic cylinder with a latex liner secured around the rim, so that warm water could be placed between the cylinder and the latex liner. The artificial vagina was connected to a plastic test tube and 5-6 ml of water at 50ºC to 55°C was introduced, ensuring a temperature of 40ºC to 42°C at the time of collection, despite heat dispersion due to the small size of the device. Ejaculate volume (ml) was recorded through gradual collection tubes. Immediately after collection, concentration (X 10^9 sperm/ml.), motility (%), livability (%), primary and secondary abnormalities (%) and acrosomal integrity (%) were initially assessed in raw semen.

2.5. Semen characteristics assessment and dilution

A phase-contrast microscope (Lica, type DMLS) with a warm stage adjusted at 37^0C was used for the assessment of sperm motility in five different fields at 400X magnification. A digital Cole Parmer pH meter (pH 500 series) was used for pH recording, sperm livability (live and dead sperm, %) and abnormalities (primary and secondary abnormalities) were examined using the differential staining technique. Acrosomal reaction was examined following the procedure reported by (Johnson *et al*., 1976).

Collected semen ejaculates were pooled and diluted (1: 3) dilution rate, with Tris base extender, composed of 250 mM of tris-hydroxymethyl aminomethane, 88 mM of citric acid, 47 mM of glucose and 80 mg/L, 20% egg yolk in up to 100 ml distilled water according to (Di Iorio *et al.*, 2014), unless stated otherwise, all chemicals and reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA). Diluted semen was re-evaluated prior insemination using a phase-contrast microscope (Leica) at 400 X magnification with a 37^0C hot stage for assessment of forward progressive motility (%), freshly diluted pooled ejaculates with progressive motility percentages (≥ 80%) were immediately used for AI (0.5 ml /doe).

2.6. Statistical analysis

The data of sexual receptivity and conception rate were analyzed by the Chi-square test, (SAS, 2006). While semen parameters and litter size data were evaluated by ANOVA, the significance of differences between means was tested using t-test.

3. RESULTS

Physical characteristics (mean±SE) of raw semen collected from mature New Zealand rabbit bucks during the breeding season are presented in Table (2), where volume showed a mean volume 0.68±0.07 ml., concentration 543.57±12.94 (X10^9 sperm/ml), pH 7.62±0.32, motility 87.55±3.44% and live spermatozoa 89.25±1.17%. Primary, secondary abnormalities and acrosomal integrity showed percentage values 5.00±0.68, 9.91±0.99 and 93.12±1.57, respectively.

Sexual receptivity examination based on the morphological appearance of hormonally treated sub-fertile New Zealand doe rabbits is shown in table (3), the results showed that, for red color vulva, the first group (PMSG) showed the highest (P<0.05) receptivity (69.57%), followed by the ($PGF_{2\alpha}$ + PMSG) treatment used in the second group with 40.00 % with significant difference in between. Thus, both the first and the second groups were significantly higher than the third group treatment (SF + PMSG) with only (10%) receptivity. The overall mean for the effect of different treatments regardless of the vulva colour showed superiority of the (PMSG) treatment (47.91%), as compared to the ($PGF_{2\alpha}$ + PMSG) treatment receptivity (31.25%) and finally the (SF + PMSG) treatment with an overall receptivity (20.8%).

On the other hand, the red color vulva seemed to be a high indicator of high receptivity of New Zealand doe rabbits (66.66%), regardless of the hormonal treatment effect, compared to the white or pink colour of the vulva, showing receptivity percentage of 31.25% and 20.83%, respectively.

The effect of different hormonal treatments for improving of sub-fertile New Zealand doe rabbits for conception rate percentage and litter size are shown in table (4). The PMSG treated group and the second group ($PGF_{2\alpha}$ + PMSG) showed significant (P<0.05) superiority (73.91% and 66.67%, respectively) compared to the (SF + PMSG) treatment as represented in group (3) with only (20%) conception rate, mathematically, the PMSG treatment in group (1) surpassed all other groups with (7.24%) for group (2) and (53.91%) for the third group.

The former aforementioned trend in conception rate percentage, was typically repeated for the litter size at birth, where non-significant superiority was recorded for the PMSG group with litter size of (5.08±0.71) compared to the second group ($PGF_{2\alpha}$ + PMSG) with litter size of 4.47±0.88. Thus, both former treatments significantly (P<0.05) showed significant superiority upon the (SF + PMSG) treatment representing the third group with 1.4±1.07 litter size.

Table (1): Analysis of basal content pelleted diet.	
Ingredients	**Values**
Crude protein (%)	16.5
Fat (%)	0.6
Digestible energy (k cal/ kg)	2630
Minerals mixture (%)	13.1
Crude fiber (%)	2.5

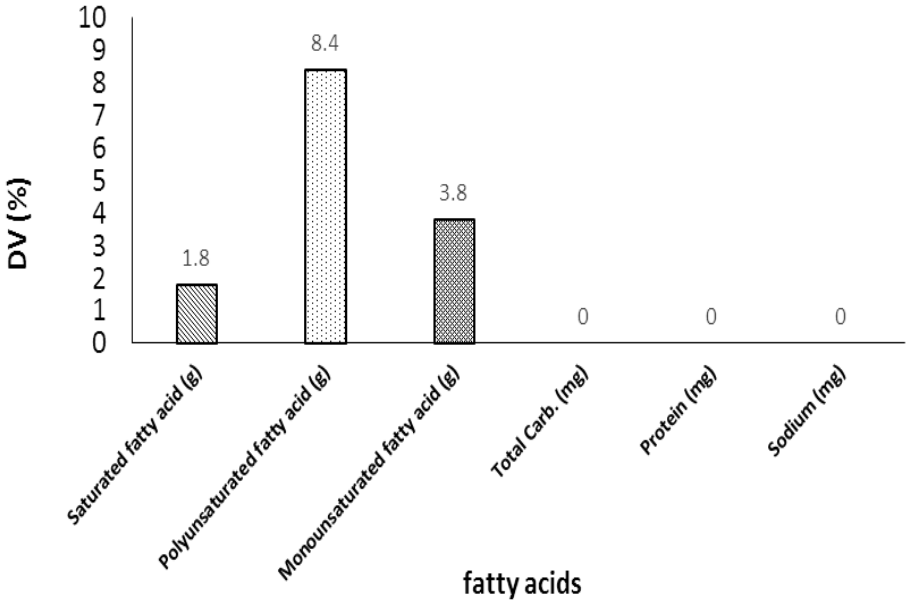
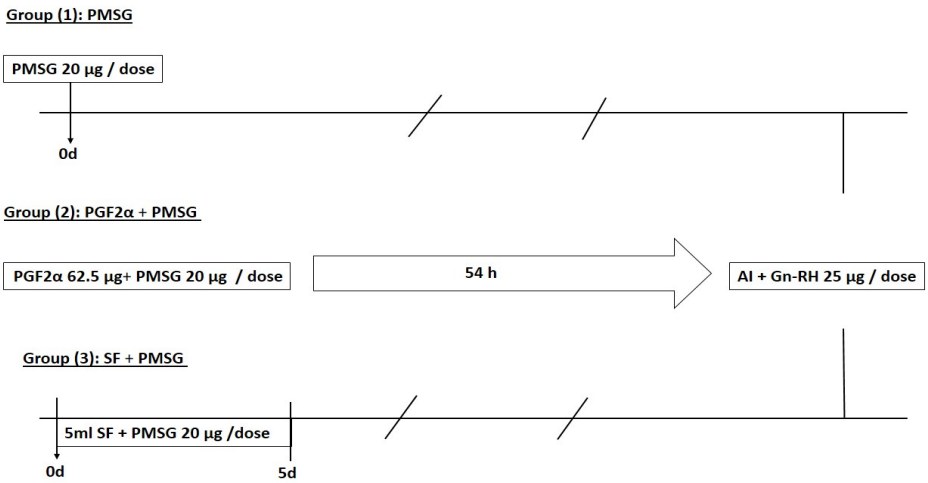


Figure 1: Chemical composition of sunflowers oil (g/k Calories), percentage of daily values (DV) are based on a 2000 calorie.



Experimental groups, different hormonal regimes and experimental design.

Table (2): Raw semen characteristics (mean ± SE) of New Zealand rabbit bucks.	
Semen parameters	**Values**
Volume (ml)	0.68 ± 0.07
Concentration ($\times 10^9$ sperm/ml)	543.57 ± 12.94
pH	7.62 ± 0.32
Sperm motility (%)	87.55 ± 3.44
Live spermatozoa (%)	89.25 ± 1.17
Primary sperm abnormalities (%)	5.00 ± 0.68
Secondary sperm abnormality (%)	9.91 ± 0.99
Acrosomal integrity (%)	93.12 ± 1.57

Table (3): Sub-fertile New Zealand doe rabbits receptivity (based on valve morphological appearance) under different hormonal regimes.

Receptivity	*PMSG Group (n=23)*	*$PGF_{2\alpha}$ + PMSG Group (n=15)*	*PMSG + SF Group (n=10)*	*Overall*	*P ≤*
White (%)	17.39 (4/23)^{C}	33.33 (5/15)^{B}	60 (6/10)^{A}	31.25 (15/48)	0.05
Red (%)	69.57 (16/23)^{A}	40.00 (6/15)^{B}	10 (1/10)^{C}	66.66 (32/48)	
Pink (%)	13.04 (3/23)^{C}	26.67 (4/15)^{AB}	30 (3/10)^{A}	20.83 (10/48)	
Overall	47.91 (23/48)	31.25 (15/48)	20.83 (10/48)		

- **$^{A-C}$ values within the same rows with different letters differ (P < 0.05)**
- **Overall means were mathematically calculated.**
- **$^{A-B}$ values within the same rows with different letters differ (P < 0.05).**
- Conception rate= (number of pregnant does/ number of mated does) × 100
- The litter size = (total number of kit) were recorded after birth.

Table (4): Conception rate & litter size of artificially inseminated Sub-fertile New Zealand doe rabbits under different hormonal regimes.

Item	*PMSG Group (n=23)*	*$PGF_{2\alpha}$ + PMSG Group (n=15)*	*PMSG + SF Group (n=10)*	*P ≤*
Conception rate (%)	73.91 (17/23)^{A}	66.67 (10/15)^{A}	20 (2/10)^{B}	0.05
Litter size (Number)	5.08 ± 0.71^{A}	4.47 ± 0.88^{A}	1.4 ± 1.07^{B}	

4. DISCUSSION

The percentage of red vulva colour was significant *(P ≤ 0.05)* higher 69.57% in doe rabbits treated by (PMSG) compared to other group treated by ($PGF_{2\alpha}$ + PMSG) 26.67% or (PMSG + SF) 10%. While the pink vulva colour was significant *(P ≤ 0.05)* higher in does treated by $PGF_{2\alpha}$+ PMSG or PMSG + SF compared with the PMSG treated group (Table 2), these results were in agreement with (Quintela, *et al*., 2001; Gogol, 2004; Gogol, 2009; Abdel-Azeem, 2010; Elkomy & El-Speiy, 2015).

Pregnant Mare Serum Gonadotrophin (PMSG) and Prostaglandin $PGF_{2\alpha}$ are used in the synchronization of doe rabbits (Castellini and Battaglini, 1991). PMSG has stimulating growing of ovarian follicles (Vanna, *et al*., 1996), while $PGF_{2\alpha}$ may have some relevant physiological role, one of the most frequently cited hypotheses relies on the luteolytic effect of $PGF_{2\alpha}$ acting on pseudo-pregnant does. Thus, $PGF_{2\alpha}$ leads to the regression of existing corpora lutea and consequently withdraws the inhibition of progesterone notably on estrogen secretion, therefore allowing a new reproductive cycle (Boiti, *et al*., 2006; Theau-Clement, *et al*., 2008; Gogol, 2009). Theau-Clément and Roustan (1992) noted that red and pink vulva colors are a very important conceded to correspond maximal receptivity and fertility, which give an indicator of receptivity and determined by the serum estradiol levels secreted by the growing ovarian follicles (Castellini, 1996). Herein this trail, almost all experimental animals were suffering from infertility referring to their previous history or either repeated of mating or very low receptivity and refusing of the natural mating process, in this regard, the AI was recommended depending on the morphological appearance of the vulva colour as a clear indication of receptivity of females (Quintela *et al.*, 2001).

The percentage of white vulva colour was significant *(P ≤ 0.05)* higher in rabbits treated by PMSG + SF 60 % and PMSG + $PGF_{2\alpha}$ 33.33% compared to other group treated with PMSG 17.39% and this was an indicator for the low receptivity of the doe. These results may be attributed to the small size of ovarian follicles, as agreement to the reported results reported by Theau-Clément and Roustan (1992), who observed less than 35% of females with a white vulva after hormonal synchronized treatment. These results may also be due to the high negative energy balance, consequently decrease in IGF1 secretion which stimulate growing of ovarian follicles (Jones, *et al*., 2008).

In this study no statistical differences (*P* = 0.8) in conception rate at 10th day after insemination of does treated with PMSG and the second group of animals treated with $PGF_{2\alpha}$ + PMSG as shown in Table 3. However the conception rate in the first group tended to increase (73.91%) compared to ($PGF_{2\alpha}$ + PMSG) treatment (66.67%). The results of this investigation were completely in agreement with the results previously reported by (Gogol, 2009; Quintela, *et al*., 2001; Facchine, *et al*., 1998; Alaphilippe and Bernard 1998; Alvariño, *et al*., 1995; Facchine, *et al*., 1992), as they reported that both hormones (PMSG) and ($PGF_{2\alpha}$) had improved the reproductive efficiency in does inseminated after parturition. As the $PGF_{2\alpha}$ may have an indirect action only on pseudo-pregnant does and may be involved in the ovulatory process by stimulating the

enzymatic proteolytic cascade which leads to the disruption of the follicular wall (Myazaki *et al*., 1991), while PMSG has a direct action on the ovaries, promoting the follicular growth and improving both quality and quantity of matured follicles.

The conception rate increased ($P \leq 0.05$) in both experimental groups 1 & 2 treated by (PMSG) and ($PGF_{2\alpha}$ + PMSG), being (73.91% and 66.67%, respectively) compared with group 3 does treated by PMSG + SF (20%). In agreement with our results Mollo, *et al*., (2003) reported that, PMSG and $PGF_{2\alpha}$ used for estrus synchronization after postpartum can be a treatment for conception rate improvement as well as litter size in doe rabbits. In our results the conception rate was lower in (PMSG + SF) group. This finding is in agreement with previous reports on dairy cow by Carroll, *et al*., (1990) and Sklan, *et al*., (1994) as they reported that conception rates were lower at first AI for primiparous cows fed calcium soaps fatty acids CSFA compared with free CSFA diet served to the control group. Many studies of PUSFA effect on farm animal's reproductive performance showed that, no significant differences were detected in conception rates or pregnancy rates of AI (Moallem, *et al*., 1997). The lower conception rate in (PMSG + SF) group may be attributed to the different types of fat with varying PUFA content to females of cattle and other mammals, which may have a role in altering the number and size of follicles, ovulation rate, progesterone production by the corpus luteum, timing of luteolysis and gestational length (McNamara, *et al*., 2003).

In this experiment, it could be observed that, using of (USFA) as sunflower oil as alternatives to hormones had a weak effect on the improvement of the reproductive efficiency of sub-fertile does. More research is needed with larger numbers of does to explain the mechanisms of $PGF_{2\alpha}$ or PUSFA action on ovarian activity in rabbits. Specially that rabbit does are not cyclic females and the reproductive response could be mediated by the ovary: if the doe presents different ovary situations, the effect of $PGF_{2\alpha}$ could be very different (luteolytic or luteotrophic).

5. CONCLUSION

Using PMSG and $PGF_{2\alpha}$ + PMSG for oestrus synchronization can increase the sexual receptivity to sub-fertile doe rabbits with low or non-conception rate, specially does with low receptivity and repeated refusals of natural mating. An appropriate hormonal treatment and application of AI using fresh diluted semen may be an adequate application to fertility improvement and expanding fertility potential through increasing of receptivity, conception rate and litter size of sub-fertile doe rabbits under semi-arid conditions that may affect the normal reproductive behavior and the reproductive efficiency of rabbit herds.

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