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# Impact of Doublesynch and Ovsynch Protocols, Preovulatory Follicle, Estradiol and Luteal Profiles on Fertility in Cyclic Murrah Buffaloes during Breeding and Compromised-Breeding Season

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## *ABSTRACT*

The present study designed to evaluate the efficacy of Doublesynch and Ovsynch estrus synchronization protocols, preovulatory follicle (POF) and luteal profile in cyclic Murrah buffaloes (n=83) in summer and winter season at farm and field level. In Ovsynch protocol, buffaloes were administered (i.m.) two injections of 10µg, Buserelin acetate on days 0 and 9, and an injection of 500µg, Cloprostenol on day 7. In Doublesynch protocol, an additional injection (i.m.) of 500µg, Cloprostenol 2 days before the start of Ovsynch protocol was administered. The ultrasonography (USG) and blood sampling was performed on each day of treatment, on day of estrus and 10 days post-AI. The conception rate in Ovsynch treatment group during summer and winter season was 20% and 27.8%, respectively and in Doublesynch groups, it was 40% and 48% in summer and winter season, respectively. Overall mean diameter of POF on day of estrus was larger ($p<0.05$) in pregnant than non-pregnant buffalo (14.6 ± 0.4 vs 12.7 ± 0.3 mm, respectively). The POF diameter was positively correlated with estradiol concentration on the day of AI and corpus luteum (CL) diameter on day 10 post-AI (E2: $r=0.84$, $p<0.05$; CL: $r=0.72$, $p<0.05$). Doublesynch group's buffaloes had higher ($p<0.05$) ovulation rate after first GnRH injection than Ovsynch group than Ovsynch in both season. It was concluded that Doublesynch protocol can be used as estrus synchronization protocol in both seasons to achieve acceptable conception rate with size of preovulatory follicle greatly affecting the fertility by influencing estradiol levels and luteal profile in buffalo.

***Key words:*** Conception rate, Murrah buffalo, Ovsynch, PGF2α, POF.

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# INTRODUCTION

Optimum fertility of buffaloes is the key determinant for the profitable dairy farming. Poor reproductive performance of the animals leads to economic losses due to reduced production and additional cost on management (Mulligan *et al*., 2006). Sound reproductive rhythm in each buffalo is essential for ensuring regularity of calving with a narrow dry period to have profitable dairy farming. Buffaloes have tendency of poor expression of estrus in compromised breeding season i.e. summer and increased sexual activity in breeding season i.e. winter (Madan *et al*., 1996; Singh *et al*., 2000). Nevertheless, the buffaloes are capable of breeding throughout the year with low ovarian activity during summer (Perera, 2011). Detection of estrus in buffaloes is a difficult task due to the lack of expert personnel, high incidence of silent estrus, poor estrus behaviour, lack of homosexual behaviour as shown by cattle, variations in duration of estrus cycle i.e.17 to 26 days, with a mean of approximately 21 days (Thakur *et al*., 2013) and variable duration of estrus from 4 to 64h (Baruselli *et al*., 2001), and the difficulty encountered in predicting the time of ovulation, limits the success rate of artificial insemination (AI) in buffaloes. Such reproductive problems reduce a farmer's financial returns due to a reduced number of pregnancies. In the last decades, various ovulation synchronization protocols have been developed which are able to precisely synchronize the time of ovulation within a stipulated time period and thus, avoid the need for heat detection with variable results regarding conception rate. In 1995, the Ovsynch protocol, a milestone protocol for estrus synchronization having sequence of a combination of GnRH-$PGF_{2\alpha}$-GnRH injections was developed to synchronize follicular development, luteal regression and ovulation and doing so theAI can be done at a fixed-time without the need of estrus detection (Pursley *et al.*, 1995). The success rate of the Ovsynch protocol is primarily dependent on the stage of estrous cycle at the time of onset of the protocol (Galina and Orihuela, 2007). Ovsynch protocol applied between days 13 and 17 or early in the estrous cycle (days 2–4) led to a reduced pregnancy rate (Moreira *et al.,* 2000 and Vasconcelos *et al.,* 1999).To overcome this problem, a novel estrus synchronization protocol named Doublesynch includes the administration of an additional $PGF_{2\alpha}$ injection 48 h before start of Ovsynch protocol has an advantage over ovsynch protocol that can be used irrespective of stages of estrous cycle (Cirit *et al*., 2007). Until now, not a single study carried out to see the impact of Double-synch and Ovsynch protocol during breeding (winter season) and compromised breeding season due to heat stress in summer under field and farm conditions in buffalo. Therefore, the current investigation was designed with the objectives: 1) to compare the effect of Ovsynch and Doublesynch estrus synchronization protocol on conception rates at farm and field condition during breeding (winter) and compromised-breeding (summer) season; 2) to establish correlation between preovulatory follicle, luteal profiles and conception rates in buffalo.

# MATERIALS AND METHODS

The study was conducted on 83 Murrah buffaloes (parity: 2-4; >2 months postpartum; BCS: 3-4 on 5-point scale) during summer (between May and June) and winter (between December and January). These buffaloes were divided into eight groups and each group comprising buffaloes as following:-

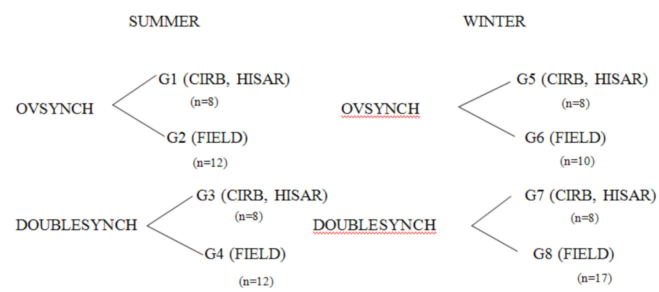


**Fig. 1:** Classification of the experimental groups based on the place of study (Field and Farm), season (Summer and Winter) and estrous synchronization protocol (Ovsynch and Doublesynch).

All the buffaloes were screened twice through ultrasound at 11 days apart to confirm the cyclicity and to rule out any abnormality in reproductive organs. Further, blood samples were also collected twice at 11 days interval to analyze the progesterone levels. The buffalo that had CL on either ovary at any day of ultrasound done twice were considered as cyclic buffalo. Further, the plasma progesterone if found >1ng/mL in one sample of two days collection at 11 days apart was also considered cyclic buffalo. The Estrous cycle of all buffaloes was synchronized by using Ovsynch and Doublesynch protocol as shown in schematic diagram (Figure 1).

In ovsynch protocol, buffaloes were administered (i.m.) two injections of a GnRH analogue (10µg, Buserelin acetate, Gynarich, Intas pharmaceutical ltd., India) on days 0 and 9, and an injection of a synthetic $PGF_{2\alpha}$ analogue (500µg, Cloprostenol Sodium, Vetmate™, Vetcare, Bangaluru, India) on day 7 (Pursley *et al*., 1995).In Doublesynch protocol, it is similar to the Ovsynch protocol but an additional injection (i.m.) of a synthetic $PGF_{2\alpha}$ analogue (500µg, Cloprostenol Sodium, Vetmate™, Vetcare, Bangaluru, India) on 2 days before the start of Ovsynch protocol was administered (Cirit *et al*., 2007). Fix timed

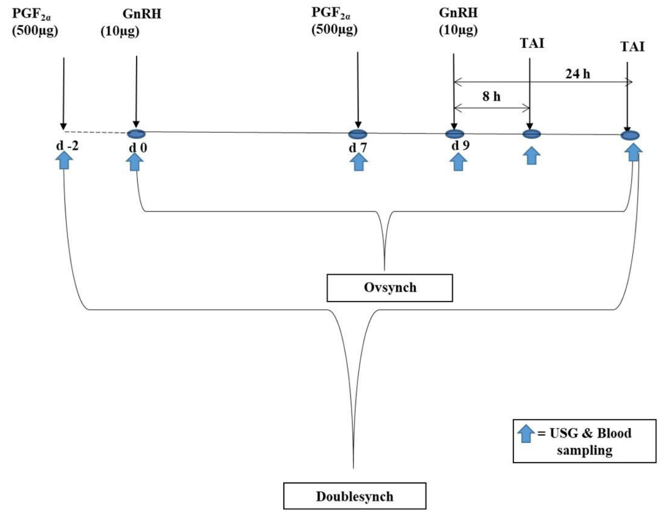


**Fig.2:** Schematic diagram of treatment schedule for estrous synchronization, AI and PD in buffalo; Ovsynch: Inj. GnRH (Buserelin acetate, 10µg) i.m. on day 0 and 9, PGF2α (Cloprostenol, 500µg) i.m. on day 7, AI at 8 and 24 hours after 2nd GnRH; Doublesynch: Same as Ovsynch except for additional Inj. PGF2α (Cloprostenol, 500 µg) i.m. 2 days before the start of Ovsynch (d: Day; h: Hour; TAI: Timed Artificial Insemination; PD: Pregnancy Diagnosis).

**Table 1:** Conception rates in cyclic buffaloes according to estrus synchronization protocol (Doublesynch and Ovsynch) at farm and field in breeding (winter) and compromised-breeding (summer) season.

| Group | Protocol | Total No. of animals | No. of Pregnant Animals | Conception rate (%) | Combined (farm+field) conception rate (%) |
|---|---|---|---|---|---|
| G1(Farm, Summer) | Ovsynch | 8 | 1 | 12.5 | 20 |
| G2(Field, Summer) | Ovsynch | 12 | 3 | 25 | |
| G3(Farm, Summer) | Doublesynch | 8 | 3 | 37.5 | 40 |
| G4(Field, Summer) | Doublesynch | 12 | 5 | 41.7 | |
| G5 (Farm, Winter) | Ovsynch | 8 | 2 | 25 | 27.8 |
| G6 (Field, Winter) | Ovsynch | 10 | 3 | 30 | |
| G7 (Farm, Winter) | Doublesynch | 8 | 4 | 50 | 48 |
| G8 (Field, Winter) | Doublesynch | 17 | 8 | 47 | |

artificial insemination (AI) twice with frozen thawed semen was carried out in all the groups at 8 and 24 hours after the second GnRH injection(Figure 2).

Transrectal ovarian ultrasonography was carried out using a battery-operated B-mode ultrasound scanner (Exago, ECM, Angouleme, France) equipped with inbuilt interchangeable 5/7.5 MHz linear-array rectal transducer. Scanning was carried out from the day of start of the protocol, during the time of each treatment (farm only), at the time of AI and thereafter at 24h interval until ovulation. On day 7 and day after induced estrus, sonographic scanning was performed to detect the ovulation rate following 1st and 2nd GnRH injections of each protocol. Ovaries were systematically scanned and the size of the follicles/CL was determined as per our previous study (Pandey *et al.*, 2011 and 2018). The ovulatory follicle diameter was, retrospectively, the diameter recorded on the day just before the day of disappearance (ovulation) of follicle (Pandey *et al.*, 2011 and 2018). In addition, CL diameter was measured through sonography on day 10 post-AI also. Pregnancy was confirmed on day 45 post-AI.

Thirteen ml of blood was collected from the jugular vein in heparinised 15 ml centrifuge vials on each day of treatment, the day of 1st AI, and on day 10 post-AI. Collected blood was centrifuged at 1500×g for 15 minutes to separate plasma, and plasma aliquots were stored at -20$^0$C until Estradiol-17β ($E_2$) and Progesterone ($P_4$) hormone analysis through ELISA using commercially available Direct Immuno-enzymatic Assay ELISA Kit (Italy).

All numerical data are represented as mean ± SE. Student's t-test was used to compare variation in ovulatory follicle diameter, plasma estradiol on the day of estrus, corpus luteum (CL) diameter and plasma progesterone profiles between groups of summer and winter seasons under farm and field conditions. The relationship between the diameter of preovulatory follicle (POF) and plasma estradiol ($E_2$) on the day of estrus, preovulatory follicle and CL (day 10 post-AI), thereafter CL and plasma progesterone on the day 10 post-AI were analyzed using Pearson's correlation coefficient. The conception rate was defined as the number of buffaloes diagnosed as pregnant on day 45 post-AI divided by the total number of buffaloes in a particular group. Differences at a p-value less than 5% ($p<0.05$) were considered to be statistically significant. All statistical analyses were performed using the SPSS (16.0) system for windows.

# RESULTS AND DISCUSSION

The study was conducted on cyclic buffaloes at farm and field level in summer and winter seasons. In summer season at farm and field level, the conception rate in Ovsynch treated groups was 12.5% (1/8) and 25% (3/12), respectively, while in the Doublesynch group, conception rate was 37.5% (3/8) and 41.7% (5/12), respectively. During winter season at farm and field level, in Ovsynch treated groups, the conception rate was 25% (2/8) and 30% (3/10), respectively, while in Double-synch groups, conception rate was 50% (4/8) and 47.1% (8/17), respectively (Table 1).

In the present study, the conception rate was recorded higher in Doublesynch protocol treated buffaloes as compared to Ovsynch treated buffaloes in both seasons. This could be attributed to the effect of $PGF_{2\alpha}$ injection which was administered 48 hours before the first GnRH, solely or by synergic effect together with GnRH, which might have overcome partially or largely the factors causing the reduction of ovulation and/or pregnancy rates in anestrous cows (Öztürk *et al.*, 2010). Similar to present findings, several authors have found increased conception rates in cattle (Cirit *et al.*, 2007 and Öztürk *et al.*, 2010) and buffaloes (Dhindsa *et al.*, 2016 and Mirmahmoudi and Prakash, 2012). The presynch-Ovsynch protocol (GnRH and $PGF_{2\alpha}$ simultaneously 7 days before ovsynch) than cows synchronized estrus with ovsynch alone resulted in a greater pregnancy rate in cyclic cows (Saini *et al.*, 2023). These mentioned studies concluded that the Doublesynch protocol is better than the Ovsynch protocol in breeding as well as non-breeding seasons. These conclusions are concurrent with the findings of the present study. But, the conception rate in present study was lower as compared to the above studies in cattle and buffaloes. This might be due to differences in parity, management and environmental factors as compared to the above studies. In the present study, buffaloes subjected to the Ovsynch protocol in the summer season showed 50% and 100% ovulation rates after the first and second GnRH, respectively, while in winter season, the ovulation rates were 62.5 and 100%, respectively. In the Doublesynch protocol, ovulation rate was 75 and 100% after the first and second GnRH during the summer season and in the winter season it was 87.5 and 100% after the first and second GnRH. Similar to the present study, higher ovulation rates were obtained after the first and second GnRH treatments (86.6% and 93.3%, respectively) following Ovsynch protocol on the crossbred (Murrah×Mediterranean) cyclic buffaloes (deAraujo *et al.*, 2002). The Ovulation occurred in 90% and 100% buffaloes after the first and second GnRH treatments in the Doublesynch protocol, respectively (Mirmahmoudi and Prakash, 2012). Cirit *et al.* (2007) reported 88.9% and 94.5% ovulation rates after the first and second GnRH treatments in cows treated with the Doublesynch protocol, respectively. The present study showed similar findings of ovulation rate as described earlier. Further, data analysis revealed that pregnancy rate in Doublesynch protocol was higher than the Ovsynch groups in both seasons might be due to a higher ovulation rate after the first GnRH treatment in the Doublesynch groups.

## Correlation of preovulatory follicle (POF) diameter on the day of estrus with subsequent conception rate and post-ovulation luteal profiles (corpus luteum diameter and plasma progesterone)

There were no differences found in diameter of POF between the Ovsynch and Doublesynch treated groups. Therefore, the data from all buffaloes (n=83) were clubbed together and used to establish a correlation between POF diameter and plasma $E_2$ concentrations (on the day of estrus), and subsequent luteal profiles. Out of 83 buffaloes, 29 (34.9%) were diagnosed as pregnant on day 45 post-AI. The mean diameter of POF on the day of estrus was larger ($p<0.05$) in buffaloes that were diagnosed pregnant as compared to their non-pregnant counterparts (14.6 ± 0.4 *vs* 12.7 ± 0.3 mm, respectively). On the day of estrus, a positive correlation was found between the POF diameter and plasma estradiol ($r=0.84$, $p<0.05$). Retrospective analysis of the data revealed higher ($p<0.05$) plasma estradiol on the day of estrus in the buffaloes that conceived as compared to their non-conceiving counterparts (35.3 ± 1.0 *vs* 27.9 ± 1.2 pg/ml).Earlier studies have also reported similar findings in buffalo (Pandey *et al.*, 2011, 2018; Rahman *et al.*, 2012 and Singh *et al.*, 2022). The larger diameter of POF was positively associated with the conception rate. In cows, ovulating larger POF had more chance of pregnancy as compared to cows ovulating smaller follicles and subsequently, luteal profile was better in larger POF than in smaller follicles (Perry *et al.*, 2007). Recently, Singh *et al.* (2022) reported a positive correlation between POF, subsequent CL development and conception rates in buffalo. In contrast, others have found an inverse relationship between the diameter of the ovulatory follicle and embryo survival (Lynch *et al.*, 2010 and Vasconcelos *et al.*, 1999). These studies suggested that as the follicle grows continuously, the oocyte becomes aged which could be attributed to a lower pregnancy rate in larger sized POF. Lynch *et al.* (2010) recorded a lower pregnancy rate of the follicle ovulated >15.1 mm in size in cows. In the present study, further analysis of conception rate on the basis of categorization of POF diameter into four categories (I: 9 to $\leq$12; II: >12.0 to $\leq$15.0; III: >15.0 to $\leq$ 18.0 and IV: >18mm), we recorded 8.3, 43.9, 41.7 and 66.7 % conception rate, respectively. Another study, conducted by Rahman *et al.* (2012) observed that buffaloes having small preovulatory follicles (9 to < 12mm) had low pregnancy rates as compared to medium size (12 to14mm) and large sizes (>14mm to 16mm) POF i.e. 9.1%, 70% and 87 %, respectively. Singh *et al.* (2022) also reported greater pregnancy rates (61.76% and 55.55%) in the POF size ranges from >11.0 to $\leq$14.0 mm and >14.0 to $\leq$17.0 mm, respectively. Retrospective analysis of current data for conception rate based upon the category of follicles confirms the previous reports in buffaloes and suggests that large POF is more competent than smaller to establish a pregnancy.

In current study, POF diameter had a positive correlation with plasma $E_2$ concentrations on the day of estrus. These results are concurrent with the findings of many researchers (Lopes *et al.*, 2007; Lynch *et al.*, 2010; Noseir, 2003; Perry *et al.*, 2007; Pandey *et al.*, 2011, 2018 and Singh *et al.*, 2022). They found higher plasma estradiol in pregnant as compared to the non-pregnant animals while others have observed an absence of correlation between POF diameter and plasma estradiol concentration on the day of estrus (Busch *et al.*, 2008; Perry *et al.*, 2005).

In the present study, CL diameter on day 10 post-AI, was positively correlated (CL: r=0.72, p<0.05) with POF diameter on the day of estrus. In addition, plasma progesterone was also positively correlated with the ($P_4$: r=0.59, p<0.05) day 10 post-AI CL diameter. Furthermore, CL diameter and plasma progesterone were found to be significantly higher (p<0.05) in pregnant as compared to non-pregnant buffaloes (p<0.05). Results of the present study showed that POF had a positive correlation with a subsequent luteal profile on day 10 post-AI. The ability of the CL to produce progesterone depends on the number of granulosa cells in the ovulatory follicle, blood flow to the CL, an adequate number of LH receptors on granulosa and theca cells (Niswender *et al.*, 2000) and the number of granulosa cells depends upon the size of POF (Perry *et al.*, 2005 and Smith *et al.*, 1994). Previous studies in dairy cattle and buffalo have also revealed that a larger POF generates a larger CL which subsequently leads to higher plasma progesterone concentration that results in a higher pregnancy rate (Binelli *et al.,* 2009; Busch *et*

**Table 2:** Ovarian and endocrine parameters in buffaloes (n=83) that were either diagnosed pregnant or non-pregnant in breeding (winter) and compromised-breeding (summer) season, irrespective of the estrous synchronization protocol.

| | **Summer (n=40)** | | **Winter (n=43)** | |
|---|---|---|---|---|
| **Parameters** | **NP (n=27)** | **P (n=13)** | **NP (n=27)** | **P (n=16)** |
| POF (mm) | 13.1 ± 0.6 | 16.1 ± 0.6* | 12.2 ± 0.3 | 13.4 ± 0.4* |
| Estradiol-17β (pg/ml) | 30.2 ± 1.6 | 38.1±2.1* | 25.5 ± 1.2 | 33.3 ± 1.2* |
| CL diameter (mm) | 15.8 ± 0.6 | 18.2± 0.6* | 14.7 ± 0.4 | 16 ± 0.7 |
| Progesterone (ng/ml) | 4.9 ± 0.3 | 6.4 ± 0.2* | 4.7 ± 0.3 | 5.8 ± 0.3* |

**(p<0.05): Between pregnant and non-pregnant within same season. (CL: Corpus luteum, NP: Non-pregnant, P: Pregnant, POF: Preovulatory follicle).*

**Table 3:** Plasma progesterone concentration (ng/ml) in buffaloes during Ovsynch (n=16) and Doublesynch (n=16)estrus synchronization protocol in breeding (winter) and compromised-breeding (summer) season.

| | | **Days** | | | | |
|---|---|---|---|---|---|---|
| **Protocol** | **Season** | **D-2** | **D0** | **D7** | **D9** | **Day of AI** |
| Ovsynch(n=16) | Summer(n=8) | | 3.9 ± 0.5 | 2.7 ± 0.5 | 0.9 ± 0.3 | 0.4 ± 0.1 |
| | Winter(n=8) | | 4.6 ± 0.5 | 4.0 ± 0.5 | 0.8 ± 0.2 | 0.3 ± 0.1 |
| Doublesynch(n=16) | Summer(n=8) | 4.6 ± 0.2 | 0.8 ± 0.3 | 3.1 ± 0.5 | 0.6 ± 0.2 | 0.3 ± 0.1 |
| | Winter(n=8) | 4.8 ± 0.3 | 1.3 ± 0.5 | 3.7 ± 0.4 | 0.6 ± 0.1 | 0.3 ± 0.1 |

*(Ovsynch: 10μg, Buserelin acetate on day 0 and 9 (i.m.) and 500μg, Cloprostenol Sodium on day 7 (i.m.); Doublesynch: similar to the Ovsynch but an additional injection of 500μg, Cloprostenol Sodium (i.m.) on 2 days before the start of Ovsynch. AI: Artificial insemination; D: Day)*

*al.*, 2008; Lopes *et al.*, 2007; Pandey *et al.*, 2011, 2018; Pfeifer *et al.*, 2009; Saini *et al.*, 2023 and Vasconcelos *et al.*, 2001).On contrary, Lynch *et al.* (2010) found no correlation between the diameter of POF and subsequent CL diameter as well as with progesterone on day 7 post-estrus in cattle. Nevertheless, CL diameter was positively correlated with progesterone on day 5 and it was lost on day 12 post-ovulation in buffaloes (Pandey *et al.*, 2011).

Furthermore, analysis of data based on summer and winter seasons revealed that the size of POF, plasma $E_2$ concentration and subsequent luteal profiles (CL diameter and progesterone concentration) on day10 post-AI were higher ($p<0.05$) in pregnant buffaloes as compared to their non-pregnant counterparts (Table 2). However, Ovsynch and the Double-synch treated buffaloes in the summer and winter season showed non-significant ($p>0.05$) variation in plasma progesterone among days of treatment (summer *vs* winter) in the respective protocols (Table 3). Pregnant buffaloes had higher progesterone concentrations on Day 10 post-AI than non-pregnant buffaloes (Campanile *et al.*, 2005) while in another study, Campanile *et al.* (2007) reported no difference in progesterone concentration on day 10 post-AI between pregnant and non-pregnant buffaloes but it reached to the higher levels on day 20 post AI for the former than latter. The findings of present study suggest that the greater POF size, estradiol concentration on the day of estrus, subsequent CL diameter and progesterone profile on day 10 post-AI greatly affected pregnancy outcome in both breedings (winter) and compromised breeding (summer) season.

## CONCLUSIONS

In conclusion, estrous synchronization of cyclic buffaloes with the Doublesynch protocol resulted in higher conception rate than the Ovsynch protocol. However, a study is warranted in large number of buffalo population to validate the results. Irrespective of the season and estrous synchronization protocol, the preovulatory follicle size was observed as an important determinant that governs the estradiol concentrations on the day of estrus, subsequent luteal profile and conception rate in cyclic buffaloes. The Doublesynch protocol could be preferred over the Ovsynch protocol in cyclic buffalo during breeding and compromised-breeding season.

## CONFLICT OF INTEREST

The authors declare no conflict of interest among themselves.

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