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Manuscript should be sent to Dr. J.P. Ravindra (jpravindra@yahoo.com) Chief Editor, Indian Journal of Animal Physiology, ICAR-NIANP, Hosur Road, Adugodi, Bangalore 560030, India.

From president's desk

I am happy to note that SAPI is able to publish our journal again after a gap of about seven years. This has become possible due to the untiring efforts by the Editor Dr. J.P. Ravindra and his team. My congratulation to the Editorial team.

Journal of any association is a window into the activities of the association and reflects the quality of the work being carried out by the members of the association. While good quality research is being carried out by our members, unfortunately most of it gets published in other journals. There are any number of reasons for this and irregular publication certainly is the most important one I believe. Therefore it would be our endeavour from now on to publish the journal regularly at least one issue in a year and perhaps more with good support from members. This is in fact is a relatively simple task if all our members resolve to publish one paper every two years in our own journal. I am sure if this is done in about 5 years – relatively small time frame in the life of an association of professionals and their journal - our journal shall get the necessary international rating to attract more and more contributions. Let us all work towards that goal in the coming years!

With best wishes

Humbly yours

Dr. V.H. Rao

Editorial

An attempt has been made here to revive the Indian Journal of Animal Physiology that has not seen any publication after 2009. Financial constraints and lack of enough number of articles of acceptable standards have been the reasons. Not being able to continuously publish may have subsequently discouraged researchers from submitting their articles to IJAP. This issue is being brought out as an e-publication linked to the SAPI website. It is hoped that this fills confidence in you all, that the society is capable of bringing out the journal in future and thus encourages you to submit articles for future issues. With more articles and improved funds situation, it is hoped to bring out the future issues in print form and work towards getting NAAS rating. The editorial committee thanks all the contributors and acknowledges all the referees who have reviewed and communicated their comments in time, the manuscripts published in this issue.

J.P. RAVINDRA

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Review article

Synch protocols along with TAI: An effective tool for augmentation reproduction in buffaloes

Kajal Sankar Roy*

Principal Scientist, ICAR-National Institute of Animal Nutrition and Physiology, Adugodi, Bangalore- 560 030, India. (E-mail:ksroy123star@gmail.com)

Introduction:

India has world's best buffalo breeds and provides superior germ-plasm throughout the world. In spite of being about one third in numbers compared to the cattle population, buffaloes contribute more than 50 percent of total milk production in India. India possesses about 56 % of the world buffalo population. However, a large population of buffaloes remains unproductive during their productive life due to subestrus/anoestrus condition. The buffalo however, has certain inherent limitations which are contributing to its poor fertility. Among limitations, which are responsible for poor fertility in buffaloes include – (a) late maturity (b) poor estrus expressivity or silent estrus (c) poor conception rates (d) long postpartum calving period and (e) summer infertility due to heat stress in the buffalo inhabiting areas during hot summer months, are the factors most responsible for reduced reproductive efficiency in buffaloes (Kanai and Shimizu, 1983; Prakash, 2002; Paul and Prakash, 2005; Roy and Prakash, 2009a, b). Various reasons have been ascribed for these limitations but no long lasting solutions have been found to solve these problems except the presently acclaimed 'Synch Protocols along with TAI'.

What is the problem in buffaloes?

An accurate detection of estrus is essential in any breeding program using artificial insemination (AI) to capitalize on efficient utilization of superior sires available. As the estrus signs in buffaloes are less obvious than in cattle (Hafez, 1954; El-Sheikh and El-Fouly, 1971; Kanai and Shimizu, 1982, 1983), the accuracy of estrus detection is one of the major problems, hence limiting the use of AI in this species. Though the use of vasectomized bull (teaser) for estrus detection has solved the problem of weak estrus symptoms, but it requires labour, frequent parading of bull and its upkeep as well as time for proper heat detection. One of the main problems for improvement of buffalo production is the low reproductive efficiency. Among other factors influencing the reproductive performance, poor heat expression and seasonal breeding are the important contributory factors (Madan, 1988). Drost *et al*. (1985) reported that the estrus behaviour of buffaloes differs from that of domestic cattle in the putative lack of expression of homosexual behaviour and the secretion of copious amounts of clear estrus mucus. Swelling of the vulva, increased frequency of urination, restlessness and repeated vocalization are suggestive, though not reliable indicators of estrus. As noted by Jainudeen (1986), it has been commonly believed that 'silent estrus' (ovulation unaccompanied by estrus) is a major problem in buffalo breeding. Prakash (2002) evaluated the incidence of silent heat occurrences in

buffaloes by milk progesterone monitoring of Murrah buffaloes throughout the year with an objective to study the influence of changing environmental temperatures on heat occurrences. Out of total 292 estruses recorded by milk progesterone monitoring, 108 estruses (37%) went unobserved. The incidence of silent heat was lowest in December (10.5%) while the peak was seen in April (70%). Due to the high incidence of silent heat large numbers of buffaloes are left unbred and substantially contribute to a prolonged service period in this animal. Determination of when a buffalo is in estrus is difficult because often the animal shows few external signs of "heat". This increases the chances of missing a cycle, especially for artificial insemination. The usual weak symptoms of estrus in the normal breeding season (October to February in India) become still weaker during the hot months of summer (Prakash, 2002; Roy and Prakash, 2007a, b). Therefore, to reduce the time of estrus detection, a few estrus synchronization protocols have been devised both in cattle (Pursley, 1995; 1997a, b) as well as in buffaloes (Odde, 1990; Singh *et al*., 2000, Paul and Prakash, 2005; Roy and Prakash, 2009a, b).

Evolving 'Synchronization Protocol and Timed Artificial Insemination (TAI)':

"Synchronization of estrus has been defined by Roberts (1971) as regulation of estrous cycle at will". The use of pharmacological agents to regulate the estrous cycle has helped to synchronize animals in estrus, and to allow a short period of time to study intensively the behavioural symptoms of heat among the herd.

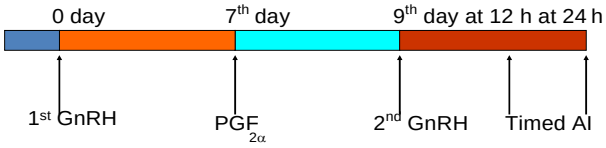
As in many domestic species of animals, including buffaloes, the duration of estrous cycle is controlled by the life span of corpus luteum (CL) in each cycle (Odde, 1990; Singh and Madan, 1991; Singh *et al.*, 2000). Progesterone secreted by the CL imposes a block for the estrus and ovulation. Therefore, if estrus and ovulations are desired the luteal function must be terminated. With this objective, various methods for regression of CL and induction of estrus have been developed but were beset with their own advantages and disadvantages. Ideally, an estrus synchronization system should elicit a fertile, tightly synchronized estrus response in a high percentage of treated females. Methods of evaluating synchronization systems include estrus response (percentage of females showing estrus), synchronized conception rate (percentage of females conceiving) and pregnancy rates during different seasons. Enhanced precision of estrus depends on controlling the life of CL and follicular development (Fogwell *et al*., 1986). An increase in the basic understanding as well as the development of treatment regimes to manipulate ovarian follicular growth and corpus luteum regulation over the last two decades have resulted in development of novel Ovsynch protocol (Pursley *et al*. 1995) for estrus synchronization which is based on elimination of the dominant follicle and initiation of new follicular wave followed by synchronization of ovulation and fixed-time artificial insemination. The technique has also been successfully applied for estrus synchronization and timed A.I. in lactating buffaloes and repeat breeding heifers (Paul and Prakash, 2005; Roy and Prakash, 2009a,

b). Ovsynch protocol successfully produces pregnancy rates equal to those after detected estrus (cows 37.8% and heifers 35.1%, Pursley *et al.* 1997). Reproduction in buffaloes is influenced by season and fertility and is reduced in the period of the year increasing day light hours (Baruselli *et al.,* 2001;2003) due to an environmental effects on the activity of hypothalamo-hypophyseal axis (Perera, 2011). However, various studies indicated that the majority of acyclic buffaloes display variable degree of ovarian activity, characterized by follicular turn over in one or both ovaries and associated to absence of estrus signs. To rescue acyclic animals, several hormonal protocols have been employed (Saini *et al.*, 1986; Rao *et al.*, 1985; Chohan *et al.*, 1995). The estrous cycle in buffaloes can vary from 16 to 28 days (Campanile et.al, 2010) and the duration of estrus is typically 8 to 20 hours (Mohan et.al, 2010). They show waves of ovarian follicular development during estrous cycles similar to cattle, and the number of follicular waves during an estrous cycle can vary from 1 to 3, with 2 waves being the most common (Campanile et.al,2010). Ultrasonic imaging of buffalo ovaries indicates mature follicles range in size from 13 to 16 mm in diameter (Campanile et.al, 2010; Baruseli et.al, 1997, 2003; Perera, 2011; Yindee et.al., 2010). As the reproductive efficiency of buffalo is hampered by poor estrus expression, prolonged calving intervals, and delayed maturity (Madan and Prakash,2007), understanding the expression of estrus behaviors and their relationship with the hormonal injections and associated endocrine changes that control estrus are also fundamental for the creation of strategies aimed at improving the detection of estrus and fertility improvement. It has been reported that estrogen acts as a key regulator of the endocrine and behavioral events associated with the natural estrous cycle and induces estrus behavior, the release of GnRH, an LH surge, and finally ovulation (Hafez and Hafez,2000). In India, currently, anestrous is one of the major causes of economic losses in both dairy and beef industries. To enhance the reproductive efficiency of buffaloes, during the last two decades several researchers were trying to develop the biotechniques through studying their reproductive endocrinology and ovarian function. For instance, after the development of Ovsynch and Heatsynch protocols in cattle (Pursely, et.al. 1995; Pancarci et.al., 2002; Paul and Prakash, 2005; Roy and Prakash, 2009a, b) and Mohan, et al. (2009) successfully performed the Ovsynch and Heatsynch programs in Murrah buffaloes for synchronization of ovulation and timed artificial insemination (TAI) with a range of success in pregnancy rates from 30% to 40%. Further, Cirit, et al. (2007) developed a new synchronization protocol (Doublesynch) by administration of an additional prostaglandin F2α (PGF2α) 48 hour before the Ovsynch program. It has been reported that estradiol benzoate (EB) has some advantages compared with GnRH (*i.e.* cheaper hormone price, easier scheduling and implementation for injection and insemination, greater uterine tone, ease of insemination, and occurrence of estrus as has been recorded using the Heatsynch protocol (Mohan, et al., 2009; Mohan, et al.,2010; Thatcher et.al., 2002). Later, Öztürk, et al. (2010) confirmed the success of

pregnancy rates (43% units more in relation to Ovsynch) of Doublesynch protocol in both cyclic and anestrus cows. The Doublesynch and Estra-Double synch protocol in both cycling and anestrus Murrah buffaloes has also been successfully performed (Mirmahmoudi and Prakash, 2012; 2014). It has been found an increase in the pregnancy rate after TAI using the Doublesynch and Estra-double synch protocol compared with AI after the

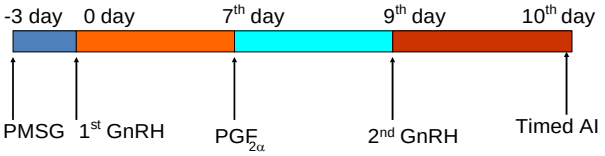
Fig. Different Synchronization Protocol presented in tabular form as ready reckoner

1. Ovsynch protocol with timed AI



- 0 day starts at any day of the month
- GnRH(Receptal dose): 10 µg
- Second GnRH injection is just after 48 hours of $PGF_{2\alpha}$ injection
- $PGF_{2\alpha}$ (Lutalyse dose): 25 mg
- For GnRH and $PGF_{2\alpha}$ other commercial product or salt dose needs to be calculated accordingly

2. Ovsynch Plus protocol with timed AI

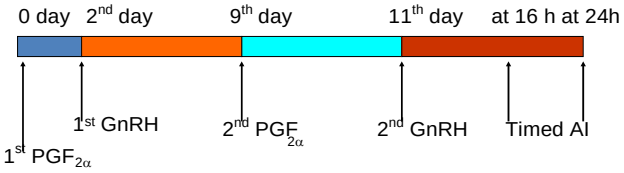


- 0 day starts at any day of the month
- GnRH(Receptal dose): 10 µg
- Second GnRH injection is just after 48 hours of $PGF_{2\alpha}$ injection
- $PGF_{2\alpha}$ (Lutalyse dose): 25 mg
- PMSG : 400 IU
- For GnRH and $PGF_{2\alpha}$ other commercial product or salt dose needs to be calculated accordingly

3. Heatsynch protocol with timed AI

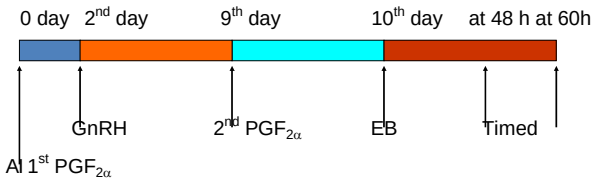
- 0 day starts at any day of the month
- GnRH(Receptal dose): 10 µg
- Second GnRH injection is just after 48 hours of $PGF_{2\alpha}$ injection
- $PGF_{2\alpha}$ (Lutalyse dose): 25 mg
- Estradiol Benzoate(EB) : 1 mg
- For GnRH and $PGF_{2\alpha}$ other commercial product or salt dose needs to be calculated accordingly

4. Doublesynch protocol with timed AI



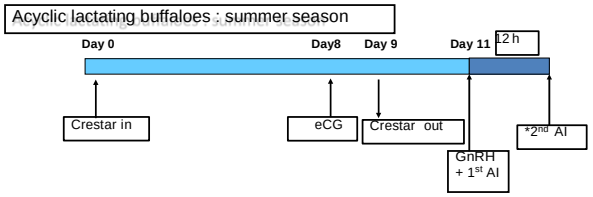
- 0 day starts at any day of the month
- GnRH(Receptal dose): 10 µg
- Second GnRH injection is just after 48 hours of $PGF_{2\alpha}$ injection
- $PGF_{2\alpha}$ (Lutalyse dose): 25 mg
- For GnRH and $PGF_{2\alpha}$ other commercial product or salt dose needs to be calculated accordingly

5. Estradoublesynch protocol with timed AI in cyclic buffaloes:



- 0 day starts at any day of the month
- GnRH(Receptal dose): 10 µg
- $PGF_{2\alpha}$ (Lutalyse dose): 25 mg
- Estradiol Benzoate(EB): 1 mg
- For GnRH and $PGF_{2\alpha}$ other commercial product or salt dose needs to be calculated accordingly

6. Synthetic progestagens - Crestar



- 0 day starts at any day of the month
- GnRH(Receptal dose): 10 µg
- GnRH other commercial product or salt dose needs to be calculated accordingly.
- eCG: 500 IU
- * 2nd AI is optional but advisable after 12 hr.

spontaneous detection of estrus. This higher pregnancy rate to the high rates of ovulation detected after both the first and second GnRH treatments, that itself was owing to the high release of LH after both GnRH injections (Mirmahmoudi and Prakash,2012; 2014). Presently, in an ICAR sponsored "All India Coordinated Research Project on Nutritional and Physiological interventions for enhancing reproductive performance in animals", we are performing these 'Synch Protocols with TAI' on cows and buffaloes at twelve different centers across India. The different methods of estrus synchronization protocols utilized here are represented in schematic diagram as follows:

Conclusions:

The above stated estrous synchronization protocols have proven as most effective and useful tool for reproductive management in buffalo and cattle; during different seasons, across the country. However, if proper levels of nutrition, body condition and health are not maintained, the program may not be highly successful. So, required improvements in amenities and management may be necessary before implementing an estrous synchronization program. This in turn can bring substantial and significant economic return to the dairy farmers.

References and suggested reading:

Baruselli PS, Mucciolo R, Visintin GA, Viana VC, Arruda RP, Madureira EH. 1997. Ovarian follicular dynamics during the estrous cycle in buffalo (Bubalus bubalis). *Theriogenology*. **47**: 1531-47.

Baruselli PS, Maduriera HE, Barnabe VH, Barnabe RC, De Araujo Berber RC. 2003. Evaluation of synchronization of ovulation for fixed timed insemination in buffaloes (Bubalus bubalis). *Brazilian J. Vet. Res. Anim. Sci*. **40**: 431-42.

Campanile G, Baruselli PS, Neglia G, Vecchio D, Gasparrini B, Gimenes LU. 2010. Ovarian function in the buffalo and implications for embryo development and assisted reproduction. *Anim. Reprod. Sci*. **121**:1-11.

Chohan, KR, Iqbal, J, Chaudhary, RA, Khan, AH. 1995. Oestrus response and fertility in true anoestrus buffaloes following hormonal treatment during summer. *Pakistan Vet. J.* **15**: 6-8.

Cirit Ü, Ak K, Ileri IK. 2007. New strategies to improve the efficiency of the Ovsynch protocol in primiparous dairy cows. *Bull. Vet. Inst. Pulawy*. **51**: 47-51.

Drost, M, Cripe, WS and Richter, AR. 1985. Oestrus detection in buffaloes (Bubalus bubalis): Use of an androgenized female. *Buffalo Journal*. **1**: 159 -169.

El-Sheikh, AS and El-Fouly, M A. 1971. Estrus, estrus cycle and time of ovulation in herd of buffalo heifers. *Alex. J. Agriculture*. **19**: 9-14.

Fogwell, RL, Kanyima, BM, Villa- Gody, A, Enright,WJ and Ireland, J. 1986. Enhanced precision of estrus and luteinizing hormone after progesterone and prostaglandin in heifers. *J. Dairy Sci*. **69**: 2179.

Hafez, ESE and Hafez, B. 2000. *Reproduction In Farm Animals*. 7th

Edition, Indian Edition, K.M.Varghese Company, Bombay.

Hafez, ESE. 1954Oestrus and some related phenomena in the buffalo. *J. Agric. Sci. Cambridge*. **44**: 165-172.

Jainudeen, MR.1986. Reproduction in water buffalo. *In: Current Therapy in Theriogenology* (D.A. Morrow, ed.). W.B. Saunders, Philadelphia, pp.443 – 449.

Kanai, Y and Shimizu, H. 1982. Some observations of estrus cycle in Swamp buffaloes. *Japanese J. Anim. Reprod*. **28**: 154-158.

Kanai, Y and Shimizu, H. Characteristics of the estrous cycle of the Swamp buffalo under temperate conditions. Theriogenology 1983; 19(4): 593 – 602.

Madan ML and Prakash BS. 2007. Reproductive endocrinology and biotechnology applications among buffaloes. *Soc Reprod and Fertil Suppl*; **64**: 261–81.

Madan ML. 1988. Status of reproduction in female buffalo. *In: Buffalo Reproduction Health: A Compendium of Latest Research Information based on Indian Studies*. ICAR Publication, New Delhi, India, pp. 89-100.

Mirmahmoudi R and Prakash BS. 2012. The endocrine changes, timing of ovulation and efficacy of the Doublesynch protocol in the Murrah buffaloes (Bubalus bubalis). *Gen.Comp. Endocrinol.* **177**: 153-159.

Mirmahmoudi R. Souri M, and Prakash BS. 2014. Endocrine changes, timing of ovulation, ovarian follicular growth and efficacy of a novel protocol (Estra-doublesynch) for synchronization of ovulation and timed artificial insemination in Murrah buffaloes (Bubalus bubalis). *Theriogenology*. **81**: 237-242.

Mohan K, Kumar V, Sarkar M, Prakash BS. 2010. Temporal changes in endogenous estrogens and expression of behaviors associated with estrus during the periovulaory period in Murrah buffaloes (Bubalus bubalis). *Trop. Anim. Health Prod*. *42*: 21-26.

Mohan K, Prakash BS. 2010. Changes in endogenous estrogens and expression of behaviors associated with estrus during the periovulatory period in Heatsynch treated Murrah buffaloes (Bubalus bubalis). *Trop. Anim. Health. Prod*. **42**: 947-952.

Mohan K, Sarkar M and Prakash B S. 2009. Efficiency of Heatsynch protocol in estrus synchronization, ovulation and conception of dairy buffaloes (Bubalus bubalis). *Asian-Australasian J. Anim. Sci*. **22**: 774-780.

Odde KG. *1990*. A review of synchronization of estrus in postpartum cattle. *J. Anim. Sci.;* **68**: 817-830.

Öztürk ÖA, Cirit Ü, Baran A, Ak K. 2010. Is Doublesynch protocol a new alternative for timed artificial insemination in anestrous dairy cows? *Theriogenology*. **73**: 568-76.

Pancarci SM, Jordan ER, Risco CA, Schouten MJ, Lopes FL, Moreira F, Thatcher WW. 2002. Use of estradiol cypionate in a presynchronized timed artificial insemination program for lactating dairy cattle. *J. Dairy Sci.* **85**:122-31.

Paul V and BS. Prakash. 2005. Efficacy of the Ovsynch protocol for synchronization of ovulation and fixed-time artificial insemination in Murrah buffaloes (Bubalus bubalis). *Theriogenology*. **64**:1049-1060.

Perera BMAO. 2011. Reproductive cycles of buffalo. *Anim. Reprod. Sci*. **124**:124-99.

Prakash BS. 2002. Influence of environment on animal reproduction. Invited Paper; National Workshop on Animal Climate Interaction, held at Izatnagar, India, pp. 33 -47.

Pursley JR, Mee MO, Wiltbank MC. 1995. Synchronization of ovulation in dairy cows using PGF2α and GnRH. *Theriogenology*. **44**: 915-23.

Pursley JR, Kosorok MR and Wiltbank MC. 1997 Reproductive management of lactating dairy cows using synchronization of ovulation. J. Dairy Sci.; **80**: 301-306.

Pursley JR, Wiltbank MC, Stevenson JS, Ottobre JS, Garverick HA and Anderson LL. 1997. Pregnancy rates per artificial insemination for cows and heifers inseminated at a synchronised ovulation or synchronised estrus. J. Dairy Sci. **80**: 295- 300.

Rao, AVN. 1985. The evaluation of progestogens and / or prostaglandin for the induction of fertile oestrus in an oestrus buffaloes during normal and low breeding season. *First World Buffalo Congress*, Cairo, pp.969-970.

Roberts, SJ. 1971. Veterinary Obstetrics and Genital Diseases. CBS Publishers and Distributors, India.

Roy KS and Prakash BS. 2007a. Development and validation of a simple sensitive enzyme immunoassay (EIA) for quantification of prolactin in buffalo plasma; *Theriogenology*. **67**: 572-579.

Roy KS and Prakash BS. 2007b. Seasonal variation and circadian rhythmicity of prolactin profile during summer months in repeat breeding Murrah buffalo heifers; *Reprod. Fertil. Dev*. **19**: 569-575.

Roy K S and Prakash B S. 2009a. Changes in endocrine profiles during ovsynch and ovsynch plus norprolac treatment in Murrah buffalo heifers at hot summer season; *Trop. Anim. Health Prod*. **41**: 677-687.

Roy KS and Prakash BS. 2009b. Plasma progesterone, estradiol-17β and total estrogen profiles in relation to estrous behavior during ovsynch treatment in Murrah buffalo heifers; *J. Anim. Physiol. and Anim. Nut*. **93**: 486-495.

Saini, MS, Galbotra, MM, Kaker, ML and Razdan, MN. 1986. Induction of oestrus and ovulation in non-cyclic buffalo (*Bubalus bubalis*) heifers with progesterone-releasing device and pregnant mare serum gonadotrophin and their gonadotrophin profile. *Theriogenology*. 26: 49-55.

Singh J, Nanda AS and Adams GP. 2000 The reproductive pattern and efficiency of female buffaloes. *Anim. Reprod. Sci.* **60-61**: 593-604.

Singh M and Madan M.L. 1991. Prostaglandin and buffalo reproduction - A review. *Agric. Rev.* **12**: 107-114.

Thatcher WW, Moreira F, Pancarci SM, Bartolome JA, Santos JEP. 2002. Strategies to optimize reproductive

efficiency by regulation of ovarian function. *Dom. Anim. Endocrinol*. **23**: 243–54.

Yindee M, Techakumphu M, Lohachit C, Sirivaidyapong S, Na-Chiangmai A, Rodriguez-Martinez H. 2010. Follicular dynamics and oestrous detection in Thai postpartum swamp buffaloes (Bubalus bubalis). *Reprod. Dom. Anim.* **46**: 91–6.

Review article

Molecular and cellular basis of fetal wound regeneration and adult wound repair

Ramesh H.S[1], Girish Kumar V[1], Mamatha N[2], Tripathi SK[3] and Nandi S[3]

[1]*Veterinary College, Hebbal, Bangalore-560024,* [2]*Department of cooperation, Bangalore,* [3]*ICAR-National Institute of Animal Nutrition and Physiology, Adugodi, Bangalore*

Abstract

Fetal wound Repair consists of a physiologic adjustment in order to create continuity aiming at rebuilding of injured tissue with an exact copy in order to restore tissue morphology as well as functionality. Adult mammalian wound is repaired by means of an inflammatory and fibrotic response that leads to accumulation of scar where repair is achieved through four precisely and highly programmed phases: Hemostasis, Inflammation, Proliferation and Remodeling. In contrast, fetal wound healing is more of a regenerative process with minimal or no scar formation. Fetal wound heal rapidly without any apparent dedifferentiation of cellular components. In general, the scarless character of fetal wound repair persists until middle of the third trimester at which point a transition to the adult scar-forming pattern of wound repair occurs. Early fetal repair mimics the process of organ morphogenesis by cells or tissues that are less differentiated and results in organ regeneration without scar formation. In fact fetal wounds heal with reduced inflammatory reaction, faster production of ECM components, high hyaluronic acid content, high profiles of TGF-β3, IL-10, fetal cells and non-canonical Wnt/PCP signalling pathway. However understanding fetal wound repair and regeneration will impact adult repair in the future and may lead to the reduction or even prevention in the formation of scar tissue in a number of organs.

Keywords: Wound, Scar, Scarless, Repair, Regeneration, Hyaluronic acid

Introduction

Repair consists of a physiologic adjustment after disruption in order to create continuity without aiming at reconstitution of the original tissue in adults. Regeneration consists of rebuilding injured tissue with an exact copy in order to restore tissue morphology as well as functionality in fetus. Adult mammalian wound does not regenerate but is repaired by means of an inflammation which leads to scar formation. Scar formation results in rapid sealing of an injured area leading to persistent pathology in the organism. For example, scar formation after tendon repair will limit their gliding ability, intra-abdominal scar lead to small bowel obstruction which needs surgical intervention. Scar then represents a significant source of morbidity in the animals. In contrast to adults, mammalian fetal wounds heal rapidly without scarring until late in gestation (Hantash et al., 2008). Fetal wounds heal rapidly without any dedifferentiation of cellular components (Hess., 1954) and restoration of normal architecture. The scarless character of fetal wound repair persists until middle of the third trimester at which point a transition to the adult scar-forming pattern of wound repair occurs (Lorenz and Adzick., 1993). This is evidenced by

the transplantation of adult sheep skin or late gestation fetal lamb skin onto fetal lambs, here skin will continue to heal with a scar even if the repair takes place in a fetal environment, so early gestation fetal lambs with skin grafts from adult skin produces scars upon wounding (Morykwas., 1991). There are numerous intrinsic and extrinsic differences between the fetus and adult that influences wound healing.

The Biology of Adult Wound Healing:

Adult wounds heal by repair which occurs by a sequence of events in which cellular and matrix components act in concert to tissue repair. The healing response occurs in four broad phases-1.Hemostasis, 2.Inflammation, 3.Proliferation and 4.Remodelling or resolution.

Hemostasis and Inflammatory Phase

At the time of injury, hemostatic mechanisms like vasoconstriction, platelet aggregation, platelet α-degranulation of vesicles containing both clotting and growth factors including fibrin deposition are stimulated to prevent local haemorrhage. The initial wound matrix of fibrin acts as a scaffold on which inflammatory cells enter the wound (Schilling et al., 1976). Degranulation of Platelet alpha results in the release of platelet derived growth factor (PDGF) and transforming growth factor beta (TGF-β) (Roberts et al., 1986), which acts as chemo attractants, mitogens and stimulators of collagen deposition for cells that will enter the wound. These growth factors make chemotactic stimulus for neutrophils, fibroblasts, and monocytes and finally results in extracellular matrix (ECM) synthesis (Sporn and Roberts., 1992). Neutrophils are first responders to these chemotactic agents and starts to infiltrate to the site of injury before the fibroblasts and monocytes. They are important for phagocytosis of bacteria and functional debridements of injured tissue by secreting additional proinflammatory cytokines. Persistent neutrophil rich inflammatory response results in the presence of foreign body or infection which leads to poor wound healing and excess fibrosis in sheep (Kumta., 1994). Persistent macrophage response may also lead to excess scar formation in case of extended neutrophilic infiltrate (Robson et al., 1992). In response to a variety of chemo-attractants monocytes become macrophages, which are considered the principle coordinators of adult wound healing (Diegelmann et al., 1981). As a chronic inflammatory infiltrate is established, neutrophils are replaced by Macrophages and then lymphocytes. Macrophages secrete growth factors like PDGF and TGF- β which enhance fibrosis. These factors further stimulates the flux of fibroblasts into the wound where they produce collagen (Roberts et al., 1986). Finally, fibroblasts replace the initial wound fibrin matrix by depositing glycosaminoglycans, proteoglycans, and ECM proteins such as fibronectin and tenascin. These multiple factors involved in wound healing and finally scar formation. Ultimately initial fibrin matrix is replaced by collagen due to continued activity of fibroblasts. Finally adult wound healing is a replacement of normal tissue by a collagenous scar which lacks the ordered structure and is an imperfect process where healed

wounds never regain the full tensile strength of uninjured tissue.

Proliferative Phase

The formation of granulation tissue having macrophages and fibroblasts that replaces fibrin clot is a feature of the proliferative phase of adult wound healing. This granulation tissue is responsible for the active wound contraction in the proliferative phase of adult wound healing, a contraction affected chiefly by myofibroblasts which is derivative of fibroblasts. The rate of granulation tissue formation depends on interaction of the fibronectin with fibroblast integrin receptor (Xu et al., 1996). The initial fibrin clot functions as a chemokine to infiltrate macrophages and fibroblasts into the wound space. Fibroblasts apply traction to the wound periphery assisting in its contraction and ultimate closure whereas macrophages provide a continuing source of growth factors needed for angiogenesis [Hunt et al., 1980] and also fibroblasts lay down a provisional matrix mainly composed of collagen and proteoglycans. Increased expression of the TGF-β1 and TGF-β2 isoforms occurs in adult wounds compared to TGF-β3. As repair process proceeds, fibroblasts shows myofibroblast phenotype with more of alpha-smooth muscle actin (α-SMA) expression which is stimulated by TGF-β1 and TGF-β2 as well as by PDGF (Montesano et al., 1988). Fibroblasts and myofibroblasts work in concert to close the wound and also contribute to the synthesis and alignment of collagen fibers (Hinz., 2007).

Remodeling Phase

In the remodeling phase synthesis, degradation, cross-linking, and reorientation of collagen occurs to form the mature scar. The healing and remodeling tissue shows increase in tensile strength, however the resultant scar will never attain physical properties same as that of uninjured tissue (Mast et al., 1992). In Remodeling phase removal of accumulated connective tissue occurs due to presence of matrix metalloproteinases (MMPs) which is under the control of a cytokine network (Parks., 1999). As wound heals fibroblasts and myofibroblasts within the maturing wound bed are reduced by apoptosis, which may be precipitated by the withdrawal of cytokines (Desmoulière et al., 1995). With continued remodeling the outgrowth of capillaries is halted, blood flow and metabolic activity in the area is reduced and resulting in hypocellular and hypovascular scar. Scar is a condition in which the dermis is dysfunctional, and this in turn affects the epidermis and its accessory organs, as well as the vasculature and nerves. Poor healing is characterized by excess accumulation of collagen that is weaker compared to unwounded skin. The wound healing process in adult mammals is imperfect and less restorative than in the juvenile or in the embryo.

The Biology of Fetal Wound Healing

Fetal wound healing occurs due to regenerative process with minimal or no scar formation due to acute inflammation is absent, hyaluronic acid is a prominent component of the wound matrix, changed profiles of growth factor expression and collagen deposition occurs in a highly organized with

scarless manner resulting in restoration of tissue integrity. It is a complex process involving cell migration, proliferation, differentiation, apoptosis, and the synthesis and remodeling of the extra cellular matrix (ECM). The factors which favours tissue repair include cell-cell interactions, cell-matrix interactions, number of different cell types, growth factors and cytokines. Indeed, wounds in early mammalian embryos have the ability to repair without scar formation and with complete restitution of the physiological architecture. In an embryonic wound site level of TGF β3 is higher compared to (TGF)-β1 and -β2. Another difference is fetal wounds close through an actin cable, wherein adult wound closure occurs by active movement of connective tissue and epidermis in order to bring two wounded edges in close proximity to allow the epidermis to migrate and cover the exposed connective tissue. The fetal wounds heal rapidly and are characterized by a complete regeneration of dermal, epithelial tissue and epidermal appendages with identical collagen pattern as like that of uninjured tissue whereas adult wounds heal by a fibroproliferative response that emphasizes repair over regeneration and it results in the formation of scar tissue which is consists largely of an unorganized dense collagen meshwork. If wounds are made at an even later stage, histological scarring remains. Since blood vessels are yet to form during early stages of gestation due to which there is no bleeding or clot formation to initiate inflammation. Switch over from scarless to scar forming repair is accomplished by development and maturation of blood vessels, inflammatory system. Due to haemostatic response, a fibrin clot is formed at the site of injury leads to infiltration of variety of cells, i.e., existing fibroblasts, mast cells, leukocytes and fibrocytes in case of sheep (Burrington., 1971**)** .

Fetal regeneration of wound occurs due to cells or tissues that are less differentiated having greater phenotypic plasticity leading to organ regeneration without scar formation. Even though late fetal repair is participated by both local and distant stem or progenitor cells but it occurs in a matured organ environment resulting in scar formation. Fetal regeneration is cell specific and show a regenerative phenotype but not due to the moist, sterile environment of the uterus. The regenerative process of fetus shows difference in a number of processes like, inflammation, extracellular matrix, Myofibroblasts and Contraction, Growth Factors, stem cells, Fetal environment, Cell Signalling, Transcription, and Gene Expression compared to adult scar wound healing.

Inflammation

Fetal wound healing shows lower inflammatory response because of lower immune cells like macrophages which are less activated having short life span compared to the adult scar wound healing (Hopkinson et al., 1994). Early mammalian fetus is significantly neutropenic and lacks self/nonself immunologic identity. This reduced fetal inflammatory process play important role in scarless wound regeneration due to decreased platelet aggregation, degranulation and lower levels of cytokines release from fetal platelets by reducing the recruitment of

inflammatory cells to fetal wounds (Olutoyeet al., 1996). Reduced inflammatory response is due to age dependent defect in the ability of fetal neutrophils to phagocytose pathogenic bacteria where early fetal neutrophils are physiologically distinct from those present at the end of gestation or postnatal cells in fetal sheep (Jennings et al., 1991). Thus there are many factors which responsible for reduced inflammatory response during scarless fetal wound healing. Proinflammatory cytokines like interleukin-6 (IL-6) and interleukin-8 (IL-8) reduces whereas IL-10 upregulated which inhibits the migration of inflammatory cells to sites of injury during scarless fetal repair. Low levels of Cyclooxygenase-2 (COX-2) and prostaglandin-2 (PGE2) occurs in scarless wound healing.

The Fetal Extra Cellular Matrix (ECM)

Scarless fetal wound healing depends on the organization of extracellular matrix (ECM). Macromolecular constituents of fetal ECM is different from adult ECM. The ECM play a role in regulating growth factors, cytokines and alter cell behaviour which are important for wound healing. Increased levels of glycosaminoglycans such as hyaluronic acid (HA) and chondroitin sulfate, which are long unbranched polysaccharides comprising of repeating disaccharides observed on the cell surface or in the ECM in case of fetal wound healing. Prolonged maintenance of an environment rich in hyaluronic acid (HA) favours cell mobility, cell proliferation and regeneration in fetal wounds in scarless manner. Because of prolonged presence of hyaluronic-acid-stimulating activity (HASA) in fetal serum, amniotic fluid, wound fluid, and a number of fetal tissues which results in a prolonged HA-rich response in fetal wound. Eventhough early deposition of HA occurs (Weigel et al., 1986) in case of adult wound but it is removed by hyaluronidase leading to deposition of sulfated glycosaminoglycans and finally collagen is laid down in a scar pattern. While in case of fetal wounds due to persistent presence of HASA causes prolong presence of HA. Thus an HA-rich ECM provides a permissive environment for the orderly deposition of collagen. HA influences the reorganization of wound collagen by acting as-a carrier for biologically active proteins.

Increased expression of HA is due to the reduced activity of hyaluronidase in the fetus, which promotes both the proliferation and migration of a number of cell types (West et al., 1997) and ultimately reduces the formation of scar tissue. Prolong presence of hyaluronic acid in fetal wounds provides the matrix signal orchestrating healing by regeneration rather than by scarring. Fetus is bathed in amniotic fluid that contains high concentration of hyaluronic acid and HA stimulating activity which enables cell motility and proliferation unique to fetal scarless wound healing. Various adhesion glycoproteins mediate interaction of cellular components such as fibronectin, laminin, and tenascin C and thrombospondin which bind to specific integrins or cellular receptor to make cell migration, proliferation and attachment. Fibronectin plays important role in migration of a number of cells like fibroblasts, keratinocytes, and

endothelial cells and show an earlier expression in fetal wounds. Fibronectin shows temporal and spatial expression in both fetal and adult sheep and mice (Whitby et al., 1991) but earlier expression in case of rabbit (Longaker et al., 1989). Tenascin C causes rapid re-epithelisation in fetal wounds (Whitby and Ferguson., 1991).

The proteoglycans, decorin and fibromodulin play role in fetal wound healing by influencing collagen fibrillogenesis, growth factor activity and cellular proliferation wherein upregulation of decorin occurs in adult scar wound healing while fibromodulin in case of scarless wounds (Soo et al., 2000). Fetal fibroblast synthesize more of type III and IV collagen compared to adult due to the action of Prolyl hydroxylase activity which causes orderly deposition of collagen fibrils with fine reticular or basket weave pattern similar to uninjured tissue in a HA-rich matrix environment leading to scarless wound healing. Fetal and adult wounds show a number of differences in collagen synthesis like speed of deposition, variations in collagen ratios and quantity of collagen. The collagen deposited by fetuses is less mature with less cross-linking because of lower expression of lysyl oxidase which has reducing rigidity without affecting the tensile strength (Lovvorn et al., 1999). IL10 protects against excess deposition of collagen, maintains elevated hyaluronic levels, enhances fibroblast function, prevents differentiation of fibroblast to myofibroblasts and increases survival of endothelial progenitor cells and angiogenesis.

Fibroblasts and Myofibroblasts in Fetal Wound Healing

Fibroblasts are the main cell type responsible to know whether scarless or fibrotic healing. Regenerative healing mainly depends on the ability of fetal fibroblasts to produce and arrange new collagen and other ECM components in similar quantities and ratios to unwounded skin. Fetal fibroblast leads to scarless healing when transplanted to an adult (Lorenz et al., 1995). During fetal scarless wound repair conversion of fibroblast into contractile myofibroblasts not occurs (McCluskey and Martin., 1995) where as it occurs in late gestation fetal wounds leading to scar formation (Chen et al., 2007) due to the effect of TGF-β on fibroblastic cells to make the expression of α-SMA and conversion to the myofibroblast leading to scar formation (Desmouliere et al., 1993).

Fetal wounds close through an actin cable which acts like a purse string whereas myosin acts in a zipper-like manner to close wounds in fetal skin [Cowin et al., 2003]. Myofibroblast is responsible for adult wound contraction.

Growth Factors

Growth factor profile in fetal healing differs significantly from adult wound healing. Several mediators released by mast cells can induce scar formation when introduced into fetal wounds of pig (Behm et al., 2008). These mediators include TGF-β family members shown to have a major role in fibrosis [Roberts et al., 1986]. The profibrotic isoform TGF-β1reduces in early fetal wounds whereas the anti-fibrotic isoform TGF-β3 increases in scarlessl fetal wound healing (Shah et al., 1995). Epidermal growth factor

(EGF) is mitogenic for a number of cell types including fibroblasts and keratinocytes which decreases with increasing gestational age leading to scar formation (Peled et al., 2001) and it causes dedifferentiation of cell whereas PDGF in the fetal wound results in scarless wound healing (Haynes et al., 1994) which is due to binding of the highly cationic factor to components of the ECM such as glycosaminoglycans at the site of the wound. The fibroblast growth factors (FGF) causes proliferation and differentiation of cells. FGF isoforms 1, 2, 5, 7 and 10 were increases in adult wound healing whereas FGF isoforms 7 and 10 are decreases in scarless healing. Overall there will be diminished FGF expression and signaling occurs during scarless wound healing (Dang et al., 2003) whereas VEGF expression reduces in scarless fetal wounds compared to fibrotic fetal wounds (Wilgus et al., 2008).

Interleukins plays important role during scarless wound healing where proinflammatory mediators like IL-6 and IL-8 are reduces in fetus whereas anti-inflammatory agent called IL-10 is increases leading to fetal scarless wound healing (Peranteau et al., 2008). Collectively it is the balance of various cytokines rather than one factor responsible for the scarless repair in the fetal tissue.

Keratinocytes and Re-Epithelialization in Fetal Wound Healing

Fetal keratinocytes plays important role in the process of fetal re-epithelialization and also involved in rapid healing of fetal wounds (Whiteby et al., 1991). Adult wounds re-epithelialize through extension of lamellipodia followed by epidermal cells at the wound edge crawling over the wound bed but embryonic wounds exhibit no signs of lamellipodia or filopodial extensions, Instead epidermal cells makes an actin “cable” at the edge of wounds that functions like a purse-string to close the wound (Martin et al., 2003) due to presence of GTP-binding protein Rho which causes proper assembly of the actin cable and re-epithelialization of fetal wounds.

Stem cells in fetal scarless wound healing

Fetal stem cell function is one of the key mechanisms underlying scarless wound healing. Fetal scarless wound healing is due to change in the pattern of fetal stem cell division and the orientation of the mitotic spindle during development. Fetal cell divisions occurs in symmetric and parallel to the basement membrane, which allows for growth and maintenance of the surface of the developing embryo as a single layer during the early stages of fetal development. During later stages of fetal stem cells undergo asymmetric division leading to scar formation.

Mesenchymal stem cells (MSCs) plays key role during scarless healing. MSCs are present in fetal extraembryonic tissues and fluids like placenta, umbilical cord, amniotic membrane, amniotic fluid, and umbilical cord blood and fetal bone marrow. These cells are similar to embryonic stem cells having greater plasticity and lower immunogenicity compared to adult derived MSCs. In addition both fetal and extraembryonic MSCs supports neovascularization and tissue

regeneration compared with adult derived MSCs.

Recently new fetal skin stem cell were identified in fetal and adult mice, as well as in humans, that plays a key role in fetal scarless wound healing. They are found in circulating blood, fetal dermis and epidermis. They are distributed in many tissues and causes differentiation in embryonic development and having ability to diffuse to the wound site where it differentiated by expressing fibroblast growth factor-2 which is antagonist to fibrosis.

Fetal environment

Fetal environment plays important role in fetal scarless wound healing due to its continuous bathing in warm, sterile amniotic fluid rich in growth factors and ECM components such as hyaluronic acid and fibronectin. Amniotic fluid makes scarless fetal wound repair due to presence of HA, fibronectin and growth factors which causes fetal wound cells to make a unique environment. Fetal tissue oxygenation is different from adult where fetus has a very low PO2 due to large transplacental oxygen gradient between maternal arterial and umbilical venous blood. For normal adult wound healing oxygen essential, however the foetus is hypoxic which influences fetal tissue repair (Pritchard et al., 1985). Amniotic fluid provides a sterile, weightless environment which is protective and thermally stable. Systemic effects of amniotic fluid is possible because the fetus swallows the fluid which makes absorption of a variety of potential mediators and also has a trophic effect which supports fetal tissue repair (Mulvihill et al., 1986). It has thus been shown that the fetal environment is not the main determinant of regeneration, but the fetal tissue itself is the key to regeneration. However, this is not simply due to the aseptic environment or the amniotic fluid, as this regenerative capacity wanes in the third trimester. Even more remarkable is that the scarless fetal wound repair is organs pecific, occurring mainly in skin and bone.

Cell Signalling, Transcription, and Gene Expression

TGF-β1 signalling pathway demonstrated to be downregulated in fetus during wound healing and some intracellular signalling proteins differ between fetal and adult wound healing [Rolfe et al., 2007] like Smad 2 or Smad 3 in case of mice. Fetal wound healing requires the expression of a number of genes like Hox genes which are influenced by number of transcription factors such as activator protein 1 (AP1), Fos and Jun in fetal mouse skin. Upregulation of AP-1 and c-Fos is influenced by Rho and GTPase which causes formation of actin cable in scarless fetal wound healing. However c-Fos and c-jun expression is transient while AP-1 expression is persistent in fetal scarrless wound repair [Gangnuss et al., 2004]. Hox protein activity is essential during embryogenesis and in limb regeneration. Totally 53 different genes expresses between early gestational to late gestational in rats out of which upregulation of 27 genes occurs including FGF8, follistatin and downregulation of 26 genes occurs including beta-catenin. A proposed key mediator of scarring is COX2 that functions by producing prostaglandins

which is downregulated in scarless healing (Wilgus et al., 2008).

Organ Specificity

In ealy foetuses regeneration is organ specific like cutaneous wounds heals with perfect regeneration whereas other organs such as the gut heals with the formation of scar tissue in case of marsupial embryo (Monodelphis domestica). Apart from skin and bone tissue other tissue organs failed to regenerate without scar formation even in early gestation (nerve, stomach, trachea, myocardium and diaphragm) therefore fetal wound healing is an organ specific response (Wulff et al., 2012).

References:

Behm GCL, Hildebrand KA and Hart DA 2008. The mast cell stabilizer ketotifen prevents development of excessive skin wound contraction and fibrosis in red Duroc pigs. *Wound Repair Regeneration*. **16:** 226-233.

Burrington JD 1971.Wound healing in the fetal lamb. *J. Pediatric Surg*. **6**: 523-528

Chen J, Li H, Sundarraj, N and Wang JHC 2007. Alpha-smooth muscle actin expression enhances cell traction force. *Cell Motility and the Cytoskeleton*. **64:** 248-257.

Cowin AJ, Hatzirodos N, Teusner JT and Belford DA 2003. Differential effect of wounding on actin and its associated proteins, paxillin and gelsolin, in fetal skin explants. *J. Invest. Derm*. **120:**1118-1129.

Dang CM, Beanes SR, Soo c, Ting K, Benhaim P, Hedrick MH and Lorenz HP 2003. Decreased expression of FIbroblast and keratinocyte growth factor isoforms and receptors during scarless repair. *Plastic and Recon. Surg*. **111:**1969-1979.

Desmouliere A, Redard M, Darby I and Gabbiani G 1995. Apoptosis mediates the decrease in cellularity during the transition between granulation tissue and scar. *Am. J. Path.* **146:** 56-66.

Desmouliere A, Geinoz A, Gabbiani F and Gabbiani G 1993. Transforming growth factor-β1 induces α-smooth muscle actin expression in granulation tissue myofibroblasts and in quiescent and growing cultured fibroblasts. *J. Cell Biol*. **122:** 103-111.

Diegelmann RF, Cohen IK and Kaplan AM 1981.The role of macrophages in wound repair: a review. *Plastic and Recon. Surg*. **68:** 107-113.

Ehrismann CR, Kalla P, Pearson CA, Beck k and Chiquet M 1988. Tenascin interferes with fibronectin action. *Cell*. **53:** 383-390.

Gangnuss S, Cowin AJ, Daehn IS 2004. Regulation of MAPK activation, AP-1 transcription factor expression and keratinocyte differentiation in wounded fetal skin. J. Invest. Derm. **122:** 791-804.

Hantash BM, Zhao L, Knowles JA and Lorenz HP 2008. Adult and fetal wound healing. *Frontiers in Biosci.* **13:**51–61

Haynes JH, Johnson DE, Mast BA, Diegelmann RF, Salzberg DA, Cohen IK and Krummel TM 1994. Platelet-derived growth factor induces fetal wound Fibrosis. *J. Pediatric Surg.* **29:**1405–1408.

Hess A 1954. Reactions of mammalian fetal tissues to injury. II. Skin. *The Anat.Rec.* **119**:435–447.

Hinz B 2007. Formation and function of the myofibroblast during tissue repair. *J. Invest. Derm.* **127:**526–537.

Hopkinson JW, d. Hughes DS, Gordon and Martin P 1994. Macrophage recruitment during limb development and wound healing in the embryonic and foetal mouse. *J. Cell Sci.* **107:**1159–1167.

Hunt TK 1980. Wound Healing and Wound Infection: Theory and Surgical Practice, Appleton-Century-Cross, New York, NY, USA.

Jennings RW, Adzick, NS, Longaker MT, Duncan BW, Scheuenstuhl H and Hunt TK 1991. Ontogeny of fetal sheep polymorphonuclear leukocyte phagocytosis. *J. Ped. Surg.* **26:**853–855.

Kumta S 1994. Acute inflammation in foetal and adult sheep: the response to subcutaneous injection of turpentine and carrageenan. *British J. Plastic Surg*. **47:** 360-368.

Longaker MT, Whitby DJ and Ferguson MWJ 1989. Studies in fetal wound healing: III. Early deposition of fibronectin distinguishes fetal from adult wound healing. *J. Ped. Surg*. **24:** 799–805.

Lorenz HP and Adzick NS 1993. Scarless skin wound repair in the fetus. *Western J. Med.* **159:**350–355.

Lorenz HP, Lin RY, Longaker, MT, Whitby, DJ and Adzick, NS 1995. The fetal fibroblast: the effector cell of scarless fetal skin repair. *Plastic and Recon. Surg.* **96:**1251–1259.

Lovvorn HN, Cheung DT, Nimni M, Perelman N, Estes JM and Adzick NS 1999. Relative distribution and crosslinking of collagen distinguish fetal from adult sheep wound repair. *J. Ped. Surg*. **34:**218–223.

Martin P, Dsouza D, Martin J, Grose R, Cooper L, Maki R and Mckercher SR 2003. Wound healing in the PU.1 null mouse—tissue repair is not dependent on inflammatory cells. *Current Biol*. **13:** 1122-1128.

Mast BA 1992. The skin in Wound Healing: Biochemical and Chemical Aspects.

Mccluskey J and Martin P 1995. Analysis of the tissue movements of embryonic wound healing-DiI studies in the limb bud stage mouse embryo. *Dev.Biol*. **170:**102–114.

Montesano R and Orci L 1988. Transforming growth factor beta stimulates collagen-matrix contraction by fibroblasts: implications for wound healing," *Proc. Natl. Acad. Sci. U S A*. **85:**4894–4897.

Morykwas MJ 1991. Cellular inflammation of fetal excisional wounds: effects of amniotic fluid exclusion. *Inflammation*. **15:**173-180.

Mulvihill S, Stone MM and Fonkalsrud EW 1986. Trophic effects of amniotic fluid on fetal gastrointestinal development. *Surg.Res.***40:**291-296.

Olutoyeet OO, Yager DR, Cohen IK and Diegelmann RF 1996. Lower cytokine release by fetal porcine platelets: a possible explanation for reduced inflammation after fetal wounding. *J. Ped. Surg*. **31:**91–95.

Peled ZM, Rhee SJ, Hsu M, Chang J, Krummel TM and Longaker MT 2001. The ontogeny of scarless healing II: EGF and PDGF-B gene expression in fetal rat skin and fibroblasts as a function of gestational age. Ann Plastic Surg. **47:**417–424.

Parks WC 1999. Matrix metalloproteinases in repair. *Wound Repair and Regeneration*. **7:**423–432.

Peranteau WH, Zhang L, Muvarak N, Badillo AT, Radu A, Zoltick PW and Liechty 2008. IL-10 overexpression decreases inflammatory mediators and promotes regenerative healing in an adult model of scar formation. *J. Invest. Derm*. **128:**1852–1860.

Pritchard A, Macdonald PC and Gant NF 1985. The morphologic and functional development of fetus. In: Williams Obstetrics. **13:**145-180.

Roberts AB, SporN MB and Assoian RK 1986. Transforming growth factor type β: rapid induction of fibrosis and angiogenesis in vivo and stimulation of collagen formation in vitro. *Proc. Natl. Acad. Sci. U S A*. **83**:4167–4171.

Robson MC, Barnett RA, Leitch IO and Hayward PG 1992. Prevention and treatment of postburn scars and contracture. *World J Surg*. **16:**87–96.

Rolfe AD, Cambrey J, Richardson LM, Irvine AO, Grobbelaar and Linge C 2007. Dermal fibroblasts derived from fetal and postnatal humans exhibit distinct responses to insulin like growth factors. *BMC Dev. Biol.* **7:**124.

Schilling A 1976. Wound healing. Surgery clinic. **56**:869-874.

Shah, M, Foreman DM and Ferguson MWJ 1995. Neutralisation of TGF-β and TGF-β or exogenous addition of TGF-β to cutaneous rat wounds reduces scarring. *J. Cell Sci.* **108:**985–1002.

Sporn MB and Roberts AB 1992. Transforming growth factor-β: recent progress and new challenges. *J. Cell Biol.* **119:**1017–1022.

Soo CF, Hu Y and Zhang X 2000. Differential expression of fibromodulin, a transforming growth factor-β modulator, in fetal skin development and scarless repair. *Am.J. Path.* **157:**423–433.

Weigel PH, Fuller GM and Leboeuf RD 1986. A model for the role of hyaluronic acid and fibrin in the early events during the inflammatory response and wound healing. *Journal of Theoretical Biology*. **119:**219-226.

West DC, Sha DM, Lorenz P, Adzick NS and Longaker MT 1997. Fibrotic healing of adult and late gestation fetal wounds correlates with increased hyaluronidase activity and removal of hyaluronan. *Int. J Biochem. Cell Biol.* **29:** 201–210.

Whitby DJ and Ferguson MWJ 1991. The extracellular matrix of lip wounds in fetal, neonatal and adult mice. *Development*. **112:** 651–668.

Wilgus TA, Ferreira AM, Oberyszyn TM, Bergdall VK and Dipietro LA 2008. Regulation of scar formation by vascular endothelial growth factor. *Lab. Invest.* **88:** 579–590.

Wilgus TA, Bergdall VK and Tober KL 2008. The impact of cyclooxygenase-2 mediated inflammation on scarless fetal

wound healing. *Am. J. Path.* **165:**753–761.

Wulff, BC, Parent, AE, Meleski MA, Dipietro LA, Schrementi ME and Wilgus, TA 2012. Mast cells contribute to scar formation during fetal wound healing. *J. Invest. Derm.* **132**:458–465.

Xu X and Clark RAF 1996. Extracellular matrix alters PDGF regulation of fibroblast integrins. *J. Cell Biol.* **132:**239–249.

Review article

Global gene expression in growing and atretic ovarian follicles

Kavya V[1*], Ramesh HS[1], Nandi S[2] and Girish Kumar V[1]
[1]Department of Veterinary Biochemistry, Veterinary College, Bangalore, India
[2]ICAR- National Institute of Animal Nutrition and Physiology, Bangalore-560030, India
*Corresponding Author:**Email: kavyavenki1234@gmail.com**

Abstract

Folliculogenesis is a regulated process, which includes a dramatic proliferation and controlled differentiation of both the somatic and germ cell. The follicle development involves many numerous molecular mechanisms and metabolic pathways, which starts from the primordial germ cells, proliferate by mitosis and form primary follicle and continues its growth to form either dominant follicle or atretic follicle. Within the growing follicles, some of the genes are upregulated and few genes are downregulated, which is essential for the development of dominant follicle to undergo the ovulation. The concept of three developmental phase of follicle defined on their fate like, either ovulation or atresia; growing phase, plateau phase, atretic phase. Many studies revealed that significant differences were observed between the growth phases. Granulosa cells from follicles in the growing phase had increased expression of RELN (Reelin). On the other hand, genes regulating the response to oxidative stress VNN1(Vanin) and angiogenesis ANGPT2 (Angiopoietin) were significantly increased in granulosa cells from atretic follicles. This article overviews and analyze transcriptomic profiles or differently expressed genes between growing and atretic ovarian follicle.

Keywords : Global gene expression, Ovarian follicle , Granulosa cells, oocytes

Introduction

The important function of an ovary is to produce oocytes for fertilisation as eggs and to maintain environment for beginning of gestation (Bonnet, *et al*.,2013). These oocytes originate from Primordial germ cells (PGCs). PGCs originate in extra embryonic development mesoderm. PGCs undergo migration, proliferation and colonization of PGCs to the developing gonads.(Sanchez and Smitz.,2012) After colonization will under phase of mitotic proliferation with local paracrine factors like cytokines, leading to the formation of 'germ cell cysts' or ' germ cell nests (Flor and Johan 2012). Around the meiotic arrest, germ cell nests breakdown to initiate follicle formation. Oocytes then attach to the epithelial cells (pre-granulosa cells) derived from surface epithelium that subsequently become pregranulosa cells and form primordial follicle. The first follicle separates them by producing a basement membrane at the base of the ovigerous cords.

Once primordial follicle is formed they are committed to gonadotropin–independent growth (Girard *et al*., 2015). As a primordial grows continuously lead to the formation of primary stage, the granulose cells increase in size number and change in shape i.e from squamous flattened to cuboidal. In ewe, the number of granulosa cells increase to a range of 30-520. The oocyte also changes, with 5 folds increases in the

volume of smooth endoplasmic reticulum, mitochondria, ribosomes and lipid droplets, and the zona pellucida, absent in primordial follicles (Cain *et al*., 1995).

While activation of primordial follicles' growth is a continuous process, recruitment of antral follicles cohorts follows a cyclical pattern. Follicles are recruited in wave-like manner in response to an increase in FSH levels (Girard *et al*., 2015 ; Flor and Johan 2012). The dominant and largest subordinate follicles of the ovulatory wave undergo a common growth phase in mares and cows. The dominant follicle begins to diverge as it continuous to grow while the subordinate follicles undergo atresia. The dominant follicles grow by suppressing the growth of subordinate follicles of the same wave and suppressed emergence of the next follicular wave through an inhibitory effect on circulating FSH concentrations. The increased FSH is requisite for recruitment of the follicular cohort, where post-surge decline in FSH is a critical factor in selection of the dominant follicles (Girard *et al*., 2015). The presence of progesterone will determine the fate of the follicle. In absence of this hormone, the growing follicle will proceed to ovulation but in high progesterone, the follicle will enter to atresia (Bonnet, *et al*., 2013). The growing phases are associated with expression of different genes, global gene analysis of follicles will provide markers to determine their physiological status. Recent studies revealed that transcriptomic profiles of granulosa cells proved that variety of genes are linked to follicular development (Girard *et al*., 2015 ; Adrienne *et al*. 2008).This article summarizes recent knowledge on the transcriptomic analysis in the development stages of follicle.

Transcriptomic profile of growing and atretic ovarian follicle

One of the most exciting new concepts is to characterise gene profiles of growing and atretic ovarian follicle. As follicles grow from primordial stage to preantral follicle stage, many cytokines and local paracrine factors play an important role in this process. Recent studies of globle gene expression in follicles revealed that analysis of gene functions indicated that there was clear rise of cell signalling and apoptosis in early stage of plateau stage of follicle and downregulation of genes which is responsible for cell viability was seen (Scaramuzzi et al 2011). Recent studies revealed that 761 differentially expressed genes (DEG) between the growing and the plateau phase, of which 468 were specific to that transition. Between the plateau and the atretic phase, 1,709 genes were differentially expressed with 1,556 of them being specific to that transition. There were 153 genes modified in both contrasts, but only 14 of them underwent continuous change between the three states (Eppig *et al*, 1989, 1994).

Biomarkers

Biomarker or biological marker, generally refers to a measurable indicator of biological state or condition. The term also used to refer to a substance whose detection indicates the presence of a living organism. Some of the genes play an important role during transition from growing to plateau phase and from plateau to atretic phase of follicle; *ACE2, ANGPT2, ANK3, ANKRD1, APOA1, BMP4, BUB1, CCNB1, CD36, CKS2, JAM2, MT2A, NMB, NR4A1, NRP1, PRC1, PTTG1,*

RARRES1, RELN, SERPINE1, STAR, TRIB2, TUBB6, TYRO3 and VNN (Table 1-3, see abbreviation below).

Several biomarker candidates showed no significant differences between stages (Albertini *et al* 2001, Girard *et al.,* 2015). Most of them are linked to LH response or to progesterone was higher in the plateau group than in the two others *RARRES1, SERPINE1, STAR, TRIB2, CYP19A1, APOA1, BMP4* (Sriraman *et al* .,2010). This may be an indicator of a more diversified population of follicles. This assumption would be coherent with our understanding of this particular stage, as we supposed that the follicles from the plateau phase can take two directions: proceed toward ovulation or enter atresia. The physiological differences between those two options may explain the important variation observed among samples of the plateau phase.

Markers of Growing Follicles

Some of the recent studies revealed that growing stages of the follicle share common characteristics with the plateau stage. RELN is a ligand of the very- low density lipoprotein receptor (VLDR), has the antiproliferative activity (Eresheim *et al.*,2014). CCND2 transcript levels were found to be significantly higher in the G and P follicles, while PCNA was more highly expressed in G follicles. The mRNA and protein levels CCND2 and PCNA genes are increased in response to FSH (Douville *et al* .,2014). Genes like gona- dotropin receptor (FSHR), steroidogenic enzymes (CYP17, CYP19 and HSD17B1) and inhibin- activin-follistatin system (INHA, INHBA and FST). It is well demonstrated that mRNA expression for FSHR, CYP17, CYP19, INHA and INHBA increases with the progress of bovine follicular development and is greater in growing follicle. Nerve Growth Factor and Receptors, C-Kit and Kit ligand, ALK3, BMP, ALK5, GDF9, and AKT are the genes which are significantly increased. NGF may play a role in differentiation of flat pre-granulosa cells into the cuboidal cells that characterize primary follicles. Kit- ligand produced by immature granulosa cells appears to promote thecal cell organization. Induces primordial follicle development and initiate folliculogenesis ALK3, BMP 6 , ALK 5- regulates primordial follicle formation by promoting germ cell to oocyte transition and somatic cell to pregranulosa cell formation. Growth differentiation factor 9 (GDF9) is a member of the transforming growth factor-β superfamily. It directly acts on granulosa cell proliferation and differentiation.

Markers of Plateau Follicles.

Only few genes had an expression level between the growing stage and plateau phase and other phases. TYRO3 and JAM2 are main genes which are upregulated and downregulated. TYRO3 (protein tyrosine kinase 3) involved in controlling cell proliferation and cell survival.TYRO3 *is* a receptor of GAS6 (growth arrest specific 6). This receptor and ligand are abundantly expressed in differentiating stem cells (Pierce *et al*., 2011). On the other hand *JAMs* are family genes that encode protein s that are localized at the junction between cells. The protein encoded by this gene is a type I membrane protein that is localized at the tight junctions of both epithelial and endothelial cells. They have multiple functions including

angiogenesis, cell migration and proliferation (**Luissant** *et al.,* 2014). In plateau phase, downregulation of *JAM2* leads to slow proliferation. Hence, the expression pattern of *TYRO3* and *JAM2* is physiologically distinct from growing and atretic phases (Girard *et al.,* 2015). The proliferation of cells function was decreased in P vs. G, was CKS1 and 2CDC28 protein kinase regulatory subunit 1B (CKS1B; also known as CKS1). CKS1B in mouse embryonic fibroblasts (MEF) cells resulted in the cessation of cell proliferation (Douville and Sirard 2014).

Markers of Follicular Atresia

Follicular atresia refers to the failure of a follicle to rupture or ovulate. The process of follicular atresia involves several local paracrine factors, and cytokines. Recent studies show that several genes were differentially expressed between the atretic phase and other phase. The blood vessel network is important during folliculogenesis, as more delivery of gonadotropins (Zeleznik *et al.,* 1989*),* and thecal blood vessel regression increases the vascularisation which is linked with the atresia of the follicle.

The genes related to angiogenesis such has ANGPT2 and CD36, are the upregulated genes in this group. The protein encoded by this gene is an antagonist of angiopoietin 1 (ANGPT2) and endothelial TEK tyrosine kinase (TIE-2, TEK). The encoded protein disrupts the vascular remodelling ability of ANGPT2 and may induce endothelial cell apoptosis**.** The both ANGPT2 and CD36 have an anti-angiogenic property. (Hale *et al.,* 2012). ANK3 gene which actively participates in the coordination of the cell membrane assembly by interacting with E-cadherin at contact sites between cells. (Wang and Genet, 2006).

Some of the genes are down regulated or decreased their expression of BUB1, CCNB1, CSK2, TUBB6, PRC1 genes play an important role in the atresia of the follicle during folliculogenesis. BUB1 is a regulator of chromosomal segregation and spindle assembly checkpoint during mitosis and meiosis (Marchetti and Venkatachalam, 2010). TUBB6 helps in cell cycle, also major constituent of microtubules. Microtubules form the spindle fibers for separating chromosomes during mitosis. PRC1 gene is present at high levels during S and G2/M phase of mitosis helps to control proliferation by regulating DNA replication (girard et al. CSK1 gene serves as a cell cycle checkpoint for the S/G2transition. (Shen *et al.,* 2013). *VNN1* is an enzyme which inhibits reduced glutathione (GSH) synthesis. Vanin promotes inflammation and tissue injury partly inducing by oxidative stress and decreases tissue resistance to oxidative stress. Vanin expression is consistent with the increase in apoptosis during follicular atresia. ID3 is more expressed in Atretic phase relative to Plateau phase and Growing phase. TGM2 is believed to sensitize cells to apoptosis by hyperpolarizing mitochondria, an event which precedes the loss of transmembrane potential, a decrease in GSH levels and consequently an increase in the production of reactive oxygen species (ROS). B-cell lymphoma 2 (BCL2), a marker for apoptosis resistance was higher in atretic follicle.

Tumor necrosis factor receptor superfamily, member 21 (TNFRSF21,

also known as death receptor 6: DR6), much like other members of the TNFR family, has been shown to induce apoptosis when over expressed. Tumour necrosis factor receptor (TNFR), interferon (IFN) and TNF-related apoptosis-inducing ligand (TRAIL) receptors which acts through the death domain receptor present on the cell membrane mediates via extrinsic apoptosis pathway(Pavanashree *et al.*,2014). NFE2L2, nuclear factor (erythroid-derived like 2) is a transcription factor responds to environment insult including reactive oxygen species (ROS). It was observed that ROS generated by the mitochondria play an important role in the release of cytochrome c and other molecules which lead to the activation of apoptosis. (Douville and Sirard, 2014). IGFBP5 mRNA expression dramatically increased in bovine atretic follicles compared with the growing follicles.

Table 1. List of biomarkers in 3 stages of follicle

Stage of follicle	Molecules significantly expressed in the process.
Growing follicle	RELN, CCND2, PCNA, FSHR, CYP17, CYP19, HSD17B1, INHA, INHBA and FST, BCL-2, Bcl-XL, Boo, Gata -6, C-KIT, SCF, Integrin, TGF-2,3, Smad 2.4.
Plateau follicle	TYRO3, JAM2, CKS1, CDC28, GDF, NOBOX, IGF, FSHR, AHR.
Atretic follicle	ANGPT2, CD36, ANK3, BUB1, CCNB1, CSK2, TUBB6, PRC1, VNN1, ID3, TGM2, BCL2, TNFRSF21,TNFR, NFE2L2, CCL2, GADD45A, IGFBP5, PLAUR, SELP, SPP1,TMP, TSP2

Table 2. List of biomarkers in growing and atretic follicles with their mechanism of action

Prosurvival molecules	Mechanism of action
RELN	ligand of the very-low-density lipoprotein receptor (vLDLR)
CCND2	Acts as regulator of cell cycle proteins affecting SAMHD1- mediated HIV-1 restriction in non- proliferating macrophages.
PCNA	act as processivity factor for DNA polymerase
BCL-2	apaf-1/caspases/bax interactions
BOO	mcl-1/apaf-1/caspase9 interactions
GATA -6	gata-6/gonadotrophin interaction
C-KIT	interaction with SCF
SCF	c-kit interaction
INTEGRIN	interaction with other extracellular matrix proteins
TGF-2,3	Interaction with smad/transcription factors
SMAD 2.4.	interactions with transcriptional regulators
TYRO3	a receptor of gas6 (growth arrest specific 6),
JAM2	adhesive ligand for interacting with a variety of immune cell types
CKS1	Protein kinase regulatory subunit 1B.

CDC28	cyclin dependent protein kinase
GDF	transforming growth factor-β superfamily
NOBOX	interaction with gdf-9
IGF	Transforming growth factor family- β superfamily
FSHR	interact with follicle stimulating hormone and represents a G-protein coupled receptor (GPCR)
AHR	interaction with polycyclic aromatic hydrocarbon

Table 3.List of biomarkers in atretic phase

Proapoptopic Molecules	**Mechanism of action**
ANGPT2	antiangiogenic effects
CD36	antiangiogenic effects
ANK3	Participates in the coordination of cell membrane assembly
BUB1	spindle assembly checkpoint and chromsomal aggregation
BAX	acts through the death domain receptor present on the cell membrane
CKS2	serve in the checkpoint protein for the S/G2 transition
TUBB6	isoform of tubulin which is essential for cell cycle.
PRC1	regulates cell proliferation
TGM2	hyperpolarizing mitochondria, an event which precedes the loss of transmembrane potential
TNFR, IFN, TRAIL	act through the death domain receptor present on the cell membrane.
Caspases	Apaf-1 interaction
Fas and Fasl	Caspase interaction
P53	Bcl-2/bax/cAMP interaction

Abbreviations:

ACE2: Angiotensin I converting enzyme 2; ANGPT2: Angiopoietin 2; ANK3: Ankyrin 3; ANKRD1: Ankyrin repeat domain 1; APOA1: Apolipoprotein A-I; BGN: Biglycan; BMP4: Bone morphogenetic protein 4; BUB1: Budding uninhibited by benzimidazoles 1 (BUB1 mitotic checkpoint serine/threonine kinase); CCNB1: Cyclin B1; CD36: Cluster of Differentiation 36 (fatty acid translocase); CDH11: Cadherin 11; CDKN1A: Cyclin-dependent kinase inhibitor 1A; CCND2 : cyclin D2 ; CDKN2A: Cyclin-dependant kinase inhibitor; cDNA: Complementary DNA; CEBPA: CCAAT/enhancer binding protein(C/EBP)alpha; CEBPB: CCAAT/enhancer binding protein(C/EBP) beta; CKS2: CDC28 protein kinase regulatory subunit 2; COL1A1: Collagen type I alpha 1; COL1A2: Collagen type 1 alpha 2; COL3A1: Collagen type III alpha 1; CREB1: cAMP responsive element binding protein 1; CTNNB1: Catenin cadherin-associated protein beta 1; ID3: DNA binding protein inhibitor -3; JAM2: Junction adhesion molecule 2; JAM3: Junctional adhesion molecule, PCNA: Proliferating cell nuclear antigen: RELN: Reelin, STAR: Steroidogenic acute regulatory protein. TYRO3: Tyrosine-protein kinase receptor TYRO3; Vegf: Vascular endothelial growth factor A; VLDLR: Very-low-density lipoprotein receptor; VNN1: Vanin 1; WIPF1: WAS/ WASL interacting protein family member 1.

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References

Adrienne B, Béatrice MP, Cédric C, Elodie P, Eric P and Corinne C. 2008. Identification of transcripts involved in meiosis and follicle formation during ovine ovary development. *BMC Genomics*. **9**:436.

Albertini DF, Combelles CM, Benecchi E and Arabatsos MJ 2001. Cellular basis for paracrine regulation of ovarian follicle development. *Reproduction*. **121**:647-53.

Berruyer C, Martin F, Castellano R, Macone A, Malergue F, Garrido-Urbani S. Vanin. 2004. Mice exhibit a glutathione-mediated tissue resistance to oxidative stress. *Mol Cell Biol*.**24**:7214–24.

Bonnet A, Cedric C, Olivier B, Julien S, Nathalie M, Sylvain F, Florent W, Philippe M and Beatrice MP. 2013. An overview of gene expression dynamics during early ovarian folliculogenesis, specificity of follicular compartments and bi-directional dialog. *BMC Genomics*. **14**:904

Cain L, Chatterjee S and Collins TJ 1995. In vitro folliculogenesis of rat follicles. *Endocrinology*. **36**: 3369-77.

Douville G, and Sirard MA. 2014. Changes in granulosa cells gene expression associated with growth, plateau and atretic phases in medium bovine follicles. *J. of Ovarian Res*. **7**:50

Eppig JJ, Hulshof SCJ, Van Den Hurk R, Nusgens B, Bevers MM and Ectors FJ 1994. Preservation of oocyte and granulosa cell morphology in bovine preantral follicles cultured in vitro. *Theriogenology*. **41**:1303-10.

Eppig, JJ and Schroeder AC 1989. Capacity of mouse oocytes from preantral follicles to undergo embryogenesis and development to live young after growth, maturation and fertilization in vitro. *Biol. Reprod*., **41**: 268-76

Eresheim C, Leeb C, Buchegger P and Nimp J. 2014. Signaling by the Extracellular Matrix Protein Reelin Promotes Granulosa Cell Proliferation in the Chicken Follicle. *The J. Biol. Chem*. **289** (14):10182–10191.

Girard A, Isabelle D, Gabriel D and Sirard MA 2015. Global gene expression in granulosa cells of growing, plateau and atretic dominant follicles in cattle. *Reprod.Biol. and Endocrin.* **13**:17.

Hale J, Sinyuk M, Dechent WJ. 2012. Context Dependent Role of the CD36 - Thrombospondin - Histidine-Rich Glycoprotein Axis in tumor angiogenesis and growth. *Plos One*. **7**: 40033.

Luissint CA, Nusrat A, and Charles A. Parko. 2014. JAM related proteins in mucosal homeostasis and inflammation. *Semin Immunopathol*. **36** (2): 211–226.

Marchetti F, Venkatachalam S. 2010. The multiple roles of Bub1 in chromosome segregation during

mitosis and meiosis. *Cell Cycle*. **9**:58–63.

Pavanashree US, Nandi S and Girish kumar V. 2014. Survival and Apoptotic Signaling Pathways in Ovarian Follicle and Oocyte. *Development*. **8** (1): 83-90.

Pierce A, Xu1 M, Bliesne B, Liu Z, Richards J, Tobet S, and Margaret E. Wierman. 2011. Hypothalamic but not pituitary or ovarian defects underlie the reproductive abnormalities in Axl/Tyro3 null mice. *Mol Cell Endocrinol*. **339** (1-2): 151–158.

Robker RL, Richards JS: 1998Hormone-induced proliferation and differentiation of granulosa cells: a coordinated balance of the cell cycle regulators cyclin D2 and p27Kip1. Mol Endocrinol. **12** (7):924–940.

Sanchez F and Smitz J. Molecular control of oogenesis 2012. *Biochemica et. Biophysica Acta.* **1822**:1896-1912.

Scaramuzzi RJ, Baird DT, Campbell BL, Driancourt MA and Dupount J. 2011.Regulation of folliculogenesis and determination of ovulation rate in ruminants. *Reprod. Fertil. Dev*. **23:**444-467.

Shen DY, Zhan YH, Wang QM, Rui G, Zhang ZM. 2013. Oncogenic potential of cyclin kinase subunit-2 in cholangiocarcinoma. *Liver Int*. **33**:137–48.

Sriraman V, Sinha M, and Richards JS. 2010. Progesterone Receptor-Induced Gene Expression in Primary Mouse Granulosa Cell Cultures. *Biol. Reprod*. **82**, 402–412.

Wang J and Genet H. 2006. Ankyrin G overexpression in Hutchinson-Gilford progeria syndrome fibroblasts identified through biological filtering of expression profiles. *J Hum Genet*. **51**(11):934-42. Epub 2006 Oct 11.

Zeleznik AJ, Schuler HM, Reichert LE. 1981. Gonadotropin binding sites in the rhesus monkey ovary. Role of the vasculature in the elective distribution of human chorionic gonadotropin to the preovulatory follicle. *Endocrinology*.**109**: 356-62.

Research article

Ovarian follicles in live non-descriptive buffalo (*Bubalus bubalis*) heifers during different seasons as observed by ultrasonography

P.S.P.Gupta*, S.Nandi and R.K. Veeranna
Animal Physiology Div. National Institute of Animal Nutrition and Physiology, Bangalore
*Corresponding author: Email:pspgupta@hotmail.com

Abstract

Ovum pick up technique (OPU) is fast emerging as an alternate tool for the in vitro embryo production technology. In OPU, preferably, the medium and large ovarian follicles are aspirated. The aim of the present study was to study the ultrasound recording of ovarian follicles of two categories i.e. medium (3-8 mm in diameter) and large (> 8 mm in diameter) follicles during peak (October to March) and low (April to September) breeding seasons in non-descriptive buffalo (*Bubalus bubalis*) heifers of south India. The number of medium sized follicles (once a week session) in the low breeding season and the peak breeding season were 0.50 ± 0.13 and 1.02±0.17, respectively. And the corresponding numbers for large follicles were 0.41 ±0.08 and 0.31±0.08, respectively. Only in case of medium sized follicles, the number of follicles was significantly more ($P<0.05$) in peak breeding season compared to the low breeding season.

Key words: ovarian follicles, Ultrasonography, buffalo heifers

Introduction

Availability of ovarian follicles for aspirating oocytes for embryo production are very less in number in buffaloes compared to cattle due to the low germ cell reserve (Danell, 1987). This limits the rapid multiplication of elite germ plasm in this species (Nandi et al., 2003). There also exists species and breed difference in the ovarian follicular population. Detailed studies on the availability of ovarian follicles in live buffaloes using latest techniques like ultrasonography are scarce in non-descriptive buffaloes of south Indian region. There are few earlier studies on the ovum pick up (OPU) in local non-descriptive buffaloes as reported by Manjunatha et al., 2008 and 2009. Studies on the availability of ovarian follicles suitable for aspiration of oocytes (i.e. medium and large size follicles) for the embryo production would help in further propagation of the OPU technology in buffaloes. Hence, this study was conducted to assess the ovarian follicular population in local non-descriptive breed of buffaloes during different seasons, which may facilitate for effective planning of ovum pick up and in vitro embryo production outcome.

Materials and Methods

Seventeen healthy, cycling heifers between 3 and 4 years of age, with normal reproductive function were selected for the study. Non-descriptive Buffalo heifers were procured from Salem / Erode region of Tamil Nadu, India. They were maintained at the Experimental Livestock Unit of our institute at Bangalore, India under standard management conditions. The

location of the study area was at an elevation of 900m from the mean sea level at 12.97^{0}N 77.56^{0}E. They were fed with 3 kg of compound pellet feed of Karnataka Milk Federation along with green fodder and water ad lib. Ultrasound recording of ovarian follicles both in peak breeding season (October to March) and low breeding season (April to September) was made during July, 2010 to March, 2011. The mean high temperatures ranged between 27^{0}C and 34^{0}C and the average low temperatures ranged between 15^{0}C and 22^{0}C. Using ultrasonography, observations on the number of medium (3-8mm) and large (>8mm) ovarian follicles were recorded every week in the buffalo heifers. The ovaries were scanned with an ultrasound equipment (Aloka, Japan) attached with a 7.5MHz transrectal rectal linear probe.

Statistical analysis

The differences between the number of follicles during the two different seasons were analyzed by 't' test using statistical software (Graph Pad PRISM, Graph Pad Software Inc., San Diego, USA). Differences between efficiency of the ovarian follicles in the non-descriptive buffaloes (Manjunatha et al. 2008 and 2009).

In an earlier study conducted by our group, the total number of ovarian follicles observed were less in non-descriptive buffaloes in low breeding season compared to the peak breeding season (Manjunatha et al., 2009). In another earlier study by our group (Manjunatha et al., 2008) we observed a mean total number of 3.62 ovarian follicles per primiparous buffalo. In those studies, while calculating the total number of follicles, we had taken in to consideration even the smaller follicles the mean values were considered significant when the P values were less than 0.05.

Results and Discussion

The number of medium sized follicles (once a week per animal) in the low breeding season and the peak breeding season were 0.50 ± 0.13 and 1.02±0.17, respectively and the corresponding numbers for large follicles (Fig.1) were 0.41 ±0.08 and 0.31±0.08, respectively. The corresponding numbers for total number of follicles were 0.91 ±0.13 and 1.33 ± 0.19, respectively. The number of follicles under medium size category was significantly more ($P<0.05$) in peak breeding season compared to the low breeding season (Fig.2). In the present study, only follicles of medium and large category were considered because of practical importance. Only these categories of ovarian follicles are useful for the aspiration of oocytes for the embryo production and the small size category (< 3mm in diameter) are not useful for the purpose. Ovarian follicles can be aspirated weekly once without compromising on the production (i.e < 3mm in diameter) also. Since the animals examined in the present study were heifers and the total number of follicles did not include smaller follicles, the total number observed was comparatively lower, which might be the reason for the difference in the observations.

The Bangalore city, where the experimental animals were maintained experienced comparatively uniform temperatures throughout the year. The mean high temperatures ranged between 27 ^{0}C and 34 ^{0}C and the mean low temperature ranged between 15 ^{0}C and 22 ^{0}C. This may be the reason for

the no significant difference in the number of large and total ovarian follicles. In a recent study conducted in Nepal in buffaloes, it was observed that the ovarian inactivity and anoestrus were more during non-breeding season (March to June) (Devkota et al., 2012). The region where the study was conducted has comparatively more variation in the temperatures between the seasons. It is to be noted that buffaloes are polyoestrus and are capable of breeding throughout the year (Perera, 2008). However, summer stress in certain regions and high prolactin concentration was identified as a factor for the acyclicity (Roy and Prakash, 2007).

The data obtained in the present study could be useful for planning the ovum pick up technology in indigenous buffaloes. This study indicates that the peak breeding season is the ideal period for performing OPU. Further studies with large number of animals subjected for ovum pick up are required to understand the seasonal influence on ovarian follicular status.

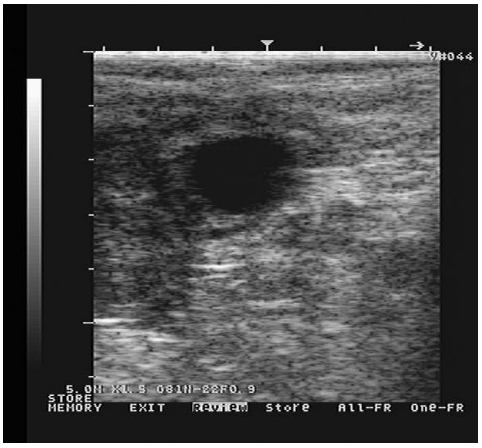

Fig.1. Large follicle (10mm) of buffalo as observed by transrectal ultrasonography

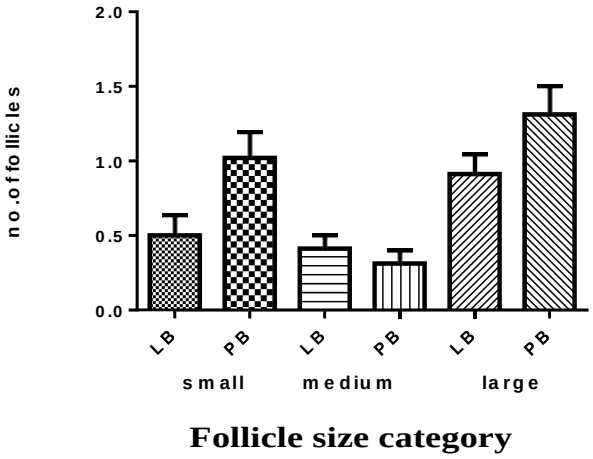

Fig.2. Availability of different size (mm) categories of ovarian follicles during low (LB) and peak (PB) breeding seasons in buffalo heifers as observed by ultrasonography.

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References

Danell B. 1987. Oestrus Behavior, Ovarian Morphology and Cyclical Variation in the Follicular system and Endocrine Pattern in Water Buffalo Heifers. Ph.D Dissertation, Swedish University of Agricultural Sciences. Uppsala. Sweden.

Devkota B, Bohara T P and Yamagishi N.2012. Seasonal variation of anestrus conditions in buffaloes (Bubalus bubalis) in Southern Nepal. *Asian J. Anim.Vet. Adv*. **7**(9)**:** 910-914.

Manjunatha BM, Ravindra J P, Gupta P S P**,** Devaraj M and Nandi S. 2008. Oocyte recovery by ovum pick up and embryo production in river buffaloes (*Bubalus bubalis*). *Reprod. Dom. Anim*. **43:** 477-80.

Manjunatha BM, Ravindra J P, Gupta P S P**,** Devaraj M and Nandi S. 2009. Effect of breeding season on in vivo oocyte recovery and embryo production in non-descriptive Indian river buffaloes (Bubalus bubalis) *Anim. Reprod. Sci*. **111:**376-83.

Nandi S, Raghu HM, Ravindranatha BM and Chauhan M.S.2003. Production of Buffalo (Bubalus bubalis) embryos in vitro: Premises and promises. *Reprod. Dom. Anim.* **37:** 65-74.

Perera BMAO. 2008. Reproduction in domestic buffalo. *Reprod. Dom. Anim*. **43**(Suppl.2): 200-206.

Roy K S and Prakash BS.2007. Seasonal variation and circadian rhythmicity of the prolactin profile during the summer months in repeat-breeding Murrah buffalo heifers. *Reprod. Fertil. Dev*.**19:**569-575.

Research article

Effect of stearic acid on ovine granulosa cell growth, hormone production, apoptosis, DNA and protein content of oocytes

Mohamed Farman*, Shivkumar Tripathi, S Nandi, V Girish kumar*, PSP Gupta, S Mondal
ICAR-National Institute of Animal Nutrition and Physiology (ICAR-NIANP), Bangalore
*Department of Biochemistry, KVAFSU, Bangalore Campus, Bangalore

Abstract

The present study was undertaken to study the effect of stearic acid (SA), a nonesterified fatty acid (NEFA) on ovine granulosa cell growth, hormone production, apoptosis, DNA and protein content of oocytes. The oocytes and granulosa cells were collected from slaughter house derived ovaries and the cells were exposed to different concentrations of stearic acid to evaluate the granulosa cell growth, estrogen and progesterone production and incidence of apoptosis and some biochemical parameters (DNA and protein content) of oocytes. The cell number increment, monolayer formation rate, apoptosis, estrogen and progesterone production were significantly decreased in stearic acid group. Similarly, Protein and DNA contents of oocyte matured in 30µM stearic acid was significantly decreased compared to control group. In conclusion the metabolic stressors like stearic acid impaired the reproductive functions by impairing the functions of granulosa cell and oocytes in ewes.

Key words: Oocyte, Granulosa cells, Stearic acid, Apoptosis, DNA, Protein.

Introduction

Nutrition has a large influence reproduction and production. Energy deficit diet results in lipolysis and is characteristically featured by high non-esterified fatty acid (NEFA) in permutation with low concentration of glucose in serum (Leroy *et al.* 2004). Variation in the easiness ovarian somatic cells has of different fatty acids, saturated fatty acids being toxic and unsaturated fatty acids being comparatively harmless (Aardema *et al.* 2011). We had demonstrated that stearic acid impaired the oocyte development at the level of 30 µM (Farman et al., 2015). The present study was undertaken to study the effect of stearic acid (SA), a nonesterified fatty acid (NEFA) on ovine granulosa cell growth, hormone production, apoptosis, DNA and protein content of oocytes.

Materials and methods

Ovaries from sheep of unknown reproductive status were collected from nearby Corporation slaughter house, Bangalore. They were brought to the laboratory within two hours of slaughter in a thermos flask. Aspiration of follicles from sheep ovaries were performed with a sterile 23G needle fitted to 5ml disposable syringe containing 1-2 ml of aspiration medium. The aspirated and sliced fluid was transferred to a dish and the oocytes were searched under stereo zoom microscope (Olympus, Japan) at 110x magnification. The oocytes were collected by aspiration technique and

those having homogenous ooplasm and 4-5 layers of cumulus cells were selected. In the preliminary studies, oocytes and granulosa cells were cultured in media containing stearic acid [0, 10, 20, 30 and 40 µM]. We found that stearic acid significantly impaired the oocyte and granulosa cell functions at 30 µM level. The control granulosa cell culture medium consisted of TCM-199 + bovine serum albumin (1%) + insulin-transferrin-selenium (1%) + gentamicin (50 mg/ml). The control oocyte maturation medium consisted of TCM-199+FBS (10%) + FSH (10 µg /mL) + Gentamicin (50 µg /mL). Oocytes were cultured at 38.5°C with 5% CO2 in air in the presence of stearic acid acid (0, 30 µM) in oocyte maturation medium for 24 h. The oocytes after 24h of culture under metabolic stressor that is., 30 µM stearic acid, were stripped off the cumulus cells (cumulus-free oocytes) after 24 h by repeated pipetting mechanically in and out through pipette in 35 mm culture petridish containing TCM-199. The oocytes were than washed in normal saline and sonicated. The protein and DNA content of the oocytes were estimated as per biuret method (Reinhold, 1953) and spectrophotometric method (Labarca and Paigen, 1980) respectively. Granulosa cell isolation and culture was performed as described by Nandi *et al.*, (2008) with some modification. In brief after aspirating follicular fluid, follicular fluid was centrifuge at 2500 rpm for 6 min twice and then granulosa cell was seeded in 96 well plates. The evaluation of granulosa cell growth was as described earlier (Pavanna *et al*. 2014). For evaluation of apoptosis and necrosis, after 5 days culture GCs pellets were re-suspended in 100 ml of 4% paraformaldehyde and prefixed for 20 min at room temperature. At the end of pre-fixation, a drop of the suspension was smeared on a slide and allowed to dry. The apoptosis and the necrosis rates were calculated on the basis of total dead cells. The procedures used for hematoxylin staining were those for the conventional hematoxylin-eosin (HE) staining as described by Brauer (1955). The stained cells were observed under a light microscope. Cells with heavily stained, shrunken nuclei, or apoptotic bodies were considered as apoptotic (Liu *et al.*, 2003). The necrotic cells were swollen with irregular border. Four to six fields of each slide were analyzed. Release of estradiol and progesterone in culture media of granulosa cells on day 5 was examined by Enzyme- Linked Immunosorbent assay (ELISA) using ELIAS kits (Diagnostics Biochemicals Pvt Inc, Ontario, Canada) (Nandi et al. 2015). Progesterone measurements were recorded in ng/ ml and pg/ml for estrogen concentrations. All measurements were carried out according to the manufacturer's instructions. The intra- and inter assay coefficients of variation for all analyses were below 5%.

Statistical analysis

Results were expressed as means ± S.E.M. A value of $P < 0.05$ was considered statistically significant. Granulosa growth parameters were analysed by unpaired 't' test. The statistical package of Graph Pad Prism, San Diego, USA was used for analyzing the data.

Results

The effect of stearic acid on granulosa cell growth parameter,

hormone production, apoptosis of granulosa cells are presented in Table 1, Table 2 and Table 3. The cell number increment, monolayer formation rate, apoptosis, estrogen and progesterone production were significantly decreased in stearic acid group. No significant difference in viability of granulosa cells and necrosis were observed. The total amount of DNA and protein present in immature oocyte (µg/oocyte) were 0.90±0.04 µg and 0.52±0.02 µg respectively. Protein and DNA contents of oocyte matured in 30µM stearic acid was significantly decreased compared to control group (Table 4).

Discussion

It is well known that low fertility in ruminants is one vital factors affecting producer profitability. NEB linked with metabolic changes considered as one of the most prominent factor for low fertility in high yielding ruminants (Leroy *et al.,* 2008). The high circulating NEFA levels reflected in the follicular fluid of dominant follicles in ruminants early postpartum. Furthermore, this knowledge was applied in an *in vitro* model, in which the oocyte maturation medium was supplemented with the predominant fatty acids at concentrations observed in follicular fluid of ruminants. Results revealed that the saturated long chain fatty acids in particular (such as: palmitic and stearic acid) provoked an inhibition of maturation rate, leading to relatively low fertilization, cleavage and blastocyst formation rates (Tripathi *et al.* 2015, Farman *et al.* 2015). Parallel with the results of the present study, it has been shown earlier in other laboratory that Palmitic acid and stearic acid exert a toxic effect on bovine granulosa cell growth and function *in vitro* (Vanholder *et al.,* 2005). This demonstrated the induction of apoptosis by these acids, probably through ceramide production or through a down-regulation of the apoptosis inhibitor Bcl-2 and the up-regulation of an apoptosis mediator such as Bax. Oocytes accumulate fatty acids from their environment, potentially changing their lipid content and composition (Adamiak *et al.,* 2005). Apoptotic cell death, was non-inflammatory, and on the other hand necrotic cell death is inflammatory, and are two extremes. Our finding suggests that stearic acid causes more apoptotic cell death compared to necrosis.

Reproductive efficiency in ruminants has been decreased over the last 5 decades despite significant gains in genetic selection, improved reproductive technologies and nutritional care. In high-yielding dairy cattle, the negative energy balance (NEB) during the first week post-partum adversely affects ovarian activity, mainly follicle growth and steroidogenesis. Circulating levels of non-esterified fatty acids (NEFA) and urea are elevated during NEB and are known to be accumulated in the follicular fluid of dominant follicle where they exert adverse effect on oocyte maturation, leading to low fertilization rate and early embryonic development. The relationship between nutrition and reproduction is a topic of increasing importance and concern among farmers, veterinarians, feed dealers and extension workers. We had earlier demonstrated that the maturation, cleavage and morulae/ blastocyst production rates were significantly lowered in media containing 20µM

stearic acid. Increment of stearic acid to 30 µM in media further reduced the maturation, cleavage and morulae/ blastocyst production (Farman *et al*. 2015). Stearic acid was found to be a inhibitory stressor amongst the metabolic stressors which affect the granulosa and oocyte functions.

Table 1. Effect of stearic acid (30 µM) on granulosa cell growth parameters

Parameters	**Control**	**Stearic Acid (30 µM)**
Viability	93.4±4.14	86.1±1.14
Cell no increment	1.29×10^{5} ±0.04[a]	0.73±0.16[b]
Monolayer	1.83±0.14[a]	0.98±0.06[b]

Values with different superscripts in the same row differ significantly (P < 0.05).

Table 2. Effect of stearic acid (30 µM) on Estrogen and progesterone production by granulosa cells in culture (5 day culture)

Treatments	**Estrogen (pg/ml)**	**Progesterone (ng/ml)**
Control	43.7±2.4[a]	366.3±9.5[a]
Stearic acid	22.1±4.1[b]	152.6±7.4[b]

Values with different superscripts in the same column differ significantly (P < 0.05)

Table 3. Effect of stearic acid (30 µM) on granulosa cells on apoptosis and necrosis

Cellular events (Cell death)	**Control**	**Stearic Acid, 30 µM**
Apoptosis, %	24.6 ±3.4[a]	34.4 ±2.1[b]
Necrosis, %	7.4 ±1.7	11.4 ±3.6

Values with different superscripts in the same row differ significantly (P < 0.05).

Table 4. Effect of stearic acid (30 µM) on protein and DNA content of oocytes

Biochemical Constituents	**Immature oocytes**	**Matured oocytes Control Stearic Acid, 30µM**	
Protein	0.52±0.02	156.3±0.04[a]	136.7±1.4[b]
DNA	0.90±0.04	1.37±0.001[a]	0.92±0.11[b]

Values with different superscripts in the same row differ significantly (P < 0.05).

Acknowledgement

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References

Aardema H, Vos PL, Lolicato F, Roelen BA, Knijn HM, Vaandrager AB, Helms JB and Gadella BM 2011. Oleic Acid Prevents Detrimental Effects of Saturated Fatty Acids on Bovine Oocyte Developmental Competence. *Biol. Reprod*. **85**(1): 62–69

Adamiak S, Ewen M, Rooke J, Webb R and Sinclair K 2005. Diet and fatty acid composition of bovine plasma, granulosa cells, and cumulus-oocyte complexes. *Reprod. Fertil. Dev.* **17**: 200–201 (Abstract)

Brauer A. 1995 Staining and mounting. *In:* Laboratory Directions for Histological Technique *Edt* A., Brauer and Minneapolis. M. N., Burgess Publishing Company., *pp:*23-25.

Farman M, Nandi S, V Girish Kumar, Tripathi SK and Gupta PSP 2015. Effect of stearic acid on in-vitro formation of sheep oocytes. *Indian J. Anim. Sci*. **85**(6): 584–587.

Labarca C and Paigen K 1980 A simple, rapid and sensitive DNA assay procedure. *Analyt. Biochem*. **102**: 344-352.

Leroy JLMR, Opsomer G, Van Soom A, Goovaerts IGF and Bols PEJ. 2008. Reduced fertility in high-yielding dairy cows: are the oocyte and embryo in danger? Part II: Mechanisms linking nutrition and reduced oocyte and embryo quality in high-yielding cows. *Reprod. Dom. Anim.* **43**: 623-632.

Li, ZH, Yue KZ, Ma SF, Sun XS and Tan JH. 2003. Effects of pregnant mare serum gonadotropin (eCG) on follicle development and granulosa cell apoptosis in the pig. *Theriogenology*. **59:** 775-785.

Nandi S, Girish Kumar V, Manjunatha BMV, Ramesh HS and Gupta PSP 2008. Follicular fluid concentrations of glucose, lactate and pyruvate in buffalo and sheep, and their effects on cultured oocytes, granulosa and cumulus cells. *Theriogenology*. **69**(2): 186-196.

Pavana Shree US, Nandi S, Girish Kumar V, Gupta PSP and Chandrasekhar Murthy V 2014. Effect of GDF-9 and bFGF in combination on caprine granulosa cell growth parameters *in vitro*. *Indian J. Anim. Sci.* **84** (1): 30.

Reinhold JC 1953. Standard Methods. *In:* Clinical Chemistry *Edt.* Reiner M., J. C., Reinhold, academic press, New York. *pp* 88.

Tripathi SK, Nandi S, Gupta PSP and Mondal S 2015. Influence of Common Saturated and Unsaturated Fatty Acids on Development of Ovine Oocytes in vitro. Asian *J. Anim. Sci.* **9**(6): 420-426.

Vanholder T, Leroy JLMR, Vansoom A, Opsomer G, Maes D, Coryn M and De Kruif A 2005. Effect of non-esterified fatty acids on bovine granulosa cell steroidogenesis and proliferation *in vitro*. *Anim. Reprod. Sci*. **87:** 33

Research article

Electroencephalography (eeg) observations on goats during intravenous fluid infusion

R. Huozha and S. K. Rastogi*, J.P. Korde
Department of Veterinary Physiology and Biochemistry,
G. B. Pant University of Agriculture & Technology, Pantnagar – 263 145,
Uttarakhand,
*E-mail address: rastogisk58@gmail.com

Abstract

Electroencephalographic tracing obtained from local crossbred Jamunapari does, subjected to routine clinical distress are reported. Duplicate EEG records were obtained from 8 adult (8-12months, 10 ±2Kg body mass) does by using portable Student Physiograph. Bipolar scalp electrodes were placed at the occipital region while the neutral electrode at the tip of the nostril. Pain was inducted with insertion of needle into jugular vein then intravenous infusion of 5% dextrose and 0.9% normal saline solution. EEG recordings were obtained from before pain induction, at insertion of needle and during intravenous infusion at different flow rates (10, 30, 60 drops per minute). Results indicated that intravenous infusion of 5 % dextrose and 0.9 % normal saline exerted significant ($P<0.05$) increased in EEG frequencies and amplitudes during insertion of needle and at infusion rates of 30 and 60 drops per minute as compared to 10 drops per minute and the EEG frequency pattern was found to be beta (β) frequency band. Combination of visual behavioural changes and EEG pattern during pain induction and intravenous infusion provided a good indicator of acute pain in goat. It may be concluded that intravenous glucose infusion imposed greater EEG changes than normal saline infusion.

Key words: Electroencephalography (EEG), intravenous infusion, goat
Abbrev: mm-millimeter, μV-microvolt, s-second, Hz-frequency, h-hour, min-minute

Introduction

Electroencephalogram (EEG) is the recording techniques of electrical potential changes in brain cells that respond to various stimuli (Guyton *et al.*, 2000). It record the 'brain waves' influenced by different behavioural and physiological states using macro electrode on the scalp (Cunningham, 1997), as it indicate the whole brain activity and even chemical actions of the entire array of the neurons in the brain (Niedermeyer *et al.*, 1999). Usage of EEG enables us to know the different behavioural states, understand the feelings and thoughts of animals (Bergamascoa *et al.*, 2005). The normal and abnormal EEG changes either in frequency or amplitude and often both, may vary according to species and type of method of restraint used (Klemm, 1968).

EEG is the only tool that provides direct quantitative measure to know the degree of distress caused by a daily farm operations by behavioural expression (posture, vocalization and activity) and physiological (hormone, heart and respiration rate, body temperature) responses (Barnett, 1997; Simon and Drnec, 2009; Huozha *et al.*, 2011). These responses directly or indirectly reflect secondary stress to farm animals (Bromm, 1984; Minton, 1994; Zimmerman, 1986). The animal welfare issue derives utmost importance on the animals suffering from distress faced in different farm

conditions causing stress, which may have a negative effect on animal growth and its production (Fraser and Duncan, 1988; Bath, 1998). Animals being mute and dumb, are unable to express/ communicate their distress through behavioural expressions. EEG provides direct data from physiological state of brain tissue owing to such stresses. Scientific data on such kind of studies are scarce in the literature. The present investigation, therefore, was conducted in conscious goat to evaluate the EEG pattern during insertion of needle into jugular vein and intravenous infusion of fluids at different flow rates causing pain which are routine clinical operations in animals.

Materials and Methods

This experiment was conducted on well trained eight healthy crossbred (local and Jamunapari) does (8-12months, 10±2Kg.body wt.) maintained under standard loose housing system of management except during experiment. The selected site of brain for recording EGG was prepared by shaving the hairs and rubbed with ethanol to remove the skin oils. Local anaesthesia (xylocaine 2%) was injected subcutaneously into the underlying areas of prepared site before placement of electrodes, to minimize the artefacts arising from adjacent skeletal muscles (Holliday and Williams, 1999). Silver disc electrodes were applied on scalp using EEG paste (bentonite paste).

Single channel Student Physiograph (Biodevice, Ambala Cantt) was used for EEG recording. Physiograph was set at amplifier sensitivity of 50μV and calibration at 100μV equal to 44mm. The paper speed was adjusted to 50mm/sec (1mm equal to 0.02s) and time constant was 0.03s for EEG recording in goats. All the recordings and observations were taken after 15 minutes of acclimatization of animal and instrument by putting 'ON' position with all electrodes attached to the animal. EEG recording was done on each animal by using bipolar lead system placing on electrodes on RO-LO, i.e. right occipital and left occipital), while the neutral electrode at the tip of the nostril (Suzuki *et al.,* 1990). EEG recording were repeated after 30 minutes and at least two replicates were made for each animal.

Intravenous normal saline (0.9% NaCl) was infused into jugular vein at flow rates of 60, 30, and 10 drops per minute on separate occasions for 2 minute each. EEG recording were obtained before, during and after infusion in each animals. The same procedure and EEG recording was repeated for intravenous dextrose (5%) infusion at similar rates. The behavioural changes were noted by visual observation (Molony and Kent, 1993).

EEG tracings recorded were visually analysed for rhythm (frequency and amplitudes). The rhythm frequencies (Hz) were calculated by counting all major and minor waves (Morris *et al.,* 1997) and were expressed in number of waves per second. The amplitudes were calculated as the height of least and most waves in millimetre (mm) and then transformed into micro volt (μV) through calibration. The recorded EEG data were analysed statistically by analysis of variance (Snedecor and Cochran, 1994).

Results and Discussion

The EEG frequencies and amplitudes of intravenous infusion of 5% dextrose and 0.9% normal saline into conscious goats are presented in Table 1

The EEG frequency (Hz) during needle insertion increased significantly ($P<0.05$) from pre needle insertion value of

26.00±0.46 to post-insertion value of 40.38±0.46 and then declined to 25.38±0.38 on needle removal. Similarly amplitude (μV) during needle insertion was significantly (P<0.05) higher before needle insertion with a value of 5.69±0.09 to needle insertion value of 25.88±2.04 which further declined to 5.36±0.11 on needle removal. Marked increase in frequencies and amplitude with insertion of needle into jugular vein for infusion of any drugs caused intense brain activity signifying some sort of pain.

The EEG frequencies (Hz) during intravenous infusion of 5% dextrose @ 60, 30 and 10 drops per minute were 31.50±0.71, 26.50±0.50 and 24.25±0.37, respectively, whereas during intravenous infusion of 0.9% normal saline @ 60, 30 and 10 drops per minute were 28.00±0.60, 23.75±0.53 and 22.38±0.42, respectively. The amplitudes (μV) during intravenous infusion of 5% dextrose @ 60, 30 and 10 drops per minute were 9.15±0.55, 7.04±0.22 and 5.83 ±0.20, respectively whereas during intravenous infusion of 0.9% normal saline @ 60, 30 and 10 drops per minute were 8.49±0.57, 7.66±0.40 and 5.54±0.10 μV. Both EEG amplitude and frequency for dextrose and normal saline infusion rate at 60 drops per minute produced significantly (P<0.05) higher EEG wave than slower rate of infusion. This signifies intense cerebral cortical electric activity, which could be correlated with involvement of painful stimuli as suggest by Jongman *et al.*, (2000). The EEG waves declined in magnitude as the flow rate were reduced. It could be deduced that 5% dextrose infusion could be more painful than 0.9% normal saline infusion based on EEG pattern. These results are in agreement with Jongman *et al.*, (2000).

Visual behavioural changes observed such as restlessness, frequent movements of limbs and head with vocalization, pupil dilatation were observed in most animals. Similarly the animals also tried to attack the point of pain source making restraining difficult as it tended to escape or avoid the source of pain (Kitchell and Erickson, 1983). Similarly observations have been reported by Ong *et al.*, (1997).

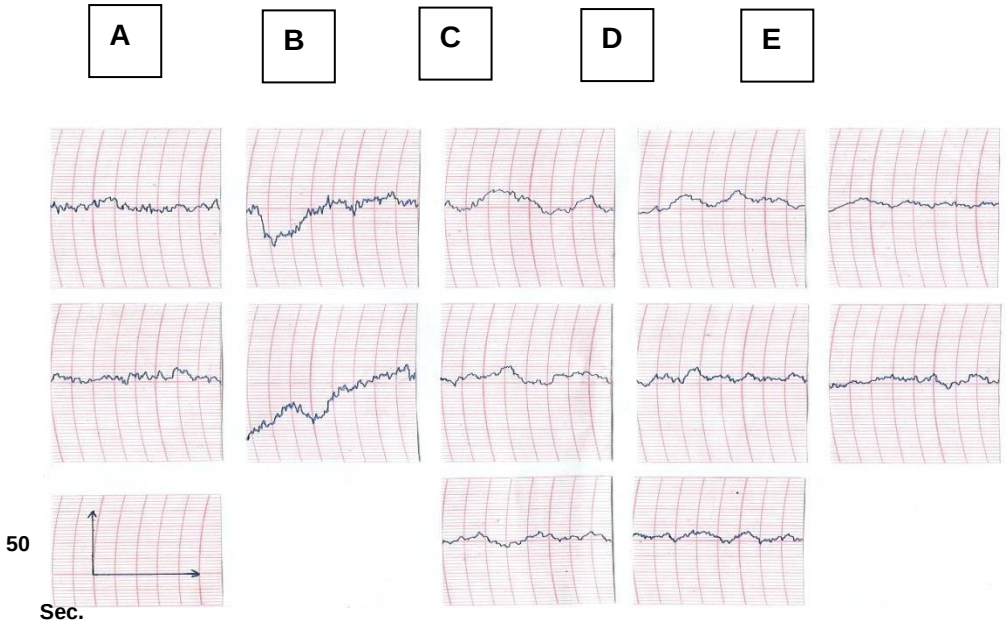
The EEG records electrical activity directly from the nervous system during the procedure and the behavioural changes depicting the states of consciousness were noticed and that it varied from each other. Hence the present study has indicated that EEG and behavioural changes together indicated a good measure to know the degree of distress caused by routine clinical operations. However, using better spectral analysis and wireless electrodes could provide better data for further study in animals. Studies on the various other kinds of distress faced by the animals is warranted.

Table 1: Electroencephalographic recording (Mean ± SE) of intravenous 5% dextrose and 0.9% Saline infusion in goats (n=8)

Experimental conditions	Frequency (Hz.)	Amplitude (μV)
- Before Infusion	26.00 ± 0.46[a]	5.69 ± 0.09[a]
- Needle Insertion	40.38 ± 0.46[b]	25.88 ± 2.04[b]
- Needle Removal	25.38 ± 0.38[a]	5.36 ± 0.11[a]
5% Dextrose Infusion		
- @ 60 drops/min	31.50 ± 0.71[a]	9.15 ± 0.55[a]
- @ 30 drops/min	26.50 ± 0.50[b]	7.04 ± 0.22[b]
- @ 10 drops/min	24.25 ± 0.37[c]	5.83 ± 0.20[c]
0.9% Saline Infusion		
- @ 60 drops/min	28.00 ± 0.60[a]	8.49 ± 0.57[a]
- @ 30 drops/min	23.75 ± 0.53[b]	7.66 ± 0.40[b]
- @ 10 drops/min	22.38 + 0.42[c]	5.54 + 0.10[c]

*1. Means with different superscripts along the column are significantly ($p<0.05$) different from each other.

Fig.1. Electroencephalographic tracings of goats during i.v. infusion of 5% dextrose and 0.9% saline



A. Before infusion
B. Needle insertion
C. 5% Dextrose infusion (60, 30, 10 drops/min.)
D. 0.9% Saline infusion (60, 30,10 drops/min.)
E. Needle removed

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References

Barnett JL, 1997. Measuring pain in animals. *Aus. Vet. J.* **75** (12): 878-879.

Bath,GF, 1998. Management of pain in production animals. *App. Anim. Behav. Sci.* **59** (1-3): 147-156.

Bergamascoa L, Macchia E, Facelloa, C, Badinob P, Odoreb R, Pagliassob S, Bellinob C. Osellab MC, and Reb G. 2005. Effect of brief maternal separation in kids on neurohormonal and electroencephalographic parameters. *Applied Animal Behavior Science*. **93**: 39 - 52.

Bromm B, 1984. The measurement of pain in man. *In*: Bromm B, editor-Pain measurement in man: Neurophysiological correlates of pain. Elsevier, New York: p 3-13.

Cunningham JG. 1997. Textbook of Veterinary Physiology. 2nd ed., W.B. Saunders Company, India.

Fraser D and Duncan IJH. 1998. "Pleasures", "pains" and animal welfare: Toward a neutral history of effect. *Anim. Welfare.* **7** (4): 383-396

Guyton AC.and Hall JE. 2000. Text book of Medical Physiology. 10th ed. W.B. Saunders Company. Elsevier. p 512-714.

Holliday TA and Williams C. 1999. Clinical Electroencephalography in dogs. *Vet Neurol Neurosurg J.* 1: 1.

Huozha R, Rastogi SK, Korde J P and Madan AK. 2011. Electroencephalographic changes during experimental pain induction in goats. *Veterinarski arhiv.* **81** (3): 359-368.

Jongman EC, Morris JP, Barnett JL and Hemsworth PH. 2000. EEG changes in 4-week-old lambs in response to castration, tail docking and mulesing. *Aust. Vet. J.* **78**: 339-343.

Klemm WR. 1968. Attempts to standardize veterinary electroencephalographic techniques. *Am. J. Vet. Res.* **29** (9): 1895-1900.

Kitchell RL and Erickson HH. 1983. Introduction: What is pain? *In*: Kitchell R.L, Erickson, HH, Carstens E, Danis LE. Editors. Animal pain: perception and alleviation. American Physiological Society, Both coda; 1983: vii-viii.

Minton JE. 1994. Function of the hypothalamic-pituitary-adrenal axis and the sympathetic nervous system in models of acute stress in domestic farm animals. *J. Am. Sci.* **72**: 1891-1898.

Morris JP, Ong RM, O'Dwyer JK, BarnettJL, Hemsworth PH, Clarke IJ and Jongman EC. 1997. Pain-related cerebral potentials in response to acute painful electrical stimulation in sheep. *Aus. Vet. J.* **75** (12): 883-886.

Molony V and Kent JE. 1993. Behavioural responses of lambs of three ages in the first three hours after three methods of castration and tail-docking. *Am. J. of Vet. Res.* **29**: 1033 - 1036.

Niedermeyer E and Lopes da Silva F. 1999. Electroencephalography, 4th Ed., Williams & Wilkins, Baltimore, MD, p.1258.

ONG RM, Morris JP, O'Dwyer JK, Barnett JL, Hemoworth PH and Clarke IJ. 1997. Behavioural and EEG changes in sheep in response to painful acute electrical stimuli. *Aus. Vet. J.* **75** (3): 189-193.

Simon W R and Drnec J Z. 2009. Quantitative Electroencephalography (EEG) to assess pain in cattle. Animal and Avian Sciences University of Maryland College Park, MD 20742.

Suzuki M, Sitizyo K, Takeuchi T and Saito T. 1990. Electroencephalogram of Japanese Black calves affected with cerebrocortical necrosis. *Jpn. J. Vet Sci.* **52**: 1077-1087.

Snedecor GW and Cochran WG. 1994. Statistical Methods, 8th ed. Iowa State University Press, Ames, Iowa.

Zimmerman M. 1986. Physiology mechanism of pain and its treatment. *Klinische Anasthesiologie und Intensivtherapie.* **32**: 1-19.

Reviewers of manuscripts for this issue

Dr. Jeyakumar, Principal Scientist, SRS_ICAR-NDRI, Bangalore

Dr. Ashish Mishra, Senior scientist, ICAR-NIANP, Bangalore

Dr. S. Selvaraju, National Fellow, ICAR-NIANP, Bangalore

Dr. K.V. Jamuna, Prof & Head, Vety. College, KVAFSU, Bangalore

Society announcements

Appeal: Corpus fund to support zonal level SAPI quiz / Madan trophy

It has been decided to create a separate corpus fund for the financial support to host Zonal level SAPI quiz. Dr. M. L. Madan and Dr. V. H. Rao have assured to donate Rs.1 lakh each to this fund and as promised, Dr. V. H. Rao has already contributed Rs.1 lakh towards the fund. All the executive members of SAPI and other life members of the society are requested to contribute for this corpus fund. The interest earned on FD of the corpus fund shall be utilized to support the hosts of different zones and the amount of support shall depend on the number of colleges participating in each zone.

Proceedings of General Body Meeting of SAPI held on 22.12.2016

The general body meeting of the Society of Animal Physiologists of India (SAPI) was held on 22.12.2016 at the College of Veterinary Science, Mhow (MP) during the XXV Annual Conference. Dr. P.S.P. Gupta, General Secretary welcomed all the members for the meeting and tabled various agenda for discussion. The items discussed and the resolutions that were made during the meeting are listed below:

1. **Confirmation of the proceedings of previous general body meeting held in Jan 2016**

   The members of the SAPI approved the proceedings of the previous general body meeting held at College of Veterinary and Animal Sciences, Guwahati during Jan 2016. The members felt to implement all the decisions made in the previous general body meeting at the earliest.

2. **Statement of Accounts of SAPI for the year 2016.**

   Dr. Ashish Mishra, the treasurer of SAPI expressed that the accounts of SAPI transactions could not be audited as the audit period is from 1st January to 31st December every year. However, he presented the statement of accounts of the society with all the details on receipts, expenditure and the balance as on 20th December, 2016. He expressed that the same would be audited after 31.12.2016. The members approved the statement of accounts and asked the treasurer to get it audited immediately after 31st December 2016 and to circulate the same to all the members through mail. *(A: Dr. Ashish Mishra)*

3. **Generation of corpus fund to support Zonal Level SAPI Quiz**

   The president and general secretary appreciated organizers of different zones for successful hosting of Zonal Level SAPI quiz. As there is a demand for the financial support for the organizer of zonal level quiz, the president highlighted the benefit of generating corpus fund exclusively to support the SAPI quiz at

zonal level. Dr. V.H. Rao and Dr. M.L. Madan announced a contribution of 1 Lakh each for this contribution. All the executive members also promised to contribute generously towards SAPI student quiz trust fund. The president requested all the members to voluntarily and generously contribute for this fund to make it at least 5 Lakhs. It was resolved to make use of only the interest earned by fixed depositing the corpus fund towards financial support for the Zonal level organizers. It was also resolved that the share of each zone shall be decided on the number of participating teams in respective zones. *(A: General Secretary, Treasurer and all the members of the society)*

4. Enhancement of Life membership fee

It was decided to enhance the life membership fee from existing Rs. 2100 to 3000 (including registration fee) which shall be in force from 1st January, 2017.

5. Online publication of SAPI journal

It was resolved to publish the SAPI journal online. It was resolved not to collect any processing fee initially for two years. (A: Dr. J P Ravindra)

6. Participation of delegates in technical sessions

The patron, president and other members expressed their concern over the poor attendance of delegates and also not presenting their paper/poster in technical sessions during the conference. The members participating in the conference were requested to attend the technical sessions and present their papers to make the conference meaningful and successful.

7. Payment of publication fee at the time of submission of abstracts.

It was decided to collect a fee of Rs. 500/- at the time of submission of abstracts from presenting author where in one presenting author can submit a maximum of 3 abstracts. This fee will be adjusted towards his/her registration fee at the time of registration to the conference. If he/she fails to register for the conference, this fee shall not be refunded but the abstracts will be published in the compendium. *(A: Org. secretary of next conference)*

8. SAPI Awards

It was resolved that the candidate applying for any of the SAPI awards must be a life member of the society at the time submitting his/her application for the award and a copy of his/her registration certificate shall be enclosed with the application/ registration number shall be clearly mentioned in the application. The delegates competing for the best oral paper/poster awards should become life member on the first day of the conference if he/she is not life member of the society.

It was also decided to start a new award **"Best Research Paper Award"** to the SAPI member for publishing a best research paper in peer reviewed national/ international journals every year covering the period January to December. The eligibility and selection criteria for this award will be notified in due course of time. *(A: General Secretary)*

9. Periodical review of progress of hosting the Annual Conference of SAPI

It was felt that periodical review of progress made in organizing Annual Conference by the host institute is necessary to support/guide the organizing secretary so that gross lapses in hosting the conference could be avoided. Therefore, it was decided to periodically visit the venue of the conference by the office bearer/executive committee member of SAPI/ organizing secretaries of previous conferences to assess the progress made in hosting the conference.

10. Venue of Next Annual Conference of SAPI

The General Secretary informed that the Registrar, Karnataka Veterinary, Animal and Fisheries Sciences University, Bidar has sent a proposal to host the Annual Conference of SAPI-2017 at Veterinary College, Bidar. It was approved to host XXVI Annual Conference of SAPI 2017-18 by Veterinary College, Bidar with Dr. Shrikant Kulkarni, Associate Professor, Dept. of Veterinary Physiology and Biochemistry as the Organizing Secretary. The meeting ended with vote of thanks by Dr. Ashish Mishra, Treasurer of SAPI.

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General Secretary, SAPI
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Indian journal of animal physiology

(An official Publication of Society of Animal Physiologists of India)

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The manuscript for publication should be submitted in duplicate to the Chief Editor (by name), Indian Journal of animal Physiology, ICAR-NIANP, Hosur Road, Adugodi, Bangalore 560070, India, with a soft copy mailed to jpravindra@yahoo.com. At least one of the authors of the research paper should be an Ordinary/Life Member of SAPI.

General

Manuscripts will be received for publication with the understanding that they have not been published, simultaneously submitted or already accepted for publication elsewhere. Submitted manuscript will be published only after reviewing by referee.

Preparation of Manuscript

The manuscript should be typed in a double-spaced on one side of white bond paper (A-4 size) with margins of 2.5 cm on all sides. Pages and lines should be n8mbered beginning with the title page.

The papers should be divided into following components in a sequence indicated below:

Title, Introduction, Material and Methods, Results, Discussion, conclusion, Acknowledgement, References, Tables, Legends for illustrations.

The titles of charts should be typed in capital letters and kept in the centre of the text. The Results and Discussion parts could be combined if the authors so desire. Subtitles if any, in each part should be underlined and suffixed with a colon. Authors should see that three should be no repetition of the data presented in the table, in the text and vice versa.

Title should include:

Title of the article, Running title of the article, Name of the author(s) with complete address and Name and address of the person to whom correspondence should be sent.

Abstract

The abstract should be written as running matter on a separate sheet. It should highlight important findings and conclusions. This text should be reproducible in abstracting journals.

Keywords

Keywords should be incorporated at the end of Abstract.

INTRODUCTION

The Introduction should give appropriate background, objectives of the work and explain briefly what is new in the paper.

MATERIALS AND METHODS

Experimental procedure of new methods should be described in detail. In case of well established methods, References are sufficient. Substantial modifications of standard methods should be described in details. Instruments, equipments, apparatus and pure chemicals/bio-chemicals used should be identified by giving the manufacturers name in parenthesis.

RESULTS

The results should be presented as concisely as possible. Tables and figures should not be used for presentation of same data.

DISCUSSION

This section should be used for the interpretation of data but data give in results should not be repeated. Conclusions should not be drawn without supporting data. New hypothesis and recommendations when appropriate may be included.

UNITS

International system of units (SI) should only be used.

ACKNOWLEDGEMENT

Authors are responsible for including the names of persons who have made substantial contributions to the study or institutions which have given financial assistance to the work.

References

The References should be arranged in an alphabetical order and should conform to those cited in the World List of Scientific, Periodicals, Butter Worths Scientific, Publications, London, 1962.

The order or presentation of reference should be as follows:

Name of the author with initials, year, Full title of article, Title of Journal, Volume no., Page no. e.g., Puri RS, Gupta NK and Ghai AK 1988. Studies on network system in Indian subcontinent. Indian Journal of Dairy Science. 14:510-515.

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Each table should be typed (double spaced0 on a separated sheet numbered consecutively in Arabic numerals at the top centre and given collectively after the References. Each table should have a brief but meaningful title.

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Figures should be drawn with Indian ink on tracing paper of size 20 cm x 14 cm to permit reduction to uniform size. Most graphs will be reduced to the width of a column and all letters should be able to withstand this reduction. The legends should be typed on a separate page. The figures should be numbered in Arabic Numerals. The photographs should be only glossy black and white and should have good contrast. The photographs and diagrams should not be larger than the sheets on which the article is typed. The figures should have titles.

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