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THE EFFECT OF LIGHT/DARK CYCLE ON BLOOD FOLLICULAR BARRIER LAMININ EXPRESSION IN OVARY OF THE MICE

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ABSTRACT : Blood-follicle barrier (BFB) is found in the developing follicles of the ovary. The BFB permeability barrier of the endothelial cells in the microvessels that surround the developing follicle is constituted by the basement membrane of the developing follicle. It provides structural support, growth and maturation.The study aim is to known role laminin á1 protein in development and maturation of blood follicular barrier in ovary of young adult female mice according to variation of dark / light cycle. A forty (40) adult healthy female mice (albino mice), aged about 12-14 weeks. These were divide into four groups according to daily illumination times (dark/light cycle). Each group have 10 female mice and were kept in special illuminating conditioned room. The group A animals were kept in normal diurnal variance, 10 hrs dark and 14 hrs light for period of one month. Group B animals were kept in room with full time light for period of one month. Group C animals were kept in room with full time of darkness for period of one month. Group D animals were kept in inverse state for normal diurnal variance (10 hr of full darkness during daylight and 14hr light during the night) for one month. Morphological appearance of histological examination by H&E reveals regress in size of ovary in dark group in comparison to light group. The follicles shows an increase in size & decrease in number of follicle ovary while the corpus lutum show increase in size and number in light group in comparison to other groups. While in reverse group, have normal value number of follicles and corpus luteum and size of ovary as in control group. This study concluded that light revealed an ovarian hyperovalution due to inhibitory effects on melatonin secretion, which will lead to stimulation of gonadotrophic hormones secretion from pituitary gland in contrast to hypo-ovulation condition of dark group mice. Immunhistochemical study reveals the light group animals they reveal a marked expression of laminin α1 at BFB in comparison to other group animals and reveals very low value in dark group, which recorded. In reverse group animals, they reveal a weak expression of laminin á1 than controlin the BFB. Conclusion: laminins α1 are an important and biologically active part in basal lamina of BFB, influencing cell adhesion, differentiation, migration and promotion of tissue survival.

Key words : Blood follicular, barrier laminin, light/dark cycle.

INTRODUCTION

The blood-follicle barrier (BFB) is one of the blood-tissue barriers in mammalian body found in developing follicles of the ovary. The BFB permeability barrier of the endothelial cells in the micro vessels that surround the developing follicle is constituted and contributed significantly by the basement membrane of the developing follicle which alters its composition rapidly during follicle development (Michelle and Cheng, 2012).

Light/dark cycle is any biological process that displays an endogenous, entrainable oscillation of about 24 hours. These 24-hour rhythms are driven by a circadian clock, and they have been widely observed in plants, animals (Edgar, 2012). It impact on a wide range of physiological systems, which impact upon reproductive capacity (Kennaway *et al*, 2012).

Laminin protein is a family of large multi domain glycoproteins, which are major component of basement membrane and extracellular matrix (ECM). The laminins are an important and biologically active part of basal lamina, influencing cell adhesion, differentiation and migration (Aumailley *et al*, 2015).

In all mammals, the reproduction depends on the function of the hypothalamus-pituitary-gonads axis. In females, gonadotropin-releasing hormone (GnRH) neurons present in the septal area of hypothalamus send their axons to median eminence. GnRH is released from hypothalamus reaches the anterior pituitary where the gonadotrophs are stimulated to secrete follicle-stimulating hormone (FSH) & luteinizing hormone (LH). In the peripheral circulation, these hormones stimulate specific cells (granulosa cells) in the ovary leading to ovulation

(Caligioni, 2009). Melatonin is synthesized and released in according to how much light reaches the eye (Macchi and Bruce, 2004). It plays an important role in regulating the endocrine system andin the regulation of many reproductive processes such as puberty, gonadal function, and pregnancy (Lampiao and Plessis, 2013). It also has the capability to suppress the release of hypothalamic gonadotropin-releasing hormone GnRH (McMillin *et al*, 2017).

MATERIALS AND METHODS

Experimental animals housing and feeding

A forty (40) adult healthy female mice (albino mice), were collected fromCancer Research Center, aged about 12-14 weeks, weighing between 20-30 g. The animals were placed in a plastic cages, easyto cleanwith free access to water (fresh tap water) & food (standard pellet diet) and 10 animals for each cage. These were kept at room temperature (20±2 ºC) in a clean and well-ventilated room.

Collections of sample

The animals were divide into four groups according to daily illumination times (dark /light cycle). Each group have 10 female mice and were kept in special illuminating conditioned room. There are 40 females mice are applied in the study were classified into group A, B, C & D according to dark day periods in which the animals were kept. The group A10 animals were kept in normal diurnal variance, 10 hrs dark and 14 hrs light for period of one month. Group B 10 animals were kept in room with full time light for period of one month. Group C 10 animals were kept in room with full time of darkness for period of one month. Group D10 animals were kept in inverse state for normal diurnal variance 10hr of full darkness during daylight and 14hr light during the night) for one month. The animals were euthanized and Median incision of anterior abdominal wall was done. With a fine scissor was used to separate and remove the whole genital system from the abdominal cavity, then with the aid of the dissecting microscope separation ovary from fallopian tube accompanied fat around the ovary. The specimens were fixed chemically by immersion in to 10% neutral buffered formalin. Then further histological procedure was done so that to obtain paraffin block of specimens for histological and immunhistochemical assessment.

The preparation of paraffin section

The ovary specimens were histologically prepared for paraffin section as follows: Beginning with the process offixation, dehydration, clearing, impregnation, embedding in tissue block, sectioning on glass slides,de-waxing, hydration,staining and mounting according to Bancroft *et al* (2018).

Table 1 : Reveals the Mean ±SE positivity of laminin expression of blood follicular barrier of ovaries in animals groups according effect to dark /light cycle.

Groups	Mean ±Std. Error pixel/(microns)²
Control	0.0538± 20.5689
Light	0.8814±0.03461
Dark	0.1978 ±0.02363
Inverse	0.3869 ±0.12768

($p \leq 0.001$).

The Harris Hematoxylin (Fisher) and Eosin-yellow (Fluke) (ready to use) this staining were applied in the histological study for their comparative simplicity.

Immunohistochemical staining

The laminin antibody (ab210954) provide by ABCAM. This antibody is applied for detection the laminin as antigen which is present in the basal lamina of primordial, primary, secondary and Griffin follicles in the ovary. It is used with immunohistochemistry detection kit called mouse and rabbit specific HRP/DAB detection IHC kit from ABCAMwith kit number (ab236466).

The prepared histological tissue slide, that stained with Heamatoxylin and esion, and IHC for laminin á1, were examined for Histological evaluation and estimation using light microscope. Image J was used the software Image J (Java-based image processing program developed at National at Institutes Health, USA).

Statistical analysis of data

For all Statistical Analysis System used the Statistical package for social sciences (SPSS). Software program, Version 19. The data are expressed as mean and standard Error of the mean (SEM). For differences between the mean percentages of the laminin $\alpha 1$ protein expression of BFB in ovary were examined for statistical significance using ANOVA shows the difference in the mean positively ± standard Error between different groups according to dark /day cycle (Babbie, 2018).

RESULTS

General morphological and numerical changes of the ovaries according to dark/ light life cycle

Morphological appearance of ovary of experimental groups reveals decrease or even regress in size of ovary in dark group while in light group they reveal a marked enlarged in size of ovary. Moreover, the follicles and corpus luteum shows an increase in size so that occupy the whole stroma of ovary in light group in comparison to other groups. In light group they shows marked decrease in number of follicle in compare to other group and the number of corpus luteum shows increase in comparison

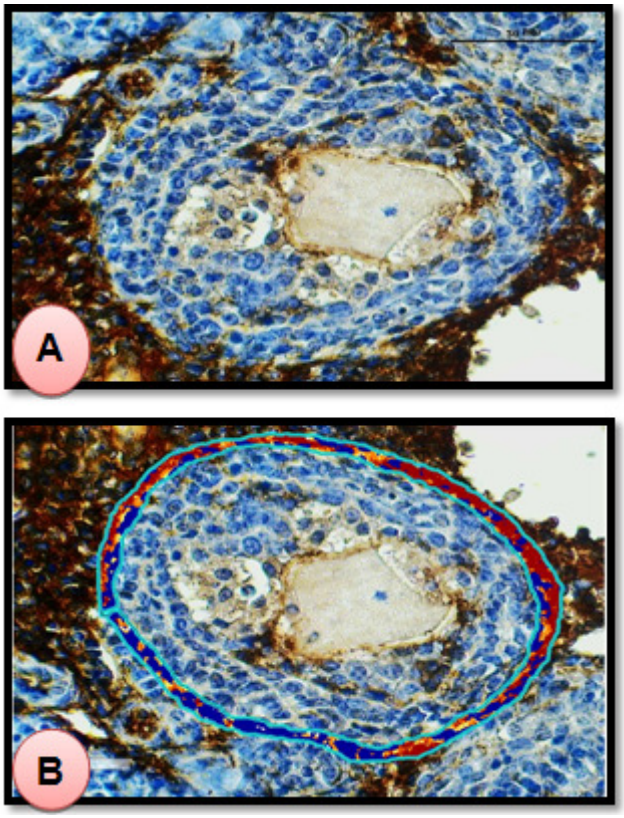

Fig. 1 : A-in cross section ovary in light group animals which show marked expression of laminin α1 at area of BFB between follicle and surrounding interstitial tissue (theca interna) 400X **B**-snap shoot for section (A) as analyzed by aprio image J software program, brown color = strong positive, orange = positive, yellow = weak positive, blue = negative.

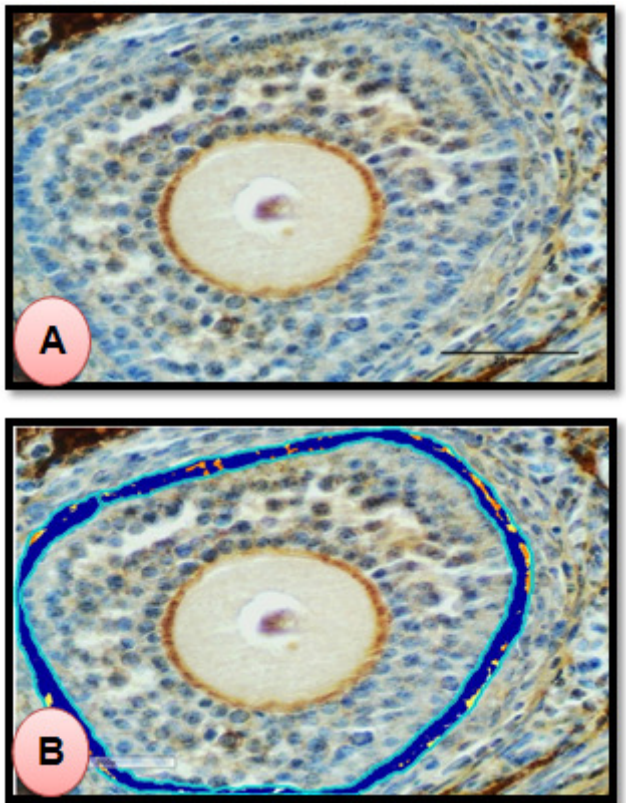

Fig. 2 : A-cross section in ovary in dark group animalswhich reveal very weak expression of laminin α1 at area of BFB between follicle and surrounding interstitial tissue (theca interna) 400X400X **B**-snap shoot for section (A) as analyzed by aprio image J soft ware program, brown color = strong positive, orange = positive,yellow = weak positive, blue = negative.

to other groups. Vise versa in dark group they reveals an absence of corpus luteum. The mean Size ±SE of ovary, Primary follicle, Secondary follicle & Corpus lutum in animals groups is recorded an increased as 119.72190±5.4936, 134.4400±5.4936, 131.4750±4.4007 & 140.2525±6.2916, respectively in light group in comparison to other group by applying image j program (Table 2).The mean number ±SE of Primary follicle, & Secondary follicle, in animals groups is revealed an decreased as 5.2000±0.32394 & 3.7000±0.32660, respectively in light group in comparison to other group by applying image j program while Corpus lutum showing increased its value (6.3000±0.39581) in comparison to other groups (Table 3). The reverse group shows the no significant differences with control group in size of ovary and number and size of follicle while the size of corps lutum decrease accordingly Comparison to other groups (Tables 2, 3).

Immunohistochemical reactivity of laminin

Lamininá1 is protein secreted by follicular cells of the ovary. It act as a receptor function of blood follicular barrier. So that it is also present at area between the follicles and interstitial area of theca interna cells. The expression of laminin á1in BFB was studied immunohistochemically by applying monoclonal primary antibody against to laminin. In the light group animals they reveal a marked expression of laminin α1 at BFB which evaluated as (0. 8814 ±0. 03461) in comparison to other group animals (control, reverse, & dark) which recorded 0.5689±0.05382, 0.3869 ± 0.12768 & 0.1978 ±0.02363, respectively (Table 1, Fig. 1 A & B). In dark group animals they reveal very low expression of laminin α1 at BFB, which estimate & evaluated as (0. 1978 ±0. 02363) (Table 1, Fig. 2 A&B). In reverse group animals they reveal a weak expression of laminin at BFB which estimate as (0. 3869 ±0. 12768), in comparison to control group (Table 1, Fig. 3 A & B). In control group animals they reveal expression of laminin α1 at BFB, which estimate as (0.5689±0.5689), which is more value as in comparison to dark & reverse group which is0. 1978 ±0.02363 & 0.3869 ±0.12768, respectively (Fig. 4 A&B].

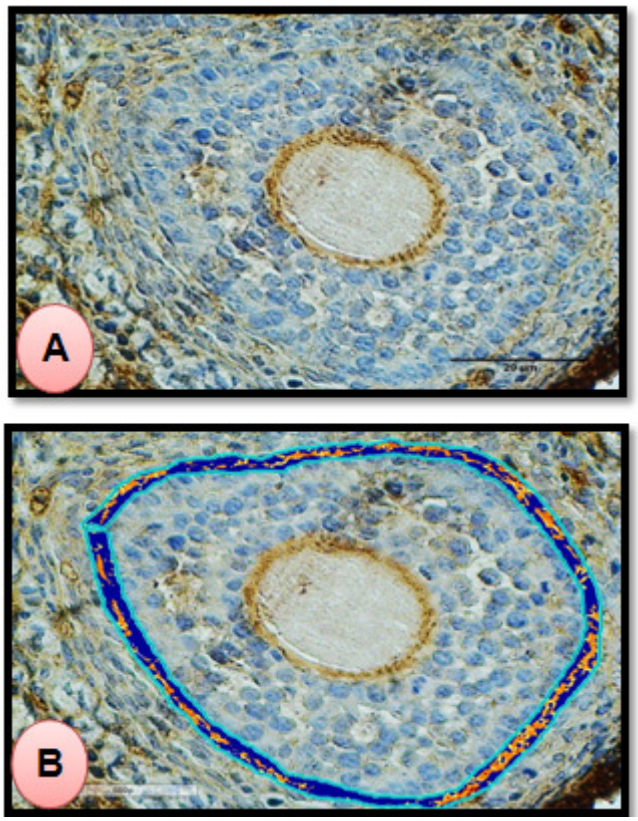

Fig. 3 : A -cross section in ovaryin reverse group animals which they reveal low expression of laminin α1 in comparison to control groupat area of BFB between follicle and surrounding interstitial tissue (theca interna) 400X. **B**-snap shoot for section (A) as analyzed by aprio image J software program, brown color = strong positive,orange = positive, yellow = weak positive, blue = negative.

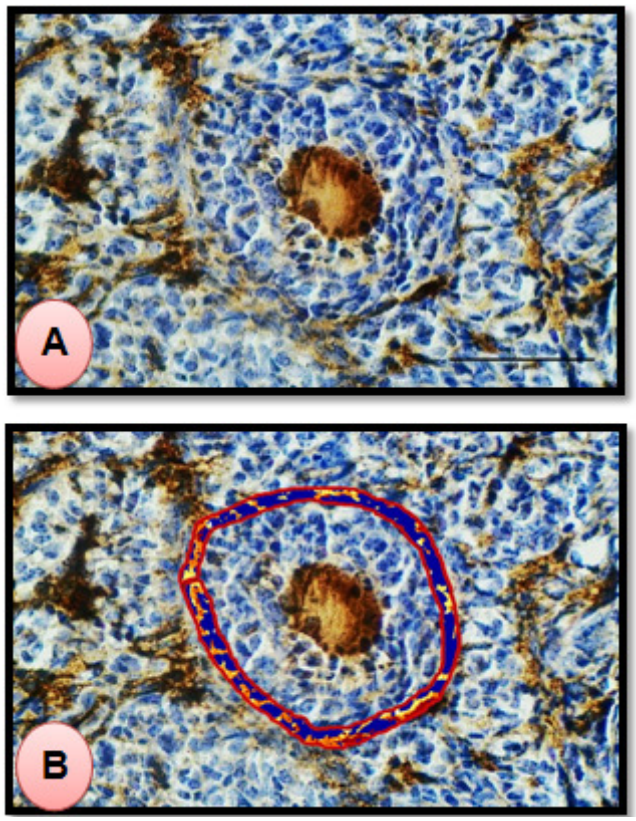

Fig. 4 : A -cross section in ovary in control group animals which reveal high expression of laminin α1 in comparison to dark & reverse group, at area of BFB between follicle and surrounding interstitial tissue (theca interna) 400X. **B**-snap shoot for section (A) as analyzed by aprio image J software program, brown color = strong positive, orange = positive,yellow = weak positive, blue = negative.

Table 2 : Reveals the Mean Size ±SE in of ovary, Primary follicle, Secondary follicle, & Corpus lutum in animals groups according to dark /light cycle by applying image j program.

Groups / Size	Control	Light	Dark	Inverse	Sig
Ovary	98.25±7.06	119.72±5.49	72.33±3.10	96.04±6.23	0.01
Primary follicle	112.28±5.42	134.44±3.44	100.64±5.65	107.98±5.45	0.01
Secondary follicle	108.26±8.26	131.47±4.40	104.72±4.08	107.94±6.85	0.02
Corpus lutum	117.63±4.12	140.25±6.29	nil	83.07±1.50	0.01

Table 3 : The Mean number ±SE of Primary follicle, Secondary follicle, & Corpus lutum in animals groups according to dark /light cycle.

Groups / No.	Control	Light	Dark	Inverse	Sig
Primary follicle	10.70±0.61	5.20±0.32	7.80±0.66	9.70±0.66	0.01
Secondary follicle	8.90±0.73	3.70±0.32	6.50±0.29	8.20±0.71	0.01
Corpus lutum	2.60±0.33	6.30±0.39	nil	2.10±0.34	0.01

DISCUSSION

Effect of illumination variance on reproductive activity of mice ovary

In the present study the routine histological assessment of Light group revealed ovarian hyperovalution due to inhibitory effects of light on melatonin secretion which lead to stimulation of gonadotrophic hormones secretion from pituitary gland in contrast to hypo-ovulation condition of dark group in which there are stimulation of melatonin secretion was found which later on lead to inhibition of gonadotrophic hormone from pituitary gland. This non ovulatory condition

is agreed with authors who found that ovulation in female mammals, which is itself stimulated by gonadotropin releasing hormone (GnRH) secretion from neurons in hypothalamus that is dueto luteinizing hormone (LH) secretion surge from the pituitary gland (Herbiso *et al*, 2008). Furthermore, other researchers found that ovulation could be stimulated by light they concluded that the increased rate of ovulation following light exposure is a consequence of faster follicle maturation. Follicle maturation is determined by the complex interrelated changes in the secretion of pituitary-ovarian hormones. They showed that LH and FSH secretions are involved in ovarian follicle growth and the ovulation, are all increased following a week of morning bright light exposure compared with dim light administered to the same women (Danilenko and Samoilova, 2007). This study agree also with other authors, who found that exposure to constant light will lead to reduce circulating melatonin concentrations and this has an increase effect on hypothalamic gonadotrophin-releasing hormone (GnRH), circulating levels of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) on ovary (Cleaver, 1991).

However, certain reporters found that Long exposure to artificial lighting leads to a reduction in endogenous melatonin exposure (Reiter *et al*, 2014). Moreover, certain researchers concluded that in very high doses of melatonin has the ability to suppress ovulation in humans, possibly by interfering with LH release (Fernando and Rombauts, 2014). Also author who performed experiments on rats, they found that high levels of melatonin have antigonadotropic effects resulting in a lack of ovulation (Starr, 2011).

Morphometric and numerical changes on ovarian tissue

The present study shows the increase size of ovary and its follicles were seen in light group as in hyperstimulated ovary in comparison to dark group, which shows a decrease in size of ovary and its follicles. This agree with authors who found that, ovary size was significantly increased by FSH/hCG (LH) stimulation (Park *et al*, 2015). Furthermore, certain authors who found that, follicle diameter were significantly increased after bright light compared to the dim light (Danilenko and Samoilova, 2007). Other, researchers recorded that ovary with superovulation is accompanied by an increases follicular size (Blondin *et al*, 1996). In present study show increase size andnumber of corps lutum in light group in contrast of dark group show absentof corpus lutum this agree with authors found that Histological analysis of ovaries following superovulation revealed large corpus lutea andoccupying a large areas in the ovary (Pasco *et al*, 2012). Consequently, this will agree with certain researchers who found that the numbers of corpus lutea of ovary were increased by PMSG/hCG treatment (Park *et al*, 2015). Also, they observed the effects of super ovulation treatments on the increment of the number of ovulated corpus luteum is more obvious (Aad and Rosadi, 2011).

The present study shows a decrease the number of follicle in light group in contrast to dark group, which reveal an increase in number of follicle and this agree with authors who found that Dark-exposed animals had more follicles. In contrast to Light group animals which reveals a reduced the number of follicles (Sanjay *et al*, 1997). These results conclude that indirect stimulant effect of light will exhaust ovary, leading to a decrease in number of follicles. Secondarily this space in the ovary, which is occupied by already existence CL will eventually end into a decrease in numbers of follicles.In present study, which reveal in reverse group have normal value number of follicles and corps luteum and size of ovary as in control group this, indicate that it is corresponding to the normal habit of mice life. It means that animals were active at night time and inactive sleepy at day time. This agree with authors who found that Laboratory mice are nocturnal active life, spending most of their daylight hours asleep, while in humans lack of sleep or sleep that occurs outside the normal circadian sleep period (as in shift work) has an adverse effects. These will lead to increased risk of metabolic disorder, cardiovascular disease, cancer, mood disorders, type II diabetes, and obesity. However, mice have sleep disruption due to their human activities, and, if so, then adverse effects may be take place affecting their diurnal (light/ dark cycle) variation of mice. This will ensure good welfare for laboratory mice and improve experimental validity (Robinson-Junker., 2016).

Presence and role of laminins as structral component in the basal lamina of follicles in ovary

The present study shows that Laminin, a proteins that is Major Ingredients of the basal lamina (layer of basement membrane), of follicles in the ovary.This agree with other reporters who concluded that Laminin protein is a family of large multi domain glycoproteins, which are major component of basement membrane and extracellular matrix (ECM). The laminins are an important and biologically active part of basal lamina, influencing cell adhesion, migration & differentiation (Aumailley *et al*, 2015). Other researchers focus on the follicular basal lamina underlying the membrane granulose, they revealed changes that it undergoes during follicular development and the ultrastructural phenotypes are related to granulosa cell shape (Irving-Rodgers and

Rodgers, 2000). Also certain researchers found that the follicular basement membrane is a specific structural protein (including laminin protein) that has to be dramatically changed during the follicular development and ovulation (Zhou *et al*, 2007). Other authors found that the outer layer of granulosa cells of ovarian follicle is closely associated with the surrounding basal lamina and has effect of follicles growth and development (Hirshfield, 1991).

Moreover, others scientists found that Laminin protein was a localized ring at the exterior of the follicular granulosa cells which marking the basement membrane, It is important in the differentiation of epithelial cells in the ovary (Woodruff and Shea, 2009). The present work it was found that laminin protein is also seen in ECM and interstitial space of ovarian stroma in cortex and medulla of ovary and that will agree with researchers who found that the laminin protein is associated with the basal lamina surrounding egg clusters and their connections were to the ovarian surface epithelium and even ovarian stroma during ovarian development. It was reported that laminin is associated with a continuous matrix which surrounds, forming primordial follicles as they are isolated from the egg clusters, also they localized Laminin in the basal lamina of primordial, primary, secondary and tertiary follicles of ovaries (Lee *et al*, 1996). This result will evaluate the presence of laminin protein in the basal lamina of BFB has a significant role in growth and differentiation of ovarian follicles. In this present study, the 5e application of Laminin α1 isotype antibody illustratethis protein in the basal lamina of all stages, which has role in development of follicles that is agreed with researchers who found that Laminin á1 is present in the mouse follicular basal lamina at all follicles, while laminin α2 is notapplied in the present study which has only been detected insome follicles and only at the preantral and antral stages (Irving-Rodgers *et al*, 2010). So that laminin α2 antibody is not apply in this study to detect various follicular stages.

Laminins protein role in steroidogenesis of ovarian follicles

The present study reveals that granulosa cells is triggered and stimulated by its communication with surrounding ECM laminin in steroidogenesis this agree with other researchers, who found that granulosa cells showed increased progesterone production when plated on laminin, fibronectin and type I collagen (Sites *et al*, 1996). And this agree with researchers who found that the extracellular matrix surrounding the follicle has been suggested to induce the expression of steroidogenic enzyme of follicular cells (Irving-Rodgers *et al*, 2010). Present study reveals that lamininá1 protein expression shows as correlation with function of follicular cell in differentiation of folliclesas increment of corpus luteum (secretory endocrine part of ovary) in light group animals in comparison to dark group and this agree with authors which demonstrated that proteins as laminin of the basal lamina can alter the steroidogenic capacity and cytoskeletal composition of mature granulosa (Lee *et al*, 1996). While other researchers found that primed preovulatory follicles, which plated on laminin become enlarged and are referred to as steroidogenic cells (Aten *et al*, 1995). This study concluded that laminin also has pivotal role in follicular cells differentiation from granulosa to luteal cells after ovulation.

Role of the laminins protein receptor potein of ovarian follicles

The present study reveals that laminin protein expression shows as receptor protein which reveals high value expression in light group animals due to indirect stimulant effect of light on cell proliferation and differentiation in folliculogensis this agree with researchers conclude that, Laminin effects are mediated by its receptors activation of a specific signal transduction pathway in the cell. Laminin signals is achieved via multiple signal transduction pathways involving various components such as focal adhesion kinase, and cytoskeleton components of follicular cell (Givant-Horwitz *et al*, 2005). Certain authors found that the ECM may modulate the physiological functions of hormones by providing binding sites like laminin protein so that they regulate downstream signaling pathways (Kim and Lee, 2014). This agree with certain researchers who found that in the rat ovary. The GnRH receptors are produced by granulosa cell itself, which has a triggering effect of the GnRH on granulosa cell proliferation as in folliculogenesis (Tamar *et al*, 2005).

Effect of light/dark cycle on laminin expression in follicules

In the present study found increase laminin expression in light groupdue to increase releaseof GNRH in contrast to that of dark group which found a decreasein laminin expression, due to low releaseof GNRH this agree with researcher, who concluded that GnRH stimulate and trigger laminin receptor that is involved in cellular adhesion and migration. Moreover, GnRH triggers laminin receptor expression that bind to laminin. Also they have clearly demonstrated that increased laminin receptor expression with GnRH will induced laminin receptor IHC expression this later onis involved in cellular adhesion and migration (Tanriverdi, 2005 and Chen *et al*, 2002). Consequently

the amount of LR (laminin recpter) on the cell surface is increased by GnRH stimulation (Poon *et al*, 2011) that high levels of GnRH expression may enhance the cellular response to GnRH stimulation, due to signal amplification or altered signaling through coupling to different G-proteins (Cheung and Wong, 2008) lamininis Scaffolding proteins can play an integral part in mediating the communication between external cues and GnRH-proliferation cells (Larco *et al*, 2018).

This agree with certain researchers found that an inhibitory effect of melatonin on the nocturnal expression of GnRH and GnRH receptors subtypes was also demonstrated. In addition, the inhibitory effect of melatonin affected the expression of hypophysiotrophic GnRH forms and GnRH receptors that exhibit day–night fluctuations, suggesting that exogenous melatonin reinforce physiological mechanisms already established. These interactions between melatoninergic and GnRH systems could be mediating photoperiod effects on reproductive physiological events (Servili *et al*, 2013).In reverse group animal they reveal high laminin protein expression than dark and less than control group this due to stimulant effect of day light on laminin expression in control group, which shows increase in size of corpus luteum in comparison to reverse group and this indicate that the normal habit of daily life is the reverse group of the mice and this agree with reporters, who conclude that the fact that mice sleep during our day (morning and afternoon), which is their night. While theyperform better during the night under a light (their day time activities) (Zhang, 2019).

CONCLUSION

The exposure to constant light has a stimulant effect on morphometric, histological and functional activity of ovarian stroma. The role of constant light on increment ofmarked expression laminin α 1 protein in BFB. The role of darkness as an inhibitory effect on morphometric, histological and functional activity this will reflect with minimum expression IHC of laminin α 1 protein in ovarian follicles. The reverse group, recorded normal value in number, size of follicles of ovary as in control group.In reverse group animals, they reveal a weak expression of laminin α 1 at BFB in comparison to control group. The presence of laminin α1 protein in the basal lamina of BFB has a significant role in growth and differentiation of ovarian follicles.

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