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Bioactivity of *human* Menopausal Gonadotrophin (*h*MG) and Deglycosylated hMG (hMGdG) from Urine of Post-Menopausal Women On *in vitro* Bovine Embryonic cleavage

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Abstract

The aim of this study was to produce hMG from the uterus of post-menopausal women and to evaluate the influence of glycan removal from hMG glycoprotein molecule underwent deglycosylation (hMGdG) on the onset of in vitro bovine embryonic cleavage. The study identified hMG from the uterus of post-menopausal women by confirmation of the glycoprotein characteristic, examined the biochemical characteristics of deglycosylated hMG using N-glycanase and determined the influence of deglycosylated hMG on the onset of in vitro bovine embryonic cleavage. Urine samples were collected from 30 post-menopausal women. The results of SDS-PAGE demonstrated that the protein bands ranged between 19.4 and 107 kDa. Western blot revealed immune-reactivity of the 30 kDa band, which was a glycoprotein. The concentration of glycoprotein was 99860.00 ug/ml (PAS), the protein was 66939.29 µg/ml (Biuret) and carbohydrate 32920.71 µg/mL (PAS). The glycoprotein, protein and carbohydrate ratio within the *h*MG molecule was 3:2:1. Chemical characteristic of hMG following enzymatic deglycosylation using N-glycanase reduced the molecular weight to 26 kDa. The deglycocylated hMG reduced the onset of in vitro cleavage of bovine embryo from 24 to 20 hours ($p < 0.01$).

Keywords: hMG, hMGdG, embryo cleavage, glycoprotein, deglycocylation, *in vitro*.

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1. Introduction

The use of human menopausal gonadotrophin (hMG) for super-ovulation in cattle was first introduced by Alcivar [1]. Subsequently, study on the use of hMG for estrous induction and pregnancy in goats was first conducted in Airlangga University and produced satisfactory results [2]. Up to the end of 1995, the world requirement of gonadotrophin

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hormone from post-menopausal women was supplied by China, Europe and Latin America. In Indonesia, however, the uterus of post-menopausal women has not been employed as the source of hMG [3].

hMG consists of a combination of FSH and LH hormones, which are required for *in vitro* embryonic production process. FSH receptors are found in ovarian granulose cells and have a direct role in follicular development during oocyte maturation [4]. LH receptors are more numerous in ovarian theca cells that have a direct role in steroidogenetic process [5]. Therefore, FSH-LH administration in balanced composition (dose μg/ml) for *in vitro* ruminant fertilization provides highly satisfactory outcome [6–8]

Human Menopouse Gonadotropin (hMG), produced from the uterus of post-menopausal women, provides a better and relevant therapy effect on recipient woman for infertility. After hMG therapy, the human *in vivo* maturation process and *in vitro* fertilization (IVF) occur immediately [9–11], triggering oocyte mitosis up to metaphase [12–14] with satisfactory embryonic development, a result almost similar to that of using recombinant human FSH (rhFSH). Therefore, hMG is effective either for infertility therapy or for pre-IVF therapy to stimulate follicular maturation process, ovulation, ovarian response, and growth of the embryo. Additionally, it is less expensive [15–17] and easily available [1]. This study describes the bioactivity of hMG on *in vitor* bovine embryonic cleavage.

2. Materials and Methods

2.1. Post-menopausal Women's Urine Sample Processing

Urine from post-menopausal women, collected in the morning at 100 ml per individual, was centrifuged 1000 xg for 15 min at 4°C [18]. The precipitate was removed before the supernatant was centrifuged again at 1000 xg at 4°C for 20 min [19]. The resulting supernatant was filtered until it became clear in the Erlenmeyer flask. The procedure was repeated on other urine samples until 50 ml of clear supernatant was obtained [20].

Similarly, urine samples were used to isolate glycoprotein. Approximately 200 μl of urine, which was homogenized by the addition of PBST–PMSF 5 times in the urine sample, sonicated for 10 min, vortexed and centrifuged 3000 xg for 15 min before cold absolute ethanol 1:1 was added and incubated within a refrigerator for 1 hour or overnight. Then it was centrifuged 5,000 xg for 15 min. The precipitate was dried until the smell of ethanol disappeared and 20 mM buffer tris Cl was added. The outcome was protein isolate, which was subsequently used for SDS-PAGE examination [21].

2.2. hMG Protein Identification by Western Blotting

The hMG band fragment, obtained following SDS-PAGE was transferred onto nitrocellulose membrane (Molecular Station, 2006). The membrane was blocked with 3% BSA in 3% BSA in 20 mM Tris-HCl pH 7.5 and 150 mM NaCl for 1 hour, and then incubated in Tris/NaCl containing 1% BSA with anti-hMG as primary antibody. It was washed with Tris-Cl containing 0.05% Tween 20. Subsequently, the membrane was incubated with secondary antibody (AP label anti-rabbit IgG, dilution 1:1000) and western blue substrate was added. The produced band was hMG band, indicating the molecular weight of hMG isolate.

2.3. hMG Protein Isolation with Electro-elution

Unstained SDS-PAGE gel was cut along the band as needed. Each gel cut was put within cellophane bag and soaked with 0.05 M phosphate buffer (PB) as much as 1-2 ml. The gel was put within electro-elution chamber containing 0.01 M phosphate buffer. Thereafter, electro-elution in cool chamber at 4°C (within a refrigerator) was performed. Power supply was turned on at 220 V, 20 mA overnight. Eluted protein could be determined by cutting acrylamide gel and stained with commasie blue stain for 20 min. De-staining was then carried out. If the band was absent, the protein should have been eluted. Subsequently, fluid containing protein within the cellophane bag was taken out, precipitated, and purified to obtain the required protein.

2.4. hMG Deglycosilation

As much as 500 µg hMF isolate was added into 45 µl incubation buffer before 2.5 µl SDS was added to the final concentration of 0.1%. Denaturation process was performed on glycoprotein through heating at 100°C for 5 min and left to cool. Then 2.5 µl of NP-40 was added to a final concentration of 0.75% and 2 µl of N-Glycanase was added before incubated overnight at 37°C. Thereafter, it was kept at -20°C. The hMGdG protein molecular weight was determined using SDS-PAGE [21]. The hMGdG was used in the *in vitro* process of maturation and fertilization.

2.5. Oocyte Collection

To observe the oocytes, the follicular fluid resulting from absorption was poured into petri dish and examined under inverted microscope. Oocytes were harvested using a

modified pasteur pipette with diameter between 300 and 500 μm and put into smaller-sized petri dish containing washing media. Then the oocytes were examined under the microscope to determine the quality [22].

2.6. Effects of hMG and hMGdG on the Rate of *in vitro* Bovine Embryonic Cleavage Formation

After identifying the quality, all oocytes were first washed in washing media for 3-4 times. The final washing was conducted using 2.5-3.0 ml Tissue Culture Medium 199 (TCM 199). Oocytes that were found to have compact cumulus were transferred into drops of 100 μl maturation medium within petri dish, each comprised 20 oocytes. Total oocytes used in this study were 100 oocytes, prepared 2 hours earlier in a 5% CO2 incubator. Each medium consisted of P1 = hMG dose I at 1 μg/ml *in vitro*; P2 = hMG dose II at 4 μg/ml *in vitro*; P3 = hMGdG dose I at 1 μg/ml *in vitro*; P4 = hMGdG dose II at 4 μg/ml *in vitro*, and K = control group receiving 15 IU PMSG *in vitro*.

Maturation medium was prepared in sterile petri dish of 35 mm in the form of drops as many as 4 drops, each of 100 μl. In each medium drop, as many as 10 oocytes were cultured before being covered with mineral oil. Culture with three media was conducted within incubator containing 5% CO_2 in humidity of 95-100% and temperature of 39°C. The media were incubated for 24 hours [22] before *in vitro* insemination (IVI) was performed. Two hours prior to the insemination, 2 mini straws containing 10-15 cells/viable sperm cells were thawed for 1 min in warm water of 37°C. At the same time, 3 ml of Earle's Balance Salt Solution (EBSS) pH 7.4 was prepared in plastic cone vial in a volume of 11 ml. Whole content of the straw was poured onto the surface of EBSS media and centrifuged at 1800 xg for 10 min. The pellet was collected. This procedure was repeated twice up to the harvested pellet. After EBSS was added, viable spermatozoa were able to swim up to the surface of EBSS media in 30 min. The weak or dead spermatozoa would remain at the base of the vial or maximally at the medial part of the media. To obtain motile spermatozoa without debris contaminant, spermatozoa were taken from the surface of the media.

In another petri dish "Rosette-Like Pattern" was prepared. This was made by placing 100 μl of EBSS media at the central part, surrounded by ± 15 μl EBSS media in 6 drops. Each drop was connected from the central to peripheral, creating a rosette. At the end of the rosette 10 oocytes, which had been incubated for 24 hours in maturation process, were inserted. Then, 30 μl solution containing a total of 1.25-1.5x10^6 motile spermatozoa was deposited at the center of the media, allowing the motile spermatozoa to search for oocytes at the end of the rosette. Fertilization was for 20-24 hours

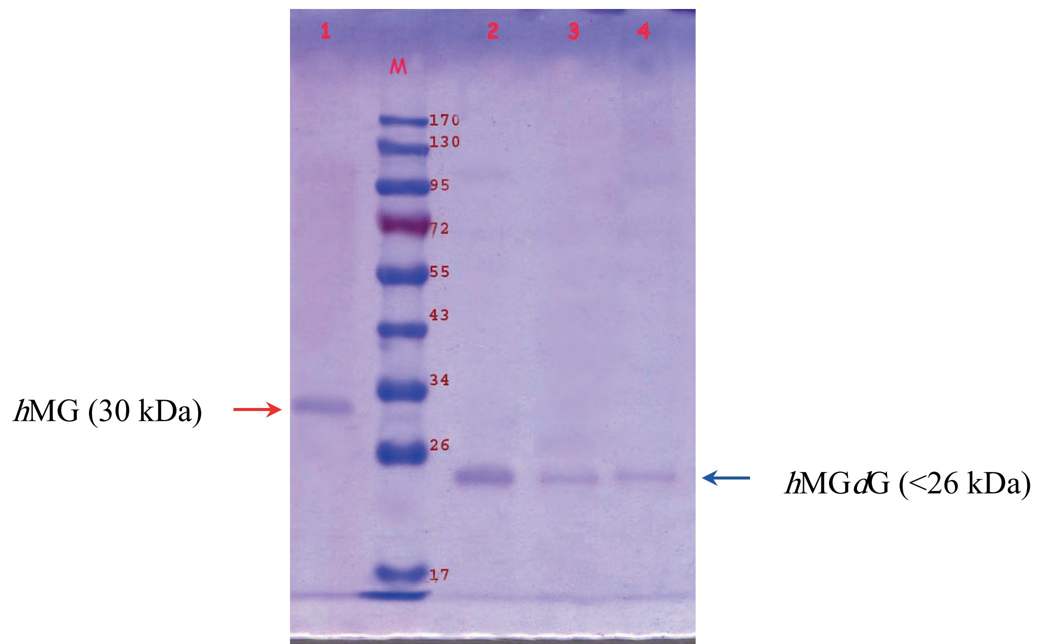

Figure 1: Results of hMG protein analysis and hMGdG deglycosilation. M = marker protein, 1 = electro-elution result on hMG, 2-4 = results of hMG deglycosilation.

within 5% CO_2 incubator at 38.5°C and the fertilized oocytes were washed with TCM 199, transferred back into maturation drop before their development was followed for 24 hours. After 24-hour fertilization, cleavage observation was observed by observing blastomer count in the embryo [22].

2.7. Data Analysis

Data regarding the percentage of fertilized oocyts and formation of *in vitro* embryonic cleavage for up to 16 and 32 cells were analyzed by factorial ANOVA and Duncan's multiple range test 5% [23].

3. Results

3.1. Characterization of hMG Glycoprotein from Uterus of Post-menopausal Women

Western blot technique on glycoprotein revealed 30 kDa band of hMG and <26kDa band of hMgdG (Fig. 1).

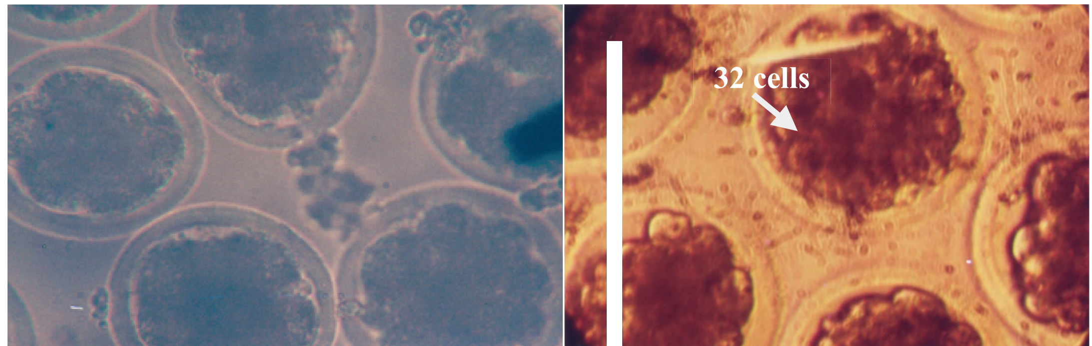

Figure 2: Development of embryos following in vitro *fertilization to 8, 16 and 32 embryonic cells. 200x.*

3.2. Rate of in vitro Bovine Embryonic Cleavage Formation

Fig. 2 shows the *in vitro* fertilization from cleavage to 8, 16 and 32 embryonic cells. The mean *in vitro* fertilized ova was significantly ($p<0.01$) different. The mean and SD of *in vitro* fertilized ova was 45.278 ± 22.716, 51.136 ± 23.784, 65.056 ± 11.754, 73.390 ± 14.689, and 44.162 ± 5.703 for hMG 1 μg/ml, hMG 4 μg/ml, hMGdG 1 μg/ml, hMGdG 4 μg/ml and 15 IU PMSG, respectively. The highest number of *in vitro* fertilized ova was obtained with 4 μg/ml hMGdG but insignificantly ($p>0.05$) different with 1 μg/ml hMGdG. The lowest number was found with 1 μg/ml hMG and 15 IU PMSG trials, which was insignificantly ($p>0.05$) different from 4 μg/ml hMG.

3.3. In Vitro Fertilized Ova Development

The mean and SD of *in vitro* fertilized ova after 20 and 24 hours were 46.534 ± 20.2956 and 65.075b ± 15.3569, respectively. This difference, however, was not significant ($p<0.01$). The mean and SD of *in vitro* fertilized ova following incubation with different dose and period were 24.333 ± 3.388, 66.222 ± 7.269, 32.273 ± 4.272, 70.0 ± 19.103, 61.667 ± 14.218, 68.444 ± 8.944, 70.234 ± 18.365, 76.545 ± 11.104, 44.162 ± 6.049 and 44.162 ± 6.049 for 1 μg/ml hMG for 20 hours, 1 μg/ml hMG for 24 hours, 4 μg/ml hMG for 20 hours, 4 μg/ml hMG for 24 hours, 1 μg/ml hMGdG for 20 hours, 1 μg/ml hMGdG for 24 hours, 4 μg/ml hMGdG for 20 hours, 4 μg/ml hMGdG for 24 hours, 15 IU PMSG for 20 hours and 15 IU PMSG for 24 hours, respectively. The interactions were significant ($p<0.01$).

Highest results were obtained with 4 μg/ml hMG for 24 hours, 4 μg/ml hMGdG for 20 and 24 hours. Similar results were found with 1 μg/ml hMG for 24 hours, 1 μg/ml hMGdG for 20 and 24 hours. The lowest was with 1 μg/ml hMG for 20 hours and 4

Table 1: The effect of dosage on *in vitro* fertilization development to 16 and 32-cell embryos.

Dosage	16 cells	32 cells
	Mean ± SD	Mean ± SD
*h*MG 1 μg/ml	14.000^{b}± 13.6606	1.250^{ab}± 3.9528
*h*MG 4 μg/ml	16.854 ^{b}± 18.2587	1.111^{ab}± 3.5136
*h*MG*d*G 1 μg/ml	10.000^{ab}± 13.3024	2.222^{ab}± 4.6849
*h*MG*d*G 4 μg/ml	20.299 ^{b}± 18.5560	7.727^{b}± 10.7842
PMSG 15 IU	0.000^{a}± 0.0000	.000^{a}±.0000

Different superscript in the same column indicates significant difference (p < 0.05).

μg/ml hMG for 20 hours, which were not significantly (p>0.05) different from 15 IU PMSG for 20 and 24 hours.

3.4. The Effect of Dosage on *In Vitro* Fertilized Ova Development

Table 1 shows the mean and SD of *in vitro* fertilized oocytes and cleavage to 16 and 32-cell embryo following treatment with hMG and hMGdG at various dosages. The highest number of *in vitro* fertilized oocytes and cleavage to 16-cell embryo was with 4 μg/ml hMGdG. Similar results (p>0.05) were observed with 1 μg/ml hMG and 4 μg/ml hMG. Significantly (p<0.05) lowest result was observed with 15 IU PMSG, which was similar (p>0.05) with 1 μg/ml hMGdG. When cleavaged to 32-cell embryo, the highest number was observed with 4 μg/ml hMGdG. Significantly (p<0.05) low number was observed with 15 IU PMSG, 1 μg/ml hMG, 4 μg/ml hMG and hMGdG 1 μg/ml (Table 1).

3.5. The Effect of Time of Incubation of Fertilized Ova Development

The number of *in vitro* fertilized oocytes and cleavage to 16-cell embryo was significantly (p<0.01) higher than following incubation at 24 hours than 20 hours, while cleavage to 32-cell embryo did not show significant difference (p > 0.05) (Table 2).

3.6. The Effect of Dosage and Time of Incubation on Fertilized Ova Development

Effect of interaction between dosage and time on the number of *in vitro* fertilized oocytes and cleavage to 16-cell embryo showed no significant difference (p > 0.05). The interaction on *in vitro* fertilized oocytes and cleavage to 32-cell embryo was also

Table 2: The effect of time of incubation on *in vitro* fertilization development to 16 and 32-cell embryos.

Time	16 cells	32 cells
	Mean ± SD	Mean ± SD
20 hours	$5.837^{a} \pm 10.2571$	1.344 ± 3.7334
24 hours	$18.624^{b} \pm 17.3734$	3.580 ± 7.8271

Different superscript in the same column indicates significant difference ($p < 0.05$).

Table 3: The effect of dosage and time on *in vitro* fertilization development to 16 and 32-cell embryos.

Trial	Time	16 cells	32 cells
		Mean ± SD	Mean ± SD
*h*MG 1 µg/ml	20 hours	$2.222^{a} \pm 4.969$	$2.500^{ab} \pm 5.590$
	24 hours	$25.778^{a} \pm 0.957$	$0.000^{a} \pm 0.000$
*h*MG 4 µg/ml	20 hours	$7.374^{a} \pm 7.280$	$2.222^{ab} \pm 4.969$
	24 hours	$26.333^{c} \pm 21.736$	$0.000^{a} \pm 0.000$
*h*MG*d*G 1 µg/ml	20 hours	$4.444^{a} \pm 9.938$	$0.000^{a} \pm 0.000$
	24 hours	$15.556^{a} \pm 14.907$	$4.444^{ab} \pm 6.086$
*h*MG*d*G 4 µg/ml	20 hours	$15.143^{a} \pm 16.800$	$2.000^{ab} \pm 4.472$
	24 hours	$25.455^{a} \pm 20.641$	$13.455^{b} \pm 12.636$
PMSG 15 IU	20 hours	$0.000^{a} \pm 0.000$	$0.000^{a} \pm 0.000$
	24 hours	$0.000^{a} \pm 0.000$	$0.000^{a} \pm 0.000$

Different superscript in the same column indicates significant difference ($p < 0.05$).

not significant ($p > 0.05$). However, the highest number of oocyts reaching embryonic development of 16 cells involved combination of 4 µg/ml hMG for 24 hours while the 32 cells involved combination of 4 µg/ml hMGdG for 24 hours incubation. Other combinations showed significantly ($p<0.05$) lower number (Table 3).

4. Discussion

The success of cleavage formation in *in vitro* fertilization depends on *in vitro* oocyte maturation efforts. One of the factors that play a role in this process is the addition of various substances in the *in vitro* media. Culture media used for oocyte maturation and fertilization is tissue culture medium (TCM 199) with addition of gonadotrophin

hormones to increase the percentage of oocyte maturation and embryonic development after the *in vitro* fertilization [24]. In this study, the added hormone was the provision of gonadotrophin hormone and deglycosilated hMG.

Fertilization involves oocyte activation process by spermatozoa, so that oocytes are able to accomplish the formation of female pronucleus and the union (syngamy) between female and male pronuclei, leading to the formation of zygote which will undergo mitotic cleavage (Yuliani, 2000). The effect of *in vitro* utilization of hMG in animals has never been published. However, in human or infertile women, hMG has been used for its therapeutic effect on IVF process (Daya *et al.*, 1995). The number of collected oocytes and the embryonic development in infertile women were found to be better in those treated with hMG [17] when 85% of the oocytes reached metaphase [12–14]. Administration of 1 μg/ml hMGdG for 20-hour maturation followed by culture for 48 hours increased the cleavage rate. This was due to the breakdown of carbohydrate chain bound to polypeptide chain in hMG glycoprotein molecule by glycanase enzyme. The carbohydrates in hMG consist of monosaccharides of mannose, glucose, fucose, sialic acid in the form of N-Acetyl-d-glucosamine and alpha-d-Mannose [25]. The *in vitro* breakdown of mannose and sialic acid increases FSH-LH activity in follicular maturation process, enhancing the formation of germinal vesicle breakdown (GVB) and increasing the granulosa and theca cells [26, 27].

Hormone administration into *in vitro* media in varied concentrations was found to have varied results. [7] added 10 μg/ml of LH and 10 μg/ml of FSH and produced 18.7% morulla and 17.4% blastula. Martino *et al*. (1994) performed IVM and fertilization on goat's oocyte by administering 10 μg/ml of FSH and 10 μg/ml of LH. To evaluate nucleus level of development, the oocytes were separated from cumulus cells into PBS that had been added with 150 IU/ml of hyaluronidase enzyme. The results revealed that 75.9% oocytes were in metaphase II of maturation while the total fertilized oocytes were 64.7%. A satisfactory result has been reported by [6] that oocyte maturation in TCM medium given FSH, LH and estrogen was 98%, with fertilization rate reaching 73%. [22] substituted the combined hormones of FSH, LH and estrogen with the combination of estrous cattle and horse serum, and found cleavage rate of 69% in bovine oocyte. [13] used hMG for *in vitro* maturation and *in vitro* fertilization, and found a success rate of 80%. In another study, success was also obtained after hMG administration, which was compared to FSH separately in IVM media, and followed with IVF [28].

It has been known that hMG and hMGdG have FSH-like activities. FSH has clear receptors in granulosa cells and has a direct role in the development during oocyte maturation [4]. LH has receptors in theca cells and has a direct role in steroidogenetic process in both receptors [5] Deglycosilating process of hMG molecule to become hMGdG involves the removal or breakdown of carbohydrate chain bound to polypeptide carbon atom, leading to enhancement of the FSH-LH action. Therefore, the *in vitro* growth of theca cells is enhanced, leading to the formation of germinal vesicle breakdown (GVB) [26, 27], allowing enhanced time for *in vitro* cleavage formation. The normal time of cleavage formation in *in vitro* fertilization is 24 hours [22]. Enzymatic breakdown utilizes the enzymes glycanase or endo-/exo-glycohydrolase [21,29,30, 31] reported that the removing process of connecting chain between mannose fucose saccharide and sialic acid in olygosaccharide band of FSH-LH-like gonadotrophin hormone (deglycosilation), like that in hMGdG, was able to enhance the activity of animal folliculogenesis and shorten the survival in blood plasma. Similar procedure was performed *in vivo* in human using deglycosilated FSH [32]. *In vitro*, FSH-LH-like substances such as hMGdG that has no correlation between manose glucose fucsoe and sialic acid, are able to enhance the induction of cAMP accumulation, which is in abundant and productive within cumulus culture-oocyte-complex (COC) of the follicle [30]. Subsequently, pronucleus development is regulated by cytoplasmic factor within the oocytes, called male pronucleus growth factor (MPGF). Nucleus maturation and cytoplasma have influence on the formation of male and female pronuclei to develop up to blastocyst stage [31] cited by [30], And hMGdG acts at cellular level when ATP is altered to cAMP under the effect of adenil cyclase enzyme, which acts directly at granulose cells in FSH receptor. FSH receptor increases activin, making granulosa cells actively produce estrogen hormone, and then FSH receptor activity reduces, and inhibin produced by granulosa cells inhibits FSH action, triggering androgen synthesis, which has effect on the formation of LH receptor in theca cells. This mechanism is designated as feed back loop, as that occurs in FSH-LH like process [31, 34].

[29] wrote that deglycosilated hMG endured the formation change and reduced molecular weight to less than 26 kDa. However, these did not alter the structure and protein weight, only the change of the weight of carbohydrate molecule, particularly sialic acid. If the latter's activity is reduced, the pH tends to alter to become alkaline, so that its activity become faster, or if it is given *in vivo* and *in vitro*, its half-life becomes faster [31]. [30] wrote that isoform FSH, such as in hMGdG that loses its sialic acid activity, may rapidly enhance follicular preantral growth to become follicular predominance. Additionally, estrogen accumulation by granulosa cells also occurs quickly.

Deglycosilated glycoprotein, particularly the FSH-LH like, indicates that the hormone is taking the other shape of its variation, which is also called as isoform, indicating a short-acting characteristic.

5. Conclusion

The breakdown of glylcan binding in FSH-LH-like molecule (hMG) from the urine of post-menopausal women produces hMGdG that is able to enhance the time of *in vitro* bovine embryonic cleavage formation. From this general conclusion, some sub-conclusions can also be drawn as follows: hMG obtained from urine of Indonesian post-menopausal women contains glycoprotein with molecular weight of 30 kDa; hMG biochemical character resulting from enzymatic deglycosilation using N-glycanase enzyme to become hMGdG leads to the reduction of ;molecular weight between 26-17 kDa; and hMGdG induction is able to enhance *in vitro* bovine embryonic cleavage formation from 24 hours to 20 hours.

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