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Immunocontraceptive Potential of Goat *Zona pellucida* as Monkey Population Control

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Review Article

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Abstract

A series of studies have explored the immunocontraceptive potential of zona pellucida-3 in local Indonesian goats. Goat *zona pellucida*-3 (gZP3) proteins have been identified and isolated. It has 82 kDa molecular weight and comprises 63.47% of the total goat zona pellucida protein. Immunofluorescence staining showed that gZP3 protein recognized goat sperm plasma membrane, whereas gZP3 antibodies recognized goat *zona pellucida* in native smear. In vitro fertilization study showed that gZP3 protein-supplemented in sperm capacitation medium and gZP3 antibody supplemented in oocyte maturation medium inhibited fertilization. It could be concluded that gZP3 was a fertilization receptor in a goat. Elisa and dot blot analysis showed that gZP3 protein was recognized by the serum of fertile unpregnant female mice. Therefore, it could be hypothesized that the gZP3 protein might be immunocontraceptive in mice. Immunization of mice with gZP3 proved that gZP3 prevented pregnancy and was dose-dependent. The contraceptive action of gZP3 was reversible after 14 estrous cycles. In vitro fertilization technique, it was proved that the antibody of gZP3 was able to prevent fertilization and decrease sperm binding rate on mice ova. The gZP3 nucleotide sequence has a homology at nearly 50% of the monkey ZP3 sequence (Macaca sp) but is foreign phylogenetically. The gZP3 protein has the potential to be developed as an immunocontraceptive substance for overpopulated monkeys to prevent agriculture disturbance in areas near forestry.

Keywords: Agriculture Disturbance; Areas Near Forestry; In Vitro Fertilization; Binding Assay; Nucleotide Sequence

Introduction

Ecosystem imbalance often conflicts with wildlife, one of them with monkeys. The overpopulation of monkeys in their habitat causes high economic losses in agriculture and a severe disturbance to villagers in areas near forestry. In recent years there have been several accidents in some areas near forestry, i.e., in 2016 in the USA (the state of Florida) and Thailand, in 2017 in India and Indonesia (Baluran National Park, and Tidar mountain at Boyolali

resident). Traditional methods (culling, trapping, poisoning) to control the monkey population are seldom cost-efficient and inhumane. These are using lethal species-specific pathogens not only more cost-effective but there are ethical and conservation implications of releasing lethal pathogens into ecosystems. Immunocontraception is one of the many alternatives. As an alternative, immunocontraception is based on reducing birth rates. This method has been used for many years. The target of this immunization can be GnRH [1-3], *zona pellucida*-3 (ZP3), or a combination of GnRH and ZP3 [4]. *Zona pellucida*, a glycoprotein that covers oocytes, especially ZP3, is a potential target of immunocontraception [5]. The administration of a vaccine that induces an adaptive immune response is expected to cause an animal to become temporarily infertile. Immunocontraception promises many advantages, including high target specificity, long-term but not permanent action, and relatively inexpensive. Theoretically lacks endocrine or metabolic side effects, easy to use and does not require insertion of an implant or device, and does not require surgical intervention. Among the candidates of immunocontraceptive substance, ZP3 is a potential antigens target because ZP3 protein is the primary receptor for sperm recognition. *Zona pellucida* protein was immunized in experimental animals in native [6], recombinant as well as deglycosylated forms [7-9].

Research models using laboratory animals have been widely conducted, among others, in mice by Wang Y, et al. [10]. Our study first investigated the immunocontraceptive potential of crude *zona pellucida* of a local goat of Java, Indonesia, called *Kambing Kacang*. Immunization of female mice with crude *zona pellucida* in Freund adjuvant prevented pregnancy in all mice when mated [11]. Based on these preliminary studies, experiments have been conducted to determine the immunocontraceptive potential of the *zona pellucida* third fraction of Indonesia's local goat, called the gZP3 in mice (*Mus musculus*) model.

Identification, Isolation, and Characterization of gZP3

Mammalian *zona pellucida* consists of several glycoproteins constituents with different molecular weights. Identify each *zona pellucida* constituent based on its molecular weight using Sodium Dodecyl Sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Furthermore, the protein band resulting from separation using SDS-PAGE was isolated and examined to ensure that the protein band was a goat fertilization receptor. In our study, the characterization of that protein consisted of measuring the molecular weight and composition of goat *zona pellucida*, immunogenicity in mice and rabbits, evaluation of gZP3 as goat sperm fertilization receptor, and immunological cross-reactivity between gZP3 with female mice serum (Table 1).

Parameters	Reference
Molecular mass of gZP3 means (±SD): 82.05 ± 6.90 Composition: 63.47% of gZP	Mustofa, et al. [12]
Immunogenic in mice and rabbit	Restiadi, et al. [13]
Proven fertilization receptor:	
- Immunofluorescence staining	Mustofa, et al. [14]
- In vitro fertilzation (ivf)	Mustofa, et al. [15]

Table 1: Identification and characterization of goat *zona pellucida*-3 (gZP3).

The Molecular Weight of gZP3 and Composition of Goat *zona pellucida*

Separating *zona pellucida* glycoprotein using SDS-PAGE commonly revealed three main constituent proteins [16]. The nomenclature of the *zona pellucida* proteins was determined by the protein mobility on the SDS-PAGE gel. Bands ordered from top to bottom with declining molecular weight were ZP1, ZP2, and ZP3. *Zona pellucida* of goat separated by SDS-PAGE on 12% gel resulted in the three most abundant bands that were separated. Based on the order of their relative molecular weight (Mr), these three bands are consecutively referred to as gZP1, gZP2, and gZP3. The molecular weight was 120, 94, and 82 kilo Dalton (kDa), respectively [12]. Densitometric analysis showed that the proportion of gZP1, gZP2, and gZP3 constituents of goat *zona pellucida* were 6.93; 29.60 and 63.47% [12].

Immunogenicity of gZP3 in Mice and Rabbits

The obtained gZP3 protein isolate was injected into female mice and male rabbits to determine its immunogenicity, and the sera were used in subsequent studies. Mice immunized with 40μg gZP3 generated an average of 640 antibody titers, which was sixteen times higher than unimmunized mice (only 44). Male rabbit pre-immune serum showed zero titers, while the average antibody titers of post-immunization with 200μg gZP3 was 7,840. The antibody titers of rabbit post-immune serum were 178 times higher than those of the unimmunized mice [3]. In the male rabbit, gZP3 was a foreign protein. The antibody titers of post-immune serum in male rabbits were higher than in female mice because the gZP3 protein was foreign to male rabbits. The antibody titers, as a measure of immune responses or immunogenicity, are mainly influenced by the foreignness of antigens by the host.

Blotting techniques (western blot and dot blot) and immunocytochemistry techniques helped analyze the immunoreactivity of ZP antibodies [17]. In our study, dot blot analysis showed that the post-immunized serum of female mice and male rabbits could recognize gZP3 protein, so it

could be concluded that the antibodies either in the post-immune serum of mice or rabbit were the antibody against gZP3. The Dot blot analysis on the pre-immune serum of male rabbits was negative, presumably caused by the absence of gZP3 homologous protein in male rabbits, so none of the IgG clones was recognized by the epitopes of gZP3 protein. That was consistent with the results of Elisa, in which the antibody titers of the male rabbit pre-immune serum were zero. Dot Blot analysis of female mice pre-immune serum showed a positive reaction with gZP3 protein. It was suspected that several gZP3 protein epitopes could be recognized by the IgG clone already present in the serum of mice (auto-antibody resulted from some amino acids homology between gZP3 and mZP3). Homologous molecules regulate fertilization in other mammals. From the recognition of gZP3 protein to the pre-and post-immune serum of mice showed in both Elisa and Dot blot, it was expected that gZP3 protein was immunocontraceptive in mice.

Evaluation of gZP3 as Fertilization Receptor

- **Immunofluorescence Staining**

The indirect immunofluorescence staining technique was used to identify the presence of fertilization receptors on goat *zona pellucida* and its ligand on goat sperm. The principle of this technique was a specific reaction of receptor-ligand, antigen-antibody, and antibody-marker labeled secondary antibody. In our studies, the receptor was called gZP3, the fertilization receptor in goats. The ligand was the sperm surface protein that could bind to gZP3. The antigen was the gZP3 protein, and the antibody was the gZP3 antibody raised in mice. One secondary antibody that could be used was the fluoro isothiocyanate (FITC) labeled rabbit anti-mouse IgG. In the examination of the existence of fertilization receptors on the *zona pellucida* histological preparation of the ovary [18]; oocytes [19]; embryos or sperm could be used [20].

The existence of egg-binding proteins in sperm could be identified by immunofluorescence techniques using anti-sperm antibodies [21]. This technique could also determine the relationship between fertilization receptors on the *zona pellucida* (sperm receptor) and their ligands. Examination of the presence of egg binding protein on the surface of sperm membrane was conducted by reacting the smear of sperm with ZP3 (receptor-ligand reaction) and followed by an exposure to ZP3 antibodies and visualization using a FITC labeled secondary antibody [20]. This technique is equal to the research on the application of in vitro sperm competition tests to evaluate the impact of ZP-derived peptides on the fertilization capacity of cat sperm. Pre-incubated cat sperm cells with ZP peptide decreased sperm binding capacity [22]. In our study, goat *zona pellucida* in control showed no fluorescence; meanwhile, without the gZP3 antibody, secondary antibodies could not recognize the goat *zona pellucida* smear. After being exposed to gZP3 antibodies and secondary antibodies, the smear of sonicated goat *zona pellucida* was fluorescing. Goat sperm smears without gZP3 showed no fluorescence, although previously exposed sequentially to gZP3 and secondary antibodies. Goat sperm smears exposed successively to gZP3, gZP3 antibodies, and secondary antibodies produced fluorescence at the plasma membrane of the sperm head [12]. This suggests that the gZP3 protein recognized the sperm plasma membrane of a goat, which subsequently had to be proven by in vitro fertilization techniques.

- **Goat in Vitro Fertilization**

In vitro fertilization consists of oocyte maturation, in vitro insemination, and in vitro culture. Examination of the potential of immunocontraceptive substances through in vitro fertilization technique could be performed at the step of oocyte maturation if the immunocontraception target was an oocyte [19]; or at the step of insemination when the immunocontraception target was sperm [23]. Our studies were carried out following this pattern. The antibody of gZP3 was supplemented into goat oocyte maturation media; on the other hand, gZP3 protein was supplemented into fertilization media containing goat sperm. This study was conducted to prove that the gZP3 protein was the fertilization receptor of goat oocytes. Inhibition of fertilization was measured based on the cleavage rate. In our study, cleavage rates in treated oocyte maturation had declined to 1/9. Meanwhile, those in treated sperm drop had declined to 1/4 compared to the control.

Our study's examination of in vitro fertilization was similar to the following publications. The previous addition of porcine *zona pellucida* (ZP) components to spermatozoa of the same species has an inhibitory effect on in vitro fertilization (IVF) [24]. Research on anti-Glutathione-S transferase antibodies on goat sperm resulted in decreased fertilization rates compared to control groups [25]. On the other hand, treated sperm using a rabbit anti-cow sperm plasma membrane proteins polyclonal antibody for in vitro fertilization also decreased the fertilization rate [23].

Immunological Cross-Reactivity between gZP3 and Female Mice Serum

There was a homology of the amino acid sequence of ZP3 in 13 vertebrate species, i.e., mice, hamsters, rabbits, pigs, wild pigs, cattle, dogs, cats, humans, bonnet, marmosets, fish, and frogs [26]. Polyclonal rabbit anti-pZP antibodies recognized ovarian sections of the dog, cat, horse, and elephant [27]. On the other hand, rabbit polyclonal antisera against carp, trout, and duck egg envelopes showed significant cross-reactions among egg envelopes of fish and birds. However, they did not show cross-reactivity with egg envelopes from

any other class [5]. The presence of ZP3 amino acid sequence homology in various species illustrated the cross-linking reaction of IgG antibody clones of ZP3 in a species with ZP3 epitopes of other species. Elisa to measure antibody titers in the serum of animals immunized with homologous antigen generated a strongly positive reaction, but cross-reactions with the other species might also occur [28]. In our study, non-pregnant-fertile mice injected only with physiological saline had antibody titers far from zero (ranging between 20-80 with a mean of 44 ± 12.65) and positive on Dot blotting [29]. It was suspected that clones of IgG in non-pregnant-fertile mice serum was recognized by gZP protein. This fact suggested the opportunity to examine the potential of gZP3 protein as an immunocontraceptive substance in mice as a model.

Immunocontraceptive Potential of gZP3 in Mice Model

Experiments to explore the immunocontraceptive potential of gZP3 in mice models were conducted in vivo and in vitro. In vivo experiments aimed to identify effectiveness, reversibility, and the possible effect on the estrous cycle, ovarian structure, and progesterone level in the follicular and luteal phases. In vitro experiment was conducted to determine the role of gZP3 antibody in preventing mice in vitro fertilization, and a binding assay of mice sperm to mice ova (Table 2).

Parameters	Reference
In vivo	
- Effective at 40 µg (dose-dependent)	Mustofa, et al. [12]
- Reversible in 14 estrous cycle	Mustofa, et al. [30]
- Not affected estrous cycle	Mulyati, et al. [31]
- Stable ovarian structure	Mustofa [29]
- Normal progesterone level	Mulyati, et al. [32]
In vitro	
- In vitro fertilization (IVF)	Mustofa [30]
- Binding assay	Mustofa I [33]

Table 2: The resume of research on the immunocontraceptive potential of gZP3 in mice model.

In Vivo: The Prevention of Pregnancy and Reversibility

In our study, the examination of gZP3 protein immunocontraceptive potential in mice models was conducted in two stages. The first stage included the determination of its potency in pregnancy inhibition, measuring litter size, and identifying the possibility of fetal malformation in pregnant mice. The second stage included the determination of the reversibility and how long the mice were back to pregnant again after immunization with gZP3.

- **Prevention of Pregnancy**

Injection of gZP3 protein could dose-dependently induce antibodies. The greater the dose of gZP3, the higher the antibody titers [13]. The data indicated that immunization with gZP3 protein could inhibit fertilization of sperm to oocyte of mice, thereby reducing the dose-dependent pregnancy rate, with up to prevent pregnancy completely (100%). This could be explained as follows. *Zona pellucida* of mammals was a layer of extracellular glycoprotein that played an essential role in the initiation of interactions between sperm and ovum [34], which in turn resulted in fertilization. In animals immunized with *zona pellucida* protein, the level of IgG in circulation was positively correlated with infertility. The IgG is bound to a sperm receptor glycoprotein of ZP3 as long as the oocyte is still in the follicle and will be augmented by the existing IgG of ZP3 in the oviduct after ovulation. This binding was steric, causing blockage of ZP3 glycoprotein, the fertilization receptor on the *zona pellucida*; therefore, sperm could not recognize the oocyte. The binding of *zona pellucida* antigen with antibodies would inhibit the fusion of gametes and cause fertilization failure.

The infertility of the experimental animals did not show an alteration of the estrous cycle [31], ovarian structure and progesterone level in the follicular nor luteal phase. These results equal the application of pZP that indicates ovarian activity amongst pZP-treated female African elephants two years after initial immunization [35].

- **Reversibility**

The reversibility of gZP3 as an immunocontraception candidate substance was examined on two groups of mice. Treatment mice were immunized with 40 µg gZP3 in Freund's adjuvant, whereas control mice received an injection of placebo at the same time as treated mice immunization. gZP3 were injected subcutaneously at 0.1 ml/per mouse at intervals of 14 days with two boosters. Seven days after the second booster, all mice were mated. Observations of vaginal plugs as a sign of the occurrence of copulation were performed every morning, followed by observation of pregnancy and parturition [30].

Control and treated mice gave birth at 26.5 ± 4.3 and 91.6 ± 4.9 days after introducing male mice. These results revealed that the treated mice had delayed parturition 3.5 times (91.6 / 26.5) longer ($p<0.05$) compared to control mice. Litter sizes were similar between the treated and control groups (7.80 ± 1.48 vs. 7.70 ± 1.34). The length of mice gestation was 21 days [36], so it could be estimated that the control mice were pregnant after 5.5 (26.5-21) days or

only one estrus cycle; meanwhile, the fertility of treated mice returned to normal and these mice got pregnant 70.6 (91.6-21) days after the introduction of the male mice. The estrous cycle is the interval from one estrus to the next estrus. In mice (*Mus musculus*) estrous cycle repeats every 4 to 5 days [37]. The estrous cycle in mice consists of four stages, namely proestrus, estrus, metestrus, and diestrus. Each of the estrous stages in mice can be observed by examining vaginal smear slides microscopically [37]. Based on the five days average of the estrous cycle in mice, the effective period of gZP3 immunocontraception in the mice model was 70.6 divided by 5, equal to approximately 14 estrus cycles. One estrous cycle in mice was analogous to one menstrual cycle in monkeys. The duration of the menstrual cycle of monkeys is roughly 30 days [38]. Thus it could be predicted that the active life of gZP3 protein as an immunocontraceptive substance in monkeys was the equivalent of 14 months. There was no significant difference in the litter size of the control and treatment groups, similar to the previous experiment in different gZP3.

In Vitro Fertilization and Sperm Binding Assay

In vitro fertilization examination of immunocontraceptive potential of gZP3 aimed to determine the effect of post-immune serum against gZP3 protein on the failure of in vitro fertilization and sperm binding assays, compared to those pre-immune sera.

- **In Vitro Fertilization**

In vitro fertilization was examined using mice pre-immune serum with the lowest titers of antibodies in the control group. In contrast, the treatment group used post-immune serum with the highest antibody titers. This study aimed to confirm the in vivo study discussed earlier that the cause of infertility in mice was the anti-gZP3 contained in the gZP3 immunized mice serum animal. The result showed that the oocytes in the fertilization drop supplemented with pre-immune serum were all fertilized. Meanwhile, none of the oocytes in fertilization drops supplemented with post-immune serum was fertilized.

Examination of antibodies performance in immunocontraception study using in vitro fertilization techniques could be performed on oocytes. If the target of immunocontraception was an oocyte, such as in this study, the oocytes were pre-incubated in a medium supplemented with the examined antibodies before inseminating in vitro [39]. This study proved that supplementation of in vitro fertilization media with 10% post-immune serum prevented fertilization of oocytes, whereas, in the control group (pre-immune serum), all the oocytes underwent fertilization, which was characterized by the degradation of cumulus cells and the formation of the second polar body.

N-glycosylation of zona glycoproteins during meiotic maturation involves sperm-*zona pellucida* interactions [40]. Gamete adhesion in mice is carbohydrate-mediated since sperm recognize and bind to certain mZP3 serine/threonine-(O-) linked oligosaccharides. On the sperm plasma membrane of mice, there was β1,4-galactosyltransferase (Galtase) which acts as a binding protein ZP3 (ligand). In contrast, on ZP3 of mice, there was receptor Gal-β(1,3)–GalNAc, it was Galactose-β-(1,3)-galactose-N-acetylgalactosamine (GalNac) [41-43]. The antibody of gZP3 supplemented into mice oocytes in vitro fertilized was also derived from mice serum. We have suggested that immunization mice with the protein produce several clones of gZP3 IgG against Gal-β(1,3)–GalNAc as specific epitopes of gZP3. Based on the recognition of serum proteins gZP3 by fertile-female mice in Elisa and Dot blot examination discussed earlier, it was believed that there was gZP3 amino acid sequence homology with the amino acid sequence mZP3. The homology led to several clones of IgG of mice immunized with gZP3 protein, resulting in adequately recognizing fertilization epitopes of mouse ZP3 (mZP3) and pregnancy prevention.

- **Sperm Binding Assay**

Like hemizona assay (HZA), sperm binding assay was a bioassay of sperm binding function to the *zona pellucida*, especially to the *zona pellucida* glycoprotein [44]. Binding assays could also be used to examine antifertility in the development of contraception methods [45]; and to examine the role of antibodies in the immunocontraception research based on ZP3 [19]. The parameter measured was the binding index, calculated by dividing the number of treated sperm bound to the *zona pellucida* by the number of control sperm bound to the *zona pellucida* multiplied by 100 [46]. In our study, the number of sperm firmly bound to the *zona pellucida* in the treatment group (incubated in media supplemented with gZP3 post-immune serum) was smaller ($p<0.05$) compared to the control group (incubated in media with pre-immune serum). Binding Index was 5.45 ± 3.88 (scale of 0-100).

The serum used in this study was homologous from immunized mice, the same as in vitro fertilization. Mice oocytes incubated in 10% pre-immune serum resulted in 35.1±3.35 binding with a range of 33–43 higher than Rankin *et al*. (1998), i.e., 17.7±2.5, but slightly lower than those reported by Naz, et al. [47] that was 42 sperm per oocyte. Mice oocytes incubated in 10% post-immune serum had an average of 1.90±1.25 sperm (ranging from 0-4) bound tightly. These results indicated that the gZP3 antibody could reduce the chances of sperm binding to the gZP3 on *zona pellucida*, causing a decrease in the fertilization rate. The results of the binding assay followed the report of Paterson, et al. [28] which proved that the homologous or heterologous ZP3 antibodies might decrease the human sperm egg binding

index by over 60%. It was also analogous to those reported by Hasegawa, et al. [19], where anti-human and anti-rabbit peptides also caused a reduction in the number of sperm bound to oocytes compared to the control group.

The Prospect of Using gZP3 as Population Control of Monkeys

Immunocontraceptive use for animal fertility has been widely practiced. In recent years it was applied to wild, zoo, farm, and domestic animals [48], i.e. in the African elephant, companion animals [49], exotic carnivores [50], and cats [51]. Another immunocontraceptive application for wildlife population control has been performed on Hokkaido Sika Deer (*Cervus nippon yesoensis*) [52], wild pigs (Sus scrofa) [53], street dogs [54], white-tailed deer (Odocoileus virginianus) [55], and captive exotic species Dall sheep (Ovis dalli dalli) and domestic goats (Capra hircus) [56]. There are even factory-made finished products, namely GonaCon (GnRH based), which among others, has been used in controlling the population of feral cattle in Hong Kong [57], dan SpayVac (ZP3 based) which, among others were effectively tried on feral horses [17]. However, the use of immunocontraception for controlling monkey populations in their habitat has yet to be reported.

Parameters	Reference
Homology	
47,35% to Macaca fascicularis and Macaca mulata	Mustofa, et al. [58]
47,62% to Macaca radiata	
Filogeny analysis: gZP3 strange protein for ZP3 of Macaca sp	

Table 3: The prospect of immunocontraceptive application of gZP3 as a control population of monkey.

The gZP3 protein expected can using for overpopulated monkeys especially *Macaca fascicularis, Macaca mulatta* and *Macaca radiate.* Geographic distribution of *Macaca fascicularis* are most commonly in Southeast Asia [59,60], *Macaca mulatta* are found in western Afghanistan, India and northern Thailand [61], and *Macaca radiata* are in southern India [62]. The nucleotide sequence of gZP3 was compared to ZP3 nucleotide sequence data of *Macaca radiata* (X82639) [63] Macaca fascicular (AS168898) [64] and Macaca mullata (XM-001114760) [65,66]. The nucleotide sequence of gZP3 has homology 47.35% to *Macaca fascicularis* and *Macaca mulatta*, and 47.62% to *Macaca radiata* (Table 3). The phylogenetic analysis showed that gZP3 was foreign to Macaca sp ZP3 protein, which qualifies as an immunogen in Macaca sp [58]. In its application in the future, immunization can be done using a DNA vaccine, as has been done by Wang, et al. [10] in the mouse. Likewise, intranasal co-delivery can make applications more easily [67-69].

Conclusion

In this study, goat *zona pellucida*-3 (gZP3) has 82 kDa molecular weight and comprises 63.47% of the total goat *zona pellucida* protein, was fertilization receptor based on immunofluorescence and in vitro fertilization studies. Immunologically, gZP3 prevented pregnancy dose-dependently in the mice model and was reversible after 14 estrous cycles. In vitro fertilization study showed that the antibody of gZP3 was able to prevent fertilization and decreased sperm binding rate on mice oocytes. The gZP3 nucleotide sequence has a homology of nearly 50% of the Macaca sp ZP3 nucleotide sequence but is foreign phylogenetically. The gZP3 protein could be developed as an immunocontraceptive substance for overpopulated monkeys.

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