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Full Length Research

Optimization of cell culture and the spread of metaphase chromosomes for cytogenetic studies using Taguchi design in the deer

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ABSTRACT: Metaphase production is an important aspect of cytogenetic studies. To achieve acceptable metaphase spreads for cytogenetic study, cells, depending on source animals, require different operating conditions. It is therefore necessary to optimize the process to produce a protocol, which achieves the best result. This study aimed to establish the growth and arrest requirements of cultured Peripheral Blood Mononuclear Cells (PBMCs) from three breeds of deer (*Axis axis*, *Rusa timorensis* and *Rusa unicolor*) using Taguchi design. Twenty-four animals (8 in each study) were used in the study. Culture medium (CM) (RPMI1640$^+$ or RPMI1640$^-$, with or without 16.6 mM glucose and 4 mM glutamine), fetal bovine serum (FBS, 10% or 20%), agitation frequency (AF, once or twice), time of colcemid introduction (col, early or late) and the duration of cells in KCL (DKCL) (20 or 30 minutes) were the factors investigated. They were set at two levels in an L8 (2^5) orthogonal array. *Rusa timorensis* had RPMI 1640$^+$, 20% FBS, AF twice per day, early col and DKCL of 20 minutes as the optimal factor levels. *Rusa unicolor* on the other hand had RPMI 1640$^+$, 20% FBS, AF once per day, late col and DKCL of 30 minutes as the optimal factor levels. *Axis axis* had RPMI 1640$^+$, 20% FBS, AF once per day, early col and DKCL 30 minutes as the optimal factor levels. This work demonstrated the utility of the Taguchi design in optimizing the production of metaphase spreads from cultured PBMC in the deer.

Keywords: Cell culture, cytogenetics, metaphase spreads, Taguchi design.

INTRODUCTION

Good cytogenetics studies depend on good slides, prepared with sufficient and acceptable metaphase spreads. This is achieved by culturing cells using specific agents, and arresting them at metaphase (Bolhaqueiro *et al*., 2019) . Cell culture, which produces good metaphase spreads depends on certain culture conditions. These conditions include; culture media, serum, availability of CO2, periodic agitation, and time during which colcemid is introduced into the culture. Other factors, which are post culture, include: duration of harvested cells in hypotonic agents, and so on.

When similar cell types from different animal breeds are cultured using these factors at the same levels, they are expected to have the same growth requirements and produce the same growth pattern (Kotsarenko *et al*., 2020). However, this is not always the case as breed is found to influence the growth requirements of similar cultured cells originating from different breeds of animals (Arora, 2013; Kerbel and Blakeslee, 1976). This makes it difficult to, in some cases, extrapolate the amount of growth factors suitable for culturing similar cell types from different breeds of animals (Chakraborty *et al*., 2019). Many laboratories painstakingly use trial and error method to arrive at optimized levels of growth requirements for culture when cells from different breeds fail to respond to an earlier optimized protocol. Others use the traditional

factorial methods, which involves testing all the levels of all factors independently and against one another. This results in conducting numerous, sometimes impossible number of experiments (Qafary *et al*., 2018). This therefore underscores the need for a study to identify and adopt a method through which optimizing the production of metaphase spreads can be universalized regardless on the breeds of animal (Klinder *et al*., 2019).

The Taguchi method is a good alternative that can be adapted to many optimization processes. It reduces time and effort by reducing the experimental trials required for optimization. It can be used with various signal-to-noise objectives such as “larger-is-better”, “smaller-is-better”, or “nominal-is- better”. The technique employs a manageable number of progressive trials to examine variables, and at the same time identify those variables with major effects on the process in question. A combination of variables that will lead to optimal performance can thereafter be predicted (Aujame et al., 2000; Han et al., 1998). Once the results of these trials are satisfactory then further trials would not been required (Kallel *et al*., 2002). This method, although popular in engineering, has been successfully applied in various fields of life science research such as; bacterial culture (Makowski *et al*., 2017), antibody production (Kallel *et al*., 2002), PCR optimization (Cobb and Clarkson, 1994; Thanakiatkrai and Welch, 2012) and the Enzyme Linked Immunosorbent Assay (ELISA) (Jeney *et al*., 1999) and in agricultural practices (Awty-Carroll *et al*., 2020). This study aimed at investigating the feasibility of applying the Taguchi method for the development of protocols for optimization of cell culture and the production of metaphase spreads for cytogenetic studies.

MATERIALS AND METHODS

The study was conducted at the Theriogenology and cytogenetics laboratory, Faculty of Veterinary Medicine, Universiti Putra Malaysia.

Animals

Three breeds of deer *Axis axis*, *Rusa timorensis* and *Rusa unicolor* were studied. Peripheral Blood Mononuclear Cells (PBMCs) were collected from twenty-four (eight individuals from each breed) apparently healthy adults of both sexes. For the females, only non-pregnant ones were included in the study. Six (6 mLs) of heparinized vacutainer tubes and venoject needle were used to collect blood from the jugular vein.

Within 6 hours of collection, 5 mL of blood, from each animal, was layered onto 5 mL of Ficoll-Paque Plus®, 1.077 g/mL (Amersham Biosciences, Buckinghamshire, UK) in a 15-mL centrifuge tubes. The samples were centrifuged for 30 minutes at 1000 x g. The PBMCs rich layer was harvested into a sterile 15 mL tube, 10 mL of sterile PBS was added and the tube was gently shaken to resuspend the cells. The PBS/ PBMC mixture was centrifuged for 5 minutes at 400 x g. The supernatant was discarded while the cells were resuspended in another 10 mL of PBS, and centrifuged for 5 minutes at 400 x g. After discarding the supernatant, the cells were finally resuspended in 2 mL of PBS and stored in a refrigerator until used (Grievink et al., 2016).

An L8 Taguchi orthogonal array was designed with five variables at two levels each (2^5) (Tables 1 and 2) for the trials: Culture medium (CM) ($RPMI1640^+$ or $RPMI1640^-$, with or without 16.6 mM glucose and 4 mM glutamine) (Kallel *et al*., 2002), fetal bovine serum (FBS, 10% or 20%), agitation frequency (AF, once or twice), time of colcemid introduction (COL, early or late) and the duration of cells in KCL (DKCL, 20 or 30 minutes) were the variables investigated.

The construction of Taguchi L8 (2^5) orthogonal array design matrix (Table 2) was performed using Minitab 17 software (Minitab, LLC, State College, Pennsylvania. USA). Because the objective of the study was to maximize the response, the signal-to-noise ratio was set at larger is better; $SN = -10 * Log10(\sum(\frac{1}{Y2})/n)$, where n = number of runs and Y = the response, this equation gives a positive signal to noise ratio, which is large, indicating that the condition is better.

Culture initiation

Five drops of cell suspension were dropped into a culture flask containing the appropriate amount of media and other supplements for each of the eight runs produced by the design. Two replicates were done for each run in each breed of deer. The cultures were incubated at 37°C and 5% CO_2 for 72 hours. Before termination, Colcemid was introduced according to requirement of the design (Table 2) to arrest the cells at metaphase. After the 72 hours duration, the culture was terminated and the suspension was centrifuged for 5 minutes at 1800 rpm. The supernatant was discarded and the sediment was resuspended in 6 ml 0.075M KCL and incubated as per the design requirement (Table 2).

Slide preparation

After treatment in KCL, the cells were washed in several changes of Canoy’s fixative (a mixture of glacial acetic acid and methanol, 1:3). They were centrifuged at 1800 rpm for 8 minutes after each wash. They were finally resuspended in 3 mL of Canoy’s fixative and stored at 4°C for 30 minutes before slides were prepared. 50µL of cell suspension was dropped onto a clean grease-free, prechilled microscope slide from a distance of 15 cm; the slides were fixed over steam for 30 seconds and then stained in 5-10% Giemsa stain for 7-10 minutes. The slides were viewed under light

Table 1. The levels of production variables for optimization of cell culture and the spread of metaphase chromosomes using Taguchi design.

Factors	CM	FBS (%)	AF/day	Col (mins)	DKCL (mins)
Level 1	RPMI 1640$^-$	10	twice	60 (late)	20
Level 2	RPMI 1640$^+$	20	once	90 (early)	30

NB. Culture medium (CM), fetal bovine serum (FBS) agitation frequency (AF), time of colcemid introduction (col), duration of cells in KCL (DKCL).

Table 2. Taguchi L8 (2^5) orthogonal array design matrix for optimization of cell culture and the spread of metaphase chromosomes.

Trial no.	Variables				
	CM	FBS (%)	AF/day	Col (mins)	DKCL (mins)
1	Level 1	Level 1	Level 1	Level 1	Level 1
2	Level 1	Level 1	Level 2	Level 2	Level 2
3	Level 1	Level 2	Level 1	Level 1	Level 2
4	Level 1	Level 2	Level 2	Level 2	Level 1
5	Level 2	Level 1	Level 1	Level 2	Level 1
6	Level 2	Level 1	Level 2	Level 1	Level 2
7	Level 2	Level 2	Level 1	Level 2	Level 2
8	Level 2	Level 2	Level 2	Level 1	Level 1

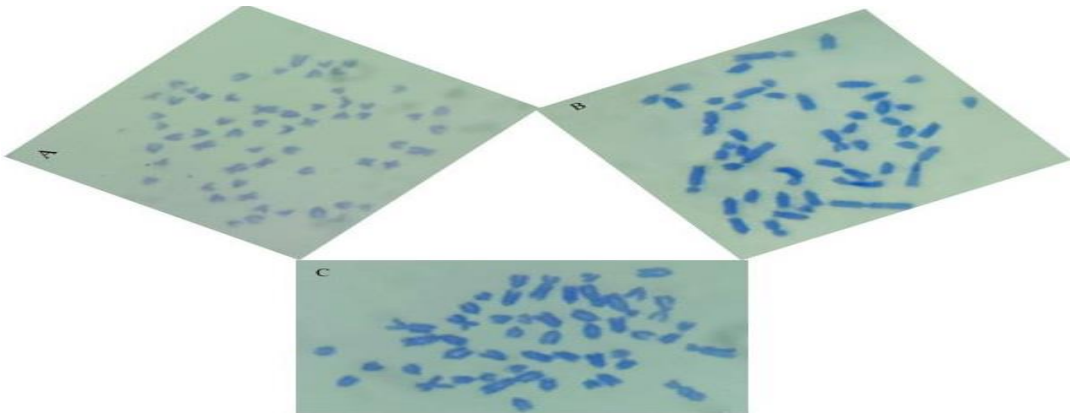

Figure 1. Metaphase spreads obtained from the current study (A) *Axis axis*, (B) *Rusa timorensis* and (C) *Rusa unicolor.*

microscope at X40 to count the number of metaphases spreads. This procedure was replicated for all animals.

Statistical analysis

Data were analysed using one sample T-test at p = 0.05 significance level using GraphPad InStat v3.1 (GraphPad Software, 2365 Northside Dr. Suite 560, San Diego, CA 92108).

RESULTS

Optimization of cell culture and the production of metaphase spreads using Taguchi design yielded significantly higher number and quantity of metaphase spreads in all the three groups compared to the traditional protocol of the laboratory (Figure 1). The mean (± SD) average number of metaphases is shown on Table 3. There was significant statistical between experimental runs in all three studies. In study 1 (*A. axis*), the mean (± SD) was 36.688 ± 20.408, indicating a significant (p = 0.0014) variability between the runs. Studies 2 (*R. timorensis*) and 3 (*R. unicolor*) had mean (± SD) 21.313 ± 17.308, p = 0.0102 and mean (± SD) 28 ± 13.384, p = 0.0006, respectively.

Table 4 shows the ranking of factors according to the

Table 3. Average number of metaphases obtained from L8 orthogonal array (2^5) Taguchi design in the deer.

Runs	Responses		
	***Axis axis* (study 1)**	***Rusa timorensis* (Study 2)**	***Rusa unicolor* (Study 3)**
1	4	4	7.5
2	12	7	16.5
3	28.5	8	43
4	44.5	16	27
5	37.5	22.5	31.5
6	52	19	17.5
7	59.5	41.5	45
8	55.5	52.5	36
Mean ±SD	36.688 ± 20.408	21.313 ± 17.308	28 ± 13.384
p-Values	0.0014	0.0102	0.0006

Table 4. Ranking of the effects of factors in the production of metaphase spread in three breeds of deer (*A. axis*, Study 1; *R. timorensis*, Study 2; *R. unicolor* Study 3).

Factors	CM	FBS	AF	Col	DKCL
***Axis axis* (Study 1)**					
Level 1	22.25	26.38	32.38	35.00	35.38
Level 2	51.13	47.00	41.00	38.38	37.50
Difference	28.88	20.62	8.62	3.38	2.12
Rank	1	2	3	4	5
***Rusa timorensis* (Study 2)**					
Level 1	8.75	13.125	18.125	21.75	22.625
Level 2	33.875	29.5	24.5	20.875	19.75
Difference	25.13	16.37	6.37	0.87	2.88
Rank	1	2	3	5	4
***Rusa unicolor* (Study 3)**					
Level 1	23.5	18.25	23.63	27.5	27
Level 2	32.5	37.5	25.75	28.5	29
Difference	9	19.25	2.12	1	2
Rank	2	1	3	5	4

amount of effect they contributed individually during the process. In study 1 (*Axis axis*), the factor with the most significant effect was the culture medium (CM) followed by the serum (FBS). Agitation frequency (AF), colcemid introduction time (Col) and duration of KCL treatment (DKCL) ranked third to fifth respectively. Similar incident was encountered in study 2 (*R. timorensis*) except that in this case DKCL and Col ranked fourth and fifth respectively. In study 3, the factor with the most effect was the FBS followed by the CM, while the AF, DKCL and Col ranked third, fourth and fifth respectively.

Figure 2 show the factors at their various levels, showing how each factor contributed at its lowest, average and highest levels. It should be noted that the two balls at the ends of the arrows, up and down, indicate the exact number of metaphases produced by level 2 and level 1 respectively. The length of the arrows on the other hand indicates the amount of influence a factor had on the production process and the longer and arrow is, the more its influence on the process.

Table 5 shows a summary of the optimized protocols from the three studies. In study, the protocol had CM, FBS, AF, Col and DKCL all at level 2. In study 2, the factors were at levels 2, 2, 1, 2, 1, while in study 3 they were at levels 2, 2, 2 1, 2.

DISCUSSION

Process optimization is a recurring decimal in scientific research; it allows the discovery of levels at which variables produce optimal results in experiments. In life

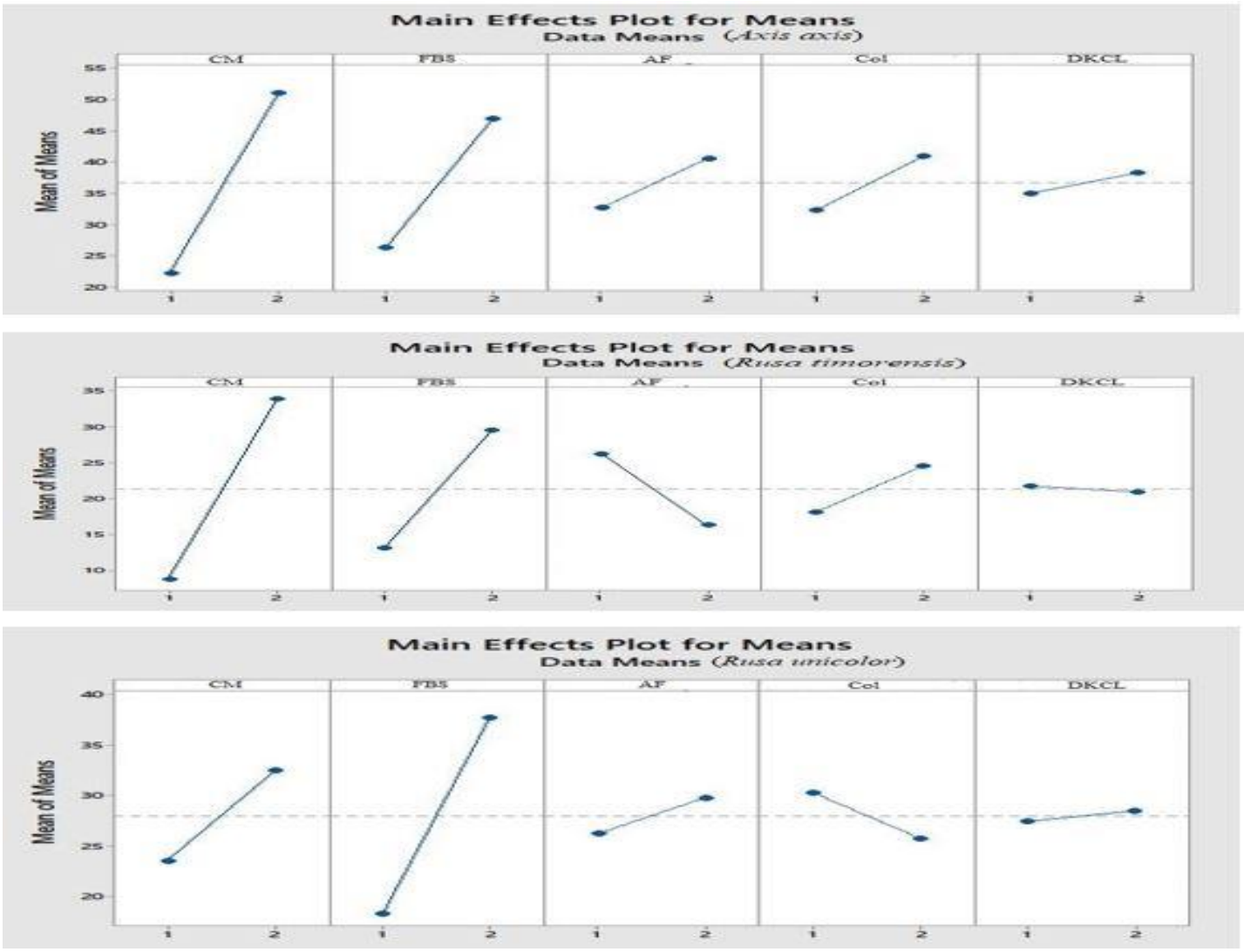

Figure 2. Combination of Factors levels and their various effects in metaphase spread production from PBMCs culture in three breeds of deer.

Table 5. Optimized combination of factor levels in the production of metaphase spread in three breeds of deer (*A. axis*, Study 1; *R*. *timorensis*, Study 2; *R. unicolor* Study 3).

Parameters	Optimized factor levels		
	***Axis axis* (study 1)**	***Rusa timorensis* (study 2)**	***Rusa unicolor* (study 3)**
CM	Level 2	Level 2	Level 2
FBS	Level 2	Level 2	Level 2
AF	Level 2	Level 1	Level 2
Col	Level 2	Level 2	Level 1
DKCL	Level 2	Level 1	Level 2

sciences and agriculture, this is generally attempted through the application of the traditional factorial method (Makowski *et al*., 2017). This involves testing all levels of all factors independently and against one another, resulting in the conduct of numerous experiments (Pundir *et al*., 2018). Taguchi method is an alternative approach, which reduces time and effort by reducing the experimental runs required (Atil and Unver, 2020). In this

study, the production of metaphase spreads from PBMcs, in three breeds of deer, *Axis axis*, *Rusa timorensis* and *Rusa unicolor*, was optimized using Taguchi design in three parallel studies. The results of the average number of metaphases show the robustness of the design matrix to produce the combination of variables that are the best based on the purpose of the research. In these studies, the bigger the better signal-to-noise (SN) ratio was the chosen parameter; therefore, the optimization presents the best combination of variables. Similar observations were made (Kallel *et al*., 2002) where a similar SN was desired to grow hybridoma cell line as well as produce antibodies from them. It can be seen that the design produced results that were not dependent on the experimental runs but on the combination of variables (Atil and Unver, 2020).

Factors do not always contribute the same amount of effects in experiments, which is to say that they do not have the same significance in the process. This makes it necessary to identify those factors with significant effect on the process, in case further adjustment may be required. In the three studies presented here, all the breeds have CM, FBS and AF ranking first to third in the order of their significance. Col and DKCL ranked fourth and fifth in *A. axis* but the same variables ranked fifth and fourth respectively in both *R. timorensis* and *R. unicolor*. In similar works where Taguchi method was used to produce some microorganisms, glucose and sucrose were ranked the most critical variables in the process (Makowski *et al*., 2017). In the light of the existing literature, the variable we selected for use in optimization were reported to be of major effect in similar studies. For example, RPMI 1640 enriched with glucose and glutamine have been found to be more critical in the production of hybridoma cells than other operating variables (Asghar *et al*., 2019; Kallel *et al*., 2002).

Conclusion

In this study, Taguchi method was used to identify the combination of factor levels, which produced the highest number of metaphase spreads. Also, the factors that are the most critical in the process were identified. Culture medium, serum and agitation frequency were the most important variables to be considered followed by the time at which colcemid is introduced and the duration of cells in KCL. The observations made here can be used as a basis for optimizing the production metaphase spreads in other breeds of animals. The study has also revealed the enormous potential of using the Taguchi methods in both optimization of factor levels as well as ranking the factors to identify which ones are more critical in the process.

CONFLICT OF INTEREST

The authors declared that they have no conflict of interest.

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