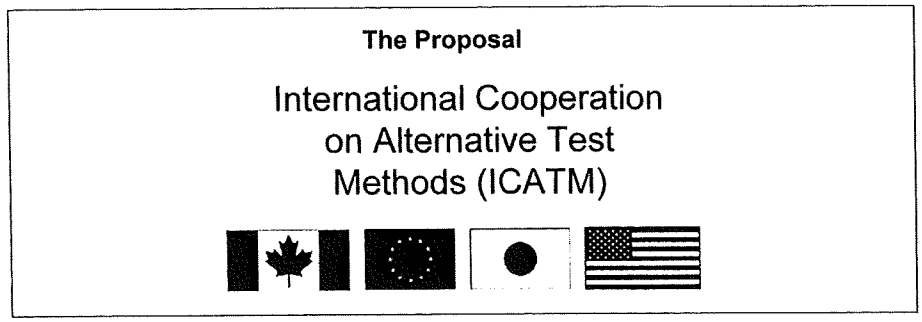


図11　代替実験法における国際協調

表14　この目的は，各国が協力して，バリデーション研究のデザイン，試験方法の検討や最適化，及びピアレビューについて高いレベルを確立することにある．また，国々のバリデーションのオーガナイゼーションによる勧告を，より実現性のあるようなものにしていく狙いがある．

表14　ICATMの目的

| ICATM Purpose |
|---|
| To promote international cooperation, collaboration, and communication among national validation organizations in order to : |
| ・**Ensure optimal design and conduct of validation studies** |
| —That will support national and international regulatory decisions on alternative methods proposed for regulatory testing. |
| ・**Ensure high quality independent scientific peer reviews** |
| —Provide for transparency and the opportunity for stakeholder involvement |
| ・**Enhance likelihood of harmonized recommendations by national validation organizations** |
| —More rapid international adoption of alternative methods |
| ・**Avoid duplication of effort and leverage limited resources to achieve greater efficiency and effectiveness** |

表15　ICATMはボランタリーな機関であり，それぞれの国ごとに置かれた財政基盤が異なる．日本がその会議に参加してバリデーションの分担を依頼されても，予算も限られており，できる範囲で協力することとなっているが，それなりの義務を果たすためにはさらに社会の認識を高め，国としての支援を得るための努力が必要である．

表15 ICATMの組織

| ICATM Proposed Membership |
|---|
| a. ICATM is a *voluntary* international group of validation organizations from the United States, Japan, the European Union, and Canada. |
| b. The four initial ICATM members are: |
| －NICEATM-ICCVAM |
| －ECVAM-ESAC |
| －JaCVAM |
| －Health Canada |
| c. The inclusion of other members and their appropriate status can be decided by consensus by the members. |

このような協力のための枠組みができると，試験法のバリデーションの段階やピアレビューの段階，公式試験方法の勧告作成の段階，それぞれの段階で国際協力する枠組みができて非常に意義がある．

今後，動物福祉に考慮し，社会に受け入れやすい安全性試験法が開発され，それを最短期間で行政的に受け入れることにより，科学の進展が加速されることが期待される．

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〈抄録〉第29回 日本臨床薬理学会年会 2008年12月4～6日 東京
シンポジウム1：早期臨床試験のあり方を考える

# 1．マイクロドーズ臨床試験指針と早期探索的臨床試験への期待

大野泰雄*

## 1．はじめに

医薬品開発のなるべく早期にヒト試験を行い，最適化合物を選択し，第Ⅰ相から第Ⅲ相の臨床試験段階での成功率を上げることは，開発経費を節減するとともに，有用な医薬品を1日も早く患者に届けるために極めて重要である．そこで，欧米ではマイクロドーズ臨床試験（MD試験）を初めとする探索的臨床試験を導入するなど，国レベルで積極的な施策を採ってきた[1,2]．わが国においても，2008年6月3日にMD試験が導入された[3]（薬食審査発第0603001号：以下「指針」と略す）．

## 2．MD試験とは

指針では，「マイクロドーズ臨床試験とは，ヒトにおいて薬理作用を発現すると推定される投与量の1/100を超えない用量又は100 μgのいずれか少ない用量の被験物質を，健康な被験者に単回投与することにより行われる臨床試験をいう」と定義された．目的は，「被験物質のヒトにおける薬物動態に関する情報を医薬品の臨床開発の初期段階に得ることである．具体的には，被験物質の吸収や血中動態，排泄特性，ヒトにおける代謝物プロファイル等を明らかにすること，分子イメージング技術を用いて被験物質の体内における局在に関する情報を得ること等である」．MD試験を医薬品開発の初期に実施し，薬物動態学的に適正と思われるものを選択しておくことにより，その後の臨床開発において，体内動態が原因で開発が失敗する可能性を削減できるものと期待される．また，分子イメージング技術と組み合わせることにより，ヒトでの体内分布の様子が明らかになり，ヒト組織を用いた *in vitro* 試験結果と組み合わせることにより，標的部位での薬力学的作用の推定も確かになると思われる．

## 3．MD試験に必要な非臨床試験データ

MD試験の実施に際しては，拡張型単回投与毒性試験を実施するとともに，局所刺激性と適切な *in vivo/in vitro* 試験による，治療標的に関連した薬理作用，主たる薬理作用，および薬効発現量を明らかにしておくことが必要である．遺伝毒性試験は必ずしも要求されない．

拡張型単回投与試験で良いとした理由は，MD試験で使用される100 μgという用量で重篤な毒性を現す低分子化合物はほとんどないこと，また，あったとしてもそれらの毒性は単回投与毒性試験で検出可能なものであることによる．主たる薬理作用の検討は薬理作用の延長による毒性を未然に防止するために必要である．なお，主たる薬理作用はスクリーニングの段階で当然明らかになるものである．また，適切な毒性試験動物種の選択にも資するものである．遺伝毒性試験を必要ないとしたのは，たとえ遺伝毒性物質であっても，0.15 μg/day以下であれば一生涯摂取しても発癌リスクは$1/10^6$であり，実質的に安全であると見なすことができ[4]，1カ月以内の反復投与試験であれば，120 μg/day投与でも$1/10^6$のリスクを超えない確率は93%であることによる[5]．

MD試験のために行われる拡張型単回投与毒性試験は，通常，1種類の哺乳類の雌雄を用いて行われ，予定臨床投与経路での最大無毒性量（NOAEL）および最小毒性発現量を確立するか，または適切な安全域（通常，体表面積換算で100倍以上）を確立するように用量段階が設定される．観察期間は2週間で，毒性徴候の種類，程度，発現，推移および可逆性について，用量および時間との関連で観察し記録される．また，適切な時期（通常，投与翌日および2週間の観察期間終了時）に血液検査，血液生化学検査および病理組織学的検査が行われる．局所刺激性については，この試験における投与局所の観察で行ってもよい．

ヒトと動物との間の重篤な毒性発現における種差の程度は体表面積換算で，せいぜい100倍程度である[6]．しかし，バイオ医薬品やリシン類，fialuridineなどのように，これ以上に大きな種差を示すものもある．それらについては，一般的な動物実験結果からのみでは適切な予想は不可能である．化学構造や薬理作用からそのような可能性を考察するとともに，ヒト組織やヒト型標本を用いた検討結果も踏まえて適切な動物種を選択すべきである．また，必要に応じて，静脈内投与試験を行うことも必要と考えられる．

## 4．MD試験の意義と期待

MD試験の実施により早期に最適化合物を選択できることは，新薬開発の効率化とコスト削減につながるとともに，以下に示すような意義があると考えられる．

1）開発早期における薬物動態情報獲得に資する．
2）候補物質の評価が迅速化される．

* 国立医薬品食品衛生研究所
〒158-8501 東京都世田谷区上用賀 1-18-1

3）少量の被験物質で評価が可能であり，1ロットでの合成で非臨床試験からMD試験に必要な被験物質を確保できることから，GMPで通常求められる複数ロット間の品質の恒常性（連続性）を保証するためのバリデーションが不要である．
4）失敗に終わる臨床試験を減らし，志願者の協力に応えることができる．また，動物実験を削減でき，3Rの尊重に資する．
5）ヒトでの体内動態が確認されることによる医薬品候補物質の価値向上

なお，MD試験実施に際しては，以下のような考えが示された[3,7]．

1）放射性同位元素（RI）標識体を志願者に投与することについて，第三者の独立した委員会において適切な曝露評価を行うことにより，被験者への安全性についての懸念を除くことが望ましい．
2）インフォームドコンセントを受ける際に，試験の目的や事前に得られている動物実験等の非臨床試験データは第Ⅰ相試験の場合に比べ限定的であることなど，通常の臨床試験とは異なる点についてわかりやすい言葉で説明する必要がある．
3）PETに用いられるRI標識化合物の品質確保については，半減期が極めて短い核種が用いられることから，最終製剤で確認できる検査項目には限界がある．
4）治験薬の交付については，やむをえない事由があるときを除き，「治験依頼者は治験薬について第三者を介在させることなく，直接実施医療機関に交付しなければならない」とされているが，MD試験においては，放射性標識体の合成等の被験物質の製造および実施医療機関への交付について，外部事業者に行わせざるをえない場合や被験物質の製造を実施医療機関において行わなければならない場合もある．そこで，このような場合が上記の「やむをえない事由」として例示された．

## 5．その他

MD試験での用量と臨床用量との間には100倍以上の用量差があることから，その間の関係が線形であるか否かについての疑問がある．日本薬物動態学会[8]は「薬物濃度が代謝酵素，トランスポーターなどへのKm値に比べて十分に低いところでは，線形性が保たれる」ことは当然であり，それを否定する根拠はないと考えている．また，今日治療に用いられている医薬品の多くにおいて，臨床投与量では，溶解度が原因である場合を除けば薬物動態が原因で非線形性を生じる例は少ない[9]．吸収段階でのトランスポーターが臨床レベルで飽和するなど特別な場合を除き，低用量レベルでは理論的に線形となると考えられる．実際，Lappinら[10]による研究では5検体中3検体で線形性が見られた．しかし，実際の試験で証明した事例は少なく，今後，さらに事例を積み重ねることが重要と思われる．

薬効用量推定方法については，非臨床におけるモデル動物におけるPK/PD関係，ヒト組織やヒト酵素等の発現系を用いた*in vitro*試験等，ヒトへの外挿に適切と思われる評価系での結果を基に推定する必要があり，指針では基本的な考えが示されている．

なお，指針は主として低分子化合物を適用範囲としたものであるが，必ずしもそれ以外の物質についてMD試験実施を否定しているものではないが，その安全性について，投与方法や候補物質の特性を十分理解したうえで，より慎重な考察を行ったうえで実施することが求められる．

## 6．最後に

現在，ICHでは「医薬品の臨床試験のための非臨床安全性試験の実施時期についてのガイドライン」の見直しが行われており，2008年6月にStep 2案の合意がなされた[11]．そのなかでMD試験も含む早期探索的臨床試験について記述されている．この内容の骨子は，今回の指針と異なっており，Step 4の合意ができた時点で，それに合わせてガイダンスの内容も変更されるべきものである．すなわち，ICH案ではMD試験を必ずしも単回投与に制限せず，5回までの反復投与を許容し，5回の総投与量が100 μg以下のものと，500 μg以下のものに分け，それぞれについて必要な非臨床試験の範囲を定めている．

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*Original Article*

# The predictivity of preliminary embryo-fetal development (EFD) studies: results of a retrospective survey in Japanese pharmaceutical companies

**Noriko Okahashi[1,2], Takashi Ikeda[1,3], Shuichi Kai[1,4], Shinichi Komatsu[1,5], Hajime Matsui[1,6], Yasuhiro Yamashita[1,7], Keiji Yamamoto[1,8] and Fumio Sagami[1,9]**

*[1]Non-clinical Evaluation Subcommittee, Drug Evaluation Committee, Japan Pharmaceutical Manufactures Association, 3-4-1 Nihonbashi Honcho, Chuo-ku, Tokyo 103-0023, Japan*
*[2]Dainippon Sumitomo Pharma Co., Ltd., 33-94 Enoki-cho, Suita, Osaka 564-0053, Japan*
*[3]ASKA Pharmaceutical Co., Ltd., 1604 Shimosakunobe, Takatsu-ku, Kawasaki, Kanagawa 213-8522, Japan*
*[4]Bristol-Myers K.K., 5-1 Nishi-Shinjuku 6-chome, Shinjuku-ku, Tokyo 163-1328, Japan*
*[5]GlaxoSmithKline K. K., 6-15 Sendagaya 4-chome, Shibuya-ku, Tokyo 151-8566, Japan*
*[6]The Chemo-Sero-Therapeutic Research Institute, Kyokushi, Kikuchi, Kumamoto 869-1298, Japan*
*[7]Nippon Shinyaku Co., Ltd., 14 Nishinosho-monguchi-cho, Kisshoin, Minami-ku, Kyoto 601-8550, Japan*
*[8]Takeda Pharmaceutical Co., Ltd., 17-85 Jusohonmachi 2-chome, Yodogawa-ku, Osaka 532-8686, Japan*
*[9]Eisai Co., Ltd., 1-3 Tokodai 5-chome, Tsukuba-shi, Ibaraki 300-2635, Japan*

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**ABSTRACT** — To explore the predictivity of dose range-finding (DRF) studies, we conducted a survey by sending out questionnaires to 72 Japanese pharmaceutical companies. The survey yielded data for 108 and 85 compounds for which any embryo-fetal development (EFD) toxicities were observed in the definitive studies in rodents and non-rodents, respectively. As a result of the analysis, 83% of studies in rodents and 80% in non-rodents showed EFD effects in the DRF studies. When focusing on teratogenicity, 91% of studies in rodents and 100% in non-rodents were judged "positive" in the DRF studies when all EFD toxicities were used as markers. When the effects of both the rodent and non-rodent studies were evaluated together, the combination predictive value in the DRF studies was 96% for EFD toxicants and 100% for teratogens. To evaluate the influence of the examination items, the predictive value was analyzed using 54 compounds for which full examinations (external, visceral and skeletal examination) were conducted in both rodent and non-rodent DRF studies. When the results were judged by including or excluding skeletal and visceral examinations results, the predictive values were not significantly different. In conclusion, the results of this survey showed that a pair of the DRF studies in the rodents and non-rodents is useful to increase the predictivity of DRF studies. In addition the inclusion of observations such as fetal survival, body weight and external examination into the DRF studies are important to predict effects in the definitive studies.

**Key words:** EFD toxicity studies, Predictivity, Preliminary or DRF studies, Survey

## INTRODUCTION

In drug development, the need to avoid the risk to the embryo or fetus is imperative when including women of childbearing potential (WOCBP) in clinical trials. To achieve this objective, use of highly effective methods of birth control or pregnancy test before and during the clinical study is essential. In addition to taking precautions to prevent pregnancy during clinical trials, conducting embryo-fetal development (EFD) studies can be useful to characterize the inherent risk of a compound so as to be able to better inform of the risks if an exposure during pregnancy occurs, and to make an assessment of the suitability of the compound as a development candidate. The International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals

Correspondence: Noriko Okahashi (E-mail: noriko-okahashi@ds-pharma.co.jp)

for Human Use (ICH) has published ICH-M3 guideline (Non-Clinical Safety Studies for the Conduct of Human Clinical Trials for Pharmaceuticals) which addresses the principles for the development of non-clinical strategies and on the timing of toxicity studies in relation to the conduct of clinical trials. In M3 (R1) guideline, there had been regional differences in the timing of the EFD toxicity studies to support the inclusion of WOCBP in clinical trials, that is, EFD studies should be completed prior to the inclusion of WOCBP in any type of clinical trial in Japan and the EU, whereas, in the US, WOCBP may be included in early, carefully monitored studies without EFD studies (ICH harmonized tripartite guideline, 2000).

EFD studies are time-consuming and require technical expertise and large number of animals. The methods and endpoints for EFD studies are stated in the ICH-S5 (R2) guideline (Detection of toxicity to reproduction for medicinal products & toxicity to male fertility). In accordance with this guideline, the definitive EFD studies should be conducted with about 20 dams per group in 2 species (rodents and non-rodents) in compliance with good laboratory practice (GLP). From the point of view of appropriate animal usage in research, studies should be conducted at a time that is necessary to support clinical safety. However, one could question the need for such studies early in clinical trials where pregnancy is extremely unlikely while on clinical study. In contrast, the pregnancy rate in the clinical studies might be increased as clinical trials get longer and larger and less daily oversight is likely to occur. Considering these points together the balance between the scales of the clinical studies (duration of the treatment and number of the volunteers) and the sizes of the non-clinical studies are most important.

Generally, many pharmaceutical companies conduct preliminary or dose range-finding (DRF) studies before the definitive EFD studies. The DRF studies are conducted in the early stages of the drug development on a small scale and with limited endpoints. The results from the DRF studies have not been utilized for the evaluation of EFD toxicities, since the purpose was to set the dose for the definitive studies. However, they do routinely use pregnant animals. The DRF studies may vary between the companies and/or compounds because DRF studies are not regulated by the guideline. Moreover, the DRF methods, endpoints and/or predictivity for EFD effects are unknown because the details of the DRF studies have not been published.

To explore the possibility of using the information from DRF studies, we conducted a survey about the EFD studies done in the pharmaceutical companies which were members of the Japan Pharmaceutical Manufactures Association (JPMA) in order to investigate the predictivity of the DRF studies.

## MATERIALS AND METHODS

Questionnaires were distributed to 72 member pharmaceutical companies of the JPMA, and 24 responses were received (response rate 33%). The first survey was conducted during April 6-20, 2007 and the follow-up survey was conducted during October 16, 2007- January 15, 2008.

The questionnaires were provided by the ICH M3 expert working group to obtain a set of summaries of the results of preliminary or DRF and definitive EFD studies, using a formatted answer sheet including questions on the study design (fetal examination items and the number of the litters per group) and the maternal/fetal effects (effective dose levels and the types of fetal effects) (Fig. 1). The data set was sought for compounds which met all the following criteria: (1) an EFD study had been conducted within the past 10 years, (2) both the DRF and definitive studies had been performed, and (3) any EFD toxicities were observed in the definitive studies. The "any EFD toxicities" were defined as effects such as embryo-fetal death, decreased fetal body weight, fetal malformation or variation (external, visceral and/or skeletal) and/or fetal retardation of ossification. The therapeutic areas were not limited. The data sets, consisting of data from the preliminary or DRF study and the definitive study, were obtained for 108 studies for rodents (5 in mice and 103 in rats) and 85 studies for non-rodents (rabbits). The net number of the analyzed compounds was 136 since 57 studies among 193 studies (108 in rodents and 85 in non-rodents) were obtained from the same compounds. The numbers of the compounds for each company are shown in Table 1.

Persons in each company who were knowledgeable about reproductive and developmental toxicity were requested to respond. The answer sheets were returned to the JPMA staff who replaced company names with code numbers to blind the analysis that was then conducted by the authors. The follow-up survey for the compounds in which teratogenic effects were not detected in the DRF studies was done to clarify the details using a interview with each company.

The data were analyzed using the identical code assigned by the allocated company number, compound number and test species. The end points of the analysis were as follows; (1) study designs of the preliminary or DRF studies, (2) predictive value of the preliminary or DRF study to detect any of the embryo-fetal toxicities in each rodent or non-rodent definitive study, (3) predic-

**EMBRYO-FETAL STUDIES**

Please provide the number of compounds in each of the following 4 categories either for studies conducted over the past 10 years or for the last 50 compounds, whichever is lower.

Number of compounds for which NEITHER the exploratory/RF study NOR the definitive study had treatment-related fetal findings and/or malformations:
Number of compounds for which treatment-related fetal findings and/or malformations were present BOTH in the definitive study AND in the exploratory/RF study:
Number of compounds for which treatment-related fetal findings and/or malformations were present in the definitive study but NOT the exploratory/RF study:
Number of compounds for which treatment-related fetal findings and/or malformations were present in the exploratory/RF study but NOT the definitive study:

Please complete this table for compounds having treatment-related fetal findings and/or malformations in the definitive study.

| | | RF/Explor Study | | | | | Definitive Study | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Cmpd ID Code | Species[a] | Extent of F1 Exam (E/V/S)[b] | # F/gp | High Dose MFD or Produced Maternal Toxicity? | F1 Generation Findings[c] | Did Results Influence Inclusion of WCBP in Clinical Studies. If Yes, How? | Extent of F1 Exam (E/V/S)[b] | # F/gp | High Dose MFD or Produced Maternal Toxicity? | $F_1$ Generation Findings[c] | Did Results Influence Inclusion of WCBP in Clinical Studies. If Yes, How? |
| 1 | Rat | 100%E/ V,S:Not Exam | 7 | HD1,HD2:Maternal toxicity | HD1,HD2: whole litter loss, MD: Fetal death, low body wt | No | 100%E/ 50%V/50%S | 20 | No (Fetal lethal dose) | HD: Fetal death, low body wt, Visceral malformation (Ventricular septum defect) | Y: Contraindication in pregnancy |
| 1 | In rabbit studies, NEITHER the exploratory/RF study NOR the definitive study had treatment-related fetal findings and/or malformations. | | | | | | | | | | |
| 2 | Rat | 100%E/ 50%V/50%S | 7 | HD: Maternal death MD: Maternal toxicity | HD:Not Exam(No live fetuses) MD: low body wt, skeletal retardation | No | 100%E/ 50%V/50%S | 20 | HD: Maternal toxicity | MD: low body wt, skeletal retardation, skeletal variation(14th rib) | Y: Contraindication in pregnancy |
| 2 | Rabbit | 100%E/ 100%V/100%S | 6 | HD1,HD2:Maternal toxicity | None | | 100%E/ 100%V/100%S | 22 | HD, MD: Maternal toxicity | HD: Abortion, Fetal death, low body wt, skeletal retardation | |

[a] If evaluated in only one species, indicate reason.
[b] E/V/S = External/Visceral/Skeletal. Indicate % evaluated in each litter (e.g., 100%E / 50%V).
[c] Indicate findings by dose level using HD (High Dose), MD (Mid Dose), and LD (Low Dose) and include multiple of clinical dose or of systemic exposure.
MFD = Maximum Feasible or Limit Dose. WCBP=Women of Childbearing Potential

*16-Mar-2007

**Fig. 1.** Example of the answer sheet.

tive value of the preliminary or DRF studies to detect any of the embryo-fetal toxicities by the combination of the rodent and non-rodent studies, (4) influence of the items examined in the preliminary or DRF studies on the predictive value of EFD toxicities. The complete dataset and analysis was also reviewed by the ICH M3 working group.

## RESULTS

### Study design of the preliminary or DRF studies

The methods used in the DRF studies varied between the studies. In almost all the studies, caesarian section was conducted with examination for embryo-fetal death, and fetal body weights were measured. However, the number of dams per group and fetal examination items such as external, visceral and/or skeletal examinations were different between the studies. Regarding the number of the dams per group, 45 out of 108 studies used six dams per group in rodents. The range of the dam numbers per group was 3-13 (minimum −max) in rodents. In non-rodents, 41 out of 85 studies used six dams per group and the range of the dams was 3-8 (minimum −max) (Fig. 2). In the fetal morphological examinations, in about a half of the studies (44%, 48 out of 108 studies in rodents and 53%, 45 out of 85 studies in non-rodents) were only external examinations done in the DRF studies. In the remaining half of the studies (51%, 55 out of 108 studies in rodents and 41%, 35 out of 85 studies in non-rodents) full examinations (external, visceral and skeletal) in the DRF studies were done (Fig. 3).

In the results for maternal toxicity, 24 out of 108 compounds showed maternal deaths in the DRF studies, compared with 6 out of 108 compounds in the definitive studies in rodents. In non-rodent studies, 27 out of 85 compounds showed maternal deaths in the DRF studies, compared with 16 out of 85 compounds in the definitive studies.

### Predictivity of the preliminary or DRF studies to detect any EFD toxicities in the definitive studies

As described above, this analysis was conducted with the definitive studies showed some EFD toxicities. If any EFD toxicities including embryo-fetal death, decreased

**Table 1.** The number of compounds

| Company Code | Number of the studies in rodents | | Number of the studies in non-rodents | Number of the compounds |
|---|---|---|---|---|
| | Rats | Mice | Rabbits | |
| 1 | 2 | 0 | 2 | 2 |
| 2 | 2 | 0 | 1 | 2 |
| 3 | 3 | 0 | 1 | 3 |
| 4 | 1 | 0 | 1 | 2 |
| 5 | 1 | 0 | 3 | 3 |
| 6 | 2 | 0 | 2 | 3 |
| 7 | 0 | 1 | 0 | 1 |
| 8 | 26 | 1 | 23 | 33 |
| 9 | 2 | 0 | 1 | 2 |
| 10 | 9 | 0 | 4 | 9 |
| 11 | 2 | 0 | 2 | 3 |
| 12 | 2 | 0 | 4 | 4 |
| 13 | 9 | 1 | 11 | 14 |
| 14 | 1[a] | 1[a] | 0 | 1[a] |
| 15 | 1 | 0 | 0 | 1 |
| 16 | 1 | 0 | 0 | 1 |
| 17 | 7 | 0 | 2 | 8 |
| 18 | 0 | 0 | 1 | 1 |
| 19 | 6 | 0 | 4 | 7 |
| 20 | 2 | 0 | 1 | 2 |
| 21 | 1 | 0 | 0 | 1 |
| 22 | 13 | 1 | 12 | 21 |
| 23 | 2 | 0 | 2 | 2 |
| 24 | 8 | 0 | 8 | 9 |
| Total | 108 | | 85 | 136 |

[a]: The studies in rats and mouse were conducted with the same compound.

fetal body weight, fetal malformation or variation (external, visceral and/or skeletal) and/or retardation of ossification were detected in the DRF studies, the studies were regarded as "positive". The predictive value was calculated by the following formula: (the number of "positive" studies in the DRF studies/ the number of the analyzed studies)*100.

In the results of the analysis, the predictive values of DRF studies for EFD toxicities were 83% (90 out of 108 studies) and 80% (68 out of 85 studies) in rodents and non-rodents, respectively (Fig. 4).

## Predictivity of the preliminary or DRF studies to detect fetal "malformation" in the definitive studies

Focusing on the fetal malformations, the same analyses were conducted for the studies in which any malformations (external, visceral and/or skeletal) were detected in the definitive studies. The number of the analyzed studies was 34 for rodents and 21 for non-rodents. In these studies, malformations were detected in 53% (18 out of 34 studies) and 57% (12 out of 21studies) by the DRF studies in rodents and non-rodents, respectively (Fig. 5).

Another analysis was conducted in which the studies were regarded as "positive" when any EFD toxicity including embryo-fetal death, decreased fetal body

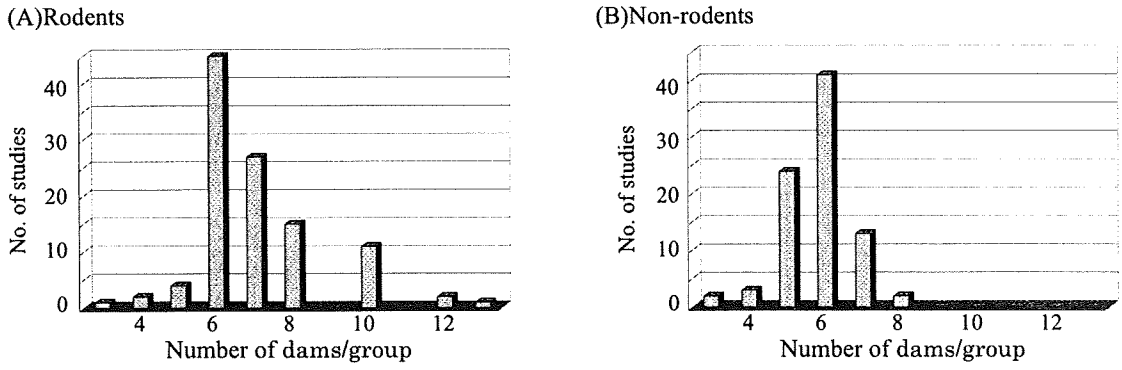


**Fig. 2.** The number of the studies in each number of the dams per group in the preliminary or DRF studies with rodents (A, Number of analyzed studies was 108.) and non-rodents (B, Number of analyzed studies was 85.).

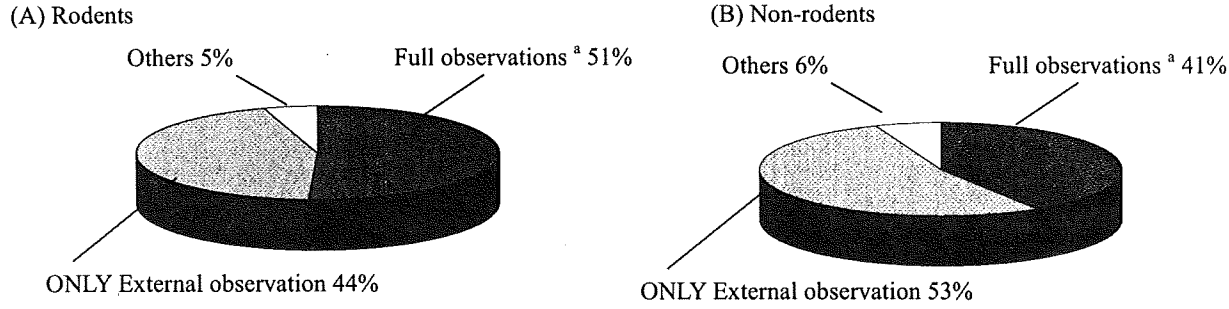


**Fig. 3.** The percentages of the studies with the observation items for the fetuses in the preliminary or DRF studies with rodents (A, Number of analyzed studies was 108.) and non-rodents (B, Number of analyzed studies was 85.).
[a]: Full observation means external, visceral and skeletal observations were performed.

weight, fetal malformation or variation (external, visceral and/or skeletal) and/or fetal retardation of ossification was detected in the DRF studies. In the results of the analysis, 91% (31 out of 34 studies) in rodents and 100% (21 out of 21 studies) in non-rodents were judged "positive" (Fig. 6).

Only 3 studies in rodents exhibited no EFD toxicity in the DRF studies because of a low incidence of malformations (2 studies; they had no effects on fetal death or body weights in both DRF and definitive studies.) or the lack of the appropriate examination (1 study; visceral and skeletal examinations were not conducted and only visceral malformation was observed in the definitive study.) in the DRF studies.

## Predictivity of the preliminary or DRF studies to detect any EFD toxicity or fetal "malformation" in the definitive studies by the combination of the rodent and non-rodent studies

When the effects of both the rodent and non-rodent studies were evaluated together, 56 compounds had both rodent and non-rodent data sets, that is, these 56 compounds showed EFD toxicity in both rodents and non-rodents in the definitive studies.

First, the analysis was conducted with "EFD toxicants", in which any EFD toxicity including embryo-fetal death, decreased fetal body weight, fetal malformation or variation (external, visceral and/or skeletal) and/or retardation of fetal ossification were observed in both the rodent or non-rodent definitive studies. The predictive value was calculated by the following formula: (the number of "positive" compounds in the DRF studies/ the number of the analyzed compounds)*100 In this formu-

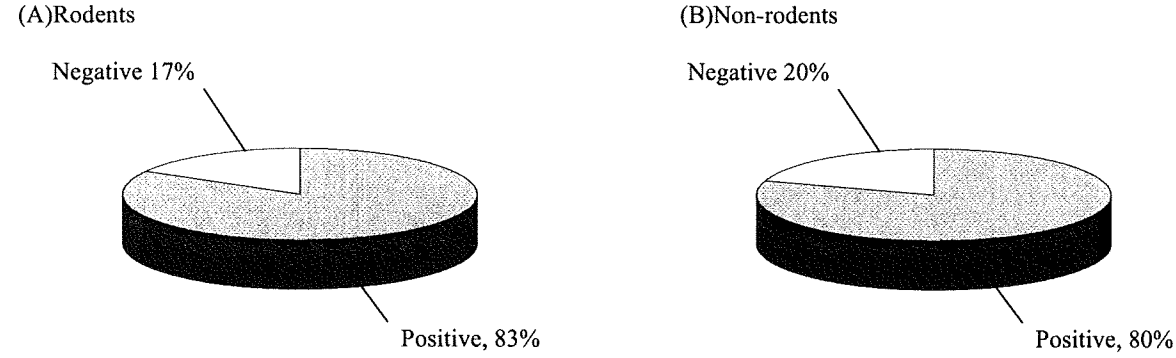


**Fig. 4.** The percentages of "positive" or "negative" studies in the preliminary or DRF studies for any EFD effects in rodents (A, Number of analyzed studies was 108.) and non-rodents (B, Number of analyzed studies was 85).
Positive: Effects on the any EFD were detected in the preliminary or DRF studies.
Negative: No effects on the EFD were observed in the preliminary or DRF studies.

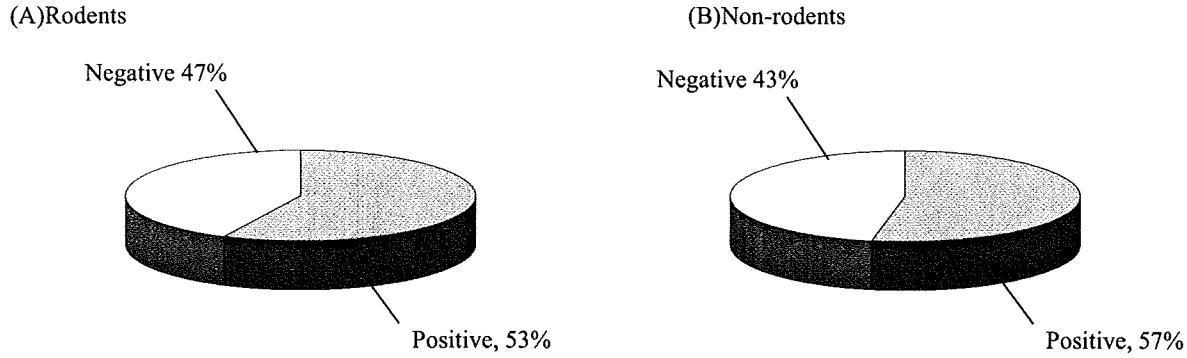


**Fig. 5.** The percentages of "positive" or "negative" studies in the preliminary or DRF studies for any fetal malformations (external, visceral and/or skeletal) in rodents (A, Number of analyzed studies was 34.) and non-rodents (B, Number of analyzed studies was 21.).
Positive: Any malformations were detected in the preliminary or DRF studies.
Negative: No malformations were observed in the preliminary or DRF studies.

la, "positive" meant any EFD effects were detected in both or either rodent and non-rodent preliminary or DRF studies. The combination predictive value for "EFD toxicants" was 96% (54 out of 56 compounds).

Next, focusing on the fetal malformations, the same analyses were conducted for the "teratogens" in which any malformations (external, visceral and/or skeletal) were detected in the definitive studies on both or either rodents or non-rodents. The combination predictive value for the teratogens which had both rodent and non-rodent data sets was 100% (23 out of 23 compounds) (Table 2).

## Influence of the examination items in the predictivity of the preliminary or DRF studies

To analyze the influence of the items examined, the predictive value was calculated assuming that fetal skeletal and visceral examinations were not conducted, that is, the EFD effects were evaluated only by embryo-fetal death, decreased fetal body weight and/or external examinations.

There were 54 analyzed compounds for which full examinations (external, visceral and skeletal examination) were conducted in both rodent and non-rodent DRF studies. The analysis was conducted in the same manner as the combination analysis described above. The combination

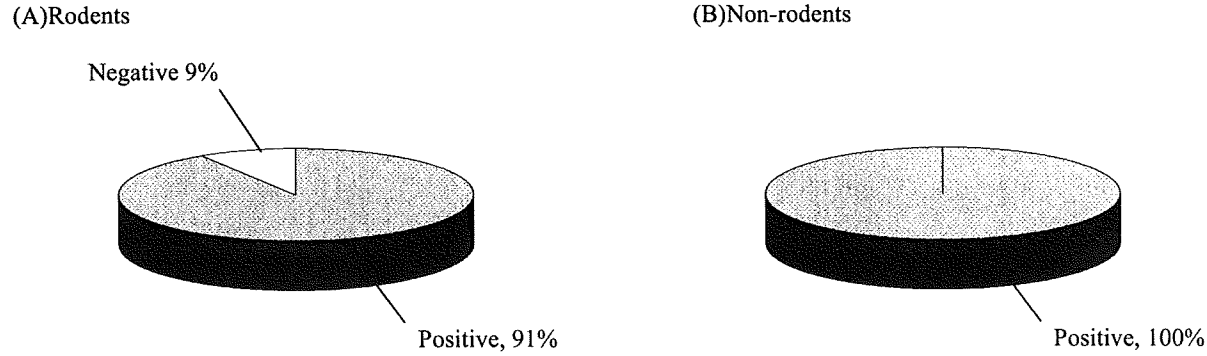


**Fig. 6.** The percentages of "positive" or "negative" studies in the preliminary or DRF studies for any fetal malformations (external, visceral and/or skeletal) in rodents (A, Number of analyzed studies was 34) and non-rodents (B, Number of analyzed studies was 21.).
Positive: Effects on EFD were detected in the preliminary or DRF studies.
Negative: No effects on EFD were observed in the preliminary or DRF studies.

**Table 2.** The Predictivity of preliminary or DRF studies by the combination of rodent and non-rodent studies

| | EFD toxicants[a] | | | Teratogens[b] | | |
|---|---|---|---|---|---|---|
| | Number of compounds | Number of "positive" compounds in the DRF studies | Predictivity (%)[c] | Number of compounds | Number of "positive" compounds in the DRF studies | Predictivity (%)[c] |
| Both rodent and non-rodent studies showed the EFD effects in the definitive studies | 56[d] | 54 | 96 | 23 | 23 | 100 |

[a]: Any EFD toxic findings including embryo-fetal death, decreased fetal body weight, fetal malformation or variation (external, visceral and/or skeletal) and/or fetal retardation of ossification were observed in both rodent and non-rodent definitive studies.
Positive: Any EFD effects were detected in both or either rodent and non-rodent preliminary or DRF studies.
[b]: Any fetal malformations were observed in both or either rodent or non-rodent definitive studies.
Positive: Any EFD effects were detected in both or either rodent and non-rodent preliminary or DRF studies.
[c]: Predictivity (%) = The number of "positive" compounds in the preliminary or DRF studies/ the number of the analyzed compounds*100.
[d]: Including the one compound which included two rodent data sets ( rats and mice) and a non-rodent data set.

predictive value was 93% (50 out of 54 compounds) and 87% (47 out of 54 compounds) for the "EFD toxicants", with full examination and without visceral and skeletal examinations results, respectively (Table 3). That is, the EFD effects could not be detected in 3 compounds without visceral and skeletal examinations results; two compounds showed only skeletal variation as a fetal effect in the DRF studies in rodents, and one compound showed only skeletal retardation as a fetal effect in the DRF studies in non-rodents.

Focusing on the fetal malformations, the combination predictive value was 95% (20 out of 21 compounds) for the "teratogens" in both situations (Table 3). Differences in the predictivity between the values including and excluding fetal visceral or skeletal examinations results were not evident.

In addition, another analysis was conducted to estimate the influence of the examination items. For the compounds for which full observations in the DRF studies were performed and teratogenicity in the definitive studies were noted, the results in the DRF studies and the definitive studies were compared (Tables 4 and 5). If external observation, fetal death and low fetal body weight are used as markers, only 1 of 17 compounds could not be

**Table 3.** Predictivity of preliminary or DRF studies by the combination of rodent and non-rodent studies In the case in which the embryo-fetal effects were evaluated only by embryo-fetal death and/or decreased fetal body weight

| | EFD toxicants [a] | | | Teratogens [b] | | |
|---|---|---|---|---|---|---|
| | Number of compounds | Number of the "positive" compounds | Predictivity (%) [c] | Number of compounds | Number of "positive" compounds | Predictivity (%) [c] |
| Evaluation by full examination | 54 | 50 | 93 | 21 | 20 | 95 |
| Evaluation only by embryo-fetal death, decreased fetal body weight, and/or external examination | 54 | 47 | 87 | 21 | 20 | 95 |

[a]: Any EFD toxic findings including embryo-fetal death, decreased fetal body weight, fetal malformation or variation (external, visceral and/or skeletal) and/or fetal retardation of ossification were observed in both rodent or non-rodent definitive studies.

[b]: Any fetal malformations were observed in both or either rodent or non-rodent definitive studies.

[c]: Predictivity (%) = The number of "positive" compounds in the preliminary or DRF studies/ the number of compounds analyzed *100.

detected the EFD toxicity in the DRF study in rodents. The compound showed no effects on skeletal and visceral observations so it would not be positive even if skeletal and visceral examinations were performed. In non-rodents, no compound (0 of 10 compounds) was missed.

## DISCUSSION

In this survey among member companies of the JPMA, we assessed the design of the DRF study in the rodents and non-rodents and their predictivity by comparing with the results of the definitive EFD toxicity studies conducted to GLP.

As for the study design in the DRF studies, six dams per group were most common and about a half of the studies were conducted with only external examination for fetal morphology in both the rodents and non-rodents. These results may indicate that the DRF studies are simply designed to set the dose range for the definitive studies rather to detect the detailed information about the EFD toxicity.

The number of the DRF studies showing maternal death was higher than that in the definitive studies in both rodents and non-rodents. These results may suggest that the maximum dose tested was relatively high in the DRF studies compared to the definitive studies. This is probably because the DRF studies are conducted with limited information.

Under these study conditions, the predictive values of the DRF studies to detect any EFD toxicities in the definitive studies was more than 80% in both rodent and non-rodent studies. There was no obvious difference between species for the predictivity.

For the teratogens which showed malformations in the definitive studies, the predictive value of the DRF study in which the malformations were detected was only about 50% in both the rodents and non-rodents. This may indicate that negative teratogenic findings in the DRF study do not preclude teratogenic findings in the definitive study where larger numbers of animals and more refined dose levels are used. However, the predictive value for the teratogenic findings in the definitive studies was higher (91% in rodents and 100% in non-rodents) when any developmental effects including fetal death and/or low fetal body weight in the DRF studies were used as potential predictive markers for adverse fetal effects. Therefore, the evaluation including the endpoints of fetal body weight and embryo-fetal death was considered to be important to evaluate potential teratogenicity. There were 3 compounds which showed no EFD effects in the DRF study but showed malformation in the definitive studies. The DRF studies for two compounds were conducted in the small scale as usual and the incidences of malformation in the definitive studies were very low, and one DRF study didn't conduct the full observation, thus, the DRF studies were likely underpowered to detect the effects.

The combination analysis with rodent and non-rodent results showed that the predictive values for the combination was higher than that for each result by a complementary effect and the values were 96% for "EFD toxicants" and 100% for teratogens. This result suggests that the predictivity becomes very high if a compound is studied in the DRF in 2 species.

The analysis of the influence of the observation items

**Table 4.** The compounds in which full observations in the DRF studies were performed and which showed teratogenicity in the definitive studies in rodents

| Company No. | Cmpd ID Code | DRF studies | | | | | Definitive studies | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Fetal death | Low fetal body weight | External malformation | Visceral malformation | Skeletal malformation | Fetal death | Low fetal body weight | External malformation | Visceral malformation | Skeletal malformation |
| 7 | 2 | | | X | | | | | X | | |
| 8 | 1 | | X | X | X | X | | X | | X | X |
| 8 | 26 | X | X | X | X | X | | X | | X | |
| 8 | 29 | X | X | X | | | | X | X | | |
| 8 | 35 | X | X | | | X | X | X | | X | X |
| 8 | 38 | | X | | | X | X | X | | X | X |
| 8 | 43 | X | X | X | X | X | | | | | X |
| 10 | 3 | X | | | | X | | | | | X |
| 10 | 6 | | X | X | | X | | X | X | | X |
| 10 | 13 | | | | | | | | X | X | X |
| 12 | 2 | X | | | | | | X | X | X | |
| 13 | 5 | X | | X | X | X | | X | X | X | X |
| 13 | 10 | X | | | | | X | X | X | X | X |
| 20 | 1 | X | X | X | | | X | X | X | | |
| 20 | 2 | X | X | X | X | X | X | | X | X | X |
| 22 | 2 | | X | | X | X | X | X | | X | X |
| 24 | 5 | | X | | | | | X | | | X |

X: The effects were observed.

**Table 5.** The compounds in which full observations in the DRF studies were performed and showed teratogenicity in the definitive studies in non-rodents

| Company No. | Cmpd ID Code | DRF studies | | | | | | Definitive studies | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Fetal death | Abortion | Low fetal body weight | External malform. | Visceral malform. | Skeletal malform. | Fetal death | Abortion | Low fetal body weight | External malform. | Visceral malform. | Skeletal malform. |
| 8 | 29 | X | | X | X | X | X | | | X | X | X | X |
| 8 | 39 | X | | | | | | X | | | | | X |
| 8 | 41 | X | | X | | | | X | | X | | X | |
| 8 | 43 | X | | | X | X | X | | | | X | | X |
| 10 | 6 | | | | X | | X | | | | X | | X |
| 12 | 1 | X | | | | | X | | | X | X | X | X |
| 12 | 2 | X | | X | X | X | X | X | | X | X | X | X |
| 18 | 1 | X | | | | X | | X | X | | | X | |
| 22 | 21 | X | | X | | | X | | | X | | X | X |
| 24 | 8 | X | X | X | X | X | X | X | X | | X | X | X |

X: The effects were observed.

revealed that the differences in the predictive value between the limited examinations (i.e., external observation only) and full examinations were not significant for teratogens and EFD toxicants. Moreover, there were no compounds which were detected only by visceral examination. It is well known that many EFD toxicants represent a continuum of increasing toxicity, with low doses producing growth retardation (low fetal body weight) and increasing doses producing malformations and then lethality (Rogers and Kavlock, 2007). Consequently, it is reasonable that the end points of fetal body weight and embryo-fetal death are useful markers to detect the embryo-fetal toxicants.

In conclusion, the results of this survey showed that a pair of the DRF studies in the rodent and non-rodent species are considered to be able to provide critical information which could be comparable to those from the definitive study if adequate dose levels, numbers of dams, appropriate observation items such as fetal survival, body weight and external examination are used. It should be noted that fetal lethality and decreased fetal body weights should be regarded not only as the signal of the developmental retardation but also as a signal that may herald teratogenic potential.

### Limitation of interpretation

There may be limitations to the generalization of the interpretation of this analysis because: 1) the numbers of the compounds and the companies are limited; 2) the interpretation was based on the hypothesis that there is no difference in methodology between the DRF study and definitive study except for dosage, number of dams and examination items; however, there could be unknown differences such as toxicokinetics in the dams. However, such differences are considered small because the companies have used these data to make their internal decisions on dose selection for subsequent studies. On the other hand, this survey focused on the sensitivity of the DRF study and therefore, the specificity of DRF studies has not been elucidated through this survey. The proposed setting of supporting early clinical development, however, sensitivity is considered to be more important for the purpose of early identification of the potential EFD toxicity. It is concluded from the above analysis that the DRF studies are sufficiently useful to support early clinical trails when strategies to minimize pregnancy rates are incorporated into the clinical design.

## ACKNOWLEDGMENTS

We acknowledge all of the researchers of the JPMA members replying willingly to the questions.

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—Full Paper—

# Long-Term Treatment with Bromocriptine Inhibits Endometrial Adenocarcinoma Development in Rats

**Midori YOSHIDA[1], Gen WATANABE[2], Tomo SUZUKI[3], Kaoru INOUE[1], Miwa TAKAHASHI[1], Akihiko MAEKAWA[4], Kazuyoshi TAYA[2] and Akiyoshi NISHIKAWA[1]**

*[1]Division of Pathology, National Institute of Health Sciences, Tokyo 158-8501, [2]Veterinary Physiology, Tokyo University of Agriculture and Technology, Fuchu, Tokyo 183-0054, [3]Pathology Group, Yakult Central Institute, Kunitachi 186-0011 and [4]Safety Assessment Division, Chemical Management Center, National Institute of Technology and Evaluation, Tokyo 151-0066, Japan*

**Abstract.** The effects of long-term blockade of prolactin (PRL) action by bromocriptine (BRC) treatment on uterine carcinogenesis and on related ovarian physiology were investigated using a rat uterine cancer model. Ten-week-old cycling female Donryu rats, a high yield strain for uterine corpus tumors (endometrial adenocarcinomas), were treated with *N-ethyl-N'-nitro-N-nitrosoguanidine* (ENNG), as a tumor initiator, and injected with 1 mg/kg body weight BRC subcutaneously 4 times per week until 14.5 months of age to block the proestrus PRL surge. The study was terminated at 15 months of age, and the results showed that long-term BRC treatment significantly inhibited endometrial adenocarcinoma development in terms of both incidence (34.6% to 13.0% with significant difference at 5%) and multiplicity (0.35 to 0.18 with significant difference at 5%), which indicates the number of adenocarcinomas per animals. While BRC did not affect estrous cyclicity in the treated animals, a significant decline was evident in the serum 17$\beta$-estradiol (E2) to progesterone (P) ratio (E: P ratio), and the serum E2 level showed a decreased tendency at 15 months of age. While the precise pathway to the inhibitory effect could not be determined; the pathway by which ovarian hormonal imbalance decreases the serum E: P ratio most likely plays a crucial role.

**Key words:** Bromocripchne, Long-term treatment, Prolactin blockade, Rat, Uteirne carcinogenesis

**(J. Reprod. Dev. 55:** 105–109, 2009)

Ovarian steroidhormone imbalance contributes to uterine carcinogenesis in human beings [1]. Maekawa *et al.* [2–4] have provided evidence that a similar imbalance, especially elevated 17$\beta$-estradiol (E2) levels relative to progesterone (P) levels (the E:P ratio), plays a crucial role in promoting endometrial adenocarcinoma development in rodents. While it is well-established that E2 and estrogenic chemicals play supportive or sometimes initiative roles in uterine carcinogenesis in rats as well as women, P may exert an inhibitory influence on human uterine cancer development [5–7]. However, the effects of other hormones related to the pituitary or ovaries on uterine carcinogenesis are not well known.

Prolactin (PRL) is one of the important regulators of the corpus luteum as well as luteinizing hormone (LH), prostaglandin and vascular endothelial growth factors and platelet derived growth factors in rats [8–10]. The function of PRL as a luteotrophic and luteolytic hormone, however, is very complex [11–17]. Repeated injection of PRL has been shown to stimulate rapid regression of the persistent corpus luteum, with a concomitant decline in total steroidogenic capacity [12, 18]. These studies indicate the possibility that the modulation of P production induced by PRL or its inhibitor affect uterine carcinogenesis through ovarian hormonal imbalance. On the other hand, PRL receptors, especially long-form ones (PRLR-L) [19], are present in the uteri of rats. PRL is known to play important roles in adenomyosis formation in Swiss mice [20–22]. Bromocriptine (BRC), a dopamine agonist, inhibits inverted growth of the uterine epithelium to the muscle layer in the uterine cervix in ovarietomized mice treated with E2 and PRL [23]. These reports indicate that direct action of PRL on the uterus should be considered as one factor related to uterine carcinogenicity in rats. Blockade of the proestrus PRL surge by treatment with BRC increases the weight of the ovary and the number of corpora lutea in rats without affecting estrous cyclicity [16, 24]. However the long-term effects of BRC treatment on the ovary or uterus have not yet been fully investigated.

In the present study, we therefore focused on the long-term effects of BRC on the ovary and uterine carcinogenesis using the Donryu rat, a high yield strain for endometrial adenocarcinomas.

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Correspondence: M. Yoshida (e-mail: midoriy@nihs.go.jp)

## Materials and Methods

*Animals and housing conditions*

A total of eighty female Crj:Donryu rats were purchased from Charles River Japan (Yokohama, Japan) at 7–8 weeks of age. The animals were maintained under conditions of controlled temperature (24 ± 2C), humidity (55 ± 10%), and lighting (12-h light/dark cycle). They were housed in plastic cages (3 or 4 animals/cage). Commercial powder diet (CRF-1, Oriental Yeast, Tokyo, Japan) and drinking water were available *ad libitum* throughout the study. Animal care and use were handled in accordance with the guidelines for the care and use of laboratory animals established by the Ethics Committee for Animal Experiments of Sasaki Institute and followed the NIH Guide for the Care and Use of Laboratory

Animals.

*Chemicals and selection of the dosing for bromocriptine*

BRC (α-ergocryptine; Sigma-Aldrich, St. Lous, MO, USA) was dissolved in DMSO (Wako Pure Chemicals, Osaka, Japan) to produce a dose of 1 mg/kg body weight for subcutaneous administration in 1 ml DMSO/kg body weight. This dose level was selected since it is known to block the proestrus PRL surge [18]. Control animals were administered DMSO only in the same manner.

*Impact on uterine carcinogenesis of long-term treatment with BRC*

Assessment of the long-term effects of BRC on uterine carcinogenicity was performed using the Donryu rat initiation-promotion assay model for uterine corpus cancer [25]. Briefly, fifty rats at 10 weeks of age were initiated with a single injection of 20 mg/kg *N-ethyl-N'-nitro-N- nitrosoguanidine* (ENNG; Nacalai Tesque, Kyoto, Japan) into one uterine horn via the vagina using a stainless steel catheter. This initiation is known to not be carcinogenetic in organs other than the uterus, and to not affect estrous cyclicity [26]. The animals were allocated into 2 groups, one receiving subcutaneous treatment with BRC five times per week up to 14.5 months of age and the other given DMSO only as the controls. Half a month before termination of the experiment, the treatment was ceased to avoid direct effects of the treatment on the serum hormone profiles. The Donryu strain rat has very regular 4-day estrous cyclicity, and about 60 to 70% of the PRL surge was estimated to be blocked in the treated animals. Clinical signs, body weight changes and estrous cyclicity were checked throughout the study. At 15 months of age, all surviving animals were euthanized by decapitation and necropsied for histopathological assessment and hormone assays as described below. Animals euthanized when moribund or found dead were also examined for histopathology.

*Pathology*

After complete necropsy of all rats, the ovaries, uteri, adrenals and livers were weighed. These organs and related tissues, including the pituitary, thymus, mammary gland, brain, vagina and sites with macroscopic abnormalities, were fixed in 10% neutral buffered formaldehyde solution and routinely processed for histopathological examination. Both the ovaries were dissected at the maximum transverse sections. In the present study, the upper, middle and lower parts of each uterine horn and the uterine cervix were cut into 3 pieces each in cross-section to detect uterine neoplastic lesions, and the lesions were classified into three degrees of atypical hyperplasia (slight, moderate or severe) and adenocarcinomas, according to the criteria described previously [27]. Atytical hyperplasia was considered to be a precancerous lesion of endometrial adenocarcinoma [27]. Uterine neoplastic lesions were evaluated using the following 2 indicators: the number of animals bearing the most serious neoplastic lesions and the frequency of each neoplastic lesion per animal were expressed as the incidence and multiplicity, respectively.

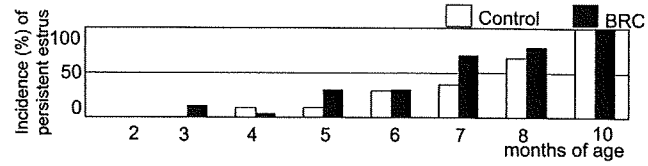


**Fig. 1.** Incidence of rats showing PE at vaginal cytology until 10 months of age, at which point all animals showed PE. White column (Control): the control group. Black column (BRC) the 1 mg/kg BRC-treated group. No significant differences were detected throughout the treatment.

*Radioimmunoassays*

Serum samples obtained after decapitation were stored at –80 C until assay. The serum concentrations of follicle-stiumulating hormone (FSH), lutenizing hormone (LH), inhibin, E2, P and PRL were determined using double-antibody radioimmunoassays and $^{125}$I-labelled radio-ligands. National Digestive and Kidney Disease (NIDDK) radioimmunoassay kits were employed for rat FSH and LH (NIAMDD, NIH, Bethesda, MD, USA) as described by Taya *et al.* [28] and Watanabe *et al.* [29], respectively. Immunoreactive inhibin in the serum was analyzed using a rabbit anti-serum, TNDH-1[31]. The serum concentrations of E2 and P were also measured as described by Taya *et al.* [29].

*Statistical analysis*

Incidences values were statistically analyzed using the Fisher's exact probability test. Other data were assessed using t-test (2 groups), and post hoc comparisons between the treated and control groups were made with the Dunnett's *t*-test.

## Results

Long-term BRC-treatment did not affect the growth curve or general condition (data not shown). With regard to estrous cyclicity, the vaginal cytology of the BRC-treated group was not different from that of the control group throughout the study. The results were similar to in regard to the occurrences of persistent estrus (PE), abnormal cyclicity including irregular estrous cycle and alterations of cellular components of vaginal smears between the control and BRC-treated groups (Fig. 1). At the end of the study, most of the ovaries were markedly atrophic in both the control and BRC-treated animals (Fig. 2), and no significant differences were detected in the absolute and relative weights of the ovary, uterus, pituitary, liver and spleen between the two groups (Table 1).

Histopathologically, the long-term BRC treatment caused significant reduction in the incidence of endometrial adenocarcinoma and the multiplicity of uterine neoplastic lesions, including both endometrial atypical hyperplasias and adenocarcinomas (Table 2). All adenocarcinomas observed in the control and treated-groups were well-differentiated; poorly- or moderately-differentiated ones were not observed in the BRC-treated animals. The incidence of adenocarinomas was decreased in BRC-treated animals, whereas the incidence of severe atypical endometrial hyperplasia was

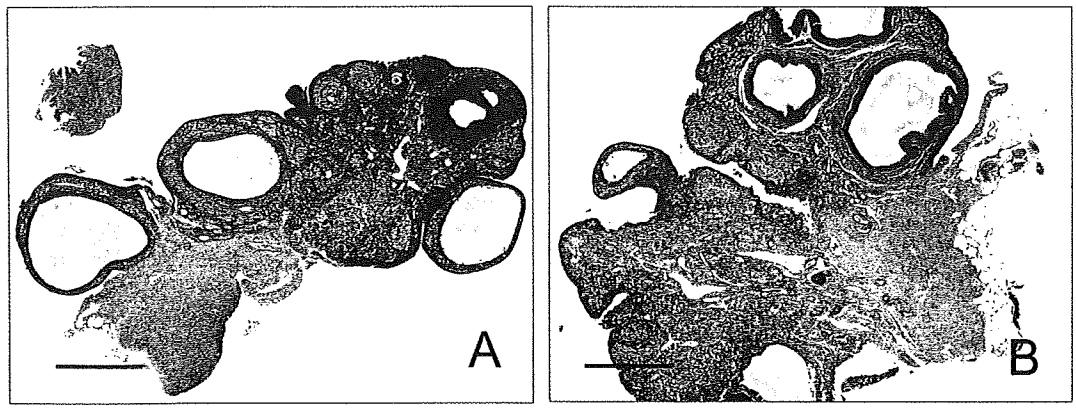


**Fig. 2.** Histopathology of the ovaries of rats in the control (A) and long-term BRC treatment (B) groups. Both ovaries showed atrophy with cystic atretic follicles and lack of corpora lutea. Bars represent 500 $\mu$m. Hematoxylin-eosin staining.

**Table 1.** Relative organ weights[a] after long-term of treatment with BRC

| | Control | BRC-treated |
|---|---|---|
| No. of rats at termination | 25 | 23 |
| Pituitary | 3.18 ± 2.11[b] | 2.14 ± 0.31 |
| Ovaries | 12.9 ± 0.72 | 14.0 ± 0.38 |
| Uterus | 325.4 ± 40.1 | 308.5 ± 16.4 |
| Spleen | 173.0 ± 25.1 | 186.1 ± 14.9 |
| Liver | 348.0 ± 48.9 | 332.1 ± 43.2 |

[a] Organ weights (mg)/body weight (g) × 100.
[b] Mean ± SD.

increased in this group. The atrophic ovaries of both the BRC-treatment and control groups exhibited similar degrees of cystic or atretic follicles associated with few or no corpora lutea. The occurrences of adenomas and/or adenocarcinomas in the anterior pituitaries or mammary fibroadenomas in the BRC-treated group were comparable to those in the controls. The incidences and intensities of other neoplasms and precancerous lesions were also comparable in the BRC-treated animals and control animals.

At the end of study, the levels of ovarian-derived hormones, such as E2, P and inhibin, at 15 months of age were not significantly different between the BRC-treated and control animals. The E2 levels showed a tendency of decrease in the BRC-treated group (Fig. 3). The ratio of E2 to P was significantly depressed in the BRC-treatment group. The values of pituitary derived hormones, such as PRL, FSH and LH, varied and showed no particular trends.

## Discussion

In the present study, long-term BRC treatment significantly inhibited uterine endometrial adenocarcinoma development with regard to both incidence and multiplicity. The incidence of severe atypical endometrial hyperplasia accepted as a precancerous lesion in multi-step tumorigenesis was paradoxically increased, indicating that BRC treatment might inhibit some process in the development of precancerous lesions and step up cancer formation, although the precise mechanism of this was not determined.

Considering the mechanisms underlying the inhibitory effects, decrease of the serum E:P ratio via through ovarian hormonal imbalance most likely plays a crucial role, since the hormonal changes, including the tendency of the serum E2 level to decrease and the significant decrease in the serum E:P ratio, were observed

**Table 2.** Incidence and multiplicity of uterine neoplastic lesions

| Group | No. of rats | None | Hyperplasia | | | Adenocarcinoma | |
|---|---|---|---|---|---|---|---|
| | | | Slight | Moderate | Severe | | |
| Incidence | | | | | | | |
| Control | 26 | 2 | 3 | 7 | 5 | 9 | |
| BRC-treated | 23 | 0 | 2 | 7 | 11* | 3* | |
| | | | Hyperplasia | | | Adenocarcinoma | Average number of neoplastic lesions |
| | | | Slight | Moderate | Severe | | |
| Multiplicity | | | | | | | |
| Control | | | 0.23 ± 0.51 | 0.64 ± 0.64 | 0.38 ± 0.64 | 0.35 ± 0.49 | 1.58 ± 0.81 |
| BRC-treated | | | 0.23 ± 0.51 | 0.17 ± 0.49 | 0.52 ± 0.59 | 0.18 ± 0.49* | 1.35 ± 0.49 |

* Significantly different from the control groups ($P<0.05$).

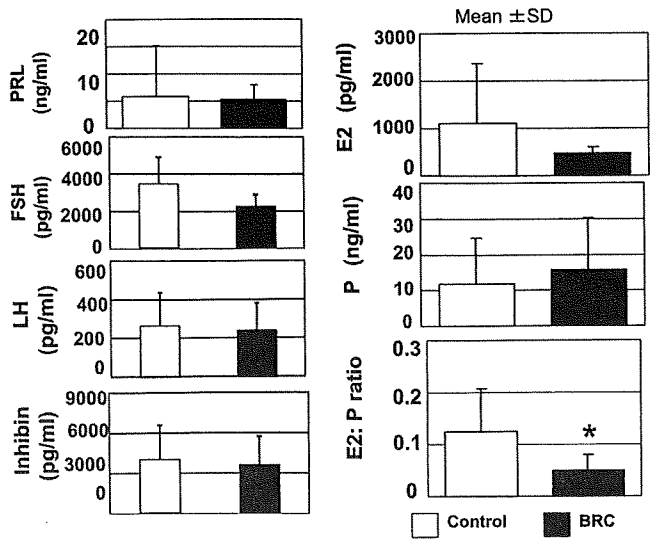


**Fig. 3.** Serum hormone profiles at 15 months of age, the end of the study. White column (Control): the control group. Black column (BRC): 1 mg/kg BRC-treated group. The E2: P ratio was calculated as E2 (pg/ml) divided by P (ng/ml). Although the FSH, LH, inhibin and progesterone levels were not different between the control and BRC-treated groups, the E2: P ratio was significantly lower in the BRC-treated group. The E2 levels of the rats in the BRC-treated group tended to increase, but there was not significant difference. *P<0.05.

in the present study. The precise mechanism, however, was not determined in the present study. The Donryu strain rat used here is a useful animal model for endometrial adenocarcinoma in the uterine corpus, particularly the endometrioid type [4]. This strain of rat is not only a high yield strain, but also has the following 3 similarities to humans [1, 4]: 1) multi-step development from atypical hyperplasia of the glandular epithelium to adenocarcinoma; 2) change in morphological and gene expression profiles; and 3) consistent elevation of the serum E2: P ratio manifested by early occurrence of atrophic ovaries with cystic atretic follicles and lack of a corpus luteum, resulting in persistent estrus (PE) on vaginal cytology [2, 3]. These features indicated that ovarian hormonal imbalance is crucial for uterine carcinogenesis in rats as well as humans [2–4]. Many previous studies have provided evidence that delayed onset of PE and/or depression of the serum E2 level can prevent adenocarcinoma development in rats [31–33]. The present study supports our hypothesis that an increased E:P ratio is very important for promoting effects on uterine carcinogenesis. There are a number of studies showing that PRL, E2 and/or their receptors control each other through endocrine and autocrine/paracrine mechanisms [34–36]. While the BRC treatment did not affect estrous cyclicity in the present study, the decrease in the serum E2: P ratio and tendency of the serum E2 levels to decrease might be related to the inhibitory effect on uterine cancer development. Subcutaneous treatment with 1 mg/kg body weight BRC did not modulate regular estrous cyclicity in the present study. The sensitivity of the response to BRC treatment in the aged ovary under PE conditions might be different from that in adults with normal estrous cyclicity.

BRC might act directly on the uterus in related to uterine carcinogenesis, because PRLR-L is predominantly located in the rat uterus [19]. The effects of PRL and BRC on the uterine proliferating lesions in the uteri of mice are controversial [23]. Mori *et al.* reported that an increase in the plasma level of PRL induces adenomyosis in mice through increased expression of PRL receptor mRNA in the uterus [20–22]. On the other hand, BRC-treatment for 30 days induces proliferation of endometrial epithelial cells in mice [37]. The present study did not provide any clear evidence showing long-term BRC treatment has any direct action on uterine carcinogenesis in rats.

When rodent model data are extrapolated for human carcinogenicity predictions, it is very important to pay attention to the differences in modes of action between rodents and humans. The functional effect of PRL on the rat ovary is quite different from that in humans. Whereas PRL directly leads to atrophic corpora lutea or luteolysis in rodents, this does not occur in women [38, 39]. Although BRC has been used clinically for therapy in patients with prolactinomas for long-term, there is little information available showing thatt therapeutic BRC affects ovarian and uterine function, and this suggests that the influence observed in the present study may be restricted to the rat. However, there remain unclear points in regard to the effects of BRC on uterine carcinogenesis in the rat model. Therefore, further investigation is necessary to clarify the differences between the data collected from animals and women.

In conclusion, the present results indicate that long-term BRC treatment inhibits uterine cancer development. The major pathway to the inhibitory effect could not be determined; however, there is a very plausible link to ovarian hormonal alterations resulting in a decrease in the serum E: P ratio.

## Acknowledgements

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