# Blood Collection Procedure of Laboratory Primates: A Neglected Variable in Biomedical Research

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A survey of 75 biomedical articles dealing with stress-dependent blood parameters in caged primates revealed that the conditions under which blood collection occurred were in most cased described either not at all or so haphazardly that it would be impossible to determine if humane handling procedures were used and basic principles of scientific methodology applied. These findings were unexpected because not only is there ample scientific evidence that stress-sensitive research data are influenced by traditional blood sampling procedures, but also that those data-biasing effects can be avoided. If dependent variables of the blood collection procedure are not controlled, data variability will increase thereby automatically also increasing the number of animals needed for statistical analysis. For ethical and scientific reasons, it was recommended that editors of biomedical journals should require that authors provide sufficient information of the blood collection – and when applicable also of the sedative injection – procedure to assure that the experiment was done with the smallest number of animals possible to achieve statistical significance, and that the investigation can be replicated reliably in another laboratory and the research data interpreted with reasonable accuracy.

Blood collection is a procedure to which laboratory primates are commonly subjected, as most biomedical research parameters are extracted from blood specimens. Usually, forceful manual or mechanical restraint is applied (e.g., Moreland, 1965, Fig. 7; Fielder, & Casmer, 1966, Fig. 3; Schultea, & Stein, 1981, Fig. 1; Reinhardt, 1998, Fig. 2a) or ketamine [a sedative/anesthetic] injected to immobilize the subject (International Association of Microbiological Societies, 1969; Fowler, 1995), because it is traditionally believed that the animals are difficult and even dangerous to handle (e.g., Gisler, Benson, & Young, 1960; Ackerley, & Stones, 1969; Valerio, Miller, Innes, Courtney, Pallotta, & Guttmacher, 1969; Altman, 1970; Whitney, Johnson, & Cole, 1973; Henrickson, 1976; Robbinson, Zwick, Leedy, & Stearns, 1986), and that it would take too long to train subjects to voluntarily cooperate – rather than resist – during the procedure (e.g., Klein & Murray, 1995; Hrapkiewicz, Medina, & Holmes, 1998). Needless to say, forceful restraint is usually also employed for ketamine injection (e.g., Ackerley, & Stones, 1969, Fig. 3). In either way, there is a high probability that being restrained and subjected to a potentially painful handling procedure is a distressing experience not only for a human but also for a nonhuman primate (cf. Public Health Service, 1996), particularly when the operation takes place in a treatment room rather than in the familiar home environment (cf. National Research Council, 1998). The stress reactions affect normal physiological functions making it impossible to interpret research data accurately. It's very simple: "Stressed animals do not make good research subjects" (American Medical Association, 1992, p. 18). Many biomedical investigators using primates as research models have specified this methodological concern.

Bush, Custer, Smeller, and Bush (1977) cautioned that the stress placed on a primate during blood collection involving restraint must be recognized as a data-influencing variable if the experimental procedure is to be considered scientifically reliable. Morton, Abbot, Barclay, Close, Ewbank, Gask, Heath, Mattic, Poole, Seamer, Southee, Thompson, Trussell, West, and Jennings (1993) recommended that it is in the interest of good science, as well as of animal welfare, to check the method of blood sampling for any physiological changes associated with restraint stress. Ferin, Carmel, Warren, Himsworth, and Frantz (1976) and Wickings and Nieschlag (1980) observed that primates are readily alarmed by restraint, and that it may be difficult to obtain stable control levels for hormones which are influenced by stress.

Mason, Wool, Wherry, Pennington, Brady, and Beer (1968) and Herndon, Turner, Perachio, Blank, and Collins (1984) demonstrated that growth hormone secretion is extremely sensitive to the psychological disturbance associated with restraint, complicating the interpretation of blood sample measurements. Fuller, Hobson, Reyes, Winter, and Faiman (1984) warned that determination of basal levels of reproductive hormones presents a problem since restraint for venipuncture may introduce sufficient stress to change endocrine secretion. The authors found that ketamine doesnot eliminate the stress which results from restraint during injection of the drug. Elvidge, Challis, Robinson, Roper, and Thorburn (1976) and Line, Markowitz, Morgan, and Strong (1991) made a similar observation, and Crockett, Bowers, Sackett, and Bowden (1993) recommended that this technique of sedation should be used judiciously because of its impact on research data. Aidara, Tahiri-Zagret, and Robyn (1981) noticed that enforced injection of ketamine resulted in a significant increase in serum prolactin, and assumed that the stress related to the injection could have been the cause of this increase. Findings of Castro, Rose, Green, Lehner, Peterson, and Taub (1981) made it clear that ketamine itself is unlikely to affect endocrine parameters.

Loomis, Henrickson, and Anderson (1980) and Wall, Worthman, and Else (1985) underscored that restraining a macaque to facilitate venipuncture or injection represents a stressful situation, which results in hematological alterations and, therefore, must be taken into account when assessing normal values and when interpreting experimental data. Ives and Dack (1956) termed this phenomenon as "alarm reaction" (p. 728). Verlangieri, De Priest, and Kapeghian (1985) found undesirable variations in serum biochemical parameters (cf. Cope & Polis, 1959) and proposed that incongruities between values presented in different reports may partly be due to the method of restraint during blood collection. Streett and Jonas (1982) cautioned that restraint may introduce major variables preventing the establishment of baseline plasma glucose tolerance curves.

Mason (1972) and Mason, Mougey, and Kenion (1973) noted that macaques show significantly higher urinary cortisol and catecholamine levels when they are restrained in a strange versus a familiar environment. The authors inferred that the familiarity of the location in which the restraint procedure occurs is a dependent variable which contributes to the variance of research data. This implies that the stress response to being removed from the home cage affects subsequently collected scientific data even before the animal is exposed to the additional stress associated with venipuncture and the actual experiment. Reinhardt, Cowley, Eisele, and Scheffler (1991) took blood from macaques on different days in their home cage and in a treatment cage located in the hallway. Cortisol response to venipuncture was significantly higher in the hallway than in the home cage, suggesting that removing an individual from the usual environment may already skew the interpretation of experimental findings independently of the stress that may accrue from venipuncture. This inference is supported by the fact that the animals exhibit behavioral signs of distress (Mitchell & Gomber, 1976) – including supression of activity, sleep and appetite (Crockett, Bowers, Sackett, & Bowden, 1993) – and elevated costisol levels when being removed from their home cages (Line, Clarke, & Markowitz, 1987) and transferred to identical cages in a different room (Phoenix & Chambers, 1984; Crockett, Bowers, Shimoji, Leu, Bowden, & Sackett, 1995).

As a result of adverse conditioning (Nahon, 1968; Robbins, Zwick, Leedy, & Stearns, 1986), primates used for biomedical research typically show signs of apprehension, fear and excitation when an investigator or technician enters their room (Tatoyan & Cherkovich, 1972; Malinow, Hill, & Ochsner, 1974; Manuck, Kaplan, and Clarkson, 1983; Line, Morgan, Markowitz, & Strong, 1989; Schnell, 1997; Boinski, Gross, & Davis, 1999; cf. Reinhardt & Reinhardt, 2000a, Figs. 63 & 64). The magnitude of the alarm reaction and its impact on research findings will depend on the time lapse from entry until the ketamine injection or venipuncture occurs. Often, several animals of a room are handled in succession, and the time lapse will therefore differ for each subject, introducing uncontrolled variability into the reseach data. Disturbance time – or "the `queue' effect of treating animals sequentially" (Fox, 1986, p. 36) – is a critical variable which needs to be taken into consideration for data-quality reason. Flow and Jaques (1997), for example, cautioned that disturbance time can affect thyroid hormone levels in macaques and hence has important implications for research studies that are designed to detect differences in thryoid function among experimental groups. Boccia, Broussard, Scanlan, and Laudenslager (1992), demonstrated that cortisol measures are highly correlated with disturbance time. Capitanio, Mendoza, and McChesney (1996) found that the numbers of CD8+ lymphocytes [a stress-sensitive hematological parameter] were 50% higher in macaques sampled 6-9 minutes after the investigor's initial entry into the room than in subjects sampled after a time lapse of `only' 3-6 minutes. The authors suggested that such variation might lead to inaccurate research results.

There are a few reports which offer solutions to the problem of data-confounding stress effects resulting from the blood collection procedure.

Dettmer, Phillips, Rager, Bernstein, and Fragaszy (1996) carefully habituated capuchin monkeys to the mechanical restraint during venipuncture; subjects exhibited no significant cortisol response as opposed to experienced but non-habituated subjects. Yeoman, Williams, Hazelton, Ricker, and Abee (1988) avoided in squirrel monkeys elevations of estradiol and luteinizing hormone levels resulting from ketamine injection by thoroughly habituating the animals to blood collection involving manual restraint without prior sedation. Findings of Golub and Anderson (1986), Line, Markowitz, Morgan, and Strong (1991) and Schnell and Wood (1993) indicate that rhesus macaques and marmosets are more difficult to habituate to being restrained for injection or venipuncture. Streett and Jonas (1982), however, were able to habituate stump-tailed macaques with a positive reinforcement method so well to being restrained during blood collection that reliable glucose tolerance testing could be performed. Hearn (1982) habituated marmosets to a restraint device for blood sampling and states – without providing supportive data – "that measurements of adrenocortical hormones and prolactin show that the animals are not affected by stress" (p. 139), or at least that the apparatus allows a small monkey to be restrained with a "minimum of stress" (Hearn, 1977, p. 262).

Several authors have reported that they worked with laboratory primates who were trained to voluntarily present a limb for injection: baboons (Levison, Fester, Nieman, & Findley, 1964), mandrills (Priest, 1991), and chimpanzee (Byrd, 1977; Laule, Thurston, Alford, & Bloomsmith, 1996; Reichard, & Shellaberger, 1992).

However, as yet there is no experimental evidence that cooperative animals show less physiological stess reactions than subjects who are restrained during injection. Photographs and videotape scenes of unrestrained rhesus macaques who were trained to cooperate during injection in the home cage demonstrate very clearly that the subjects experienced, at least, no psychological stress (Reinhardt, & Dodsworth, 1989, Scenes 11 & 22; Reinhardt, 1992, Fig. 3; Reinhardt, 1997, Fig. 18; Reinhardt, & Reinhardt, 2000b, Fig. 2).

Elvidge, Challis, Robinson, Roper, and Thorburn (1976) trained macaques to offer a leg for blood sampling, and achieved cortisol values which were significantly lower than those of untrained or ketamine-injected animals. Michael, Setchell, and Plant (1974) evaluated cortisol concentrations in macaques who were trained to enter a restraining apparatus and permit venipuncture without showing behavioral signs of stress. Values were 30% lower than had previously been reported for untrained animals. Reinhardt (1992) observed that the typical, significant cortisol response and acute fear reactions during blood collection on treatment tables or in treatment cages can be avoided if the subject is trained to cooperate in his/her familiar home cage. Vertein and Reinhardt (1989), Reinhardt (1991) and Reinhardt and Cowley (1992) spent less than a cumulative total of 45 minutes per subject to train adult rhesus and stump-tailed macaques to cooperate during in-home cage blood sampling. The time investment quickly paid off in a strict control of the time lapse from initial entry into the room until completion of blood collection: The range of disturbance time was only 1-2 minutes, compared to 1-20 minutes reported for untrained animals (e.g., Capitanio, Mendoza, & McChesney, 1996).

Successful training for cooperation in venipuncture has been reported by numerous investigors not only for macaques (cf. Bernstein, Rose, & Gordon, 1977; Rosenblum & Coulston, 1981; Bunyak, Harvey, Rhine, & Wilson, 1982; Hein, Schatorje, Frencken, & Segers, 1989; Scallet, McKay, Bailey, Ali, Paule, Slikker, & Rayford, 1989; Phillippi-Falkenstein & Clarke, 1992; Eaton, Kelley, Axthelm, Iliff-Sizemore, & Shiigi, 1994) but also for vervet monkeys (Suleman, Njugana, & Anderson, 1988), baboons (Chambers, Gibson, Bindman, Guillou, Herbert, Mayes, Pool, Wade, & Wood, 1992), mandrills (Priest, 1990), chimpanzees (T-W-Fiennes, 1972; McGinnis & Kraemer, 1979; Laule, Thurston, Alford, & Bloomsmith, 1996), and marmosets (Moseley & Davis, 1989). The Home Office (1989), the International Primatological Society (1989) and the National Research Council (1998) underline the importance of training in order to reduce the stress of restraint and to promote quality data (cf. Klein & Murray, 1995; Baskerville, 1999; Laule, 1999). In addition, training nonhuman primates to cooperate rather than resist during blood collection increases the safety of the personnel since the research subject is no longer given any reason for self-defense reactions such as biting or scratching (Anderson, & Houghton, 1983; Reinhardt, 1991; Reichard, & Laule, 1993).

One of the fundamental principles of scientific biomedical methodology is the rigorous control of extraneous variables which might influence the variability of research data and hamper the replication of the study by other laboratories (Claassen, 1994; Woolley, 1997). "Unless these requirements are met, an experiment is not considered scientifically valid" (American Medical Association, 1992, p. 5). This is one reason why laboratory animals – including nonhuman primates – are often kept under standardized, extremely boring housing conditions. The present article tests the hypothesis that biomedical investigators also take variables into account which pertain to the blood collection procedure as an assurance for scientific validity of the research methodology.

## METHOD

A survey was conducted of all scientific articles published since inception in the *Journal of Medical Primatology* (Vols. 1-28) and the *Journal of American Primatology* (Vols. 1-49). Studies assessessing stress-sensitive blood parameters were screened for the following methodological details (variables):

1. Was the subject sedated with ketamine prior to venipuncture?
2. How long did it take from entering the animal room to injection of ketamine and venipuncture, respectively?
3. Where did the injection of ketamine, and where did venipuncture take place?
4. How was the subject handled during injection and venipunture, respectively?

## RESULTS

A total of 75 scientific articles were dealing with one or several stress-sensitive parameters (Table 1) in blood collected from caged macaques (58), vervet monkeys (7), capuchin monkeys (4), marmosets (3), squirrel monkeys. (2), and baboons (1). Regarding the first variable, 76 % of the studies specify whether or not the research subjects were injected with ketamine prior to venipuncture (Table 2). Twelve percent of the studies mention the time lapse from the investigator's entry into the animal room to ketamine injection (1%) or venipuncture (11%; Table 2). Sixteen percent of the studies disclose if the research subjects were injected or venipunctured in their home cages or away from them in corridors or treatment areas (Table 2), and 27% of the studies explain if the research subjects were habituated or trained for injection or venipuncture, or if they were forcefully restrained during these procedures (Table 2). Seventy-two percent of the articles provide no information (16) or mention only one (38) of the four variables pertaining to the blood collection procedure. Twenty-eight percent (21/75) of the articles elaborate on two (9), three (10) or all four (2) of these variables.

**TABLE 1**

| PARAMETERS ASSESSED IN ARTICLES SURVEYED | |
|---|---|
| Reproductive hormones | 48 |
| Cortisol | 12 |
| Adrenocorticotrophic hormone | 4 |
| Catecholamine | 1 |
| Insuline | 2 |
| Growth hormone | 1 |
| Immune stress response | 4 |
| Hematology | 9 |
| Serum chemistry | 9 |

**TABLE 2**

| DETAILS OF BLOOD COLLECTION PROCEDURE DISCLOSED IN ARTICLES SURVEYED | |
|---|---|
| **(1) Is the subject injected with Ketamine prior to venipuncture?** | |
| (a) Yes<br>(b) No<br>(c) Not mentioned | 24<br>33<br>18 (24%) |
| **(2) What it the time lapse from intry into the room to injection/venipuncture?** | |
| (a) 2 minutes (injection)<br>(b) 1-20 minutes (venipuncture)<br>(c) Not mentioned | 1<br>8<br>66 (88%) |
| **(3) Where does injection/venipuncture take place?** | |
| (a) In home cage<br>(b) Away form home cage<br>(c) Not mentioned | 6<br>6<br>63 (84%) |
| **(4) How is the subject handled during injection/venipuncture?** | |
| (a) Subject is habituated<br>(b) Subject is trained to cooperate<br>(c) Subject is restrained<br>(d) Not mentioned | 4<br>6<br>10<br>55 (73%) |

## DISCUSSION

The present survey indicates that many primatological investigators are unaware of (c.f Arluke, 1988) or do not think it is important or critical how their research subjects are handled (cf. Traystman, 1987). They disregard the fact that the blood collection procedure can be a stressful event which could affect scientific results, or at least they do not adequately describe the dependent variables which are tied to it. The conditions under which blood samping occurred were described in most instances so haphazardly or not at all that it would be impossible to determine if stress-related variables have distorted the research findings. This circumstance also makes it difficult, if not impossible, to replicate the experiment regardless of its scientific merit (cf. Claassen, 1994; Öbrink & Rehbinder, 1999). The same situation has been highlighted by Davis, Bennett, Berkson, Lang, Snyder, and Pick (1973) for laboratory rodents. The authors concluded from a survey of 191 scientific articles that "It is most distressing to find that the frequency of handling the animals [for procedures] was mentioned in only a single article [0.5%] of the entire lot" (p. 3).

The primatological literature testifies that some investigators are aware that enforced restraint during injection, enforced restraint during venipucture, unfamiliarity of environment, and disturbance time are variables of the blood collection procedure which are relevant for a meaningful interpretation of stress-sensitive research data. To ignor these variables while ascertaining at the same time that `basal' or `normal' values of stress-sensitive blood paramters were determined, would contravene basic scientific rules. The literature also documents numerous reports of researchers who have made successful attempts to control these variables by working with animals who were trained to cooperate during swift blood collection in their familiar home cages.

To safeguard the integrity of scientific methodology and the humane treatment of primates used in research, the following guidelines should be observed:

1. Investigators should consider how physiological changes resulting from compulsory restraint affect their proposed experiments (Olfert, Cross, & McWilliam, 1993).
2. Editors of scientific journals should require that authors provide sufficient information on the blood collection – and when applicable also of the ketamine injection procedure so that the investigation can be replicated reliably in another laboratory (cf. Davis, Bennett, Berkson, Lang, Snyder, & Pick, 1973; Öbrink & Rehbinder, 1999) and the research data interpreted with reasonable accuracy.

It is not only a scientific but also an ethical obligation to describe variables of the blood collection procedure unless it has been proven beforehand or documented in the scientific literature that these do not stress the research subject and that they do not influence the data collected from it. Uncontrolled variables introduced by ketamine injection or venipuncture automatically increase data variability and hence the number of research subjects needed to achieve statistical significance (cf. Russell, & Burch, 1959; Brockway, Hassler, & Hicks, 1993; Hau & Craver, 1994). This is not necessary if published refinement techniques are applied which allow the research primate to be a cooperative partner rather than a subdued subject during the handling procedure (cf. T-W-Fiennes, 1972; Schnell & Gerber, 1997). Since "scientists themselves have adopted the principle: `Good Animal Care and Good Science Go Hand in Hand'" and since institutions are "obliged to adhere to the highest possible standards for the humane care and responsible use of laboratory animals" (Public Health Service, 1994, p. 12), it must be considered unethical if an investigator through carelessness or ignorance uses more animals for a project and hence inflicts more suffering than is absolutely necessary (cf. Öbrink & Rehbinder, 1999).

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